CCAAT enhancer binding protein β and hepatocyte nuclear factor 3 β are necessary and sufficient to mediate dexamethasone-induced up-regulation of α_2 HS-glycoprotein/fetuin-A gene expression

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Abstract

α_2 HS-glycoprotein/fetuin-A (Ahsg) is a serum protein preventing soft tissue calcification. In trauma and inflammation, Ahsg is down-regulated and therefore considered a negative acute phase protein. Enhancement of Ahsg expression as a protective serum protein is desirable in several diseases including tissue remodelling after trauma and infection, kidney and heart failure, and cancer. Using reporter gene assays in hepatoma cells combined with electrophoretic mobility shift assays we determined that dexamethasone up-regulates hepatic Ahsg. A steroid response unit at position –146/–119 within the mouse Ahsg promoter mediates the glucocorticoid-induced increase of Ahsg mRNA. It binds the hepatocyte nuclear factor 3 β and CCAAT enhancer binding protein β (C/EBP- β). The up-regulation is mediated indirectly via glucocorticoid hormone-induced transcriptional up-regulation in C/EBP- β protein. A high degree of sequence identity in mouse, rat and human Ahsg promoters suggests that the promoter is similarly up-regulated by dexamethasone in all three species. Therefore, our findings suggest that glucocorticoids may be used to enhance the level of Ahsg protein circulating in serum.

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Introduction

α_2 HS-glycoprotein/fetuin-A (Ahsg) is a major serum protein in mammals. Homologous proteins exist in marsupials, reptiles, fish and *Drosophila*. During fetal development Ahsg serum levels are usually higher than in adult life. Towards adulthood the serum concentration decreases to about 1% of the total serum proteins (0.5–1 g/l) (Dickson *et al.* 1983, Dziegielewska *et al.* 1987, Yang *et al.* 1992, Terkelsen *et al.* 1998) and the protein is more than 95% liver-derived. Thus, Ahsg is a major hepatic serum protein. A thorough understanding of Ahsg gene regulation during inflammation and recovery is important for several reasons. A well-documented physiological function of Ahsg is the systemic inhibition of ectopic calcification. This was recently confirmed in a study with Ahsg knockout mice linking Ahsg deficiency to a particularly severe form of ectopic calcification, calciphylaxis (Jahn-Dechent *et al.* 1997, Heiss *et al.* 2003, Ketteler *et al.* 2003b, Schäfer *et al.* 2003) and cardiovascular calcification (Merx *et al.* 2005, Moe *et al.* 2005). We recently showed that Ahsg is a novel uraemia- and inflammation-related mortality risk factor in haemodialysis patients (Ketteler *et al.* 2003a). Pharmacological

elevation of Ahsg serum levels was suggested as one therapeutic approach to alleviate the described ill effects associated with low Ahsg serum levels. Another well-documented function of Ahsg is antagonism of transforming growth factor β (TGF- β) and related cytokines. This function was confirmed by studying the regulation of postnatal bone growth and remodelling in Ahsg-deficient mice (Binkert *et al.* 1999, Szweras *et al.* 2002). TGF- β is a potent anti-inflammatory agent and modulation of its activity has been demonstrated both in cell culture and in animal models. Thus, Ahsg itself can influence the inflammatory response. This conclusion was strengthened by a series of experiments using lipopolysaccharide (LPS)-treated macrophages and carrageenan-treated rats. Ahsg blunted the pro-inflammatory action of tumour necrosis factor α (TNF- α) through enhancement of the uptake of natural anti-inflammatory polyamines like spermidine and related drugs (Wang *et al.* 1997, 1998, Ombrellino *et al.* 2001). These results warrant a thorough study of the inter-dependency of inflammation and Ahsg expression.

Injury, trauma or infection triggers a complex series of local and systemic reactions known as the acute phase response (APR). The APR prevents excessive tissue

damage, mediates the isolation and destruction of invading microorganisms and activates repair reactions necessary to restore homeostasis. The systemic APR is associated with fever, neutrophilia, changes in lipid metabolism, increased gluconeogenesis, activation of the complement pathways, hormonal changes and induction of acute phase proteins (APP) (Baumann & Gauldie 1994). APPs are classified as positive APPs when their serum concentration increases during APR. This is the case with serum amyloid A, C-reactive protein, and complement C3 and C5. In contrast, albumin, transferrin, transthyretin and *Ahsg* (Gabay & Kushner 1999) are all considered as negative APPs, because their serum concentration decreases by at least 25% during inflammatory disorders (Morley & Kushner 1982). Up-regulation of positive APPs during inflammatory episodes and the underlying regulatory mechanisms are well understood. Proteins are classified into type 1 and type 2 APPs corresponding to the inducing cytokines (interleukin (IL)-1-like cytokines and IL-6-like cytokines respectively, reviewed in Ramadori & Christ 1999). For example, the down-regulation of the transthyretin gene expression during APR is caused by a reduction in hepatocyte nuclear factor (HNF)-3 α and correlates with a decrease of its target gene, transthyretin (Qian *et al.* 1995). The reduced expression of the negative APP albumin is mediated by a TNF- α -induced phosphorylation on Ser239 within the nuclear localization signal of CCAAT enhancer binding protein β (C/EBP- β), thus inducing nuclear export and disrupting transcription (Buck *et al.* 2001). The mechanism for APR-induced negative regulation of the *Ahsg* gene (serum level decreased by 50–70% in humans (Lebreton *et al.* 1979), rats (Daveau *et al.* 1990) and mice (Gangneux *et al.* 2003)) was linked to a cytokine-induced replacement of long C/EBP isoforms (maintaining strong basal *Ahsg* gene transcription) by short C/EBP isoforms, which were unable to transactivate *Ahsg* gene transcription (Gangneux *et al.* 2003). Thus, today, the down-regulation of *Ahsg* is mechanistically well understood. In contrast, our knowledge of molecular mechanisms governing the subsequent reactivation of *Ahsg* is limited.

Here, we studied the influence of glucocorticoids on the mouse *Ahsg* promoter activity. Glucocorticoids are potent anti-inflammatory reagents, which become naturally elevated after the initial acute phase response thus counteracting the adverse effects of inflammation. Our working hypothesis was that glucocorticoids may enhance *Ahsg* expression on the transcriptional level and may therefore be useful in up-regulating *Ahsg* during inflammation. We report that dexamethasone up-regulates *Ahsg* gene transcription through specific DNA elements in the promoter region of the mouse *Ahsg* gene in an indirect fashion. Dexamethasone treatment increased cellular C/EBP- β thus enhancing a transcriptional complex comprising C/EBP- β and HNF-3 β

mediating the transcriptional activation of the *Ahsg* gene.

