Cytokine Regulation of the Liver Transcription Factor Hepatocyte Nuclear Factor-3β Is Mediated by the C/EBP Family and Interferon Regulatory Factor 1

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Abstract

Three distinct hepatocyte nuclear factor-3 (HNF-3) proteins (α, β, and γ) regulate the transcription of numerous liver-enriched genes. The HNF-3 proteins bind DNA via a homologous winged helix motif common to a number of proteins known to be critical for determination and differentiation in embryogenesis. We have demonstrated previously that two binding sites in the −184 HNF-3β promoter are recognized by widely distributed factors and that there is also a critical autoregulatory site present that is recognized by members of the HNF-3 family. Adjacent to the autoregulatory site, we identified a binding site for a cell-specific factor, LF-H3β, that may function in restricting HNF-3β gene expression to hepatocytes. Our present study demonstrates that members of the C/EBP and proline and acidic amino acid-rich subfamilies of basic region leucine zipper transcription factors bind the LF-H3β site, and cotransfection of HepG2 cells shows that these factors are able to activate an HNF-3β promoter reporter construct. The LF-H3β-C/EBP binding sequence also confers HNF-3β promoter stimulation in response to interleukin (IL)-1 and IL-6. Upstream of this HNF-3β proximal promoter region, an IFN-stimulated response element core sequence (−231 to −210) was found that mediates transcriptional induction by IFN-γ but not IFN-α. Gel mobility supershift assay demonstrates that an IFN-γ-induced protein-DNA complex is disrupted by an antibody specific for interferon regulatory factor-1/interferon-stimulated gene factor-2. Consistent with this finding, we observed that IFN-γ induction requires ongoing protein synthesis. Surprisingly, the effect of the three cytokines (IL-1, IL-6, and IFN-γ) in combination as assayed by the same model is not synergistic. HNF-3β joins the C/EBP family on the list of liver-enriched transcription factors, the expression of which is modulated by cytokines.

Introduction

Analysis of regulatory regions governing hepatocyte-specific transcription suggests that five distinct families of liver-enriched factors participate in coordinate regulation of liver gene expression via combinatorial interaction on multiple DNA sites (1). These liver transcription factor families include: the HNFα-3α, HNF-3β, and HNF-3γ proteins (2, 3) that use the winged helix DNA-binding motif (4); the steroid hormone receptor family members, HNF-4 (5) and ApoAl regulatory protein-1 (6); the POU-homeodomain members, HNF-1α and HNF-1β (also known as LF-B1 and vHNF-1); Refs. 7 and 8); the C/EBP bZIP family members, C/EBPβ (9, 10), C/EBPβ (also known as liver-enriched activator protein, IL-6DBP, NF-IL6, C/EBP-related protein 2, and AGP/EBP; Refs. 11–16), and C/EBPβ (also known as C/EBP-related protein 3 and NF-IL6; Refs. 12, 16, and 17); and the PAR bZIP family members, DBP (18), VBP/thyrotroph embryonic factor (19, 20), and hepatic leukemia factor (21, 22).

Work in our laboratory focuses on the HNF-3 proteins, which were originally identified as factors mediating liver-specific transcription of the TTR (23, 24) gene and were later shown to control the expression of numerous hepatocyte-specific genes (1, 25). Ang et al. (26), DiPersio et al. (27), and Liu et al. (28) further demonstrated that HNF-3α is the critical factor in maintaining the differentiative potential of H2.35 cells. HNF-3α also participates with HNF-4 in the hierarchical transcriptional activation of HNF-1 in the hepatocyte, verifying its role in establishing hepatocyte differentiation (29). The HNF-3 proteins bind to the consensus DNA-binding site AA/TTTRTT/G/IYTY as a monomer using the 100-amino acid winged helix DNA-binding domain (4, 30). Mammalian HNF-3 (2, 3) and the Drosophila homeotic gene fork head (31) are prototypes of a family of transcription factors that share homology in their winged helix motif (4). The HNF-3/fork head family is comprised of

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4 The abbreviations used are: HNF, hepatocyte nuclear factor; TTR, transthyretin; C/EBP, CAAT/enhancer binding protein; Stat, signal transducers and activators of transcription; IRF-1, interferon response factor-1; ISGF, interferon stimulatory gene factor; ISRE, interferon-stimulated response element; GAS, IFN-γ activation sequence; UF, ubiquitous factor; Hpx, hemopexin; VBP, vitellogenin gene-binding protein; NF, nuclear factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bZIP, basic region leucine zipper; PAR, proline and acidic amino acid-rich; DBP, albumin D-box binding protein.
more than 30 distinct genes that are expressed in a diverse group of mammalian cell lineages (25, 26, 32–41).

