"Investigation of glucocorticoid and dissociated glucocorticoid activity in hepatoma cell lines with specific reference to regulation of the corticosteroid binding globulin (CBG) proximal promoter."

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Declaration:

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

This study investigated the effect of several hormones on the rat corticosteroid binding globulin proximal promotor and for the first time showed that modulation occurs at the promotor level and can be correlated with changes in corticosteroid binding globulin mRNA and protein levels. The effect of various physical and psychological stressors on rat liver corticosteroid binding globulin mRNA levels was also tested and it was shown that voluntary running had no effect on rat corticosteroid binding globulin levels but that involuntary swimming and immobilization decreased rat corticosteroid binding globulin mRNA levels. Glucocorticoid responsiveness of the corticosteroid binding globulin promoter was investigated further by using truncated contructs of the corticosteroid binding globulin proximal promoter. Glucocorticoid responsiveness was delineated to between –296 and –145bp from the transcription start site an area that contains putative binding sites for D-site binding protein, hepatic nuclear factor-3 and CAAT/enhancer binding protein suggesting that these transcription factors may be involved in glucocorticoid responsiveness of the corticosteroid binding globulin proximal promoter.

The dissociative glucocorticoid activity of medroxyprogesterone acetate and Compound A, both putative dissociated glucocorticoids, was compared to standard glucocorticoids by examining transactivation of glucocorticoid response element-containing reporter constructs and transrepression of corticosteroid binding globulin gene expression in hepatic cell lines. Results showed that medroxyprogesterone acetate, but not Compound A, transactivates only in the presence, but not in the absence, of co-transfected glucocorticoid receptor. Medroxyprogesterone acetate down modulated dexamethasone transactivation while the modulatory effect of Compound A depends on the order of

addition of Compound A. If added together Compound A has no effect on dexamethasone transactivation, however, if Compound A was added before dexamethasone, Compound A significantly decreased dexamethasone transactivation. Both medroxyprogesterone acetate and Compound A, like glucocorticoids, transrepressed the rat corticosteroid binding globulin proximal promoter. The potency of repression was similar but Compound A repressed with a higher efficacy than medroxyprogesterone acetate. We conclude that Compound A is a completely dissociated glucocorticoid in contrast to medroxyprogesterone acetate that displays only partial dissociation, which is dependent on glucocorticoid receptor levels.

OPSOMMING

Tydens hierdie ondersoek is die effek van verskeie hormone op die rot kortikosteroied bindings globulien proksimale promoter ondersoek en vir die eerste keer is getoon dat modulering plaasvind op promoter-vlak en dat repressie korrileer met die verandering in kortikosteroied bindings globulien mRNA-en proteinvlakke. Die effek van verskeie fisiese en fisiologiese stressors op rotlewer kortikosteroied bindings globulien-mRNA-vlakke is ook getoets en daar is getoon dat willikeurige hardloop geen effek op rot kortikosteroied bindings globulien-mRNA-vlakke het nie maar dat gedwonge swem en immobilisering rot kortikosteroied bindings globulien-mRNA-vlakke verlaag. Glukokortikoied responsiewiteit van die kortikosteroied bindings globulien proksimale promoter is verder ondersoek deur verkorte konstrukte van die kortikosteroied bindings globulien te toets. Glukokotikoied responsiewiteit is afgebaken tot tussen –296 en –145bp vanaf die transkripsie beginplek 'n area wat beweerde bindings setels vir D-setel bindings protein, hepatosiet faktoor-3 en CCAAT-bindings protein-2 bevat en dus suggereer dat hierdie transkripsie faktore betrokke mag wees met glukokortikoied effekte op die kortikosteroied bindings globulien-proksimale promoter.

Die dissosiatiewe glukokortikoied aktiwiteit van medroksiprogesteroon asetaat en Verbinding A, beide beweerde dissosiatiewe glukokortikoiede, relatief tot standaard glukokortikoiede is vergelyk deur transaktivering van glukokortikoied reseptor elelment-bevattende konstrukte en onderdrukking van kortikosteroied bindings globulien geen ekspressie in lewersellyne te bestudeer. Medroksiprogesteroon asetaat, maar nie Verbinding A nie, transaktiveer slegs in die teenwoordigheid, maar nie in die afwesigheid, van ko-getransfekteerde glukokortikoied reseptore. Medroksiprogesteroon asetaat moduleer deksametasoon transaktivering afwaarts terwyl die modulerende effek

van Verbinding A afhanklik van die orde van Verbinding A byvoeging is. Indien saam bygevoeg het Verbinding A geen effek op deksametasoon transaktivering nie, maar indien Verbinding A voor deksametasoon bygevoeg word verlaag Verbinding A deksametasoon transaktivering. Beide medroksiprogesteroon asetaat and Verbinding A, soos glukokortikoiede, onderdruk die rot kortikosteroied bindings globulien-proksimale promoter. Die sterkte van onderdrukking is dieselfde maar Verbinding A onderdruk met 'n hoër effektiwiteit as medroksiprogesteroon asetaat. Ons toon dat Verbinding A 'n totale dissosiatiewe glukokortikoied is in teenstelling met medroksiprogesteroon asetaat, wat slegs gedeeltelik dissosiatief is afhangende van glukokortikoied reseptor-vlakke.

To my family

For your love and support

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ABBREVIATIONS

ABRs	acute-booster reactants
α1-ACT	α1-antichymotrypsin
ACTH	adrenocorticotropic hormone
AF-1	activation function-1
AF-2	activation function-2
AFs	accessory factor-binding sites
AP-1	activating protein-1
APPs	acute phase proteins
APR	acute phase response
AR	androgen receptor
α1-AT	α1-antitrypsin
ATR	antitrypsin related sequence
AVP	arginine vasopressin
11betaHSD2	11-beta-hydroxy-steroid dehydrogenase
BMI	body mass index
bp	base pairs
CBG	corticosteroid binding globulin
Cbg	CBG gene
CBP	CREB-binding protein
Cdk	cyclin-dependent kinase
C/EBP	CAAT/enhancer binding protein
COUP-TF	chicken ovalbumin upstream transcription factor
CP-2	CCAAT-binding protein-2
CpdA	**Compound A:** 2-(4-acetoxyphenyl)-2-chloro-N-Methyl-ethylammonium chloride
CRE	cAMP reponse element
CREB	CRE binding protein
CRH	corticotropin releasing hormone
DBD	DNA-binding domain
DBP	D-site binding protein
DRIP	vitamin D3 receptor-interacting protein
E2	17β-estradiol

EMSAs	electrophoretic mobility shift assays
ER	estrogen receptor
FCS	fetal calf serum
GC(s)	glucocorticoid(s)
GFP	green fluorescent protein
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
GRE(s)	glucocorticoid response element(s)
nGRE(s)	negative glucocorticoid response element(s)
GRIP-1	GR interacting protein-1
GRU(s)	glucocorticoid response unit(s)
HAT	histone acetyltransferase
HNF-1	hepatic nuclear factor-1
HNF-3	hepatic nuclear factor-3
HNF-4	hepatic nuclear factor-4
HOMA	fasting insulin resistance index
HPA axis	hypothalamic-pituitary-adrenal axis
HRE	hormone response element
Hsp90	heat shock protein 90
ICAMI	intercellular adhesion molecule
IGF-1	insulin-like growth factor-1
IGFBP-1	insulin-like growth factor-binding protein-1
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
JNK	c-Jun NH_2-terminal kinase
KAL	kallistatatin
KREs	keratin response elements
LBD	ligand-binding domain
LH	luteinizing hormone
MAPK	mitogen activated protein kinase
MMTV	mouse mammary tumor virus
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor

NCoR	nuclear hormone receptor corepressor
NES	nuclear export signal
NF-κB	nuclear factor-κB
NL	nuclear localization
NLS	nuclear localization signal
NUC	nucleosome region
P/CAF	p300/CBP-associated factor
PCI	protein C inhibitor
PDE	phosphodiesterase
PEPCK	phospoenolpyruvate carboxykinase
PEPCK-C	cytosolic form of PEPCK
PI	acidic isoelectric point
α1-PI	α1-proteinase inhibitor
POMC	pro-opiomelanocortin gene
RIA	radio-immuno assay
PP5	phosphatase type 5
PPARγ2	peroxisome proliferator-activated receptor γ2
PR	progesterone receptor
RAR	retinoic acid receptor
RXR	retinoic X receptor
SEGRAs	selective GR agonist
SERPINS	serine protease inhibitor gene family
SLPI	secretory leucocyte protease inhibitor
SMRT	silence mediator of retinoic and thyroid receptors
SPI-3	serine protease inhibitor-3
SRC-1	steroid receptor coactivator-1
STAT3	signal transducers and activators of transcription3
STAT5	signal transducers and activators of transcription5
SWI/SNF	family of switch/sucrose non-fermentable proteins
TAT	tyrosine aminotransferase
TBG	T4–binding protein / thyroxin binding globulin
TBP	TATA box binding protein
TIF	transcriptional intermediary factor

TIF2	transcriptional intermediary factor 2
TR	thyroid receptor
VDR	vitamin D receptor
WHR	waist to hip ratio

CHAPTER 1

Aim and strategy of study

Glucocorticoids (GCs) have a wide variety of functions in the body. Chapter 2 provides an extensive background on GCs and glucocorticoid receptor (GR)-mediated gene regulation. GCs are also used pharmacologically for the suppression of inflammation in chronic inflammatory diseases. Unfortunately this use is often accompanied by side-effects. This chapter also reviews the literature on the synthetic dissociative GCs tested up to now in the search for a drug with less side-effects. GCs are transported by corticosteroid binding globulin (CBG) and the second part of this literature review summarizes the function and gene organization of CBG and focuses on the regulation of CBG. This broad and extensive background is necessary to explain the results obtained in this study, to compare the results with what is known in the literature, and to place the results within the context of exciting paradigms.

CBG protein and mRNA levels are regulated by numerous hormones, conditions and factors, however whether this regulation is at the transcriptional level is unknown. To address this question we therefore investigated as described in chapter 3, the regulation of the CBG proximal promotor by several hormones reported to influence CBG production and attempted to correlate this regulation with CBG mRNA and protein levels. Experimentally this was carried out in human and mouse hepatoma cell-lines as CBG is produced primarily by the liver. In addition, some work with rats was also carried out. Northern blotting was used to determine CBG mRNA levels and CBG protein levels were determined by radio-immuno assay (RIA) while promoter regulation was studied by using transiently and stably transfected promoter-reporter constructs of the rat CBG proximal promoter. Further work focused on GC regulation of CBG levels and transfection experiments with truncated proximal promoter reporter constructs were performed to delineate the GC responsiveness on the rat CBG promoter. We also

investigated whether GC responsiveness of the CBG promoter was dependent on the levels of GR by transiently co-transfecting a GR expression vector and by evaluating the effect of the GR antagonist, RU486 on GC repression. The results with the truncated CBG promoter constructs implicated three transcription factors in GC responsiveness of the CBG promoter. To test for mutual transrepression the effect of these putative transcription factors on transactivation of a glucocorticoid response element (GRE)-containing promoter by liganded GR was then investigated.

GCs, both endogenous and exogenous, have been shown to decrease CBG expression resulting in an increase in free GCs thus amplifying the effect of endogenous levels of GCs or pharmacological analogues. Therefore we used transrepression of a rat CBG proximal promoter reporter construct and transactivation of GRE-containing reporters, as the two parameters to evaluate dissociated GC activity. As described in chapter 4 we investigated and compared the dissociative GC activity of the steroidal compound, medroxyprogesterone acetate (MPA) and the non-steroidal compound, (2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride) (Compound A) relative to conventional GCs. To test whether the transrepressive or transactivation potential was influenced by the GR expression levels, GR expression vectors were co-transfected and to strengthen the case that the observed repression of the CBG promoter reporter construct by CpdA was mediated by the GR the effect of the glucocorticoid antagonist, RU486, on transrepression was also investigated. In addition, the effect of the test compounds on CBG mRNA levels in the absence and presence of co-transfected GR and co-transfected dimerization deficient GR, was investigated to establish if these effects correlated with the CBG promoter reporter results. Dimerization deficient GR was used to check for a dimerization requirement in transrepression of CBG mRNA as

transrepression may be mediated through direct binding of the GR to nGREs that requires dimerization or via a tethering mechanism that does not require DNA binding or dimerization by the GR.

The results of this study are summarized in chapter 5 where conclusions and possible explanations for the results obtained are presented as well as a discussion of future experiments. In chapter 6 the methods of all experiments performed in this study are described in detail. Data from this study has been presented inpart at several national and international conferences and at the end of the thesis a list of conference abstracts may be found.

CHAPTER 2

Literature review

2.1 Glucocorticoid receptor mediated gene regulation

2.1.1 Mechanism of action

Endogenous GCs are important in maintaining basal and stress-related homeostasis and preventing excessive immune responses to antigenic challenges [Chrousos and Gold 1992]. Pharmacologically, GCs are also the most effective therapy in the long-term treatment of inflammation and autoimmune diseases [Cato and Wade 1996].

GCs exert their effects by binding to the GR and the mineralocorticoid receptor (MR) [Beato *et al* 1995 & Beato and Sachez-Pacheco 1996b]. While the GR is widely expressed in most cells of the organism, the MR is restricted to certain organs such as the brain, the kidney (epithelial cells), the colon and exocrine glands [Kellendonk *et al* 2002]. In addition, although the MR binds GCs with higher affinity than the GR, the presence of 11-beta-hydroxy-steroid dehydrogenase (11betaHSD2), which inactivates GCs, provides functional distinction in most tissues, with the exception of the heart and brain [De Kloet *et al* 1998]. Most biological effects of endogenous GCs are thus mediated by the GR, as are the effects of modern synthetic GCs, which are for the most part GR selective [Newton 2000].

The inactive cytoplasmic GR is bound to a protein complex, that includes two subunits of the heat shock protein hsp90 plus a number of other proteins such as p23, preventing the nuclear localization of unoccupied GR [Bresnick *et al* 1989; DeRijk *et al* 2002]. Upon hormone binding the GR, a ligand-dependent transcription factor, dissociates from these cytoplasmic chaperone molecules and translocates to the nucleus, where the activated receptor can transactivate or transrepress specific genes [Beato *et al* 2000]. The different models of GR transcriptional modulation are illustrated in Fig. 2.1 and the genes regulated by these models are summarized in Table 2.1.

Transactivation is mediated by binding of a GR dimer to GREs in the promoter region of GC responsive genes, followed by recruitment of coactivators, chromatin remodelling, and increased gene transcription (Fig. 2.1A) [Adcock 2000]. Examples of genes upregulated by GCs via simple GREs include lipocortin I (now known as annexin I), and p11/calpactin binding protein, which are involved in suppressing the release of arachidonic acid during inflammation [Flower and Rothwell 1994; Pitzalis *et al* 2002; Yao *et al* 1999]. Other examples of genes that are upregulated by GCs via GREs include, the β_2-adrenergic receptors [Collins 1988 *et al*; Mak *et al* 1995; Cornett *et al* 1998], secretory leucocyte protease inhibitor (SLPI) [Abbinante-Nissen *et al* 1995], the decoy interleukin-1 (IL-1) type II receptor [Colotta *et al* 1993; Muller *et al* 2002], the tyrosine aminotransferase (TAT) gene [Sassi *et al* 1998] and the mouse mammary tumour virus (MMTV) gene [Lee *et al* 1998].

The GR can act in synergy with other transcription factors to transactivate or repress from composite elements (also called glucocorticoid response units - GRUs) with DNA binding of both the GR and the other transcription factors (Fig. 2.1B & G) [Diamond *et al* 1990; Pearce and Yamamoto 1993; Pearce *et al* 1998]. The term "composite" GRE thus describes a non-consensus sequence that binds GR with low affinity and transactivates (Fig 2.1B) or transrepresses (Fig 2.1G) [Diamond *et al* 1990]. This "composite" DNA binding element may have binding sites for GR and other transcription factors and GCs may activate or repress transcription from these binding sites, depending on cell type. The phospoenolpyruvate carboxykinase (PEPCK) gene is an example of a gene containing a composite GRE, through which it is upregulated, in liver and kidney, or downregulated, in adipose tissue, by GCs [Scott *et al* 1998; Wang *et al* 1999; Stafford *et al* 2001a; Stafford *et al* 2001b; Hanson and Reshef 1997]. Induction

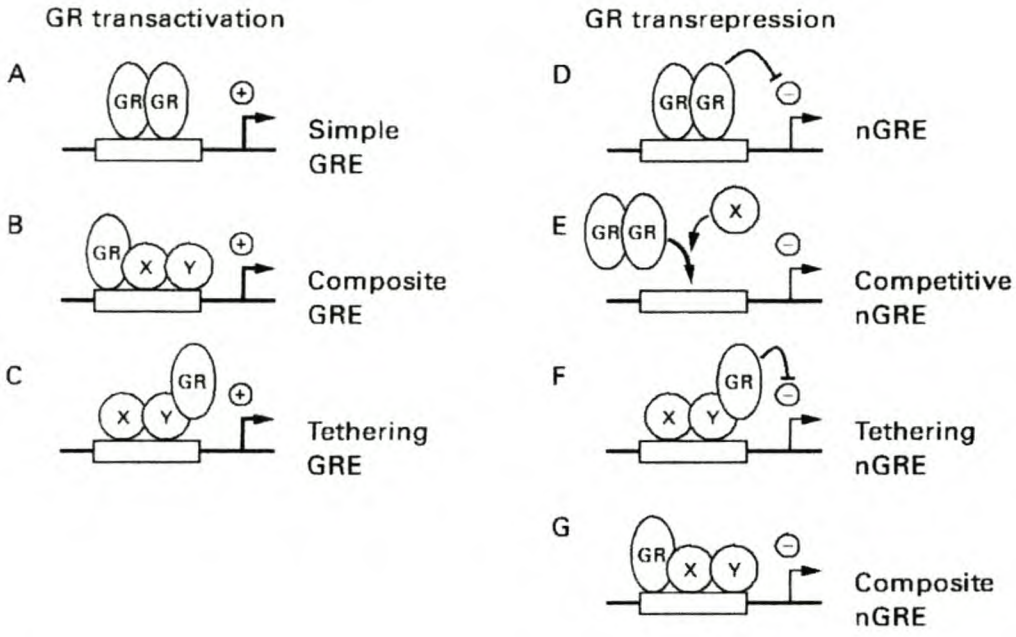


Figure 2.1. Models of GR transcriptional modulation. (A) Homodimers of GR bind cooperatively to classical GRE sites to activate transcription. (B) Interaction of GR with another transcription factor can activate transcription from composite binding sites with DNA binding of both factors. (C) Interaction of GR with a second transcription factor may result in activation of transcription without DNA binding of GR. (D) Homodimers of GR repress transcription from a nGRE. (E) At a competitive nGRE, binding of GR to the GRE site prevents binding of factors that are necessary for transcriptional regulation and thus cause repression. (F) Interaction of GR with a second transcription factor may result in repression of transcription without needing DNA binding of GR. (G) Interaction of GR with a second transcription factor can repress transcription from composite binding sites in a manner that involves DNA binding of both factors [taken from Newton 2000].

of the PEPCK gene in liver by GCs is achieved through a complex GRU consisting of two low affinity GR-binding sites, GR1 and GR2, and binding sites for transcription factors that act as accessory factors for the glucocorticoid response. Three accessory factor-binding sites (AFs), AF1, AF2 and AF3, have been identified in this GRU. The proteins that mediate accessory activities through AF1 have been identified as hepatic nuclear factor-4 (HNF-4) and chicken ovalbumin upstream transcription factor (COUP-TF) [Hall *et al* 1995]. Members of the hepatic nuclear factor-3 (HNF-3) family bind to AF2 and COUP-TF acts as the accessory factor through AF3 [Wang *et al* 1996; Scott *et al* 1996]. All three AF elements as well as a downstream element, the cAMP response

element (CRE), are needed for a complete GC transactivation response. The GR can also interact with other transcription factors, resulting in repression of transcription from composite binding sites that involve DNA binding of both factors (Fig. 2.1G). For example, as discussed above, transcription of the cytosolic form of PEPCK (PEPCK-C) gene is repressed in white adipose tissue via two nonconsensus sequence GREs that bind GR with a low affinity [Olswang *et al* 2003]. The GR, in the presence of ligand, inhibits activation of the PEPCK-C gene promoter by CAAT/enhancer binding protein (C/EBP)α or C/EBPβ in adipose tissue, most probably through binding to the AF2 element. There is evidence that this may not be a true composite negative GRE (nGRE), as DNA-binding of the GR may not be required for transrepression [Olswang *et al* 2003].

The third mechanism of GR transactivation involves the interaction of the GR with another transcription factor without GR binding to DNA. This is illustrated by the synergistic induction of the rat serine protease inhibitor-3 (SPI-3) gene by interleukin-6 (IL-6) and dexamethasone that involve the interaction of GR with signal transducers and activators of transcription 3 (STAT3) and C/EBP, without GR binding to DNA (Fig. 2.1C) [Kordula and Travis 1996]. This synergy between the transcription factor STAT5 and the GR appears to involve direct interaction between STAT5 and GR without requiring GR DNA binding, as shown for the β–casein gene in mammary cells [Stöcklin *et al* 1996; Cella *et al* 1998].

Transrepression may also be mediated via direct binding of the GR to DNA via nGREs (Fig. 2.1D & E). nGREs are present in various genes and include the corticotropin releasing hormone (CRH) gene promoter [Malkoski and Dorin 1999; King *et al* 2002],

the pro-opiomelanocortin (POMC) gene [Turney and Kovacs 2001], the prolactin gene [Subramaniam *et al* 1998], the IL-1 gene [Zhang *et al* 1997] and the osteocalcin gene [Aslam *et al* 1995]. The POMC gene is an example of a promoter repressed by GCs where multimers of GR were shown to bind a nGRE causing repression of transcription [Charron and Drouin 1986; Drouin *et al* 1993, Turney and Kovacs 2001]. Specifically, this involves binding of a GR homodimer followed by binding of a GR monomer. The mechanism of action involves protein-protein interactions with other factors (for example NUR77) on the promoter or direct inhibition due to steric hindrance, which may be due to the close proximity to the TATA box and the transcription start site. The binding of GR to the nGRE may block binding of positive factors and thus cause transcriptional repression (Fig. 2.1E) [Murphy and Conneely 1997]. Another example of a gene repressed by GCs is the glycoprotein hormone α subunit gene [Akkerblom *et al* 1988; Stauber *et al* 1992]. This promoter contains overlapping binding sites for a CRE-binding protein (CREB) as well as GR and is positively regulated by CREB. DNA binding by GR is thought to inhibit transcriptional activation by preventing binding of CREB (Fig. 2.1E). The osteocalcin promoter is a further example of repression through a competitive nGRE that overlaps the TATA box [Stromstedt *et al* 1991; Meyer *et al* 1997]. Binding of the GR may prevent binding of the basal transcription factor, TATA binding protein (TBP), which will result in repression of transcription. Furthermore, in the bovine prolactin gene GR-dependent repression may be via competition between factors that bind or via the inhibition of positive activation by binding of GR to the nGRE [Sakai *et al* 1988; Subramaniam *et al* 1998]. The GR was also shown to suppress keratin gene expression through interaction of nGREs with four GR monomers [Radoja *et al* 2000]. In all of these examples, DNA binding by the GR is necessary for repression.

Table 2.1 Specific examples of genes to illustrate models of GR transcriptional modulation.

MECHANISM (numbering of models as in Fig.2.1)	GENES REGULATED	REFERENCE
Simple GRE (2.1A)	lipocortin I	Flower and Rothwell 1994; Pitzalis *et al* 2002;
	p11/calpactin binding protein	Yao *et al* 1999
	β_2-adrenergic receptors	Collins 1988 *et al*; Mak *et al* 1995; Cornett *et al* 1998
	SLPI	Abbinante-Nissen *et al* 1995
	decoy IL-1 type II receptor	Colotta *et al* 1993; Muller *et al* 2002
	TAT gene	Sassi *et al* 1998
	MMTV gene	Lee *et al* 1998
Composite GRE (2.1B)	PEPCK gene	Scott *et al* 1998; Wang *et al* 1999; Stafford *et al* 2001a; Stafford *et al* 2001b; Hanson and Reshef 1997
Tethering GRE (2.1C)	rat SPI-3 gene	Kordula and Travis 1996
	β–casein gene	Stöcklin *et al* 1996; Cella *et al* 1998
nGRE (2.1D)	CRH gene promoter	Malkoski and Dorin 1999; King *et al* 2002
	POMC gene	Turney and Kovacs 2001
	prolactin gene	Subramaniam *et al* 1998
	IL-1 gene	Zhang *et al* 1997
	osteocalcin gene	Aslam *et al* 1995
	keratin gene	Radoja *et al* 2000
Competitive nGRE (2.1E)	glycoprotein hormone α subunit gene	Akkerblom *et al* 1988; Stauber *et al* 1992
	osteocalcin gene	Stromstedt *et al* 1991; Meyer *et al* 1997
	bovine prolactin gene	Sakai *et al* 1988; Subramaniam *et al* 1998

Tethering nGRE (2.1F)	IL-8 gene	Nissen *et al* 2000
	ICAM1 gene	Nissen *et al* 2000
	collagenase-3 gene	Rogatsky *et al* 2001
Composite nGRE (2.1G)	PEPCK-C	Olswang *et al* 2003

Alternatively, transrepression may proceed without direct DNA-binding by the GR, but via protein-protein interactions. This may only require direct or indirect binding of the GR monomer to other transcription factors such as nuclear factor-κB (NF-κB), activating protein-1 (AP-1), STAT proteins and C/EBP (Fig. 2.1F) [McEwan *et al* 1997; Webster and Cidlowski 1999]. As this effect does not require direct binding of GR to DNA, the term "tethering GRE" is used to describe these elements. Most of the anti-inflammatory effects of GCs are mediated through repression of inflammatory and immune genes that do not contain GREs in their promoters [Cato and Wade 1996]. These inhibitory effects of GCs are mostly due to protein-protein interactions between activated GR and transcription factors such as AP-1, NF-κB and C/EBPβ, which mediate expression of inflammatory genes [Barnes 1998]. GCs have been shown to repress NF-κB-mediated activation of proinflammatory genes like interleukin-8 (IL-8) and intercellular adhesion molecule (ICAM1) via protein-protein interactions between NF-κB and the GR [Nissen *et al* 2000]. In addition, the collagenase-3 gene repression by GCs is mediated by the GR forming protein-protein interactions with AP-1 [Rogatsky *et al* 2001].

2.1.2 The GR

The GR is a ligand-activated transcription factor and a member of the nuclear hormone receptor superfamily, which includes the MR, the progesterone receptor (PR), the estrogen receptor (ER), the androgen receptor (AR), the thyroid hormone receptor (TR),

the retinoic acid receptor, and the vitamin D receptor (VDR) [Beato *et al* 1991; Mangelsdorf *et al* 1995; Aranda and Pascual 2001]. The GR, AR, PR and ER share over 50% identity in their amino acid sequences, have a similar three-dimensional structure and are classified as steroid receptors or Class III nuclear receptors [Tsai and O'Malley 1994; Bledsoe *et al* 2002; Aranda and Pascual 2001]. Phylogenetic analysis and sequence alignments show that the GR, MR, PR and AR form a subfamily of oxosteroid receptors that are separate from the ER subfamily.

2.1.2.1 Structure of the GR

2.1.2.1.1 Human GR gene

GRs are expressed in most cell types and their density varies from 2000-30000 molecules per cell [Adcock *et al* 1996]. Cloning of the GR from different species revealed that it consists of about 800 amino acid residues. The human GR (hGR) has 777 amino acids [Hollenberg *et al* 1985] and the gene is located on chromosome 5 (locus q11-q13) and consists of 9 exons [Encio and Detera-Wadleigh 1991; Yudt and Cidlowski 2002]. Exon 1 and the first part of exon 2 contain the 5'UTR, exon 2-9 the coding sequences and exon 9 the 3'UTR. It has also been shown that two more exons exist, called exon 1A and 1B, located upstream from the original exon 1 (now exon 1C) [Breslin *et al* 2001]. The activity of the mouse and human GR-gene is regulated by three promoters of which the first two promoters are located in the 3 kb region upstream of exon 1C (promoter 1B and 1C). Both promoters contain a few GC boxes, which are binding sites for the transcription factor SP-1. Promoter 1B also contains YY1 sites and promoter 1C contains a putative AP-2 site but no TATA or CAAT elements are present in these promoters [Encio and Detera-Wadleigh 1991]. Promoter 1A is located further

upstream and contains a putative interferon regulatory factor-binding element, as well as a sequence that resembles a GRE [Breslin *et al* 2001]. The structure of the hGR is shown in Fig. 2.2.

2.1.2.1.2 GR isoforms and subtypes

An additional level of complexity of steroid hormone receptors is the presence of multiple receptor subtypes and isoforms. For the human GR, two classes of cDNAs have been described which are the result of alternative splicing of a single gene transcript and that encode the hGRα and hGRβ isoforms of the receptor [Hollenberg *et al* 1985; Bamberger *et al* 1995]. Both mRNA transcripts contain exons 1-8 and differ only in that the hGRα mRNA (5.5kb) contains exon 9α while hGRβ (4.3kb) contains exon 9β [DeRijk *et al* 2002]. In addition, a third mRNA transcript (7.0kb) has been identified that is thought to also code for the hGRα [Oakley *et al* 1996].

Both GR protein isoforms are identical up to 727 amino acids, with hGRα (777 amino acids) larger than hGRβ (742 amino acids). GRβ cannot bind GCs and has a dominant negative effect on the transcriptional effects of GRα. The β-isoform was first thought to be a cloning artefact but was later shown to be expressed at varying levels in different tissues.

In the blood of patients with GC-resistant asthma, higher numbers of hGRβ positive cells were found, further suggesting a dominant negative function of the human GRβ [Leung *et al* 1997]. Evidence for a physiological role for the GRβ isoform in neutrophils was recently found. The GC sensitivity of human neutrophils was compared

to that of peripheral blood cells and the presence of GRα/GRβ heterodimers was only observed in neutrophils [Strickland *et al* 2001]. Also, transfection of neutrophils from

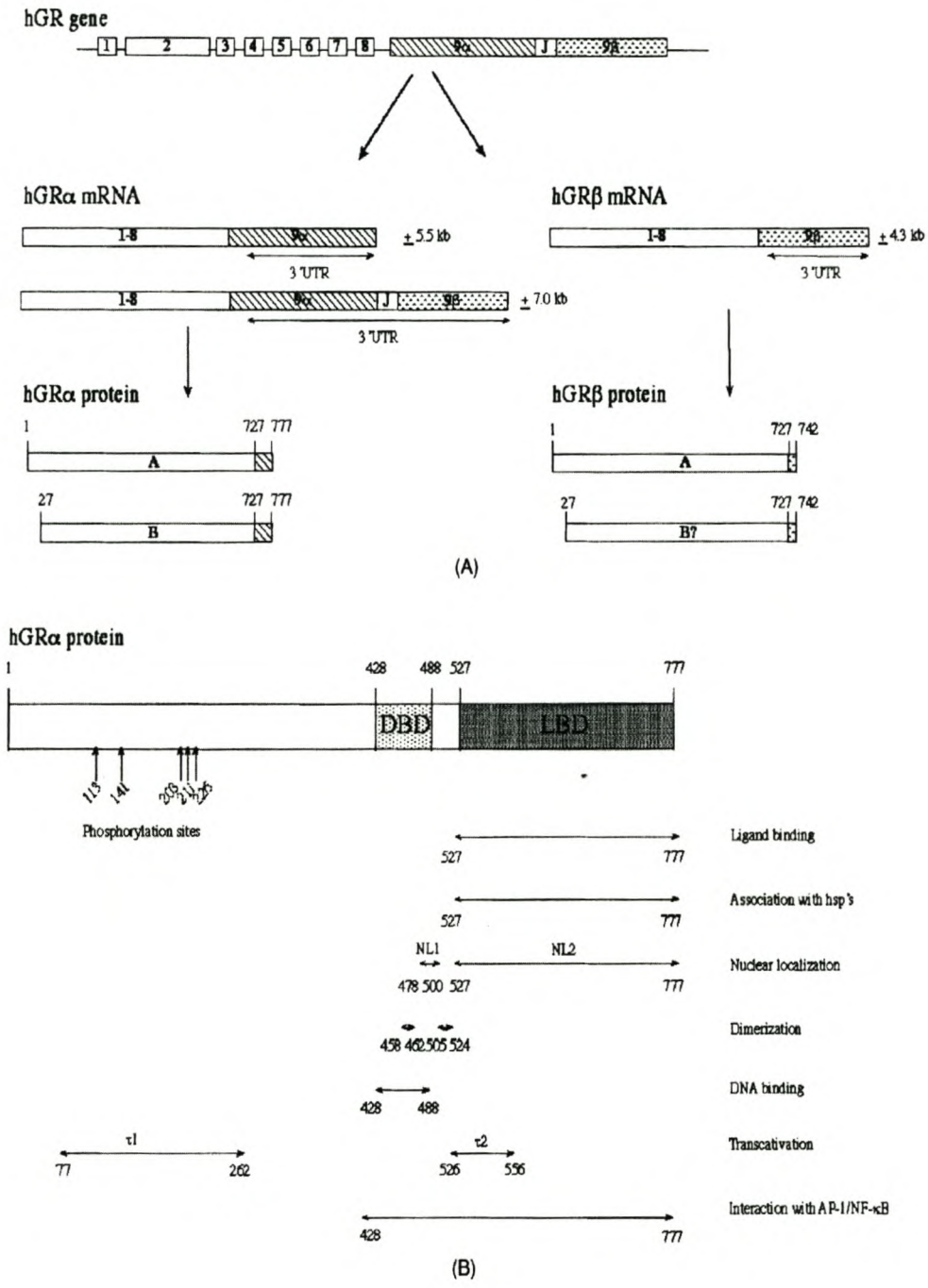


Figure 2.2. Structure of the hGR. (A) Schematic representation of the hGR gene, the mRNAs encoding hGRα and hGRβ as well as the A and B-isoforms. (B) Structure of the hGRα protein. The different domains of the receptor and several functions mapped to regions of the protein are indicated [taken from DeRijk *et al* 2002].

mice with a functional hGRβ led to an inhibition of GC induced apoptosis. The GRα/GRβ heterodimers may thus be functionally inactive, but their expression might limit the GC responsiveness mediated via the GRα homodimer.

In addition, two forms of hGRα are produced by alternative translation of the same mRNA and are called GR-A and GR-B [Yudt and Cidlowski 2001]. The longer GR, GR-A (aa 1-777; 94kDa) is generated from the initial ATG start codon (methionine 1) and the shorter GR, GR-B (aa 27-777; 91kDa) is translated from the internal ATG corresponding to methionine 27. Both receptors display similar subcellular localization and nuclear translocation after ligand activation and are present in several human cell lines and rodent tissues. Functional assays of these splice variants with GC responsive promoters showed that the shorter hGR-B is nearly twice as effective as the longer hGR-A in transactivation, but not in transrepression [Yudt and Cidlowski 2001]. A similar mechanism of alternative translation initiation has been proposed, but not yet proven, for the hGRβ [Yudt and Cidlowski 2001].

2.1.2.1.3 Functional domains of the GR

The GR, like other steroid receptors, has several functional domains, namely a ligand-binding domain (LBD), a N-terminal domain and a DNA-binding domain (DBD) (Fig. 2.2B, p15) [Giguere 1986]. The N-terminal domain in the hGRα consists of amino acids 1-427 and the amino acids 428-488 form the DBD. The C-terminal LBD, separated from the DBD by a hinge region, consists of amino acids 527-777, of which 50 amino acids at the end are encoded by exon 9α. The LBD of the hGRβ is the same as that of hGRα up to amino acids 727. After that the structures of hGRα and hGRβ diverge. In

hGRβ the last 15 amino acids are encoded by exon 9β and form the C-terminal part of the LBD of hGRβ, which does not in fact bind ligand [DeRijk *et al* 2002].