Materials and methods

Isolation and culture of primary mouse hepatocytes

Mouse hepatocytes were isolated by an adaptation of the collagenase method for the isolation of rat hepatocytes (Le Cam 1995). Briefly mice were anaesthetized with avertin and the liver was perfused *in situ* with collagenase (Gibco BRL, Karlsruhe, Germany) through the inferior vena cava. After a digestion period of 10 min, the liver capsule was ruptured with forceps and liver cells were passed through a cell strainer. Cells were washed using William's medium E (Sigma) and centrifuged twice at 16 *g* for 4 min at room temperature. Finally, cells were suspended in William's medium E containing 5% fetal calf serum (FCS), 100 U/ml streptomycin and 100 mg/ml penicillin at a density of 1×10^5 cells/ml. One millilitre of the cell suspension was cultured in 0.2% gelatin-coated 12-well tissue culture dishes. After 1 h at 37 °C in 5% CO₂, non-adherent cells were removed, and fresh medium was added. After an overnight incubation, the hepatocytes were washed twice and cultured under serum-free conditions for 24 h. Hepatocytes were subsequently stimulated with dexamethasone in fresh medium for an additional 24 h and 48 h. To block translation, the cells were treated with cycloheximide (10 μ g/ml) for 24 h prior to RNA isolation.

RNA preparation and real-time PCR

Total RNA was prepared from cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) combined with an on-column RNase-free DNase digestion according to the manufacturer's instructions. The integrity and the amount of total RNA were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). cDNA was reverse transcribed from 200 ng total cellular RNA using random nonamer primers and a Reverse Transcriptase Core Kit (Eurogentec, Seraing, Belgium). Quantification of cDNA was performed with real-time PCR according to the manufacturer's instructions using the qPCR Core Kit for SYBR Green I (Eurogentec) containing 2 μ l template cDNA from reverse transcription, 3.5 mM MgCl₂, 0.2 mM dNTP solution, 100 nM fluorescence labelled *Ahsg* probe (Eurogentec), 0.45 units Taq-Polymerase and 0.3 μ M of each oligonucleotide primer (MWG Biotech AG, Ebersberg, Germany). Real-time PCR was performed with the sequence detection system ABI 7700 (PE, Applied Biosystems, Foster City, CA, USA). The 18S ribosomal RNA standard (Applied Biosystems, Warrington, Cheshire, UK) was used as an

Table 1 Oligonucleotide primers used for real-time PCR

Target gene	5' to 3' sequence		Amplicon length
	Sense	Antisense	
Ahsg	CCTGACTCCGTTCAACGATACC	GAGTAGACACTGGGAGAGGCACA	139 bp
	Probe	FAM-CCGTCAACACTGCCCTGGCTGC-TAMRA	

internal standard to control for variability in amplification caused by differences in the amount of input mRNA and/or cDNA. Oligonucleotide primers used for real-time PCR are shown in Table 1. Relative mRNA changes were reported as fold changes over background expression in unstimulated control cells.

Ahsg promoter luciferase reporter constructs

A pUC18 HindIII-SalI clone containing 9.5 kb genomic DNA including 7988 bp of 5'-upstream sequences, exon 1 and a part of intron 1 of the mouse Ahsg gene was used to generate Ahsg luciferase reporter constructs (see Fig. 2). This plasmid served as a template for PCR employing Pfu DNA polymerase (Stratagene, Heidelberg, Germany). We used Ahsg gene-specific primers gen-mAhsg1 (5'-CAGCCTCCTCAGTAGCTACAATTAGGA-3', nucleotide (nt) -2634 to -2607) and gen-mAhsg2 (5'-ATGGTTGCTCGAGAGAGGCCTTGGA-3', nt +75 to +50) to introduce a XhoI site (underlined) into the PCR product by exchanging a C to G (bold). A StuI-XhoI fragment of the PCR product was inserted into SmaI/XhoI digested luciferase reporter plasmid pGL3 basic (Promega, Mannheim, Germany) and termed pGL3 basic-mAhsg-2575/+68. Then a SacI-NdeI fragment was cut out of pUC18 HindIII-Sal 9.5 Ahsg and ligated into the SacI and NdeI sites within pGL3 basic-Ahsg-2575/+68 resulting in a plasmid named pGL3 basic-Ahsg-7988/+68 according to the inserted number of nucleotides 5' of the Ahsg transcription start. Additional deletion constructs were made by cutting the pGL3 basic-mAhsg-2575/+68 plasmid at two single restriction enzyme sites and re-ligating the corresponding ends. Restriction sites leading to 5' deletion constructs were SacI/NdeI (-2065/+68), SacI/EspI (-1318/+68), SacI/Age (-521/+68), SacI/Eco72I (-314/+68) and SacI/BalI (-260/+68). All restriction and modifying enzymes were purchased from MBI Fermentas (St Leon-Rot, Germany). A second set of deletion constructs (-107/+68, -65/+68 and -41/+68) were generated by using Pfu DNA polymerase, specific forward primers (5'-CCAGAGCAGCTGTTTGCAAGG-3', 5'-CGTCCGGGCCTTACGCAATT-3' and 5'-TTCCCCGGGCCTTACGCAATT-3', respectively) and the reverse primer gen-Ahsg2. The PCR products were cut with

PvuII/XhoI (-107/+68) or SmaI/XhoI (-65/+68 and -41/+68) and ligated into pGL3 basic linearized by SmaI/XhoI. The deletion construct pGL3 basic-mAhsg-260ΔSRU/+68 lacking the putative steroid response unit (SRU) was cloned by ligating a 105 bp fragment from the pGL3 basic-mAhsg-260/+68 plasmid into the KpnI site and the Klenow fragment blunted NheI restriction sites of the pGL3 basic-mAhsg-260/+68 plasmid. All deletion constructs are verified by DNA sequencing.

