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# Single nucleotide polymorphisms in the human corticosteroid-binding globulin promoter alter transcriptional activity

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Corticosteroid-binding globulin (CBG) is a high-affinity plasma protein that transports glucocorticoids and progesterone. Others and we have reported non-synonymous single nucleotide polymorphisms (SNPs) that influence CBG production or steroid-binding activity. However, no promoter polymorphisms affecting the transcription of human CBG gene (*Cbg*) have been reported. In the present study we investigated function implications of six promoter SNPs, including −26 C/G, −54 C/T, −144 G/C, −161 A/G, −205 C/A, and −443/−444 AG/−, five of which are located within the first 205 base pairs of 5′-flanking region and close to the highly conserved footprinted elements, TATA-box, or CCAAT-box. Luciferase reporter assays demonstrated that basal activity of the promoter carrying −54 T or −161 G was significantly enhanced. The first three polymorphisms, −26 C/G, −54 C/T, and −144 G/C located close to the putative hepatic nuclear factor (HNF) 1 binding elements, altered the transactivation effect of HNF1β. We also found a negative promoter response to dexamethasone-activated glucocorticoid receptor (GR) α, although none of the SNPs affected its transrepression function. Our results suggest that human *Cbg* −26 C/G, −54 C/T, −144 G/C, and −161 A/G promoter polymorphisms alter transcriptional activity, and further studies are awaited to explore their association with physiological and pathological conditions.

**corticosteroid-binding globulin, single nucleotide polymorphism, hepatic nuclear factor 1**

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Corticosteroid-binding globulin (CBG) is a steroid-binding protein that transports glucocorticoids and progesterone in the blood [1]. The human CBG gene (*SERPINA6*, *Cbg*) resides on chromosome 14q32.1 within a cluster of related serine proteinase inhibitor (SERPIN) genes [2]. However, unlike other serpins, CBG lacks proteinase inhibitor activity [3]. CBG binds with high affinities the circulating glucocorticoids [4] and progesterone [5], both of which are well-known hormones for pregnancy maintenance or labor onset. While CBG mainly binds >80% of cortisol in human peripheral blood [3], at the maternal-fetal interface CBG will be occupied by the very high concentrations of progesterone, which is several fold higher than those of cortisol [6]. In the circulation of women during mid-late pregnancy, approximately 10% of CBG steroid-binding sites are occupied by progesterone, versus <1% in non-pregnant women [5]. Plasma CBG levels rise progressively through gestation until term pregnancy [7], and so do blood cortisol and progesterone levels. Maternal plasma CBG, total and free cortisol concentrations are reduced in pre-eclampsia and gestational hypertension patients, and this may be a consequence of decreased *Cbg* synthesis driven by cytokines or increased degradation driven by inflammation [7].

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Besides its role in systemic transport of glucocorticoids and progesterone, CBG also plays direct roles in their local delivery, thereby modulating their access to target tissues [8]. Except for the liver, the main organ where CBG is synthesized, CBG is also expressed in fetal exocrine pancreas and the proximal convoluted tubules of postnatally developing kidney, where CBG may influence their glucocorticoid-dependent maturation [9,10]. The release of ligands from CBG at their sites of action is controlled in a targeted manner, as illustrated by crystal structure studies [1]. The best example for this is at sites of inflammation neutrophil elastase cleaves CBG at a single site in a surface exposed reactive center loop, which causes a significant rearrangement in the tertiary structure of CBG that disrupts steroid-binding, thus providing a highly efficient mechanism for the local release of cortisol [11–13]. As there is evidence that CBG is the target of other proteinases [9], CBG accumulated at the maternal-fetal interface [6] can be cleaved by placenta-specific proteinases and thus involved in regulating local progesterone bioavailability and action.

Thus far, seven non-synonymous single nucleotide polymorphisms (SNPs) within the SERPINA6 sequence, including CBG W11Stop, CBG Santiago, A51V, L93H, CBG Lyon, G237V, and E102G, have been identified that influence CBG production [14–16] or its cortisol-binding activity [16–19]. Among these, all the variants are considered rare except for recently reported CBG A51V by us [16], which occurs at a frequency of ~1:37 in Han Chinese and will allow clinical consequences of CBG deficiencies to be assessed in large populations. However, to our knowledge, relatively little is known regarding the gene regulatory mechanism of *Cbg*, and the SNPs in the promoter region that might affect *Cbg* transcription have not been reported.

The proximal rat *Cbg* promoter which is located within −295 base pair (bp) has been cloned, and DNase I footprinting experiment has identified five *cis*-acting elements/footprinted (FP) regions (FP1 to FP5), which are highly conserved in the human *Cbg* promoter [20]. Regions FP1 to FP5 resemble recognition sequences for hepatocyte nuclear factor 1 (HNF1), transcription factor CP-2 (TFCP2), D site of albumin promoter (albumin D-box) binding protein (DBP), HNF3 and CCAAT-enhancer-binding protein (C/EBP), respectively, and electrophoretic mobility shift assays (EMSAs) indicate that the FP1 element binds HNF1 [20,21]. Nucleotide (nt) −295 to −52 is a positive component of rat *Cbg* transcriptional activity, whereas sequences located between nt −295 and −800 repress transcription, and this repressor effect is partially overcome by the addition of sequences up to −1200 [20].