The N-terminal domain (τ1 or AF-1) is involved in transactivation after DNA binding has taken place and may also be involved in binding to other transcription factors. The human GR also contains a second transactivation domain (τ2 or AF-2), which is adjacent to the GC binding domain. This region is also important for nuclear translocation of the receptor.

The three-dimensional structure of the DBD of the GR by 2D-NMR showed that the two zinc fingers form a single structural domain consisting of two α-helices, one adjacent to each zinc finger. The GR is folded in a manner so that two zinc fingers protrude out from the surface, which enables it to interact with DNA. All receptors with a conserved DBD have these two zinc fingers, which are expressed by separate exons (Fig. 2.3) [Evans 1988].

The one zinc finger is responsible for receptor-DNA contact by binding a specific hormone response element (HRE) and the other one is involved in receptor dimerization. The important amino acid residues responsible for the DNA-receptor interaction in the wild type GR are situated in the P-box of the first zinc finger as well as in the interfinger region. A change in the secondary structure from a β–strand to an α–helix, near the P-box, is observed and was called a DNA-recognition helix [Luisi *et al* 1991]. The D-box of the second zinc finger up to the amino acid D485 builds an extended coil, which is followed by a second helix. The tetrameric co-ordinative binding of four cysteins with a central Zn^{2+} ion determines the molecular shape of the

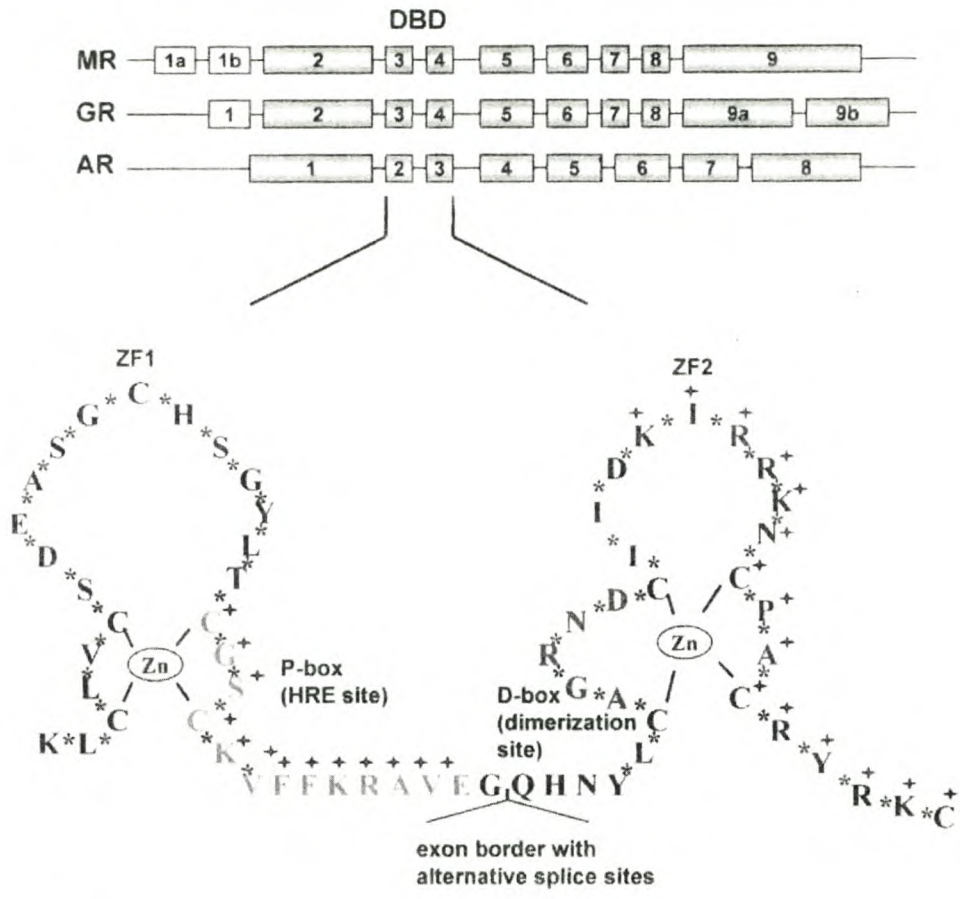


Figure 2.3. Organization and structure of the DNA binding domain of the MR, GR and AR. White boxes, untranslated exons; grey boxes, translated exons. Amino acid sequence in one letter code: grey letters, DNA identification sequence. Amino acids marked with a plus sign form α helices. ZF, zinc finger [Wickert and Selbig 2002].

two zinc fingers [Luisi *et al* 1991]. The conserved amino acid sequence and the resulting 3D structure provides for the optimal, specific DNA binding and is a requirement for regulating target gene expression by steroid hormone receptors. Alternative splicing of exon 3 and 4 of the GR as well as the MR leads to mRNAs with an additional inframe insertion in the interfinger region between the two zinc fingers of the DBD as shown in Fig. 2.3. GR receptors from different species with an insertion of one (GR+1 in primates) or nine (GR+9 in fish) additional amino acid residues have been described [Brandon *et al* 1991; Ducouret *et al* 1995]. The interfinger region of the GR DBD seems to be flexible and can cope with insertions of up to 27 amino acid

residues [Zandi *et al* 1993]. Structure predictions by Wickert *et al* indicated that the hinge region of the zinc finger coding exons of the GR could be flexible due to the insertion of several amino acids [Wickert and Selbig 2002].

2.1.2.2 Function of the GR

The GR is predominantly localised in the cytoplasm of target cells and upon binding of GCs to the GR it translocates to the nucleus where it dimerizes and modulates transcription of GC-responsive genes [Oakley *et al* 1999; Beato *et al* 1996b].

2.1.2.2.1 Ligand binding

The LBD is located at the carboxyl terminus of the molecule and is separated from the DBD by a hinge region. The LBD contains 12 α-helices, which are folded to form a hydrophobic pocket for the ligand [Baumann *et al* 1993]. Ligand binding results in a conformational change, in which the helix 12 folds back and "closes" the pocket. The LBD also contains a ligand-dependent activation function (AF-2), which is regulated by hormone binding. In the absence of hormone, the GR is retained in the cytoplasm by association with chaperone proteins like hsp90, which binds to the LBD. After hormone binding the chaperone proteins are released from the GR and this allows for dimerization and translocation into the nucleus. Receptor dimerization is partly mediated through the LBD. In the nucleus the GR can bind to DNA elements and can either activate or repress transcription, both requiring the intact function of the LBD. Mutations of the receptor at Tyrosine735, have identified this amino acid as being important for ligand binding and ligand-dependent transactivation [Ray *et al* 1999]. Tyr735 interacts with the D ring of the synthetic glucocorticoid agonist, dexamethasone,

and contributes to the conformational change of helix 12, which is predicted to be necessary for efficient recruitment of transcriptional coactivator molecules.

Previously, the crystal structures of nuclear receptor LBDs in the apo-state or bound to agonist or antagonist revealed that the LBDs fold into a canonical three-layer helical sandwich that embeds a hydrophobic pocket for ligand binding. It also emphasizes the importance of the C-terminal (AF-2) helix in ligand-dependent regulation [Bourquet *et al* 1995; Brzozowski *et al* 1997; Wagner *et al* 1995]. In the apo-or antagonistic bound receptor, the AF-2 helix is destabilized from its active conformation, which allows the LBD to interact with corepressors, such as nuclear corepressor (N-CoR) [Chen *et al* 1995; Horlein *et al* 1995]. Agonist binding on the other hand, induces a conformational change of the AF-2 helix which stabilises the receptor in an active conformation to facilitate its association with coactivator proteins like steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor 2 (TIF2) [Onate *et al* 1995; Voegel *et al* 1996]. Crystal structures of receptor/coactivator complexes revealed that the coactivator LXXLL motifs adopt a two-turn α–helix and both helical ends are stabilised by a charge clamp formed partly by a conserved acidic residue from the AF-2 helix [Darimont *et al* 1998; Nolte *et al* 1998; Shiau *et al* 1998].

Recently, the crystal structure of the LBD bound to dexamethasone revealed that the LBD adopts a dimer configuration, which involves the formation of an intermolecular beta sheet that could be important for GR-mediated activation [Bledsoe *et al* 2002]. Bledsoe *et al* also found that the GR uses two charge clamps to define its sequence-specific binding to the TIF third motif [Bledsoe *et al* 2002]. The first charge clamp consists of the E755 from the AF-2 helix and K579 from helix 3, which cap the

backbone amides and carbonyls of the coactivator helix. Both of these residues are highly conserved in the nuclear hormone receptor superfamily and also share a common binding mode for all LXXLL motifs. The second charge clamp consists of R585 and D590 that form hydrogen bonds with the side chains of residues R+2 and D+6, which are present in the TIF2 third motif but not in the first or second motif. The interactions with the second charge clamp are partly responsible for the binding specificity of the GR for the TIF2 third motif. The residues from the second charge clamp are conserved in the GR, AR, PR and MR but are not found in the ER and thus may be responsible for the differential binding of coactivator motifs by nuclear receptors. Structures of the endogenous steroid hormones are shown in Fig. 2.4. Comparing the structures of the GR, AR, PR and ER provides information into how functional specificity is achieved by steroid receptors. There is 50% homology in amino acid sequences between these steroid receptors and they also share a similar three-dimensional structure. In these structures, the core steroid template (A, B, C and D rings) assumes a general orientation with the A ring orientated towards the conserved arginine from helix 5 and the D ring toward the AF-2 helix. Small differences in the secondary structure and the ligand binding pockets exist in steroid receptors. In the GR, helices 6 and 7 differ from the ER, PR and AR and produce a unique side pocket in the GR. This unique side pocket may explain the GR selectivity for GCs, which like mineralocorticoids that bind MR, have larger substituents at the C17α position in comparison with estrogen, progesterone and testosterone. The polar substituents in steroid hormones are mostly situated at positions C3 or C17. In the GR structure, but also conserved in the AR and PR, the C3 ketone accepts hydrogen bonds from Q570 and R611. In the ER, the glutamine is replaced by a glutamate, which prefers to accept a hydrogen bond from the ligand. This accounts for the ERs selectivity for a hydroxyl group at the C3 position. The differences in hydrogen

bond formation may explain why the GR, AR, PR and MR prefer steroid hormones with a ketone at position C3, whereas the ER favours a hydroxyl group (Fig. 2.4) Although the GR and MR have a similar side pocket, the MR selectivity for mineralocorticosteroids may be due to differences in hydrogen bonding patterns between the receptors and the ligands. The MR selective steroids all lack the 17α-hydroxyl group, which forms a specific hydrogen bond with Q642 in the GR structure.

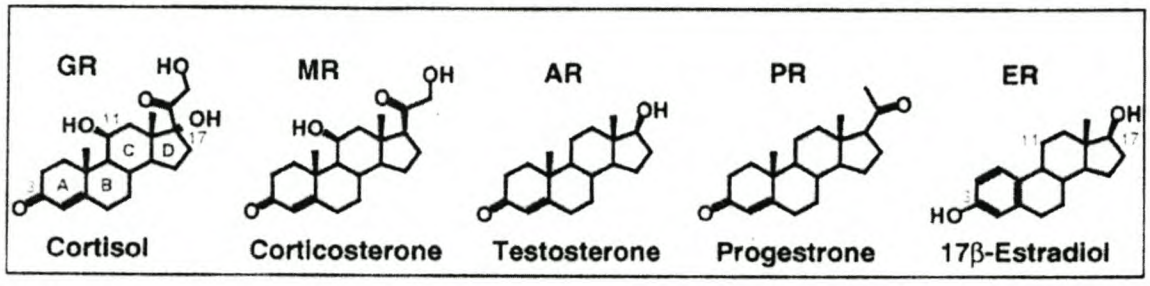


Fig. 2.4. Structures of the endogenous steroid hormones [taken from Bledsoe *et al* 2002].

Several diseases like Cushing's syndrome, autoimmune diseases and numerous cancers have been associated with missense mutations in the GR LBD, which may be explained by the structure of the GR LBD [Bledsoe *et al* 2002]. These mutations can be classified into two groups, based on their location in the GR structure. The first group includes the G507C, M601L, M604P, M646T, Y735S, C736S and L753F mutations. In the GR structure, these residues make direct contacts with dexamethasone and this results in a GR defective in ligand binding. The second group includes P541A, I559D, C638Y, V729I, Y764N and F774A mutations. These residues are involved in hydrophobic interactions within the GR protein structure and their mutations may destabilize the protein. With the availability of the GR LBD structure [Bledsoe *et al* 2002], correlations

between the location of mutations and various diseases associated with these mutations may now be understood.

2.1.2.2.2 Dissociation from heat shock proteins

Inactive GR is bound to a protein complex (300 kDa), which includes two subunits of heat shock protein hsp90. They act as molecular chaperones preventing nuclear localization of unoccupied GR [Bresnick *et al* 1989]. Hsp90 associates with the LBD of the GR (Fig. 2.2, p15), and this association keeps the receptor in a conformation that can bind steroid [Picard *et al* 1990]. Other factors, present in the protein complex and required for formation of a stable GR-hsp90 complex are p23, p60, hsp40, hsp70 as well as several immunophilins, for example hsp56. The immunophilins appear not to be needed for complex formation [reviewed by Pratt and Toft 1997]. Hsp90 complexation with GRs also opens up the LBD to permit the attachment of thiol residues by a thiol-derivatizing agent as well as the proteolytic cleavage at basic amino acids by trypsin [Stancato *et al* 1996; Modarress *et al* 1997]. These results support the idea that the hsp90-based chaperone machinery directs the partial unfolding of the LBD and thus the opening of the hydrophobic steroid-binding cavity for access by the steroid [Pratt 1993].

A seven amino acid sequence (547-553) of the rat GR is essential for hsp90 binding as well as steroid binding [Giannoukos *et al* 1999]. Steroid binding activity depends on a LXXLL motif at amino acids 550-554 and the LXXLL motifs have been previously described to mediate protein-protein interactions of transcriptional co-factors with steroid receptors [Heery *et al* 1997; Ding *et al* 1998]. Recently, Kaul *et al*, using GR LBD fusion proteins, demonstrated that the presence, but not the sequence of the seven-amino acid segment (position 547-553) within helix 1 of the rat GR LBD, is necessary

for LBD-hsp90 heterocomplex assembly as well as steroid binding activity [Kaul *et al* 2002]. Additional support for this came from the findings that when a GR fragment ending in this segment (547-553) is fused to the complementary retinoic acid receptor LBD fragment, the chimera is complexed with hsp90 and undergoes cytoplasmic/nuclear translocation in response to retinoic acid [Mackem *et al* 2001]. Thus, the GR LBD confers hsp90 binding to the retinoic acid receptor, which normally does not bind hsp90. After GC binding to GR, hsp90 dissociates which allows nuclear localization of the activated GR-GC complex [Truss and Beato 1993].

2.1.2.2.3 Phosphorylation

The GR-mediated transcription is regulated positively and negatively by phosphorylation. Although ligand binding is important for activation of the GR, the receptor is also subject to post-translational modification through phosphorylation [Bodwell *et al* 1998]. In the absence of hormone, the GR is phosphorylated and becomes hyperphosphorylated in the presence of agonist [Orti *et al* 1993]. Hormone-dependent phosphorylation of GR may determine target promoter specificity, cofactor interaction, receptor signalling and receptor stability.

Eight phosphorylation sites have been identified in the mouse GR (mGR), of which five are conserved in the hGR [Almlof *et al* 1995]. All five phosphorylation sites are located in the τ1 region in the N-terminal domain of the receptor (Fig. 2.2B, p15). The GR has been shown to be poly-phosphorylated on serine and threonine residues [Yudt and Cidlowski 2002]. The rat GR (rGR) is phosphorylated at Thr171, Ser224, Ser232 and Ser246 [Krstic *et al* 1997]. Mitogen activated protein kinase (MAPK) phosphorylates the rat GR at Thr171 and Ser246 while cyclin-dependent kinase (Cdk) phosphorylates it

at Ser224 and Ser232. Cdk positively regulates GR-mediated transcription whereas MAPK negatively regulates GR-mediated transcription [Krstic *et al* 1997]. In the rGR Ser224 and Ser232 correspond to Ser203 and Ser211 in the hGR. In the absence of hormone, the level of GR phosphorylation at Ser211 is low compared to phosphorylation at Ser203 but phosphorylation of both sites increase with addition of the agonist, dexamethasone [Wang *et al* 2002]. Biochemical fractionation studies and immunofluorescence showed that the Ser203-phosphorylated form of the receptor is predominantly located in the cytoplasm, whereas Ser211–phosphorylated GR is present in the nucleus. These results suggest that differently phosphorylated receptors are located in separate subcellular compartments and may modulate distinct receptor functions [Wang *et al* 2002].

It has been reported that phosphorylation of Thr171 by glycogen synthase kinase-3 and Ser246 by c-Jun NH_2-terminal kinase (JNK) inhibits rat GR-mediated activation of transcription [Rogatsky *et al* 1998a; Rogatsky *et al* 1998b]. The molecular mechanism by which JNK negatively regulates GR-mediated signals remains unclear, but JNK phosphorylation of GR might modulate ligand-dependent nuclear-cytoplasmic shuttling of GR. Recently, Itoh *et al* showed that JNK-mediated phosphorylation of the hGR at Ser226 (corresponds to Ser246 in rGR) enhances GR nuclear export and may contribute to the inhibition of GR-mediated transcription [Itoh *et al* 2002]. However, the biological effect of GR phosphorylation remains unknown.

2.1.2.2.4 Nuclear translocation

Ligand-bound GRα is transported into the nucleus with the help of two nuclear localization (NL) signals, NL1 and NL2 [Savory *et al* 1999]. NL1 is located in the C-

terminal part of the DBD, extending into the hinge between DBD and LBD and is located between amino acid 478 and 500 [Brink *et al* 1992]. NL1 catalyzes rapid transport of the GR through the nuclear pore via the classic importin pathway. This region is conserved (100%) between the rGR and hGR [Baumann *et al* 1993]. NL2 is located in the LBD of GRα and contributes to a slower traffic via an unknown mechanism [Picard and Yamamoto 1987].

Several N-terminal GR fusion chimeras to green fluorescent protein (GFP) were constructed to investigate the importance of the LBD for intracellular GR trafficking [Kino *et al* 2001]. GFP-hGRα was transported within 12 min from the cytoplasm to the nucleus after addition of dexamethasone. GFP-hGR514, which does not contain a LBD and does not bind hsp, as well as GFP-hGRβ, which has a unique LBD and low affinity for hsp, entered the nucleus immediately. hGR514 contains only NL1 and this signal may thus be responsible for fast translocation. Therefore, it can be concluded that hGRα, hGR514 and hGRβ, through their N1 region, interact with importin α and can enter the nucleus through the nucleur pores via the classical importin pathway. The GFP-hGRαI559N mutant receptor, in which Ile was replaced by Asn at position 559, which is located close to NL1, was situated mostly in the cytoplasm. Only high dose or prolonged exposure of dexamethasone led to transport of this mutant into the nucleus in about 180 min. This mutation appears to impair the NL1 function of the receptor and change the nucleocytoplasmic shuttling activity of the mutant receptor [Kino *et al* 2001]. Another group also using a chimera of GFP (GFP-GR), showed dynamic cytoplasmic to nucleus translocation of GR upon ligand binding [Htun *et al* 1996]. Subcellular localization of the GR was also compared to the MR in living cells and

whereas unliganded GR was predominantly in the cytoplasm, the GFP-MR was distributed both in the cytoplasm and nucleus [Nishi *et al* 2001].

Although little is known about what regulates steroid receptor movement through the cytoplasm and into the nucleus, several studies suggested that movement is influenced by reversible phosphorylation. Treatment with okadaic acid, a potent Ser/Thr protein phosphatase inhibitor, results in inefficient nuclear retention of agonist-bound GR as well as the cytoplasmic "trapping" of GR which is then unable to recycle [DeFranco *et al* 1991]. More studies also indicated that phosphatase type 5 (PP5) associates with the GR-hsp90 complex, which suggests that PP5 might influence GR actions [Silverstein *et al* 1997]. In the absence of ligand, GR-GFP is localised in the cytoplasm, but treatment with dexamethasone resulted in translocation of GR-GFPs into the nucleus. In contrast, nuclear accumulation of GR-GFP was observed in the absence of GCs, when the expression of PP5 was suppressed by treatment with ISIS 15534 [Dean *et al* 2001]. The results suggest that PP5 participates in the regulation of GR shuttling between the cytoplasm and nucleus and that the GR-induced transcriptional activity observed during suppression of PP5 expression results from nuclear accumulation of GR. This form of GR is capable of binding DNA but still needs agonist for maximal transcriptional activation [Dean *et al* 2001]. Previous studies demonstrated that antidepressants could enhance GR translocation and function, possibly through activation of cAMP and downstream cAMP-dependent protein kinases. Therefore, GR function was investigated in cells treated with rolipram, a phosphodiesterase (PDE) type 4 inhibitor, which antagonises cAMP breakdown [Miller *et al* 2002]. Rolipram alone, or in combination with dexamethasone, enhanced GR function in reporter genes driven by a GRE. This enhancement of GR function by rolipram was reversed by RU486 and was associated

with reduced cytosolic GR binding, which indicated rolipram enhanced GR nuclear translocation.

Nucleocytoplasmic shuttling is predicted to be a general property of nuclear receptors and this would integrate cytoplasmic signalling events with the regulation of transcription [Doucas *et al* 2000]. Nuclear import involves recognition of a nuclear localization signal (NLS) by importins, which mediated translocation into the nucleus. The well-characterised nuclear export pathway uses Crm as a receptor for proteins that contain a leucine-rich nuclear export signal (NES) [Black *et al* 2001]. Nuclear receptors like GR, AR, TR and PR lack a leucine-rich NES and export is insensitive to leptomycin B, which indicates that nuclear export is not mediated by Crm1. Results showed that all 60 amino acids of the GR DBD are important and necessary for export [Black *et al* 2001]. Further analysis revealed that a 15 amino acid sequence between the two zinc-binding loops in the GR-DBD confers nuclear export to a GFP reporter protein. NLS-mediated import and DBD-mediated export was proposed to form a shuttling cycle, which integrates the compartmentalization and activity of nuclear receptors.

2.1.2.2.5 Dimerization and DNA binding

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes known as hormone response elements (HREs). After the GR is activated, it forms a dimer, which binds to DNA at consensus sites called GREs in the promoter region of target genes [Adcock 2000]. This interaction leads to activation or repression of these genes.

The rGR was shown to interact in solution as a dimer and this interaction is delimited to a novel interface within the hinge region (488-527) of GR, which may be essential and sufficient for direct protein-protein contacts between receptor monomers [Savory *et al* 2001]. Using GR homodimers isolated from rat liver cytosol it was shown that dimerization of the GR involves both coiled-coil dimerization interfaces and the conserved heptad repeats of leucine [Jaya *et al* 2001]. These leucine-rich regions of proteins, called leucine zippers, are heptad repeats which contain leucine at every seventh amino acid residue and were proposed as possible sites of protein dimerization [Landshulz *et al* 1988]. Receptor dimerization is mediated partly through the LBD.

The functional significance of the GR dimer interface was confirmed by mutagenesis studies [Bledsoe *et al* 2002]. Mutations in the dimer interface, mutant I628A and P625A, decrease the transactivation potential of the GR LBD but have different effects on transrepression. The P625A GR is inactive in repression and defective in nuclear translocation upon ligand binding, whereas the I628A mutant has normal effects on transrepression as well as nuclear localization. The I628A mutation decreases the LBD-LBD dimerization affinity. The effects seen with the I628A mutant correspond to those observed with the mouse GR^{dim} mutant, which showed that a defect in DBD dimerization results in GR activation function loss but has no affect on transrepression activity [Reichardt *et al* 1998]. Therefore, a mutation in the dimer interface can selectively decrease the potency for transactivation by the GR LBD.

The consensus sequence for GRE binding is a palindrome of the 15 base pair (bp) sequence GGTACAnnnTGTTCT (n can be any nucleotide). The nGRE that leads to repression of transcription has a more variable sequence (ATYACnnTnTGATCn)

[Truss and Beato 1993]. The GREs that exhibit the highest affinity for GR are imperfect palindromes separated by 3 bp, although many regulatory elements only contain one half of the palindrome. The GR affinity for these half-GREs depends on the dimerization status as well as on the GR interaction with other sequences. Optimal binding, however, is seen with homodimers [Truss and Beato 1993]. The number of GREs as well as their position relative to the transcription start site may be important in determining the magnitude of the transcriptional response to GCs [Jantzen *et al* 1987; Wieland *et al* 1990]. An increase in the number of GREs and their location relative to the TATA box appears to increase the GC inducibility of a gene. However, other transcription factors binding in the vicinity of the GRE may also influence the GC inducibility.

DNA binding domain structure models of alternatively spliced or mutated GR variants were constructed by QUANTA-and MODELLER based modelling, using the crystallographic analysis of the GR DBD as template [Wickert and Selbig 2002]. Receptor function was based on the preservation or destruction of secondary structure. The results showed that amino acid insertions of one, four or nine residues of existing receptor splice variants did not have any effect on the secondary structure. In contrast, a deletion of four amino acids at the splice site junction leads to changes in the secondary structure of the DNA-recognition helix, which disturbs the receptor-DNA interaction. Comparison of receptor function based on the molecular models, with experimental results from *in vitro* studies, revealed that the two are closely related.

2.1.2.2.6 Coactivators

The transcriptional activation of nuclear receptors involves the participation of coactivators and corepressors, which function as amplifiers for receptor-mediated transcriptional activation and repression, respectively. Coactivators and corepressors interact with nuclear hormone receptors in a ligand dependent manner. In the hGRα two domains have been found to be important for optimal transactivation of target genes [Hollenberg *et al* 1988; DeRijk *et al* 2002]. The first one τ1 (or AF1 domain), is hormone independent and located in the N-terminal part of the receptor, between amino acid 77 and 262. The τ2 (or AF2 domain), also has transactivation properties and is located in the LBD in the C-terminal region of the receptor. Hormone binding is necessary for the activity of τ2. Binding of hGRα to GREs leads to the initiation of transcription through a very complex mechanism involving the basic transcription machinery, consisting of RNA polymerase II and transcription factors such as TBP, which have to be recruited to the promoter [Beato and Sachez-Pacheco 1996a]. The transcriptional activity of the hGRα is dependent on coactivators that facilitate the recruitment of the basal transcription machinery or remodel chromatin [Jenkins *et al* 2001].

Coactivators can be divided into two classes, namely members of the family of switch/sucrose non-fermentable (SWI/SNF) proteins and members of the histone acetyltransferase (HAT) family (summarised in table 2.2) [reviewed by Collingwood *et al* 1999]. Both classes of coactivators are able to modify the chromatin environment, thus facilitating transcription indirectly by alleviating the repressive effects of histone-DNA contacts. They can also influence the activity of the basal transcriptional machinery directly through protein-protein contacts. The SWI/SNF proteins were first

Table 2.2. Nuclear receptor cofactors (taken from Collingwood *et al* 1999).

Protein	**Nuclear receptor target** ***in vitro***	**Function**
Coactivators		
SRC-1, NCoA-1	ER, GR, PR, TR, RXR	Acetyltransferase Interacts with CBP
TIF2, GRIP1, NCoA2	ER, AR, GR, PR	Interacts with CBP
P/CIP, ACTR, RAC3	ER, PR, TR, RAR	Acetyltransferase
AIB1, TRAM1	RXR, VDR	Interacts with CBP, PCAF
PCAF	RAR, RXR	Acetyltransferase Interacts with p300/CBP
p300/CBP	ER, TR, RAR, RXR	Acetyltransferase Interacts with SRC-1/PCAF
Corepressors		
NCoR, SMRT	TR, RAR, RXR ER + hormone antagonist PR + hormone antagonist	Interacts with SIN3 to recruit histone deacetylase
TIF1α, TIF1β	RAR	Interacts with HP1 and histone deacetylase

characterized in yeast. Ligand-dependent transcriptional activation by the GR in yeast is facilitated by several SWI gene products which are all part of a large SWI/SNF

chromatin remodelling complex [Yoshinaga *et al* 1992]. Histone acetyltransferase coactivators were identified by their interaction with the LBD of nuclear receptors in the presence of receptor ligands and their HAT activity [Spencer *et al* 1997]. One group of HAT coactivators is the p160 family of which multiple variants have been isolated. Their structural features are represented by SRC-1a. Three distinct members of the p160 family have been isolated which exist as multiple splice variants and include SRC/NCoA-1, TIF2/GRIP1/NCoA-2 and p/CIP/ACTR/RAC3/AIB1/TRAM-1. Coactivators formed by CREB-binding protein (CBP) and p300 were shown to interact directly with τ1 and indirectly with τ2 of the hGRα [Jenkins *et al* 2001]. The coactivators have HAT activity, allowing them to acetylate histones in the promoter, which enhances transcription. CBP/p300 can be recruited to GR and other nuclear receptors directly through AF1, or indirectly through coactivators interacting with AF2 (example the p160 coactivator family) [Jenkins *et al* 2001]. Analysis of coactivators revealed the presence of multiple highly conserved LXXLL helical motifs [Heery *et al* 1997]. These motifs are important for mediating the interaction between coactivators and receptors by associating with critical residues in a coactivator interface region of the receptor LBD. Mutation of these residues leads to abrogation of coactivator recruitment and transactivation [Collingwood *et al* 1997].

Only a few coactivators have been investigated for GR interaction and include the SWI/SNF complex, the p300/CBP-associated factor (P/CAF) complex, CBP/p300, the p160 coactivators and components of the vitamin D3 receptor-interacting protein (DRIP) complex [Jenkins *et al* 2001]. Components of the SWI/SNF complex interact directly with the GR AF-1, and were shown to potentiate GR activity in yeast and mammalian cells and *in vitro*. Members of the P/CAF complex were shown to be

important for GR-dependent transcriptional activation in yeast and mammalian cells. The SWI/SNF complex and the P/CAF complex interact with AF1 of the GR. Recently, the coactivator, SRC-1, was shown to be downregulated by dexamethasone *in vivo* and *in vitro,* indicating that ligand-mediated downregulation of SRC-1 plays an important role in GC action [Kurihara *et al* 2002]. However, dexamethasone had no effect on the other coactivactors tested. The AF2 transactivation domain in GR is the only domain needed for hepatic PEPCK gene expression and mutation of this domain disrupts the direct interaction of GR with SRC-1 [Kucera *et al* 2002]. TIF2 was shown to interact directly with the GR in a hormone dependent manner and requires the receptor interaction domains of TIF2 [He *et al* 2002].

2.1.2.2.7 Corepressors

Coactivators facilitate activation of transcription by ligand-bound receptors whereas corepressors repress transcription depending on the presence of specific receptors in the absence of ligand (summarised in table 2.2) [reviewed by Collingwood *et al* 1999]. Many components of chromatin can non-specifically repress transcription but corepressors are important in targeting the receptor. Two classes of corepressors have been identified namely NCoR and the silence mediator of retinoic and thyroid receptors (SMRT) [Chen *et al* 1995]. Corepressors, like coactivators, appear to exert important functions through the modification of chromatin. Both classes of corepressors recruit histone deacetylase to the receptor. The NCoR and SMRT corepressors interact with the unliganded TR and RAR when associated with the retinoic X receptor (RXR) [Chen *et al* 1995]. A region called the CoR box, in the hinge between the receptor DBD and the LBD, has been identified as important for association with corepressors [Horlein *et al* 1995]. The TIF1α and β family of corerepressors were identified by the ability of

TIF1α to interact with the LBD of the RAR in the presence of hormone [Le Douarin *et al* 1996]. The GR antagonist, RU486 promotes interaction between the GR and the receptor interacting domains of NcoR [Schulz *et al* 2002]. Ligand independent interactions between the GR N-terminus and NCoR were identified and dexamethasone and RU486 were found to recruit NCoR to the GR C terminus. Multiple NCoR motifs were identified as being capable of interacting with the GR [Schulz *et al* 2002]. Substitution of Tyr735 in the human GR selectively impairs GC transactivation but not transrepression, via reducing interaction with SRC-1, both basally and in response to agonist binding. Recently, one of the three nuclear receptor interacting domains (NCoR-N1) of NcoR was found to interact with the GR C terminus in an RU486-specific manner [Stevens *et al* 2003]. Substitution of Tyr735phe, Tyr735val and Tyr735ser, which impairs SRC-1 interaction, enhanced NCoR-N1 recruitment, basally and with RU486. As SRC-1 and NCoR are predicted to recognize a common hydrophobic cleft in the GR, it may be that changes favorable to one interaction are not favourable to the other one, thus identifying a molecular switch [Stevens *et al* 2003].

2.1.2.2.8 Chromatin remodeling

Steroid-induced transcription of eukaryotic genes is tightly controlled and a critical regulatory mechanism is the organization of the gene into chromatin [reviewed by Collingwood *et al* 1999]. In the nucleus, DNA is wrapped around histone proteins, forming chromatin. Highly compact regions of chromatin are associated with low transcriptional activity, whereas less compact regions show higher transcriptional activity [Elgin 1988]. Histone modification and HAT activity might be required to disrupt chromatin or act subsequently to chromatin disruption [Aranda and Pascual 2001]. HAT activity is discussed in more detail under coactivators. DNA packaged as

chromatin is resistant to nuclease attack and transcription factors have limited access to their binding sites [Wolffe and Heyes 1999]. Therefore, the chromatin structure of promoters is an obstacle to transcription that steroid hormone receptors must overcome. Cells contain multiple distinct chromatin remodeling complexes, of which each have a central ATPase subunit. The first complex characterized was the yeast SWI/SNF complex, which contains 11 known subunits [Cairns *et al* 1994]. In the mammalian analogue, named SWI2/SNF2, two distinct ATPase subunits have been identified, termed hBRM and BRG1 [Muchardt and Yaniv 1993]. These chromatin remodeling complexes have been shown to enhance transcriptional activity by the GR in some genes [Muchardt and Yaniv 1993].

The GR has been used as a model to study the effect of chromatin on steroid induced transcription [Wallberg *et al* 2000]. The MMTV promotor provided general information on the mechanism of GR-mediated transcription from a chromatin template [Deroo and Archer 2001b]. In this promoter, chromatin remodeling by the GR is associated with activation of transcription. When integrated in cellular chromosomes, the MMTV promoter adopts a specific chromatin organization which consists of six positioned nucleosome families called nucleosome region A (Nuc-A) to Nuc-F [Fragoso *et al* 1995]. Activation of the MMTV promoter by the GR is associated with a chromatin structural transition in Nuc-B of the long terminal repeat. It was later shown that this transition extends upstream of the B nucleosome, which includes a region larger than a single nucleosome (LTR). Results also showed that GR-dependent chromatin remodeling is a multistep process [Fletcher *et al* 2000]. In the absence of ATP, GR binds to multiple sites on the chromatin array and prevents restriction enzyme access to recognition sites. Upon addition of ATP, the GR induces remodeling and an increase in

access to enzyme sites in the transition region. These results suggest a model in which the GR binds to chromatin after ligand activation, recruits remodeling activity and is then lost from the template. This model corresponds to the "hit and run" mechanism for GR action in living cells which suggests that the receptor undergoes constant and rapid exchange with HREs in the presence of ligand [McNally *et al* 2000]. Recently, Fletcher *et al* further showed that human SWI/SNF was recruited to MMTV chromatin by GR and that the GR was actively displaced from the chromatin template during the remodeling process but still participated in binding of the secondary transcription factor, nuclear factor 1 [Fletcher *et al* 2002]. These results fit in with the model (hit and run) where GR first binds to chromatin after ligand activation, recruits a remodeling activity, facilitates transcription factor binding and is lost from the template.