In situ hybridization studies of stage-specific embryos suggest that the HNF-3 proteins function in endodermal determination as well as in the formation of the neural tube and notochordal mesoderm (26, 40, 42, 43). In support of the importance of HNF-3 expression in these developing structures, HNF-3β mutant embryos lack properly formed node and notochord, leading to defects in neurotube and somite organization (44, 45). Although these mutants are not disrupted in definitive endoderm, they fail to form gut endoderm, which gives rise to the liver, lung, pancreas, and intestine. Furthermore, ectopic hindbrain expression of the HNF-3β gene in transgenic mice mediates the conversion of hindbrain to the floorplate, as evidenced by activation of the endogenous HNF-3 and floorplate marker genes (46).

The HNF-3α gene is also transiently induced during retinoic acid differentiation of F9 embryonic stem cells toward both the parietal and visceral endoderm lineages (47).

Hepatocyte-specific genes are also transcriptionally responsive to diverse signaling pathways. For example, trauma or infection results in the release of proinflammatory cytokines (e.g., IL-6, IL-1, and tumor necrosis factor), which interact with hepatocyte receptors and elicit expression of acute phase proteins, resulting in significant changes in serum protein composition (48). The activation of acute phase gene expression is mediated by induction of combinations of transcription factors that recognize distinct target sequences in acute phase-responsive promoter regions (48, 49). Among these induced transcription factors are the NF-κB family (50), the acute phase responsive factor of Stat family members, and M, 91,000 polypeptide (51–54). In acute phase livers, expression of the bZIP C/EBPβ and C/EBPα proteins is also increased, while C/EBPα expression is reciprocally decreased (11, 15, 17, 55–58). IFN-γ is also secreted by activated T lymphocytes during the immune response. Recognition of a specific receptor results in tyrosine phosphorylation and subsequent activation of a preexisting cytoplasmic Stat1α (M, 91,000) protein (59). Stat1α coordinately activates specific sets of genes via recognition of an GAS contained within the promoter regions of the target genes. Stat1α (59) also mediates the activation of IRF-1 (also called ISGF-2, Refs. 60–63). Newly synthesized ISGF-2/IRF-1 protein activates promoters through the 9-bp core of the larger ISRE (63–66). This binding site is distinct from the GAS element that mediates a rapid response to IFN-γ. Thus, IFN-γ is able to induce a temporal cascade of transcription factors that regulate diverse sets of genes.

To elucidate the mechanisms restricting HNF-3β gene expression, we previously identified four protein binding sites in the −184 proximal promoter region that are required for HNF-3β expression in HepG2 cells (67). Two of the HNF-3β promoter sites are recognized by proteins present in a variety of tissue extracts and designated UF1-H3β and UF2-H3β. A third element binds the HNF-3 proteins and plays an autoregulatory role in sustaining expression, a feature shared with other transcription factors involved in differentiation (68). Adjacent to the autoregulatory site, we identified a binding site for a cell-specific factor, LF-H3β, that appears critical for restricting the adult expression pattern of the HNF-3β gene (Fig. 1). Mutation of this site results in a 60% decrease in HNF-3β promoter activity (67). In this study, we used the LF-H3β binding site to screen a liver cDNA expression library and found that this protein binding activity is encoded by the cytokine-regulated C/EBPβ gene. Further binding studies reveal that the LF-H3β site is also recognized by the cytokine-responsive C/EBPβ as well as C/EBPα and a member of the PAR subfamily of bZIP transcription factors. We show that factors from these two bZIP subfamilies are capable of activating the HNF-3β promoter in cotransfection assays via the LF-H3β site in HepG2 cells. This sequence also confers responsiveness to the combination of IL-1 and IL-6 cytokines. Furthermore, an IRF-1 binding site (−231 to −210) was identified upstream of the minimal HNF-3β promoter region, which elicited stimulation by IFN-γ. We discuss these results with respect to liver-specific transcription of the HNF-3β gene and to maintenance of HNF-3β expression during the acute phase response.

Results

A Family of C/EBP Proteins Binds to the HNF-3β Promoter.