Genome-wide sequencing projects (http://www.ncbi.nlm.nih.gov/snp) have identified several single nucleotide polymorphisms (SNPs) in the proximal human *Cbg* promoter. The SNPs involved in this study include C/G SNP at −26 bp (rs188371806), C/T SNP at −54 bp (rs115833113), G/C SNP at −144 bp (rs180685032), A/G SNP at −161 bp (rs186384700), C/A SNP at −205 bp (rs77990214), and AG/– SNP at −443/−444 bp (rs80219741 and rs77992119). In order to determine whether these SNPs affect human *Cbg* gene transcription, a series of plasmids containing the firefly luciferase gene under the control of various human *Cbg* promoter fragments (ph*Cbg*) were constructed, binding of transcription factors that these SNPs may affect were predicted by using TESS (Transcription Element Search System, http://www.cbil.upenn.edu/cgi-bin/tess/tess), Genomatix/MatInspector (http://www.genomatix.de/) and ConSite (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/), and effects of these SNPs on the basal promoter activity and regulation of *Cbg* transcription were investigated.

## 1 Materials and methods

### 1.1 Cell culture

The human hepatoblastoma cell line HepG2 was a generous gift from Prof. Geoffrey L. Hammond (Department of Obstetrics and Gynecology, University of British Columbia, and Child and Family Research Institute). HepG2 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U penicillin $mL^{-1}$ and 100 μg streptomycin $mL^{-1}$ at 37°C in a 5% $CO_2$ atmosphere.

### 1.2 Constructs

Expression plasmids for human HNF1α [22] and HNF1β [23], in pcDNA3.1/HisC and pcDNA3.1/HisB, respectively, were generous gifts from Dr. Lise Bjørkhaug and Dr. Pål Rasmus Njølstad (University of Bergen, Norway). Expression plasmids of human c-Jun and c-Fos were kind gifts from Dr. John E. Coligan (NIAID, National Institutes of Health, USA). Human specificity protein 1 (Sp1) expression plasmid was generously provided by Dr. Guntram Suske (Institute of Molecular Biology and Tumor Research, Germany). Expression vectors of glucocorticoid receptor (GR) α and β, pRShGRα and pRShGRβ, and an empty vector pRSV were from Dr. Ronald M. Evans (Salk Institute for Biological Studies, USA). Coding sequences of nuclear factor [24] -IC and -YA were PCR-cloned and inserted into pcDNA3 expression vector. All the constructs were sequenced to confirm their authenticities.

### 1.3 Cloning of 5′-flanking regions of the human *Cbg* promoter into firefly luciferase reporter vector

Different 5′-flanking regions (−291, −501, −833, or −1347) of the human *Cbg* promoter upstream of the transcription start site (+1) extending to part of non-coding exon I (+54) were obtained by PCR from HepG2 genomic DNA using

the primers (ph*Cbg*–291for/–501for/–833for/–1347for as forward primers and ph*Cbg*rev as the reverse primer) shown in Table 1. Among these, the –291 to +54 construct corresponds to the proximal human *Cbg* promoter, which includes the five footprinted region [20]. The PCR products were subcloned into *Xho* I-*Hin*d III sites of the pGL3-basic luciferase reporter vector. All constructs were sequenced to confirm their authenticity.

### 1.4 Site-directed mutagenesis

To obtain the human *Cbg* promoter reporter plasmids that carry different SNPs (–26C/G, –54C/T, –144G/C, –161A/G, –205C/A, –443/–444AG/–), appropriate plasmids were subjected to site-directed mutagenesis using the QuikChange® Lightning Site-Directed Mutagenesis Kit (catalog No. 200516; Stratagene). The forward (for) and reverse (rev) primers for mutagenesis are listed in Table 1. All constructs were sequenced to confirm that only the targeted mutations had occurred.

### 1.5 Luciferase assay

About $1\times10^6$ cells/well were seeded in six-well plates to reach 50%–60% confluence one day before transfection. Cells were co-transfected with 2 μg reporter plasmid and 100 ng Renilla luciferase reporter vector or 300 ng pCMVlacZ control plasmid (in the case when Renilla luciferase activity changed upon co-transfection with other transcription factors or administration of dexamethasone) [25] per well using QuickShuttle transfection reagents (catalog No. KX0110041; Beijing Kang Bi Quan Biotech. Co. Ltd.), according to the manufacturer's instructions. For transactivation or transrepression experiments, 200 ng expression vectors for transcription factors or the corresponding empty vectors were co-transfected. Dexamethasone (100 nmol $L^{-1}$) treatment was performed together with co-transfection of GR plasmids, with ethanol as the vehicle control. 48 h after transfection, the cells were washed three times with cold PBS buffer and harvested by scraping and centrifuging at 5000 r $min^{-1}$ for 3 min. The cell pellets were re-suspended in 150 μL lysis buffer and lysed by two cycles of freezing and thawing. The activities of firefly and Renilla luciferases were measured using Dual-Luciferase® Reporter Assay System (catalog No. E1960; Promega), according to the manufacturer's instructions. The β-galactosidase activities were determined by OD reading at 405 nmol $L^{-1}$. The firefly luciferase activity was normalized by the activity of Renilla luciferase or β-galactosidase.

### 1.6 Statistics

Data were analyzed using Student's *t*-test, and results were expressed as the means±SD. *P*-values of <0.05 were considered significant.