The SWI/SNF complex has been shown to alter nucleosome conformation in an ATP-dependent way, which leads to increased accessibility of nucleosomal DNA to transcription factors. In yeast and mammalian cells this complex potentiates the activity of the GR through the N-terminal transactivation domain, τ1 [Wallberg *et al* 2000]. GR-τ1 can directly interact with the SWI/SNF complex and τ1 mutations affect the transactivation activity *in vivo* as well as affecting τ1 interaction with SWI/SNF. The SWI/SNF can also stimulate τ1-driven transcription from chromatin templates *in vitro*. These findings suggested that the GR could directly recruit the SWI/SNF complex to target promoters during gene activation by GCs. Using domain mutations of the GR, the domains required for activation of the MMTV promoter were divided into two nucleoprotein configurations [Keeton *et al* 2002]. For activation of a stable template, both transcriptional domains of the GR (τ1 and the LBD τ2) are required, but for activation of the transient template, only the LBD is needed. Two cDNA clones were

isolated, hOsa1 and hOsa2, which encode the larger subunits of human SWI/SNF [Inoue *et al* 2002]. In mammalian cells, hOsa1 and hOsa2 stimulate transcription by the GR, ER and AR, which suggest that they participate in promoting transcriptional activation by the steroid hormone receptors.

The effect of GCs on the *in vivo* chromatin structure of the GC-responsive IκBα gene promoter was also investigated. In the absence of GCs the promoter assembles into regularly positioned nucleosomes, which does not change in the presence of GCs. However, GCs may be required for transcription factor binding [Deroo and Archer 2001a]. IκBα is an inhibitor of the transcription factor NF-κB, which regulates many immune system genes. GCs inhibit NF-κB activity in some tissues by increasing IκBα. IκBα appears preset for transcription and requires GCs to activate transcription but not to remodel chromatin. The GR-mediated activation of the IκBα promoter may be a novel mechanism by which steroid hormone receptors stimulate gene expression from single copy genes within chromosomes.

2.1.3 Physiological role of GCs

GCs are synthesized in the zonae fasciculata/reticularis of the adrenal cortex. They are released into the circulation in response to a wide variety of stimuli for example: pain, starvation, surgery, trauma, stress, extreme heat or cold, inflammation and cellular damage [McKay and Cidlowski 1999 & Newton 2000]. The release of GCs is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases CRH and arginine vasopressin (AVP), which acts on the anterior pituitary to induce synthesis and release of adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to release GCs. GCs are transported in plasma bound to CBG with high affinity

and to albumin with low affinity [Hammond 1990a & Hammond *et al* 1991]. Once in the blood, GCs are transported to target organs where they have numerous physiological effects.

GCs have a wide variety of functions in the body and affect many physiological systems such as metabolism, immune function, bone density, the cardiovascular system, behaviour and the HPA axis itself. The metabolic effects of GCs include the increase in blood glucose levels, stimulation of gluconeogenesis in the liver and the mobilisation of amino and fatty acids [Rosmond *et al* 1998] (Fig. 2.5). In addition, GCs have an important effect on the immune system, as they are potent suppressors of the immune response and inflammation. They are known to suppress the expression of proinflammatory cytokines, which are the key regulators of the immune response [De Kloet *et al* 1994]. Synthetic GCs are widely used in the treatment of a broad range of autoimmune and inflammatory disorders such as rheumatoid arthritis and asthma. They are also used during organ transplants to suppress the immune system and to prevent organ rejection [De Kloet *et al* 1994]. Although GCs are frequently used neonatally for the prevention of chronic lung disease in premature infants, concern has arisen about the long-term consequences of these treatments as animal research has shown that neonatal GC treatment enhances susceptibility to autoimmune diseases in adult life [Bakker *et al* 2001].

Certain cardiovascular diseases like arteriosclerosis are considered to be the result of a chronic inflammatory process. Evidence that repeated episodes of acute stress or chronic physiological stress can induce a chronic inflammatory process have recently been reviewed [Black and Garbutt 2002]. Stress activates the sympathetic nervous

system, the HPA axis and the renin-angiotensin system, which causes the release of various stress hormones like catecholamines, GCs, glucagons, growth hormones, rennin and high levels of homocysteine. These hormones induce a heightened condition of cardiovascular activity, injured endothelium and induction of adhesion molecules on endothelial cells to which inflammatory cells adhere and translocate to the arterial wall.

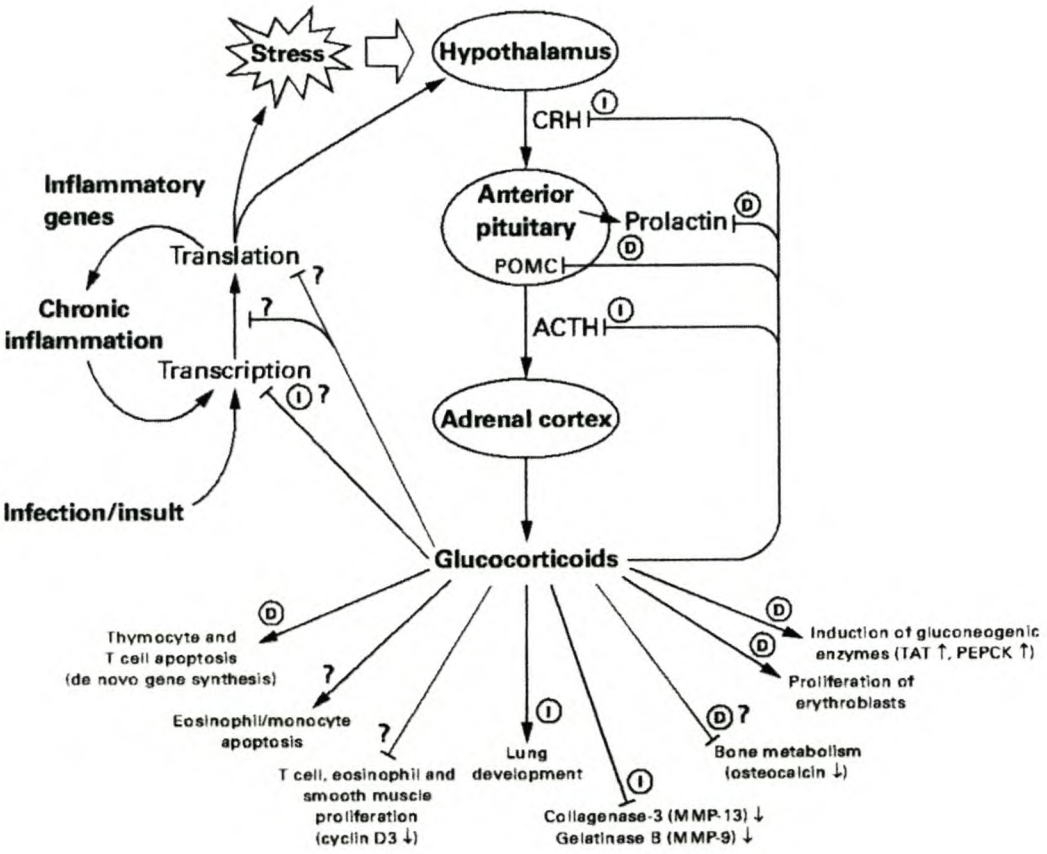


Figure 2.5. Effects of GCs on the HPA axis. This scheme illustrates the sites of synthesis and action of the main HPA hormones and the targets of GC action. Based on analysis of dimerization defective mice, most of the effects of GCs are labelled as dependent on (D) or independent of (I) GR DNA binding. Question marks indicate those whose mechanism of action is uncertain [taken from Newton 2000].

An acute phase response (APR), characterised by macrophage and mast cell activation and production of cytokines, other inflammatory mediators, and acute phase proteins (APPs), is turned on which promotes the inflammatory process. The APR is defined as

the body's response to any type of tissue damage and infection which causes a series of physiological reactions to repair the damage, contain the organisms, promote wound healing or recruit host defence mechanisms. The APPs are induced in the liver and may be positive (upregulated) or negative (down regulated) in response to injury or infection. The positive APPs are important during the inflammatory process while the negative APPs are important carrier proteins like albumin and CBG. Cytokines are the main inducers of the APR whereas GCs and catecholamines, the major stress mediators, can enhance the induction to a greater or lesser extent [Baumann and Gauldie 1994]. GCs can also directly stimulate the expression of most of the APPs.

2.1.4 Dissociative GCs

GCs are widely used for the suppression of inflammation in chronic inflammatory diseases like asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases [Barnes 1998]. All of these inflammatory diseases are associated with an increase in the expression of inflammatory genes that can effectively be suppressed by GCs. A large number of side-effects such as diabetes, weight gain, hypertension and osteoporosis are, however, associated with the long-term use of GCs [reviewed by Schacke *et al* 2002a and summarized in table 2.3]. This has led to a search for novel GCs with improved specificity for anti-inflammatory activity that dissociate between transrepression and transactivation because many of the side effects of conventional GCs may be attributed to transactivation [Adcock 2000, Dumont *et al* 1998, Cato and Wade 1996].

According to the ability of GCs to induce or inhibit certain functions of the GR they may be divided into three classes namely agonists and type I and type II antagonists. Agonists induce AF-2 in the LBD with varying efficiency [Garcia *et al* 1992].

Table 2.3. Summary of typical GC side-effects with the affected organs [taken from Schacke *et al* 2002a].

Skin	Atrophy, stria rubrae distensae
	Delayed wound healing
	Steroid acne, perioral dermatitis
	Erythema, teleangiectasia, petechia, hypertrichosis
Skeleton and muscle	Muscle atrophy/myopathy
	Osteoporosis
	Bone necrosis
Eye	Glaucoma
	Cataract
CNS	Disturbances in mood, behavior, memory and cognition
	"steroid psychosis", steroid dependence
	Cerebral atrophy
Electrolytes, metabolism, endocrine system	Cushing's syndrome
	Diabetes mellitus
	Adrenal atrophy
	Growth retardation
	Hypogonadism, delayed puberty
	Increased Na^+ retention and K^+ excretion
Cardiovasculer system	Hypertension
	Dyslipidemia
	Thrombosis
	Vasculitis
Immune system	Increased risk of infection (e.g. *Candida*)
	Re-activation of latent viruses (e.g. CMV)
Gastrointestinal	Peptic ulcer
	Gastrointestinal bleeding
	Pancreatitis

In contrast in the presence of a type I antagonist, for example RU486, GR AF-2 will be inactive. These antagonists, however, do not impair the activity of AF-1 located in the N-terminal region of the GR nor do they affect DNA-binding of the liganded receptor and therefore may act as cell-specific partial agonists [Garcia *et al* 1992]. Type II antagonists, like RU43044, on the other hand are able to impair DNA binding of GR and do not induce transcriptional activity of GR target genes [Bocquel *et al* 1993].

A fourth, novel, class of GR ligands have come to the fore lately. These are called dissociated GCs or selective GR agonists (SEGRAs) [Schacke *et al* 2002b]. Some 'dissociated GCs' that transrepress but do not transactivate have been described [Vayssierre *et al* 1997, Vanden Berghe *et al* 1999] and it has been suggested that dissociation may be due to inhibition of GR dimerization [Reichart *et al* 1998, Heck *et al* 1994]. The proposed molecular mechanism of action of dissociative GCs compared to full agonist is illustrated in Fig. 2.6. The dissociated GCs that have been described include the synthetic GC, RU24858 (Fig. 2.7), that transrepresses AP-1 activity in HeLA cells but only weakly activates GRE-driven promoters [Vayssierre *et al* 1997]. *In vivo* RU24858 also exhibits anti-inflammatory activity. However transactivation still occurred with the same potency as for a standard steroid [Belvisi *et al* 2001]. This suggests that the *in vitro* dissociation of transrepression from transactivation activity does not necessarily mean an increased theurapeutic ratio for GCs *in vivo* or alternatively that some of the adverse side effects of GCs may also be due to transrepression.

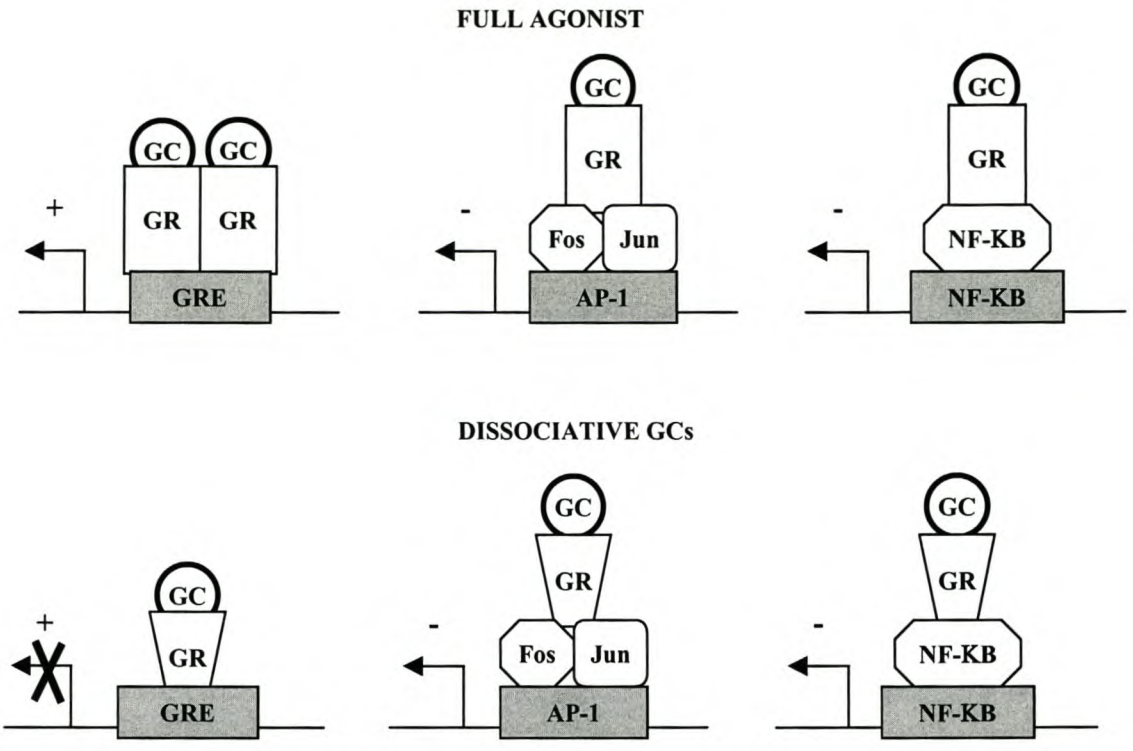


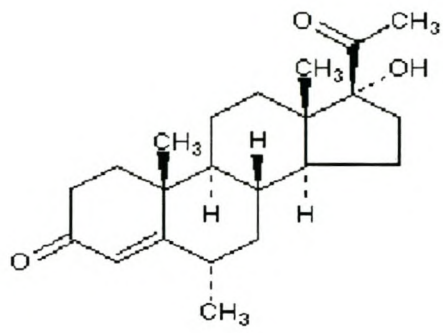
Figure 2.6. The proposed mechanism of action of dissociated GCs compared to full GR agonists.

Miner *et al* reported a selective, non-steroidal GR antagonist, AL082DO6 (DO6), which binds to the GR but not to the MR, AR, ER and PR [Miner *et al* 2003]. This compound inhibits GC-mediated transcriptional regulation, partially blocks GR translocation to the nucleus and completely blocks DNA binding by the receptor. Another non-steroidal GR ligand, A276575, with high affinity for the GR was described by the same group. A276575 repressed IL-1 induced IL-6 production and antagonised transactivation of a MMTV GRE reporter construct [Lin *et al* 2002]. Another non-steroidal GR ligand, AL-438 (Fig. 2.7), was able to selectively repress and activate genes normally regulated by GCs [Coghlan *et al* 2003]. In addition, this compound was also tested *in vivo* and retains full anti-inflammatory efficacy and potency compared to steroids, but has less negative effects on bone metabolism and glucose control. Schacke *et al* also reported that non-steroidal ligands that bind to the GR showed a good separation between transactivation

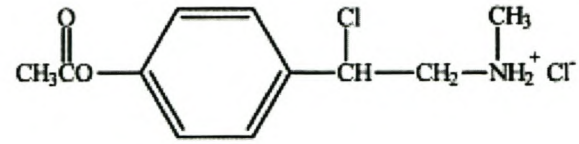
of a TAT GRE reporter construct and transrepression of IL-8 protein [Schacke *et al* 2002b].

MPA (Fig. 2.7), an injectable contraceptive with GR agonist activity [Kontula *et al* 1983], has recently been shown to exert dissociative GC activity in normal human lymphocytes [Bamberger *et al* 1999]. MPA treatment caused pronounced transrepression of IL-2 gene expression whereas MPA-mediated transactivation of a dexamethasone inducible GRE-dependent promoter was marginal. MPA displayed a transrepression/transactivation ratio of 6.6 relative to dexamethasone (ratio of 1) indicating that it possessed significant dissociated properties [Bamberger and Schulte 2000]. MPA is also used in the treatment of metastatic endometrial, breast and renal cancer but few studies have looked at the effect of MPA on the immune response. A reduction in T-cell numbers in female breast cancer patients who received high doses of MPA treatment was observed [Mallmann *et al* 1990]. It was also shown that MPA inhibits lymphocyte proliferation [Kontula *et al* 1983].

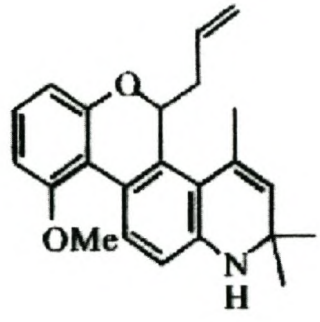
Another compound reported to exert dissociative properties is a phenyl aziridine precursor, Compound A (Fig. 2.7) [De Bosscher unpublished results]. Compound A (CpdA) is a more stable chemical analogue of the active compound in the shrub, *Salsola tuberculatiformis* Botsch. The scrub is a a medicinal plant which has traditionally been used by the Kalahari Bushmen for contraceptive purposes, is contraceptive in rats, and causes prolonged gestation in sheep [Basson *et al* 1969]. The active fraction from the shrub contains a highly labile hydroxyphenyl aziridine or precursor. The stable analogue, CpdA, also cyclizes to the corresponding aziridine under physiological



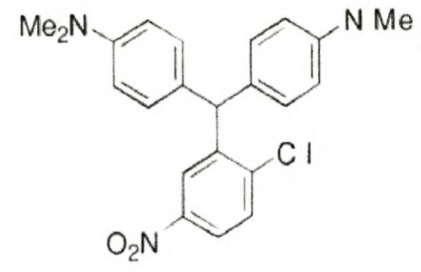
MPA



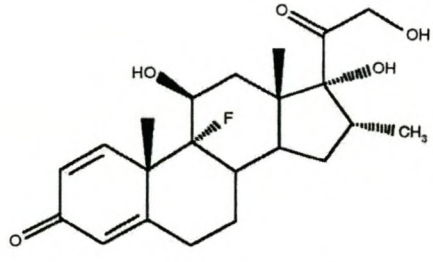
CpdA



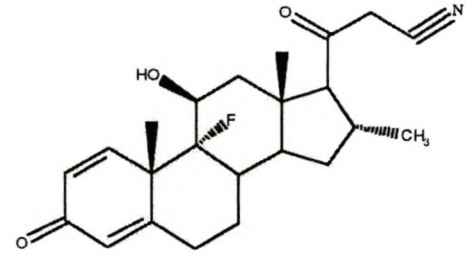
AL-438



DO6



DEXAMETHASONE



RU 24858

Figure 2.7. Structures of MPA, CpdA, AL-438, DO6, dexamethasone and RU24858 [taken from Vayssiere *et al* 1997; Coghlan *et al* 2003 and Miner *et al* 2003].

conditions. However, in plasma, binding to CBG stabilized the precursor. CpdA and the active fraction from the shrub, inhibits P450c11, the enzyme responsible for the final step in the synthesis of corticosterone and cortisol [Louw *et al* 1997]. In addition, CpdA

has contraceptive properties in rats by blocking the estrus cycle of these rats, and also decreases CBG levels and increases free corticosterone levels in rats [Louw and Swart 1999]. CpdA also decreased ACTH and luteinizing hormone (LH) levels in rats. CpdA repressed androgen-induced activation of both specific and non-specific androgen response elements but does not compete with androgen for binding to the AR [Tanner *et al* 2003]. This compound was shown to exert anti-inflammatory potential by down modulating TNF-induced IL-6 gene expression, but it did not activate GRE-driven promoters [De Bosscher unpublished results]. CpdA is therefore a potential non-steroidal anti-inflammatory agent.

The search for dissociated GCs has up to now focused on the transrepressive effects on NF-κB and AP-1 mediated gene regulation [Dumont *et al* 1998; Adcock 2000; Vayssierre *et al* 1997; Vanden Berghe *et al* 1999; Bamberger *et al* 1999]. The regulation of CBG function, as a key modulator of GC action may, however, also be important for full evaluation of any such drugs.

2.2 Corticosteroid binding globulin (CBG)

2.2.1 Function of CBG

In plasma GCs are bound to CBG, which when released translocate across the plasma membrane and exert their effects by binding to a cytoplasmic GR. CBG is a steroid-binding protein that binds steroid hormones, primarily GCs and progesterone, with high affinity [Hammond 1990a & Hammond *et al* 1991]. The steroid-binding protein, albumin binds these steroid hormones with low affinity but high capacity. Current theory holds that plasma steroid-binding proteins transport steroid hormones in the blood and regulate their access to target tissues [Sandberg *et al* 1966]. Only the

unbound fraction of steroid hormones can enter target cells. Thus, steroid hormones exist in two states in plasma, namely free and reversibly bound to proteins. The free, unbound hormone is the biologically active fraction, which regulates the bio-availability and metabolic clearance of GCs [Westphal 1986; Mendel 1989]. Binding to CBG has been shown to affect the metabolic clearance rate of steroid hormones and thereby protects steroid hormones from rapid metabolism [Siiteri *et al* 1982].

CBG identified in various cell types (Table 2.4), can arrive at these cells by active cellular uptake over the cell membrane or by extra-hepatic synthesis. CBG is taken up by cells, by binding of the CBG-GC complex to a membrane CBG receptor, which transports the CBG-GC complex over the membrane or it is internalized by endocytosis. Inside the cell, CBG can be cleaved to release GCs causing an increase in the amount of free GCs available to activate intracellular receptors. CBG binding sites or likely receptors have been identified in the human liver, decidual endometrium, placental syncytiotrophoblast and prostate, rat spleen and liver and rhesus monkey kidney, spleen, liver and uterus (Table 2.4) [Hryb *et al* 1986; Singer *et al* 1988; Strel'chyonok and Avvakumov 1991; Maitra *et al* 1993]. Internalization of CBG has been detected in human breast cancer and rat and human hepatoma cells [Kuhn 1988; Nakhla *et al* 1988; Khan *et al* 1984]. It is interesting to note that all reported sites of CBG receptors correlate with sites where CBG has been reported to occur intracellularly lending authority to the suggestion that the CBG receptor may be involved in cellular uptake of CBG (Table 2.4). There is also proof that CBG, bound to its membrane receptor, can activate intracellular second-messenger systems resulting in increased cAMP within the cell. These studies were done in a breast cancer cell-line, MCF7, as well as in

trophoblast cells from human embryos [Nakhla *et al* 1988; Strel'chyonok and Avvakumov 1991].

Comparison of the human CBG with other protein sequences revealed that it was structurally unrelated to any other steroid-binding proteins but shared remarkable sequence similarity with several members of the serine protease inhibitor (SERPIN) gene family [Hammond *et al* 1987]. These proteins may have arisen from a common ancestral gene by a process of gene duplication [Underhill and Hammond 1989]. α1-proteinase inhibitor (α1-PI), α1-antichymotrypsin (α1-ACT) and thyroxin binding globulin (TBG) are the SERPINs the closest related to CBG. The members of the SERPIN family contain a functional domain, which interacts with a specific serine protease. The target enzymes for α1-PI and α1-ACT are elastase and cathepsin G, respectively, and both are produced by activated neutrophils. Therefore, it was predicted that CBG might act as a substrate for serine proteases at sites of inflammation. Neutrophil elastase indeed does cleave the CBG molecule at a position, which is closely related to the elastase binding site on α1-PI [Pemberton *et al* 1988]. This results in a dramatic decrease in CBG steroid binding activity. Elastase cleavage of CBG occurs on the surface of activated neutrophils from patients with acute inflammatory diseases like sepsis [Hammond *et al* 1990b]. This may be an important role for CBG in the delivery of GCs to the site of inflammation. The formation of free radicals by activated neutrophils may oxidize an important methionine in the active site of α1-PI, which will destroy its capability to inhibit neutrophil elastase. The elastases would then attack CBG, which results in the release of GCs directly to the inflammatory cells. Thus in addition to its function as transporter of steroid hormones CBG may also play a more

active role in delivering GCs to specific cells via its membrane receptors or at sites of inflammation via elastase cleavage.

2.2.2 Structural analysis

CBG is a monomeric glycoprotein with a molecular weight of 50-60 kDa [Strel'chyonok and Avvakumov 1990; Rosner 1991]. The polypeptide consists of 383 amino acids and contains only one steroid-binding site per molecule [Hammond *et al* 1987]. The amino acid composition of CBG indicates that the molecule is rich in methionine and contains a few cysteine residues [Westphal 1986]. It also contains an abundance of negatively charged amino acids that account in part for its acidic isoelectric point (pI) and may be accentuated by the presence of carbohydrates. A consensus sequence for N-glycosylation was identified at residue 9 (Asn) in the sequence of the mature protein. The protein contains six consensus sequences for the attachment of sugar chains of which five are used for glycosylation [Strel'chyonok and Avvakumov 1990]. The presence of carbohydrates influences the half-life of plasma proteins and removal of sialic acid residues by neuramidase clearly increases the clearance of CBG by the asialo-glycoprotein receptor of the liver. In addition, variations in carbohydrate composition may modify the interaction between CBG and target cell membranes, and may thereby influence any biological response [Avvakumov and Strel'chyonok1988].

2.2.3 CBG synthesis

The liver is the major organ involved in the synthesis of CBG and more than 90% of circulating GCs are bound to CBG in humans and rats [Dunn *et al* 1981]. Although the liver is the major site of CBG synthesis, other cells have also been reported to synthesize (extra-hepatic) CBG (Table 2.4). Extra-hepatic synthesis of CBG has been

Table 2.4. Presence of CBG and CBG receptors in organs, tissue and cell lines.

	CBG		RECEPTORS	
Organs, Tissue or cell lines	**Species**	**References**	**Species**	**References**
Brain	human rat	Kuhn 1988; Turner 1986		
Breast (normal, neoplastic, MCF7 cells)	human	Amaral and Werthamer 1976		
Heart	rat	Turner *et al* 1986		
Kidney	human rat	Siiteri *et al* 1982; Kuhn 1988; Seralini 1996; Turner 1986; Hammond 1990a; Feldman *et al* 1973	rhesus monkey	Strel'chyonok and Avvakumov 1991; Maitra *et al* 1993
Liver	human rat guinea pig	Siiteri *et al* 1982; Kuhn 1988; Seralini 1996; Turner 1986; Hammond 1990a; Perrot-Applanat *et al* 1981	human rat rhesus monkey	Strel'chyonok and Avvakumov 1991; Maitra *et al* 1993
Lung	human rat	Siiteri *et al* 1982; Kuhn 1988; Seralini 1996; Hammond 1990a		
Lymphocytes	human	Kuhn 1988; Werthamer *et al* 1973		
Muscle	human	Siiteri *et al* 1982; Kuhn 1988		
Ovary	human pig	Seralini 1996; Misao *et al* 1999a; Misao *et al* 1999b		
Pituatary	human rat	De Kloet and McEwan 1976; Koch *et al* 1976; Perrot-Applanat *et al* 1984; Kuhn 1988; Siiteri *et al* 1982; Seralini 1996; Turner 1986		
Placenta (syncytiotro-phoblast	human	Seralini 1996	human	Strel'chyonok and Avvakumov 1991
Prostate			human	Hryb *et al* 1986

Uterus (endometrium & Ishikawa cells)	human rat	Siiteri *et al* 1982; Kuhn 1988; Seralini 1996; Milgrom *et al* 1970; Rosenthal *et al* 1974; Hammond 1990a; Al-Khouri *et al* 1980; Misao *et al* 1994	rhesus monkey human	Strel'chyonok and Avvakumov 1991
Spleen		Seralini 1996	rat rhesus monkey	Singer *et al* 1988
Testis	human monkey	Seralini 1996		
Thyroid	rat	Kuhn 1988		

reported in lung, ovary, endometrium, trophoblast cells, foetal pancreas, neonatal kidney and foetal pituitary [Hammond *et al* 1987; Scrocchi *et al* 1993; Berdusco *et al* 1995; Misao *et al* 1995; Misao *et al* 1999b; Seralini 1996; Benassayag *et al* 2001]. CBG mRNA and protein in the mammalian pituitary have been localized to the corticotropes [Berdusco *et al* 1995]. The localization of CBG to different tissues may be required to modulate GC action in these tissues.

2.2.4 CBG gene

2.2.4.1 CBG gene organization

The CBG gene is located on chromosome 14q32.1 [Seralini 1990]. The human and rat CBG genes (*Cbg*) have been cloned [Underhill and Hammond 1989; Underhill and Hammond 1995]. The human protein was found to be encoded by a single gene consisting of five exons (exon I-V) distributed over about 19 kb of genomic DNA [Underhill and Hammond 1988; Underhill and Hammond 1989]. The first exon spans 70 base pairs (bp) while exons II-V, span about 10.5 kb and contain the complete coding sequence for CBG. Exon II contains a 19 kb untranslated sequence, which is

followed by a coding region for 205 amino acids that includes the translation initiation site and signal peptide for the CBG precursor. The translation stop codon is located in exon V and the 3'-boundary, which is defined by the polyadenylation site. Sequences that resemble TATA and CAAT-box motifs were centred 28 bp and 73 bp, respectively, upstream of the transcription start site.

The nucleotide sequence of the 5'-flanking region of the human CBG gene was compared with the promoter region of other liver genes (mouse albumin and rat α–fetoprotein). The results showed a similarity between the promoter regions of CBG and the human and mouse albumin genes [Lichsteiner *et al* 1987]. Both contain six (A-F) highly conserved sequence elements that in the mouse albumin promoter interact with nuclear proteins for efficient, liver-specific expression. However, these binding sites occur in a different order in the human CBG promoter and sites C, F and E are repeated. Thus, the promoter regions of the albumin and CBG genes could have quite different effects on gene expression.

The CBG gene was also compared to other SERPIN genes and was found to be the closest related to the human α1-proteinase inhibitor and α1-antichymotrypsin genes. These proteins may therefore be derived from a common ancestral gene [Underhill and Hammond 1989]. The human genes encoding α1-antitrypsin (α1-AT), CBG, α1–ACT, protein C inhibitor (PCI), kallistatatin (KAL), as well as an antitrypsin related sequence (ATR) all map to chromosome 14q32.1. An approximately 370-kb cosmid contig, which includes all six SERPIN genes, has been characterised [Rollini and Fournier 1997]. These SERPIN genes are organised into two discrete subclusters of three genes each that are separated by more or less 170 kb. The distal subcluster includes KAL, PCI

and α1–ACT, occupying 63 kb of DNA while the proximal subcluster includes α1-AT, ATR and CBG, occupying 90 kb of genomic DNA. These genes are all transcribed in a distal-to-proximal orientation. The organization of the 14q32.1 gene cluster has implications for the potential regulation of SERPIN gene expression in this region. The relative proximity of SERPIN genes within each subcluster suggests that they might share regulatory elements whereas the large distance between the two subclusters may indicate that they are independently regulated [Rollini and Fournier 1997].

No consensus steroid hormone response elements seem to be present in the rat or human CBG proximal promoter, although it has been shown that estrogens increase [Westphal 1971; Coolens *et al* 1987] and GCs repress [Westphal 1971; Smith and Hammond 1992] the CBG gene, suggesting that the steroid receptors may be acting through a tethering mechanism that may not involve direct binding to the DNA. For example, the GR may repress transcription via protein-protein interactions with other transcription factors rather than by direct DNA binding [Boruk *et al* 1998; Rabek and Papaconstantinou 1999; Wang *et al* 1999]. However, transrepression via direct binding of the GR to DNA, via nGREs, cannot be excluded as nGREs have a very degenerate sequence [Malkoski and Dorin 1999; King *et al* 2002; Turney and Kovacs 2001; Subramaniam *et al* 1998; Zhang *et al* 1997; Aslam *et al* 1995]. Steroid hormone response elements may also be located further upstream or within the introns, which have not been sequenced.

DNase I footprinting of the rat CBG proximal promoter identified five protein-binding sites (P1-P5) within 236 bp immediately 5' of the transcription start site in rat liver nuclear extract [Underhill and Hammond 1995]. These five protein-binding sites are

highly conserved in the human CBG gene. They resemble recognition sequences for hepatic nuclear factor-1 (HNF-1), CCAAT-binding protein-2 (CP-2), D-site binding protein (DBP), HNF-3 and C/EBP or NF/IL6, respectively.

Various luciferase reporter constructs were constructed by Underhill and Hammond to identify cis-domains that play a role in the regulation of CBG gene expression in H4IIEC3 rat hepatoma cells [Underhill and Hammond 1995]. The ratCBG295Luc construct, which contains P1 to P5 binding sites, was the most active of the constructs tested. Deletion of the P3 to P5 sites in the ratCBG145Luc construct caused a twofold reduction in promoter activity. Also, removal of all five binding sites in ratCBG52Luc resulted in a 13-fold loss in luciferase activity, with a further loss of function with the extending of the promoter region in the ratCBG439Luc construct as well as in the ratCBG800Luc construct. The repression is partially overcome by addition of sequences in the ratCBG1200Luc construct. Thus, it was concluded that nt –295 to –52 represent a positive component of *Cbg* transcription whereas additional 5' sequences repress activity.

Electrophoretic mobility shift assays (EMSAs) confirmed that footprint one (P1) binds to HNF-1β [Underhill and Hammond 1995] and footprint two (P2) binds to CP2 [Zhao *et al* 1997]. Many other genes predominantly expressed in liver, like CBG, also contain an HNF-1 site in their proximal promoters. This includes several related serine proteinase inhibitor genes, such as α1-antitrypsin, and thyroxine-binding globulin [De Simone *et al* 1987; Tani *et al* 1994]. The CBG proximal promoter, containing only P1, in addition to the TATA and CAAT box sequences, is insufficient to activate transcription of a luciferase promoter gene. The presence of an additional 5' portion of

the promoter that includes P2 is needed for transcriptional activity. To determine whether this is due to synergy between factors binding to P1 and P2, or whether P2 is the main determinant of CBG transcription, P1 was mutated, which prevented HNF-1 from binding [Zhao *et al* 1997]. The disruption of the HNF-1 site had no effect on transcriptional activity of the CBG proximal promoter that only contains P1 and P2 but it may be important for CBG expression *in vivo* because it is perfectly conserved in the human and mouse. However, only the involvement of P1 in the context of the CBG proximal promoter was studied. Interactions between HNF-1β bound to P1 and other upstream factors may affect the overall expression of the rat CBG promoter. The fact that deletion of P2 from the CBG proximal promoter totally abolishes transcriptional activity, suggests that P2 and the CCAAT-binding protein that interact with it, may be important determinants of how under different physiological conditions the CBG gene is expressed.

2.2.4.2 CBG gene abnormalities

A few rare deficiencies of CBG have been reported in humans. Three separate mutations in the CBG gene have been described thus far (Fig. 2.8) [Van Baelen *et al* 1982; Emptoz-Bonneton *et al* 2000; Torpy *et al* 2001]. Sequence analysis of the first mutant, called transcortin-Leuven, showed several mutations within the coding sequence [Van Baelen *et al* 1982]. One of the mutations results in an amino acid substitution and is located within exon 2. It alters the codon (CTC) associated with Leu-93 in the CBG gene to a codon (CAC) for histidine in the mutant gene. The second mutation, CBG-Lyon, located in exon 5, alters the codon for residue GAC to AAC, which results in Asp^{367} substitution for Asn [Emptoz-Bonneton *et al* 2000]. The patient with this mutation had normal urinary cortisol excretion and normal circadian ACTH

variations, which indicates that the HPA-axis was unimpaired but low blood pressure was detected. Both of these mutations lead to reduced cortisol-binding affinity. The third mutant describes a novel complete loss of function (null) mutation of the CBG gene [Torpy *et al* 2001]. This novel exon 2 mutation is a single base substitution (c.121G to A), which leads to a premature termination codon at residue –12 of the pro-CBG molecule. This mutation is associated with low blood pressure and fatigue but ACTH and cortisol levels were normal.