Cell culture, transient transfection and luciferase assays

Mouse hepatoma cells Hepal-6 (DMSZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium containing 10% FCS, glutamate, 100 units/ml penicillin and 100 mg/ml streptomycin. For transient transfection of Hepal-6 cells 1×10^5 cells were seeded on 12-well tissue culture dishes and allowed to grow overnight. Medium was replaced and cells were transfected with 1.8 µg plasmid DNA using the calcium phosphate method (Chen & Okayama 1987). After 16–18 h, medium was renewed and cells were allowed to grow in the presence of 5 µM dexamethasone or dexamethasone/mifepristone (RU486) 5 µM each for 48 h before harvesting. Harvesting and luciferase assays were performed according to the protocol of the Steady-Glo Luciferase Assay System (Promega). Cotransfection of the pSV-β-galactosidase control vector (Promega) was used to normalize basal luciferase results for variation in transfection efficiency. For some experiments we used the Dual-Glo Luciferase Assay System (Promega) and cotransfected the renilla luciferase reporter vector phRL-TK Vector as the internal control. Each data point represents the average of a minimum of three independent experiments performed in triplicate. All error bars represent the standard error of the mean (S.E.M.).

Nuclear extracts

For extraction of nuclear proteins, mouse hepatoma cells Hepal-6 were washed twice with ice-cold phosphate buffered saline (PBS) containing 100 µM Na₃VO₄. Cells

were harvested in 1 ml hypotonic buffer A (10 mM Hepes-KOH, pH 7.6, 2 mM MgCl_2 , 15 mM KCl, 100 μM Na_3VO_4 , 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT)) using a rubber policeman and lysed for 10 min at 4 °C. After centrifugation (700 *g* at 4 °C) for 5 min the supernatant was discarded. The pellet was resuspended in buffer C (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 100 μM Na_3VO_4 , 0.5 mM PMSF, 0.5 mM DTT), incubated for 20 min at 4 °C and centrifuged at 16 000 *g* for 10 min yielding nuclear extracts for further processing. Protein concentrations of nuclear extracts were measured with the Roti-Nanoquant protein assay (Roth, Karlsruhe, Germany).

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing the putative glucocorticoid response unit of the mouse *Ahsg* promoter were generated by annealing two single-stranded oligonucleotides (see Table 2; MWG-Biotech AG, Ebersberg, Germany). The oligonucleotide probes were labelled using Klenow enzyme and [α - ^{32}P]dATP (10 mCi/ml, 3000 Ci/nmol). For each EMSA reaction 10 μg protein of nuclear extract were incubated with 100 fmol probe in EMSA incubation buffer (10 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM MgCl_2 , 5% (v/v) glycerol, 10 mM DTT) in 20 μl reaction volume containing 1 μg poly(dI-dC) for 30 min at room temperature. All oligonucleotides used for EMSAs were designed with the same overhanging 5'- and 3'-sequences for cloning purposes (sense: 5' ctagccgcggg, 3' c; antisense: 5' tcgag, 3' ccgcggg). The entire sequences were scanned for putative transcription factor binding sites using public domain software (Transcription Element Search Software, URL: <http://www.cbil.upenn.edu/tess>; TFBlast of TRANSFAC 6.0 – Public, URL: <http://www.gene-regulation.com/cgi-bin/pub/programs/tfbblast/tfbblast.cgi>). For supershift experiments nuclear extracts were pre-incubated with antibodies against the glucocorticoid receptor (GR), C/EBP- α , C/EBP- β and HNF-3 β ('P-20', 'N-19', 'C-19', 'M-20' respectively; Santa Cruz Biotechnology, Heidelberg, Germany) for an additional 30 min at room temperature. EMSAs were performed in reaction buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) as recommended by Santa Cruz. When indicated, 10-molar and 100-molar excess of unlabelled oligonucleotides were included in the binding reaction. All protein-DNA complexes were resolved on a 6% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris-HCl, pH 8.0, 20 mM boric acid, 0.5 mM EDTA). Gels were dried, exposed to phosphor screens overnight and evaluated using the Molecular Imager FX (BIORAD, München, Germany).

Immunoblotting

Protein extracts from Hepal-6 and primary mouse hepatocytes were diluted in reducing SDS-PAGE sample buffer containing 5% 2-mercaptoethanol, separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membrane (Protran NC, Schleicher & Schuell, Dassel, Germany) by semi-dry electro-blotting (1 h, 1.5 mA/cm²). After blocking the membranes with buffer (PBS containing 1.5% nonfat milk) for 30 min at 37 °C, membranes were incubated for 30 min at 37 °C with the primary antibodies against the GR, C/EBP- α , C/EBP- β and HNF-3 β ('P-20', 'N-19', 'C-19', 'M-20' respectively, Santa Cruz Biotechnology) dissolved in blocking buffer. Membranes were washed four times with buffer (PBS containing 0.05% NP40) and incubated for 30 min at 37 °C with peroxidase-conjugated polyclonal IgG (goat anti-rabbit for 'P-20' and 'C-19'; rabbit anti-goat for 'N-19' and 'M20') dissolved in blocking buffer. Goat anti-rabbit IgG was purchased from Jackson ImmunoResearch (Westgrove, PA, USA) and rabbit anti-goat IgG was purchased from Sigma (Taufkirchen, Germany). After washing four times with washing buffer, proteins were detected by enhanced chemiluminescence (ECL reagent, Amersham Biotech, Freiburg, Germany).

Decoy oligonucleotide approach

Generally, in the decoy approach double-stranded oligonucleotides with specific binding sequences for transcription factors are brought into living cells to selectively disrupt the function of these factors. In the cells, transcription factors then interact with an excess of decoy oligonucleotides instead of binding to the natural regulatory motifs of genes (Bielinska *et al.* 1990, Kraus *et al.* 2003). Hepal-6 cells were seeded in 12-well tissue culture dishes. After 24 h, cells were washed once with PBS and kept in culture medium without FCS. Decoy oligonucleotides (5 μM) were directly added to the medium and after 24 h cells were stimulated with dexamethasone (5 μM) for an additional 24 h. The oligonucleotides had no chemical modifications. To exclude unspecific effects of decoy oligonucleotides that might be a result solely of their presence, 5 μM of a randomly designed oligonucleotide were used as a control. RNA preparation and real-time PCR were performed as described above.

Statistical analysis

For statistical evaluation, Student's *t*-tests (two sided and for independent samples) were performed. Significance levels are indicated (* P <0.05, ** P <0.01).

Figure 1 Effect of dexamethasone on mouse Ahsg mRNA levels. Total RNA was isolated from primary mouse hepatocytes (black columns) exposed to different concentrations of dexamethasone (0.1 to 50 µM) for 48 h. Ahsg mRNA was measured using real-time PCR. Data represent the mean of two independent primary mouse hepatocyte preparations both analysed in duplicate. Hepa1-6 cells (grey columns) were treated with 5.0 µM dexamethasone according to the treatment of the primary hepatocytes. Ahsg mRNA quantities are given as fold induction of untreated controls. For Hepa1-6 cells data represent the mean of two independent experiments.

