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Hepatocyte nuclear factor-4α interacts with other hepatocyte nuclear factors in regulating transthyretin gene expression

Zhongyan Wang and **Peter A. Burke***
Department of Surgery, Boston University School of Medicine, Boston, Massachusetts 02118, USA

Abstract

Transthyretin (TTR) is a negative acute phase protein whose serum level decreases during the acute phase response (APR). TTR gene expression in liver is regulated at the transcriptional level, and controlled by hepatocyte nuclear factor (HNF)-4α and other HNFs. The site-directed mutagenesis of the HNF-4, HNF-1, HNF-3 or HNF-6 binding sites in the TTR proximal promoter dramatically decreased TTR promoter activity. Interestingly, the mutation of HNF-4 binding site not only abolished the response to HNF-4α, but also significantly reduced the response to other HNFs. However, mutation of the HNF-4 binding site merely affected the specific binding of HNF-4α, but not other HNFs, suggesting that an intact HNF-4 binding site provides not only a platform for specific interaction with HNF-4α, but also facilitates the interaction of HNF-4α with other HNFs. In a cytokine-induced APR cell culture model, we observed a significant reduction in the binding of HNF-4α, HNF-1α, HNF-3β and HNF-6α to the TTR promoter, which correlates with the decrease in the TTR expression after injury. These findings provide a new insight into the mechanism of the negative transcriptional regulation of TTR gene after injury caused by a decrease in HNFs' binding and a modulation in their coordinated interactions.

Keywords

hepatocyte nuclear factor; transthyretin; gene transcription; acute phase response; HepG2 cell

Introduction

The acute phase response (APR) is characterized by rapid and dramatic changes in the pattern of proteins produced and released by liver cells in response to a series of pathological conditions such as inflammations, infections and traumas [1,2]. The APR constitutes an ideal system for the study of the regulation of gene expression. In the liver, the APR is characterized by significant changes in its gene and protein expression profile, resulting in the up-regulation of positive acute phase proteins (APPs) such as C-reactive protein, as well as in the down-regulation of negative APPs such as transthyretin (TTR) and albumin. The hepatic APR is mediated by several cytokines including interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) [3]. While the APR is primarily a protective mechanism, prolonged exposure to acute phase condition has been correlated with destructive inflammatory syndromes such as sepsis and multiple organ failure [4,5]. Consequently, clarification and understanding of the transcriptional regulation of specific APPs and the potential to modulate their expressions have obvious clinical benefits. The study of the APR and the transcriptional changes in the APR genes provides an excellent

*Corresponding author: Peter A Burke, MD. Boston University School of Medicine, Boston Medical Center, 850 Harrison Avenue, Dowling 2 South, Boston, MA 02118; Telephone: 617-414-8056; Fax: 617-414-7398; peter.burke@bmc.org.

system to dissect the basic molecular mechanisms involved in the modulation of gene expression.

TTR is a classic negative acute phase protein whose serum level decreases during acute inflammation, infection, and surgical stress. It also plays an important role in plasma transport of thyroxin and retinol [6]. Human TTR is a 55-kDa tetramer protein in which each subunit is composed of 127 amino acids [7]. The main source of plasma TTR comes from the liver [8]. The TTR gene is regulated by a proximal promoter of 200 base pairs (bp) and a distal 100 nucleotide enhancer located about 2 kilobases (kb) from the initiation site [9]. These two regions are necessary and sufficient for hepatoma-specific expression in transient transfection assays and also elicit the normal hepatic expression pattern in transgenic mice [10,11]. Analysis of the TTR proximal promoter sequence has revealed DNA binding sites for multiple hepatocyte-enriched nuclear factors (HNFs), including HNF-1, HNF-3, HNF-4, and HNF-6. Interestingly, HNF-3 and HNF-6 recognize the same DNA-binding site in the TTR proximal promoter. However the specific base pairs required to maximize binding efficiency are different [12]. These HNFs have been shown to play pivotal roles in both the establishment and maintenance of the hepatic phenotype [13,14]. They are part of a complex regulatory network, which is responsible for the activation of most liver specific genes [13–15]. However, how these transcription factors coordinately contribute to the gene expression of TTR and the effects on the injury response need to be defined.

HNF-4α has been known to regulate TTR gene expression. Previous work by our lab has demonstrated that HNF-4α binding ability is rapidly and significantly reduced in a burn injury mouse model and a cytokine-induced injury cell culture model [16,17]. We have also shown that utilizing a cell culture model the decrease in HNF-4α binding activity affects its ability to transactivate target genes [17]. The current study was undertaken to investigate the mechanism of interaction of HNFs within the TTR's proximal promoter and the impact of each of these factors on the activity of this promoter. Our findings suggest that HNFs (HNF-1α, HNF-3α/β, HNF-4α, and HNF-6α) are indispensable for TTR transcription. A coordinated interaction of these factors with the TTR promoter is required for maximal promoter activity. Effective interaction requires the integrity of HNF binding as well as a component of protein-protein interactions between the factors.

Results

Functional analysis of the proximal promoter region of TTR gene

It has been reported that the proximal promoter of TTR contains binding sites for HNF-4, HNF-1, HNF-3 and HNF-6 [12]. In order to identify the functional importance of these HNFs' binding sites and their transcription factors, we performed site-directed mutagenesis of the proximal promoter to modify these sites in such way that they are unable efficiently to bind their respective *trans*-acting factors (Fig. 1). The wild-type (WT) or mutated TTR promoter was linked to the luciferase gene and cotransfected with expression plasmid carrying HNF-4α, HNF-1α, HNF-3α/β and HNF-6α into HepG2 cells. The results, given in terms of transcriptional activity relative to the native promoter (WT), are depicted in Fig. 2. A significantly higher expression of the WT promoter was seen compared to the promoterless pGL4.11 [luc2P] vector (pGL4) ($p<0.01$). Introduction of the mutations in the HNF-3 and HNF-6 binding sites reduced the promoter activity to 17% and 40% of the WT value, respectively. Mutation of the HNF-4, or the HNF-1 site, and mutation of both HNF-3 and HNF-6 (HNF-3/6) binding sites together led to a dramatic decrease of the activity to near background level (pGL4) (Fig. 2A). The isolated overexpression of HNF-4α, HNF-1α, HNF-3α or HNF-6α resulted in a significant increase in WT promoter activity compared to non-overexpressed WT control ($p<0.05$), while overexpression of HNF-3β had little effect

on the activity ($p>0.05$) (Fig. 2B). Taken these data together suggest that all of the HNFs tested are essential for positive maximal transcription of the TTR gene in HepG2 cells.