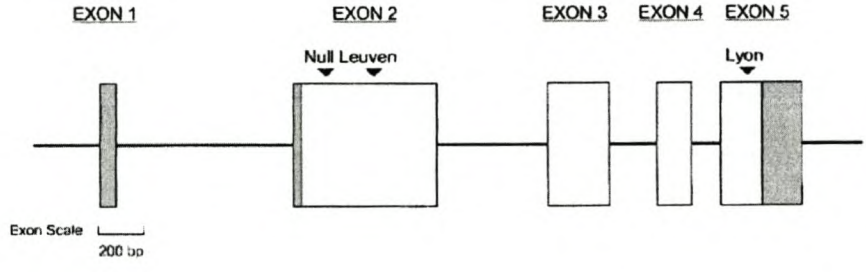


Figure 2.8. Schematic diagram of the CBG gene (24-kb region) with the three mutations described to date in humans. Darker shaded regions of the exons are untranslated. The mutations include CBG null: c.121G→A; Trp-12x, transcortin Leuven: c.433T→A; $Leu^{93}His$, and CBG Lyon: c1254G→A; $Asp^{367}Asn$ [Torpy *et al* 2001].

2.2.5 Regulation of CBG

CBG protein and RNA are regulated by numerous hormones, conditions and factors which are summarized in Table 2.5 .

Table 2.5: Summary of the hormones, conditions and factors known to regulate CBG protein and mRNA levels.

Effect	Hormone, condition or factor	Level of effect	Species	Site	References
Decrease	Cushing's syndrome	protein	human	plasma	Westphal 1971; Coolens *et al* 1987
decrease	GCs	protein	rat; human	plasma	Westphal 1971; Smith and Hammond 1992; Schlechte *et al* 1987
increase	adrenalectomy	protein	rat	plasma	Westphal 1971; Feldman *et al* 1979; Grasa *et al* 1998
decrease	stress	protein	rat	plasma	Tinnikov *et al* 1993; Tinnikov *et al* 1999; Fleshner *et al* 1995; Deak *et al* 1997; Leonhardt *et al* 2002
decrease	septic shock	protein	human	plasma	Pugeat *et al* 1989; Westphal 1986; Beishuizen *et al* 2001
decrease	burn patients	protein	human	plasma	Garrel 1996; Bernier *et al* 1998
decrease	coronary artery bypass patients; open hart surgery major elective surgery	protein	human	plasma	Roth-Isigkeit *et al* 2000; Tinnikov *et al* 1993; Tinnikov *et al* 1996 Le Roux *et al* 2003
no effect	GCs	mRNA; protein	human	HepG2 cells	Emptoz-Bonneton *et al* 1997
decrease	IL-6	mRNA; protein	human	HepG2 cells	Emptoz-Bonneton *et al* 1997; Baratlena *et al* 1993
decrease	IL-6 + GCs	mRNA; protein	human	HepG2 cells	Emptoz-Bonneton *et al* 1997
increase	GCs	protein; mRNA	foetal sheep	plasma & liver	Berdusco *et al* 1993; Berdusco *et al* 1994; Jeffray *et al* 1995
decrease	GCs	protein; mRNA	Adult pregnant sheep	plasma & liver	Berdusco *et al* 1993; Berdusco *et al* 1994
decrease	GCs	mRNA; protein	baboon foetus	plasma & liver	Pepe *et al* 1996
no effect	GCs	protein	adult pregnant baboon	plasma	Pepe *et al* 1996
no effect	GCs	protein	human foetus	plasma	Jeffray *et al* 1999
no effect	GCs	protein	adult pregnant human	plasma	Jeffray *et al* 1999

decrease	GCs	protein	human amniotic fluid	plasma	Jeffray *et al* 1999
increase	progesterone	protein	rat	plasma	Westphal 1971
no effect	progesterone	Protein; mRNA	rat	plasma & liver	Smith and Hammond 1992
increase	progesterone	mRNA	human	Luteal cells	Misao *et al* 1999a
decrease	progesterone/MPA	mRNA	human	Ishikawa cells/normal endometrium	Misao *et al* 1994; Misao *et al* 1998
increase	pregnancy	protein	rat; human	plasma	Westphal 1971; Westphal 1986; Abou-Samra *et al* 1984; Moore *et al* 1978; Potter *et al* 1987; Robinson *et al* 1985; Hau *et al* 1983
no effect	estrogen	protein; mRNA	female rat	plasma	Westphal 1971; Mataradze *et al* 1992
increase	estrogen	protein	male rat; human	plasma	Westphal 1986; Hammond 1990a; Coolens *et al* 1987; Misao 1999a; Darj *et al* 1993; Feldman *et al* 1979
increase	estrogen	mRNA	human	Ishikawa cells; ovarian corpus luteum	Misao *et al* 1998; Misao *et al* 1999a
decrease	ovariectomy	protein	female rats	plasma	Westphal 1971
decrease	testosterone	protein	female rats; castrated rats and men	plasma	Westphal 1971; Westphal 1986 Mataradze *et al* 1992
no effect	5α-dihydrotestosterone	protein	rat	plasma	Smith and Hammond 1992
increase	castration	protein	rat	plasma	Westphal 1971; Mataradze *et al* 1992; Nock *et al* 2000
increase	thyroxine	mRNA; protein	rat	plasma	Smith and Hammond 1992; D'Agostino and Henning 1982
no effect	thyroxine	protein	human	HepG2 cells	Rosner *et al* 1984
increase	hyperthyrodism	protein	human	plasma	Westphal 1986; Rosner 1991
increase	triiodothyronine	mRNA	rat foetus	hepatocyte	Seralini 1996; Elfahime *et al* 1994
decrease	triiodothyronine	mRNA; protein	human	HepG2 cells	Barlow *et al* 1994
decrease	Retinoic acid	mRNA	rat foetus	hepatocytes	Seralini 1996; Elfahime *et al* 1994
decrease	insulin & insulin-like growth factor (IGF-1)	mRNA; protein	human	HepG2 cells	Crave *et al* 1995

decrease	obesity	protein	human	plasma	Fernandez-Real *et al* 2001; Fernandez-Real *et al* 2002
decrease	liver disease	protein	human	plasma	Westphal 1971; Westphal 1986; Rosner 1991

2.2.5.1 GCs and cytokines

GCs, the main steroid transported by CBG, regulate the levels of its own transport protein in a negative feedback loop. Thus, plasma levels of CBG are suppressed during prolonged exposure to GCs, whether endogenous as in Cushing's syndrome [Westphal 1971; Coolens *et al* 1987] or exogenous as during administration of synthetic GCs [Westphal 1971; Smith and Hammond 1992; Schlecte *et al* 1987]. In contrast, the removal of endogenous GCs by adrenalectomy has been shown to increase rat plasma CBG levels [Westphal 1971; Grasa *et al* 1998; Feldman *et al* 1979]. A number of studies in rats also indicate that physiological and physical stressors down-regulate CBG production [Tinnikov *et al* 1993; Tinnikov 1999; Fleshner *et al* 1995; Deak *et al* 1997; Leonhardt *et al* 2002] while the dramatic fall of human CBG levels during stress, with concomitant substantial (2 to 20-fold) increases in free GC levels, merits its classification as a negative acute-phase protein [Pugeat *et al* 1989; Garrel 1996; Ingenbleek and Bernstein 1999]. Recent reviews also summarize the important role played by CBG during the stress reaction [Black and Garbutt 2002; Breuner and Orchinik 2002]. GCs, released during the stress response, take part in the early stages of the stress response, including the inflammatory reaction.

A dramatic decrease in serum CBG levels is associated with septic shock in humans. The decrease in the concentration of CBG during septic shock is associated with a low binding activity for cortisol in serum [Pugeat *et al* 1989; Beishuizen *et al* 2001]. Major

stress, for example in burn patients with elevated serum IL-6 concentration, also results in an decrease in CBG levels with a concomitant rise in free cortisol [Garrel 1996; Bernier *et al* 1998]. In addition, major elective surgery, which is a severe stress that occurs in a controlled environment, causes a rise in serum total cortisol and a decrease in serum CBG [Le Roux *et al* 2003]. During coronary artery bypass [Roth-Isigkeit *et al* 2000] and open-heart surgery [Tinnikov *et al* 1996] a rise in serum cortisol was also observed due to a decrease in CBG concentration. Cardiac surgery induces a systemic inflammatory response, which results in the secretion of mediators of the endocrine and immunological stress reaction with cortisol playing an important part in the endocrine stress reaction [Udelsman and Holbrook 1994]. The reason for this stress-induced decrease in CBG is not fully understood but it has been hypothesized that CBG plays a role in the delivery of cortisol to the sites of inflammation [Pugeat *et al* 1989]. This dramatic decrease in CBG levels has been associated with impaired immune function. In this regard, pro-inflammatory cytokines alone or in synergy with GCs have also been shown to decrease CBG protein and hepatic mRNA levels in HepG2 cells, a human hepatoma cell-line which secretes CBG, while GCs alone appear to have no significant effect in this cell line [Emptoz-Bonneton *et al* 1997].

The regulation of CBG by GCs alone varies in mammals. GCs increased CBG protein and mRNA in fetal sheep, while it decreased CBG protein and mRNA levels in adult pregnant sheep [Berdusco *et al* 1993a; Berdusco *et al* 1994; Jeffray *et al* 1995]. The ontogeny of CBG in primates differs from that in the ovine. In baboons, GC treatment between early and midgestation decreased fetal but not maternal CBG concentrations, which were unaffected by betamethasone treatment [Pepe *et al* 1996]. Women with preterm labour usually receive synthetic GCs, which cross the placenta and promote

fetal lung maturity. Fetal and maternal human plasma CBG levels were found to be unaffected by GCs but amniotic fluid CBG concentration decreased significantly [Jeffray *et al* 1999].

IL-6, the main mediator of the acute phase response regulates protein gene transcription, either positively or negatively [Bartalena *et al* 1992; Morrone *et al* 1988]. Its action appears to be mediated by the interaction of one or more transcription factors with IL-6 responsive elements identified in the cis-regulatory region of several acute phase protein genes. The expression of CBG, TBG, AT and ACT genes is influenced by IL-6 in very different ways although the genes for these proteins are very closely related in terms of structural organization and are also derived from a common ancestral gene. IL-6 increases α1-AT and α1-ACT synthesis and decreases T4-binding globulin synthesis in a human hepatoblastoma-derived cell-line (HepG2), predominantly at a transcriptional level [Bartalena *et al* 1993]. Hepatic CBG synthesis and CBG mRNA steady state levels are dose-and time-dependently decreased by IL-6 [Bartalena *et al* 1993]. No detectable change in the rate of CBG gene transcription was observed. Thus, IL-6 may affect CBG synthesis at a posttranscriptional, but pretranslational level. The fact that changes in CBG mRNA levels preceded by several hours the changes in secreted protein further supports the pretranslational action of IL-6. In addition, no variations in electrophoretic mobility of protein occurred in presence of IL-6, and the kinetics of CBG secretion was unaffected.

2.2.5.2 Progesterone

Progesterone, like GCs, binds to plasma CBG with high affinity, and changes in CBG concentration are also thought to modify the bio-availability of this steroid. In addition, like GCs, progesterone also regulates CBG levels. In rats results are contradictory: one study reports that rat serum CBG or hepatic CBG mRNA levels were not influenced by progesterone treatment [Smith and Hammond 1992] whereas in another study CBG protein levels in rat plasma were increased by progesterone [Westphal 1971].

High dose progestin treatment is used in the treatment of advanced or recurrent endometrial cancers as the cancers are estrogen dependent and regress in response to progestin therapy. Endometrial adenocarcinomas express a high level of steroid receptors and CBG [Ehrlich *et al* 1981; Misao *et al* 1995]. It was shown that high dose progesterone or MPA (10^{-6}M) suppresses CBG mRNA expression in a well-differentiated endometrial cancer cell-line, Ishikawa, and in normal human endometrium that contains estrogen and progesterone receptors [Misao *et al* 1994; Misao *et al* 1998]. Physiological concentrations of progesterone (10^{-8}M), however, had no effect on CBG levels. In contrast, human luteal CBG mRNA appears to be upregulated by progesterone treatment [Misao *et al* 1999a]. In addition, formation of colonies of Ishikawa cells treated with a high dose of MPA did not differ from cells treated with MPA in combination with an antiprogestin (RU486). This indicates that the anti-tumour effects of high dose progestins may not be brought about via the progesterone receptor cascade, but rather via cytotoxic effects on the tumour cells. Thus, the suppression of CBG mRNA expression might be related to the cytotoxic effects [Misao *et al* 1999a].

2.2.5.3 Estrogen and pregnancy

CBG plasma protein levels increase during pregnancy in rats and humans [Westphal 1971; Westphal 1986; Abou-Samra *et al* 1984; Moore *et al* 1978; Potter *et al* 1987; Robinson *et al* 1985; Hau *et al* 1983] or after administration of natural or synthetic estrogens in humans [Westphal 1971; Westphal 1986; Hammond 1990a; Coolens *et al* 1987; Misao *et al* 1999b; Darj *et al* 1993]. Treatment of rats with natural estrogens stimulated the rate of CBG production in male rats [Feldman *et al* 1979] but had no significant effect on serum CBG or hepatic CBG mRNA levels in female rats despite the fact that the serum CBG level of mature female rats was more than double that of male rats [Mataradze *et al* 1992]. In addition, 17β-estradiol (E2) significantly increased CBG mRNA expression in a dose- dependant manner, in a well-differentiated endometrial cancer cell-line, Ishikawa, and was positively correlated with human ovarian corpus luteum CBG mRNA levels [Misao *et al* 1998; Misao *et al* 1999a].

2.2.5.4 Androgens

Testosterone decreases CBG protein levels in female or castrated male rats and men [Mataradze *et al* 1992; Westphal 1971; Westphal 1986] while castration just before or after puberty increased CBG concentration in male rats [Nock *et al* 2000]. The androgen 5α-dihydrotestosterone, however, has no effect on serum CBG nor on hepatic CBG mRNA levels in rats [Smith and Hammond 1992].

2.2.5.5 Thyroid hormones and retinoic acid

Thyroxine administration to intact rats increases serum CBG as well as hepatic CBG mRNA levels [Smith and Hammond 1992; D'Agostino and Henning 1982]. It has, however, been suggested that the increase in CBG mRNA steady state levels may be due to increased mRNA stability rather than increased transcription [Smith and

Hammond 1992]. In contrast, in a human hepatoma cell line thyroxine did not affect CBG levels [Rosner *et al* 1984] but hyperthyroidism did increase plasma CBG levels [Rosner 1991; Westphal 1986].

CBG gene expression is regulated in an antagonistic way by triiodothyronine and retinoic acid in fetal rat hepatocytes [Seralini 1996]. Triiodothyronine activates while retinoic acid inhibits CBG gene expression [Elfahime *et al* 1994]. In a human hepatoblastoma-derived cell line, triiodothyronine attenuates CBG gene expression and CBG protein levels [Barlow *et al* 1994].

2.2.5.6 Insulin, insulin-like growth factor (IGF-1), insulin resistance and obesity

Insulin and IGF-1 were shown to dose-dependently inhibit CBG mRNA and protein levels in HepG2 cells [Crave *et al* 1995]. In one study, however, no correlation was found between insulin and CBG levels in the case of insulin variations during diabetes or obesity [Seralini 1996]. Recently, in other studies, decreased CBG levels were, however, associated with obesity and indexes of insulin resistance such as body mass index (BMI), waist to hip ratio (WHR) and fasting insulin resistance index (HOMA) in both men and women [Fernandez-Real *et al* 2001; Fernandez-Real *et al* 2002].

CBG, a member of the SERPIN family, is a substrate for elastase, which is expressed at the surface of neutrophils [Rosner *et al* 1990; Hammond 1990b]. The change in circulating CBG levels during obesity might be due to the cleavage of CBG by activated neutrophils [Hammond 1990b] as an increase in peripheral white blood cell count and neutrophils are found in obesity [Targher *et al* 1996] and insulin resistance [Fernandes-Real *et al* 2000]. This might facilitate serine protease availability and CBG cleavage. This mechanism could contribute to the reduced CBG levels observed during obesity

and insulin resistance. A 50% decrease in CBG levels was recently found in a woman with obesity and chronic fatigue, due to a CBG gene mutation [Emptoz-Bonneton *et al* 2000]. These findings suggest that low CBG levels may not only be a result of obesity but could contribute to the development of obesity. Thus, low CBG could be a marker for the development of type 2 diabetes as well as cardiovascular disease.

CHAPTER 3

Corticosteroid binding globulin gene regulation

3.1 INTRODUCTION

CBG, produced primarily in the liver, transports and modulates the bioavailability of glucocorticoids and progesterone in plasma [Hammond 1990a; Hammond *et al* 1991; Seralini 1996]. According to the free hormone hypothesis only the free fraction of steroid hormone is biologically active and able to diffuse across the plasma membrane of target tissues. The ratio between free and bound steroids depends on the number of binding sites (concentration of plasma CBG) and the affinity (Kd) for the binding sites. This implies that any changes in the levels of CBG would modify the distribution of steroids to target tissues [Westphal 1986; Mendel 1989]. Several factors influence CBG production as discussed briefly below but in more detail under section 2.2.5.

Plasma levels of CBG are suppressed during prolonged exposure to GCs, endogenously as in Cushing's syndrome [Westphal 1971; Coolens *et al* 1987] as well as exogenously as during administration of synthetic GCs [Westphal 1971; Smith and Hammond 1992; Schlecte *et al* 1987]. In contrast, adrenalectomy has been shown to increase rat plasma CBG levels [Westphal 1971; Grasa *et al* 1998; Feldman *et al* 1979]. In rats physiological and physical stressors, such as fasting, physical exercise, cold exposure and water deprivation, down-regulate CBG production [Tinnikov *et al* 1993; Tinnikov *et al* 1999; Fleshner *et al* 1995; Deak *et al* 1997; Leonhardt *et al* 2002]. In humans, CBG levels are dramatically decreased in septic shock [Pugeat *et al* 1989; Beishuizen *et al* 2001; Westphal 1986], severe burn [Garrel 1996; Bernier *et al* 1998], coronary artery bypass patients [Roth-Isigkeit *et al* 2000] and open-heart surgery [Tinnikov *et al* 1993; Tinnikov *et al* 1996]. Pro-inflammatory cytokines alone, or in synergy with GCs, have also been shown to decrease CBG protein and mRNA levels in HepG2 cells, a human

hepatoma cell-line which secretes CBG, while GCs alone appear to have no significant effect in this cell line [Emptoz-Bonneton *et al* 1997].

Progesterone or MPA suppresses CBG mRNA expression both in a well-differentiated human endometrial cancer cell line, Ishikawa, and in normal human endometrium [Misao *et al* 1994; Misao *et al* 1998]. However, in human luteal cells CBG mRNA appears to be upregulated by progesterone treatment [Misao *et al* 1999a]. In rats, one study reports that rat serum CBG or hepatic CBG mRNA levels were not influenced by progesterone treatment [Smith and Hammond 1992] whereas in another study CBG protein levels in rat plasma were increased by progesterone [Westphal 1971].

CBG plasma protein levels increase during pregnancy in rats and humans [Westphal 1971; Westphal 1986; Abou-Samra *et al* 1984; Moore *et al* 1978; Potter *et al* 1987; Robinson *et al* 1985; Hau *et al* 1983] or after administration of natural or synthetic estrogens in humans [Westphal 1971; Westphal 1986; Hammond 1990a; Coolens *et al* 1987; Misao *et al* 1999a; Darj *et al* 1993]. Testosterone on the other hand decreases CBG protein levels in female or castrated male rats and men [Mataradze *et al* 1992; Westphal 1971; Westphal 1986], while castration per se increased CBG concentrations in male rats [Nock *et al* 2000]. Insulin and IGF-1 were shown to inhibit CBG mRNA and protein levels in HepG2 cells [Crave *et al* 1995] and recently, insulin was also associated with decreased serum CBG levels in humans, as was obesity [Fernandez-Real *et al* 2001; Fernandez-Real *et al* 2002].

Despite the fact that both the human and rat CBG genes (*Cbg*) have been cloned, no regulatory studies to identify possible cis-acting sequence elements involved in their

regulation by hormones have been done [Underhill and Hammond 1989; Underhill and Hammond 1995]. DNase I footprinting of the rat CBG proximal promoter, has identified five protein-binding sites (P1-P5) within 236 bp immediately 5' of the transcription start site in rat liver nuclear extract [Underhill and Hammond 1995]. These five protein-binding sites are also highly conserved in the human CBG gene. They resemble recognition sequences for HNF-1, CP-2, DBP, HNF-3 and C/EBP or NF/IL6, respectively [Underhill and Hammond 1995]. Organization of the CBG gene is discussed in more detail under section 2.2.4.1.

Although CBG transcription is modulated by steroid hormones no steroid hormone response elements seem to be present in the rat or human CBG proximal promoter, suggesting that the steroid receptors may be acting through a tethering mechanism that may not involve direct binding to the DNA (see section 2.1.1 for a full discussion of mechanisms whereby GR regulate gene transcription). For example, the GR may repress transcription via protein-protein interactions with other transcription factors rather than by binding to GREs directly. Footprint P3-P5 resemble recognition sequences for DBP, HNF-3 and C/EBP or NF-IL6 and, although binding of these transcription factors have not been confirmed, it is interesting to note that HNF-3 and C/EBP have been reported to form protein-protein interactions with the GR [Boruk *et al* 1998; Rabek and Papaconstantinou 1999; Wang *et al* 1999; Hocke *et al* 1992].

3.2 AIM OF STUDY

In the present study I investigated the effect of various physical stressors on rat liver CBG mRNA production as well as the effect of several hormones reported to influence CBG production on CBG mRNA and protein levels in a hepatic cell line. I also

determined if the mRNA and protein results correlate with the transcriptional regulation of the rat CBG proximal promoter. Only GC transrepression was investigated in more detail. Further experiments were done to delineate the GC responsiveness on the rat CBG promoter and to determine if the GC responsiveness is GR dependent.

3.3 RESULTS

3.3.1 Regulation of CBG mRNA by physical stressors in rats

Physical stressors like exercise have been shown to down-regulate CBG production with a concomitant increase in free GC levels. In female rats physical exercise, influencing metabolic homeostasis, like swimming and fasting were shown to decrease circulating CBG protein levels [Tinnikov 1999]. They proposed that the need for substantial mobilization of spare fuel (that takes place during physical exercise or fasting) is critical in involving CBG in the stress response. Recently, stressful conditions such as malnutrition and restraint were also shown to decrease CBG protein levels in rats [Lesage *et al* 2002]. These data in malnourished rats suggests that an increase in basal concentration of corticosterone, after reduced CBG levels, will lead to a larger impact of corticosterone on target cells that mediate the negative-feedback mechanism on the activity of the HPA axis. In a collaborative study (Physiological Sciences) we obtained livers from rats that were subjected to exercise stress. This allowed us the opportunity to investigate the effect of exercise stress on CBG mRNA levels. In addition we included an extra group of rats to study the effect of CpdA on CBG mRNA levels and to compare the effect of CpdA with exercise stress (results in chapter 4).

Voluntary exercise causes many positive biological effects whereas endurance training because of its excessive physical demand can cause injuries, reproductive disturbances, impaired immunity and chronic stress-like changes in the HPA-axis [Droste *et al* 2003]. In this study we investigated the effect of voluntary running, involuntary swimming and immobilization compared to the sedentary group, which was used as the control, on rat mRNA CBG levels (Fig. 3.1).

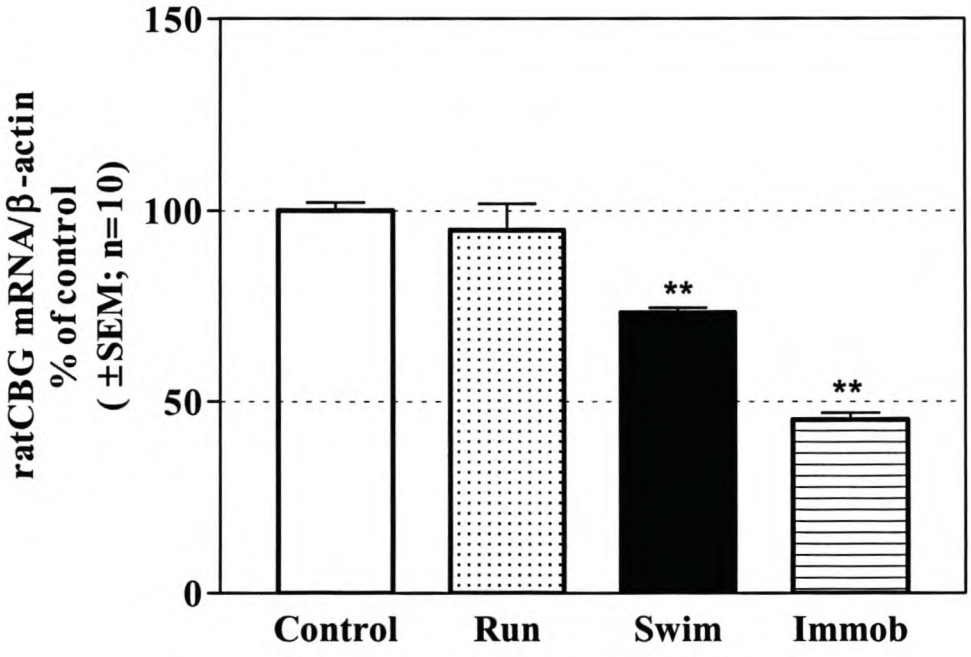


Figure 3.1. Regulation of rat CBG mRNA levels by physiological stressors. Adult male Wistar rats were subjected to voluntary running (Run), involuntary swimming (Swim) or immobilization (Immob) treatment for ten days (see materials and methods for details). Rats were decapitated and livers removed for RNA isolation. Statistical analysis was done to compare values in the presence of test conditions relative to the corresponding control (sedentary rats) using one-way ANOVA followed by Dunnett's multiple comparison's posttest (**: $P<0.01$).

The experiment protocol and RNA isolation from rat livers are explained under the materials and methods section 6.5.2. CBG mRNA levels were determined by Northern blotting using a rCBG probe and β–actin was used as an internal control (materials and methods section 6.5.4). Results showed that voluntary running had no significant ($P>0.05$) effect on rat CBG mRNA levels but psychological stressors like involuntary

swimming and immobilization significantly decreased ($P<0.01$) rat CBG mRNA levels compared to the control and the volutary running group.

3.3.2 Regulation of CBG mRNA and protein levels in hepatic cells

Glucocorticoid levels increase during exercise and are a marker of the physical stress associated with this activity [Duclos *et al* 2003; Peijie *et al* 2003]. Thus, to follow up on the rat study and specifically to evaluate the effects of glucocorticoids we investigated the effect of dexamethasone, a potent synthetic glucocorticoid, on CBG mRNA and extra-cellular protein levels in HepG2 cells, a hepatic cell line that secretes CBG. CBG mRNA levels were determined by Northern blotting using a hCBG probe and β–actin was used as an internal control (materials and methods 6.5.4). Extra-cellular CBG protein levels were determined by a RIA. E2 was included in the study as a positive control whereas insulin was included as a negative control, because previous results had showed that E2 increased [Misao *et al* 1998] whereas insulin decreased human CBG mRNA levels [Crave *et al* 1995]. The study was conducted in the presence and absence of fetal calf serum (FCS) (Fig. 3.2A & B). This was done to study the effect of the hormones on CBG mRNA and protein levels in the absence (-FCS) as well as in the presence (+FCS) of exogenous steroids and growth factors [Botti *et al* 1997; Lamb *et al* 1999].

Dexamethasone and insulin significantly decreased ($P<0.01$) CBG mRNA levels with or without FCS in HepG2 cells with insulin repression being more pronounced in the presence of FCS. E2 on the other hand significantly increased ($P<0.01$) only CBG mRNA levels in the presence of FCS. The fact that FCS enhanced the effect of insulin and E2 on CBG mRNA levels may be due to the presence of steroids and growth factors

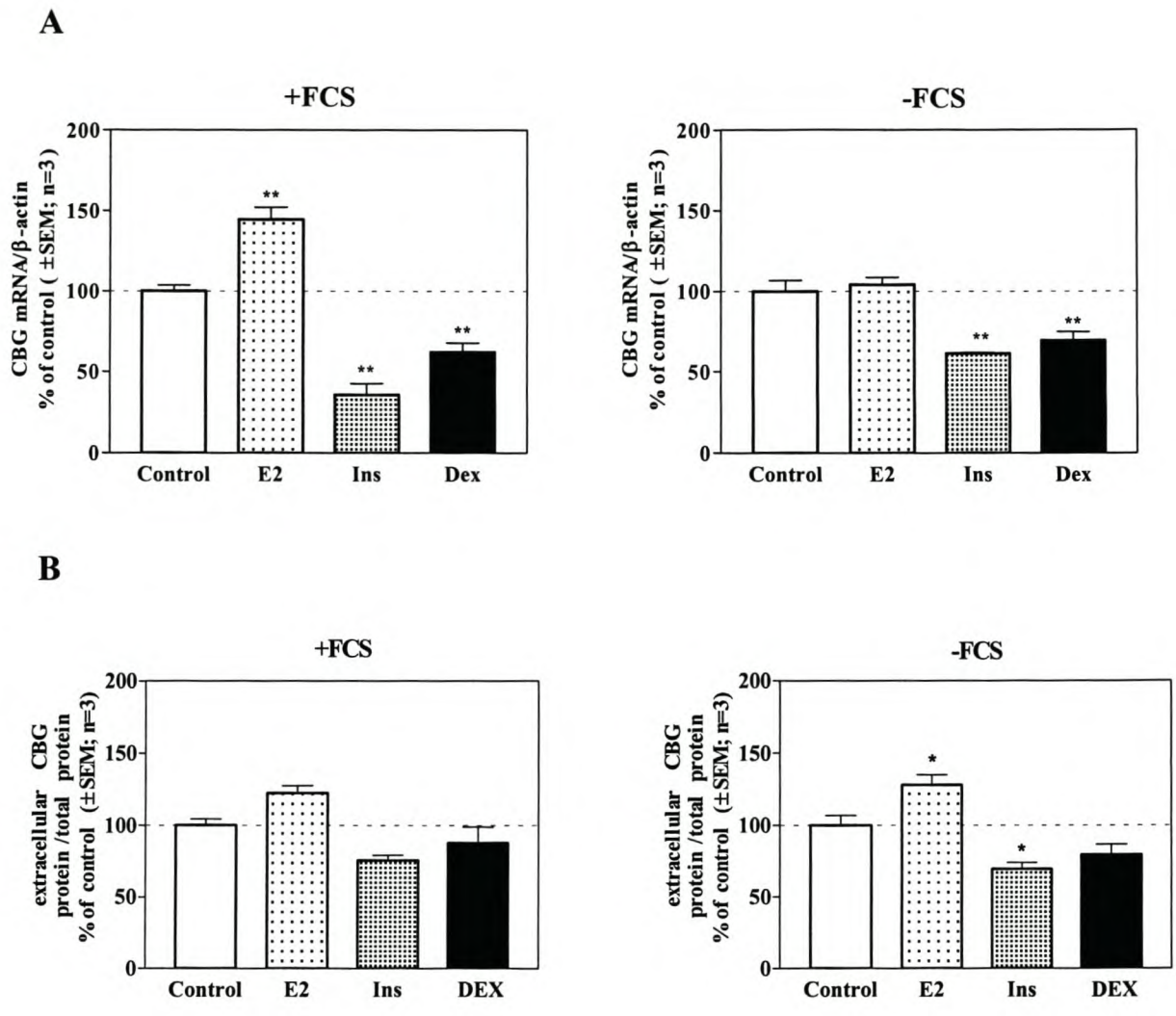


Figure 3.2. Regulation of CBG (A) mRNA levels and (B) extracellular protein levels. The effect of dexamethasone (Dex), β-estradiol (E2) and insulin (Ins) on CBG mRNA and extracellular protein levels was investigated in a human hepatoma cell-line (HepG2) in the presence or absence of FCS. HepG2 cells were cultured as described in materials and methods and incubated with test compounds for 72 hrs. The concentration of all hormones was 1 μM. Control wells received an equal amount of ethanol. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$).

in FCS, which would increase the concentration of insulin and estrogen present [Botti *et al* 1997; Lamb *et al* 1999]. Although controversy exists about whether HepG2 cells contain endogenous ER, with some authors suggesting that it does [Tam *et al* 1986] while others show that only low levels [Marino *et al* 2001] or no ER [Barkhem *et al*

1997] is present, our results seem to indicate that the cells we are using do contain endogenous ER. However, it has also been shown that HepG2 with low levels of ER may mediate non-genomic actions of ER and this should not be discounted in our system [Marino 2001]. To establish whether our HepG2 cell line contains endogenous ER Western blots and transactivation studies with ERE-containing promoters should be conducted.

In the absence of FCS, the expression of E2 receptors [Medrano *et al* 1990] has been shown to be increased but the presence of FCS down- regulates insulin receptors [Hwang *et al* 1985]. Also, when human breast cancer cells are grown without E2 addition, a progressive and transient increase of ER RNA was observed, but when cells are cultured with E2 the ER RNA increased early and remained higher than in the absence of E2 [Piva *et al* 1988]. These results from the literature do not, however, explain why FCS enhanced the effect of insulin and E2 on CBG mRNA levels. This study was performed in a human hepatoma cell-line and regulation in this cell-line may differ. Extracellular CBG protein levels were slightly increased by E2 and decreased by insulin but only significantly ($P<0.05$) in the absence of serum. Dexamethasone also slightly decreased CBG protein levels, both with and without FCS, although not significantly.

Previous studies done by Crave and Emptoz-Bonneton in HepG2 cells also used 1 μM hormones (E2, insulin and dexamethasone), as we did, but were only done in the absence of FCS [Crave *et al* 1995; Emptoz-Bonneton *et al* 1997]. In the current study 1 μM dexamethasone significantly decreased CBG mRNA levels in contrast to the study by Emtoz-Bonneton *et al* that showed no effect [Emptoz-Bonneton *et al* 1997]. In their

study cells were incubated with steroids for 48 hrs whereas in our study the incubation time was 72 hrs. This may account for the difference in the results. Crave *et al* also tested the effect of 1 μM E2 on CBG mRNA levels in the absence of FCS and like in this study found no effect on CBG mRNA levels in the absence of FCS [Crave *et al* 1995]. The decrease in CBG mRNA levels by insulin in their study in the absence of FCS also corresponds to the decrease in CBG mRNA levels by insulin found in this study.

3.3.3 Regulation of the rat CBG proximal promoter

To determine if the results of the protein and mRNA determinations correlated with transcriptional regulation of the rat CBG promoter, mouse hepatoma BWTG3 cells, were transiently transfected with the full length rat CBG proximal promoter reporter construct (ratCBG1200Luc) as well as truncated versions thereof (ratCBG295Luc and ratCBG145Luc) as described in materials and methods section 6.6.1 (Fig. 3.3A). The ratCBG295Luc promoter reporter construct contains the five protein-binding sites identified by DNase I foot printing namely P1-P5 [Underhill and Hammond 1995]. The transcription factor binding at P1 has been identified as HNF-1 [Underhill and Hammond 1995] and at P2 as CCAAT-binding protein-2 (CP-2) [Zhao *et al* 1997] while for P3-P5 the putative binding factors are DBP, HNF-3 and C/EBP, respectively. The ratCBG145Luc promoter reporter construct contains only the binding sites for HNF-1 and CP-2 [Underhill and Hammond 1995].