Results

Glucocorticoid hormone up-regulates mouse mRNA levels

The effect of glucocorticoid hormones on Ahsg gene expression was investigated using the stable analogue, dexamethasone. Ahsg mRNA levels were analysed using real-time PCR in untreated primary mouse hepatocytes and in hepatocytes treated with dexamethasone as well as in treated and untreated mouse hepatoma cells (Hepa1-6). Figure 1 illustrates the dexamethasone-stimulated Ahsg mRNA production in mouse hepatocytes. The lowest concentration of dexamethasone used (0.1 µM) induced a 3.9-fold higher transcription of Ahsg. Increasing the concentrations of dexamethasone up to 1 µM and 5 µM resulted in an eight-fold and seven-fold stimulation of Ahsg gene transcription. The dexamethasone response was saturable because dexamethasone concentrations exceeding 5 µM did not further increase the Ahsg gene transcription in primary mouse hepatocytes. For further evaluation of the regulatory mechanisms underlying this up-regulation we used mouse hepatoma cells (Hepa1-6) as a model system. We first determined the expression of Ahsg in Hepa1-6 cells and compared the transcript amounts of Ahsg mRNA with those expressed in primary mouse hepatocytes. In comparison, primary mouse hepatocytes expressed much more Ahsg than Hepa1-6 cells (2764 vs 4 copies Ahsg/10 million copies 18S respectively; data not shown). Stimulation of Hepa1-6 cells with 5 µM dexamethasone enhanced the Ahsg gene transcription about 15.2-fold compared with untreated Hepa1-6 cells (Fig. 1) indicating that despite low basal expression Hepa1-6 cells regulate their Ahsg production like

primary mouse hepatocytes and thus are well suited for the study of the mouse Ahsg promoter regulation.

Glucocorticoid responsiveness of the mouse Ahsg promoter

To detect regulatory elements conferring glucocorticoid responsiveness to the mouse Ahsg gene promoter we performed a computer sequence search for putative glucocorticoid receptor binding sites. We determined two possible sequences within the mouse Ahsg promoter sharing 80% sequence identity with a classical glucocorticoid response element (GRE) sequence and also containing the core sequence 5'-TGTNC-3' at the 3' end of the imperfect palindromic classical GRE (Beato *et al.* 1989). The putative Ahsg elements (termed E1 and E2) and the classical GRE sequence are listed in Table 2. To investigate if these elements were involved in the observed mRNA up-regulation of the Ahsg gene in primary mouse hepatocytes, we subcloned the proximal 7988 bp of the 5' promoter/regulatory sequence of the mouse gene (Genbank/EMBL AF007900) into plasmid pGL3 basic upstream of a luciferase reporter gene. When this construct (pGL3 basic-Ahsg - 7988/+68) was transiently transfected into Hepa1-6 cells subsequently treated with 5 µM dexamethasone, luciferase activity was stimulated 10-fold as shown in Fig. 2. Progressive shortening of the Ahsg promoter by 5'-deletions did not affect the induced reporter gene activity, as long as both putative elements remained intact (up to pGL3 basic-Ahsg - 314/+68). A reporter construct lacking E1 (pGL3 basic-Ahsg - 260/+68) but containing the second putative element E2 (nt - 146) like the longer constructs, still showed 23-fold

Table 2 Putative glucocorticoid response elements within the Ahsg promoter. The sequences of the two putative Ahsg GRE sites with sequence homology to the classical GRE (Beato *et al.* 1989) are shown (underlined). The location of the elements is given with respect to the transcription initiation site of the Ahsg gene

Putative element	Position	Sequence	Homology compared to consensus
E1	−277	<u>GCTGCGCTCTGTGCT</u>	12/15 (80%)
E2	−146	<u>GAGAGATGATGTCCT</u>	12/15 (80%)
GRE consensus		GGTACANNNTGTNCT	

induction of luciferase activity following stimulation of transfected cells by dexamethasone (Fig. 2B). In contrast, deleting E2 by 5' deletion or in the context of the pGL3 basic-Ahsg −260/+68 construct completely abolished the increased reporter gene activity following stimulation with dexamethasone (Fig. 2B). This finding indicates that the putative element E2 may mediate the up-regulation of the Ahsg promoter reporter gene constructs by dexamethasone.

The enhancement of Ahsg promoter transcriptional activity by dexamethasone is glucocorticoid receptor dependent

To establish that the enhancement of the Ahsg promoter transcriptional activity by dexamethasone was mediated by the glucocorticoid receptor, dexamethasone treatments similar to those described above were conducted in the presence of the glucocorticoid receptor antagonist, RU486. Hepa1-6 cells transfected with pGL3 basic-Ahsg reporter constructs (−7988/+68, −260/+68, −107/+68 and pGL3 promoter) were grown in the presence or absence of 5 μM dexamethasone with or without 5 μM RU486 for 48 h (Fig. 3). As in the experiments reported in Fig. 2, dexamethasone stimulation induced the luciferase reporter gene activity. When RU486 was administered to the cells together with dexamethasone, reporter gene activity for pGL3 basic-Ahsg −7988/+68 was still up-regulated threefold. The promoter construct pGL3 basic-Ahsg −260/+68 was most strongly up-regulated after dexamethasone treatment (30-fold). The concomitant addition of RU486 abolished the stimulatory effect of dexamethasone. In contrast, reporter construct activity of the E2-less pGL3 basic-Ahsg −107/+68 and pGL3 promoter was not influenced by any treatment. These experiments suggested that RU486, a strong and specific glucocorticoid receptor antagonist, blocked the stimulatory action of dexamethasone on Ahsg gene expression, indicating that the glucocorticoid receptor directly or indirectly mediated the dexamethasone-induced enhancement of Ahsg transcriptional activity.

Cycloheximide pretreatment abolishes the dexamethasone-induced up-regulation of the Ahsg gene transcription

To clarify whether Ahsg gene transcription was up-regulated by direct binding of the GR to the Ahsg gene promoter or via induction of newly transcribed transcription factor(s), cells were treated with dexamethasone and the protein synthesis inhibitor cycloheximide simultaneously for 24 h. As already shown for dexamethasone treatment for 48 h (Fig. 1), Ahsg mRNA levels were also up-regulated by dexamethasone after 24 h. Cycloheximide treatment alone had no effect on Ahsg gene transcription but treatment of the cells with dexamethasone in combination with cycloheximide for 24 h completely abolished the stimulatory effect of dexamethasone (Fig. 4). Hence, the glucocorticoid stimulated up-regulation of Ahsg transcription was indirect and required *de novo* protein synthesis.

