

Mechanism of negative regulation of rat glutathione S-transferase A2 by the cytokine interleukin 6

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A decrease in concentration of some liver proteins, including the detoxification enzyme glutathione S-transferase A2 (rGSTA2), occurs during the acute-phase response. Interleukin 6 (IL-6) with dexamethasone (DEX) decreases transcription of rGSTA2 in rat hepatocytes. The promoter region that mediates suppression of rGSTA2 was localized to 150 bp. These 150 bp were divided and used for electrophoretic mobility-shift assays. Induction of a protein that specifically bound to an oligonucleotide from this region required new protein synthesis and IL-6 with DEX in the culture media. The protein bound to part of the hepatocyte

nuclear factor 1 (HNF1) site but was different from and did not displace HNF1. A core sequence, TGATT, was required for binding. The protein also bound to an HNF1 site in the albumin promoter. We hypothesize that IL-6 along with DEX induced a novel protein that decreased transcription of rGSTA2 and possibly albumin by interfering with the transactivating function of HNF1. The protein may be an important negative regulator of transcription during the acute-phase response.

Key words: acute-phase response, dexamethasone, transcription.

INTRODUCTION

The response of an organism to injury by infection, burns and endotoxaemia can lead to a co-ordinated series of physiological and metabolic changes called the acute-phase response. Among the changes are positive and negative variations in the concentrations of serum and non-serum proteins that are produced by the liver. Most of the changes in serum proteins reflect changes in gene expression within the liver [1–3]. Interleukins 1 and 6 (IL-1 and IL-6) are the principal mediators of changes in protein expression during the acute-phase response. Type I proteins are induced by IL-1-like cytokines and include serum amyloid A and C reactive protein. Type II proteins are induced by IL-6-like cytokines and include fibrinogen, haptoglobin and α_1 -antitrypsin. Proteins (C-reactive protein and fibrinogen) whose concentrations increase within 2–6 h after initiation of the acute-phase response are called positive acute-phase proteins, whereas proteins (albumin and transferrin) whose concentrations decrease are termed negative acute-phase proteins [3]. Glucocorticoids are also necessary for the change in expression of some acute-phase proteins and play a permissive or synergistic role [1,4].

The mechanism behind the alterations in expression of most acute-phase proteins appears to be cytokine-mediated changes in the levels of transcription factors such as CCAAT/enhancer binding protein β (C/EBP β), nuclear factor κ B (NF- κ B), signal transduction and activation of transcription (STAT) proteins and activator protein 1 (AP-1). The genes of some positive acute-phase proteins contain response elements in their promoters that bind one or more members of the above transcription factor families leading to an increase in transcriptional activity [3,5]. The mechanisms that cause a decrease in the levels of negative acute-phase proteins are not well understood. Recent works [6,7]

have focused on changes in hepatocyte nuclear factor 1 (HNF1). HNF1 is a transcription factor that is essential for the basal and inducible transcription of many hepatocyte genes, including albumin and the bile acid transporter *ntcp*. Changes in the levels of HNF1 may account for the decline in transcriptional activity of the *ntcp* gene observed during the acute-phase response [8]. A similar mechanism has been described for the down-regulation of murine growth-hormone receptor by lipopolysaccharide or by tumour necrosis factor α [9]. Decreased binding of the transcription factors sp1 and sp3 to the growth-hormone receptor-promoter results in lower expression of the growth-hormone receptor.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are an important family of detoxification enzymes. Most of the well characterized liver GSTs can be grouped into five classes of soluble proteins termed Alpha, Mu, Pi, Theta and Sigma [10–14]. Two additional classes of soluble GSTs with unusual enzymic activities, Omega and Zeta, were described recently in a variety of organisms, including mammals [15,16]. A previous study [17] had shown that in rats, concentrations of several isoenzymes of hepatic GST, especially those in the Alpha class, decrease during the acute-phase response. Studies of rat hepatocytes in primary culture showed that IL-6, in the presence but not in the absence of dexamethasone (DEX), decreased the expression of rGSTA2 (an Alpha-class GST). The effect of IL-6 was mediated through elements in the promoter of rGSTA2 [18]. In other studies, IL-1 β also decreased the expression of GSTs in cultured hepatocytes. However, a decrease in the message stability rather than a decrease in transcriptional activity appeared to account for the drop in mRNA levels [19]. Administration of IL-6 to animals caused a decrease in the levels of activity of some, but not all forms of cytochrome P450 (CYP) [20]. Moreover, studies of CYP

Abbreviations used: AP-1, activator protein 1; CAT, chloramphenicol acetyltransferase; C/EBP β , CCAAT/enhancer binding protein β ; CYP, cytochrome P450; DEX, dexamethasone; EMSA, electrophoretic mobility-shift assay; GATA, family of transcription factors that bind to (A/T)GATA(A/G) sequence of DNA; GR, glucocorticoid receptor; GRE, GR element; GST, glutathione S-transferase; HNF1, hepatocyte nuclear factor 1; IL-1, interleukin 1; NP, nuclear protein; STAT, signal transduction and activation of transcription.

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in cultured cells have shown that cytokines suppress the induction of CYP by xenobiotics at both transcriptional and post-transcriptional levels [21]. Therefore several important families of drug-metabolizing enzymes are negative acute-phase proteins.

In the present study, we used chloramphenicol acetyltransferase (CAT) reporter constructs to localize the region in the promoter of rGSTA2 that mediates the response to IL-6/DEX. This region was then used in electrophoretic mobility-shift assays (EMSA) to identify an inducible factor that binds to a portion of the HNF1 site, but does not displace HNF1.

EXPERIMENTAL

Materials

All chemicals used were of analytical grade and unless otherwise noted were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, U.S.A.). Restriction enzymes were from Promega (Madison, WI, U.S.A.) or New England Biolabs (Beverly, MA, U.S.A.), and radiolabelled nucleotides [α - 32 P]ATP, [γ - 32 P]ATP and [14 C] chloramphenicol were from New England Nuclear (Boston, MA, U.S.A.). Collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Recombinant murine IL-6 was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, U.S.A.). Antibodies raised against transcription factors *c-jun* (sc-44X), *c-fos* (sc-447X), C/EBP β (sc-746X), glucocorticoid receptor (GR; sc-1004X), family of transcription factors that bind to (A/T)GATA(A/G) sequence of DNA (GATA) 4, 5, 6 (sc-1237X, sc-7280X, sc-7244X), HNF1 α (sc-6547X), HNF1 β (sc-7411X) and consensus oligonucleotides for different response elements [AP-1, C/EBP, GATA, GR element (GRE), STAT3] were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and their sequences are shown below.