## 2 Results

### 2.1 Phylogenetic analysis of the human *Cbg* proximal promoter

The human *Cbg* (NC_000014.8)) and mouse *Cbg* (NC_000078.6) proximal promoters from –500 bp upstream of the transcription start site were compared with the rat *Cbg* (NC_005105.3) promoter containing the five footprinted regions (FP1-FP5) [20]. In the rat and human *Cbg* promoter, two perfectly conserved elements before the

**Table 1** Primer sequences used for PCR amplification[a)]

| Primer | Sequence (5′–3′) |
|---|---|
| ph*Cbg* –291for | CGGCTCGAGCAAGTATCTGCACCTTGGTGC |
| ph*Cbg* –501for | CGGCTCGAGACTTCCATGCAGCTCAGTTTCC |
| ph*Cbg* –833for | CGGCTCGAGCCAGAGCTACTGTGGCCACAT |
| ph*Cbg* –1347for | CCGCTCGAGCAGGACTTATCAAAGGGAG |
| ph*Cbg* +54rev | CGGAAGCTTGCTTGGTCCTGCTGTCCTGG |
| –26C/Gfor | ACCATTAACCACCCAGGAAGGTGGCAAACAAAATTTAACAGG |
| –26C/Grev | CCTGTTAAATTTTGTTTGCCACCTTCCTGGGTGGTTAATGGT |
| –54C/Tfor | GGTGAAGGCCACTGGTCCCTCTAACCATTAACC |
| –54C/Trev | GGTTAATGGTTAGAGGGACCAGTGGCCTTCACC |
| –144G/Cfor | CAGAGCAAAGTTAATTTGCACCATTTCTGTGTTTACTCAGC |
| –144G/Crev | GCTGAGTAAACACAGAAATGGTGCAAATTAACTTTGCTCTG |
| –161A/Gfor | CTTCATAGAATTGAACGCAGGGCAAAGTTAATTTGCAGCA |
| –161A/Grev | TGCTGCAAATTAACTTTGCCCTGCGTTCAATTCTATGAAG |
| –205C/Afor | CAACCACATTGATGCAACTCCAGACACATTTTGGTATTAAAAAAAGAC |
| –205C/Arev | GTCTTTTTTTAATACCAAAATGTGTCTGGAGTTGCATCAATGTGGTTG |
| –443/–444AG/–for | GGTACCCACTTCACAGTCATTGTGAAGATTTCG |
| –443/–444AG/–rev | CGAAATCTTCACAATGACTGTGAAGTGGGTACC |

a) Characters underlined represent mutagenesis sites.

transcription start site, ATTTA and CCACT, are suggested as a TATA-box and CCAAT-box, respectively [20,26]. As shown in Figure 1, the five footprinted regions, TATA-box, and CCAAT-box are highly conserved in the human, rat, and mouse *Cbg* promoters. Moreover, the human, rat, and mouse *Cbg* promoters are highly conserved from approximately −300 bp relative to the transcription start site to the non-coding exon I, which supports that the rat *Cbg* promoter from the first 295 bp of the 5′-flanking region encompassing the five footprinted regions has the most efficient promoter activity [20].

### 2.2 Cloning and identification of human *Cbg* promoter

We cloned a series of the human *Cbg* promoter fragments encompassing different 5'-flanking regions, −291, −501, −833, or −1347 bp relative to the transcription start site (+1), and to +54 bp in non-coding exon I (Figure 2A), into the firefly luciferase reporter plasmid pGL3-basic. As shown in Figure 2A, the proximal ph*Cbg* located within the first 291 bp of the 5′-flanking region, which includes the FP1 to FP5 regions, had a strong activity. Extending the promoter region upstream from −291 to −501 resulted in a slight loss of

