IL-4 Suppresses the Expression and the Replication of Hepatitis B Virus in the Hepatocellular Carcinoma Cell Line Hep3B

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IL-4 Suppresses the Expression and the Replication of Hepatitis B Virus in the Hepatocellular Carcinoma Cell Line Hep3B

Sue-Jane Lin,* Pei-Yun Shu,† Chungming Chang,*‡ Ah-Kau Ng,‡§ and Cheng-po Hu‡§

IL-4 has been known as a Th2 cytokine and can act on B cells, T cells, and monocytes. In this study we demonstrate that IL-4Rs are expressed on human hepatocellular carcinoma (HCC) cells. We found that IL-4 suppresses hepatitis B surface Ag (HBsAg) mRNA and HBsAg production in the Hep3B cell line, which contains an integrated hepatitis B virus (HBV) genome and constitutively secretes HBsAg. When Hep3B cells are further transfected with the plasmid pHBV3.6 that contains >1 U of HBV genome, IL-4 could suppress the production of all HBV RNA and secreted HBsAg and hepatitis B virus e Ag. Furthermore, an endogenous DNA polymerase activity assay shows a decrease in HBV DNA after IL-4 treatment. Using luciferase reporter assays we have demonstrated that IL-4 could suppress the activity of the surface promoter II and the core promoter (CP). To delineate how IL-4 suppressed the transcription of HBV genes, we have examined the effect of IL-4 on the expression of transcription factors that are known to bind to the core upstream regulatory sequence, which colocalizes with enhancer II of the HBV genome. Our results demonstrate that IL-4 suppresses the expression of C/EBPα. Furthermore, overexpression of C/EBPα blocked 43 and 30% of the IL-4-mediated suppression of CP activity and IL-4-induced suppression of pregenomic RNA, respectively. Finally, we have demonstrated that mutations affecting the C/EBPα-binding sites on core upstream regulatory sequence/enhancer II completely abolish the IL-4-mediated suppression of CP activity. Thus, down-regulation of C/EBPα may be involved in the anti-HBV effect of IL-4 in Hep3B cells. The Journal of Immunology, 2003, 171: 4708–4716.

Cytokines are known to contribute directly or indirectly to antiviral responses (1, 2). The direct protective effects of IFNs against viruses are mediated through the downstream signals delivered by IFN receptors, which block one or more steps of the life cycle of various viruses. TNF-α has antiviral properties that are often synergistic with IFN-γ; however, its direct intracellular antiviral mechanisms are unclear. IL-12 and IL-18, on the other hand, have an indirect antiviral effect through the induction of IFN-γ after infection with HSV or HIV (3, 4). Other cytokines, such as IL-1, IL-6, and some chemokines, have been reported to have antiviral activities during viral infections (5–7). IL-4 has not been considered an antiviral cytokine; however, it has been shown to suppress PMA-induced HIV expression at the transcriptional level in monocyte U1 cells (8). The related cytokine, IL-13, has also been found to inhibit HIV production in human macrophages (9).

IL-4 is produced by Th2 cells, basophils, mast cells, and NK T (NKT)³ cells. It has a plethora of biological properties, including the up-regulation of class II MHC expression, the activation of B cells, and IgE class switching. IL-4 also plays a central role in regulating the differentiation of T cells. Moreover, it inhibits proinflammatory cytokine and chemokine production in monocytes and enhances the expression of VCAM-1 on endothelial cells. The IL-4R complex has been detected on several cell types, including B cells, T cells, monocytes, endothelial cells, and hepatocytes, a finding in keeping with the broad range of biological effects of IL-4 (10).

The hepatitis B virus (HBV) is a 3.2-kb, partially dsDNA, noncytopathic virus. Patients with a persistent infection of HBV in hepatocytes are at a high risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (11, 12). It is generally believed that the elimination of HBV is mainly mediated by CD8⁺ CTL. A balance between viral spread and the strength of the CTL response determines whether the immune responses result in the clearance of the virus or immunopathology. Substantial evidence obtained from studies in chimpanzees during acute HBV infection (13), in HBV transgenic mice following adoptive transfer of HBV-specific CTL (14), and in chronically HBV-infected patients (15) indicated that CTL may control HBV replication without the development of hepatic immunopathology (16, 17). It has also been shown that cytokines released from CTL contribute to noncytopathic antiviral activity against HBV (14). For instance, injection of IFN-α/IFN-β inducer poly (I-C) (18) or rIL-12 and rIL-18 eliminated hepatocellular HBV replicative intermediates in HBV transgenic mice (19, 20). Recombinant duck IFN-γ and IFN-α inhibited the replication of duck HBV in primary duck hepatocytes (21, 22). In addition, NKT cells, which make up for 30–50% of resident intrahepatic T lymphocytes, can rapidly produce high levels of IL-4 and IFN-γ after activation (23) and inhibit HBV replication in HBV transgenic mice (24). Thus, an antiviral effect of the noncytokytic cytokines seems to play an important role in the control of HBV in the host.

In this study we found that the IL-4R complex was expressed on all human HCC cell lines tested. To address the effect of IL-4 on...
HBV, a well-differentiated HCC cell line, Hep3B, which contains an integrated HBV genome in its chromosome and continuously produces hepatitis B surface Ag (HBsAg) (25, 26), was chosen for this study. Our results indicate that IL-4 suppresses the production of HBsAg in Hep3B as well as the replication of HBV in a transiently transfected cell system. We also demonstrate that the transcription factor C/EBPα is involved in IL-4-mediated suppression of HBV.