An initial experiment was conducted to determine the basal transcriptional activity of each of the constructs (Fig. 3.3B). Of the three constructs tested the ratCBG145Luc

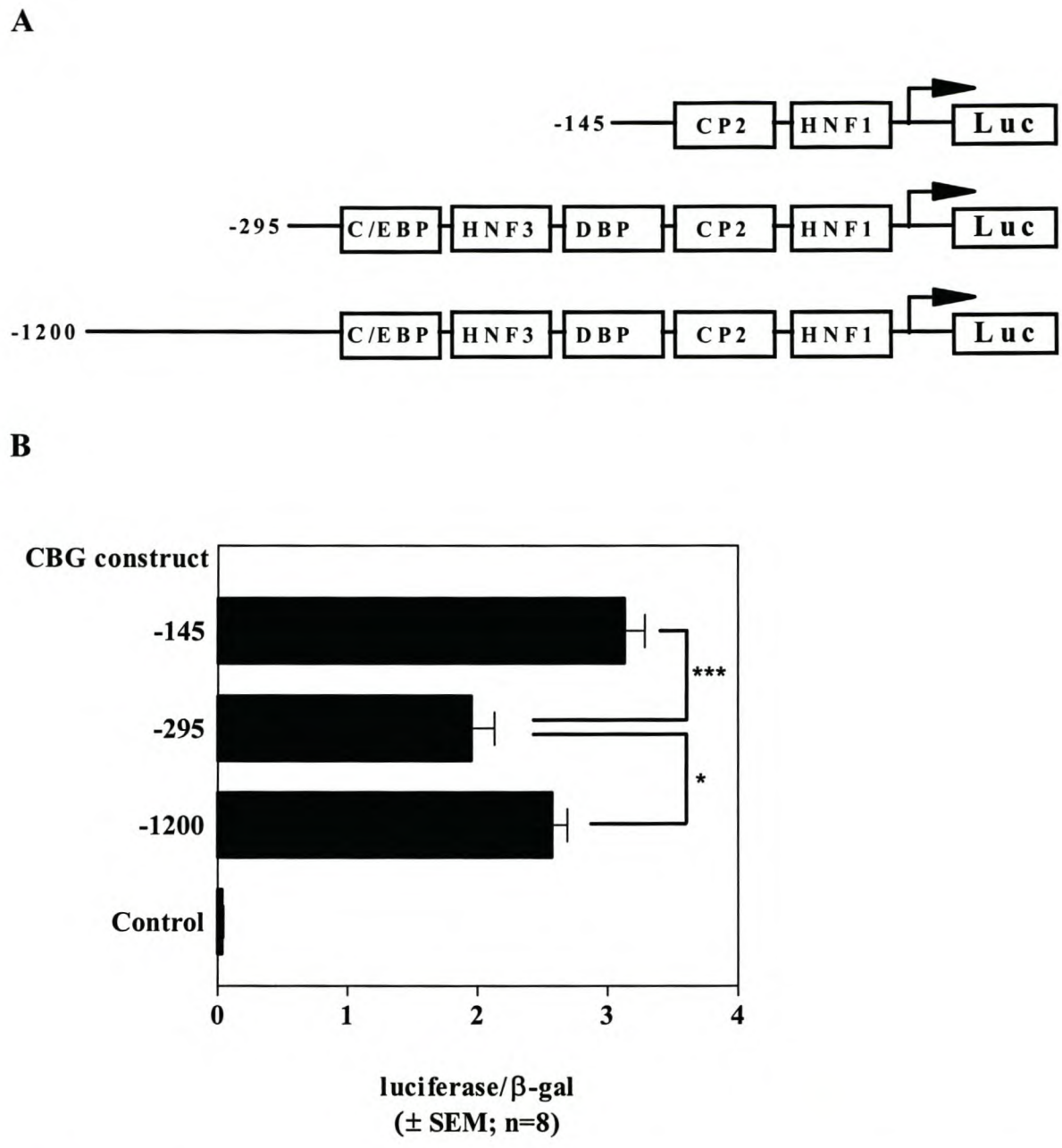


Figure 3.3. Rat CBG proximal promoter. *(A) A schematic presentation of the full-length rat CBG proximal promoter reporter construct (ratCBG1200Luc) and truncated rat CBG promoter reporter constructs (ratCBG295Luc and ratCBG145Luc) indicating the position of the five protein-binding sites P1-P5 by showing boxes with the putative transcription factors binding at these sites. (B) Basal expression levels of the three constructs represented in (A) were investigated in BWTG3 cells.* BWTG3 cells were cultured as described in materials and methods. The cells were transiently transfected with the indicated promoter reporter constructs. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. Luciferase activity was determined in the cell lysates 24 h after induction and values were normalized for β–galactosidase. Statistical analysis was done to compare basal expression levels between constructs using one-way ANOVA followed by Bonferonni's multiple comparison's posttest comparing all columns (*: $P<0.05$; ***: $P<0.001$). All constructs were significantly ($P<0.001$) different from the control.

promoter reporter construct had the highest basal activity followed by the ratCBG1200Luc construct, while the ratCBG295Luc promoter reporter construct had the lowest basal activity (Fig. 3.3B). The ratCBG1200Luc construct differs significantly from the ratCBG295Luc promoter reporter construct ($P<0.05$). There is also a significant difference between the ratCBG295Luc construct and the ratCBG145Luc promoter reporter construct ($P<0.001$). Reporter studies by Underhill *et al*, using the same constructs showed the opposite, with the ratCBG295Luc promoter reporter construct having the highest basal activity followed by the ratCBG1200Luc and then the ratCBG145Luc promoter construct [Underhill and Hammond 1995]. They used a rat cell-line (H4IIEC3) to transfect these constructs whereas in this study a mouse cell-line (BWTG3) was used, which may influence the basal expression of these constructs.

We then investigated the effect of various test compounds (dexamethasone, hydrocortisone, progesterone, insulin, IL-6 and E2) on the transcriptional regulation of each of the CBG reporter promoter constructs transiently transfected into BWTG3 cells, as well on a stable transfection of the ratCBG295Luc construct in BWTG3 cells (Fig. 3.4). Dexamethasone, as a potent synthetic glucocorticoid, and hydrocortisone, as an endogenous glucocorticoid, was chosen to further explore the effects of glucocorticoids on CBG transcription. Insulin and estradiol were chosen because we had also previously (Fig. 3.2) shown effects on CBG mRNA levels. Finally, IL-6 and progesterone were included because others had shown that they regulate CBG levels [Emptoz-Bonneton *et al* 1997; Misao *et al* 1994; Misao *et al* 1998].

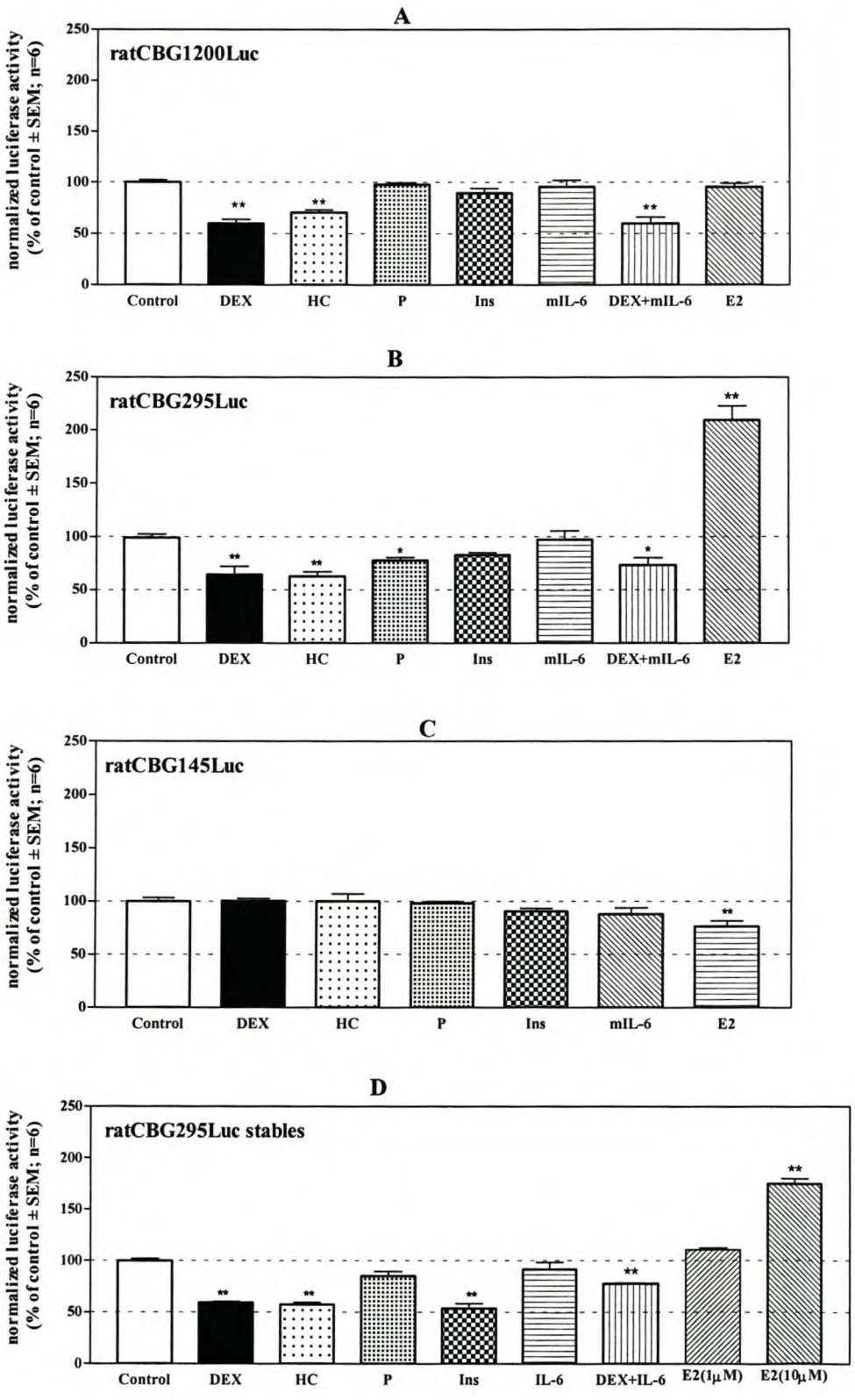


Figure 3.4. Hormonal regulation of the rat CBG proximal promoter and deletion constructs. The effect of the glucocorticoids, dexamethasone (DEX) and hydrocorticosterone (HC), as well as progesterone (P), insulin (Ins), mouse interleukin-6 (mIL-6) and 17β-estradiol (E2) on the transiently transfected (A) full length rat CBG

proximal promoter construct (ratCBG1200Luc) and truncated rat CBG promoter reporter constructs, (B) ratCBG295Luc and (C) ratCBG145Luc, was investigated in BWTG3 cells. BWTG3 cells were cultured as described in material and methods. The cells were transiently transfected with the indicated promoter reporter constructs. BWTG3 cells were also (D) stably transfected with the ratCBG295Luc construct. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. Twenty-four hrs after transfection hormones were added at a concentration of 1 μM, except in (D) where 10 μM E2 was also used. Control wells received an equal amount of ethanol. After a further 24 hr incubation cells were lysed and luciferase activity was determined. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to compare values in presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$).

Progesterone (1 μM) only transrepressed (with 22.4 ± 2.7 % inhibition) the transiently transfected ratCBG295Luc reporter construct to a significant degree ($P<0.05$) while in stable transfections repression was seen but not to a significant degree.

Insulin (1 μM) also transrepressed all constructs except the ratCBG145Luc promoter reporter construct. However, only transrepression (46.5 ± 4.7 % inhibition) of the stably transfected ratCBG295Luc is significant ($P<0.01$) and compares well with the decrease in CBG mRNA levels (64.6 ± 4.1 % inhibition) by insulin. In transient transfections, the expression vectors are introduced into cultured cells and expressed transiently, without replication or integration into the cellular genome [Smith and Hager 1997]. However, in stable transfections, gene promoters are integrated into mammalian cells and are subjected to effects from surrounding chromatin. There was no difference in the effect of insulin between the transiently and stably transfected CBG promoter reporter constructs, which suggests that insulin regulation of the CBG promoter may not involve chromatin remodelling.

Mouse interleukin 6 (mIL-6) at 1 μM had no significant effect on any of these CBG promoter reporter constructs whether transiently or stably transfected. However, mIL-6 together with dexamethasone (1 μM) significantly transrepressed the ratCBG1200Luc ($P<0.01$) and ratCBG295Luc ($P<0.05$) promoter reporter constructs transiently transfected as well as the stably transfected ratCBG295Luc ($P<0.01$). This repression may only be due to dexamethasone alone as there is no significant ($P>0.05$) difference between the repression by dexamethasone only as compared to the repression by dexamethasone together with mIL-6.

The transiently transfected ratCBG1200Luc reporter construct was unaffected by E2 (1 μM), whereas the ratCBG295Luc promoter reporter construct was significantly transactivated ($P<0.01$). The ratCBG145Luc was, however, significantly repressed by E2 ($P<0.01$). Only at higher concentrations of E2 (10 μM) was transactivation significantly increased ($P<0.01$) when the ratCBG295Luc promoter reporter construct was stably transfected. This indicates that when the ratCBG295Luc was stably transfected it is less reactive to activation by E2. The differences between transient and stable transfections could account for the variations in effects observed.

The fact that the transiently or stably transfected ratCBG295Luc construct was significantly transactivated ($P<0.01$) by E2 (209.5 ± 13.5 % activation of the transiently (1 μM E2) transfected ratCBG295Luc and 174.8 ± 5.3 % activation of the stably (10 μM E2) transfected ratCBG295Luc) while transactivation was abrogated with the ratCBG1200Luc reporter construct indicates that transcription factors further downstream of the ratCBG295Luc may prevent activation by E2. The activation of the ratCBG295Luc promoter reporter construct (174-209 % activation) by E2 corresponds

to the increase in CBG mRNA levels (144.5 ± 5.3 % activation) by E2. However, the comparison between the mRNA effects and the promoter experiments should be treated with caution as these experiments were done in two different cell lines, one human and the other mouse.

The GCs, dexamethasone and hydrocortisone, at 1 μM, significantly transrepressed ($P<0.01$) the transiently transfected ratCBG1200Luc and ratCBG295Luc promoter reporter constructs as well as the stably transfected ratCBG295Luc construct (Fig. 3.4 A, B and D). However, GCs had no effect on the ratCBG145Luc promoter reporter construct (Fig. 3.4C). Dexamethasone resulted in a 35.8 ± 7.7 % inhibition ($P<0.01$) and hydrocortisone in a 37.4 ± 4.4 % inhibition ($P<0.01$) of the transient ratCBG295Luc construct. For the ratCBG1200Luc construct, dexamethasone resulted in a 40.3 ± 4.3 % inhibition ($P<0.01$) and hydrocortisone in a 29.3 ± 2.2 % inhibition ($P<0.01$). Also, dexamethasone resulted in a 41.1 ± 1.1 % inhibition ($P<0.01$) and hydrocortisone in a 43.2 ± 2.3 % inhibition ($P<0.01$) of the stably transfected ratCBG295Luc construct. There is no significant difference between the percentage inhibition of the reporter constructs by dexamethasone and hydrocortisone observed with the ratCBG295Luc construct and the ratCBG1200Luc construct nor with the ratCBG295Luc stably transfected. The fact that results for the transient versus stably transfected ratCBG295Luc promoter reporter construct, do not differ, indicates that GC regulation of the CBG promoter does not involve chromatin remodelling. Dexamethasone repression of the ratCBG295Luc promoter reporter construct correlates with the decrease in CBG mRNA levels by dexamethasone (Table 3.1).

Despite the fact that the mRNA experiments were done in a human hepatoma cell line (HepG2) and the promoter experiments in a mouse hepatoma cell line (BWTG3) we obtained good correspondence (Table 3.1) between promoter reporter constructs' responses, especially with the stably transfected ratCBG295Luc construct, and CBG protein and mRNA levels in the presence of several of the hormones tested (E2, insulin, and dexamethasone). We, however, chose to focus further investigation on the transrepression of the rat CBG proximal promoter by GCs. In the case of the GCs specifically there was also no significant difference between the stably and transiently transfected ratCBG295Luc construct and thus further studies were performed using transient transfections.

Table 3.1. Comparison of the percentage expression of CBG mRNA levels vs ratCBG295Luc promoter activity by 1μM test compounds. Control levels are 100%; levels > 100% indicate activation while levels < 100% indicate inhibition.

	CBG mRNA levels (Fig. 3.2)	**ratCBG295Luc promoter (Fig. 3.4)**
E2	144.5 ± 5.3 %	209.5 ± 13.5 % (transient) 174.8 ± 5.3 % (stable at 10μM)
Insulin	64.6 ± 4.1 %	46.5 ± 4.7 % (stable)
Dexamethasone	37.8 ± 3.2 %	35.8 ± 7.7 % (transient) 41.1 ± 1.1 % (stable)

3.3.4 Delineation of GC responsiveness within the rat CBG proximal promoter reporter construct

To delineate the region of GC responsiveness on the CBG proximal promoter, BWTG3 cells were transiently transfected with the ratCBG1200Luc, ratCBG295Luc and ratCBG145Luc promoter constructs as discussed in materials and methods section 6.6.1

(Fig. 3.5A). Dexamethasone produced a 53 ± 2.7 % inhibition ($P<0.01$) of the ratCBG1200Luc and a 32.5 ± 1.6 % inhibition ($P<0.01$) of the ratCBG295Luc construct. Thus dexamethasone (1 μM) significantly ($P<0.01$) transrepressed both the ratCBG1200Luc and ratCBG295Luc promoter reporter constructs whereas the ratCBG145Luc construct was unaffected. This indicates that the binding sites for DBP, HNF3 and C/EBP present in the ratCBG295Luc construct but not in the ratCBG145Luc construct may be involved in the GC responsiveness of the CBG proximal promoter. The ratCBG1200Luc construct was transrepressed to a greater extent by dexamethasone than the ratCBG295Luc construct, which indicates that transcription factors further upstream of the ratCBG295Luc may also be involved in modulating transrepression by GCs.

To further characterize the effect of GCs on the CBG proximal promoter, dose response experiments for transrepression by dexamethasone and hydrocortisone of the ratCBG1200Luc and ratCBG295Luc promoter reporter constructs transiently transfected, as well as the ratCBG295Luc stably transfected into BWTG3 cells, were performed (Fig. 3.5B). EC50 values are defined as the concentration of agonist that evokes a response half way between the baseline (bottem) and maximal response (top). The EC50s for dexamethasone and hydrocortisone transrepression were 3.7 nM and 6.9 nM, respectively for the transiently transfected ratCBG295Luc, 2.8 nM and 10.6 nM for the transiently transfected ratCBG1200Luc and 3.1 nM and 1.2 nM for the stably transfected ratCBG295Luc (Table 3.2). There was no significant difference ($P>0.05$) in the EC50s of dexamethasone for the transiently transfected ratCBG1200Luc and ratCBG295Luc promoter reporter constructs as well as the stably transfected ratCBG295Luc construct. There is also no significant difference ($P>0.05$) in the EC50s

of hydrocortisone between the transiently transfected ratCBG1200Luc versus the ratCBG295Luc promoter reporter constructs, but both were significantly different ($P<0.001$) from the EC50 obtained with the stably transfected ratCBG295Luc construct.

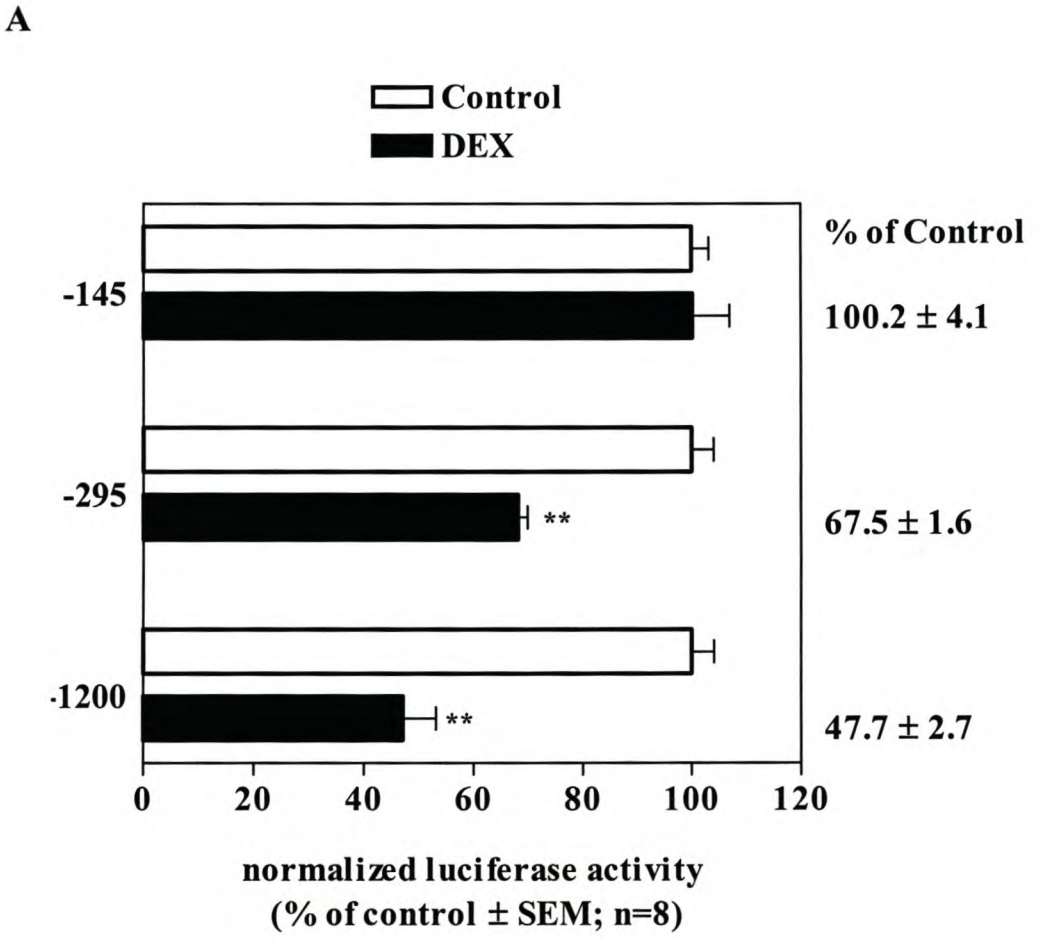
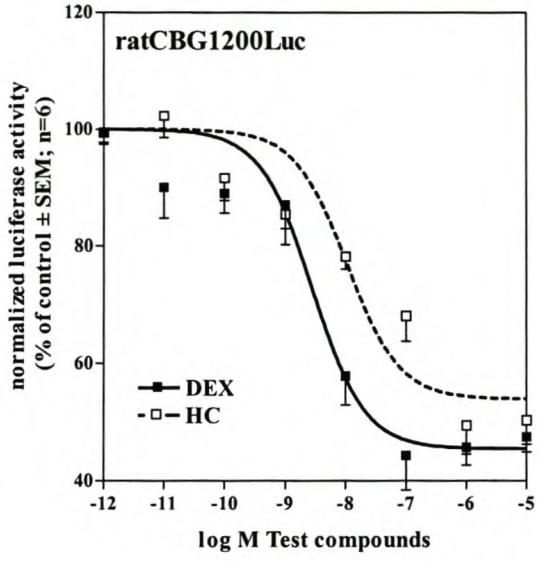
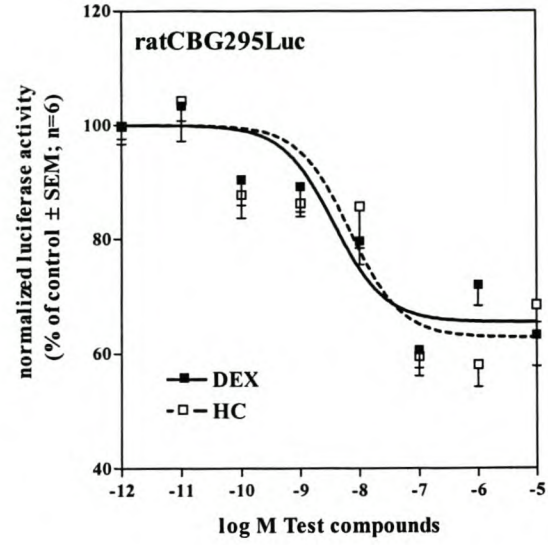
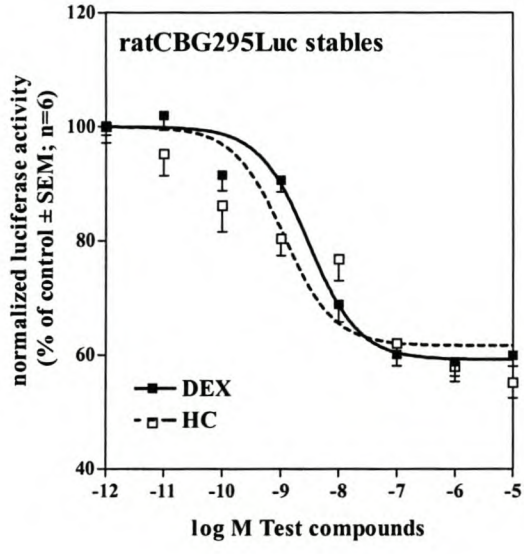


Figure 3.5. Characterization of the glucocorticoid responsiveness of the rat CBG promoter reporter construct. ***(A) Localization of glucocorticoid responsiveness.*** The ratCBG1200Luc, ratCBG295Luc and ratCBGl45Luc promoter reporter constructs were transiently transfected into BWTG3 cells. BWTG3 cells were cultured and transfected as described in materials and methods. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. The concentration of dexamethasone added was 1 μM. Control wells received an equal amount of ethanol. Luciferase activity was determined in the cell lysates after 24 h. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to compare values in presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$).

B







(B) Dose response curves of dexamethasone (DEX) and hydrocorticosterone (HC) transrepression with transiently transfected ratCBG1200Luc and ratCBG295Luc promoter reporter constructs as well as stably transfected ratCBG295Luc. RatCBG1200Luc and ratCBG295Luc promoter reporter constructs were transiently transfected into BWTG3 cells. A cell line stably transfected with the ratCBG295Luc promoter reporter construct was also investigated. Cells were treated with increasing concentrations of hormone as indicated and lysed after 24 hrs. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. EC50 and maximal repression values were determined by fitting a dose response curve with one site competition.

Table 3.2. Summary of EC50s and maximal repression for dexamethasone and hydrocortisone of constructs tested. Statistical analysis was done on results obtained in Fig 5B to compare EC50s and maximal repression between different constructs for each compound tested using one-way ANOVA followed by Bonferroni multiple comparison's posttest (a: $P<0.05$; b: $P<0.01$; c $P<0.001$).

	EC50		Maximal repression	
	DEX	**HC**	**DEX**	**HC**
ratCBG1200Luc	2.8 nM	10.6 nM	54.6 ± 2.9%	46.1 ± 2.5%
ratCBG295Luc	3.7 nM	6.9 nM	34.5 ± 2.7% [c]	37.2 ± 3.0% [c]
ratCBG295Luc stables	3.1 nM	1.2 nM [c]	40.8 ± 1.5% [c]	38.4 ± 2.0% [c]

The maximal transrepression by dexamethasone and hydrocortisone was 34.5 ± 2.7% and 37.2 ± 3.0%, respectively, for the transiently transfected ratCBG295Luc construct, 54.6 ± 2.9% and 46.1 ± 2.5% for the transiently transfected ratCBG1200Luc construct and 40.8 ± 1.5% and 38.4 ± 2.0% for the stably transfected ratCBG295Luc construct (Table 3.2). The maximal repression by dexamethasone and hydrocortisone of the ratCBG295Luc contruct transiently and stably transfected was significantly different ($P<0.001$) from the maximal transrepression obtained with the ratCBG1200Luc construct transiently transfected. The fact that the ratCBG1200Luc construct was transrepressed to a greater extent than the ratCBG295Luc construct and that the maximal repression between the two constructs was significantly different, further indicates that transcription factors further upstream of the ratCBG295Luc construct may also be involved in transrepression by GCs.

3.3.5 Effect of co-transfected GR and RU486 on glucocorticoid repression of the ratCBG295Luc promoter reporter construct.

The ligand-activated GR has been shown to repress transcription of various genes, through protein-protein interactions with other transcription factors. The steroid induction properties of a transiently transfected gene were found to be sensitive to the concentration of steroid receptor [Szapary *et al* 1996]. Therefore whether the transrepression potential is dependent on the GR expression level, was also investigated. BWTG3 cells were transiently transfected with the ratCBG295Luc promoter reporter construct and without and with the rGRα expression vector (Fig. 3.6A). Co-transfection with rGRα in BWTG3 cells significantly increased the transrepression response of both dexamethasone [from 35.75 ± 15.4% to 57.4 ± 2.6% ($P<0.05$)] and hydrocortisone [from 32.5 ± 7.8% to 49.9 ± 8.4% ($P<0.05$)], while transrepression by progesterone was unaffected.

To strengthen the case that the observed dexamethasone repression of the ratCBG295Luc promoter reporter construct was mediated by the GR the effect of the GC antagonist, RU486, on transrepression was investigated (Fig. 3.6B). As shown before dexamethasone (1 μM) significantly ($P<0.01$) transrepressed the CBG promoter construct and indeed RU486 (20 μM) could relieve this repression.

A

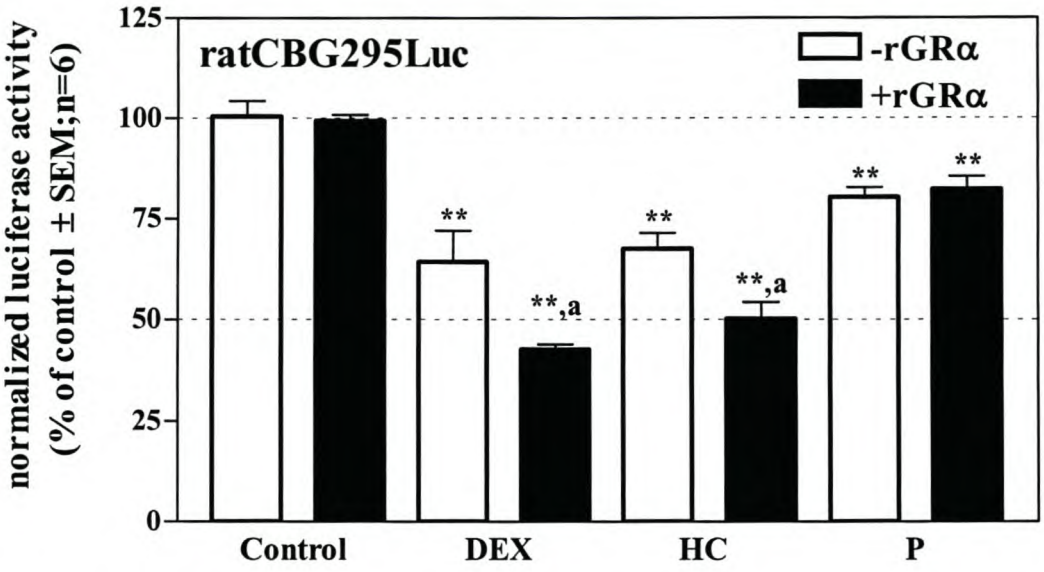
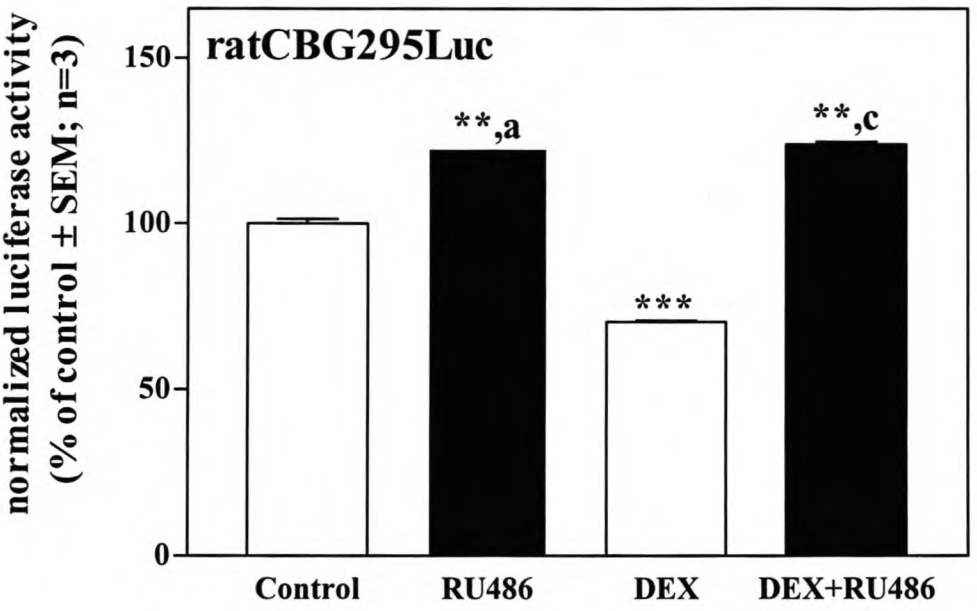


Figure 3.6. Effect of co-transfected GR and RU486 on glucocorticoid repression of the ratCBG295Luc promoter reporter construct. ***(A) Effect of co-transfected GR expression vector on transrepression by dexamethasone, hydrocortisone and progesterone.*** Transrepression of a ratCBG295Luc promoter construct by the glucocorticoids, hydrocortisone (HC) and dexamethasone (DEX), and progesterone (P) was investigated in BWTG3 cells, in the absence or presence of co-transfected rGRα. BWTG3 cells were cultured as described in materials and methods. The cells were transiently transfected with a ratCBG295Luc, with or without the rat GRα expression vector, pSVGR1. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. Twenty-four hrs after transfection hormones were added at a concentration of 1 μM. Control wells received an equal amount of ethanol. After a further 24 hr incubation cells were lysed and luciferase activity was determined. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$) and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) for each compound tested using a two-tailed unpaired t-test (a: $P<0.05$).

B



(B) Effect of the glucocorticoid antagonist, RU486, on transrepression. A ratCBG295Luc promoter reporter construct was transiently transfected into mouse hepatoma cells (BWTG3). BWTG3 cells were cultured as described in materials and methods. Twenty-four hrs after transfection cells were treated with 1 μM dexamethasone and 20 μM RU486 as indicated. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values are plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in presence of test compounds relative to the corresponding control using one-way ANOVA followed by Bonferonni's multiple comparison's posttest comparing all columns (**: $P<0.01$; ***: $P<0.001$) and to (ii) compare values of each compound tested relative to transrepression in the presence of RU486 using one-tailed unpaired t-test (a: $P<0.05$; c: $P<0.001$).

3.3.6 Does HNF3α, DBP and C/EBPβ influence transactivation by a GRE-containing promoter reporter construct?

This study has indicated that the transcription factors, C/EBP, HNF3 and DBP may be involved in the GC responsiveness of the CBG proximal promoter. C/EBP and HNF3 have been shown to form protein-protein interactions with the GR [Rabek and Papaconstantinou 1999; Wang *et al* 1999]. In NF-κB mediated transcription that is transrepressed by GCs, NF-κB has also been shown to physically interact with the GR.

This interaction results in mutual transcriptional antagonism, which is solely mediated through the p65 subunit of NF-κB, and co-expression of the p65 subunit dose-dependently represses GR transactivation [McKay *et al* 1998].

To test whether transactivation of a GRE-containing promoter by dexamethasone is influenced by the transcription factors, C/EBP, HNF3 and DBP, the mouse hepatoma cell-line, BWTG3, was transiently transfected with a $(GRE)_2$tkLuc promoter reporter construct with or without expression vectors for the transcription factors and treated with 1 μM dexamethasone (Fig. 3.7). The results show that dexamethasone significantly ($P<0.01$) transactivated the GRE-dependent promoter reporter construct but that in the presence of co-transfected HNF3α, DBP and C/EBPβ the transactivation was significantly decreased ($P<0.01$ for HNF3α and $P<0.001$ for DBP and C/EBPβ). These results suggest that an increase in the transcription factors HNF3α, DBP and C/EBPβ partially inhibits transactivation of a GRE-containing promoter by dexamethasone. This indicates that these transcription factors influence GR-mediated transcriptional regulation most probably by forming protein-protein interactions with the GR. Thus these transcription factors may potentially be involved in transrepression of the CBG promoter by GCs although further work is required to elucidate the specific factor involved and the precise mechanism involved. This experiment can also be repeated on the CBG constructs and could show whether co-transfection with HNF3α, DBP and C/EBPβ would influence transrepression of the CBG proximal promoter.