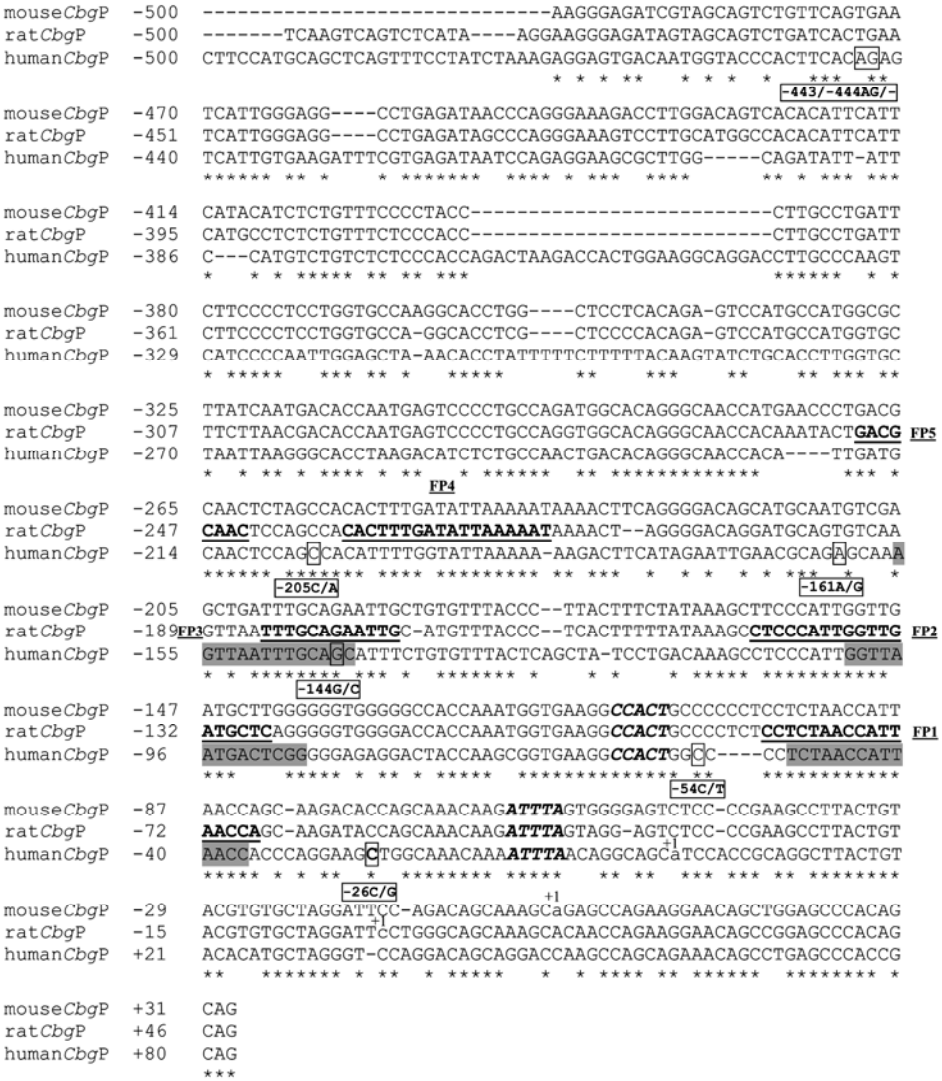


**Figure 1** Phylogenetic comparison of the mouse, rat, and human *Cbg* proximal promoter sequences and location of SNPs in the human *Cbg* promoter. The mouse *Cbg* promoter from −500 to +33 bp (NC_000078.6), the rat *Cbg* promoter from −500 to +48 bp (NC_005105.3), and the human *Cbg* promoter from −500 to +82 bp (NC_000014.8) relative to the transcription start site (+1, in lowercase lettering) are aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The positions of the SNPs in the human *Cbg* promoter investigated in the present study, including −26 bp C/G (rs188371806), −54 bp C/T (rs115833113), −144 bp G/C (rs180685032), −161 bp A/G (rs186384700), −205 bp C/A (rs77990214), and −443/-444 bp AG/– (rs80219741 and rs77992119), are denoted as empty boxes. DNase I footprinted regions (FP1–FP5) previously identified within the rat *Cbg* promoter [20] are illustrated by bold and underlined letters. Sequences that resemble TATA- and CCAAT-box motifs are shown in bold and italic fonts. Putative HNF1 binding elements in the human *Cbg* promoter are marked in shaded boxes.

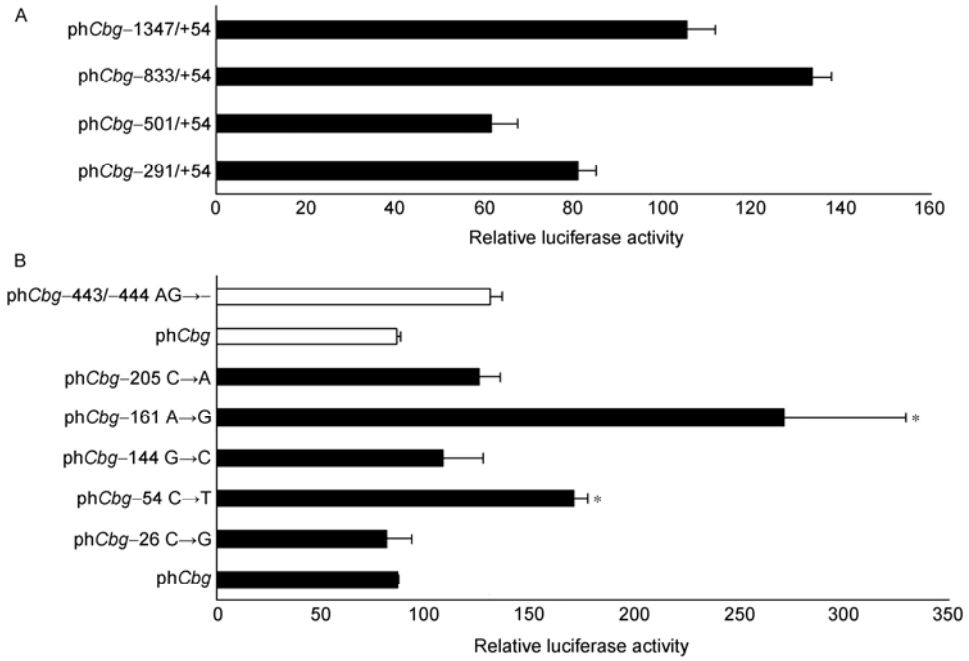


**Figure 2** Functional comparison of human *Cbg* promoters with different SNPs in their basal promoter activities. A, Activities of different-length human *Cbg* promoters fused into pGL3-basic, including −291 to +54 bp (−291/+54), −501 to +54 bp (−501/+54), −833 to +54 bp (−833/+54), −1347 to +54 bp (−1347/+54), relative to the transcription start site. B, Promoter reporter plasmid (ph*Cbg*−291/+54, closed bars) was subjected to site-directed mutagenesis to introduce different SNPs (−26 C/G, −54 C/T, −144 G/C, −161 A/G, and −205 C/A), ph*Cbg*−501/+54 (open bars) was mutated to delete the AG SNP (−443/−444 AG/−), and their promoter activities were examined in parallel. ph*Cbg*, promoter of human *Cbg*. The transcriptional potentials are indicated as relative firefly luciferase activities normalized by corresponding Renilla luciferase activities. Data points are shown as mean±SD of three independent experiments. *, $P<0.05$ as compared to unmutated ph*Cbg*−291/+54.

activity that was again overcome by further inclusion of sequences present in ph*Cbg*−833 and ph*Cbg*−1347. Thus, unlike rat *Cbg* promoter p*Cbg*295, which is the most active construct [20], the human *Cbg* promoter ph*Cbg*−833/+54 represented the most positive component of *Cbg* transcriptional activity. However, the activity of ph*Cbg*−291/+54 was comparable to that of ph*Cbg*−833/+54 and therefore still supports that the most active promoter resides within the region encompassing the five footprinted regions, i.e., *cis*-regulatory elements.