Sequence alignment of the proximal promoters of the Ahsg genes

Next, we asked which transcription factors might be involved in regulating the glucocorticoid-mediated transcriptional up-regulation of the Ahsg gene. To this end we performed a sequence analysis of the published 5' promoter regions of the Ahsg genes in humans, rats and mice. Our analysis revealed that the regulatory sequences were highly conserved in the promoter region bearing the SRU in all three species (Fig. 5). All three promoter sequences contained a putative GRE with an overlapping C/EBP-β binding site (Falquerho *et al.* 1992) and a putative HNF-3 binding site with high sequence homology to known functional HNF-3β sites and the consensus sequence (Table 3). Mouse and rat SRUs showed 96.4% sequence identity, whereas the SRU in the human Ahsg promoter (nt −162) showed 79.6% sequence identity. This finding suggested a common regulatory mechanism in all three species.

Figure 2 Transient expression of reporter gene constructs in Hepa1–6 cells. (A) A cartoon at the top shows the promoter of the Ahsg gene with the relative location of the two putative glucocorticoid response elements (E1, nt –277; E2, nt –146). The first exon of the Ahsg gene is shown as a box and the transcription start site (tsp) as an arrow. On the left side, the Ahsg promoter reporter gene constructs are indicated relative to their position within the gene. On the right side, basal luciferase activities for the respective Ahsg promoter constructs together with the SV40 promoter bearing the positive control plasmid (pGL3 promoter) and the promoterless vector (pGL3 basic) are shown. Hepa1–6 cells were transiently transfected with Ahsg promoter constructs and basal luciferase activity was monitored after 24 h. Reporter gene activities were expressed as luciferase units normalized to β -galactosidase activity, which was co-transfected in each case. (B) Transiently transfected Hepa1–6 cells were stimulated with dexamethasone (5 μ M) for 48 h (black columns). Ahsg promoter-dependent luciferase activity was expressed as fold induction in comparison with unstimulated controls (white columns). Data represent the means $\pm$ s.e.m. derived from at least three independent experiments measured in triplicate. For statistical evaluation, Student's *t*-tests were performed. Significance levels are indicated as ***P* < 0.01.

Figure 3 Influence of RU486 on dexamethasone-induced Ahsg reporter activity. Transiently transfected cells were treated for 48 h with dexamethasone (5 µM) alone or with dexamethasone and RU486 (5 µM). Luciferase activity was expressed as fold induction for dexamethasone-treated transfected cells (black columns) and for dexamethasone/RU486-treated transfected cells (grey columns) compared with untreated controls (white columns): pGL3 promoter vector (prom) was used as a control. Data represent the means ± S.E.M. derived from four independent experiments measured in triplicate. For statistical evaluation, Student's *t*-tests were performed. Significance levels are indicated as ***P* < 0.01.

C/EBP-β and HNF-3β bind to the SRU within the Ahsg promoter

Next, we performed EMSAs to identify the transcription factors binding to the putative recognition sites within the SRU of the mouse Ahsg gene promoter. We analysed if the glucocorticoid receptor itself was involved in transcriptional up-regulation of the mouse Ahsg gene following treatment with dexamethasone or whether additional components or factors were also involved (Fig. 6A). To this end, we incubated nuclear extracts isolated from dexamethasone-treated or untreated Hepa1-6 cells with a ³²P-labelled fragment of the mouse Ahsg gene promoter (Ahsg -150/-113). This fragment contained a putative GRE as well as the overlapping C/EBP binding site identified in the rat Ahsg promoter. Additionally, the fragment also contained a putative HNF-3 binding site located 3' of the C/EBP site as shown in Fig. 5. Incubation of radioactively labelled Ahsg -150/-113 with extracts from untreated Hepa1-6 cells caused a distinct bandshift (Fig. 6A, lane 1, arrow). In nuclear extracts of dexamethasone-treated cells the intensity of the band detected in lane 1 was slightly increased (Fig. 6A, lane 2). To reveal the identity of the transcription factors bound to the SRU we used specific antibodies. Pre-incubation with a glucocorticoid receptor antibody (Fig. 6A, lane 3) slightly decreased and

Figure 4 Effect of the protein synthesis inhibitor, cycloheximide, on dexamethasone-induced Ahsg gene transcription. Total RNA was isolated from primary mouse hepatocytes exposed to 5 µM dexamethasone (dex) for 24 h in combination with 10 µg/ml cycloheximide (chx) as shown in the scheme. After reverse transcription, cDNA was amplified with Ahsg specific primers using real-time PCR. Data represent the mean of two independent primary mouse hepatocyte preparations both analysed in duplicate. Relative Ahsg mRNA quantities are given as fold induction of untreated controls.

Figure 5 Schematic drawing of SRU organization and sequence homology of the Ahsg SRU. The C/EBP binding site (Falquerho *et al.* 1992) and putative transcription factor binding sites for glucocorticoid receptor and HNF-3 within the Ahsg promoter, which might be responsible for dexamethasone-induced transcriptional up-regulation, are shown. A high degree of sequence identity exists in the SRUs of mouse, rat and human Ahsg. The location of the SRUs is given with respect to the transcription initiation sites of the three Ahsg genes (Genbank accession numbers: human Ahsg: AB038689; rat Ahsg: M36547; mouse Ahsg: AF007900). Consensus sequences for putative transcription factors are shown in italics. Transcription factor binding sites within the SRU are highlighted; GRE, boxed; C/EBP, capital letters, and HNF-3, boldface.

an antibody against C/EBP- α (Fig. 6A, lane 4) did not affect the intensity of the shifted complexes in nuclear extracts of dexamethasone-treated Hepa1-6 cells. However, pre-incubation of nuclear extracts with a specific antibody against C/EBP- β resulted in a complete loss of the shifted complex (Fig. 6A, lane 5) suggesting that binding of C/EBP- β was essential for the formation of the transcriptional complex within the SRU of the Ahsg gene promoter. Furthermore, addition of a specific HNF-3 β antibody (Fig. 6A, lane 6) caused a supershift (arrowhead) of the entire complex, indicating that C/EBP- β and HNF-3 β were both present in the shifted complex. These results confirmed the results presented in Fig. 2B in that only the sequence comprising GRE-E2, C/EBP- β and HNF-3 β served as

functional transcription factor binding sites whereas the putative GRE-E1 was not functional in up-regulating Ahsg transcription.