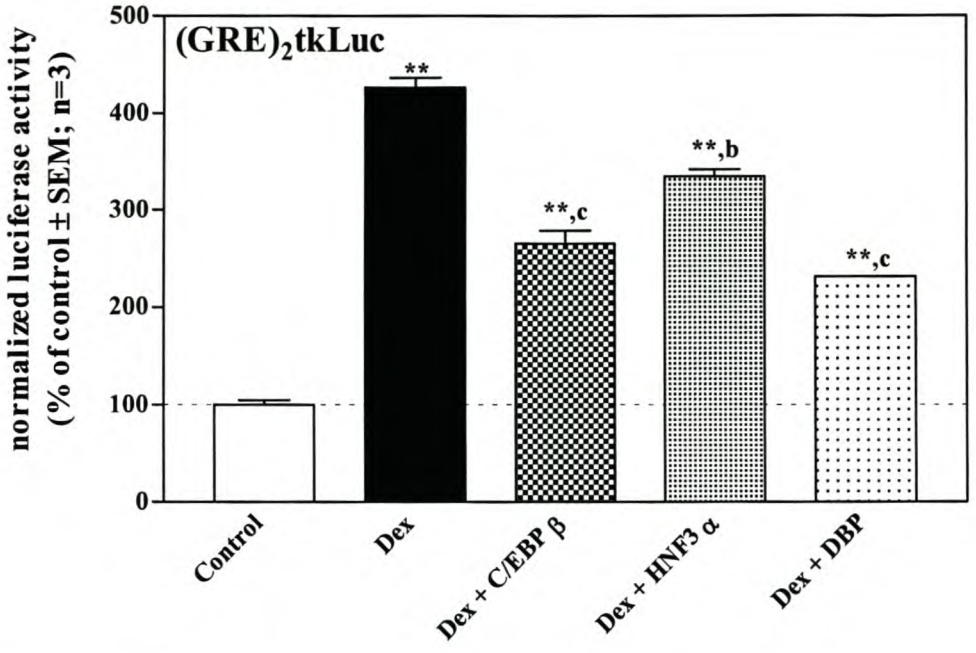


Figure 3.7. Effects of co-transfected C/EBPβ, HNF3α and DBP on dexamethasone transactivation of a GRE-containing promoter reporter construct. The effect co-transfection of the transcription factors, C/EBPβ, HNF3α and DBP on transactivation of a GRE-containing promoter reporter construct by dexamethasone (DEX) was investigated in BWTG3 cells. BWTG3 cells were cultured as described in materials and methods. The cells were transiently transfected with $(GRE)_2$tkLuc with or without co-transfected CAAT/enhancer binding protein (C/EBP or NF/IL6), hepatocyte nuclear factor-3 (HNF-3) and D-site binding protein (DBP). In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. The concentration of dexamethasone added was 1 μM. Control wells received an equal amount of ethanol. Luciferase activity was determined in the cell lysates after 24 hrs. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in presence of test compounds relative to the control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$) and to (ii) compare values of each construct tested relative to transactivation by dexamethasone alone using one-tailed unpaired t-test (b: $P<0.01$; c $P<0.001$).

3.4 DISCUSSION

This study investigated the hormonal regulation of CBG by determining effects on mRNA levels and on the transcriptional regulation of the CBG proximal promoter. In addition, transcriptional regulation was compared with effects on mRNA levels to establish whether a correlation existed.

Exercise like swimming and stressful conditions such as malnutrition and restraint, were previously shown to decrease plasma CBG levels in rats resulting in an increase in free GC levels which are a marker of the physical stress associated with this activity [Duclos *et al* 2003; Peijie *et al* 2003; Tinnikov 1999; Lesage *et al* 2002]. In the present study, voluntary running had no effect on rat CBG levels but involuntary swimming and immobilization decreased rat CBG mRNA levels. The decrease in CBG mRNA levels with physical stressors like involuntary swimming and immobilization observed in this study corresponds to the decrease in circulating plasma CBG levels observed during exercise stress in the literature [Tinnikov 1999].

To evaluate the effects of glucocorticoids we investigated the effect of dexamethasone as well as various other hormones, known to regulate CBG levels, on CBG mRNA and protein levels in a human hepatic cell line, HepG2. A previous study had shown that dexamethasone alone had no effect on CBG mRNA and protein levels in HepG2 cells [Emptoz-Bonneton *et al* 1997]. However, in the present study dexamethasone decreased CBG mRNA and protein levels, albeit only significantly in the case of the mRNA. In both studies the same cells and concentration of steroids were used but the incubation time differed. In the present study cells were incubated with steroids for 72 hrs whereas in the study by Emptoz-Bonneton *et al* cells were incubated for 48 hrs. This might account for the differences in GC effects observed. The results obtained in this study could be the result of indirect effects due to the longer incubation time. After 72 hrs incubation with steroids, other proteins might be upregulated, which could influence CBG mRNA levels. As expected from the literature E2 increased [Misao *et al* 1998] and insulin decreased [Crave *et al* 1995] CBG mRNA and protein levels in HepG2 cells.

We also found that dexamethasone and hydrocortisone repressed the full-length, ratCBG1200Luc promoter reporter construct and the truncated ratCBG295Luc promoter reporter construct as well as the latter construct stably transfected into BWTG3 cells. This is in accordance with the decrease in CBG mRNA and protein levels observed in HepG2. Although caution should be excercised in comparing results from different cell-lines, especially if they originate form different species as is the case here, the fact that the mRNA work from both the HepG2 (human liver cell line) and the rat livers correlate with the promoter work in BWTG3 cells (mouse liver cell line) does lend weight to the evidence that GCs probably do repress CBG at the transcriptional level. The results with the promoter reporter constructs can only be compared to mRNA and protein levels in the literature as no promoter work has been done before. In one study dexamethasone alone had been shown to have no effect on CBG mRNA and protein levels in HepG2 cells. However, together with IL-6 dexamethasone synergised in repressing mRNA and protein, while IL-6 alone also decreased CBG mRNA and protein levels [Emptoz-Bonneton *et al* 1997]. IL-6 had no effect on any of the promoter reporter constructs we tested but IL-6 together with dexamethasone repressed the CBG promoter reporter constructs. Our work, however, does not suggest a synergy but rather that there is no significant difference between the effect seen with dexamethasone alone and dexamethasone together with IL-6.

Progesterone only repressed the ratCBG295Luc promoter reporter construct, which corresponds to the decrease in CBG mRNA levels by progesterone observed in Ishikawa cells [Misao *et al* 1994; Misao *et al* 1998] but not with previous work in rats suggesting that progesterone either has no effect [Smith and Hammond 1992] or increases plasma CBG levels [Westphal 1971]. Insulin decreased, although not

significantly, the ratCBG1200Luc and the ratCBG295Luc promoter reporter constructs. Significant repression was only seen with the stably transfected ratCBG295Luc construct and matches the decrease in CBG mRNA and protein levels in HepG2 cells observed by us and others [Crave *et al* 1995]. Once again caution is required in comparing the mRNA work with the promoter work as these were done in different cell lines. An increase in the expression of the ratCBG295Luc promoter reporter construct, transiently and stably transfected, by E2 was observed in our study and correlates with our mRNA results in HepG2 cells (+FCS) and results described in the literature that show an increase in CBG mRNA levels in Ishikawa cells [Misao *et al* 1998; Misao *et al* 1999a] and plasma levels in male rats and humans [Westphal 1986; Hammond 1990a; Coolens *et al* 1987; Misao 1999a; Darj *et al* 1993; Feldman *et al* 1979] but not with the results in female rats [Westphal 1971; Mataradze *et al* 1992] that showed no effect.

In our study we found a good correlation between the effects of hormones on the mRNA levels and the expression of the ratCBG295Luc promoter construct (Table 3.1) despite the fact, as mentioned previously, that the mRNA experiments were done in a human hepatoma cell line (HepG2) and the promoter experiments in a mouse hepatoma cell line (BWTG3). For dexamethasone and E2 the correlation was found both with the transiently and stably transfected systems, while for insulin the effect was only evident with the stably transfected construct. The study of mammalian genes using transient transfection assays has helped us understand transcriptional mechanisms. However, transfected promoter constructs are not always the right stand-in for endogenous genes, especially in cases where chromatin remodeling must take place [Smith and Hager 1997]. Functional differences between transiently transfected and stably replicating

templates can thus be used to identify and characterise regulatory mechanisms that involve chromatin components. In our study there was no significant difference between the EC50 and maximal repression values of dexamethasone obtained with the transiently versus stably transfected ratCBG295Luc promoter reporter constructs (Table 3.2). Although the results with hydrocortisone are not so clear-cut the results with dexamethasone certainly suggest that GC regulation of the CBG promoter may not involve chromatin remodelling. A precedent for GC effects that do not require chromatin remodelling is found in the induction of IκBα by GCs [Deroo and Archer 2001a].

Further investigation of the glucocorticoid responsiveness of the CBG proximal promoter, showed that dexamethasone transrepressed both the ratCBG1200Luc and ratCBG295Luc promoter constructs, whereas the ratCBG145Luc construct was unaffected. This indicates that the protein-binding sites for DBP, HNF3 and C/EBP present in the ratCBG295Luc construct but not in the ratCBG145Luc construct may be involved in the glucocorticoid responsiveness of the CBG proximal promoter. The ratCBG1200Luc construct was transrepressed to a greater extent by dexamethasone than the ratCBG295Luc construct, which suggests that transcription factors further upstream of the ratCBG295Luc may also be involved in modulating transrepression by glucocorticoids. An example of such an effect may be seen in the osteocalcin gene promoter in which GC suppression of 1.25-dihidroxyvitamin D3-stimulated transcription occurs independently of distal or proximal GREs [Aslam *et al* 1995]. Promoter deletion analysis revealed an additional GRE in the distal promoter that function to suppress osteocalcin transcription.

Although we clearly demonstrate that CBG transcription is modulated by GCs no nGREs appear to be present in the rat or human CBG proximal promoter. nGRE's are present in various genes and the consensus sequence of the nGRE assembled from the sequences of these genes has been suggested to be very degenerate and may thus not be easily identified. On the other hand it may suggest that the GR is acting through a tethering mechanism that does not involve direct binding to the DNA. The GR can repress transcription via protein-protein interactions with other transcription factors rather than by binding to a nGRE. GCs have, for example, been shown to repress NF-κB-mediated activation of proinflammatory genes like IL-8 and ICAM1 through protein-protein interactions between the GR and NF-κB [Nissen *et al* 2000]. The collagenase-3 gene repression by GCs is also mediated by the GR forming protein-protein interactions with AP-1 [Rogatsky *et al* 2001]. Interestingly HNF3 and C/EBP, present in the ratCBG295Luc construct, have been reported to form protein-protein interactions with the GR [Boruk *et al* 1998; Rabek and Papaconstantinou 1999; Wang *et al* 1999; Hocke *et al* 1992]. Although DBP, involved in the circadian rhythm, has not been shown to form protein-protein interaction with the GR, GCs do induce DBP expression in liver, kidney and heart [Balsalobre *et al* 2000]. In this study we showed that an increase in the levels of the transcription factors HNF3α, DBP and C/EBPβ partially inhibits transactivation of a GRE-containing promoter by dexamethasone, which indicates that these transcription factors interact with the GR. Thus the converse may also be valid with the GR negatively modulating the activity of these transcription factors on the CBG promoter. In this regard the p65 subunit of NF-κB has been shown to physically interact with the GR, resulting in mutual transcriptional antagonism, which dose-dependently represses GR transactivation [McKay *et al* 1998]. Future experiments should include co-transfection experiments with the three possible factors interacting

with the GR, namely HNF3α, DBP and C/EBPβ, to ascertain if the inhibitory effect of GR on the CBG promoter can modulated by increasing concentrations of these interacting factors.

The C/EBP family of transcription factors plays a key role in the development and maintenance of metabolically important processes [Croniger *et al* 1998]. Cross-talk between the C/EBP family members and GR has been documented in various systems and they have also been shown to bind directly to the LBD of nuclear receptors including the GR [Boruk *et al* 1998]. These interactions result in induction or inhibition of target genes but do not always involve binding of GR to DNA [Boruk *et al* 1998]. The GC-dependent induction of transcription from the herpes simplex virus thymidine kinase gene promoter was for example localized to a binding site for C/EBPβ and was independent of GR binding to DNA [Boruk *et al* 1998]. In addition, the GR, through its DNA-binding domain, has been shown to suppress the binding of C/EBPβ on the rainbow trout ER promoter by protein-protein interactions and thereby prevents the enhancer effect of this transcription factor [Lethimonier *et al* 2002]. Recently, the PEPCK-C gene was shown to be regulated by GCs and was induced in the liver and kidney but repressed in white adipose tissue [Olswang *et al* 2003]. The repression of the PEPCK-C gene transcription by GR in adipocytes was shown to be via inhibition of C/EBP-mediated activation and does not require DNA binding of the receptor. Thus, the GR probably inhibits PEPCK-C gene transcription via protein-protein interactions. Furthermore, the carbamoylphosphate synthetase gene is expressed in the hepatocytes of the liver and is controlled by GCs and intracellular cAMP levels [Christoffels *et al* 2000]. A 102-bp GC response unit, containing binding sites for HNF3, C/EBP and the GR, was shown to be the main determinant of hepatocyte-specific and hormone-

controlled activity. Genes coding for the acute-phase proteins are induced by IL-6 through the human transcription factor NF-IL-6 and its rat homologue IL-6-DBP/LAP [Hocke *et al* 1992]. It is also synergistically induced by IL-6 plus GCs but the synergism is blocked by RU486 and was thus dependent on the GR.

Members of the HNF3 family (HNF3α, β and γ) play an important role in the regulation of genes involved in various aspects of cellular metabolism [Zimmermann *et al* 1997; O'Brien *et al* 1995; Roux *et al* 1995]. This regulation is accomplished through N-and C-terminal transactivation domains of which the C-terminal terminal domain is needed for accessory factor activity in the PEPCK gene GRU [Wang *et al* 1999]. HNF3 also functions as an accessory factor in other GRU's for example those located in the tyrosine aminotransferase, insulin-like growth factor-binding protein-1 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoters [Zimmermann *et al* 1997; O'Brien *et al* 1995; Roux *et al* 1995]. The adenovirus-mediated expression of an HNF3β protein with a deleted C-terminal transactivation domain reduces the GC-induced expression of the PEPCK and glucose-6-phosphotase genes in H4IIE hepatoma cells [Wang *et al* 2000]. Thus the functional interaction between HNF3 and GR appears to be a common mechanism for GC-regulated gene transcription, especially in the liver. These findings, together with the results of the present study, suggest that C/EBP and HNF3 forming protein-protein interactions with the GR, may be involved in GC repression of the CBG promoter.

This study has for the first time provided information on hormonal regulation of the CBG proximal promoter as well as correlating transcriptional regulation with mRNA levels in the case of GCs, insulin and estrogen. This suggests that differences in levels

of mRNA are due to transcriptional effects and not RNA stability or transport. In addition, the GC responsiveness of the proximal promoter was delineated to between –145 and –295bp relative to the transcription start site. This area on the promoter contains putative binding sites for C/EBP, HNF3 and DBP. These transcription factors are probably involved in the GR-mediated regulation of the CBG promoter. However, further work is needed to ascertain which of the three transcription factors identified in this study are specifically responsible for the GR-mediated regulation of the CBG proximal promoter and to discover the mechanism of action involved.

CHAPTER 4

Dissociative glucocorticoid activity of MPA and Compound A, a non-steroidal plant analogue, in hepatoma cell lines.

4.1 INTRODUCTION

Endogenous GCs are important in maintaining basal and stress-related homeostasis and preventing excessive immune responses to antigenic challenges [Chrousos and Gold 1992]. GCs are also the most effective therapy in the long-term treatment of inflammation and autoimmune diseases [Cato and Wade 1996]. Upon hormone binding the GR, translocates to the nucleus, where the activated receptor can transactivate or transrepress specific genes [Beato *et al* 2000]. GR mediated gene regulation is discussed in detail in section 2.1. Transactivation is generally mediated by binding of a GR dimer to GREs in the promoter region of GC responsive genes, followed by recruitment of coactivators, chromatin remodeling, and increased gene transcription [Adcock 2000]. Transrepression may also be mediated via direct binding to DNA, via nGREs. Alternatively, transrepression may proceed without direct DNA-binding by the GR but via protein-protein interactions, that may only require binding of the GR monomer, with other transcription factors such as NF-κB, AP-1, STAT proteins and C/EBP [McEwan *et al* 1997; Webster and Cidlowski 1999].

Most of the anti-inflammatory effects of GCs are mediated through repression of inflammatory and immune genes that do not contain GREs in their promoters [Cato and Wade 1996]. The inhibitory effect of GCs are thus mostly due to protein-protein interactions between activated GR and transcription factors such as AP-1, NF-κB and C/EBPβ, which mediates expression of inflammatory genes [Barnes 1998]. A large number of side-effects such as diabetes, weight gain, hypertension and osteoporosis are, however, associated with the long-term use of GCs [Barnes 1998]. This has led to a search for novel GCs with improved specificity for anti-inflammatory activity that dissociate between transrepression and transactivation because many of the side effects

of conventional GCs may be attributed to transactivation (Table 2.3 and Fig. 2.6) [Adcock 2000; Dumont *et al* 1998; Cato and Wade 1996]. Some such 'dissociated glucocorticoids' have been described as dicussed in section 2.1.4 [Vayssierre *et al* 1997; Vanden Berghe *et al* 1999] and dissociation may be due to inhibition of GR dimerization [Reichart *et al* 1998; Heck *et al* 1994]. MPA has recently been shown to exert dissociative GC activity in normal human lymphocytes [Bamberger *et al* 1999]. MPA has also been shown to bind to both the GR and PR [Pridjian *et al* 1987; Kontula *et al* 1983]. Another compound reported to exert dissociative properties is a phenyl aziridine precursor, Compound A [De Bosscher unpublished results]. CpdA decreases CBG levels and increases free corticosterone levels in rats [Louw and Swart 1999]. The dissociated GC activities of MPA and CpdA will be further described in this chapter.

The search for dissociated glucocorticoids has focused on the transrepressive effects on NF-κB and AP-1 mediated gene regulation [Dumont *et al* 1998; Adcock 2000]. We suggest that regulation of CBG function, as a key modulator of glucocorticoid action, is imperative for full evaluation of any such drugs. CBG is a central player in glucocorticoid disposition. In plasma, GCs are bound to CBG that regulates the free fraction (2- 4%) of glucocorticoids able to diffuse across the plasma membrane of target tissues and exert biological activity. This implies that any changes in the levels of CBG would modify the distribution of glucocorticoids to the target tissues [Hammond *et al* 1990b]. Although the dramatic fall of CBG levels during stress, with concomitant substantial (2 to 20-fold) increases in free glucocorticoid levels, merits its classification as a negative acute-phase protein [Black and Garbutt 2002] investigators have up to now neglected its role in stress and modulation of the acute phase response [Beishuizen *et al* 2001]. Recent results, however, indicate that the decrease in CBG during the acute

phase response releases GCs that actively participate to strengthen all stages of the stress response and the designation of acute-booster reactants (ABRs) to depict these modulating properties has been proposed for molecules such as CBG [Ingenbleek and Bernstein 1999]. In addition, the rapid decline in CBG activity during open-heart surgery in adults and children suggest a more active role for this acute phase protein in the stress responses of the body [Tinnikov *et al* 1996]. Thus, a thorough evaluation of GC action, including dissociated GC action, is not complete without an investigation of the effect on CBG. We have shown in chapter 3 that GCs suppress CBG levels by transrepressing hepatic transcription and that transrepression of the CBG promoter reporter construct (rat295CBGLuc) is evident in the presence of GCs.

4.2 AIM OF STUDY

In the present study I investigated and compared the dissociative GC activity of MPA and CpdA relative to conventional GCs by examining transactivation of GRE-containing reporters and transrepression of CBG gene expression in hepatic cell lines. In addition, effects on CBG mRNA levels were investigated to establish if these effects correlate with the CBG promoter reporter results. The effect of these test compounds were further investigated in the absence and presence of hGR as well as in the presence of hGR^{dim} to check for dimerization requirement.

4.3 RESULTS

4.3.1 MPA, but not CpdA, transactivates GRE-containing promoters in the presence of co-transfected rGRα

Dissociative glucocorticoid activity requires that a compound can dissociate between transrepression and transactivation [Adcock 2000]. Thus to compare the dissociative activity of MPA with that of CpdA, we initially investigated and compared the transactivation of two GRE-containing promoters. Both of these constructs contain no other binding sites. The mouse hepatoma cell-line, BWTG3, was transiently transfected with a $(GRE)_2$tkLuc or a $(GRE)_2$50hIL6PLuc promoter reporter construct as described in section 6.6.1 (Fig. 4.1A & B, white bars). The result with both constructs show that, at 1 μM, dexamethasone and hydrocortisone significantly ($P<0.01$) transactivate the GRE-dependent promoter constructs while MPA at 1 μM, progesterone at 1 μM and CpdA at 10 μM have no effect. Dexamethasone results in a 1.6 ± 0.04-fold ($P<0.01$) induction of the $(GRE)_2$tkLuc and a 9.3 ± 1.2-fold ($P<0.01$) induction of the $(GRE)_2$50hIL6PLuc construct, while hydrocortisone induces a 1.4 ± 0.09-fold ($P<0.01$) induction of the $(GRE)_2$tkLuc and a 7.4 ± 0.3-fold ($P<0.01$) induction of the $(GRE)_2$50hIL6PLuc construct.

To further investigate transactivation of the GRE-containing promoters, dose response experiments of transactivation with the $(GRE)_2$tkLuc and $(GRE)_2$50hIL6PLuc promoter reporter constructs by dexamethasone, hydrocortisone, MPA, progesterone and CpdA (Fig. 4.2A & B) were performed in BWTG3 cells. The EC50s for dexamethasone and hydrocortisone were 0.1 μM and 0.6 μM, respectively, for the $(GRE)_2$tkLuc construct, and 0.02 μM and 0.2 μM, respectively, for the $(GRE)_2$50hIL6Pluc construct suggesting

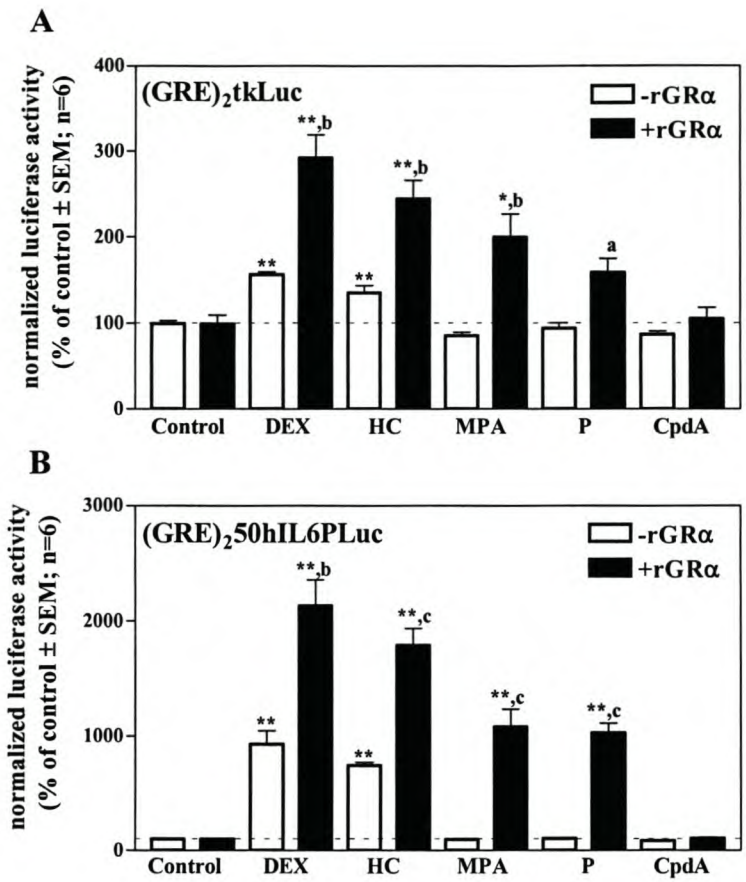


Figure 4.1. Comparison of the effects of MPA and CpdA, in the absence and presence of co-transfected GR, on transactivation. *Transactivation of a (A) $(GRE)_2$tkLuc or a (B) $(GRE)_2$50hIL6PLuc promoter reporter construct by medroxyprogesterone acetate (MPA) and Compound A (CpdA) was compared to the conventional glucocorticoids, hydrocortisone (HC) and dexamethasone (DEX), and to progesterone (P) in BWTG3 cells, in the absence or presence of co-transfected rGRα.* BWTG3 cells were cultured as described in materials and methods and cells were transiently transfected with the indicated constructs. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. Twenty-four hours after transfection hormones were added at a concentration of 1 μM for all hormones except for CpdA, which was added at 10 μM. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$) and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) for each compound tested using a two-tailed unpaired t-test (a: $P<0.05$; b: $P<0.01$; c $P<0.001$).

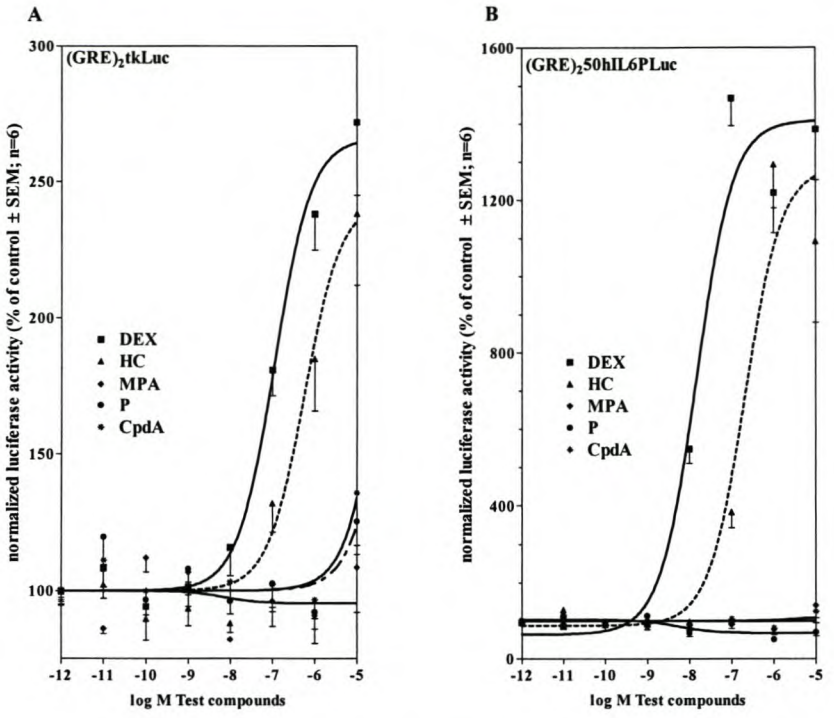


Figure 4.2. Dose response curves of transactivation of GRE-containing promoters (A: $(GRE)_2$tkLuc or B: $(GRE)_2$50hIL6Pluc) by dexamethasone (DEX), hydrocorticosterone (HC), medroxyprogesterone acetate (MPA), Progesterone (P), and Compound A (CpdA). Reporter constructs were transiently transfected into BWTG3 cells as described in the materials and methods. Cells were treated with increasing concentrations of hormone as indicated and lysed after 24 hrs. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. EC50s were determined by fitting a dose response curve with variable slope.

that dexamethasone is significantly ($P<0.001$) more potent (6 to 10 times) than hydrocortisone in transactivating via the GRE-containing promoter reporter constructs used.

To test whether the transactivation potential is dependent on the glucocorticoid receptor expression level, as has been suggested for MPA, [Bamberger *et al* 1999], BWTG3 cells were co-transfected with the rat GR alpha (rGRα) expression vector in the presence of the GRE-containing promoter reporter constructs (Fig. 4.1A & B, solid bars). Co-transfection with rGRα almost doubled the transactivation response of both

dexamethasone (from 1.6 ± 0.04-fold to 2.9 ± 0.3-fold ($P<0.01$) for $(GRE)_2$tkLuc and from 9.3 ± 1.2-fold to 21.3 ± 2.3-fold ($P<0.01$) for $(GRE)_2$50hIL6Pluc) and hydrocortisone (from 1.4 ± 0.09-fold to 2.4 ± 0.2-fold ($P<0.01$) for $(GRE)_2$tkLuc and from 7.4 ± 0.3-fold to 17.9 ± 1.5-fold ($P<0.001$) for $(GRE)_2$50hIL6Pluc). In addition, co-transfection with rGRα resulted in significant transactivation by MPA (2.0 ± 0.3-fold ($P<0.01$) for the $(GRE)_2$tkLuc and 10.8 ± 1.6-fold ($P<0.001$) for the $(GRE)_2$50hIL6Pluc) and progesterone (1.6 ± 0.2-fold ($P<0.05$) for the $(GRE)_2$tkLuc and 10.2 ± 0.9-fold ($P<0.001$) for the $(GRE)_2$50hIL6Pluc). MPA thus transactivates in the presence of higher GR levels and in our system results in transactivation levels that are not significantly ($P>0.05$) different from that of dexamethasone in the absence of co-transfected GR. CpdA, however, even at ten times the concentration of the other test compounds (10 μM), did not transactivate even when GR was co-transfected.

4.3.2 CpdA transrepresses the rat CBG proximal promoter with a similar potency (EC50) but with a higher efficacy (maximal repression) than MPA

Next we investigated the second requirement for dissociative glucocorticoids namely that the compound displays strong transrepression activity. We thus compared the ability of MPA with that of CpdA in transrepressing the CBG promoter. BWTG3 cells were transiently transfected with a ratCBG295Luc promoter reporter construct as discussed in section 6.6.1 (Fig. 4.3, white bars). The results show that, at 1 μM, the glucocorticoids, dexamethasone and hydrocortisone, significantly transrepress ($P<0.01$) this CBG promoter reporter construct. MPA (1 μM), progesterone (1 μM) and CpdA (1 μM) also transrepress but to a lesser degree than the glucocorticoids.

Dexamethasone resulted in a 42.7 ± 4.7 % inhibition (P<0.01) and hydrocortisone in a 37.4 ± 4.4 % inhibition (P<0.01) of the ratCBG295Luc construct while MPA caused 22.3 ± 1.9 % inhibition (P<0.01), progesterone a 19.8 ± 2.5 % inhibition (P<0.01) and CpdA a 9.8 ± 2.3 % inhibition.

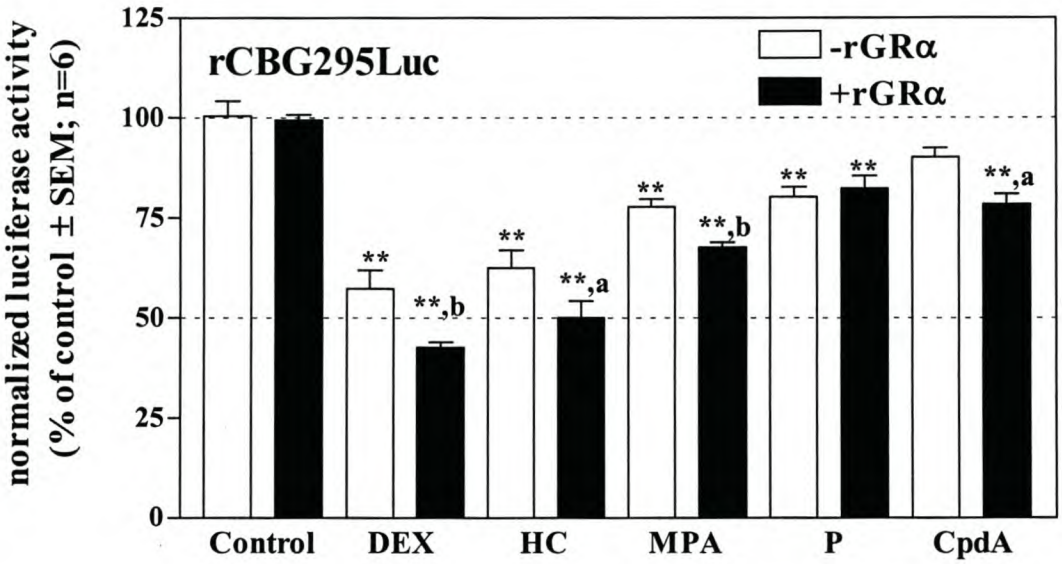


Figure 4.3. Comparison of the effects of MPA and CpdA, in the absence and presence of co-transfected GR, on transrepression. *Transrepression of a ratCBG295Luc promoter reporter construct by medroxyprogesterone acetate (MPA) and Compound A (CpdA) was compared to the conventional glucocorticoids, hydrocortisone (HC) and dexamethasone (DEX), and to progesterone (P) in BWTG3 cells, in the absence or presence of co-transfected rGRα.* BWTG3 cells were cultured as described in materials and methods and cells were transiently transfected with the ratCBG295Luc promoter reporter construct. In addition a β-galactosidase expression plasmid (pPGKβGopbA) was co-transfected to monitor for transfection efficiency. Twenty-four hours after transfection hormones were added at a concentration of 1 μM. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: P<0.05; **: P<0.01) and to (ii) compare values without GR (–rGRα) to values with co-transfected GR (+rGRα) for each compound tested using a two-tailed unpaired t-test (a: P<0.05; b: P<0.01).

Dose response curves of transrepresion with the ratCBG295Luc promoter reporter construct by dexamethasone, hydrocortisone, MPA, and CpdA (Fig. 4.4) were performed. The EC50s for dexamethasone, hydrocortisone, MPA and CpdA were 3.3 nM, 3.3 nM, 0.11 nM and 0.99 nM, respectively. The maximal repression for dexamethasone, hydrocortisone, MPA and CpdA were 35.7 ± 3.7%, 38.1 ± 4.4%, 15.6 ± 2.2% and 23.7 ± 3.6%, respectively. Statistical analysis reveals no significant ($P>0.05$) difference between the potency (logEC50) of the compounds tested. However, analysis of the efficacy (maximal repression) shows that the order of repressive efficacy is HC=DEX>CpdA>MPA and that CpdA has a significantly ($P<0.05$) higher efficacy of repression than MPA.

To test whether the transrepression potential is dependent on the GR expression level, BWTG3 cells were co-transfected with the rGRα expression vector in the presence of the ratCBG295Luc reporter construct (Fig. 4.3, solid bars). Co-transfection with rGRα in BWTG3 cells significantly increased the transrepression response of both dexamethasone [from 42.7 ± 4.7% to 57.4 ± 1.3% ($P<0.01$)] and hydrocortisone [from 37.4 ± 4.4% to 49.9 ± 4.2% ($P<0.05$)]. Co-transfection with rGRα also significantly increased the transrepression by MPA [from 22.3 ± 1.9% to 32.3 ± 1% ($P<0.01$)] and CpdA [from 9.8 ± 2.3% to 21.8 ± 2.7% ($P<0.05$)] while transrepression by progesterone was unaffected.