To isolate the LF-H3β cDNA, a liver cDNA eukaryotic expression library was transfected into HeLa cells and screened for LF-H3β binding by gel shift assay (see "Materials and Methods"). HeLa cells lack endogenous LF-H3β binding activity and, therefore, provide a suitable cell line in which to screen for LF-H3β protein binding (67). Forty

![Fig. 1. HNF-3β promoter and 6X LF-H3β TATA reporter constructs and DNA binding site oligonucleotides. Schematically shown is the HNF-3β CAT reporter plasmid illustrating the position of the DNA-binding proteins recognizing this promoter region (67). These include the autoregulatory HNF-3 site as well as two sites that bind factors that are widely distributed (UF1-H3β and UF2-H3β). The LF-H3β promoter site binds factors that are enriched in lung and liver nuclear extracts, and we show in Fig. 2 that the LF-H3β site is recognized by the C/EBP family. Also shown schematically is the 6X LF-H3β TATA CAT reporter construct and the sequence of the double-stranded oligonucleotides used in this study. The sequence of the promoter and enhancer oligonucleotides are derived from the following references: HNF-3β (67), Hpx (15), VBP (20), and TTR-3 (72).](image-url)
different pools of 3000 cDNA expression clones were transfected into HeLa cells, from which nuclear extracts were prepared 36 h later and tested for LF-H3β binding activity (Fig. 1). Positive pools of expression cDNA clones were then diluted and rescreened for binding activity to purify the cDNA clone encoding the LF-H3β protein. Comparison of the LF-H3β cDNA sequence with the Genbank database revealed that it encoded the bZIP transcription factor C/EBPB.

Because members of the C/EBP family play an important role in mediating cytokine-induced transcription, we wanted to compare C/EBP binding properties of the LF-H3β site (HNF-3β; −97 to −67 bp; Ref. 67) with those of a C/EBP-like cytokine-response element from the Hpx promoter (Fig. 1; Ref. 15). We carried out gel shift assays with DNA probes containing these sites and recombinant C/EBPa and C/EBPB protein expressed in HeLa cells (Fig. 2A; see “Materials and Methods”). Both of the probes formed identical C/EBP protein-DNA complexes that were both self- and cross-competed (Fig. 2A), indicating that the LF-H3β element is an efficient C/EBP protein binding site. Multiple C/EBP complexes are probably due to differential use of internal translational initiation sites as described by Descombes and Schibler (69).

Inspection of the LF-H3β sequence revealed that this site is similar, not only to a canonical C/EBP site (ATTGCC-CAAT), but also to the site recognized by the related PAR subfamily of bZIP proteins (ATTAGCTAAT; Fig. 1; Ref. 70). In addition, it has been observed that C/EBPa has affinity for certain PAR-like sites (71) and that the PAR factor VBP can bind to mixed sites composed of PAR and C/EBP-like half-sites (70). In view of the potential cross-talk between LF-H3 and PAR bZIP protein-binding sites, we sought to further investigate the ability of C/EBPa, C/EBPB, C/EBPA, as well as VBP, to bind to the HNF-3β promoter site by cross-competing with the C/EBP and VBP sites shown in Fig. 1. These competition studies revealed that the C/EBPB (the two most slowly migrating complexes are shown), C/EBPA, and VBP proteins possessed approximately 4-fold greater binding affinity for the Hpx promoter site than they did for the LF-H3β sequence (Fig. 2B). C/EBPa, however, exhibited nearly identical binding affinities for both sequences. By contrast, the VBP-binding site from the chicken vitellogenin promoter was not an effective competitor for binding of the C/EBP family members and thus appeared to be selectively bound by the VBP protein (Fig. 2B). Finally, a C/EBP binding site in the transgelatin enhancer region (TTR-3; Ref. 72)

![Fig. 2](image_url). The LF-H3β site in the HNF-3β promoter is recognized by multiple C/EBP family members. A, recombinant C/EBP proteins exhibit identical recognition properties with C/EBP binding sites derived from the HNF-3β and acute phase Hpx promoters. Oligonucleotides containing C/EBP recognition sites from the HNF-3β (LF-H3β site) and the Hpx promoters were used for gel retardation experiments with recombinant C/EBPa and C/EBPB proteins produced in HeLa cells. Included were lanes with 200-fold molar excess of oligonucleotide containing the C/EBP recognition sites from either the HNF-3β or Hpx promoters. B, the HNF-3β promoter is recognized by several members of the C/EBP family. The top portion of the gel is shown depicting the protein-DNA complex formation between the C/EBP site situated in the HNF-3β promoter region and recombinant C/EBPa, C/EBPB, and VBP proteins expressed in HeLa cells and C/EBPa expressed in E. coli (see Fig. 1). For the C/EBPs competition studies, only the two slowly migrating bands are shown from A, which reacted with C/EBPB antisera in supershift assays (data not shown). Competition with the indicated fold excess of C/EBP-binding site oligonucleotides derived from HNF-3β, Hpx, VBP (chicken vitellogenin promoter), and TTR-3 (TTR enhancer) regulatory regions (see Fig. 1).
competed poorly for C/EBP recognition of the LF-H3β site, but it did inhibit C/EBPβ complex formation when a 300-fold molar excess was used (Fig. 2B).