Materials and Methods

Cell culture and reagents

HepG2, Hep3B, HuH7, and HA22T/VGH (27) are human HCC cell lines. An EBV-transformed lymphoblastoid cell line (LCL) and a Burkitt’s lymphoma cell line (Raji) are human B cell lines. These cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% FCS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM t-glutamine, and 1% nonessential amino acids at 37°C in 5% CO₂. Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN). Lipofectamine was obtained from Life Technologies (Gaithersburg, MD). Actinomycin D and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO). 125I-labeled IL-4 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and anti-C/EBPα Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Binding of 125I-labeled IL-4 to HCC cell lines

HCC cells and Raji cells (2 × 10⁶) were resuspended in 0.2 ml of DMEM containing 1% BSA and incubated with 125I-labeled IL-4 and various concentrations (3–500 nM) of unlabeled IL-4 at 4°C for 1 h. The separation of cell-bound 125I-labeled IL-4 from unbound 125I-labeled IL-4 was by centrifugation of cells through a cushion in phthalate oils. The numbers of bound IL-4 molecule per cell and the binding affinities were determined by Scatchard plot analysis.

Measurement of HBsAg and HBeAg

Hep3B cells were seeded in 24-well plates at a concentration of 6 × 10⁴ cells/well. After overnight incubation, the culture was refed with serum-free (SF) medium for 48 h, followed by treatment with various concentrations of IL-4 (10–300 ng/ml) for another 48 h. Cell numbers were determined by trypsin blue exclusion. HBsAg and hepatitis B virus e Ag (HBeAg) in the culture medium were determined by ELISA (General Biological, Taiwan, Republic of China). The OD values were normalized with cell numbers.

Plasmids and transient transfection assay

Plasmid pHBV3.6, containing >1 U of HBV genome, was used for transient transfection experiments because the viral gene expression and the replication of pHBV3.6 are similar to HBV infection in vivo (28). To generate pSPl–Luc, pSPlII–Luc, pBCP–Luc, and pCP–Luc, the XbaI–HindIII fragments containing the surface promoter I (SPI), surface promoter II (SPlII), basal core promoter (BCP), or core promoter (CP) from pA3SPICAT, pA3SPIICAT, pA3BCPCAT, or pA3CPCAT, respectively, were inserted into the NheI–HindIII site of the pG3L-Basic vector (Promega, Madison, WI). The E4BP4 antisense plasmid pE4BP4-AS was generated by inserting the 378-bp BamHI-XhoI fragment, which corresponded to the 5' end region of E4BP4 from nt 117–494, into the BamHI-XhoI site of the pCDNA3.1 expression vector. To express C/EBPα in the expression vector, the BamHI–HindIII fragment containing the C/EBPα open reading frame from pCMV-C/EBPα was inserted into the BamHI–HindIII site of the pCDNA3.1 expression vector. pCMVβ, a β-galactosidase (β-gal) expression plasmid, was used for standardization of transfection efficiency. To destroy the C/EBPα-binding sites (underlined) on the CP (29, 30), the sequence of 5'-GTCTTACATAAAGAGGACTCTTGGACTC CGACGAAATGTCAACGAC-3' was mutated to 5'-GTCTTACATCCCG TGACTCTTTGACTCAATATAGCAGCAC-3'. The plasmid pmC/EBPα-C/EBPα-Luc was then generated by inserting the mutated CP sequence into the KpnI–HindIII site of pG3L-3Basic vector. Hep3B cells (4 × 10⁴) were transfected with 10 μg of plasmid DNA, washed three times with SF medium and incubated for 3 h with various amounts of plasmid DNA and Lipofectamine (Life Technologies, Grand Island, NY) as indicated for each experiment. The culture medium was then changed to fresh DMEM containing 10% FCS. After 18 h the transfected cells were resuspended, pooled, and reseeded at a density of 2.5 × 10⁶ cells/plate. After overnight incubation, the cells were cultured in SF for 2 days and then refreshed with SF medium with or without 150 ng/ml of IL-4 for an additional 2 days.

RNA isolation and Northern blot analysis

Total cellular RNA was extracted by the RNAzol B method (Biotechnology Laboratories, Houston, TX). RNA samples were electrophoresed on 1.2% formaldehyde agarose gels and transferred to nylon membranes (Hybond-N⁺; Amersham Pharmacia Biotech, Piscataway, NJ) by a vacuum blotter (model 785; Bio-Rad, Hercules, CA). Membranes were prehybridized at 60°C for 6 h in Quick-Hyb solution (Merck, Darmstadt, Germany) and then hybridized in the same solution by adding 2 × 10⁵ cpm/μg of 32P-labeled DNA probes prepared by random oligonucleotide priming of the whole HBV genome, IL-4R, IL-13Rα1, γc, E4BP4, C/EBPα, or a G3PDH gene fragment. After 24 h the membranes were washed with 0.2× SSC and 0.2% SDS at 60°C three times, 20 min each time, and finally autoradiographed at −70°C.