### 2.3 −54 C/T and −161 A/G SNP affected the basal human *Cbg* promoter activity

The SNPs in the human *Cbg* promoter investigated in the present study include C/G SNP at −26 bp (close to the TATA-box ), C/T SNP at −54 bp (located beside FP1 and CCAAT-box), G/C SNP at −144 bp (located in the middle of FP3), A/G SNP at −161 bp (located beside FP3), C/A SNP at −205 bp (located between FP4 and FP5), and AG/− SNP at −443/−444 bp, the locations of which are shown in Figure 1. By site-directed mutagenesis, we introduced each individual SNP into the proximal human *Cbg* promoter. To minimize the effects of upstream repressor or enhancer sequences, all the proximal SNPs, including −26 C/G, −54 C/T, −144 G/C, −161 A/G, −205 C/A, were introduced into ph*Cbg*−291/+54, and −443/−444 AG nucleotides were removed on top of ph*Cbg*−501/+54. Functional analysis of different human *Cbg* promoters showed that the promoter with −54 T or −161 G caused a nearly two- to three-fold induction in their basal promoter activities (Figure 2B). The changes caused by other SNPs were less than two-fold and therefore were not considered significant (Figure 2B).

### 2.4 HNF1α and HNF1β had distinct effects on the transcription of the human *Cbg* gene and promoter SNPs alter HNF1β response

Sequence of the proximal human *Cbg* promoter was searched in the databases for putative transcription factor binding elements, and three regions with a high degree of identity to known HNF1 consensus element (5′-GGTTA-ATNATTAA$_{/C}$A$_{/C}$) [27,28] were revealed. The sequence in FP1 is perfectly conserved in human, rat and mouse *Cbg* promoters and it contains the HNF1 consensus element (TCTAACCATTAACC) with deviations at only two positions (Figure 1). It has been previously demonstrated by EMSA experiments that in the rat *Cbg* promoter FP1 binds HNF1 [20,21]. The other two putative HNF1 binding sites were found in FP2 and part of FP3 (Figure 1).

To explore the functional impact of the two HNF1 isoforms, HNF1α and HNF1β homodimers or heterodimers,

on the human *Cbg* gene transcription, we co-transfected human *Cbg* promoter reporter plasmid along with HNF1α and HNF1β expression plasmids individually or together. As shown in Figure 3, the human *Cbg* proximal promoter with the C SNP at −26 bp (Figure 3A, closed bars), the C SNP at −54 bp (Figure 3B, closed bars), or the G SNP at −144 bp (Figure 3C, closed bars), was significantly activated in response to over-expressed HNF1α by at least two-fold. However, when HNF1β was over-expressed, only a slight response was detected in the above promoters (Figure 3, closed bars). Co-expression of HNF1β with HNF1α was not additive to transcriptional activation (Figure 3, closed bars). Therefore, HNF1α and HNF1β had different effects on the transcription of the human *Cbg* gene.

As −26 C/G, −54 C/T, and −144 G/C are the first three proximal SNPs located close to HNF1 binding element in