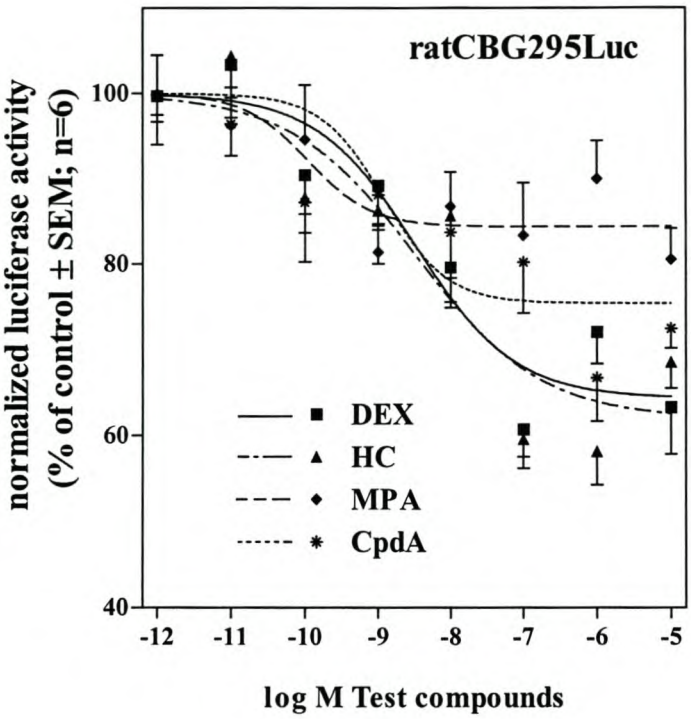


Figure 4.4. Dose response curves of transrepression of the ratCBG295Luc promoter by dexamethasone (DEX), hydrocorticosterone (HC), medroxyprogesterone acetate (MPA), progesterone (P), and Compound A (CpdA). The ratCBG295Luc promoter reporter construct was transiently transfected into BWTG3 cells as described in the materials and methods. Cells were treated with increasing concentrations of hormone as indicated and lysed after 24 hrs. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values plotted as a percentage of the average control. EC50s and maximal repression values were determined by fitting a dose response curve with variable slope.

4.3.3 Further investigation of the effects of MPA and CpdA on the transactivation of GRE-containing reporter constructs

Previous results (Fig. 4.1, solid bars) showed that MPA, but not CpdA, could transactivate via GRE-containing promoters in the presence of co-transfected GR in the mouse hepatoma cell-line, BWTG3. This effect was investigated further (Fig. 4.5): (a) by comparing effects in a human cell-line, (b) by investigating the transactivation responses in the presence of the GR antagonist, RU486, and (c) by investigating the modulating effects of MPA, progesterone and CpdA on dexamethasone transactivation.

Both cell-lines were transiently transfected with a $(GRE)_2$tkLuc promoter reporter construct and the appropriate GR expression vector as described in section 6.6.1.

(a) The results comparing transactivation in the mouse vs human (Fig. 4.5A) cell-lines, showed that dexamethasone significantly ($P<0.01$) transactivates the $(GRE)_2$tkLuc promoter reporter construct in the presence of co-transfected GRα expression vector in both cell-lines but to different extents (6.9 ± 0.8 fold in BWTG3 cells and 124.4 ± 9.4 fold in HepG2 cells). MPA also significantly ($P<0.01$) transactivates the $(GRE)_2$tkLuc promoter reporter construct in both cell lines and like dexamathasone to different extents (4.6 ± 0.5 fold in BWTG3 cells and 40.6 ± 2.1 fold in HepG2 cells). However, if MPA transactivation is compared to that of dexamethasone it is interesting to note that in BWTG3 cells MPA transactivation is 66% of dexamethasone activation while in HepG2 cells it is only 33%. This is unlikely to be due to higher levels of GR as dexamethasone activation relative to control is higher in HepG2 cells (124.4 ± 9.4 fold) than in BWTG3 cells (6.9 ± 0.8 fold). Progesterone also significantly ($P<0.01$) transactivates the $(GRE)_2$tkLuc promoter reporter construct but only in BWTG3 cells and to a similar extent (4.5 ± 0.6 fold) as MPA. Interestingly, progesterone does not transactivate the $(GRE)_2$tkLuc promoter reporter construct in HepG2 cells. The increased transactivation, relative to dexamethasone, of MPA in BWTG3 cells may thus be due to the presence of the progesterone receptor for which MPA acts as an agonist [Kontula *et al* 1983; Selman *et al* 1996]. CpdA on the other hand, does not activate this $(GRE)_2$tkLuc promoter reporter construct on its own in either of the cell-lines confirming its inability to transactivate via the $(GRE)_2$tkLuc promoter reporter construct.

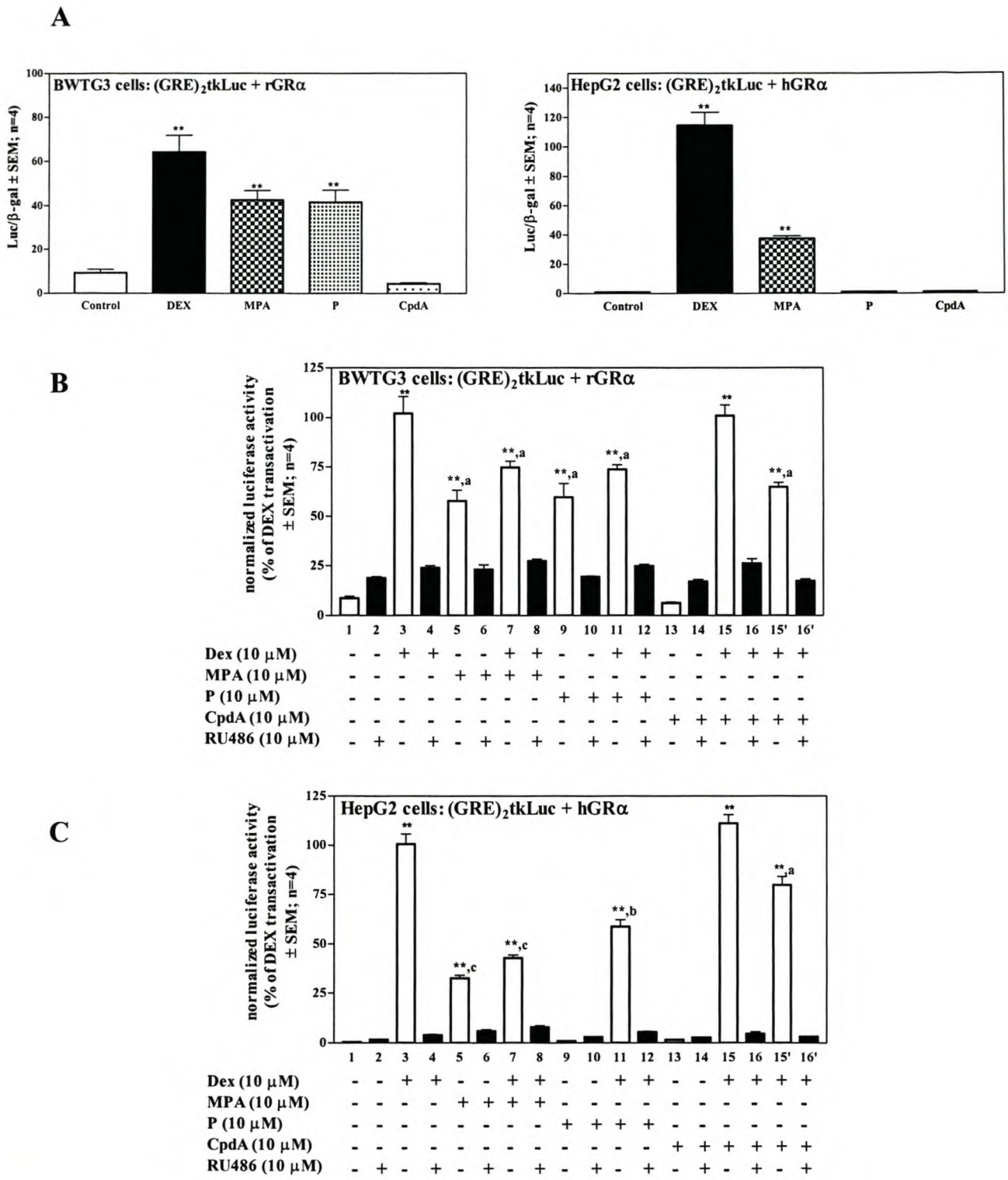


Figure 4.5. Further investigation of the effects of MPA and CpdA on transactivation by *(a) comparing effects in a human cell-line, (b) investigating the transactivation responses in the presence of the GR antagonist, RU486, and (c) investigating the modulating effects of MPA, P and CpdA on dexamethasone transactivation.* The $(GRE)_2$tkLuc promoter reporter construct together with a rat GRα (pSVGR1), or a human GRα (pRS-hGRα), expression vector was transiently transfected into mouse hepatoma cells, BWTG3 (Fig. 5A and B), and also into human

hepatoma cells, HepG2 (Fig. 5A and C) as described in the materials and methods. Cells were treated with 10 μM hormones as indicated. Control wells received an equal amount of ethanol. For Fig. 5B & C lanes 1-16, compounds were added to the cells at the same time. To investigate the effects of the order of addition of CpdA we first added CpdA (Fig. 5A & B, lanes 15' & 16') to the cells and after 10 minutes added dexamethasone (lane 15') or dexamethasone and RU486 (lanes 16'). Luciferase values were normalized for β–galactosidase and (A) values plotted directly or (B and C) plotted as a percentage of the average transactivation by dexamethasone alone. Statistical analysis was done to (i) compare values in presence of test compounds relative to the corresponding controls (control for A, and lane 1 or 2 for B and C) using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$) and to (ii) compare values of each compound tested relative to transactivation by dexamethasone alone (lane 3 for B and C) using a one-tailed unpaired t-test (a: $P<0.05$; b: $P<0.01$; c: $P<0.001$).

(b) The glucocorticoid antagonist, RU486, abolishes the dexamethasone (Fig. 4.5B and C compare lanes 3 and 4), MPA (Fig. 4.5B and C compare lanes 5 and 6), and progesterone (Fig. 4.5B compare lanes 9 and 10) transactivation of the $(GRE)_2$tkLuc promoter reporter construct. RU486 is also a PR antagonist and thus will also abolish the effects of progesterone via the PR [Crains *et al* 1993].

(c) Finally, we examined the ability of the test compounds to modulate dexamethasone transactivation of the $(GRE)_2$tkLuc promoter reporter construct. MPA significantly decreased dexamethasone transactivation of the $(GRE)_2$tkLuc promoter reporter construct in both cell-lines: in BWTG3 cells (Fig. 4.5B compare lane 3 with 7) to 74.7 ± 3.2% of dexamethasone activation ($P<0.05$) and in HepG2 cells (Fig. 4.5C compare lane 3 with 7) to 42.8 ± 1.6 % ($P<0.001$). Progesterone also decreased dexamethasone transactivation in both cell lines: in BWTG3 cells (Fig. 4.5B compare lane 3 with 11) to 73.8 ± 2.5 % of dexamethasone activation ($P<0.05$) and in HepG2 cells (Fig. 4.5C compare lane 3 with 11) to 58.8 ± 3.5 % ($P<0.01$).

The modulatory effect of CpdA on dexamethasone transactivation of the $(GRE)_2$tkLuc promoter construct is more complex in that it depends on the order of addition of CpdA. If dexamethasone was added together with CpdA (as in other lanes), CpdA has no effect on dexamethasone transactivation (Fig. 4.5B and C compare lanes 3 and 15). On the other hand, if CpdA was added before dexamethasone, CpdA significantly ($P<0.05$) decreased dexamethasone transactivation: in BWTG3 cells (Fig. 4.5B compare lanes 3 and 15') to 64.8 ± 2.3 % of dexamethasone activation and in HepG2 cells (Fig. 4.5C compare lanes 3 and 15') to 79.9 ± 4.2 %. In all cases RU486 could abolish the transactivation of test compounds in the presence of dexamethasone (Fig. 4.5B and C lanes 8, 12, 16 and 16').

4.3.4 RU486 relieved transrepression by dexamethasone and CpdA

To strengthen the case that the observed repression of the ratCBG295Luc promoter reporter construct by CpdA was mediated by the GR the effect of the glucocorticoid antagonist, RU486, on transrepression in BWTG3 cells was also investigated (Fig. 4.6). The ratCBG295Luc promoter reporter construct is slightly increased by RU486. RU486 has been shown to exhibit partial agonist activities in a cell-type-dependent manner [Liu *et al* 2002]. The effect on transcription correlated with different ratios of endogenous coactivators and corepressors in cells [Schulz *et al* 2002]. As shown before dexamethasone (1 μM) significantly ($P<0.01$) transrepressed the CBG promoter construct and indeed RU486 (20 μM) could relieve this repression (Fig. 4.6 compare lanes 3 and 4). The significant ($P<0.05$) transrepression by CpdA (10 μM) could also be relieved by RU486 (Fig. 4.6 compare lanes 5 and 6). In addition, CpdA (10 μM) added together with dexamethasone (1 μM) slightly increases, although not significantly

(P>0.05), the transrepression seen by dexamethasone alone (Fig. 4.6 compare lanes 3 and 7). Once again, RU486 could relieve the combined repression of CpdA and dexamethasone. These results therefore strongly suggest that repression of the ratCBG295Luc promoter reporter construct by dexamethasone as well as CpdA is mediated by the GR.

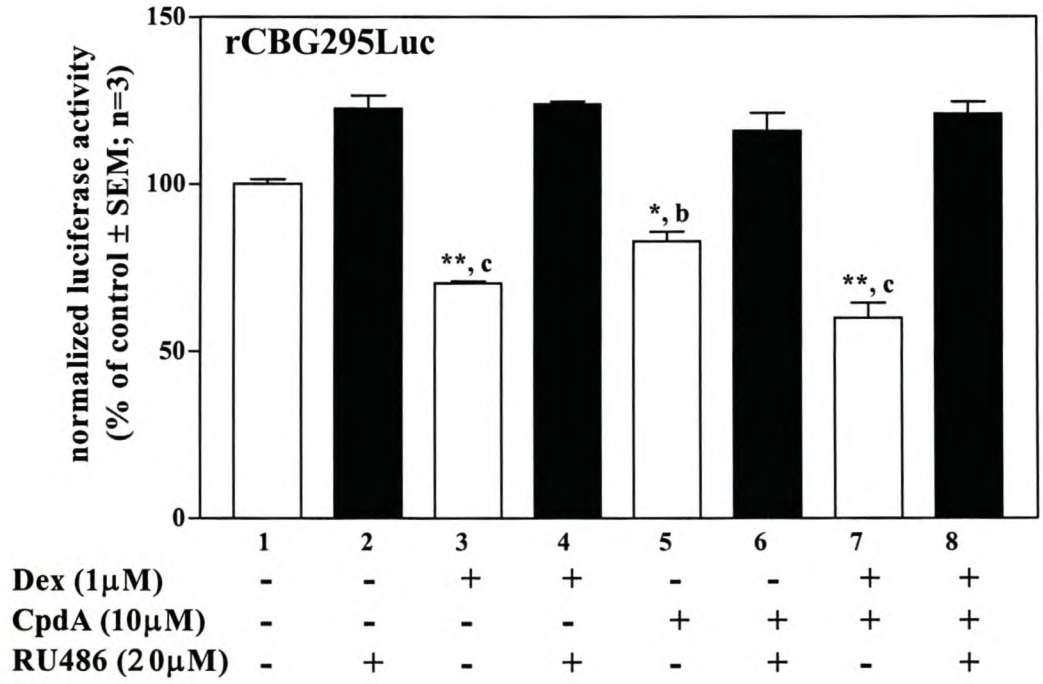


Figure 4.6. Effect of the glucocorticoid antagonist, RU486, on transrepression. A ratCBG295Luc promoter reporter construct was transiently transfected into mouse hepatoma cells (BWTG3). BWTG3 cells were cultured as described in materials and methods. Cells were transfected as described in the materials and methods and treated with the compounds as indicated. Control wells received an equal amount of ethanol. Luciferase values were normalized for β–galactosidase and values are plotted as a percentage of the average control. Statistical analysis was done to (i) compare values in presence of test compounds relative to the corresponding control (lanes 1 or 2) using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: P<0.05; **: P<0.01) and to (ii) compare values of each compound tested relative to transrepression in the presence of RU486 using a one-tailed unpaired t-test (b: P<0.01; c: P<0.001).

4.3.5 Regulation of CBG mRNA levels in hepatic cells.

To determine if the transcriptional regulation of the rat CBG promoter correlates with the regulation on mRNA level, the effect of dexamethasone, progesterone, MPA and CpdA was investigated on CBG mRNA levels in a human hepatoma cell-line (HepG2) in the absence (-hGR) and presence of transfected hGR (+GR) or hGR^{dim} (+hGR^{dim}) (Fig. 4.7) (Materials and methods section 6.6.1). The hGR^{dim} receptor was constructed by inserting the point mutation A458T into the GR dimerization domain (D-loop); this prevents dimerization and activation of GRE promoters [Reichardt *et al* 2001]. The effect of the test compounds was thus tested in the presence of hGR^{dim} to check for a dimerization requirement in transrepression of CBG mRNA [Reichardt *et al* 2001]. Transrepression may be mediated through direct binding of the GR to nGREs or via a tethering mechanism that does not require DNA binding by the GR. The first mechanism is reported to require dimerization of the GR while the latter mechanism may be mediated by monomeric GR [Schacke *et al* 2002a].

Dexamethasone resulted in a 23.1 ± 8.8 % inhibition of CBG mRNA levels in the absence of transfected hGR (endogenous) and a 19.5 ± 2.7 % inhibition in the presence of co-transfected hGR. The presence of transfected hGR therefore did not significantly ($P>0.05$) increase the percentage inhibition of CBG mRNA levels. Co-transfected hGR^{dim}, however, practically abolished dexamethasone repression and only resulted in a 1.3 ± 7.9 % inhibition of CBG mRNA levels. In spite of this, statistical analysis reveals that not only are none of the mRNA values significantly ($P>0.05$) repressed relative to the control values but that there is no significant ($P>0.05$) difference between the different GR conditions (Table 4.1) tested for dexamethasone.

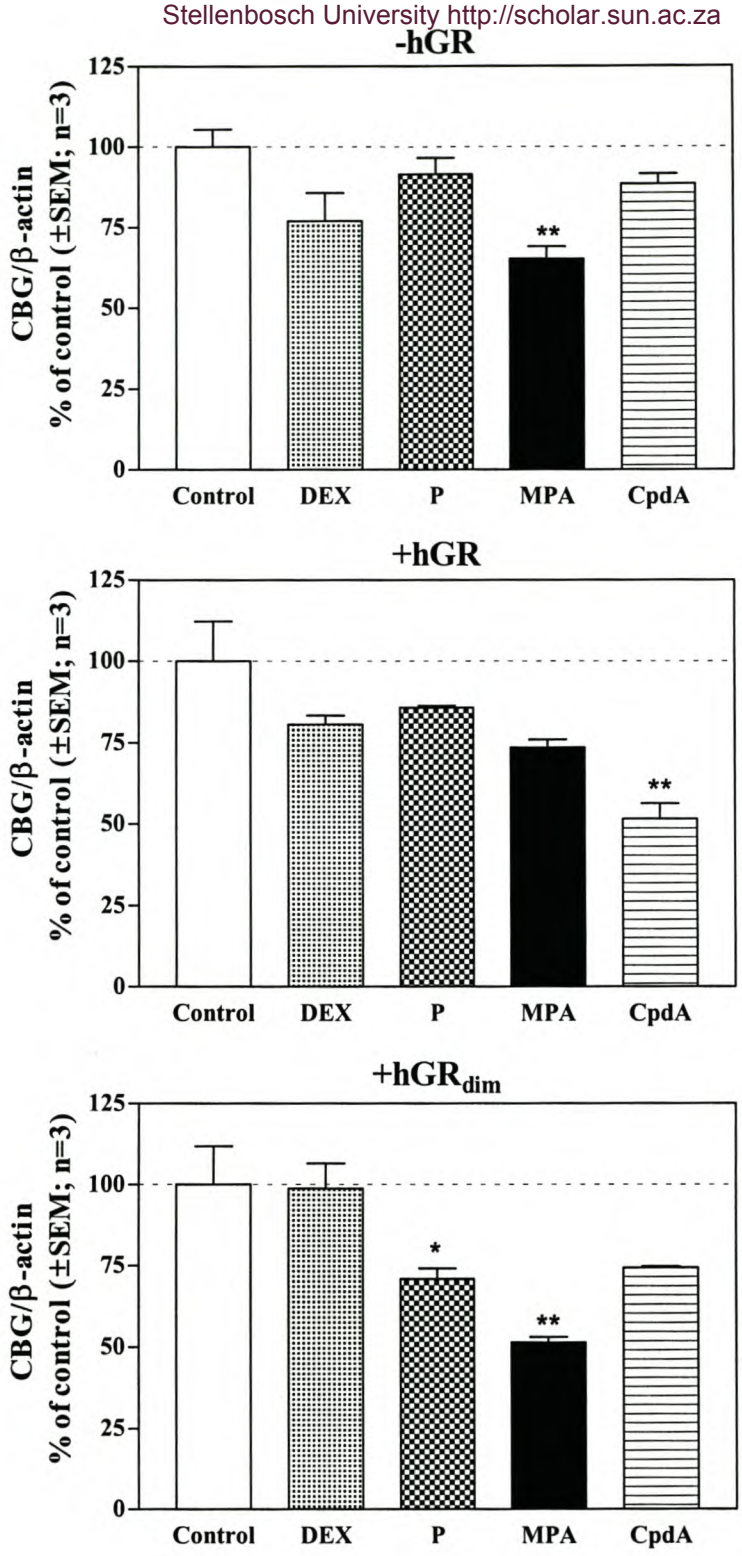


Figure 4.7. Regulation of CBG mRNA levels in HepG2 cells. The effect of dexamethasone (Dex), progesterone (P), medroxy-progesterone acetate (MPA) and Compound A (CpdA) on CBG mRNA levels was investigated in the absence (-hGR), and presence of transfected hGR (+GR) or hGR^{dim} ($+hGR^{dim}$) in a human hepatoma cell-line (HepG2). HepG2 cells were cultured as described in materials and methods. The concentration of dexamethasone added was 1 μM and of that of MPA, P and CpdA 10 μM. Statistical analysis was done to compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$).

Progesterone resulted in 8.5 ± 5.0 % inhibition in the absence of transfected hGR, a 14.4 ± 0.7 % inhibition in the presence of transfected hGR and 29.2 ± 3.2 % inhibition in the presence of transfected hGR^{dim}. Inhibition by progesterone was only significantly ($P<0.05$) different in the presence of transfected hGR^{dim}.

MPA, on the other hand, significantly ($P<0.01$) inhibited CBG mRNA levels in the absence of transfected hGR (34.7 ± 3.8 % inhibition) as well as in the presence of transfected hGR^{dim} (48.7 ± 1.7 % inhibition). Statistical analysis, however, indicate that although MPA in the presence of transfected hGR resulted in 26.5 ± 2.5 % inhibition this is not significantly ($P>0.05$) different from the control. Comparison and statistical analysis of the effect of the different GR constructs on repression by MPA (Table 4.1) shows that only hGR^{dim} differs significantly ($P<0.05$) from repression with endogenous hGR.

Table 4.1: Summary of percentage inhibition of CBG mRNA levels. Statistical analysis was done on results obtained in Fig. 4.7 to (i) compare values in the presence of test compounds relative to the corresponding control using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$) and to (ii) compare values without transfected GR (–rGRα) to values with transfected GR (+rGRα and hGR^{dim}) for each compound tested using a two-tailed unpaired t-test (a: $P<0.05$; b: $P<0.01$; c $P<0.001$).

	-hGR	**+hGR**	**+GR^{dim}**
Control	0 ± 5.4	0 ± 12.3	0 ± 11.8
DEX	23.1 ± 8.8	19.5 ± 2.7	1.3 ± 7.9
P	8.5 ± 5.0	14.4 ± 0.7	29.2 ± 3.2*
MPA	34.7 ± 3.8**	26.5 ± 2.5	48.7 ± 1.7**,[a]
CpdA	11.6 ± 3.1	48.5 ± 4.8**,[a]	25.7 ± 0.4

Cpd A caused a small and insignificant (11.6 ± 3.1 %) inhibition of CBG mRNA levels in the presence of endogenous hGR while CpdA in the presence of transfected hGR resulted in a significant ($P<0.01$) inhibition (48.5 ± 4.8 % inhibition) of CBG mRNA levels. The presence of transfected hGR^{dim} resulted in a 25.7 ± 0.35 % inhibition of CBG mRNA levels, which is not significantly different from the control. Comparison and statistical analysis of the effect of the different GR constructs on repression by CpdA (Table 4.1) shows that only co-transfection with wild type hGR and not hGR^{dim} differs significantly ($P<0.05$) from repression with endogenous hGR.

Previous results from the literature has shown that GCs had no effect on CBG mRNA levels in human liver cells (HepG2) [Emptoz-Bonneton *et al* 1997] although they decreased plasma levels in both rats and humans [Westphal 1971; Smith and Hammond 1992; Schlechte *et al* 1987] whereas progesterone and MPA decreased CBG mRNA levels in a human endometrial cancer cell-line (Ishikawa cells) [Misao *et al* 1994; Misao *et al* 1998] but had no effect on rat plasma CBG levels [Smith and Hammond 1992] or increased rat plasma levels [Westphal 1971]. We showed that co-transfection with rGR in BWTG3 cells increased repression of the CBG promoter (Fig. 4.3, solid bars) by all the test compounds, except progesterone, but transfection with hGR in HepG2 cells only increased repression of CBG mRNA levels by CpdA. Despite the fact that no significant repression was shown with CpdA in HepG2 cells (in the presence of endogenous GR) we found significant repression ($P<0.01$) of CBG mRNA levels in rat livers by CpdA (Fig. 4.8). This could support the idea that CBG mRNA levels may be differently regulated in different species but caution must be exercised in interpreting these results as we did not measure effects on CBG mRNA in BWTG3 cells nor did we examine promoter-reporter studies in HepG2 cells.

Although the effect of transfected GR^{dim} was investigated to ascertain a requirement or not for GR dimerization, the results are inconclusive. Despite the fact that dexamethasone and CpdA decreased CBG mRNA more in the presence of hGR than in the presence of GR^{dim} ($P<0.05$), this occurred within the background of endogenous GR. It has to be acknowledged that this experiment has certain limitations since the amount of endogenous GR present as well as the proportion of cells transfected with the above mentioned constructs is unknown. Thus the dexamethasone results of endogenous versus GR^{dim}, where transrepression is abrogated do not correspond to those with CpdA where transrepression is increased and interpretation is difficult. A possible hypothesis for these results are that dexamethasone may be acting via a dimer and CpdA acts via a monomer but this is a highly unlikely hypothesis. Dimerization requirements for transrepression of the CBG gene could be more convincingly tested by using the GR^{dim} mice constructed by Reichardt *et al* [Reichardt *et al* 2001]. In these mice, no wild-type GR would be present that could influence the results, like in the currrent sytem.

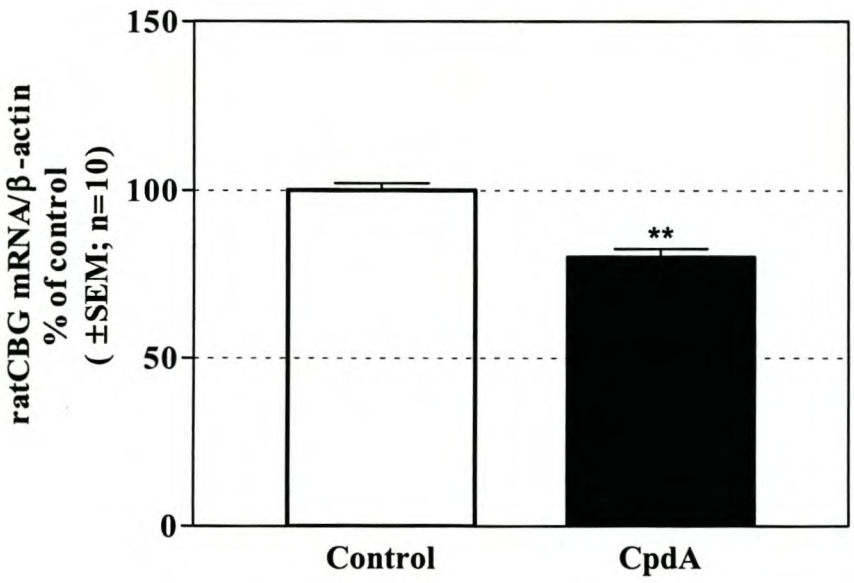


Figure 4.8. Regulation of rat CBG mRNA levels by CpdA. Adult male Wistar rats were injected with CpdA (see materials and methods for details). Rats were decapitated

and livers removed for RNA isolation. Statistical analysis was done to compare values in the presence of CpdA relative to the corresponding control (sedentary rats) using one-way ANOVA followed by Dunnett's multiple comparison's posttest (*: $P<0.05$; **: $P<0.01$).

4.4 DISCUSSION

High doses of synthetic GCs remain the most effective treatment of inflammatory or autoimmune diseases [Barnes 1998; Schacke *et al* 2002a]. GC therapy is used for reduction of inflammation and immune activation in asthma and allotransplantation as well as in allergic, collagen, rheumatoid, vascular, dermatological, inflammatory bowel and other systemic diseases [Schacke *et al* 2002a]. The use of GCs has increased dramatically over the years and in the United States alone about 10 million new prescriptions for oral corticosteroids are written each year.

The effects of GCs are mediated by the GR via activation and repression of gene expression. The desired anti-inflammatory effects of GCs are mainly mediated via transrepression of gene transcription. Some genes coding for anti-inflammatory proteins are induced by the GR via GR-DNA interactions [Schacke *et al* 2002a]. However, most pro-inflammatory genes like cytokines, chemokines and adhesion molecules are regulated by the transcription factors NF-κB and AP-1 and GR causes repression of the expression of these pro-inflammatory proteins by interacting, probably as a monomer [Heck *et al* 1994], with the transcription factors NF-κB and AP-1, thus inhibiting their activity [Barnes 2001]. It has been hypothesized that the above transrepression mechanism mediated by the GR monomer via GR-protein interactions is sufficient to achieve most anti-inflammatory effects [Schacke *et al* 2002a]. A large number of side-effects are, however, associated with the long-term use of GCs [Schacke *et al* 2002a]. Though most side effects are mediated via GR transactivation and DNA-binding of GR

dimers (diabetes mellitus, glaucoma) recent results suggest that some side-effects are mediated via transrepression (skin atrophy, suppression of HPA axis). In addition, the precise molecular action of some side effects is not known or may be due to both transactivation and transrepression (osteoporosis). Despite the above caveats it is still proposed that novel GCs that can dissociate between transrepression and transactivation may produce drugs with an improved therapeutic profile [Adcock 2000].

In the present study the dissociative GC activity of MPA and CpdA was investigated and compared to GCs in hepatoma cell-lines. Compared to GCs which strongly transactivate the GRE-dependent promoters, MPA and Compound A had no effect in the presence of endogenous GR. Co-transfection with GR, however, increased transactivation by MPA while CpdA did not transactivate the GRE-containing promoters even at 10 μM and in the presence of co-transfected GR. The second aspect of dissociative GC activity, which is transrepression, was investigated by studying the effect of these compounds on the CBG proximal promoter as well as on CBG mRNA levels. The rationale for this was that CBG plays an integral role in the disposition of both endogenous and administered glucocorticoids and thus any investigation of potential dissociated drugs should address effects on CBG. In addition, De Bosscher has dealt with the obvious investigation of effects on inflammatory genes. She found that CpdA exerts anti-inflammatory potential by down-modulating TNF-induced IL-6 gene expression as well as other NF-κB and AP-1 regulated genes, but that, as we found, did not activate GRE-driven promoters [De Bosscher 2003 unpublished results]. In the present study we found that, like dexamethasone and hydrocortisone, both CpdA and MPA transrepress the rat CBG proximal promoter. CpdA represses with a similar potency but with a higher efficacy than MPA. Dexamethasone, progesterone, MPA and

CpdA slightly decreased CBG mRNA levels in HepG2 cells in the presence of endogenous GR, which does not correspond well with the significant repression of the CBG promoter by these compounds. Co-transfection with GR in BWTG3 cells increased repression of the CBG promoter by these compounds but only increased repression of CBG mRNA levels by CpdA in HepG2 cells. Although the discrepancy between promotor and mRNA results may only be a matter of statistics, which may be corrected if more experiments are done, another possibility exists. The promoter experiments were performed in a mouse hepatoma cell-line (BWTG3) whereas the mRNA experiments were done in a human hepatoma cell-line (HepG2) and thus GC regulation may differ due to species differences. The amount of receptors, co-activators and co-repressors may also differ between these two cell-lines, which might be a reason for the different effects observed. However, we did in Chapter 3 show significant repression of CBG mRNA levels by dexamethasone in HepG2 cells and thus would need to expand the current study to definitively answer the question of whether dexamethasone significantly represses CBG mRNA levels in HepG2 cells.

Bamberger *et al* showed that MPA marginally transactivates in the presence of endogenous GR and, like us, found that co-transfection with GR significantly increased transactivation [Bamberger *et al* 1999]. In their study MPA displays a transrepression/transactivation ratio of 6.6 relative to dexamethasone (ratio of 1), which indicates significant dissociated properties [Bamberger and Schulte 2000]. MPA displays a transrepression/transactivation ratio of 0.8 for the $(GRE)_2$tkLuc and 1.1 for the $(GRE)_2$50hIL6Pluc reporter constructs in the presence of co-transfected GR relative to dexamethasone (ratio of 1) in this study, which suggests that it is significantly less dissociated in our system than in the one Bamberger describes. In another study MPA

repressed IL-6 and IL-8 promoter reporter constructs, as well as a GRE-containing promoter construct in L929sA cells and thereby exhibiting no dissociative GC properties [Koubovec *et al* unpublished results]. While both MPA and CpdA transrepress the CBG proximal promoter, CpdA, does not activate GRE-containing promoters on its own in either of the cell-lines tested, which makes it a better dissociative GC than MPA although it does preclude the calculation of a dissociation ratio. This compound is therefore a potential non-steroidal anti-inflammatory agent.

Although it is clear that CpdA cannot transactivate in either cell line investigated it is interesting to note that it can modulate dexamethasone transactivation of the GRE-containing promoters (Fig. 4.5). This effect, however, depends on the order of addition of CpdA. If dexamethasone was added together with CpdA, CpdA has no effect on dexamethasone transactivation but if CpdA was added before dexamethasone, CpdA decreased dexamethasone transactivation in both cell-lines. Binding of CpdA to the GR has been shown to be reversible, and therefore it can be concluded that CpdA does not covalently modify the receptors and thus prevent binding of other compounds to the GR [unpublished results]. A possible, albeit revolutionary, hypothesis to explain this effect is that CpdA may be accommodated in the ligand-binding pocket together with dexamethasone, only when CpdA is added before dexamethasone, but not when they are added together. This may then result in a liganded-GR that is unable to transactivate. GR antagonists such as RU486 and ZK 98,299 have a bulky side chain at position C11beta [Modarress *et al* 1997] that is similar in size to CpdA and thus it is conceivable that both compounds could be accommodated in the ligand-binding pocket. The structures of dexamethasone, ZK 98.299 and RU486 are shown in Fig. 4.9. This hypothesis presumes that dexamethasone has a higher affinity for the GR than CpdA

and that once bound to the GR cannot accommodate CpdA binding. Thus when CpdA and dexamethasone are added together, CpdA does not significantly compete with dexamethasone for binding to the receptor and does not reduce dexamethasone transactivation as does MPA and progesterone. In addition, the hypothesis presumes that CpdA bound GR can accommodate dexamethasone binding without displacement of already bound CpdA. Thus, if CpdA is added first, dexamethasone is not present to compete for binding and a greater proportion of the receptors would be occupied by CpdA. The CpdA-bound GR could then subsequantly bind to dexamethasone. It may be that there would be a requirement for the CpdA-occupied receptor to have a greater affinity for dexamethasone that the unoccupied recptor. To sum up the hypothesis assumes that when CpdA and dexamethasone are added together a much smaller percentage of the liganded receptors accommodate both ligands together than when CpdA is added before dexamthsone.