HNF-4α cooperates with other HNFs to induce TTR transcription

Given that multiple liver-enriched transcription factors are able to activate the TTR proximal promoter based on the transient transfection assays described above. A question is raised whether, as in other complex regulatory regions, a coordinate interaction of these factors is required for high-level transcription of the TTR gene. It has been demonstrated from our previous work that HNF-4α plays an important role in TTR expression, and the ability of HNF-4α binding to TTR proximal promoter is rapidly and significantly reduced after injury in a mouse burn model [16]. To further define the injury induced changes in transcriptional regulatory process, we focus on the interactive effect of HNF-4α with the other HNFs on TTR promoter activity. HepG2 cells were cotransfected with the luciferase reporters containing WT or mutated TTR promoter along with the corresponding HNF expression plasmid. As shown in Fig. 3, when the HNF-4 binding site was mutated, a complete loss of HNF-4α-dependent stimulation induced by either endogenous or overexpressed HNF-4α was seen in HepG2 cells (comparing column bar 1 with 3; 2 with 4 in Fig. 3). In addition to this expected result, we also noted that the mutation of HNF-4 binding site not only destroyed the active effect of HNF-4α, but also diminished the effect of exogenous HNF-1α, HNF-3α, HNF-3β and HNF-6α on TTR transcription (comparing bar 6 with 7; 9 with 10; 12 with 13; 15 with 16 in Fig. 3). The loss of responses to the overexpression of HNF-3α or HNF-3β was the most pronounced when the HNF-4 binding site was mutated and other HNFs' binding sites remained unchanged, in this case the reporter activity was comparable to the HNF-4α response level when the HNF-4 binding site was mutated (comparing bar 10 or 13 with 4 in Fig. 3). These data suggest that HNF-4α may interact with other HNFs in activating TTR gene expression, and efficient HNF-4 binding is important for effective transactivation of the TTR gene by other HNFs. Given the profound effect of altered HNF-4 binding on the HNF-3 function, we further looked for an impact of HNF-3 binding on HNF-4α function. As shown in Fig. 4A, mutation of the HNF-3 binding site eliminated HNF-3α and HNF-3β-dependent transactivations, and also abolished the response to HNF-4α. Because HNF-3 and HNF-6 recognize the same DNA-binding site in the TTR proximal promoter, a construct with both mutated HNF-3 and HNF-6 binding sequences was also tested. Similar results were seen as with the mutation of the HNF-3 site alone (Fig. 4B). These findings imply that alterations in HNF-4 binding by the mutation of the HNF-4 *cis*-element at position −151/−140 in the TTR gene reduce TTR promoter activation by two mechanisms one is directly caused by the loss of HNF-4α binding, and another is a secondary effect on TTR transactivation *via* changes in the interaction of HNF-4α with the other HNFs.

The HNFs independently bind to the TTR promoter

To identify the potential mechanism underlying the interaction of HNF-4α with the other HNFs in TTR transcription, we carried out DNA-protein binding assays to detect whether HNFs affect each other's binding ability. A biotinylated DNA probe encompassing TTR promoter with the WT or individually mutated HNF binding site was incubated with nuclear protein extracted from HepG2 cells, the DNA-protein complexes were analyzed by enzyme-linked immunosorbent assay (ELISA) with antibodies against HNF-4α, HNF-3α, HNF-3β, or HNF-6α protein. As shown in Fig. 5, the mutation of the HNF-4 binding site significantly reduced HNF-4α specific binding, but did not appear to disturb the binding of HNF-3α, HNF-3β, and HNF-6α (Fig. 5A). When the probe containing the mutated HNF-3 binding site was used (Fig. 5B), the binding of both HNF-3α and HNF-3β was greatly decreased compared to the non-mutated WT ($p<0.01$), and the binding of HNF-6α was slightly but significantly greater than WT ($p<0.05$). The increase in HNF-6α binding seen with the

HNF-3 mutation may be due in part to competition between HNF-3 and HNF-6 for the same binding site. Similar results were observed when the mutation of HNF-6 binding site was present (Fig. 5C). The binding ability of HNF-4α remained unchanged in the case of single mutation of either HNF-3 or HNF-6 ($p>0.05$, Fig. 5B and Fig. 5C), however, a small but significant decrease in HNF-4α binding was detected when the both HNF-3 and HNF-6 binding sites were mutated (Fig. 5D). To further verify the specificity of HNF-4 binding and the results in Fig. 5 assayed by ELISA method, DNA-protein complexes were immunoblotted with anti-HNF-4α antibody (Fig. 6), a strong band was detected in the complex of DNA derived from WT-TTR promoter and nuclear protein from HepG2 cells, indicating that the HNF-4α specific binding did exist. A faint band was found when the HNF-4 binding site was mutated. However, no significant difference in the HNF-4α binding intensity was found between WT and HNF-1, HNF-3, HNF-6, or HNF-3/HNF-6 mutants. These findings indicate that disruption of a specific HNF binding site in the TTR proximal promoter leads only to alteration in binding for that HNF site without affecting the other HNFs' ability to bind to their specific DNA binding sites.