AP-1: CGCTTGATGACTCAGCCGGAA

C/EBP: TGCAGATTGCGCAATCTGCA

GATA: CACTTGATAACAGAAAGTGATAACTCT

GRE: GACCCTAGAGGATCTGTACAGGATGTTCTAGAT

STAT3: GATCCTTCTGGGAATTCCTAGATC.

Dr Moshe Yaniv (Institut Pasteur, Paris, France) kindly provided an antiserum that cross-reacts with HNF1 α and HNF1 β .

Primary hepatocyte cultures

Hepatocytes were isolated from male Sprague–Dawley rats (150–200 g; Harlan Sprague–Dawley Inc., Prattville, AL, U.S.A.) by collagenase perfusion as described previously [11]. The liver was perfused for 6–10 min *in situ* through the portal vein with calcium-free buffer (127 mM NaCl/7 mM KCl/5 mM pyruvate/5 mM glutamate/5 mM fumarate/25 mM NaHCO₃/10 mM Hepes, pH 7.2/11 mM glucose). The liver was then perfused with the same buffer with 5 mM CaCl₂ and 0.03% collagenase for 6–8 min and removed. The cells were dispersed in modified Dulbecco's modified Eagle's/F12 media without serum, which was supplemented with additional amino acids and trace elements [22], insulin (10⁻⁷ M), glucagon (10⁻⁸ M), DEX (10⁻⁷ M), penicillin (100 units/ml) and streptomycin (100 μ g/ml). Hepatocytes were filtered through Nytex cloth (3-60/45 mesh; Tetko, Kansas City, MO, U.S.A.) by gravity and were allowed to settle by gravity for 5–10 min. The supernatant was discarded and hepatocytes were resuspended and allowed to settle for 5–10 min a total of three times. Hepatocytes were plated onto 60 mm plates coated with rat-tail collagen (Type I; Sigma) at a density of

2.4 \times 10⁶ cells/plate. The cells were incubated at 37 °C in humidified air plus 5% CO₂. Hepatocyte viability was determined by Trypan Blue exclusion and viability was consistently > 85% at plating.

Hepatocytes were transfected 3 h after plating by using the method of Holmen et al. [23]. Plasmid DNA (7.5 μ g/plate) was vortexed with DOTMA (*N,N,N*-trimethyl-*N*-[bis-1,2-(9-*Z*-octadecenyl)oxy]propyl ammonium chloride, 40 μ g/plate, a gift from Kevin Trimble, Roche Bioscience) in a culture medium without antibiotics and then applied to the cultured cells. After the addition of the transfection medium for 12 h, the cells were washed, fresh medium was added and the cells were classified into control and IL-6-treated groups of at least three plates each. The fresh medium contained DEX (10⁻⁷ M) and antibiotics with or without IL-6 (200 units/ml) and was changed every 24 h.

CAT assays

The reporter plasmid pGTB1.6 CAT was a gift from Dr C. Pickett and contains 1651 bp of the 5'-flanking sequence of rGSTA2 cloned into the base reporter plasmid pSV0 CAT. Deletion constructs of pGTB 1.6 CAT (Figure 1A) were also obtained from Dr C. Pickett [24]. Specific point mutations and additional deletions were made to pGTB1.6 CAT by the QuikChangeTM PCR-based mutagenesis method (Stratagene, La Jolla, CA, U.S.A.). All of the original plasmids and mutations were sequenced to confirm their identity. The base reporter plasmid pSV0 CAT was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

For CAT assays, hepatocytes were washed and harvested in 1 ml of buffer [10 mM Tris/HCl, 10 mM NaCl, 1 mM EDTA (pH 7.5)]. The cells were scraped from the plates, centrifuged (at 800 *g* for 2 min at 4 °C) and resuspended in 200 μ l of 250 mM Tris/HCl (pH 8.0) and lysed by three freeze–thaw cycles in a 2-propanol–solid CO₂ slurry. Cellular debris was removed by centrifugation (12000 *g* for 15 min at 4 °C) and 75 μ l of the supernatant was transferred to a tube and heated to 65 °C for 10 min to inactivate endogenous deacetylases. [14 C]chloramphenicol (0.25 μ Ci) and *N*-butyryl CoA (final concentration 2.5 mM) in 250 mM Tris/HCl buffer, pH 8.0 were added to produce a final volume of 100 μ l and the reaction mixture was incubated overnight at 37 °C. Then, 200 μ l of 2,6,10,14-tetramethyl-pentadecane/xylenes (2:1, v/v) was added to the reaction mixture, which was mixed vigorously and then centrifuged (16000 *g* for 15 min at 24 °C). An aliquot of the upper organic phase containing acetylated [14 C]chloramphenicol was added to scintillation fluid and counted in a liquid-scintillation counter. Similarly, a mixture prepared from untransfected cells that lacked acetylase activity was extracted as a control for the efficiency of extraction. For each treatment, plates (control or IL-6/DEX-treated) were harvested in triplicate separately for measurements of CAT activity. CAT activity was normalized to total cellular protein, and the CAT activity in cells transfected with the base reporter pSV0 CAT was subtracted from the activity obtained with pGTB1.6 CAT and its derivatives to obtain basal expression levels. CAT activities in control (no IL-6) and IL-6-treated cells were determined from the same batch of transfected hepatocytes from a single liver and are expressed as percentages relative to the control cells. It was not necessary to control for transfection efficiency with a separate reporter because the same pool of transfected hepatocytes was used to compare the effects of IL-6 and control media on CAT activity. CAT activity levels in control hepatocytes transfected with pGTB1.6 CAT were approx. 6-fold greater than activities in control hepatocytes transfected with pSV0 CAT.