Another possible hypothesis may be that CpdA is binding to a second binding site on the GR. Compounds like ketoconazole, cortivazol, ursodeoxycholic acid, β-Lapachone, carbenoxolone and free fatty acids have been proposed to bind to a second binding site on the GR [Suthers *et al* 1976; Yeakly *et al* 1980; Schmidt *et al* 1984; Svec 1988; Svec *et al* 1989; Srivastava and Thompson 1990; Valette *et al* 1991; Tanaka and Makino 1992; Soro *et al* 1997; Miura *et al* 2001; Yoshikawa *et al* 2002]. Once again some presumptions have to be made to accommodate the hypothesis: firstly that once dexamethsone is bound CpdA cannot bind to the second binding site and secondly that once CpdA has bound dexamethasone can still bind to the GR LBD. Interestingly, ketoconazole has been shown to inhibit P450c20 [Santen *et al* 1983], whereas CpdA inhibits P450c11 [Louw *et al* 1997]. Both hypotheses suggested are controversial and

we have little evidence at present to support them so they should be treated with caution until additional evidence is provided.

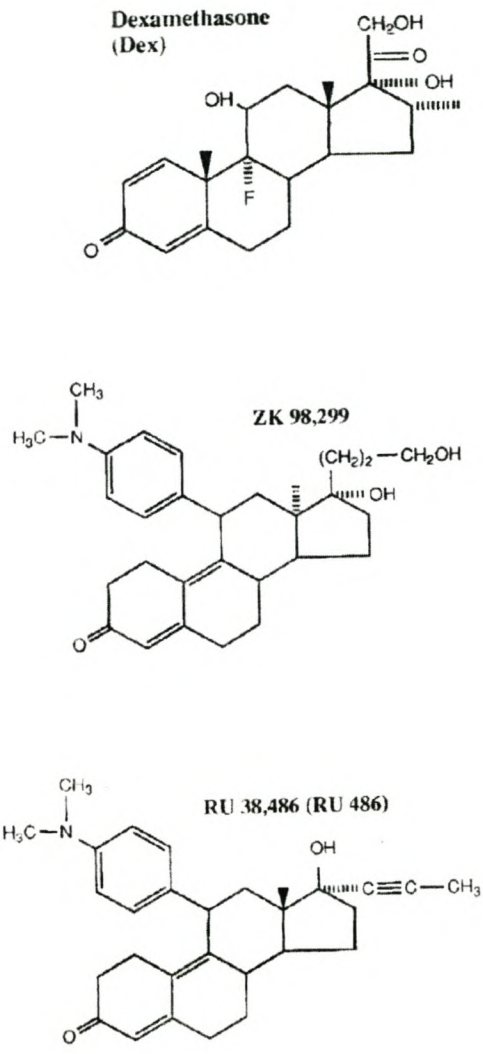


Fig. 4.9. Structures of dexamethasone, ZK 98.299 and RU486.

Progesterone significantly transactivated in BWTG3 cells but not in HepG2 cells whereas MPA transactivation relative to dexamethasone in BWTG3 cells was higher than in HepG2 cells. This is unlikely to be due to higher levels of GR in BWTG3 cells as dexamethasone activation relative to control was higher in HepG2 cells. MPA has been shown to bind to both the GR and PR [Pridjian *et al* 1987; Kontula *et al* 1983].

Thus the higher transactivation by MPA in BWTG3 cells may be due to the presence of PR in this cell-line transactivating via the GRE [Song *et al* 2001]. MPA also altered dexamethasone transactivation of the GRE-containing promoter by decreasing dexamethasone transactivation in both cell-lines. These two ligands, dexamethasone and MPA, can compete for binding to the GR and thereby explain the decrease in dexamethasone transactivation of the GRE-containing promoter in the presence of MPA. Not only is the affinity of MPA for the human [Kontula *et al* 1983] and rat [Pridjian *et al* 1987] GR receptors between 2-10 times less than for dexamethasone but MPA has also been shown to be much less potent than dexamethasone in activating HRE-tk-luciferase activity through the GR [Zhang *et al* 2000]. As already suggested the difference in the progesterone results between BWTG3 cells and HepG2 cells may be due to the presence of more PR in BWTG3 cells as both the PR and GR can bind and activate transcription from the same HRE [Song *et al* 2001]. Less obvious to explain is that progesterone like MPA decreased dexamethasone transactivation of the GRE-containing promoter in both cell-lines.

Two possible explanations are put forward. Firstly, in BWTG3 cells, where PR is proposed to be present, a possible explanation could be that it has been shown that steroid receptors can compete for coactivators and are differentially affected by corepressors. Progestin antagonism of GR-mediated transcription from the MMTV-promoter has for example been shown to be due to competition for the BRG-1 chromatin remodeling complex [Deroo and Archer 2002]. Thus, the competition between the PR and GR for binding coactivators in cells that express both of these receptors can decrease dexamethasone transactivation. It has also been shown that changes in the concentration of the corepressors NcoR and SMRT can modify the

transcriptional properties of the GR differently from the PR [Song *et al* 2001]. Secondly, in an attempt to explain the decrease of dexamethasone repression by progesterone in the HepG2 cells that we have argued does not contain PR we have to resort to a more controversial proposal: that progesterone modulated GR activity via a second binding site on the GR. Progesterone has been shown to increase the dissociation rate of dexamethasone-receptor complexes in liver and kidney cytosol [Suthers *et al* 1976; Svec *et al* 1989]. It was postulated that GC receptors have two classes of binding sites and that occupation of the second binding site increased the dissociation rate of agonist from GR. Alternatively progesterone may be activating directly via the GR as it was recently shown that progesterone and cortisol antagonism of the prostaglandin dehydrogenase gene may not require PR but that progesterone could act via the GR [Patel *et al* 2003].

Based on the molecular mechanisms of GC-mediated action, it has been hypothesized that activation of the GR with compounds that induce a predominant induction of transrepression over transactivation, should lead to the majority of the anti-inflammatory effects of GCs with less side effects. A novel class of synthetic GCs has been reported to differentiate between transactivation and AP-1 or NF-κB transrepression [Vayssiere *et al* 1997; Vanden Berghe *et al* 1999]. Belvisi *et al* investigated these compounds, specifically RU24858, *in vivo*. RU24858 was shown to be dissociative *in vitro* in human HeLA cells and rat hepatoma cells but did not show a separation between anti-inflammatory and side-effects *in vivo* in rats [Belvisi *et al* 2001]. Specifically, this compound exhibited comparable anti-inflammatory properties to standard steroids in the sephadex model of lung edema but decreased osteocalcin levels, indicating inhibition of bone turnover in rats [Belvisi *et al* 2001]. This suggests

that it is crucial to confirm any *in vitro* data of dissociative compounds with in *vivo* testing as *in vitro* separation of transrepression from transactivation does not necessarily translate to an increased therapeutic ratio for GCs. Alternatively it may suggest that more of the side-effects than thought are a result of transrepression rather than transactivation.

Non-steroidal ligands for GR have previously been reported. Miner *et al*, for example, reported that a selective, non-steroidal GR antagonist, AL082DO6 (DO6), inhibits GC-mediated transcriptional regulation, partially blocks GR translocation to the nucleus and completely blocks DNA binding by the receptor [Miner *et al* 2003]. This compound has a high affinity for the GR, binds competitively with other known GR ligands and may occupy the same hydrophobic pocket in the LBD as steroids. The receptor is in an antagonist conformation when bound to DO6 and this conformation is different from that elicited by both the partial antagonist (RU486) and the full agonist (dexamethasone) [Miner *et al* 2003]. Another non-steroidal ligand, with high affinity for the GR, A276575 repressed IL-1 induced IL-6 production and antagonised transactivation of a MMTV GRE reporter construct [Lin *et al* 2002]. Schacke *et al* also reported that non-steroidal ligands that bind to the GR showed a good separation between transactivation of a TAT GRE reporter construct and transrepression of IL-8 protein [Schacke *et al* 2002b]. Recently a non-steroidal GR ligand that does not dissociate but rather selectively activates or represses GR-regulated genes was described [Coghlan *et al* 2003]. To distinguish these compounds from steroidal dissociated GCs the term selective GR agonists (SEGRAs) was introduced and refers to compounds that are non-steroidal, partially displays the molecular effects of GCs and are dissociated in their clinical profile [Schacke *et al* 2002b]. Coghlan *et al* show that AL-438, a non-steroidal

GR ligand, is able to selectively repress and activate genes normally regulated by GCs [Coghlan *et al* 2003]. This compound was also tested *in vivo* and retains full anti-inflammatory efficacy and potency compared to steroids but has less negative effects on bone metabolism and glucose control. This is thus not a classical dissociated compound in dissociating transactivation from transrepression but rather a selective GR modulator that both transactivates and transrepresses GR-regulated genes but in a tissue-specific manner. Coghlan proposes that the selectivity of the ligand action is based on the structural changes in the receptor that influences co-activator interactions and that for AL-438 this reduces the ability of the GR to bind to the co-factor peroxisomal proliferator-activated receptor γ coactivator-1 (PGL-1), a co-factor critical for steroid-mediated glucose up-regulation in the liver, while maintaining normal interaction with GRIP-1 [Coghlan *et al* 2003]. The rate-limiting enzyme in the gluconeogenic pathway, phosphoenolpyruvate carboxy kinase, requires the action of PGC-1 to effectively respond to GCs.

The search for new dissociated glucocorticoids has up to now focused on the transrepressive effects on NF-κB and AP-1 mediated gene regulation [Dumont *et al* 1998; Adcock 2000; Vayssierre *et al* 1997; Vanden Berghe *et al* 1999; Bamberger *et al* 1999; Miner *et al* 2003; Coghlan *et al* 2003]. The results of this study showed that GCs regulate the CBG promoter and thus it may be important to consider the effect of these dissociative GCs on CBG function for full evaluation of these drugs. The primary role of CBG is to regulate the bioavailability and metabolic clearance of GCs [Westphal 1983] and synthesis of CBG, a negative acute phase protein, is down-regulated in response to injury or infection [Beishuizen *et al* 2001]. In addition, there is also growing support for CBG playing a more active role in mediating physiological responses

[Breuner and Orchinik 2002]. Thus it may be important to consider the effect of these dissociative GCs on CBG function for complete assessment of these drugs.

Presently, drug finding programs are developing dissociative GCs based on the differential molecular regulation of the major anti-inflammatory actions of GCs and their side-effects. Schacke *et al* reviewed the mechanisms involved in the side-effects of GCs, which are complex and not completely understood [Schacke *et al* 2002a]. This study has shown that *in vitro* CpdA can completely dissociate between transactivation and transrepression, which makes it an even better dissociative GC than MPA. According to Schacke's criteria CpdA could thus be classified as a SERGA but *in vivo* data is needed to confirm this. Up to now steroidal and non-steroidal ligands have been described that seems to dissociate between activation and repression *in vitro* [Vayssiere *et al* 1997; Belvisi *et al* 2001; Bamberger *et al* 1999]. *In vivo* tests showed that although they are effective anti-inflammatory agents, they do not always have a useful side-effect profile. At this stage it is unclear if simple dissociation of activation from repression in a ligand will result in a beneficial therapeutic profile as evidenced by the results of Coghlan and co-workers with AL-438 [Coghlan *et al* 2003]. Thus in addition to *in vivo* function more information is also needed on the mechanism of action of CpdA. A priority is to establish if indeed CpdA acts via the GR. The fact that RU486 reverses transrepression and co-transfection of GR enhances transrepression of the CBG promoter by CpdA is preliminary proof that CpdA is working through the GR. This can be investigated further by testing if CpdA binds to the GR, if CpdA bound to receptor can translocate to the nucleus or whether transrepression of the CBG promoter by CpdA is mediated by the GR forming protein-protein interactions with other transcription factors and thus acts via a GR monomer. In addition, to further elucidate the mechanism

of action of CpdA, the importance of the functional domains of the GR in mediating repression by CpdA can also be investigated (discussed in the introduction chapter section 2.1.2.1.3), as could the effects of CpdA on the kinetics of nuclear translocation (discussed in the introduction chapter section 2.1.2.2.4). If indeed CpdA is found to be completely dissociated, not only *in vitro* but also *in vivo*, it could be used as a drug or lead compound with less side-effects for the treatment of inflammatory and autoimmune diseases. In addition, an understanding of the means whereby CpdA elicits its effects through GR should expand our knowledge of the molecular mechanisms involved in GR-mediated gene regulation.

CHAPTER 5

Conclusion

Since 1948, it has been known that hydrocortisone (cortisol), the primary GC produced by the adrenal cortex, when administrated pharmacologically, has potent anti-inflammatory and immunosuppressive effects [Asadullah *et al* 2002]. Major progress in discovering the underlying molecular mechanisms of this effect has only been made in the last 10-15 years. Currently GCs are standard therapy for disorders like rheumatoid arthritis, allergic reactants, asthma, connective tissue diseases, psoriasis, vasculitis, inflammatory bowel disease and eczema [Schacke *et al* 2002a]. However, during prolonged treatment with GCs, their beneficial effects are overshadowed by side-effects like osteoporosis, skin atrophy and diabetes [Schacke *et al* 2002a]. Despite these complications, prednisone and dexamethasone are routinely used for a wide range of inflammatory conditions [Miner *et al* 2002]. Therefore, scientists are searching for drugs that separate the beneficial effects of GCs from their undesired effects.

Elucidation of the molecular mechanisms of GC-mediated action led to a hypothesis that activation of the GR with compounds that produce a predominant induction of transrepression over transactivation, should lead to the majority of the anti-inflammatory effects of GCs with less side effects, as the major side-effects are mediated via transactivation. Genes coding for anti-inflammatory proteins are induced by the GR via a GR-DNA interaction whereas the major pro-inflammatory genes coding for cytokines, chemokines and adhesion molecules are regulated by the transcription factors AP-1 or NF-κB [Barnes 2001]. In this case the GR interacts as a monomer with these transcription factors, inhibiting their activity and thereby repressing expression of pro-inflammatory proteins. Synthetic GCs that differentiate between transactivation and AP-1 or NF-κB transrepression *in vitro*, but not *in vivo*, have been reported [Vayssiere *et al* 1997; Vanden Berghe *et al* 1999; Belvisi *et al* 2001]. Currently, drug screening

programs are developing dissociative GCs based on the differential molecular regulation of the major anti-inflammatory actions of GCs and their side-effects [Schacke *et al* 2002a]. The term selective GR agonists (SEGRAs) distinguishes steroidal dissociated GCs from compounds that are non-steroidal, partially display the molecular effects of GCs and are dissociated in their clinical profile [Schacke *et al* 2002b]. Different groups have recently identified several promising SEGRAs. Schacke e*t al* analysed compounds with an *in vitro* dissociation between transactivation and transrepression in rodents [Schacke *et al* 2002b]. As a parameter for anti-inflammatory activity they measured the inhibition of ear edema and both classical GCs and dissociative GCs was very effective. They reached a similar efficacy compared to GCs after treatment of mice and slight differences in potency were observed. These dissociated GCs showed a good separation between inhibition of edema and TAT induction in contrast to classical GCs. Belvisi *et al* focussed on a lung edema model in rats to demonstrate anti-inflammatory activity and their compound showed a potency better than GCs *in vivo* but was as effective as GCs in induction of side-effects [Belvisi *et al* 2001]. Coghlan *et al* described what appears to be a true SEGRA. They showed that AL-438, a non-steroidal GR ligand, selectively represses and activates genes normally regulated by GCs [Coghlan *et al* 2003]. Anti-inflammatory activity was measured in rats using the carrageeman-induced paw edeema assay, which is a model of acute inflammation, as well as a more chronic model of inflammation, the adjuvant-induced arthritis [Coghlan *et al* 2003]. AL-438 retains full anti-inflammatory efficacy and potency compared to steroids but has less negative effects on bone metabolism and glucose control. Although these results are very encouraging to definitively state that a compound could dissociate between transactivation and transrepression, micro-array or proteomic screens involving GC responsive genes should be done [Komamura *et al* 2003].

In the present study we investigated and compared the dissociative GC activity of the steroidal compound, MPA, and the non-steroidal compound, CpdA, relative to GCs by examining transactivation of GRE-containing reporter constructs and transrepression of CBG gene expression in hepatic cell lines. Results showed that MPA, but not CpdA, only transactivates GRE-containing promoters in the presence, but not in the absence, of co-transfected GR. As CBG transports and modulates the bioavailability of glucocorticoids and progesterone in plasma, we studied the second aspect of dissociative GCs, namely transrepression, by examining effects on the CBG promoter [Hammond *et al* 1990b; Hammond *et al* 1991; Seralini 1996]. Only the free fraction of steroid hormones is biologically active and able to diffuse across the plasma membrane of target tissues. This implies that any changes in the levels of CBG would modify the distribution of steroids to target tissues [Westphal 1986; Mendel 1989]. Several factors have been shown to influence CBG production. Exercise and stressful conditions such as malnutrition and restraint decrease CBG levels in rats resulting in an increase in free GC levels [Duclos *et al* 2003; Peijie *et al* 2003; Tinnikov 1999; Lesage *et al* 2002]. In this study we also investigated the effect of various physical stressors on rat liver CBG mRNA levels as well as the effect of several hormones known to influence CBG production on CBG mRNA and protein levels in a human hepatic cell line. Voluntary running had no effect on rat CBG levels but involuntary swimming and immobilization decreased rat CBG mRNA levels, which corresponds to the observed decrease in circulating plasma CBG levels during exercise stress reported in the literature [Tinnikov 1999; Lesage *et al* 2002]. To support these findings we also showed that GCs decreased CBG mRNA and protein levels in a human hepatoma cell-line.

Although both the human and rat CBG genes (*Cbg*) have been cloned no work has been done to identify possible cis-acting sequence elements involved in their regulation by hormones [Underhill and Hammond 1989; Underhill and Hammond 1995]. DNase I footprinting of the rat CBG proximal promoter identified five protein-binding sites (P1-P5) within 236 bp immediately 5' of the transcription start site with rat liver nuclear extract which resemble recognition sequences for HNF-1, CP-2, DBP, HNF-3 and C/EBP or NF/IL6, respectively [Underhill and Hammond 1995]. EMSAs confirmed that footprint one (P1) binds to HNF-1β and footprint two (P2) binds to CP2 [Underhill and Hammond 1995; Zhao *et al* 1997]. Even though CBG transcription has been shown to be regulated by steroid hormones [Westphal 1972; Smith and Hammond 1992; Misao *et al* 1998; Coolens *et al* 1987] no hormone response elements seem to be present in the rat or human CBG proximal promoter. This suggests that the steroid receptors might be acting through a tethering mechanism without direct DNA binding. The GR may thus repress transcription via protein-protein interactions with other transcription factors rather than by directly binding to nGREs. The present study is the first that investigated the effect of GCs on the CBG proximal promoter. Results showed that hydrocortisone and the synthetic GC, dexamethasone, repressed the full-length, ratCBG1200Luc, proximal promoter reporter construct and the truncated ratCBG295Luc promoter reporter construct as well as the second construct stably transfected into BWTG3 cells. MPA and CpdA, like GCs, repress both the ratCBG1200Luc promoter reporter construct and the truncated ratCBG295Luc promoter reporter construct. Although MPA and CpdA both repress the CBG promoter, CpdA transrepressed the CBG proximal promoter with a similar potency but with a higher efficacy than MPA.

Further examination of glucocorticoid responsiveness on the CBG proximal promoter, showed that dexamethasone transrepressed both the ratCBG1200Luc and ratCBG295Luc promoter constructs whereas the ratCBG145Luc construct was unaffected. This indicates that the protein-binding sites for DBP, HNF3 and C/EBP present in the ratCBG295Luc construct but not in the ratCBG145Luc construct may be involved in the glucocorticoid responsiveness of the CBG proximal promoter. The transcription factors HNF-3 and C/EBP or NF-IL6 have been reported to form protein-protein interactions with the GR [Boruk *et al* 1998; Rabek and Papaconstantinou 1999; Wang *et al* 1999; Hocke *et al* 1992] and we have showed that DBP, HNF3 en C/EBP all partially inhibit transactivation of a GRE-containing promoter by dexamethasone. Thus DBP, HNF-3 and C/EBP or NF-IL6 appears to be likely candidates for mediating the repressive effect of GR on the CBG promoter. Additional work is, however, needed to determine the precise mechanism of action involved as well as which of the three transcription factors identified in this study are specifically responsible for the GR-mediated regulation of the CBG proximal promoter. Future experiments could include site-directed mutagenesis to mutate the DBP, HNF3 and C/EBP binding sites, which may identify, which one of these transcription factors is involved in GC repression of the CBG promoter. By using EMSAS it can also be confirmed if the transcription factor involved in repression of the CBG promoter forms protein-protein interaction with the GR. For example, using EMSAS, the GR has been shown to suppress the binding of C/EBPβ on the rainbow trout ER promoter by protein-protein interactions [Lethimonier *et al* 2002]. In addition, further study of upstream modulators is warranted as we found that the ratCBG1200Luc construct was transrepressed to a greater extent by dexamethasone than the ratCBG295Luc construct, which suggests that transcription factors further upstream of the ratCBG295Luc may also be involved in modulating

transrepression by glucocorticoids. The full-length CBG proximal promoter also needs to be sequenced to identify the binding sites upstream of the ratCBG295Luc construct that contributes to repression of the CBG promoter. The present study has, however, for the first time provided information on hormonal regulation of the CBG proximal promoter.

Other hormones known to regulate CBG mRNA levels include progesterone, E2, and insulin. CBG mRNA levels were shown to be suppressed by progesterone but stimulated by E2 in a human endometrial cancer cell-line, Ishikawa, whereas insulin was shown to decrease CBG mRNA levels in a human hepatoma cell-line, HepG2 [Misao *et al* 1998; Crave *et al* 1995]. The present study has shown that this regulation occurs at the level of the CBG promoter, as progesterone and insulin decreased, while E2 increased transcription from the ratCBG295Luc promoter reporter construct. Despite the fact that our mRNA experiments were done in a human hepatoma cell line (HepG2) and the promoter experiments in a mouse hepatoma cell line (BWTG3) and that results from the literature are also from different cell lines and species our promoter studies correlate nicely with the regulation of CBG mRNA levels by these hormones reported in the literature as well as with our own mRNA results.

It is important to test the effect of dissociative GCs compared to standard GCs on CBG expression as standard GCs decrease CBG expression resulting in an increase in the amount of active, free GCs available to target tissues. Repression of CBG levels may thus act to amplify the effects of administered GCs. In addition, CBG has recently been proposed to play a more active role in the stress response that goes beyond its function of regulating the free GC concentration by acting as a GC reservoir [Breuner and

Orchinik 2002]. In this regard it has been suggested that CBG may be involved in the targeted release of GCs to specific inflammation sites [Hammond *et al* 1990b], that extra-hepatic CBG may act to modulate GC action locally [Berdusco *et al* 1995], and that CBG may have intrinsic biological activity through eliciting an intracellular response via its membrane receptor [Nakhla *et al* 1988; Strel'chyonok and Avvakumov 1991]. We submit therefore, that to evaluate the full physiological effect of dissociative GCs it may be essential to investigate their effect on CBG expression.

To conclude, the results of this study showed that MPA displays partial dissociation between transactivation and transrepression whereas the non-steroidal CpdA, *in vitro*, can completely dissociate between transactivation and transrepression. According to the criteria for SEGRA's, CpdA could thus be classified as such but *in vivo* data is needed to confirm this. If CpdA is found to be completely dissociative *in vitro* and *in vivo* with less side effects than conventional GCs, it could possibly be developed as a drug for the treatment of anti-inflammatory and auto-immune diseases.

Further investigation into the mechanisms of GC-mediated effects, especially their side-effects is needed. Analyzing the side-effects of standard GCs compared to SEGRAs in $GR^{dim/dim}$ mice will help in understanding the molecular mechanisms of side-effect induction [Schake *et al* 2002a]. Most importantly, *in vivo* investigations and first clinical trials will demonstrate the safety and efficacy profile of SEGRAs and will also contribute to a better understanding of the molecular basis of GC-mediated effects.

CHAPTER 6

Materials and Methods

6.1 Materials

Dexamethasone, hydrocorticosterone, MPA, progesterone, E2, insulin, and mifepristone (Ru486) were obtained from Sigma chemical Co (South Africa). The aziridine precursor Compound A, was synthesised as described previously [Louw *et al* 1997] and the structure and purity was verified by NMR and electrospray-mass spectrometry (ESMS). The recombinant mIL-6 was provided by G. Haegeman (Department of Molecular Biology, University of Gent-VIB).

6.2 Plasmids

$(GRE)_2$50hIL6PLuc was constructed by inserting a glucocorticoid-responsive element with protruding Bg1II-PstI ends, into the multicloning site of p50hIL6Pluc [De Bosscher *et al* 2000]. The $(GRE)_2$tkLuc construct and the rat GRα (pSVGR1) expression vector was kindly provided by S. Okret (Department of Medical Nutrition, Karolinska Institute, Sweden). The rat GRα (pSVGR1) expression vector was constructed by Miesfeld *et al* (UCSF) and contains the SV40 enhancer/early promoter region, the coding region of the rat GRα cDNA and the SV40 polyA region [Miesfeld 1986]. The human GR (pRS-hGRα) was a kind gift from R.M. Evans (Howard Hughes Medical Institute) and the GR^{dim} construct from G. Haegeman (Department of Molecular Biology, University of Gent-VIB, Belgium). Various rat CBG-luciferase reporter plasmids as well as hCBG and rCBG cDNA were kindly provided by G.L. Hammond (London regional Cancer Centre, London) of which the rat CBG-295Luc construct contains the five protein-binding sites identified by DNase I footprinting [Underhill and Hammond 1995]. The β-galactosidase reporter plasmid (pPGKβGopbA) constitutively expressing a neomycin-resitant/β-galactosidase fusion protein under the

control of the pPGK promoter from the mouse houskeeping enzyme, 3-phosphoglycerate kinase was a gift from P. Soriano (Fred Hutchinson Cancer Research Centre, Seatle, WA). The hepatocyte nuclear factor-3 (HNF-3α) construct was provided by Eseng Lai (Memorial Sloan-Kettering Cancer Center, New York), the D-site binding protein construct (pBS-DBP) by Pascal Gos (Department of Molecular Biology, University of Geneve, Switzerland) and the CAAT/enhancer binding protein construct (C/EBPβ) by Weihua Xiao (NCI-Frederick Cancer Research and Development center, Frederick, USA). PGL2 basic vector was a kind gift from D. Harnish (Womens's Health Research Institute, Wyethayerst labs, Pensylvania). β–actin cDNA was provided by H. Okayama.

6.3 Preparation of test substances

Steroids were dissolved and diluted in ethanol and added to cells with medium. Only ethanol was added to control groups. Compound A (CpdA), prepared just before addition, was dissolved and diluted in ethanol, sonicated until fully dissolved and kept on ice before adding to cells. The final concentration of ethanol added to cells was 0.001%. Insulin and IL-6 were directly diluted in serum-free medium.

6.4 Tissue culture

Dulbecco's modified eagle medium (DMEM) and penicillin-streptomycin (P/S) solution were purchased from GIBCO, L-glutamine from Sigma and fetal calf serum (FCS) from Sterilab. The mouse hepatoma (BWTG3), obtained from G. Haegeman (Department of Molecular Biology, University of Gent-VIB), and the human hepatoma (HepG2), obtained from the Medical research council (MRC, Tygerberg, South Africa), cell-lines

were grown in Falcon T150 and T75 flasks at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin (40 000 U/L) and L-glutamine (30 g/L).

6.5 Protein and mRNA studies:

6.5.1 Hormone inductions, protein and RNA isolations from HepG2 cells

For the induction experiment in chapter 2, $5x10^5$ HepG2 cells were plated in 5 ml DMEM supplemented with 10% fetal calf serum (FCS), penicillin- streptomycin (40 U/ml) and L-glutamine (30 g/L) in T25 flasks. The next day medium was replaced with or without 10% FCS. In chapter 3, $2.5x10^5$ HepG2 cells were plated in 3 ml DMEM supplemented with 10% fetal calf serum (FCS), penicillin- streptomycin (40 U/ml) and L-glutamine (30 g/L) in 6 well plates. The next day cells were either transfected using Fugene^{TM}6 (Roche Molecular Biochemicals) transfection reagent (10 µl/well) with 2 µg rat GRα construct (+GR) or a rat GR^{dim} construct (+GR^{dim}) or not transfected (-GR).

After 24 h, cells were induced in DMEM with or without test compounds. For each concentration of each test compound, three culture flasks or wells were prepared. After 72 h of incubation, the medium from each flask was removed and saved for protein analysis. Total ribonucleic acid (RNA) was extracted according to the trizol method as described by the manufacturer (Sigma). After extraction, the final RNA pellet was dissolved in 50 µl formazol (Molecular research center, Inc) and kept at –70°C until used. The RNA concentration was determined by reading the absorbance at 260 nm.

6.5.2 Inductions and RNA isolations from rat livers

Carine Smith from the department of Human Physiology, University of Stellenbosch performed the rat experiment. In this study 40 adult male Wistar rats (350 g) were used. These rats were divided into four groups of ten rats each of which Group 1 (SED) acted as the control sedentary group and underwent no intervention treatment. Group 2 (RUN) was housed in rat wheels designed for this purpose and allowed to run at will and subjected to voluntary exercise stress only. Group 3 (SWIM) was subjected to involuntary swimming exercise of one hour per day for the duration of the experiment (10 days). Group 4 (IMOB) was subjected to immobilization for one hour per day in small perspex cages (8x11x17 cm) for the 10 days of the experiment. Rats were fed rat chow and water *ad libitum*, and were housed in groups of three or four in standard rat cages, except for the RUN group, which was housed individually in specially designed rat wheels. The rats were subjected to a 12-hour light-dark cycle and weighed daily. Since rats are nocturnal animals, all interventions were carried out in the late afternoon. All rats were sacrificed by decapitation on day 11 of the protocol at noon, to counter diurnal changes in endocrine measures. The livers were removed and total ribonucleic acid (RNA) was immediately extracted according to the trizol method as described by the manufacturer (Sigma). After extraction, the final RNA pellet was dissolved in 50 µl formazol (Molecular research center, Inc) and kept at –70°C until used. The RNA concentration was determined by reading the absorbance at 260 nm.

6.5.3 CBG protein levels

Extracellular CBG protein levels (from medium) were determined by using a RIA and was done by A. Emptoz-Bonneton at the Hospital De l'Antiquaille, Laboratoire de la Clinique Endocrinologique, Lyon, France [Emptoz-Bonneton 1997].

6.5.4 Northern blotting

Twenty micrograms of total RNA were loaded and run on a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham). To check RNA preparation integrity, ethidium bromide staining was used to demonstrate the presence of intact 18S and 28S ribosomal bands. RNA was fixed on the membrane by using UV crosslinking for 12 s. The membrane was prehybridized in a hybridisation oven at 50°C for 1h with prewarmed Dig easy Hyb solution (Roche). Plasmids carrying hCBG or rCBG, complementary DNAs were amplified in a DH5α competent Escherichia coli strain and digested to cut out the insert. Inserts were isolated by agarose gel electrophoresis. Hybridization was performed overnight at 50°C with 25 ng cDNA probes labelled with [α-^{32}P] dCTP (50 μCi) using the random priming technique (Amersham megaprime labelling kit). Membranes were washed twice for 5 min in 2xSSC, 0.1 % SDS at room temperature, followed by two washings for 15 min in 0.1xSSC, 0.1% SDS at 50°C. Membranes were wrapped in cling wrap and underwent autoradiography for between 24 and 48 h at –70°C. Autoradiograms were scanned using the densitometry program UN-SCAN-IT. Membranes were stripped using a hot 0.5% SDS solution and reprobed with human [^{32}P]β–actin.

6.6 Promoter reporter construct studies:

6.6.1 Transient transfections

For transient transfections $5x10^4$ cells (BWTG3 or HepG2) were plated in DMEM supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin (40 U/ml) and L-glutamine (30 g/L) in 24-well tissue culture plates. Cells were transfected 24h later using Fugene^{TM}6 transfection reagent (3 μl/well) as described by the manufacturer

(Roche Molecular Biochemicals). All reporter constructs ((GRE)$_2$50hIL6PLuc, (GRE)$_2$tkLuc, rCBG295Luc, rCBG145Luc and rCBG1200Luc) were transfected using 360 ng of Luc reporter plasmid with or without 200 ng of rat (pSVGR1) or human (pRS-hGRα) GRα expression vectors. In addition, 40 ng of the β–galactosidase reporter plasmid (pPGKβGopbA) was included in all samples as internal standard for transfection efficiency.

6.6.2 Transient transfections with HNF3α, C/EBPβ and DBP

For transient transfections 5x10^4 cells BWTG3 cells were plated in DMEM supplemented with 10% fetal calf serum (FCS), penicillin- streptomycin (40 U/ml) and L-glutamine (30 g/L) in 24-well tissue culture plates. Cells were transfected 24h later using Fugene^{TM}6 transfection reagent (3 µl/well) as described by the manufacturer (Roche Molecular Biochemicals). The (GRE)$_2$tkLuc reporter construct was transfected using 640 ng of Luc reporter plasmid, 400 ng of rat (pSVGR1) GRα expression vector and 600 ng pGL basic vector. In addition, 640 ng (GRE)$_2$tkLuc reporter constructs, 400 ng of rat (pSVGR1) GRα expression vector, 200 ng pGL basic vector with 400 ng HNF3α, C/EBPβ or DBP was transfected. In all samples 60 ng of β–galactosidase reporter plasmid (pPGKβGopbA) was included as internal standard for transfection efficiency.

6.6.3 Stable transfections

For stable transfections 7.5x10^6 cells were plated in DMEM supplemented with 10% fetal calf serum (FCS), penicillin- streptomycin (40 U/ml) and L-glutamine (30 g/L) in T75 flasks. The next day, medium was removed and replaced with 10 ml MEM-HEPES

without FCS. Next transfection solutions were prepared: A1 contains 120 µl $CaCl_2$, 480 µl TE buffer and 34.5 µg total DNA (consists of 15 µg rat CBG295Luc construct, 1.5 µg β–galactosidase reporter plasmid and 18 µg carrier DNA (genomic DNA from HeLA cells)) and B1 contains 600 µl BS-HEPES buffer. The DNA mix, A1, was then slowly dripped into B1 and incubated for 10 minutes in a waterbath at 37°C for the precipitate to form. The solution was then vortexed for 30 seconds before adding 1 ml per flask. Thereafter 100 µl cloroquine was added to each flask and incubated for 4 h at 37°C. After 4 h, DMEM containing 10% FCS was added. Cells were incubated for 48 h to grow and then plated at $2x10^6$ cells in a T150 flask in DMEM containing 10 % FCS and 1000 mM G418 antibiotic. Cells were grown for one week. After one week medium was replaced with fresh medium containing 10% FCS and 750 mM G418. Cells, which did not contain the β–galactosidase reporter plasmid died as the expression plasmid contained a resistance gene to G418. Colonies of cells were pooled and kept growing in DMEM supplemented with 10% fetal calf serum (FBS), penicillin-streptomycin (40 U/ml) and L-glutamine (30 g/L) in T75 flasks.

6.6.4 Hormone inductions, and Luciferase and β–galactosidase assays

Hormones were added to cells 24 h after transfection. After 24 h, cells were washed with phosphate buffered saline (PBS), lysed with 100 µl lysis buffer (PE Biosystems) and frozen at –20°C overnight. Luciferase and β-galactosidase activities were determined using the luciferase assay kit (Promega) and the Galacto-star assay kit (PE Biosystems) according to the instructions of the manufacturers. Light emission was measured in a luminoskan plate reader (Labsystems).

6.7 Data and statistical analysis

Data are expressed as the mean ± SEM for triplicate values of each experiment and analysed by ANOVA followed by the Dunnett's or Bonferroni's multiple comparison's posttest ($P<0.01$**; $P<0.05$*; $P>0.5$ not significant) or when comparing two groups only a one-tailed unpaired t-test was used. Each experiment was repeated at least twice, in triplicate each time. GraphPad Prism® version 3.0 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used for graphical representation and statistical analysis. The EC50s were determined using non-linear regression (curve fit) and dose response curves with variable slope in chapter 3 whereas in chapter 2 the EC50 and maximal repression was determined by fitting a dose response curve with one site competion.

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