We next compared the HNF-3β and Hpx oligonucleotides for complex formation with liver nuclear protein extracts using the gel shift assay. The HNF-3 promoter oligonucleotide exhibits two distinct protein-DNA complexes with liver nuclear extracts (67). The slowly migrating LF-H3β complexes are due to recognition by the C/EBP family, as demonstrated by effective competition with the Hpx site and antibody supershift studies (Fig. 3 and data not shown). The more rapidly migrating complexes are due to recognition by the HNF-3α, HNF-3β, and HNF-3γ proteins, which are competed by the TTR strong affinity HNF-3 site (HNF-3S). This sequence was previously shown to bind recombinant HNF-3 protein (2, 3). Reciprocal competition experiments using the Hpx probe show that the HNF-3β oligonucleotide effectively disrupts all but one of the complexes, whereas HNF-3S sequence does not compete for any of the bands (Fig. 3). Consistent with gel shift experiments using recombinant C/EBP protein, the TTR-3 site was not able to inhibit C/EBP complex formation with either probe in liver-derived extracts (Fig. 3). The use of the TTR-3 site as a C/EBP competitor precluded identification of the LF-H3β factor in our earlier work (67). These studies demonstrate that the LF-H3β sequence possesses strong affinity for members of the C/EBP and PAR subfamilies of the bZIP family of transcription factors.
55, 56-58, 73), we sought to determine whether the HNF-3β promoter responds to these cytokines. We transfected the HNF-3β CAT reporter constructs (Fig. 1) into HepG2 cells and examined cytokine-induced activation at several time intervals (see “Materials and Methods”). Shown in Fig. 5A is the average cytokine stimulation derived from at least three separate experiments; a representative experiment is shown in Fig. 5B. At the 5- or 14-h time point, treatment with IL-1 and IL-6 alone or in combination stimulated the 6X LFH3β reporter expression to approximately the same extent (Fig. 5, A and B). The TATA reporter plasmid lacking the C/EBP recognition sequence was not cytokine responsive, further demonstrating that the LFH3β site is mediating cytokine induction (data not shown).

Cytokine-induced activation of −184 HNF-3β promoter expression levels was comparable to those found in our C/EBP cotransfection studies (compare Figs. 4 and 5). At the 5-h time point, IL-6 treatment resulted in a 3-fold stimulation of HNF-3β promoter activity, which was slightly higher than that elicited by IL-1 alone (Fig. 5, A and B). In addition, combining these cytokines did not further enhance HNF-3 expression. At the longer time point, neither IL-1 or IL-6 alone resulted in significant stimulation of promoter expres-
sion. However, combining both of these cytokines caused a 3-fold increase in HNF-3β expression (Fig. 5, A and B). Furthermore, site-directed mutagenesis of the LF-H3β/C/EBP binding site in the –184 HNF-3β reporter inhibited cytokine stimulation (Fig. 5A). Taken together, these data suggest that prolonged activation of HNF-3β expression by a combination of cytokines is mediated through the LF-H3β site.

**Endogenous HNF-3β Expression in HepG2 Cells Is Stimulated by Combining Cytokines IL-1 and IL-6.** We next examined whether the endogenous HNF-3β gene is also stimulated by cytokines IL-1 and IL-6. RNA was isolated from HepG2 cells that were mock treated or treated for 12 h with various cytokine combinations and then evaluated for HNF-3 expression by RNase protection assay (see “Materials and Methods”). Consistent with the transfection experiments, the expression of the HNF-3β gene at 12 h of exposure was increased severalfold when IL-1 and IL-6 were combined, but only minimal activation was elicited when they were used separately (Fig. 5C). In contrast, GAPDH expression levels did not exhibit increases in response to cytokine exposure (Fig. 5C). Consistent with the studies of the HNF-3β promoter, cytokine-activated expression of the HNF-3β endogenous gene in HepG2 cells requires the combination of multiple cytokines.