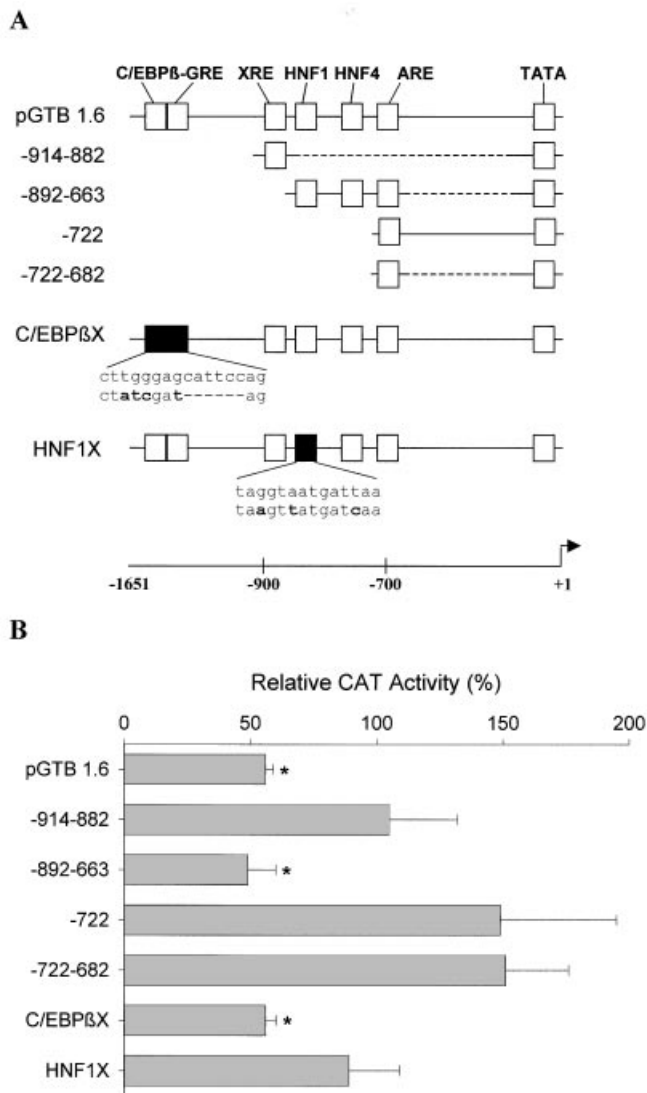


Figure 1 Effect of IL-6 on activity of CAT reporter constructs

(A) Schematic representations of CAT reporter constructs; pGTB1.6 contains the full-length rGSTA2 promoter sequence. Boxes represent known or putative promoter elements and broken lines indicate deleted sequences. Mutations made to C/EBPβX and HNF1X are indicated in boldface below the wild-type sequences. (B) The effect of IL-6 on the activity of the CAT constructs. Primary hepatocytes were transfected with the CAT reporter constructs and assayed 48 h after transfection. Each result is the means \pm S.E.M. from triplicate determinations from at least four different animals. Activities of CAT reporters from IL-6-treated cells are expressed with respect to CAT reporter activities from cells from the same animal transfected with the same CAT reporter and cultured without IL-6; all cells were cultured with DEX (10^{-7} M).

Nuclear extracts

Nuclear extracts for EMSA were prepared by the methods of Schreiber et al. [25] and Staal et al. [26] with optimizations for our cells. Briefly, hepatocytes were cultured as described above in the same medium \pm IL-6/DEX. At specified time intervals, the medium was removed and hepatocytes were washed twice with ice-cold PBS, scraped from plates and pelleted by centrifugation (800 g for 2 min at 4 °C). The supernatant was discarded and the cells were resuspended in the buffer solution [10 mM Hepes/1.5 mM MgCl₂/10 mM KCl/0.1% Nonidet P40 (pH 7.9)] and lysed for 5 min on ice. Cellular debris was pelleted by centri-

fugation (1100 g for 2 min at 4 °C) and the pellet was washed twice in the same buffer without detergent with centrifugation (800 g for 5 min at 4 °C). The pellet was resuspended in a high-salt buffer [20 mM Hepes (pH 7.9)/420 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA/25% glycerol, containing a mixture of protease inhibitors (Boehringer Mannheim)] and incubated on ice for 30 min. Finally, after centrifugation at 16000 g for 10 min at 4 °C, the supernatant containing nuclear proteins (NPs) was frozen at -80 °C until used.

EMSA

The method used for the EMSA has been described previously [27,28]. Briefly, double-stranded DNA probes (30 bp in length) were end-labelled with [γ -³²P]ATP by T4-polynucleotide kinase. Unincorporated label was removed using a Sephadex G-50 spin column. For each reaction, 5 μ g of NPs was incubated for 1 h at 4 °C with 0.3–0.6 pmol of end-labelled probe, 4.5 μ g BSA and 4 μ g poly(dI/dC) in a total volume of 20 μ l of buffer (60 mM KCl, 12 mM Hepes, 4 mM Tris/HCl, 1 mM EDTA and 48% glycerol; pH 7.9). For EMSA supershift experiments, antisera against various transcription factors were added to the reaction mixture before or after combining the nuclear extracts with the DNA. The resultant mixture was held on ice for 60 min before electrophoresis. In experiments involving cold competition, unlabelled oligonucleotide (5–100-fold greater concentration than radiolabelled probe) was mixed with radiolabelled probe for 30 min before the addition of the nuclear extracts. Samples were run on a 4% non-denaturing polyacrylamide gel (5 W, 4 °C) for approx. 3 h and the gel was dried under vacuum and autoradiographed.

UV cross-linking

Covalent binding of NPs to radiolabelled oligonucleotide probes was performed as described previously [29,30]. To make radiolabelled probes, 30-bp single-stranded templates were filled in by Klenow fragment (1 unit) using a 15-bp primer and [α -³²P]dATP, 5-bromo-2'-dUTP, dCTP and dGTP. Reaction mixtures containing hepatocyte NPs and radiolabelled probes were prepared for EMSA as described above and irradiated with UV light (254 nm) for 20 min on ice. The reaction mixture was subjected to non-denaturing PAGE. The wet gel was irradiated for 10 min with UV light and autoradiographed. Radiolabelled protein bands were cut from the gel and analysed by SDS/PAGE (10% gel). The gel was autoradiographed and the molecular mass of the cross-linked protein was determined by comparing its relative mobility with that of pre-stained protein molecular mass standards.

Effect of cycloheximide on IL6DEX-NP induction

Treatment of hepatocytes with IL-6/DEX led to the appearance of a protein that bound to the promoter sequence of rGSTA2, which we term as IL6DEX-NP (see below). We examined whether new protein synthesis was required for the appearance of the protein by pretreating hepatocytes with cycloheximide (10 μ g/ml) for 30 min before culturing with or without IL-6 for 48 h; hepatocytes were harvested and nuclear extracts were prepared as described above.

Data analysis

Each value represents the means \pm S.E.M. from at least three separate samples or animals. For each series of CAT assays, IL-

6-treated cells were compared with non-IL-6-treated cells that were isolated from the same rat liver. Statistical analyses were performed using Student's *t* test and $P \leq 0.05$ was considered significant.

RESULTS

Effect of IL-6/DEX on reporter constructs in cultured hepatocytes

Transfection of pGTB 1.6 CAT into cultured hepatocytes and treatment with IL-6/DEX reduced the rGSTA2 promoter-driven CAT expression to approx. 50% of the untreated controls [18]. Therefore pGTB 1.6 CAT contains the response element(s) that mediates decreased expression of rGSTA2 in the presence of IL-6/DEX. A consensus C/EBP β site overlapping a consensus GRE half-site is present in pGTB 1.6 CAT (-1614 to -1595). Previous studies suggested that this arrangement of binding sites is associated with decreased expression due to steric interference of transcription factor binding to the promoter [31]. We mutated both sites to eliminate the binding of C/EBP β or GR transcription factors. Mutations to or deletions of either site did not affect the suppression of CAT activity by IL-6/DEX (NFIL6X; Figure 1B).