A role of HNFs on down-regulation of TTR expression in response of cytokine stimulation

Our previous study has shown that TTR expression significantly decreased in a cytokine-induced acute phase response model [17], and that changes in HNF-4α and HNF-1α binding can be seen very rapidly in murine burn injury model [16,18]. To determine whether cytokines have an effect on the binding ability of HNFs in the context of chromatin in intact cells, we performed chromatin immunoprecipitation (ChIP) assays. Antibodies raised against HNF-4α, HNF-1α, HNF-3β and HNF-6α could efficiently immunoprecipitate the TTR promoter DNA, indicating *in vivo* association of these factors with this promoter. More importantly, cytokine treatment led to a decrease in the formation of protein-DNA complexes for all of the HNFs compared to untreated-controls ($p<0.05$) (Fig. 7). However, this decrease in protein-DNA binding is not due to the alteration of HNF concentration after treatment with cytokines, as protein levels of HNF-4α, HNF-1α, HNF-3β and HNF-6α were not significantly altered after cytokine stimulation (($p<0.05$) (Fig. 8). Taken together, these results suggest that cytokines reduce the binding abilities of HNFs affecting their ability to interact and coordinate the transcriptional activity of the TTR gene that may be responsible for negatively regulating TTR expression during the APR.

Discussion

Tissue-specific gene transcription is regulated based in part on the recognition of *cis*-elements in the noncoding regions of target genes, and is accomplished by transcription factors that have restricted tissue distributions. Transcriptional regulation, the modulation of transcription factors and their activities play an important role in somatic phenotype change such as seen after injury. Liver-specific gene expression is governed by the combinatorial action of a small set of liver-enriched transcription factors: HNF-4, a member of the steroid hormone receptor superfamily; HNF-1, a member of the POU homeobox gene family; HNF-3, the DNA binding domain, which is very similar to that of the *Drosophila* homeotic forkhead gene; and HNF-6, containing a single cut domain and a divergent homeodomain motif. These liver-enriched transcription factors constitute a complex transcriptional network responsible in part for the development and maintenance of the liver's phenotype. HNF-4α is a key member of this regulatory network [19–21].

In this work we have utilized the proximal promoter of the TTR gene as a model to determine the role of multiple HNFs in TTR gene expression and its response to injury. Several lines of evidence suggest that in HepG2 cells the TTR gene is regulated by HNF-4α and other HNFs, including HNF-1α, HNF-3α/β, and HNF-6α in a combinatorial manner. First, mutagenesis of the HNF-4, HNF-1 binding site or both HNF-3 and HNF-6 sites

together in the TTR promoter eliminated TTR transcriptional activity, whereas a separate mutation of the HNF-3 or HNF-6 site significantly reduced the activity (Fig. 2A). This may be due in part to the fact that the HNF-3 binding site (−106 to −93 bp) is overlapping with the HNF-6 binding site (−106 to −93 bp) in the TTR promoter [12]. Second, cotransfection of HNF-4α, HNF-1α, HNF-3α or HNF-6α expression plasmid with a reporter of the TTR promoter resulted in a higher level of TTR transcription comparing with the cotransfection of empty vector (Fig. 2B). Third, *in vitro* DNA-protein binding assays (Fig. 5 and 6) and *in vivo* ChIP assays (Fig. 7) reveal that these transcription factors are associated with the TTR proximal promoter. Fourth, the reduced expression of TTR gene in response to cytokine treatment [17] coincides with a large decrease in the ability of HNFs to bind to the TTR promoter (Fig. 7).

HNF-4α has been shown to be a regulator of the hepatic APR gene expression [16,17], the interactive effect of HNF-4α with other HNFs on the activity of genes such as TTR is of particularly interesting in understanding the complexity of transcriptional regulation and the liver's response to injury as the TTR gene contains several HNFs' binding sites in its promoter, and the TTR expression is modulated by injury. The results from our transactivation experiments indicate that the mutation of HNF-4 binding site not only affected the response of TTR promoter to endogenous and overexpressed HNF-4α, but also eliminated or reduced the response to the overexpressed of HNF-1α, HNF-3α/β, and HNF-6α (Fig. 3), implying that alteration in HNF-4α binding not only affects itself but also interferes with the function of other HNFs. Given the observation that a mutation in the HNF-4 binding site only destroys the binding for HNF-4α, but not for other HNFs (Fig. 5A and Fig. 6), one potential mechanism is that an intact HNF-4α/DNA binding complex is required for effective TTR transactivation and may provide a platform to maintain a stable network of various HNFs for efficient TTR transcription. Consistent with this hypothesis, it has been reported that a mutation of the TTR HNF-3/HNF-6 binding site to a sequence that only binds HNF-3 protein diminished expression of the TTR promoter in HepG2 cell transfection assays [12]. Another interpretation is that the mutation of the HNF-4α binding site may affect the promoter conformation that results in defective recruitment/sequestration of the other factors and thus a loss of factor-factor interaction, either directly or through mediation of a cofactor or another transcription factor. One of example of this is seen in that the apolipoprotein AI gene expression in liver depends on the interactions between HNF-4 and HNF-3 within a hepatocyte-specific enhancer in the 5′-flanking region of the gene. It has been proposed that an intermediary factor normally present in liver cells is recruited to the enhancer and core transcription complexes when both HNF-3 and HNF-4 occupy their binding sites but not with either of them occupying their cognate sites individually [22].

The extraordinary packing of multiple HNF binding sites within the short stretch of DNA in TTR gene as well as the availability of highly enriched HNFs in liver cells make it likely that protein-protein interactions between different HNF proteins take place and affect transactivation. The existence of multiple sites and factors also allows for a finer modulation of liver-specific genes under different physiological conditions. However, little is known in the modulation of these factors individually or in combination under changing conditions. In this study, we have utilized the TTR DNA regulatory region as a model to investigate hepatocyte-specific gene transcription during the APR. TTR has been recognized as a negative acute phase protein. During acute inflammation, the rate of TTR synthesis [23] and its mRNA level [24] decrease in the liver. This decrease is due to a reduction in the rate of transcription of this gene [25]. We have previously demonstrated that a classic acute phase response can be induced in HepG2 cells after cytokine treatment. Utilizing this cell culture model, we found that the treatment with cytokines caused a significant decrease in mRNA expression of TTR gene [17]. Evidence from our ChIP assay shows that the ability of HNF-4α, HNF-1α, HNF-3β and HNF-6α to bind to the TTR proximal promoter is all