Nuclear run-on

Cells were homogenized in the buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 5 mM DTT, 0.3 M sucrose, and 0.1% Triton X-100 by a Dounce homogenizer (Kontes, Vineland, NJ). Nuclear pellets were obtained by centrifugation at 36,000 × g at 1°C for 90 min. The pellets were washed three times with the cushion buffer (50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 5 mM MgCl₂, and 0.5 mM DTT), resuspended in 40 μl of the storage buffer (50 mM Tris-HCl (pH 8.3), 40 mM (v/v) glycerol, 5 mM MgCl₂, and 1 mM EDTA), and stored at −70°C. For run-on experiments, 2 × 10⁶ nuclei were reacted in 40 μl of transcription reaction mixture (10 mM Tris-HCl (pH 8.0), 1 mM ATP, 1 mM GTP, 1 mM CTP, 10 μl of 40 U/ml RNasin (Promega), 8% glycerol, and 5 μl of [α-32P]UTP (3000 cpm/μmol)) for 30 min at 26°C. Unincorporated [α-32P]UTP was removed by a centrifugation through Ultrafree MC Centrifugal filter units (Millipore, Bedford, MA). Nuclear RNA was isolated using the RNAzol B method and isopropanol precipitation at 4°C for 30 min. RNA was treated with 10 U/ml DNase (Roche, Mannheim, Germany) for 30 min at 37°C and 100 μg/ml proteinase K (Roche) for 30 min at 37°C. The labeled RNA products were partially degraded by treatment with 1 N NaOH for 10 min at 8°C. Membranes containing denatured HBV and β-actin control DNA were prehybridized at 42°C for 6 h in the hybridization solution (30% formamide, 0.1 M PIPES (pH 6.5), 5 mM NaCl, 10% SDS, and 100 μg/ml Escherichia coli RNA) and then hybridized with equal counts per minute of nuclear RNA per membrane at 42°C for 3 days. Membranes were washed three times with 0.2× SSC and 0.2% SDS at 60°C for 20 min and finally underwent autoradiography at −70°C.

Luciferase reporter assay

Hep3B cells were cotransfected with pSPl-Luc, pSPlII-Luc, pCP-Luc, pmC/EBPα-pCP-Luc, pBCP-Luc, and pCMVβ expression plasmids. After 24 h cells were reseeded and cultured in SF medium for 2 days, then treated with IL-4 (150 ng/ml) for the indicated periods. Cells were washed twice with PBS, and the protein concentration of cell lysates was determined by the Bradford assay (Bio-Rad). Luciferase and β-gal activity in 100 μg of cell lysates were measured by the Luciferase Assay System and the β-Galactosidase Enzyme Assay System (Promega, respectively). Transfection efficiency was normalized using β-gal activity.

HBV endogenous polymerase activity assay

To dissolve the envelope of HBV particles, equal protein amounts of lysates from pHBV3.6-transfected cells or equal volumes of the culture medium were treated with TNE buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA) containing 2% Nonidet P-40. The HBV core particles containing the incomplete plus strand of HBV DNA were then immunoprecipitated with rabbit anti-HBcAg antiserum. The endogenous DNA polymerase activity in the core particles was measured by incubating the immunoprecipitates with the polymerase buffer (50 mM Tris-HCl (pH 7.5), 40 mM NH₄Cl, 10 mM MgCl₂, 1% Nonidet P-40, 2% 2-ME; 12.5 μM each of dATP, dGTP, and dTTP; and 80 μCi [α-32P]dCTP (5000 cpm/mmol)) for 3 h at 37°C. Unlabeled dCTP (12.5 μM) was added to fill the remaining gaps. After washing, 200 μg/ml of proteinase K and 0.5% SDS were added for 4 h at 37°C. The labeled HBV DNA was extracted with phenol/chloroform and subjected to 1.2% agarose gel electrophoresis and autoradiography at −70°C.

Western blotting

Cells transfected with pC/EBPα were washed twice with ice-cold HBBS and lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, 2 mM PMSF, 2 μg/ml aprotinin, 1 μM leupeptin, 1 mM Na₃VO₄, 80 mM pyrophosphate, and 50 mM NaF) on ice. The lysates were subjected to SDS-PAGE. The separated proteins were then transferred onto nitrocellulose membranes by a semidry blotter (Bio-Rad). The membrane
was soaked in 5% skimmed milk in PBS at 4°C for 16 h and then incubated with rabbit anti-C/EBPα Ab at 37°C for 1 h. After washing three times with PBS containing 0.05% Tween 20, peroxidase-conjugated goat anti-rabbit Ig was added, and the membranes were incubated in the buffer at room temperature for 1 h. The signal was visualized by the ECL system according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Results**