We then used deletion constructs to define the region in pGTB 1.6 CAT that accounted for the IL-6/DEX effect. IL-6/DEX-mediated reductions in CAT activity that were similar to those observed with the pGTB 1.6 CAT were seen in the -892 to -633 CAT construct, but there was no reduction in the -914 to -882 CAT construct (Figure 1B). These findings suggested that the region of DNA that was responsible for the IL-6/DEX effect was present in nt -892 to -633. To test this hypothesis, constructs lacking the above region were transfected into rat

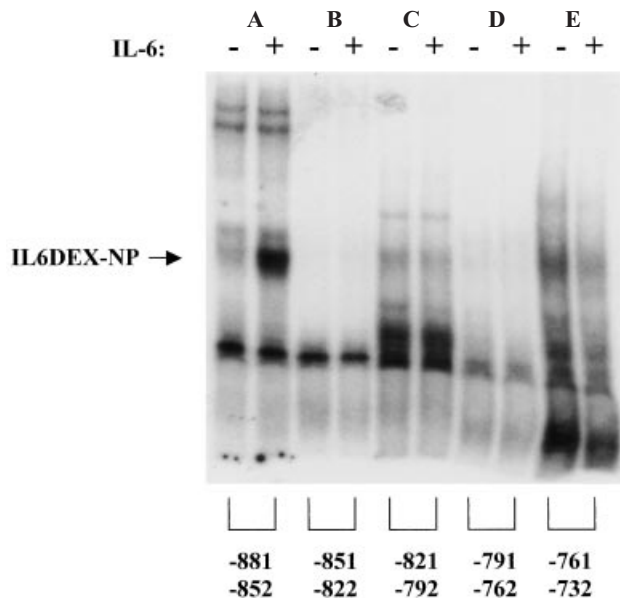


Figure 2 IL6DEX-NP binds to the -881 to -732 oligonucleotide of rGSTA2 promoter

Five radiolabelled oligonucleotides were prepared to span the promoter region involved in rGSTA2 suppression. Nuclear extracts were prepared from control (-) and IL-6-treated (+) hepatocytes cultured with DEX (10^{-7} M). The complex in the nuclear extracts from IL-6-treated cells is labelled as IL6DEX-NP. (A) -881 to -852: AGTCACTAGGTAATGATTAATAACCAAGA (B) -851 to -822: CCCATGAACCGACCCTGTAACCTAAGATCA (C) -821 to -792: GGACTCAGCATGTGGGTGTGAGTGGAGCCA (D) -791 to -762: TCGGTGATGACCTTGTGTGGATAAGATT (E) -761 to -732: CTGGTCTGAACCTGGCAGGAAGGATCAGT.

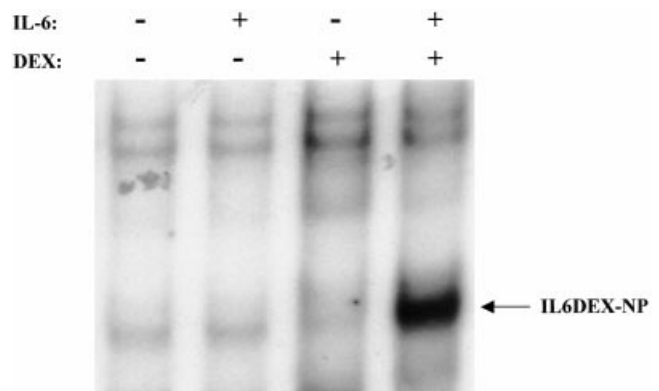


Figure 3 Appearance of IL6DEX-NP in nuclear extracts requires the presence of both IL-6 and DEX in the culture medium

Nuclear extracts from hepatocytes cultured without IL-6/DEX, IL-6 (200 units) alone, DEX (10^{-7} M) alone, or both IL-6/DEX were used for EMSA using the -881 to -852 radiolabelled oligonucleotide.

hepatocytes. Activities from -722 CAT and -722 to -682 CAT were unaffected by IL-6/DEX (Figure 1B), supporting the idea that suppression was mediated within nt -881 to -723.

Characterization of a protein that binds to the rGSTA2 promoter and is IL-6 and DEX dependent

The above studies suggested that the IL-6/DEX effect on gene transcription was mediated, at least partially, by elements in nt -881 to -723 of the promoter of rGSTA2. Five oligonucleotides were synthesized to cover this region and were used for EMSAs in combination with nuclear extracts from hepatocytes, which had been in culture for 48 h with DEX (10^{-7} M), with or without IL-6 (200 units/ml). A new DNA-protein complex was identified with the -881 to -852 oligonucleotide in nuclear extracts of IL-6-treated cells, which was not in extracts from non-IL-6-treated control cells (Figure 2). In contrast, the DNA-protein complex was absent when the four other nucleotide probes were used; extracts from IL-6-treated and IL-6-untreated cells gave similar patterns (Figure 2).

Previous studies [18] had shown that both IL-6 and DEX were required in the culture medium to decrease the expression of rGSTA2. We questioned whether the appearance of the new DNA-protein complex required the presence of both IL-6 and DEX in the culture medium. The NP (termed IL6DEX-NP for IL-6/DEX-induced NP) was present only in nuclear extracts from cells treated with both IL-6 and DEX (Figure 3). Additional oligonucleotides were made that spanned sequences across the ends and breaks between the above five oligonucleotides but no other IL-6+DEX-induced NP binding was observed.

Formation of IL6DEX-NP DNA complex is specific and IL6DEX-NP is not C/EBP, HNF1, GR or AP-1

The -881 to -852 sequence contains at least four potential transcription response elements (AP-1, HNF1, GRE and C/EBP) based on sequence analysis (AP-1 is italicized, HNF1 is underlined, GRE is in boldface and C/EBP is between asterisks): **AGATCAC*TAGGTAAT*GATTAATAACCAAGA**.