Next, we determined the minimal binding sequence responsible for transcription factor complex formation at the SRU of the Ahsg gene promoter using unlabelled oligonucleotides to compete for protein-DNA complex formation. We determined that oligonucleotides containing both the C/EBP- β and the HNF-3 β binding site ($-138/-116$, Fig. 6A, lanes 7 and 8) blocked binding of a nuclear complex to the Ahsg SRU-containing probe in a concentration-dependent manner. To determine whether both binding sites (C/EBP- β and the HNF-3 β) together were necessary for complex formation at the Ahsg SRU, an oligonucleotide containing only five

Table 3 Sequence alignment of HNF-3 β binding sites. The HNF-3 binding sites are derived from several promoters and enhancers. All sequences are aligned to the HNF-3 β consensus sequence (Overdier *et al.* 1994) (underlined letters indicate mismatches with consensus)

Gene/Reference	Recognition sequence	Similarity to consensus
Transthyretin (Welsheimer & Newbold 1996)	GATTATTGACTT	12/12
Transthyretin TTR-2 (Samadani <i>et al.</i> 1996)	ACATGTTTGAAC	9/12
Vitronectin site A (Shimizu <i>et al.</i> 2002)	CTTTATTTGCTC	11/12
Vitronectin site B (Shimizu <i>et al.</i> 2002)	CAGTGTTTGCT	10/12
Aldolase B (Raymondjean <i>et al.</i> 1991)	AGGTGTTTATTC	10/12
Surfactant Protein B (Bohinski <i>et al.</i> 1994)	ACTTGTTTGTTT	11/12
HNF-1 (Kuo <i>et al.</i> 1992)	CTCTGTTTACAT	9/12
HNF-3 β (Overdier <i>et al.</i> 1994)	CCCTGTTTGTTT	10/12
Mouse Ahsg	ACTTATTTGCTT	11/12
RAT Ahsg	ACTTATTTGCTT	11/12
Human Ahsg	ACCTGTTTGCTT	10/12
HNF-3 β consensus (Overdier <i>et al.</i> 1994)	VAWTRTTKRYTY	

Abbreviations for nucleotides: W, A or T; K, G or T; Y, C or T; R, G or A; V, A, C or G.

Figure 6 The Ahsg SRU motif binds C/EBP-β and HNF-3β. (A) We performed electrophoretic mobility shift assays with a labelled oligonucleotide containing the putative Ahsg SRU (-150/-113). Nuclear extracts from Hepa1-6 cells treated with dexamethasone for 4 h (+) or not treated (-) were incubated with the radiolabelled Ahsg SRU probe. Specific antibodies (α) against the glucocorticoid receptor (GR), C/EBP-α, C/EBP-β and HNF-3β were added to the extracts prior to probe addition to show specificity of shifted complexes. Supershifted complexes are indicated with an arrowhead. Unlabelled competitor oligonucleotides are labelled according to their position within the mouse Ahsg gene promoter and the respective sequences are shown below (putative GR, underlined; C/EBP-β, italics; and HNF-3β, boldface). (B) Binding of nuclear proteins was monitored using radiolabelled probes (-156/-127, -138/-116, and -134/-115). (C) Nuclear extracts were pre-incubated with increasing concentrations of C/EBP-β antibody shown by the black wedge.

nucleotides of the C/EBP- β site and the HNF-3 β site was used in competition experiments (–134/–115, Fig. 6A, lanes 9 and 10). This competitor DNA effected similar complete competition of the bound protein complex like oligonucleotide –138/–116 containing both the complete C/EBP- β and the HNF-3 β binding sites. In contrast, competitor DNA including the putative GR binding site E2 together with the C/EBP- β site and lacking the HNF-3 β site (–156/–127) as well as a randomly designed oligonucleotide did not compete with nuclear protein binding to the Ahsg SRU probe (Fig. 6A, lanes 11 to 14). Finally, competition with a HNF-3 β consensus site oligonucleotide with three base pairs difference from the Ahsg HNF3- β sequence showed a decrease in the bound complex (Fig. 6A, lanes 15 and 16) but less complete than that observed with competitors containing the autologous HNF-3 β site.

To verify the results of the competition experiments, oligonucleotides were radioactively labelled and used as probes in EMSA (Fig. 6B). Two probes containing the HNF-3 β site (–138/–116, –134/–115) were able to bind a protein complex consisting of C/EBP- β and HNF-3 β (Fig. 6B, lanes 5, 6, 9 and 10) as defined by pre-incubation with antibodies against C/EBP- β (Fig. 6B, lanes 7 and 11) and HNF-3 β (Fig. 6B, lanes 8 and 12). In contrast, no radioactively labelled transcription complex was detected when nuclear extracts were incubated with a probe containing the GRE-E2 together with the C/EBP- β site (–156/–127, Fig. 6B, lanes 1 to 4).

Next, we determined which transcription factors participate in SRU binding employing antibody supershift and EMSA. To this end we pre-incubated nuclear extracts with increasing concentrations of an antibody against C/EBP- β and used the –134/–115 oligonucleotide containing the half-site of C/EBP- β and the complete HNF-3 β site as a probe in EMSA. As shown in Fig. 6C, the bound protein complex (lanes 1 and 2) disappeared in a concentration-dependent manner (lanes 3 to 8).

Decoy oligonucleotides interfere with basal Ahsg gene transcription in hepatoma cells

We asked if the binding of C/EBP- β and HNF-3 β to the Ahsg SRU could also be disrupted in living cells. To this end we transfected intact hepatoma cells with the oligonucleotides used for EMSA experiments as double-strand decoy oligonucleotides and measured Ahsg gene transcription. Figure 7 shows that decoy oligonucleotides spanning the 23 nt of the Ahsg promoter (–138/–116) containing the C/EBP- β and HNF-3 β sites significantly repressed basal Ahsg gene transcription down to 53% of basal activity. In contrast, HNF-3 β consensus decoy oligonucleotides or random

Figure 7 Basal Ahsg gene transcription in Hepa1–6 cells is down-regulated by decoy oligonucleotides containing the C/EBP- β and HNF-3 β binding site (nt –138/–116) within the Ahsg promoter. Total RNA was isolated from Hepa1–6 hepatoma cells treated without (w/o) or with double-stranded decoy oligonucleotides for 24 h (random: randomly designed oligonucleotide; HNF-3 β : consensus sequence; –138/–116: Ahsg SRU containing binding site for C/EBP- β and HNF-3 β). Ahsg mRNA was measured using real-time PCR. Data represent the means $\pm$ S.E.M. derived from four independent experiments. Relative Ahsg mRNA quantities are given as fold induction of untreated cells. For statistical evaluation, Student's *t*-tests were performed. Significance levels are indicated as **P* < 0.05.

decoy oligonucleotides had no significant effect on Ahsg gene transcription (Fig. 7).