*Expression of IL-4R complex on HCC cell lines*

To demonstrate the expression of IL-4R complex in HCC cell lines, we first examined the mRNA expression of the IL-4R components, IL-4Rα-chain, common γ-chain (γc), and IL-13Rα1-chain, by Northern blotting. A 4.0-kb transcript of IL-4Rα (Fig. 1A) and a 4.2-kb transcript of IL-13Rα1 genes (Fig. 1B) were detectable in all HCC cell lines: HepG2, Hep3B, HuH7, and HA22T/VGH cells. An additional 2.0-kb transcript of IL-13Rα1 was present in HuH7 and HA22T/VGH cells, but was only just detectable in HepG2 and Hep3B cells. The 3.0- and 1.6-kb transcripts of γc (Fig. 1C) were also detected in the HCC cell lines, Hep3B and HA22T/VGH. The LCL and Raji cell lines expressed IL-4Rα, γc, and a very small amount of IL-13 Rα1 transcripts, as expected. We further characterized the IL-4 binding sites and binding affinities of HCC cell lines using 125I-labeled IL-4 binding assays. The numbers of IL-4 binding sites per cell and the Kd values are listed in Table I. The Kd values were between 10^-10 and 10^-11 M, and the numbers of IL-4 binding sites were 2250/HepG2, 2250/Hep3B, 1246/HuH7, and 664/HA22T/VGH cell. Similar to the results described in a previous report (31), Raji cells, a Burkitt’s lymphoma cell line, express 2952 IL-4 receptors/cell with a Kd of 3.1 × 10^-10 M. For comparison, Table I also shows the transcripts of the IL-4R components and the secretion of HBsAg in these cell lines.

**Effect of IL-4 on the production of HBsAg and HBeAg**

To study the effect of IL-4 on HBV, the production of HBsAg was measured in a well-differentiated human HCC cell line, Hep3B, which contains an integrated HBV genome and spontaneously secretes HBsAg. As shown in Fig. 2A, IL-4 suppresses the production of HBsAg in a dose-dependent manner. At the concentration of 150 ng/ml of IL-4, 71% of the HBsAg is suppressed in the culture medium. At a higher concentration (300 ng/ml) of IL-4, there is no further suppression of HBsAg production. To investigate whether the suppression was due to an effect on the secretion process, the amount of HBsAg in the cytosol was also measured. Our data indicated that IL-4 inhibited the production of both secreted and cytosolic HBsAg (Fig. 2A). To rule out the possibility that IL-4 might be acting on cellular regulatory elements upstream of the HBV-integrated site in Hep3B, the effect of IL-4 was also studied in Hep3B cells transiently transfected with a plasmid pHBV3.6, which contained >1-U length of the HBV genome and retains the ability to produce mature HBV virions (28). Thus, in pHBV3.6-transfected Hep3B cells, HBsAg can be encoded by both the integrated and the transfected HBV DNA, whereas HBeAg is only produced by pHBV3.6. Fig. 2B shows that the amount of HBeAg in these cells is also curtailed in the culture medium. These results show that IL-4 suppressed the production of HBV proteins encoded by both the integrated and the transiently transfected HBV genome.

**Effect of IL-4 on RNA expression and replication of HBV**

To investigate the mechanism by which IL-4 suppresses HBV, we examined the effect of IL-4 on the steady state level of HBV RNA using Northern blotting analysis. After IL-4 treatment, HBsAg mRNA was significantly reduced at 9, 12, 18, and 24 h (Fig. 3A). When Hep3B was further transfected with pHBV3.6, the 4.0-kb HBsAg mRNA transcribed from the integrated HBV DNA and the 3.5-kb precore/pregenomic RNA transcribed from pHBV3.6 could not be distinguished on the gel. However, it was evident that the transcripts with molecular sizes of 4.0/3.5 kb (HBsAg mRNA and precore/pregenomic RNA) and 2.4/2.1 kb (large HBsAg mRNA and middle/major HBsAg mRNA) were significantly suppressed by IL-4 over the various time periods studied (Fig. 3B). In addition, we measured endogenous DNA polymerase activity. The HBV core particles in the culture supernatant or in the cytoplasm of Hep3B transfected with pHBV3.6 were isolated and reacted with [α-32P]dCTP. As shown in Fig. 3C, both the nicked circular and the linear forms of HBV DNA dramatically decreased after treatment with IL-4 for 12, 24, and 36 h. These results clearly show that IL-4 suppressed HBV replication.

**Effect of IL-4 on the stability of HBsAg mRNA**

To test whether IL-4 affected the stability of HBsAg mRNA, the RNA samples obtained from actinomycin D-treated and untreated Hep3B cells were subjected to Northern blotting analysis. As shown in Fig. 4, IL-4 suppressed the expression of 4.0-kb HBsAg mRNA of untreated cells as expected. Actinomycin D alone also suppressed HBsAg mRNA; however, IL-4 did not affect the amount of HBsAg mRNA in actinomycin D-treated cells. Thus, the suppression of HBsAg mRNA production by IL-4 was not due to an effect on the stability of mRNA.

**Effect of IL-4 on the transcriptional rate of the HBsAg gene**

To test whether IL-4 affected the transcriptional rate of HBV genes, the newly synthesized transcripts were detected by a nuclear run-on experiment. There were ~52 and 56% decreases in the transcriptional rate of the HBsAg gene detected in IL-4-treated nuclei at 12 and 24 h, respectively (Fig. 5A). In addition, the transcriptional rate of HBV genes in Hep3B cells transfected with pHBV3.6 was significantly reduced after IL-4 treatment (data not shown). These results indicate that IL-4 may inhibit the transcription of HBV genes at the transcriptional level.
Independent experiments were repeated three times and showed similar results.