The HNF1 site in rGSTA2 is known to be functional [24]. When the HNF1 site was mutated (HNF1X; Figure 1A), the two

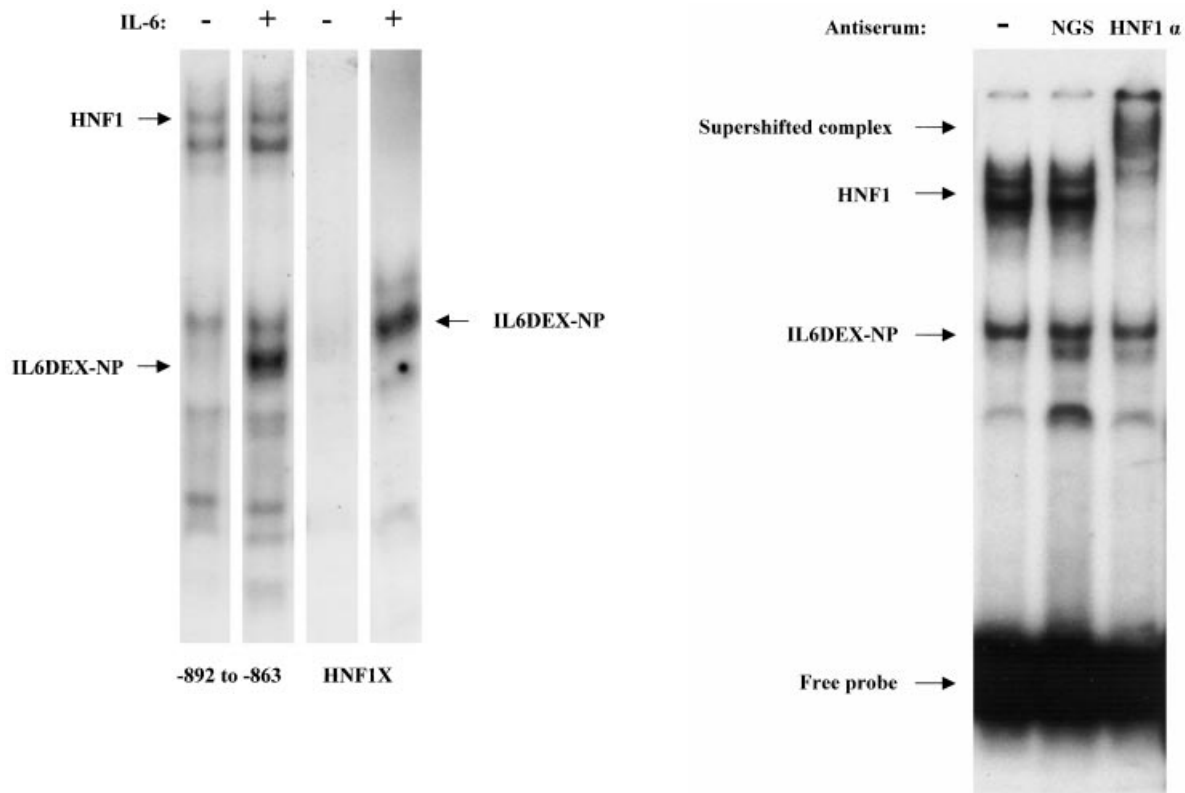


Figure 4 Identification of HNF1 NPs

Left panel: a mutant of the -881 to -852 oligonucleotide (HNF1X) was prepared and used for EMSA. Hepatocytes were cultured with DEX (10^{-7} M). Right panel: a goat antiserum raised against HNF1 α supershifted HNF1 proteins binding to the -881 to -852 oligonucleotide in EMSA, whereas control, non-immune normal goat serum (NGS) did not.

upper bands on EMSAs were lost (Figure 4, left panel). When an antiserum to HNF1 α (cross-reacts with HNF1 α and β) was added to the binding mixture with the -881 to -852 (wild-type) probe, the two upper bands were supershifted (results not shown). When a different antiserum specific for HNF1 α was used, the two upper bands were also supershifted (Figure 4, right panel); an antiserum specific for HNF1 β failed to shift any band or modify the appearance of binding complexes. Therefore the two upper bands are HNF1 α . In contrast, IL6DEX-NP binding was unaffected by either the mutation to the HNF1 site or by the antisera to HNF1 (Figure 4, left and right panel).

Unlabelled competition studies were performed using the self-sequence -881 to -852 oligonucleotide with or without a mutation that prevents HNF1 binding (HNF1X), with the sequence -67 to -43 bp of the rat albumin promoter that spans the albumin HNF1 site, and with oligonucleotide -761 to -732 as a sequence-unrelated negative control. The self-sequence -881 to -852 , HNF1X and albumin oligonucleotides competed for the binding of IL6DEX-NP, but the -761 to -732 oligonucleotide (Figure 5) did not compete.

Consensus binding site oligonucleotides for AP-1, C/EBP, GR and STAT3 were used for unlabelled competition studies, and binding of IL6DEX-NP was reduced by GR (Figure 6) but not by AP-1, C/EBP or STAT3 oligonucleotides (results not shown). Deletion of the putative GRE did not affect binding of the IL6DEX-NP complex. Moreover, neither did antiserum directed towards GR shift the IL6DEX-NP complex nor did

antisera directed towards *c-fos*, *c-jun*, C/EBP β (cross-reacts with C/EBP α , δ and ϵ ; results not shown).

Time course of appearance of the IL6DEX-NP and the need for protein synthesis

The time course of the appearance of IL6DEX-NP was determined and is shown in Figure 7(A). IL6DEX-NP was observed within 4 h of the addition of IL-6/DEX and its level increased in a 48 h period. By treating hepatocytes with cycloheximide, we also determined that a new protein synthesis was required for the appearance of IL6DEX-NP. With the prevention of a new protein synthesis, the level of IL6DEX-NP was reduced in IL-6/DEX-treated cells (Figure 7B).

IL6DEX-NP core-binding sequence

We made a series of mutations in the HNF1 site of the -881 to -852 oligonucleotide to define the nucleotides that were essential for binding of IL6DEX-NP (Figure 8). The nucleotide sequence required for the binding of IL6DEX-NP was TGATT. The lower molecular-mass complex that appeared in some of these experiments was observed whether IL-6 was present or absent in the medium, indicating that it was different from IL6DEX-NP (Figure 8). The sequence TGATT is known to bind GATA