significantly reduced after cytokine stimulation (Fig. 7), and the alteration in binding is not caused by the less protein levels of HNFs (Fig. 8). One plausible mechanism for the acute phase repression of TTR may involve an early and rapid decrease in binding ability of the HNFs, and consequently leading to alterations in their interaction with each other affecting transactivation. Because the efficient binding of HNF-4, HNF-1, HNF-3 and HNF-6 to TTR promoter is critical for TTR gene transcription (Fig. 2A), the reduction in the binding ability either from a post-translation alteration in binding efficiency or a change in HNF's availability can diminish the transcription of the TTR gene. In addition, an alternation in the binding of HNF-4 or other HNFs would be expected to affect the formation, configuration and stabilization of the multiple protein-protein interactions or recruitment of other cofactors. Support for this hypothesis comes from our transfection assays (Fig. 3 and 4), and our previous findings that transcription co-activator PGC-1α (peroxisome-proliferator-activated receptor-γ co-activator-1α) enhances the TTR transactivation, whereas cytokine treatment reduces the recruitment of PGC-1α to HNF-4α-binding sites and thereby decreases transcriptional activity [26].

In this study the results obtained from transfection assays and DNA-protein binding assays demonstrate mechanism by which the expression pattern of a hepatic gene TTR is determined by the presence of multiple *cis*-elements and their ability to effectively interact with their specific transcription factors, and is also influenced by secondary interactions among these diverse liver-specific transcription factors, which provides a new insight into the understanding of the regulation of TTR gene during variable physiological states. The promoter regions of many liver-enriched genes contain putative binding sites for more than one of the HNF factors, thus, the combinatorial transcriptional regulation seen for the TTR gene may represent a generalized mechanism of transcriptional regulation. However, in vivo interactions can differ from those observed in cell culture system, and the in vivo relevance of these mechanisms and their potential importance for regulating the overall hepatic acute phase response will require further investigation. In addition to the liver, TTR gene is also expressed at high levels in the choroid plexus [12], where the liver-specific transcription factors are generally not found. It would be interesting to study what differences there are in the regulation of TTR between the liver and the choroid plexus, and how this regulation is altered in different tissues by the global injury response.

Materials and methods

Cell culture and acute phase response

HepG2 human hepatoma cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The acute phase response in HepG2 cells was stimulated by incubation with a cytokine mixture consisting of 1 ng/ml of recombinant human IL-1β, 10 ng/ml of IL-6 and 10 ng/ml of TNF-α (PeproTech, Rocky Hill, NJ, USA) in serum-free medium for 18 h [17].

Expression and reporter plasmids

Expression plasmids for rat HNF-1α (Dr. F. Gonzalez, NCI, National Institutes of Health, Bethesda, MD, USA), rat HNF-3α and HNF-3β (Dr. D. Waxman, Boston University, Boston, MA, USA), rat HNF-4α (Dr A. Kahn, Institut Cochin, Paris, France) and rat HNF-6α (Drs. F. Lemaigre and G. Rousseau, University of Louvain Medical School, Brussels, Belgium) were obtained from the indicated individuals.

The luciferase reporter plasmids (wild type and mutants [12,27], Fig. 1) were generated by subcloning a 196-bp DNA fragment corresponding to −191 to +5 of the mouse TTR gene (nucleotide numbering relative to the transcriptional start site) (accession number M19524

[GenBank]; GenBank/EBI Data Bank) into the pGL4.11 [luc2P] vector (Promega, Madison, WI, USA) at *BglII* and *HindIII* sites. All constructs were verified by DNA sequencing.

Transient transfection and luciferase assay

For transient transfections, the cells were seeded in 48-well plates, and were transfected using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer's protocol. Typically, each wellof a 48-well tissue culture plate received a total of 400 ng of DNA, including 70 ng of firefly luciferase reporter and 330 ng of expression plasmid or empty vector. In all cases, 4 ng of *Renilla* luciferase reporter plasmid were included as an internal control for transfection efficiency. Forty-eight hours after the addition of the transfection reagent-DNA complex, cells were lysed in 1 × lysis buffer (Promega), and luciferase activity was determined using a dual reporter assay system (Promega). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalized luciferase activities (mean ± S.D. values for $n = 3$ independent transfections). Relative luciferase activities were then calculated to facilitate comparisons between samples within a given experiment.

DNA-protein binding assay

Binding of HNFs to their target DNA in the TTR proximal promoter was measured by enzyme-linked DNA–protein interaction assay using the TransFactor Colorimetric kit (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, 20 μg of nuclear extract prepared as previously described [17] were mixed with the biotinylated oligonucleotide probe (2 pmol) in 1 × TransFactor/Blocking buffer (kit provided) at room temperature for 15 min. The mixture was added in each well and incubated for 1 h at room temperature. After washing, diluted primary antibodies against various HNFs (all antibodies used were purchased from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) were added (100 μl/well) and incubated at room temperature for 1 h. After washing, diluted secondary antibody conjugated with horseradish peroxidase was added to each well and further incubated at room temperature for 30 min. After repeated washing, 100 μl of tetramethylbenzidine substrate solution were added to each well. The reaction was quenched by 100 μl of 1 M H_2SO_4/well, and binding intensity was measured as absorbance at 450 nm using a microtiter plate reader.

To further test the specificity of HNF-4α/DNA binding, Western blot analysis was performed. Nuclear extracts (200 μg) were mixed with the biotinylated oligonucleotide probe (2 μg) at room temperature for 15 min in 1xTransFactor/Blocking buffer. Fifty microliters of Dynabeads M-280 Streptavidin (Invitrogen) were mixed in by rotation for 1 h at 4°C. The Dynabeads were then collected with a magnet and washed three times with cold PBS. The trapped proteins were analyzed by Western blotting as previously described [16,17].