Effect of IL-4 on the promoter activity of HBV genes

To further investigate the mechanism by which the transcriptional suppression of IL-4 acts on HBV genes, we measured the effect of IL-4 on the activity of SPI, SPII, BCP, and CP, which makes up the BCP and the core upstream regulatory sequence (CURS). Using luciferase activity assays, we found that IL-4 significantly suppressed SPII and CP activity. As shown in Fig. 5B, IL-4 suppressed 51, 56, and 75% of the SPII activity at 12, 24, and 48 h, respectively. Similarly, 26, 47, and 74% suppressions of CP activity were seen at 12, 24, and 48 h after IL-4 treatment, respectively (Fig. 5C). In contrast, luciferase activity driven by SPI was not significantly affected (data not shown). We also found that IL-4 did not affect the activity of BCP (data not shown). Thus, IL-4 may exert its suppressive effect on CP activity through the regulation of CURS.

Suppression of HBsAg by IL-4 required de novo protein synthesis

Since the suppression of HBsAg mRNA was detected after 9 h, we examined whether de novo protein synthesis was required for the inhibitory effect of IL-4. Fig. 6 shows that IL-4 only suppressed 38% of HBsAg mRNA in cycloheximide-treated cells compared with a 93% reduction in untreated Hep3B cells, suggesting that de novo protein synthesis is required for the suppressive effect of IL-4 on HBsAg mRNA.

Effect of IL-4 on the expression of E4BP4, C/EBPα, and C/EBPβ

The CURS is colocalized with enhancer II (ENII) in the HBV genome. Thus, the sequence of CURS/ENII not only regulates CP activity and thus the expression of pregenomic RNA and precore mRNA, but it also enhances the activity of the SPI, SPII, and HBx

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### Table I. The binding sites, binding affinities, and presence of each IL-4R transcript in human HCC cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>HBV Genome</th>
<th>Receptors/Cell</th>
<th>K_a (M)</th>
<th>IL-4 Rα</th>
<th>IL-13 Rα</th>
<th>γc</th>
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<td>+</td>
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<tr>
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<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HuH7</td>
<td>-</td>
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<td>1.2 × 10^{-11}</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HA22T/VGH</td>
<td>+^b</td>
<td>664</td>
<td>3.0 × 10^{-10}</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a Hep3B contains an integrated HBV genome in its chromosome and only expresses HBsAg.

^b HA22T/VGH contains an HBV genome in its chromosome and does not produce any HBV Ags.

### FIGURE 2. IL-4 suppresses the production of HBsAg and HBeAg in Hep3B cells. A, HBsAg produced by Hep3B cells. Cells cultured in SF medium were treated with or without IL-4 (10, 40, 60, 150, and 300 ng/ml) for 2 days. The amount of HBsAg in the supernatants (○) or the cell lysates (□) was measured by ELISA. B, HBeAg produced by Hep3B cells transfected with pHBV3.6 DNA (7.5 μg) was transfected into Hep3B cells. After 18 h, the cells were pooled, reseeded, and cultured in SF medium for 2 days. IL-4 (150 ng/ml) was added to the culture for additional 2 days. The supernatant was collected, and the amount of HBsAg was measured by ELISA. The OD values of HBsAg and HBeAg were normalized with cell numbers and calculated as the mean of triplicate wells ± SD. The results are expressed as a percentage of the SF control. Independent experiments were repeated three times and showed similar results.

### FIGURE 3. IL-4 suppresses gene expression and replication of HBV. A. Northern blotting of HBsAg mRNA. Hep3B cells cultured in SF medium were treated with or without IL-4 (150 ng/ml) for 2 days. Total cellular RNA was extracted at the indicated times. Thirty micrograms of RNA were loaded in each lane and hybridized with the full-length HBV DNA. B. Northern blotting of HBV transcripts in Hep3B cells transfected with pHBV3.6. Hep3B cells were transfected and treated with IL-4 as described in Fig. 2B. Total cellular RNA was extracted at the indicated times, and Northern blotting was performed. The same membranes were rehybridized with G3PDH DNA fragment to ensure an equal amount of RNA in each lane. C. HBV endogenous polymerase activity assay. Hep3B cells were transfected with pHBV3.6 and treated with IL-4 as in B. Equal protein amounts of lysates or equal volumes of the culture medium were solubilized with Nonidet P-40. The core particles were immunoprecipitated with a rabbit anti-HBcAg antiserum at the indicated times and assayed for HBV endogenous polymerase activity. The nicked circular (NC) and linear (L) forms of HBV DNA are indicated.
promoters (32). Therefore, we focused our study on the mechanism by which IL-4 affects CP activity. It has been reported that C/EBPα, C/EBPβ, E4BP4, and HLF bind to box α within the CURS/ENII region and regulate the transcription of HBV genes (29, 33). To address whether IL-4 could regulate the expression of these transcription factors in Hep3B cells, the mRNA level of these factors was measured by Northern blotting analysis after IL-4 treatment. We found that E4BP4 mRNA was increased 3-fold, and C/EBPα mRNA was decreased 38% at 6 h after treatment with IL-4 (Fig. 7A). However, no difference could be seen after 9 h, indicating a transient regulation of these genes by IL-4. In contrast, the amount of C/EBPβ mRNA was not altered after treatment with IL-4, and the expression of HLF in Hep3B cells was below the detection level for RT-PCR of the gene (data not shown).