Mouse hepatoma cells and primary mouse hepatocytes up-regulate C/EBP- β protein following stimulation with dexamethasone

In view of the indirect action of dexamethasone in enhancing Ahsg gene transcription, we performed western blot analysis to determine if dexamethasone influenced the expression of C/EBP- β and HNF-3 β thus enhancing Ahsg gene expression. The amounts of full length C/EBP- β (38 kDa) and an isoform called liver-enriched activator protein (LAP, 34 kDa) were both increased in Hepa1–6 cells after dexamethasone treatment compared with untreated cells (Fig. 8A). C/EBP- β (38 kDa/34 kDa) protein expression was also up-regulated in primary mouse hepatocytes following dexamethasone treatment (Fig. 8B). The amounts of HNF-3 β and GR protein remained unchanged in all samples. Interestingly, Hepa1–6 cells generally showed higher amounts of a short 21 kDa C/EBP- β isoform (designated LIP), which is thought to inhibit C/EBP- β action (Fig. 8).

Figure 8 Western blot analysis of transcription factors in Hepa1-6 cells and primary mouse hepatocytes. (A) Hepa1-6 cells and (B) primary mouse hepatocytes were treated with dexamethasone for 24 h and 48 h. Cell extracts were separated by SDS-PAGE, electrotransferred to nitrocellulose, and GR, C/EBP-β and HNF-3β were detected with specific antibodies. Each protein band is labelled with name and size (kDa). In each lower panel the amount of actin is detected as loading control. Note that C/EBP-β was strongly induced following dexamethasone treatment of Hepa1-6 cells and slightly up-regulated in primary hepatocytes.

Figure 9 Over-expression of C/EBP-β or HNF-3β enhances Ahsg promoter activity. Neuroblastoma × glioma cells were transiently transfected with Ahsg promoter reporter gene constructs and cotransfected with either C/EBP-β or HNF-3β expression plasmids (1 ng). After 24 h in culture, luciferase activity was measured. Ahsg promoter-dependent luciferase activity was expressed as fold induction for C/EBP-β cotransfection (black columns) and for HNF-3β cotransfection (grey columns) in comparison with unstimulated controls (white columns). Data represent the mean values $\pm$ S.E.M. derived from at least three independent experiments measured in triplicate. For statistical evaluation, Student's *t*-tests were performed. Significance levels are indicated as **P* < 0.05 or ***P* < 0.01.

Ahsg gene promoter activity is up-regulated after over-expression of C/EBP-β and HNF-3β in neuroblastoma × glioma cells

We used forced expression of C/EBP-β and HNF-3β to confirm the positive involvement of these factors in Ahsg promoter up-regulation observed in dexamethasone-treated Hepal-6 cells. We asked if over-expression of these two transcription factors would be sufficient to alter promoter activity of Ahsg reporter constructs containing the SRU in a cell line that does not normally express Ahsg. To this end we cotransfected expression plasmids for C/EBP-β or HNF-3β with Ahsg promoter reporter gene constructs (pGL3 basic-Ahsg – 1318/+68, pGL3 basic-Ahsg – 260/+68, pGL3 basic-Ahsg – 107/+68) into neuroblastoma × glioma NG108–15 cells and luciferase activity was measured. As shown in Fig. 9, over-expression of C/EBP-β significantly increased Ahsg promoter activity of constructs bearing the SRU (– 1318/+68, 30-fold; – 260/+68, 6-fold) whereas a construct missing the SRU (– 107/+68) showed no altered luciferase activity in NG108–15 cells. Over-expression of HNF-3β significantly up-regulated Ahsg promoter activity for the constructs – 1318/+68 (2.9-fold) and – 260/+68 (2.7-fold). Promoter activity of the SRU-less construct – 107/+68 was unchanged. Cotransfection of both HNF-3β and C/EBP-β did not further enhance reporter activity. In both cases cotransfection of expression plasmids had no effects on control vector pGL3 basic activity. Together, these results indicate that increased C/EBP-β expression

and basal expression of HNF-3β confer glucocorticoid-mediated up-regulation of the Ahsg gene promoter.

Discussion

Healthy animals can resolve acute inflammatory episodes within a few days. Prolonged disturbance of regulatory mechanisms counteracting inflammation can be fatal as in septic shock, systemic inflammatory response syndrome and multi organ failure (Yao *et al.* 1998). Natural termination of the acute phase response includes (1) up-regulation of anti-inflammatory cytokines, such as IL-4, -10 and -13, (2) enhanced transcription of soluble decoy receptors of TNF-α and IL-1 or IL-1-receptor antagonist and (3) endogenous production of glucocorticoids (Koj 1998). During inflammation, glucocorticoids are induced by pro-inflammatory cytokines and they can act on the adrenal pituitary axis to generate adrenocorticotrophic hormone and, subsequently, induce the production of cortisol. This provides a negative-feedback loop on inflammation, because corticosteroids inhibit gene expression of pro-inflammatory mediators and ensure restoration of homeostasis (Fink 1997).

Here, we demonstrated that Ahsg gene transcription is up-regulated in primary mouse hepatocytes and mouse hepatoma cells after treatment with the glucocorticoid analogue, dexamethasone. This regulation was mediated by a regulatory element of 105 base pairs containing a binding site for C/EBP (Falquerho

et al. 1992, Gangneux *et al.* 2003) and a putative binding site for HNF-3 β . Therefore, we termed this region a steroid response unit (SRU). We demonstrated that this up-regulation involved the action of glucocorticoid receptor, because RU486, a specific inhibitor of glucocorticoid receptor DNA binding (Bourgeois *et al.* 1984), completely abrogated the enhanced transcription of the *Ahsg* gene. The *Ahsg* gene response to glucocorticoids was indirect for two reasons. First, EMSA did not indicate direct binding of the glucocorticoid receptor to the putative recognition sequence within the *Ahsg* gene promoter. Secondly, the protein synthesis inhibitor, cycloheximide abolished the dexamethasone-induced up-regulation of *Ahsg* gene transcription in primary mouse hepatocytes.