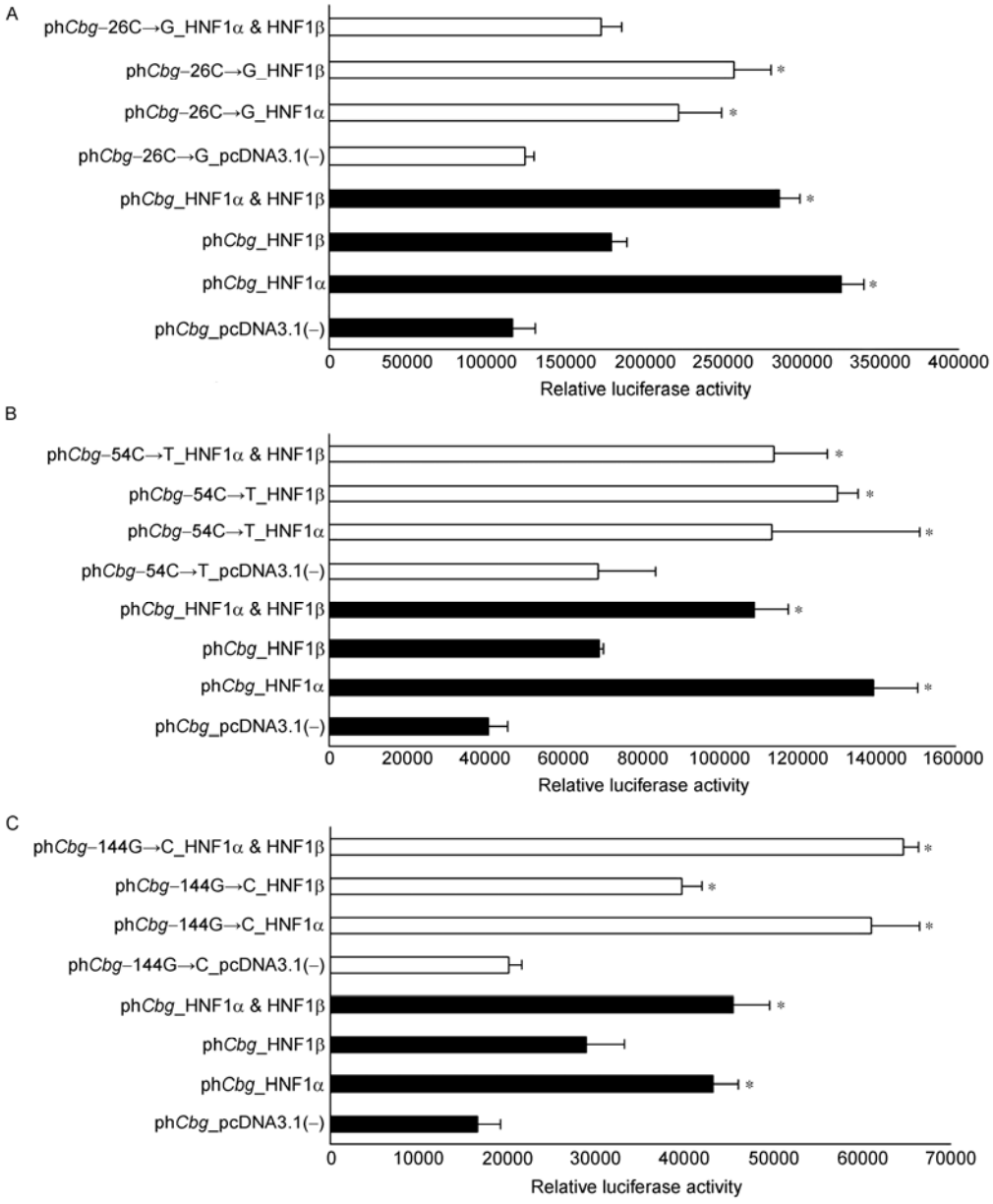


**Figure 3** Effects of SNPs, −26 C/G (panel A), −54 C/T (panel B), or −144 G/C (panel C), on the transcriptional activation of the human *Cbg* proximal promoter (ph*Cbg*−291/+54) by over-expressed HNF1α homodimers, HNF1β homodimers, or HNF1α and HNF1β heterodimers. A, Response of the human *Cbg* proximal promoter with the −26 C (closed bars) or G (open bars) SNP to over-expressed HNF1. B, Response of the human *Cbg* proximal promoter with the −54 C (closed bars) or T (open bars) SNP to over-expressed HNF1. C, Response of the human *Cbg* proximal promoter with the −144 G (closed bars) or C (open bars) SNP to over-expressed HNF1. Human *Cbg* proximal promoter plasmid was co-transfected with pcDNA3.1(−) empty vector (control), HNF1α or/and HNF1β expression plasmids with the amounts described in Material and methods. Normalized transcriptional activities by β-galactosidase activities are indicated as relative luciferase activities. Each bar represents mean±SD of three parallel experiments. *, $P<0.05$ as compared to the corresponding co-transfection with empty vector.

FP1 and FP3 (Figure 1), we introduced these three individual SNPs into the human proximal promoter ph*Cbg*–291/+54, which carried the G SNP at –26 bp (Figure 3A, open bars), the T SNP at –54 bp (Figure 3B, open bars), or the C SNP at –144bp (Figure 3C, open bars). While the introduction of the above SNPs into the promoter did not alter the transactivation effect of HNF1α or HNF1α&HNF1β heterodimer, they all caused a gain of HNF1β response by about two-fold (Figure 3, open bars).

### 2.5 GRα and GRβ had distinct effects on the transcription of the human *Cbg* gene

It has been previously demonstrated that glucocorticoids repress *Cbg* transcription in the livers of adult rats [29]. Searching in the database suggested that –161 A/G, –205 C/A, or –443/–444 AG/– might affect the binding of GR, although no consensus glucocorticoid response elements within the proximal promoter were found. As shown in Figure 4, the human *Cbg* promoter (ph*Cbg*–291/+54) with the A SNP at –161 bp (Figure 4A, closed bars), the C SNP at –205 bp (Figure 4B, closed bars), or ph*Cbg*–501/+54 with the AG SNP at –443/–444 bp (Figure 4C, closed bars), was abundantly repressed by over-expressed GRα in complex with dexamethasone, but not by inactivated GRα alone (Figure 4, closed bars). Neither GRβ nor dexamethasone-GRβ complex had an effect on promoter activity (Figure 4, closed bars). We next introduced the above three SNPs into ph*Cbg*–291/+54, which carried the G SNP at –161 bp (Figure 4A, open bars), the A SNP at –205 bp (Figure 4B, open bars), or ph*Cbg*–501/+54 with the AG SNP deleted at –443/–444 bp (Figure 4C, open bars). However, the results showed no changes in the promoter response to over-expressed GRα or GRβ in complex with dexamethasone (Figure 4, open bars).

### 2.6 Promoter responses to transcription factors NF-IC, NF-YA, Sp1, activator protein 1 (AP1) were not affected by the SNPs presented in this study

The database also predicted NF-IC, NF-YA, Sp1, and AP1 as transcription factors that might be affected. NF-IC, but not NF-YA, had a negative effect on the proximal promoter activity. Sp1 and c-Jun had a transactivation effect. However, none of the SNPs tested, including –54 C/T, –144 G/C, –161 A/G, –205 C/A, –443/–444 AG/–, affected the promoter activities in response to these transcription factors (data not shown).

## 3 Discussion

Proteolysis of CBG by neutrophil elastase serves to be the mechanism of rapid and targeted delivery of biologically active steroids to their sites of action [1]. Apart from proteolytic cleavage, hepatic biosynthesis of *Cbg* reduces considerably during acute inflammation, due to an interleukin-6 (IL-6) induced decrease in *Cbg* mRNA stability [30]. Dexamethasone-induced decreases in plasma CBG levels in adult rats have been attributed to decreased *Cbg* transcription in the liver [29]. In the present study, we firstly set out to investigate the functional effects of six proximal promoter SNPs (–26 C/G, –54 C/T, –144 G/C, –161 A/G, –205 C/A, –443/–444 AG/–), five of which are located within the first 205 bp of the 5′-flanking region, on the transcriptional control of human *Cbg*.