**E4BP4 is not involved in IL-4-mediated suppression of HBV RNA**

E4BP4 has been reported to be a negative regulator for the activity of CURS/ENII. However, its function on HBV gene expression is controversial (29, 33). To test the involvement of E4BP4 in IL-4-mediated suppression, E4BP4 antisense plasmid (pE4BP4-AS) was introduced into Hep3B cells, and CP activity and HBV RNA were measured. Our results showed no significant difference in IL-4-mediated suppression of CP activity and HBV RNA between cells transfected with pE4BP4-AS and those effects when transfected with the control plasmid pCDNA3.1 (data not shown). To further confirm whether E4BP4 plays a role in IL-4-mediated suppression, an E4BP4-overexpressed system was used. Northern blotting of HBV RNA in cells transfected with pCDNA3.1 or pE4BP4 in the absence or the presence of IL-4 is shown in Fig. 7B. After normalization of the amount of RNA using the G3PDH gene and β-gal activity, the overexpressed E4BP4 did not further reduce the amount of HBV RNA in the presence of IL-4. As shown in Fig. 7C, IL-4 suppressed 87% of 4.0/3.5-kb RNA and 77% of 2.4/2.1-kb RNA in pCDNA3.1-transfected cells, as expected. In E4BP4-overexpressed cells, IL-4-mediated suppression is not higher than that in control cells, i.e., IL-4 suppresses 79% of 4.0/3.5-kb RNA and 54% of 2.4/2.1-kb RNA. Thus, E4BP4 does not seem to play a role in IL-4-mediated suppression of HBV RNA.

**C/EBPα is involved in IL-4-mediated suppression of CP activity and HBV gene expression**

To test whether C/EBPα was involved in the regulation of CP activity by IL-4, we overexpressed C/EBPα in IL-4-treated and untreated Hep3B cells. While IL-4 could suppress 28.4% of the CP activity in pCDNA3.1-transfected cells, it only suppressed 16.0% of the CP activity in cells transfected with pCEBPα. Thus, overexpression of C/EBPα blocked 43% of the IL-4-induced suppression of CP activity (Fig. 8A). Fig. 8B shows the overexpression of C/EBPα in Hep3B cells, as demonstrated by anti-C/EBPα Ab. Fig. 8C shows the Northern blotting of HBV RNA in cells transfected with pCDNA3.1 or pCEBPα in the absence or the presence of IL-4. After normalization of the amount of RNA with G3PDH gene and β-gal activity, the relative levels of RNA are shown in Fig. 8D. Under SF conditions, the overexpression of C/EBPα was enhanced by 1.8-fold for the 4.0/3.5-kb RNA, but had no effect on the 2.4/2.1-kb mRNA (Fig. 8D, compare the two SF lanes), suggesting that C/EBPα, a transcriptional activator for HBV, activates...
the expression of 4.0/3.5-kb RNA, but not 2.4/2.1-kb RNA. In the presence of IL-4, 78% of 4.0/3.5-kb RNA was suppressed in pCDNA3.1-transfected cells, while only 55% was suppressed in C/EBPα-overexpressed cells. Thus, the overexpression of C/EBPα blocks 30% of the IL-4-induced suppressive effect of 4.0/3.5-kb RNA (78 vs 55%). On the contrary, the degree of suppression of the 2.4/2.1-kb mRNA was similar in control and C/EBPα-overexpressed cells (69 vs 72%) after treatment with IL-4. Taken together, C/EBPα seems to be involved in IL-4 suppression of the production of the 4.0/3.5-kb RNA, but not of the 2.4/2.1-kb RNA.

C/EBPα binding sites are crucial to the effect of IL-4

To further confirm the role of C/EBPα in IL-4-mediated suppression, the sequences of the two C/EBPα-binding sites on the CURS/ENII were mutated. The nucleotide sequence from nt 1649–1692 and the binding sites for the transcriptional factors are shown in the upper part of Fig. 9. Mutations of C/EBPα binding sites 1 and 2 resulted in a 6.7-fold decrease in CP activity (compare the two SF lanes in the lower panel). This indicates that the C/EBPα binding sites are important for the activity of CURS/ENII. In addition, while IL-4 could suppress 27% of the pCP-Luc activity, IL-4 did not have any suppressive effect on the activity of pmC/EBPα-CP-Luc. Thus, our results demonstrated that the C/EBPα binding sites of the CP are crucial for the IL-4-suppressive effect.

Discussion

IL-4, a cytokine produced by Th2 cells, NKT cells, basophils, and mast cells, displays a wide array of immunological functions, ranging from Th2 development, lymphocyte proliferation, and up-regulation of class II MHC expression to IgE class switch (10); it also has a role in asthma, allergy, and resistance to intestinal nematode infection (34, 35). Moreover, IL-4 has been shown to inhibit lipogenesis stimulated by TNF-α, IL-1, and IL-6 in mouse hepatocytes (36) and fibrinogen biosynthesis in the human HCC cell line HepG2 (37). In primary human hepatocytes, IL-4 could also induce the expression of cytochrome P-450 and GSTα (38, 39). Furthermore, it inhibits the production of haptoglobin, albumin, and C-reactive protein (40), but enhances the stimulatory effect of IL-1β on the production of the IL-1R antagonist in human hepatocytes (41). In exploring the effect of exogenous IL-4 on Hep3B cells and their transient transfectants, we discovered that the cytokine also had a suppressive effect on HBV gene expression. Thus, IL-4 appears to play an important, yet complex, role in regulating the expression of viral genes and in the innate defense mechanism in liver.