We showed that in dexamethasone-treated Hepal-6 cells, a complex of transcription factors is bound to the putative SRU. This complex is composed of C/EBP- β and HNF-3 β as confirmed by our supershift experiments. Astonishingly, only the HNF-3 β site directly binds the complete protein complex as shown by competitor experiments and EMSAs with shortened oligonucleotides used as probes. The fact that neutralization of C/EBP- β by pre-incubation with a specific antibody caused a complete loss of any transcription factor binding to the SRU showed that C/EBP- β was critically required for the formation of the complex of C/EBP- β and HNF-3 β at the *Ahsg* SRU. In conclusion, both factors are essential for complex formation at the *Ahsg* SRU, but only HNF-3 β seems to directly bind to the DNA. This conclusion is supported by our results of the decoy oligonucleotide approach showing that for basal *Ahsg* gene transcription both C/EBP- β and HNF-3 β are necessary. Furthermore, over-expression of either C/EBP- β or HNF-3 β was able to enhance *Ahsg* promoter activity via the SRU. In addition, results obtained with a GR antibody (Fig. 6A, lane 3) and competition EMSA with GR consensus oligonucleotides (according to Beato *et al.* 1989, data not shown) suggested a weak interaction with the GR, which might further enhance transcription.

The modulation of transcriptional regulation via protein-protein interactions of C/EBP- β has previously been shown in various systems. Hanlon and colleagues showed that the serum response factor (SRF) and p35-C/EBP- β interact *in vivo* through the DNA-binding domain of SRF and the C-terminus of C/EBP- β . They further showed that C/EBP- β was recruited to the serum response element (SRE) by protein-protein interaction with SBF (Hanlon & Sealy 1999, Hanlon *et al.* 2000). In this context, it should be mentioned that the C/EBP- β antibody used in our experiments recognized the C-terminus of C/EBP- β and therefore our findings of complete loss of complex formation in supershift experiments might be a result of blocking the interaction of C/EBP- β with the HNF-3 β protein. Another regulatory

protein-protein interaction of C/EBP- β was shown for activation of the IL-1 β gene, where C/EBP- β interacted with the transcription factor Spi-1/PU.1 (Yang *et al.* 2000).

Unlike several studies of genomic effects of dexamethasone (Cram *et al.* 1998, Ramos *et al.* 1999, Rüdiger *et al.* 2002) we did not observe a contribution of C/EBP- α in dexamethasone-mediated stimulation of the mouse *Ahsg* gene, because an antibody against C/EBP- α was unable to alter transcription factor binding at the *Ahsg* SRU.

C/EBP- β is transcribed from an intronless gene and encodes three different isoforms, including two activators (38 kDa full length and 34 kDa LAP) and a repressor (called LIP, 21 kDa). These isoforms of C/EBP- β are differentially translated from the same mRNA (Descombes & Schibler 1991). The expression of C/EBP- β isoforms is hormonally regulated during normal development (Descombes & Schibler 1991, Diehl *et al.* 1994, Raught *et al.* 1995) as well as during regeneration following liver damage (Diehl 1998) and acute inflammatory episodes (reviewed in Ruminy *et al.* 2001). Here we showed that in Hepal-6 as well as in primary mouse hepatocytes the levels of full length C/EBP- β and the LAP isoform were up-regulated after dexamethasone treatment. These results are in full accordance with previous reports stating a dexamethasone-induced increase in C/EBP- β mRNA in hepatoma cell lines (Baumann *et al.* 1992, Gotoh *et al.* 1997). HNF-3 β , the second component of the mouse *Ahsg* SRU was likewise shown to be dexamethasone responsive (Imae *et al.* 2000). In this study, however, the protein amount of HNF-3 β did not change after dexamethasone stimulation, ruling out a major stimulatory role of HNF-3 β in dexamethasone-mediated up-regulation of *Ahsg* transcription. However, our observation that the short repressor isoform, LIP was expressed in Hepal-6 cells but not in primary hepatocytes suggested that the constitutive expression of LIP may confer weak basal expression of *Ahsg* in Hepal-6 cells. We suggest that following dexamethasone treatment the inhibitory effect of LIP is overcome by increased expression of the full length and the LAP isoforms, mediating the observed transcriptional up-regulation of the *Ahsg* gene in both cell types.

The C/EBP binding site responsible for the up-regulating signal of an anti-inflammatory stimulus (like dexamethasone) presented here was also important in the down-regulation of the *Ahsg* gene during an acute phase response (Gangneux *et al.* 2003). Thus, different C/EBP isoforms are the major players in modulating the *Ahsg* gene transcription during induction and recovery from inflammation. The fact that C/EBP- β expression is regulated by IL-1-like cytokines as well as by IL-6-like cytokines via activation of the MAP kinase pathway (reviewed in Ramadori & Christ 1999) renders these transcription factors as ideal modulators for both

down-regulation of the Ahsg gene during APR and up-regulation in response to glucocorticoid hormones during the termination of APR. Furthermore, the genes for albumin (Nawa *et al.* 1986, Sidhu *et al.* 2004), transferrin (McKnight *et al.* 1980, Sidhu *et al.* 2004) and transthyretin (Sidhu *et al.* 2004) may share a common mechanism in up-regulation, because they are also glucocorticoid hormone responsive in the maturation and differentiation of hepatocytes.

In conclusion, we demonstrated a positive regulation of Ahsg gene transcription by the glucocorticoid hormone, dexamethasone and we have shown that dexamethasone strongly up-regulates the mouse Ahsg gene through a SRU comprising C/EBP- β and HNF3- β . Moreover, binding of HNF-3 β to the newly described HNF-3 β binding site within the mouse Ahsg gene promoter is important for basal promoter activity. Dexamethasone-induced Ahsg gene transcription via C/EBP- β could modulate the transcriptional complex by protein–protein interactions with the bound HNF-3 β , and thus enhance transcription of the Ahsg gene.

This mode of gene activation may apply not only to the mouse Ahsg gene but possibly also to the rat and the human Ahsg genes, because the SRU is highly conserved in all three species. Therefore, glucocorticoid treatment may be therapeutically beneficial in situations of low serum Ahsg. Interestingly, a recent bone scan study in calciphylaxis patients reported that steroid therapy appeared to be beneficial in some patients (Fine & Zacharias 2002). It will be interesting to measure serum Ahsg in these patients undergoing glucocorticoid treatment to determine if up-regulation of serum Ahsg may be beneficial in diseases associated with low serum Ahsg such as soft tissue calcification for which there is currently no adequate therapy.

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