*Cis*-acting sequence elements, i.e., footprinted regions FP1 to FP5, in the rat *Cbg* promoter have been identified [20]. These FP1-FP5 sites, TATA-box, and CCAAT-box are highly conserved in the human, rat, and mouse *Cbg* promoters. Consistent with the rat *Cbg* promoter encompassing FP1 to FP5, which is the most active construct [20], the human proximal *Cbg* promoter including the FP1 to FP5 was also among the most active constructs tested. An EMSA and an antibody-supershifting EMSA have proved that FP1 element binds HNF1 [20,21], and FP2 to FP5 have been suggested to resemble recognition sequences for TFCP2, DBP, HNF3 and C/EBP, respectively [20]. Our results demonstrated that the introduction of two promoter SNPs, the –54 T SNP and –161 G SNP, significantly enhanced the basal promoter activity, as compared to –54 C SNP and –161 A SNP. These data are not unexpected because –54 C/T SNP is located beside FP1 and the CCAAT-box [20,26] and –161 A/G SNP is located beside FP3 and the putative HNF1 binding site. As previously reported, mutations within rat FP1, which prevent HNF1 binding, do not influence the basal transcriptional activity of rat *Cbg* [21]. However, this does not mean FP1 or HNF1 is not important for transcriptional control, because different physiological conditions will drive the expression and modulate the DNA motif binding of HNF1, a major determinant of the transcription regulation of a number of hepatic genes, such as α1-antitrypsin, fibrinogen, albumin, C-reactive protein (CRP), and α-fetoprotein (AFP) [31–33].

HNF1α and HNF1β, two members of the HNF1 transcription factor family, share amino-terminal dimerization domain and POU-like DNA-binding homeo domain, but not carboxyl-terminal transcriptional activation domain, and they bind DNA as homodimers or heterodimers [24,34,35]. Our data demonstrated that in the human *Cbg* proximal promoter, HNF1α or HNF1β had different effect on the basal promoter activity. The proximal human *Cbg* promoter ph*Cbg*–291/+54 was significantly activated by over-expressed HNF1α, and –26 C/G, –54 C/T, and –144 G/C polymorphisms did not affect its transactivating function. On the contrary, –26 G, –54 T, and –144 C all probably created an HNF1β binding site, because the promoter ph*Cbg*–291/+54 with –26 C, –54 C, or –144 G was not