**An Upstream IRF-1/ISRE Core Sequence Confers IFN-γ Inducibility to the HNF-3β Promoter.** We next searched the HNF-3β promoter region for consensus binding sites to other cytokine-inducible transcription factors. Upstream of the LF-H3β site, we found in the HNF-3β promoter region (–227 to –214; Fig. 6A) two overlapping 9-bp sequences with high homology to the ISRE core sequence (RAANB-GAAA), known to be a binding site for ISGF-2/IRF-1 (63–66). However, each of the sites is immediately followed by a base that diverges from the full ISRE consensus at a position found to be absolutely conserved among binding sites that effectively bind ISGF-3 in vitro. ISGF-3 is an IFN-β-inducible multiprotein complex containing the Stat1α, Stat1β (M, 84,000), Stat2 (M, 113,000), and a M, 48,000 DNA-binding subunit (59). IFN-γ treatment results in activation of only the Stat1α (M, 91,000; Ref. 59) protein that recognizes GAS sequences and is responsible for IFN-γ induction of IRF-1/ISRE-2 expression (74). To examine which of the IFNs activated HNF-3β transcription through the IRF-1/ISRE core sequence, we used HepG2 cells to compare activation levels of the –184 and –245 HNF-3β reporter constructs. A 12-h exposure with IFN-γ resulted in a consistent 2-fold stimulation of –245 HNF-3β expression, while no activation was observed with IFN-α (Fig. 6B and data not shown). As expected the –184 HNF-3β construct that lacked the IRF-1/ISRE sequence was not stimulated by IFN-γ alone but was activated by IL-1 and IL-6 combined (Fig. 6B). Moreover, the HNF-3β IRF-1/ISRE core sequence (–237 to –210 bp) was sufficient to confer IFN-γ stimulation to a TATA box-driven CAT reporter construct demonstrating that the IRF-1/ISRE sequence is mediating IFN-γ stimulation (Fig. 6B). Furthermore, consistent with our transfection results, IFN-γ stimulated endogenous HNF-3β expression in HepG2 cells and elicited maximum expression with 4 h of IFN-γ treatment (Fig. 6C).

**The IRF-1/ISRE Core Consensus Binds the IRF-1 Protein but not Stat1α or ISGF-3 Complexes.** To distinguish which factor is binding to the HNF-3β promoter, we exploited the differences in the activation kinetics of the IRF-1 and Stat1α proteins. The Stat1α protein preexists in a latent form and is activated within minutes of IFN-γ induction, while induced IRF-1 binding activity is dependent on new protein synthesis (62, 63). Therefore, we prepared HepG2 protein extracts at various times after IFN-γ treatment and used them for gel shift assays with an oligonucleotide containing the IRF-1/ISRE core sequence from the HNF-3 promoter (Fig. 6A; –237 to –210 bp). The IFN-γ-induced complex was not induced within 15 min, suggesting that the IFN-γ responsive factor is not the Stat1α protein (Fig. 7A). This conclusion is further supported by the fact that the Stat1α antibody did not react with the IFN-γ-induced complex (Fig. 7B).

Consistent with our IFN-α transfection studies, the HNF-3β IRF-1/ISRE core site was not recognized by IFN-α-inducible proteins, and complex formation was not further induced when both IFNs were used (Fig. 7B). The IFN-γ inducible complex did react with an IRF-1/ISGF-2-specific antiserum (Fig. 7). Furthermore, inhibitors of protein synthesis prevented IFN-γ induction of the complex, providing further evidence for IRF-1 protein recognition (Fig. 7C). These data establish that the IRF-1 protein is binding to HNF-3β promoter sequences and can mediate transcriptional activation in response to IFN-γ.

**Discussion**

The HNF-3 transcription factors play an important role in maintenance of hepatocyte differentiation as well as determination of events of other embryonic layers during development. In this study, we analyzed transient expression from HNF-3β promoter constructs in a differentiated hepatoma cell line (HepG2) to gain insights about the molecular basis for HNF-3β gene regulation in adult hepatocytes.

We show that the previously identified binding site, LF-H3β (–84 to –74 bp), is recognized and activated by members of the C/EBP and PAR subfamily of bZIP transcription factors. In addition to VBP, the PAR subfamily also includes two liver-enriched members (DBP and hepatic leukemia factor), which are likely also capable of effecting HNF-3β gene expression through the LF-H3β control element (Fig. 8).

Our studies are consistent with C/EBP protein regulation of the HNF-3β promoter in adult hepatocytes, but late embryonic expression of C/EBP makes it unlikely to be a candidate for HNF-3β induction (75). Perhaps a yet uncharacterized C/EBP family member or another cell-specific factor is responsible for embryonic induction of HNF-3β expression.