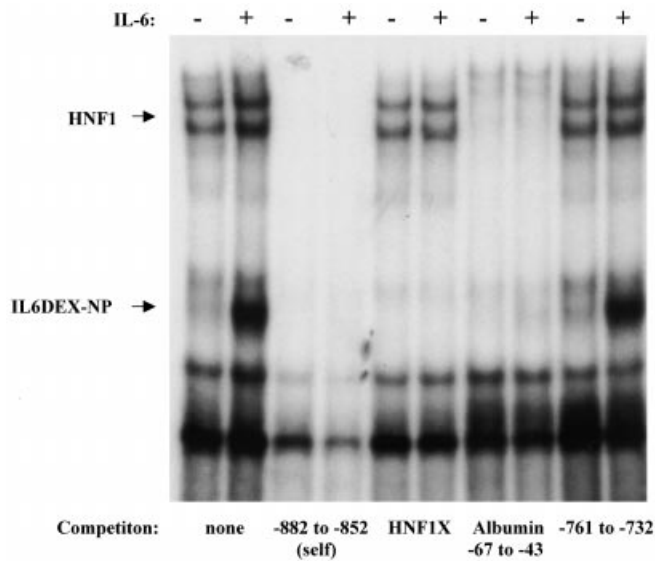


Figure 5 Interaction between IL6DEX-NP and -881 to -852 oligonucleotide is specific

Unlabelled oligonucleotide-competition experiments for NP binding to the -881 to -852 oligonucleotide of the rGSTA2 promoter were performed using nuclear extracts from IL6/DEX-treated hepatocytes and a 100-fold excess of the -882 to -852 oligonucleotide (self), HNF1 mutant oligonucleotide (HNF1X), -67 to -43 oligonucleotide from the albumin promoter, or -761 to -732 oligonucleotide from the rGSTA2 promoter as a sequence-unrelated negative control.

transcription factors [32]. However, a GATA consensus oligonucleotide that binds transcription factors GATA1-GATA6 did not compete with a radiolabelled -881 to -852 probe for binding of IL6DEX-NP (Figure 6). Antisera against the GATA transcription factors that are expressed in the gastrointestinal tract (GATA4, 5 or 6) did not supershift IL6DEX-NP (results not shown).

Molecular mass of IL6DEX-NP

The molecular masses of IL6DEX-NP and HNF1 were determined by covalently cross-linking the proteins to the -881 to

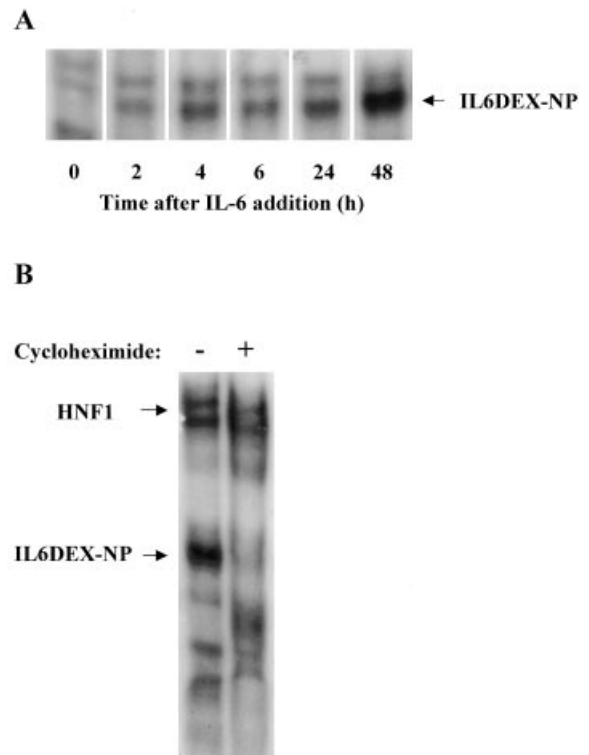


Figure 7 Time course of appearance of the IL6DEX-NP and effect of cycloheximide

(A) EMSA was performed using the -881 to -852 radiolabelled oligonucleotide. Nuclear extracts were prepared from hepatocytes cultured with IL-6/DEX for 2, 4, 6, 24 and 48 h. (B) Hepatocytes were cultured with IL6/DEX and protein synthesis was inhibited with cycloheximide.

-852 oligonucleotide. The mass of IL6DEX-NP was approx. 28 kDa after subtracting the mass of the bound oligonucleotide (Figure 9). The molecular mass of HNF1 was approx. 95 kDa, which is similar to the reported molecular mass (92 kDa) of HNF1 [33,34].

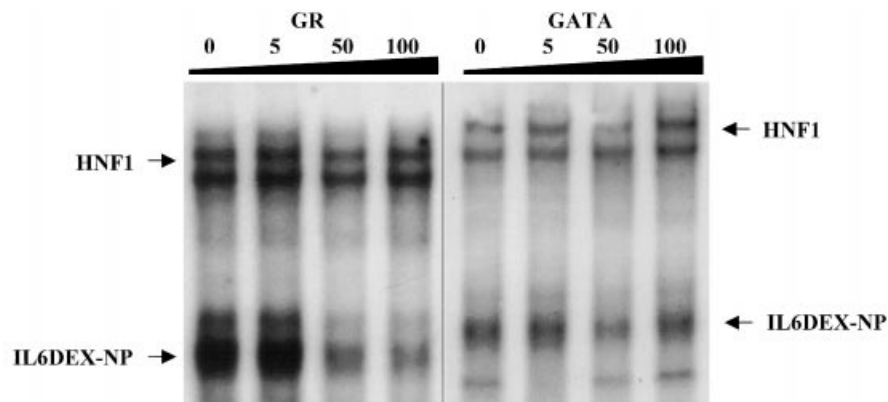


Figure 6 Competition studies using consensus oligonucleotides for GR and GATA

Unlabelled oligonucleotides (5-100-fold excess) were added to binding reactions of nuclear extracts from IL6/DEX-treated cells and radiolabelled -881 to -852 oligonucleotide.

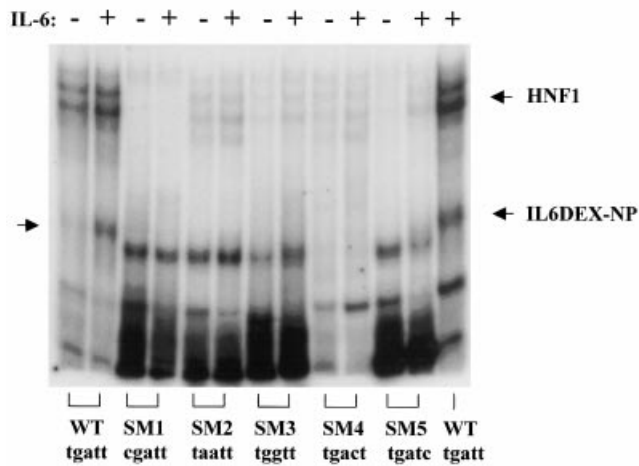


Figure 8 The core sequence required for binding of IL6DEX-NP is TGATT

EMSA was performed with nuclear extracts from IL6/DEX-treated cells and radiolabelled oligonucleotides containing single base pair mutations (SMs) of the core TGATT sequence within the HNF1 site. The complex of lower molecular mass, which appeared when some of the mutant oligonucleotides were used was present in nuclear extracts from both treated and untreated IL-6 cells and is considered non-specific. WT refers to the -881 to -852 oligonucleotide.