The biotin labeled double-strand oligonucleotide probes based on mouse TTR promoter sequence (−162 ~ −81) containing WT or mutant DNA binding sites of HNF-4, HNF-1, HNF-3 and HNF-6 used for DNA-protein binding assay are the same as those described as in Fig. 1.

Chromatin immunoprecipitation (ChIP) assay

HepG2 cells were grown in 100 mm culture dishes to 80% confluence. The cells were then untreated or treated with cytokines for 18 h. ChIP assays were performed using an EZ ChIP kit (Upstate Biotechnology, Temecula, CA, USA) following the manufacturer's protocol. Antibodies against HNF-4α, HNF-1α, HNF-3β and HNF-6α (Santa Cruz Biotechnology) were used to immunoprecipitate DNA–protein complexes, and additional mock

immunoprecipitations with normal goat or rabbit IgG (Santa Cruz Biotechnology) were utilized to detect background DNA binding. Real-time PCR was used to analyze immunoprecipitated DNA and input control DNA. TTR promoter-specific primer (Assays by Design, Applied Biosystems, Foster City, CA, USA) was designed as follows: forward primer 5′-CGAATGTTCCGATGCTCTAATCTCT-3′, reverse primer 5′-ACTGCAAACCTGCTGATTCTGATTAT-3′ and TaqMan® FAM (6-carboxyfluorescein) dye-labeled probe 5′-CATATTTGTATGGGTTACTTATT-3′. Amplification of input chromatin was used as an internal reference gene in the same reactions. Relative quantification was determined by using the comparative Ct (ΔΔCt) method.

Immunoblotting

Whole cell extracts were used for immunoblotting as previously described [16]. Antibodies against HNF-4α, HNF-1α, HNF-3β and HNF-6α were purchased from Santa Cruz Biotechnology.

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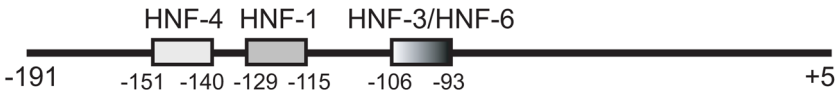
Abbreviations

APR	acute phase response
APP	acute phase protein
TTR	transthyretin
HNF	hepatocyte nuclear factor
ChIP	chromatin immunoprecipitation
ELISA	enzyme-linked immunosorbent assay
PGC-1α	peroxisome-proliferator-activated receptor-γ co-activator-1α

References

1. Kushner I. The phenomenon of the acute phase response. Ann N Y Acad Sci. 1982; 389:39–48. [PubMed: 7046585]
2. Fey GH, Fuller GM. Regulation of acute phase gene expression by inflammatory mediators. Mol Biol Med. 1987; 4:323–338. [PubMed: 2449594]
3. Baumann H, Gauldie J. The acute phase response. Immunol Today. 1994; 15:74–80. [PubMed: 7512342]
4. Cerra FB. The systemic septic response: concepts of pathogenesis. J Trauma. 1990; 30:S169–174. [PubMed: 2254978]
5. Beal AL, Cerra FB. Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction. Jama. 1994; 271:226–233. [PubMed: 8080494]
6. van Jaarsveld PP, Edelhoch H, Goodman DS, Robbins J. The interaction of human plasma retinol-binding protein and prealbumin. J Biol Chem. 1973; 248:4698–4705. [PubMed: 4718739]
7. Kanda Y, Goodman DS, Canfield RE, Morgan FJ. The amino acid sequence of human plasma prealbumin. J Biol Chem. 1974; 249:6796–6805. [PubMed: 4607556]
8. Felding P, Fex G. Cellular origin of prealbumin in the rat. Biochim Biophys Acta. 1982; 716:446–449. [PubMed: 7115763]
9. Costa RH, Lai E, Grayson DR, Darnell JE Jr. The cell-specific enhancer of the mouse transthyretin (prealbumin) gene binds a common factor at one site and a liver-specific factor(s) at two other sites. Mol Cell Biol. 1988; 8:81–90. [PubMed: 3336368]

10. Costa RH, Lai E, Darnell JE Jr. Transcriptional control of the mouse prealbumin (transthyretin) gene: both promoter sequences and a distinct enhancer are cell specific. Mol Cell Biol. 1986; 6:4697–4708. [PubMed: 3025666]
11. Yan C, Costa RH, Darnell JE Jr, Chen JD, Van Dyke TA. Distinct positive and negative elements control the limited hepatocyte and choroid plexus expression of transthyretin in transgenic mice. Embo J. 1990; 9:869–878. [PubMed: 1690125]
12. Samadani U, Costa RH. The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. Mol Cell Biol. 1996; 16:6273–6284. [PubMed: 8887657]
13. Cereghini S. Liver-enriched transcription factors and hepatocyte differentiation. Faseb J. 1996; 10:267–282. [PubMed: 8641560]
14. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. Hepatology. 2003; 38:1331–1347. [PubMed: 14647040]
15. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA. Control of pancreas and liver gene expression by HNF transcription factors. Science. 2004; 303:1378–1381. [PubMed: 14988562]
16. Li X, Salisbury-Rowswell J, Murdock AD, Forse RA, Burke PA. Hepatocyte nuclear factor 4 response to injury involves a rapid decrease in DNA binding and transactivation via a JAK2 signal transduction pathway. Biochem J. 2002; 368:203–211. [PubMed: 12106016]
17. Wang Z, Burke PA. Effects of hepatocyte nuclear factor-4alpha on the regulation of the hepatic acute phase response. J Mol Biol. 2007; 371:323–335. [PubMed: 17574576]
18. Burke PA, Luo M, Zhu J, Yaffe MB, Forse RA. Injury induces rapid changes in hepatocyte nuclear factor-1: DNA binding. Surgery. 1996; 120:374–380. discussion 380–381. [PubMed: 8751607]
19. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M. Regulation of a transcription factor network required for differentiation and metabolism. Science. 1998; 281:692–695. [PubMed: 9685261]
20. Ktistaki E, Talianidis I. Modulation of hepatic gene expression by hepatocyte nuclear factor 1. Science. 1997; 277:109–112. [PubMed: 9204893]
21. Nagaki M, Moriwaki H. Transcription factor HNF and hepatocyte differentiation. Hepatol Res. 2008; 38:961–969. [PubMed: 18462375]
22. Harnish DC, Malik S, Kilbourne E, Costa R, Karathanasis SK. Control of apolipoprotein AI gene expression through synergistic interactions between hepatocyte nuclear factors 3 and 4. J Biol Chem. 1996; 271:13621–13628. [PubMed: 8662915]
23. Dickson PW, Howlett GJ, Schreiber G. Metabolism of prealbumin in rats and changes induced by acute inflammation. Eur J Biochem. 1982; 129:289–293. [PubMed: 7151801]
24. Dickson PW, Howlett GJ, Schreiber G. Rat transthyretin (prealbumin). Molecular cloning, nucleotide sequence, and gene expression in liver and brain. J Biol Chem. 1985; 260:8214–8219. [PubMed: 3839240]
25. Birch HE, Schreiber G. Transcriptional regulation of plasma protein synthesis during inflammation. J Biol Chem. 1986; 261:8077–8080. [PubMed: 2424892]
26. Wang Z, Burke PA. Modulation of hepatocyte nuclear factor-4alpha function by the peroxisome-proliferator-activated receptor-gamma co-activator-1alpha in the acute-phase response. Biochem J. 2008; 415:289–296. [PubMed: 18510493]
27. Costa RH, Grayson DR. Site-directed mutagenesis of hepatocyte nuclear factor (HNF) binding sites in the mouse transthyretin (TTR) promoter reveal synergistic interactions with its enhancer region. Nucleic Acids Res. 1991; 19:4139–4145. [PubMed: 1870969]