In HNF-3β promoter-transfected HepG2 cells, the LF-H3β-C/EBP site conferred a 3-fold transcriptional induction upon treatment with a combination of cytokines IL-1 and IL-6 (Figs. 2–5). This result was also paralleled by the endogenous HNF-3β gene in nontransfected cells. The induction of HNF-3β expression presented here is comparable to that observed with another acute phase transcription factor, C/EBPβ, which is also present in fairly high levels in normal liver (55). We also identified an IRF-1/ISGF-2 binding site (–227 to –214 bp), which confers transcription induction in HepG2 cells in response to IFN-γ, and this induction was recapitulated by the endogenous HNF-3β gene (Figs. 6 and 7). Exposure to both IL-1 and IL-6 provided additive stimulation of HNF-3β promoter activity, consistent with the ability of C/EBPβ and C/EBPδ proteins to interact through the formation of heterodimers (17). Combination of interleukins with IFN-γ did not elicit a stronger response, suggesting that the protein structures or locations of C/EBP and IRF-1 are not favorable for collaborative interactions in HNF-3 promoter activation. The responsiveness of C/EBPβ and C/EBPδ to IL-1/IL-6 treatment was the first example of a
Fig. 6. The HNF-3β promoter contains an IFN-γ responsive element. A, HNF-3β promoter sequence depicting -250 nucleotides upstream and a portion of the first exon included in the CAT reporter construct. Indicated on the sequence are the protein-binding sites shown in Fig. 1, which were previously identified (67), as well as an IRF-1/ISRE core binding site (62, 63, 66). B, the IRF-1/ISRE binding site confers responsiveness to IFN-γ. HNF-3β reporter CAT constructs containing (-245) or lacking (-184) the ISRE/IRF-1 site were transfected into HepG2 cells and were treated (12 h) with either IFN-γ or IFN-α (data not shown) alone or with IFN-γ, IL-1, and IL-6 together (IL + IFN-γ). Cytoplasmic protein extracts were prepared and analyzed for CAT enzyme levels. Results shown are an average from at least three separate experiments. C, endogenous HNF-3β gene expression is stimulated by IFN-γ in HepG2 cells. Show is the IFN-γ induction of HNF-3β mRNA levels in HepG2 cells evaluated by RNase protection assays at different intervals of IFN-γ treatment (24). Densimetric scans of the HNF-3β band were normalized to GAPDH levels and compared to HNF-3β expression levels in untreated HepG2 cells.
Fig. 7. The HNF-3β IRF-1/ISRE core site binds the IFN-γ-inducible IRF-1 protein but not the Stat1α M, 91,000 polypeptide. A, the HNF-3β IRF-1/ISRE binding factor is induced in HepG2 cells after 2 of IFN-γ treatment. HepG2 nuclear extracts were prepared at various time points after IFN-γ treatment (500 units/ml). Alternating lanes included 200-fold molar excess of homologous competitor DNA. B, the HNF-3 IRF-1/ISRE binding factor is not recognized by the Stat1α M, 91,000 polypeptide. Nuclear protein extracts prepared from IFN-γ treated (2 h) or untreated HepG2 cells were used for gel retardation assay with the HNF-3β ISRE/IRF-1 binding site. Lanes include homologous competition and reaction with antibody specific for the IFN-γ-inducible Stat1α M, 91,000 protein (α-91 KD). Included were gel shifts with HepG2 nuclear extracts prepared from cells treated with IFN-α (2 h) alone or together with IFN-γ (14 h). C, IRF-1 protein recognizes the HNF-3β IRF-1/ISRE site and requires protein synthesis. Gel shifts assays with HNF-3β IRF-1/ISRE site and nuclear extracts from untreated or IFN treated HepG2 cells. Lanes include homologous competition, reaction with antibody specific to the IRF-1/ISGF-2 protein (α-IRF-1; Ref. 63), and nuclear extracts made from HepG2 cells inhibited for protein synthesis with cycloheximide prior to IFN-γ stimulation.
liver-enriched transcription factor activated by cytokines. This study demonstrates that HNF-3β responsiveness is the second such example and the first to implicate IFN-γ.