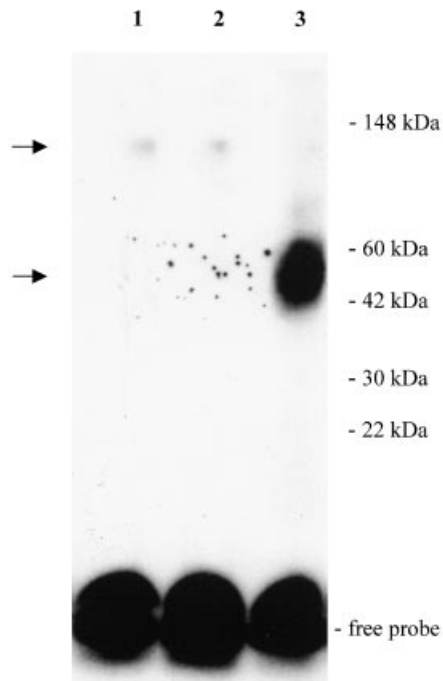


Figure 9 Molecular mass of IL6DEX-NP and of HNF1

Nuclear extracts from IL-6/DEX-treated cells were mixed with radiolabelled -881 to -852 oligonucleotide containing 5-bromo-2'-dUTP and irradiated with UV, subjected to EMSA and then irradiated again with UV. The two prominent HNF1 bands and the IL6DEX-NP band were identified by autoradiography, individually cut from the gel, and loaded on to separate lanes of an SDS/PAGE gel which was autoradiographed. Lane 1, upper HNF1 band; lane 2, lower HNF1 band; lane 3, IL6DEX-NP band. The HNF1 (upper arrow) and IL6DEX-NP (lower arrow) complexes are indicated on the autoradiogram and the positions of molecular mass (kDa) standards are shown on the right-hand side.

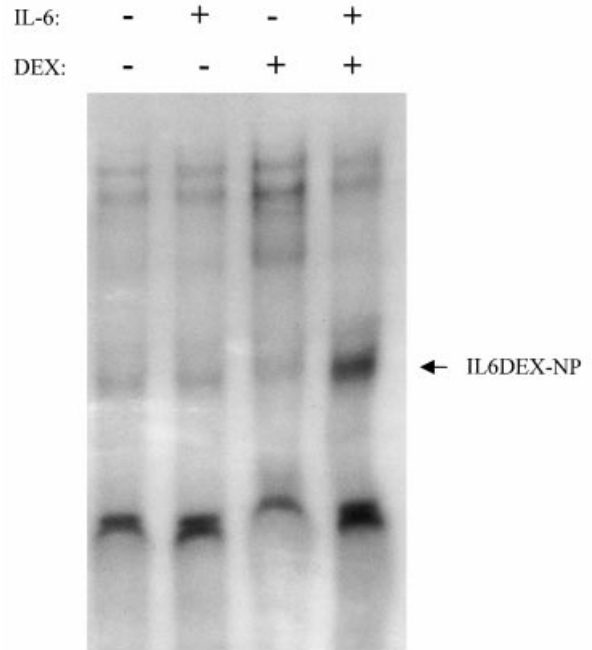


Figure 10 IL6DEX-NP binds to the HNF1 region of the albumin promoter and requires the presence of both IL-6 and DEX in the culture medium

A radiolabelled oligonucleotide of the HNF1 region from the albumin promoter, TTAGTGTGGTTA-ATGATCTACAGTT (-67 to -43) was synthesized and used for EMSA. Nuclear extracts were prepared from control and IL-6-treated hepatocytes cultured with or without DEX (10^{-7} M).

Role of HNF1 in loss of activity

Mutant oligonucleotide HNF1X does not bind HNF1 and does bind IL6DEX-NP (Figure 4, left panel). When the same mutation was made in pGTB 1.6 CAT and transfected into rat hepatocytes in primary culture, the suppressive effect of IL-6/DEX on CAT activity was lost (HNF1X CAT; Figure 1B). Hence, despite the presence of IL6DEX-NP, no suppression was observed unless HNF1 was bound to the DNA.

Binding of IL6DEX-NP to the albumin promoter

Albumin is another negative acute-phase protein [3,17]. The expression of albumin also depends on an HNF1 element in the 5'-flanking sequence of the gene [30]. We therefore made an oligonucleotide of the HNF1 region (-67 to -43) of the albumin promoter for gel-shift assays [30]. The HNF1 (underlined) portion of the albumin promoter is compared with the same region of the GST promoter below and to the IL6DEX-NP core-binding sequence (TGATT, double underlines).

rAlbumin: TTAGTGTGGGTTAATGATCTAC
rGST: CACTAGGGTAATGATTAAT.

A DNA-binding protein was identified in the nuclear extracts from the IL-6/DEX-treated cells using the albumin oligonucleotide (Figure 10) and excess oligonucleotide from the albumin sequence competed off IL6DEX-NP and HNF1 α from the -882 to -852 sequence of the rGSTA2 promoter (Figure 5). Based on competition assays, the binding affinity of IL6DEX-NP to the albumin promoter was less when compared with the rGSTA2 promoter (results not shown). This result is not unexpected

because IL6DEX-NP had less affinity when the core sequence of the rGSTA2 promoter was mutated to the sequence of the albumin promoter (TGATC; see Figure 8, SM5).

DISCUSSION

The acute-phase response is associated with decrease in the concentrations of a number of secreted and intracellular liver proteins, including albumin and detoxification enzymes such as GSTs [3,12]. IL-1 and IL-6 are among the cytokines that cause the acute-phase response. Previous studies [18] showed that IL-6 with DEX decreased the expression of the GSTs in cultured hepatocytes. The mechanism of this suppression was investigated further in the present study.

In most situations, the decrease in expression of proteins during the acute-phase response reflects changes in rates of transcription [1–3]. Consistent with those observations was the present finding that the activity of a CAT reporter construct that contains the promoter of rGSTA2 was suppressed by IL-6/DEX when transfected into hepatocytes in primary culture. Further studies localized the IL-6/DEX effect on the reporter construct to a 159 bp region of the promoter. A binding site for an IL-6/DEX-dependent DNA-binding protein (IL6DEX-NP) was identified in this region of the promoter. The induced DNA-binding protein bound to a portion of the HNF1 site but did not displace HNF1 protein (Figure 4). Dilution of the labelled oligonucleotide probe by 1/128 did not result in any sample-dependent decrease of free-probe band intensity in parallel lanes of control and IL-6-treated hepatocytes, indicating that the probe is in excess at this dilution (results not shown). Moreover, as the free probe became limited due to dilution, there was no difference in the relative HNF1 and IL6DEX-NP band intensities, indicating that both factors have similar affinity for the probe. Preliminary experiments with the limiting free probe and the use of antiserum to HNF1 to immunoprecipitate HNF1 and therefore to reduce or prevent the interaction of HNF1 with the probe did not result in an increased band intensity of IL6DEX-NP. These results, and the fact that no change in HNF1 band intensity occurs when IL6DEX-NP is induced, suggest that HNF1 and IL6DEX-NP bind to the rGSTA2 promoter simultaneously with the net result of reduced transcription.