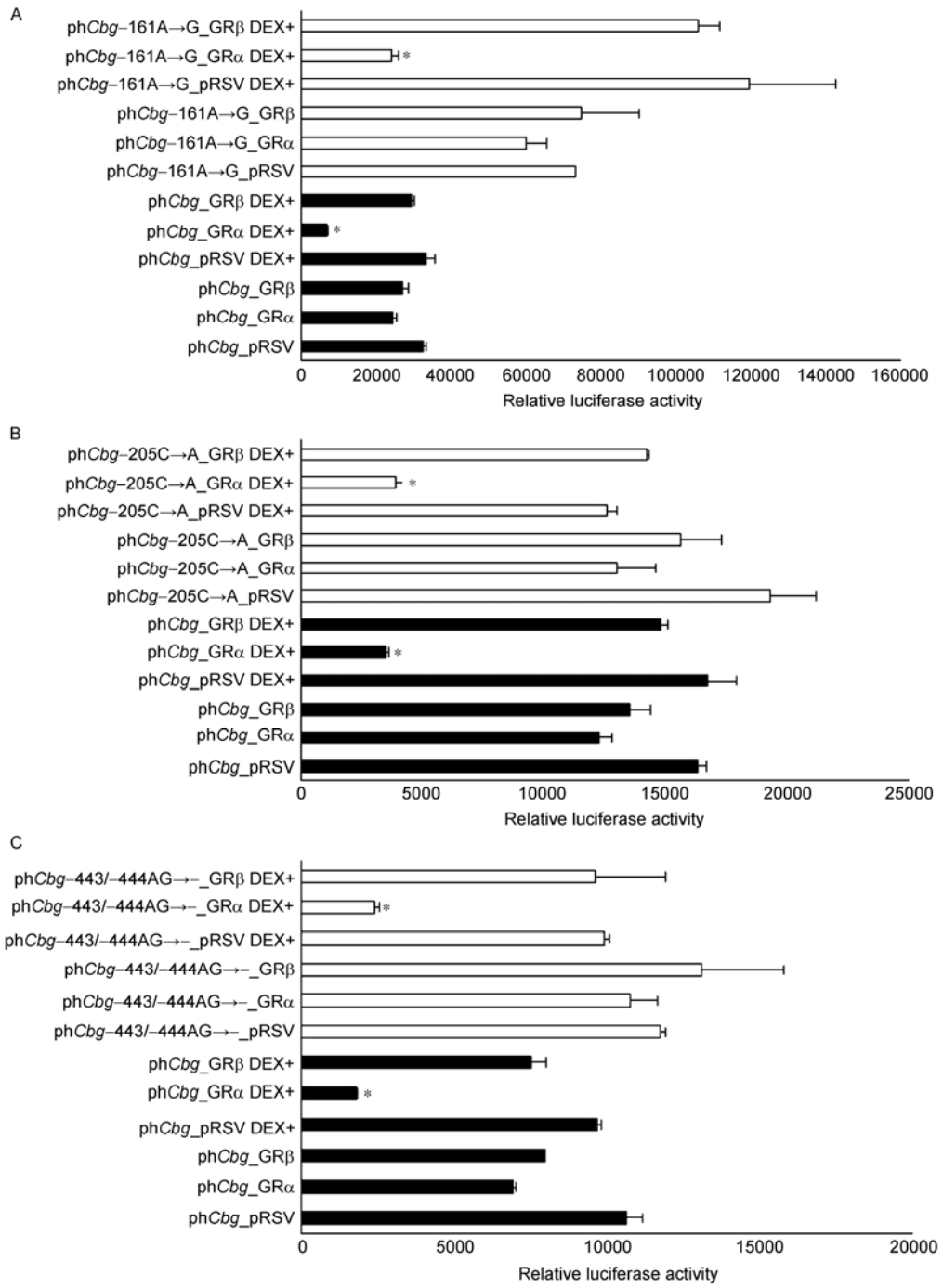


**Figure 4** Effects of SNPs, −161 A/G (panel A), −205 C/A (panel B), or −443/−444 AG/– (panel C), on the transcriptional regulation of the human *Cbg* proximal promoter by over-expressed GRα or GRβ, with or without the administration of synthetic glucocorticoid (dexamethasone, DEX). A, Response of the human *Cbg* proximal promoter (ph*Cbg*−291/+54) with the −161 A (closed bars) or G (open bars) SNP to over-expressed GR with/without DEX. B, Response of the human *Cbg* proximal promoter (ph*Cbg*−291/+54) with the −205 C (closed bars) or A (open bars) SNP to over-expressed GR with/without DEX. C, Response of the human *Cbg* proximal promoter (ph*Cbg*−501/+54) with the −443/−444 AG (closed bars) or these two nucleotides deleted (open bars) to over-expressed GR with/without DEX. Human *Cbg* proximal promoter plasmid was co-transfected with pRSV empty vector (control), GRα or GRβ expression plasmids (pRShGRα or pRShGRβ) with the amounts described in Material and methods. Normalized transcriptional activities by β-galactosidase activities are indicated as relative luciferase activities. Each bar represents mean±SD of three parallel experiments. *, $P<0.05$ as compared to the corresponding co-transfection with empty vector.

significantly stimulated by over-expressed HNF1β whereas their counterparts gained an abundant response. This is also expected because −26 C/G and −54 C/T are all close to FP1, the most important *cis*-regulatory element identified in rat *Cbg* that binds HNF1 [20], especially HNF1β [21], and −144 G/C is located within the putative HNF1 binding ele-

ment and FP3. Taken together with previously discussed −54 C/T and −161 A/G which affected basal promoter activity, the first four polymorphisms located within the first ~160 bp in 5′-flanking region either affect basal transcription activity or HNF1 transactivation. Moreover, our data demonstrated no functional effect of HNF1β on HNF1α-stimulated promoter activity, as previously reported by others [36–38]. Therefore, the significant *in vivo* reduction of CBG biosynthesis during inflammation is likely associated with decreased binding activity of HNF1 to *Cbg* promoter and subsequently decreased gene transcription initiation, and promoter SNPs might be associated with different inflammatory response among different individuals.

Accumulated evidence from both *in vivo* and *in vitro* experiments has linked CBG synthesis to the regulation by glucocorticoids. Research in different adult species has illustrated that administration of synthetic glucocorticoid dexamethasone exerts inhibitory effects on hepatic *Cbg* biosynthesis at the transcriptional level [29,39,40]. However, earlier predictive study within rat *Cbg* proximal promoter failed to reveal any glucocorticoid response element (GRE) [20]. Glucocorticoids act by binding to GR. In the absence of glucocorticoids, GR is maintained in the cytoplasm. When the GR binds to glucocorticoid, the activated glucocorticoid-GR complex enters into the nucleus and modulates gene transcription. It is becoming widely recognized that in contrast to "simple" GRE [41], which is a transactivation element, no consensus sequence for GREs that confer transcriptional repression has emerged, i.e., this ''tethering'' GRE does not contain DNA binding sites for GR, but instead contains binding sites for other transcription factors that GR can interact with [42]. In this respect, the non-existence of consensus GRE in the human *Cbg* promoter does not rule out the transrepressive activity of GR, especially because a research using GR-knockout mice revealed that GR is essential for repressing *Cbg* gene expression in the liver, and for dexamethasone-inhibited CBG expression in the adult liver [39]. We therefore performed *Cbg* promoter activity study in response to activated dexamethasone-GRα or -GRβ complex. The results showed that activated GRα, but not GRβ, caused significant transrepression effects on the activities of the human *Cbg* promoters, ph*Cbg*−291/+54 and ph*Cbg*−501/+54, although none of the SNPs, −161 A/G, −205 C/A, or −443/−444 AG/−, altered the response to activated GRα. Whether this is physiologically or pathologically relevant, for instance pregnancy maintenance, labor onset or pregnancy-related disease pre-eclampsia, remains to be further elucidated. Furthermore, in the absence of GR overexpression, neither of the human *Cbg* promoters was responsive to dexamethasone in human hepatoma HepG2 cells, which express endogenous GR [43,44]. However, in the presence of overexpressed GRα, dexamethasone treatment inhibited *Cbg* promoter activity. Similar effects have also been reported on the promoter activity of many other genes, such as sterol 27-hydroxylase (CYP27A1) [45], CYP2C19 [46], UDP-glycosyltransferase 1A6 (UGT1A6) [47] and UGT1A1 [48] in HepG2 cells. Therefore, the level of endogenous GR, which could be activated by dexamethasone and subsequently translocated into the nucleus, was insufficient for exerting a transrepression effect on human *Cbg* promoter, and this could be overcome by complementation with a GR expression vector.

In summary, by utilizing luciferase reporter assay we demonstrated that the promoter with −54 T or −161 G polymorphisms, which are located beside the CCAAT-box/FP1 and FP3, respectively, significantly enhanced the basal promoter activities. −26 C/G, −54 C/T, and −144 G/C polymorphisms altered the transactivation effect of HNF1β. A negative response of human *Cbg* promoter to dexamethasone-activated GRβ was found, but none of the SNPs, −161 A/G, −205 C/A, or −443/−444 AG/−, affected the transrepression effect of GRβ. Further association studies are needed to explore whether the first four proximal promoter SNPs that alter *Cbg* transcription activities, including −26 C/G, −54 C/T, −144 G/C, −161 A/G, are related to physiological and pathological conditions.

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