Our recent studies using lipopolysaccharide injection to induce the hepatic acute phase response have demonstrated that HNF-3α expression is dramatically decreased, while the HNF-3β protein was only slightly diminished (76). The 3-fold cytokine stimulation of HNF-3β expression in HepG2 cells observed in this study at first seems inconsistent with the small in vivo decrease in HNF-3β expression. One possible explanation stems from the 95% reduction in HNF-3α expression seen during the hepatic acute phase response (76), whereas no reduction of HNF-3α is elicited by cytokines in HepG2 cells (data not shown). Site-directed mutation of the autoregulatory HNF-3 site results in an 80% decrease in HNF-3β promoter activity (67). The importance of the autoactivation mechanism in HNF-3β transcription is further supported by elegant transgenic studies in which ectopic midbrain/hindbrain expression of the HNF-3β gene resulted in activation of the endogenous HNF-3β and HNF-3α genes in these embryonic structures (46). Therefore, the effect of diminished HNF-3α levels on the HNF-3β promoter seems to be partially compensated by activation of the C/EBP family and IRF-1. The overall result is a small transcriptional decrease. Therefore, we propose that stimulatory and inhibitory pathways converge in order to maintain HNF-3β promoter activity during the acute phase response (Fig. 8).

Materials and Methods
Cell Culture and Transfections. Human hepatoma (HepG2) and HeLa cell lines were maintained and transfected as described previously (67, 77). Briefly, HepG2 cells were grown in Ham’s F-12 media supplemented with 7% FCS, 100 units/ml penicillin/streptomycin, 0.5X MEM amino acids, and 0.5 units/ml human recombinant insulin (Eli Lilly). HeLa cells were grown in DMEM supplemented with 3% FCS, 7% calf serum, 1X nonessential amino acids, penicillin/streptomycin, and insulin. Calcium phosphate transfections were formed with the N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline method under 3% CO₂ conditions using DMEM supplemented with 7% FCS as described previously (67). All tissue culture reagents were purchased from GIBCO-BRL.

Construction of Eukaryotic Liver cDNA Expression Library and Library Screening. A liver cDNA library was synthesized using an oligo(dT) primer containing a SalI site for cDNA synthesis via superscript reverse transcriptase and second-strand synthesis as described by the manufacturer (GIBCO-BRL). EcoRI methylase (New England BioLabs) was used to inactivate internal EcoRI sites in the cDNA, followed by addition of EcoRI linker ligation, digestion of cDNA with EcoRI and SalI, and size fractionation of cDNA using cDNA spinn columns (Pharmacia). The liver cDNA library was then directionally cloned into the EcoRI and SalI sites of the CMV promoter expression plasmid (77) and transformed into DH5 competent cells (GIBCO-BRL). Pools of 3000 colonies each were grown for 40 separate DNA plasmid isolations and then used to transfect HeLa cells, from which nuclear extracts were prepared 36 h later as described earlier (77). An oligonucleotide containing two copies of the LF-H3β binding site was generated (Fig. 1) and used to screen the nuclear extracts via the electrophoretic mobility gel shift assay with self-competition as described previously (67, 77). The plasmid preparation containing LF-H3β binding activity was again transformed into DH5α cells, smaller pools of colonies were grown, and the process was repeated until a single colony containing the cDNA encoding the factor binding to LFH3β was identified. DNA sequencing of the purified cDNA clone with primers for the vector adjacent to the cDNA cloning site, and subsequent comparison with the Genbank database, revealed that it encoded the C/EBPβ protein.

Plasmids, Cytokine Treatment, and CAT Enzyme Assays. The −184 and −245 HNF-3β promoter region was linked to the CAT gene (78) as described in Pani et al. (77). The 6X LFH3β-TATA-CAT construct was generated by multimerization of the 2X LFH3β oligonucleotide (Fig. 1) and subsequent cloning into the XbaI site of the TATA CAT reporter vector. The 5X IRF-1/5SRE TATA-CAT reporter was made in the same fashion using the HNF-3β −237 to −211 oligonucleotide. For each transfection, 15 μg of reporter vector, and where indicated 2 μg of expression vector, were transfected along with 10 μg of carrier pBluescript plasmid DNA using calcium phosphate precipitation. Cotransfections also included 1 μg of CMV-driven β-galactosidase to serve as an internal marker of transfection efficiency. Cytokine treatment was executed in fresh DMEM containing reduced serum (1%) FCS for 5–14 h, unless otherwise indicated in the figure legends. We used the following cytokine concentrations determined to be optimum by titration studies: 100 units/ml IL-1, 300 units/ml IL-6, 150 units/ml IFN-γ (all from Boehringer Mannheim), and 1000 units/ml IFN-α (a generous gift of Hoffman-LaRoche).