The IL-4R complex has been characterized in various cell types. The type I receptor, which consists of IL-4Rα-chain and γc, is expressed on both T and B cells. The type II receptor, which consists of IL-4Rα and IL-13Rα1-chains, is found on endothelial cells, and most carcinoma cells, such as ovarian carcinoma and colon carcinoma (42, 43). In our study although all the HCC cell lines tested expressed the IL-4Rα and IL-13Rα1-chains, the γc were also detected on Hep3B and HA22T/VGH cells. Although with IL-4 as described, and Northern blotting analysis was performed. The β-gal activity and the intensities of the 4.0/3.5-kb, 2.4/2.1-kb, and G3PDH transcripts of each sample are listed at the bottom of B. Relative expression levels of 4.0/3.5-kb and 2.4/2.1-kb HBV RNA are shown in C. The relative level of RNA represents the ratio of the intensity of the 4.0/3.5-kb RNA (■) or the 2.4/2.1-kb RNA (□) to that of the G3PDH transcript and then normalized with the β-gal activity of each sample. % ▼, The percent decrease in the relative RNA level in cells cultured in the presence of IL-4.

Similar results were obtained in three independent experiments.

FIGURE 7. E4BP4 is not involved in the IL-4-mediated suppression of HBV gene expression. A, Northern blotting analysis of E4BP4 and C/EBPα genes. Total cellular RNA of Hep3B cells was prepared as described in Fig. 3. The ratios of E4BP4/G3PDH and C/EBPα/G3PDH represent the relative intensity of E4BP4 or C/EBPα. B, Hep3B cells were cotransfected with pHBV3.6, pCMVβ, and pCDNA3.1, pE4BP4. After 18 h, the pE4BP4-transfected cells were pooled, reseeded, and cultured in SF medium for 18 h. Cells were cultured in SF medium or were treated
most carcinoma cells do not express the \( \gamma \), it has been shown that IL-4R, IL-13R, and \( \gamma \) transcripts are expressed in HT-29 carcinoma cells (44). In addition, the \( \gamma \) has been shown to be a subunit of the IL-4R complex in HT-29 cells using a labeled IL-4 cross-linking experiment (45). It would be interesting to investigate further the expression and the functional significance of \( \gamma \) in Hep3B and HA22T/VGH cells or their clinical counterparts.

Hep3B, a well-differentiated human HCC cell line, contains an integrated HBV genome in its chromosome and continuously produces HBsAg (25, 26). In this study we clearly demonstrated that C/EBP is involved in the IL-4-mediated suppression of CP activity and HBV gene expression. A. Effect of pC/EBP on the IL-4-mediated suppression of pCP-Luc activity. Hep3B cells were cotransfected with pCMV, pCDNA3.1, and pC/EBPα. The transfected cells were cultured in SF medium or were treated with IL-4. The same amounts of cell extracts were measured for luciferase activity at 12 h. Relative luciferase activity of pCP-Luc was normalized with β-gal activity. The effect of IL-4 on the activity of pCP-Luc or pmC/EBPα-CP-Luc is determined as the percent decrease or increase in activity after treatment of IL-4.

FIGURE 8. C/EBPα is involved in the IL-4-mediated suppression of CP activity and HBV gene expression. A. Effect of pC/EBPα on the IL-4-mediated suppression of pCP-Luc activity. Hep3B cells were cotransfected with pCP-Luc, pCMVβ, and pCDNA3.1 or pC/EBPα. The transfected cells were cultured in SF medium or were treated with IL-4. The same amounts of cell extracts were measured for luciferase activity at 12 h. Relative luciferase activity of pCP-Luc was normalized with β-gal activity. The percentage of IL-4-mediated suppression of pCP-Luc activity is represented by the percent decrease in pCP-Luc activity in Hep3B cells cultured in the presence of IL-4. Blocking of IL-4-mediated suppression after overexpression of pC/EBPα. A, Western blotting of C/EBPα. Cells were transfected with pC/EBPα as described above, and cell lysates were collected after 2 days. Western blotting was performed using anti-C/EBPα Ab and goat anti-rabbit Ig and visualized by the ECL system. B, Northern blotting analysis of HBV genes. Hep3B cells were cotransfected with pHBV3.6, pCMVβ, pCDNA3.1, and pC/EBPα. The transfected cells were cultured in SF medium or treated with IL-4 as described, and Northern blotting analysis was performed. The β-gal activity and the intensities of 4.0/3.5-kb, 2.4/2.1-kb, and G3PDH transcripts of each sample are listed at the bottom of C. Relative expression levels of 4.0/3.5-kb and 2.4/2.1-kb HBV RNA are shown in D. The relative level of RNA represents the ratio of the intensity of the 4.0/3.5-kb RNA (f) or the 2.4/2.1-kb RNA (g) to the G3PDH transcript and then normalized with the β-gal activity of each sample. % ↓. The percent decrease in the relative RNA level in cells cultured in the presence of IL-4. Similar results were obtained in four independent experiments. The difference in the IL-4-mediated suppression of 4.0/3.5-kb RNA between pCDNA3.1- and pC/EBPα-transfected cells (D; ) is statistically significant (*, \( p < 0.05 \), t test.)
IL-4 could bind to its receptor on Hep3B cells with high affinity, leading to a suppression of HBV gene expression and replication. The Hep3B cell line thus presents a suitable cell model for investigating the biochemical mechanisms underlying the effect of IL-4 on HBV expression in host cells.