The appearance of IL6DEX-NP required new protein synthesis suggesting that it was induced and not translocated to the nucleus from another cellular compartment. We used specific antisera and consensus-sequence oligonucleotides to ascertain whether IL6DEX-NP was related to transcription factors that are induced by IL-6 as described previously. IL6DEX-NP was not identified as STAT3, C/EBP or AP-1 transcription factors. Although the GRE consensus oligonucleotide did compete with IL6DEX-NP, deletion of the putative GRE-binding site and lack of a supershift with the GR antiserum indicates that it is not GR. The GRE-consensus sequence has several GAT elements and may compete for IL6DEX-NP simply due to similarity in sequence. Additionally, we performed Western-blot analyses on nuclear extracts of control and IL-6/DEX-treated hepatocytes and detected a protein with the correct molecular mass of GR, but the band intensity of GR was not altered by IL-6 treatment (results not shown). The direct or indirect involvement of GR in the suppression of rGSTA2 expression is unclear. We believe therefore that IL6DEX-NP may be a novel factor.

IL6DEX-NP may be related to the GATA family of transcription factors. The GATA family of proteins are zinc-finger transcription factors that recognize the (T/A)GATA(G/A) sequence [35]. This sequence has been shown to be more general: (G/C)PuPu(G/C)NGAT(A/T)PuPy [32]. The core sequence for

the binding of IL6DEX-NP is TGATT, which is consistent with a GATA site. However, a consensus GATA oligonucleotide did not compete for binding of IL6DEX-NP with the promoter region of rGSTA2. The molecular mass of the GATA transcription factor is approx. 52 kDa [33,34], whereas the mass of IL6DEX-NP is approx. 28 kDa. In addition, antisera against the GATA transcription factors that are expressed in the gastrointestinal tract (GATA4–6) did not supershift IL6DEX-NP. Thus if IL6DEX-NP is a member of the GATA family it must be unique both in structure and in function.

Our data suggest that IL6DEX-NP mediates the decrease in rGSTA2 transcript levels observed in cultured hepatocytes treated with IL-6/DEX. We showed previously that in cultured hepatocytes there is a decrease in rGSTA2 mRNA levels only when cells are treated with both IL-6 and DEX [18]. In the present study, IL6DEX-NP appeared only in cells treated with both IL-6 and DEX (Figure 3). In addition, IL6DEX-NP bound to a region of the promoter that was shown by CAT assays to mediate the suppression of transcription by IL-6/DEX.

In studies of other HNF1-regulated genes, changes in the levels of the HNF1 proteins were associated with a decrease in transcription. Treatment of rats with endotoxin is associated with a decrease in expression of the bile acid transporter *ntcp*. The decrease in transcriptional activity is secondary to a decrease in the levels of the NPs HNF1 and FpB BP [8]. Similarly, low levels of GSTs in kidney tumour cells are correlated with low levels of HNF1 [36]. We did not detect any decrease in binding of HNF1 by EMSA in cells treated with IL-6/DEX (Figure 4, left panel and Figure 5) and therefore a decrease in the amount of HNF1 does not account for the decline in transcription of rGSTA2.

The expression of albumin is dependent upon HNF1. Albumin transcription is decreased in diabetic animals and there is a decline in abundance and binding activity of HNF1 in nuclear extracts from hepatocytes of diabetic animals. A new protein appears in diabetic animals that binds to the HNF1 site and is shifted by an HNF1 antiserum, but has a lower molecular mass than HNF1 [29]. The loss of HNF1 and the appearance of the lower molecular mass HNF1-like factor are thought to account for the decrease in albumin transcriptional activity observed in diabetic animals. In the present study, IL6DEX-NP was not shifted by HNF1 antiserum and did not bind to the HNF1 site in the same way as HNF1. These facts suggest that IL6DEX-NP is not a member of the HNF1 family of transcription factors.

IL6DEX-NP may suppress transcription by interfering with the function of HNF1. We base this hypothesis on the following observations. HNF1 is required for GST expression in hepatocytes and in other cells [24,36]. In the present study, IL-6/DEX did not decrease the binding of HNF1 to the GST promoter in extracts from cultured hepatocytes. However, when the HNF1 site in pGTB 1.6 CAT was mutated to prevent HNF1 binding, IL-6/DEX could no longer suppress CAT activity despite the presence of IL6DEX-NP (Figures 1B and 4, left panel). Thus a functional HNF1 site was required for the suppression of transcription by IL-6/DEX. If IL-6/DEX is acting by interfering with HNF1 enhancement of transcription, then the relative decrease in transcription should be similar whether the activity of HNF1 is reduced by eliminating the HNF1 site or by inhibiting its function. In the present study, IL-6/DEX decreased pGTB 1.6 CAT activity by approx. 50%, whereas deletion of the HNF1 site from this construct reduces the activity by approx. 65% [24]. Thus the relative changes in transcription rates are the same.

HNF1 homoeodomain proteins form dimers before binding to DNA [7,33]. HNF1 α and HNF1 β form the homodimers or heterodimers that bind to the DNA and enhance transcription.

HNF1 dimers associate with two molecules of another protein termed dimerization cofactor of HNF1 to form heterotetramers. This dimerization cofactor of HNF1 does not bind to DNA but stabilizes the HNF1–DNA interactions and increases transcriptional activation by HNF1 [37,38]. It is possible that IL6DEX-NP decreases the rates of transcription by interfering with the formation of the heterotetramers but further study is required to test that hypothesis.

Many other genes in the liver require HNF1 for maximum transcriptional activity. One of these genes is albumin and the HNF1 region of its promoter contains a TGAT core sequence, which is also present in the rGSTA2 promoter. IL6DEX-NP is also bound to this region of the albumin promoter (Figure 10). Thus IL6DEX-NP may also affect the transcriptional activity of the albumin gene. If IL6DEX-NP contributes to a decrease in the transcriptional activity of a number of genes whose expression decreases during the acute-phase response, we will have a better understanding of how the acute-phase response affects the gene expression.

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