Cytoplasmic extracts were prepared from HepG2 cells 72 h after transfection via the freeze-thaw method as described previously (67, 77). Extracts were normalized for transfection efficiency or total protein content via Bio-Rad protein assay before being subjected to CAT enzyme quantitation by liquid scintillation counting of n-butylated [14C]chloramphenicol (substrates from Pharmacia and ICN, respectively; liquid scintillation counting protocol by Promega). TLC CAT assays were performed to provide a visual demonstration of CAT activity.

Fig. 8. Summary of the pathways regulating HNF-3 promoter expression. Summarized are the IRF-1/SFG-2 and C/EBP pathways that are involved in conferring cytokine stimulation of HNF-3β promoter activity in HepG2 cells. We propose that the small decrease in HNF-3β in the hepatic acute phase response may be due to the 95% reduction in HNF-3β expression, the effect of which is only partially compensated via interleukin and IFN stimulatory pathways. Because the embryonic expression pattern of C/EBP commences later than that of the HNF-3β gene, we propose that C/EBP and PAR subfamily of bZIP transcription factors regulate adult hepatocyte expression and not embryonic induction. HNF-3β gene expression may also be influenced by the PAR subfamily members DBP and hepatic leukemia factor. Therefore, we hypothesize that an earlier expressed factor may be required for endodermal induction of HNF-3β gene expression.
Electrophoretic Mobility Gel Shift Assays and Antibodies. Liver nuclear extracts were prepared as described previously (52). HepG2 cells were either mock treated or treated with IFN-γ, IFN-α, or IL-6, and nuclear extracts were prepared at the indicated time periods thereafter. Cycloheximide (50 μg/ml) was added to HepG2 cells 30 min prior to the addition of IFN-γ, and nuclear extracts were prepared 2 h thereafter. The CMV promoter-driven expression vectors containing the C/EBPα, C/EBPβ, or VBP CDNAs were transfected into HeLa cells, and protein extracts were prepared 36 h later. The histidine-tagged C/EBPβ fusion protein was expressed in Escherichia coli and affinity purified using nickel affinity chromatography (Novagen). Gel shift assay consisted of a binding reaction allowing the formation of protein-DNA complexes, which were then separated from unbound probe by native gel electrophoresis (67, 77). The binding reaction was incubated at room temperature for 30 min and consisted of a 20-μl reaction mixture containing 1 ng 32P end-labeled, double-stranded oligonucleotide, 20 mM HEPES (pH 7.9), 4% Ficoll, 2 mM MgCl2, 40 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, 4 μg poly(dI-dC):poly(dI-dC) (Pharmacia), 4 μg salmon testes DNA (Sigma Chemical Co.), and 5 μg of nuclear extract. Approximately 20–40 ng of affinity purified C/EBPβ fusion protein were used for gel shift assay. A 100–300-fold molar excess of unlabelled oligonucleotide was included in the reaction when competition experiments were performed. Oligonucleotides used for gel shift analysis of the C/EBP site are listed in Fig. 1, and for the ISGF-2/IRF-1 site consisted of the −237 to −211 bp of the HNF-3β promoter. For the supershift analyses, DNA-protein complexes were formed for 30 min, followed by the addition of antisera and further incubation for 30 min prior to gel electrophoresis. mAbs to the STAT alpha p91 subunit were the gracious gift of Dr. Xin-Yuan Fu (Mount Sinai Medical Center Department of Biochemistry, New York, NY). Polyclonal antibody to the ISGF-2/IRF-1 protein purified from HeLa cells was generated in rabbits as described (63).

RNase Protection Assays. Antisense RNA probes were generated by in vitro transcription of linearized templates using either Sp6 or T7 RNA polymerase in the presence of [32P]UTP. Antisense GAPDH RNA probe was synthesized with T7 RNA polymerase (New England Biolabs) from a Sall linearized pGEM5 template containing a Psfl and Ncol subclone from the GAPDH cDNA (33). For the HNF-3β antisense probe, a plasmid subclone of a BglII fragment of HNF-3β genomic DNA containing part of intron II and 480 bp of the third exon of rat HNF-3β was digested with Stul and in vitro transcribed with SP6 RNA polymerase. RNA was purified from HepG2 cells, coprecipitated and hybridized with the GAPDH and HNF-3β probes in separate reactions, digested with T2 RNase ( Gibco-BRL), separated on an 8% denaturing acrylamide gel and visualized by autoradiography as described (23, 24).

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References

27. DiPersio, C. M., Jackson, D. A., and Zaret, K. S. The extracellular matrix coordinates modulates liver transcription factors and hepatocyte morphol
35. Hromas, R., Moore, J., Johnston, T., Socha, C., and Klemz, M. Doro
41. Weinstei
42. Weinstei
43. Weinstei