Previously, IL-2, IFN-α/β, TNF-α, and IFN-γ have been shown to down-regulate HBV genes in transgenic mice through post-transcriptional regulation (46–48). IFN-γ could also inhibit the expression level of HBV RNA in an immortalized hepatocyte cell line (HBV-MET), especially when it acts synergistically with TNF-α. These results suggested that IFN-γ and TNF-α could directly affect HBV replication in hepatocytes (49). In our study IL-4 could directly suppress the expression of the 4.0-kb HBsAg mRNA and the HBsAg in Hep3B cells. It could also suppress the 2.4- and 2.1-kb HBsAg mRNA and the 3.5-kb pregenomic RNA in Hep3B cells transfected with pHBV3.6. The 3.5-kb precore mRNA, which encodes the HBsAg, also appeared to be suppressed by IL-4, since the production of HBsAg was decreased in IL-4-treated Hep3B cells transfected with pHBV3.6. Presumably, IL-4 suppressed the production of the 3.5-kb pregenomic RNA, which not only encoded the core and polymerase proteins, but also served as a template for reverse transcription, and this resulted in the inhibition of HBV replication. We further demonstrated that IL-4 could suppress the promoter activity of SPII and CP on HBV. In the HBV genome, the CP consists of BCP and CURS that are colocalized with the ENII that regulates the activities of all promoters. Thus, CURS/ENII is one of the most important regulatory elements operating to control HBV gene transcription.

It has been demonstrated that IL-4 induced the expression of E4BP4 in mouse B cells (50). In this study we showed that IL-4 enhanced the mRNA level of E4BP4 in Hep3B cells. However, E4BP4 appears to not be involved in IL-4-mediated suppression of CP activity and HBV transcripts, as demonstrated by a series of experiments involving antisense E4BP4 and overexpressed E4BP4. Interestingly, overexpressed E4BP4 even slightly increased the expression of the 2.4/2.1-kb mRNA and decreased the suppressive effect of IL-4 on the 2.4/2.1-kb mRNA. E4BP4 has been reported to be a negative regulator for CURS/ENII activity, but its function in HBV gene expression is controversial (29, 33). While overexpression of E4BP4 completely abolishes the suppression of HBV RNA in HuH7 cells in one study (29), E4BP4 does not have any effect in another study (33). It is not clear whether E4BP4 can regulate the expression of 2.4/2.1-kb mRNA through an unknown mechanism.

C/EBPα is a transcription factor found in the liver at higher levels and serves as an activator of the CURS/ENII. Our results indicated that IL-4 decreased the mRNA level of C/EBPα, and overexpression of C/EBPα blocked 43% of IL-4’s effect on CP activity. Thus, IL-4-mediated suppression of CP activity may involve a decrease in the amount of C/EBPα. To confirm the role of C/EBPα, we demonstrated that C/EBPα binding sites on CURS/ENII are necessary for IL-4-mediated suppression of CP activity. The overexpression of C/EBPα also resulted in a 30% suppression of 4.0/3.5-kb HBV RNA, further indicating the involvement of C/EBPα in the regulation of HBV expression. Whether other transcription factors are also involved needs further investigation. In contrast, C/EBPα does not seem to be involved in IL-4-mediated suppression of the 2.4/2.1-kb HBsAg mRNA. Other factors must be responsible for IL-4-mediated suppression of SPII activity and hence the changed expression of the 2.4/2.1-kb HBsAg mRNA. Finally, the inhibitory effect of IL-4 on replication of HBV seems to be more dramatic than its effect on RNA expression. Therefore, other mechanisms may also contribute to the IL-4-mediated suppressive effect, for example, post-transcriptional regulation (51), an effect on core particle encapsidation, the prevention of nucleocapsid assembly, or an acceleration of nucleocapsid degradation (18).

The source of IL-4 in the liver may be T cells and/or NKT cells. A previous study indicated that most CD4+ and CD8+ intrahepatic T cell clones isolated from chronically HBV-infected patients produce IFN-γ and IL-4 (52). Furthermore, Schistosoma mansoni-induced Th1 and Th2 cytokines inhibited HBV replication in HBV transgenic mice, but this parasite-induced antiviral effect was only partially blocked in IFN-γ-deficient mice (53), implying an antiviral role played by other cytokines. In the normal liver, NKT cells account for 30–50% of resident intrahepatic lymphocytes, and their role in immunity has been a focus of research. Interestingly, when the intrahepatic NKT cells were activated by α-galactosylceramide-presenting hepatocytes, they predominately produced IL-4 (54). Moreover, recent studies have shown that α-galactosylceramide-activated NKT cells can inhibit HBV replication in HBV transgenic mice (24). IL-4 production by liver NKT cells, therefore, may play a role in the host defense against microbial infections of the liver, and its clinical significance remains to be further explored.

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References

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