	WT	Mutant
HNF-4	GGCAAGGTTCA	GGgcAtGcTCA
HNF-1	GTTACTTATTCTCC	GcTcgagATTCTCC
HNF-3	CTAAGTCAATAATC	CTAAaTCAATAtcg
HNF-6	CTAAGTCAATAATC	CTAAacaAAcAtTC
HNF-3 and HNF-6	CTAAGTCAATAATC	CTAtcgatATAATC

Fig. 1.

TTR proximal promoter (nt −191 to +5 region). Schematically shown the locations of HNF-4, HNF-1 and overlapped HNF-3/HNF-6 binding sites on the TTR promoter region. Shown below are the wild-type (WT) and mutated (small letter) oligonucleotide sequences of HNF-4, HNF-1, HNF-3, HNF-6 and HNF-3/HNF-6 binding sites [12,27].

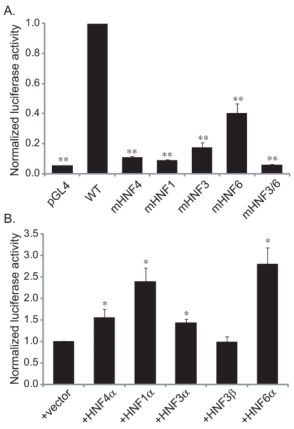


Fig. 2.

Functional analysis of the *cis*-elements in TTR promoter. (A) HepG2 cells were transfected with a luciferase construct containing the promoter region spanning from nt −191 to +5 (WT) and its derivatives carrying mutations (mHNF4, mHNF1, mHNF3, mHNF6 and mHNF3/6) as described in Fig. 1 or empty pGL4.11 [luc2P] vector (pGL4). (B) The cells were cotransfected with WT luciferase reporter and the corresponding expression plasmids. The data shown are the normalized luciferase activity representing the ratio of the firefly luciferase activity to that of *Renilla* luciferase activity, and representing mean ± S.D. of 3 independent experiments. The luciferase activity in the cells transfected with WT reporter (A) or empty expression vector (B) was set at 1. *$p<0.05$ and **$p<0.01$ indicate a significant difference compared to WT (A) or empty vector (B).

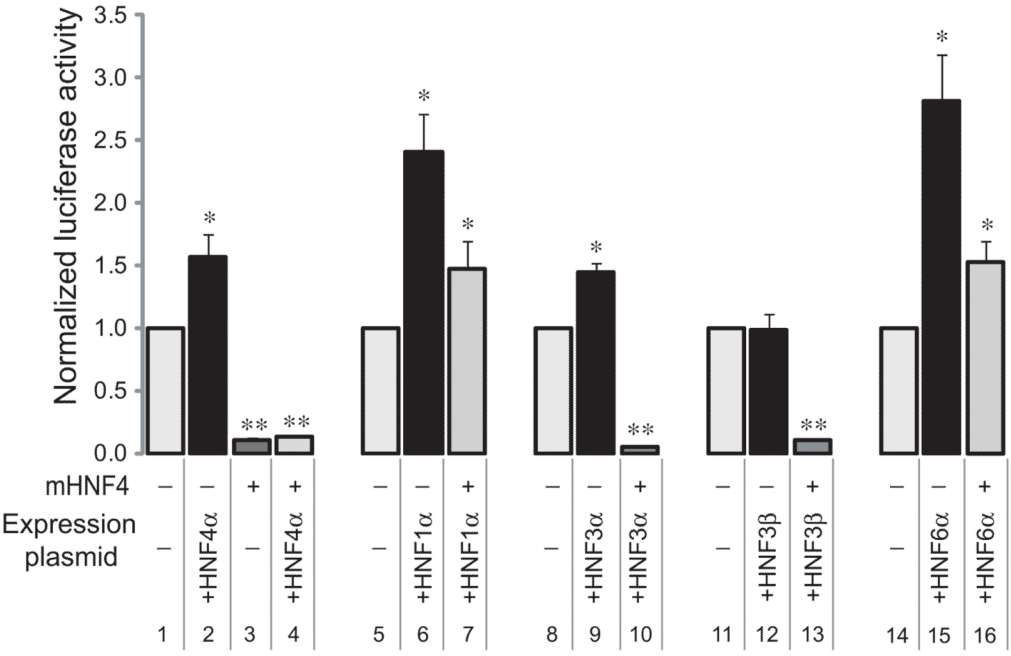


Fig. 3.

Mutation of HNF-4 binding site affects not only the response to HNF-4α and also to other HNFs in activating TTR transcription. HepG2 cells were cotransfected with the luciferase reporter containing mutated HNF-4 site (mHNF4+) or WT (mHNF4−) TTR promoter and indicated expression plasmid (+) or empty vector (−). The luciferase activity in the cells cotransfected with WT reporter and empty vector was set at 1. The data represent the mean ± S.D. of three different experiments. *$p<0.05$ and **$p<0.01$ indicate a significant difference compared to the control cells cotransfected with WT reporter and empty vector.

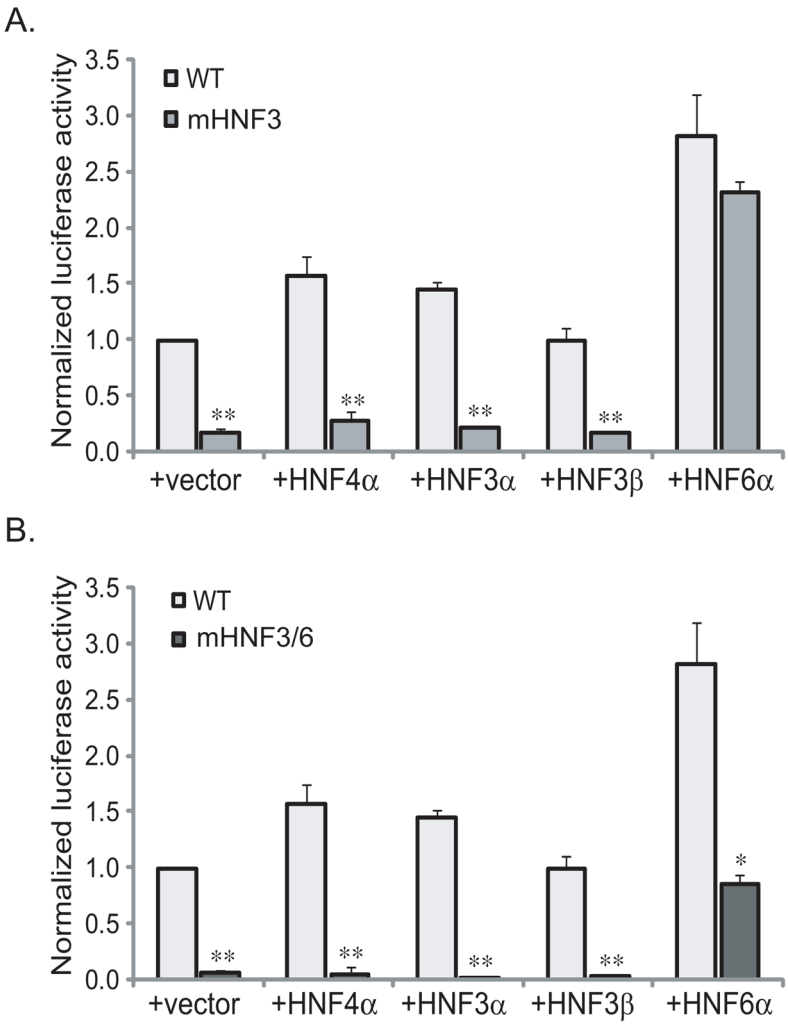


Fig. 4.

Mutation of HNF-3 or HNF-3/HNF-6 binding site affects the response to its relative HNF(s) and HNF-4α in activating TTR transcription. HepG2 cells were cotransfected with the luciferase reporter containing mutated HNF-3 (mHNF3) (A), mutated HNF-3 and HNF-6 sites (mHNF3/6) (B) or WT TTR promoter (WT) and indicated expression plasmid or empty vector (vector). The luciferase activity in the cells cotransfected with WT reporter and empty vector was set at 1. The data represent the mean ± S.D. of three different experiments. *p<0.05 and **p<0.01 indicate a significant difference between the luciferase reporter of WT and mutated promoter.

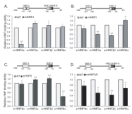


Fig. 5.

Mutation of HNF binding site mainly disrupts the corresponding HNF binding ability, not others. Nuclear extracts prepared from HepG2 cells were incubated with biotinylated DNA probe encompassing TTR promoter (−161 to −81) with the binding sites of either native (WT) or mutated HNF (mHNF4, mHNF3, mHNF6, and mHNF3/6). The complexes of DNA-HNF proteins were assayed by ELISA using antibodies (α-HNF) for detecting HNF proteins. On the top of each panel the schematic shows the location of HNF binding site and the mutated site which is marked as X. The binding ability of WT DNA probe was set at 1. Data represent mean ± S.D. from 3 independent experiments. *$p<0.05$ and **$p<0.01$ indicate a significant difference compared to WT.

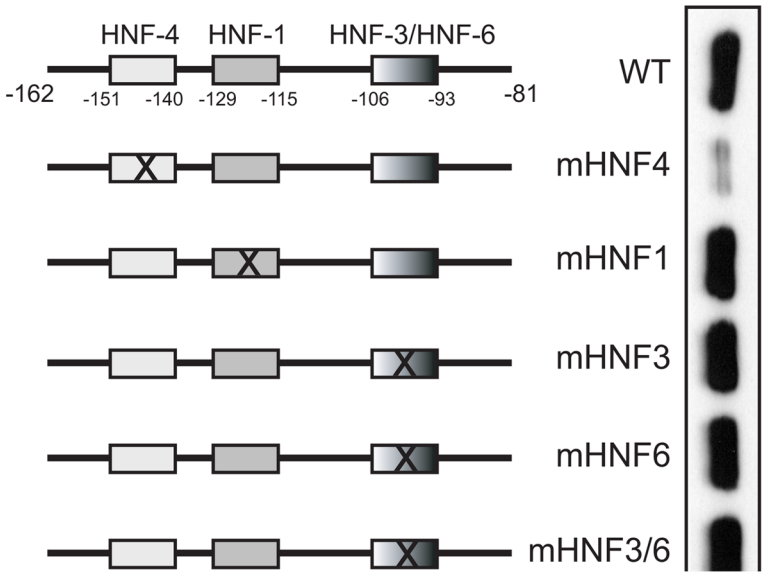


Fig. 6.

HNF-4 binding ability is only affected by the mutation in HNF-4 binding site, but not in other HNF sites. The complexes of DNA-HNF proteins were assayed by Western blot using an antibody for specifically detecting HNF-4α proteins. Schematic in the left is described as in Fig. 5.

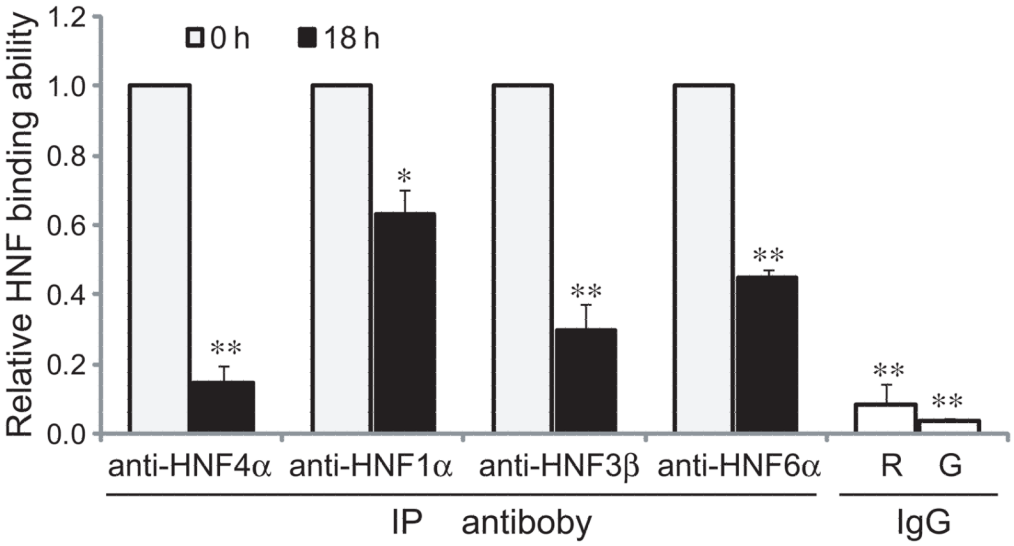


Fig. 7.

The binding abilities of HNFs are reduced by treatment with cytokines. HepG2 cells were treated with or without cytokines for 18 h. The interaction of HNF protein and DNA binding site was determined by ChIP assays with either antibodies against HNF-4α, HNF-1α, HNF-3β and HNF-6α, or rabbit (R) or goat (G) IgG (IgG). Chromatin-immunoprecipitated (IP) DNA was analyzed by real-time PCR using specific primers and probe for the TTR proximal promoter. The control samples (cytokine-untreated cells, time zero) were set at 1. The results are mean ± S.D. (n =3). **p*<0.05 and ***p*<0.01 indicate that the value is significantly different from control.

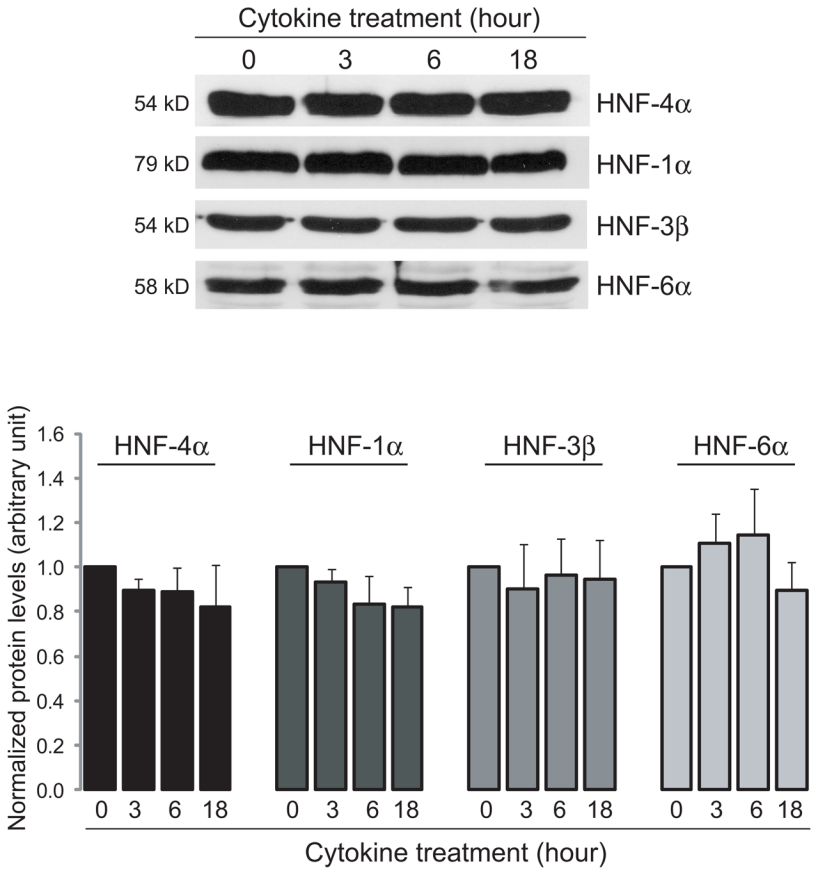


Fig. 8.

Cytokine treatment does not reduce the protein levels of HNFs. The protein lysates were extracted from HepG2 cells untreated or treated with cytokines for the indicated times. The protein levels of HNFs were determined by Western blot. Histograms showing the densitometric analyses of protein levels summarize three separate experiments. Values represent mean ± S.D., and cytokine-untreated HepG2 cells (time zero) were set at 1. No significant difference was found between untreated and treated cells ($p>0.05$).