

Human Hepatitis B Virus Enhancer 1 is Responsive to Human Interleukin-6

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Serum levels of interleukin-6 (IL-6) are elevated in acute and chronic hepatitis B patients. The effect of IL-6 and its transcription factor of NF-IL6 (a nuclear factor for IL-6) on hepatitis B virus (HBV) enhancer 1 (Enh1), which controls HBV X expression, were investigated in HepG2 cells. Twenty ng/ml of IL-6 increased 4-fold the enhancer activity of Enh1 according to the CAT assay. The IL-6 stimulation was abolished by introducing a mutation either in an AP-1-related site or a C-stretch sequence in the Enh1 sequence, demonstrating that the *cis*-elements are necessary for the IL-6 response. Co-transfection of NF-IL6 expression plasmid similarly increased the enhancer activity of Enh1 through both binding sites. Further, a specific complex formation of the Enh1 was detected using HepG2 nuclear lysates by electromobility shift assays, and the complex formation was increased in the lysates of cells treated with IL-6 and NF-IL6-transfection. In competition assays, one half of the complex formed was found to remain in the presence of 500-times excess competitor DNA fragment harboring NF-IL2 binding site, suggesting indirect binding of NF-IL6 to the Enh1 sequence. These results indicate that IL-6 increased the enhancer activity of HBV Enh1 through signal transduction pathways, indirectly involving NF-IL6, and may control HBV X expression and viral replication in HBV infected liver. *J. Med. Virol.* 52:413–418, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: HBV; IL-6; NF-IL6; HBx; enhancer activity

INTRODUCTION

Human hepatitis B virus (HBV), a member of *Hepadnaviridae*, encodes the X gene, as well as the surface, core, and polymerase genes in a compact genome [Miller et al., 1989]. The HBV X gene is conserved among mammalian hepadnaviruses [Faktor et al., 1990; Kodama et al., 1985; Mahe et al., 1991] and is essential for viral replication in the natural host [Chen

et al., 1993]. HBV X protein (HBx) transactivates many viruses and host genes through a wide variety of *cis*-elements [Aufiero et al., 1990; Avantaggiati et al., 1992; Lucito et al., 1992; Mahe et al., 1991; Seto et al., 1990; Twu et al., 1993]; moreover, HBx demonstrates oncogenic potential in a transgenic model [Kim et al., 1991]. Expression of HBV X gene is controlled by the HBV enhancer 1 (Enh1) [Dikstein et al., 1990; Guo et al., 1991] which is responsive to HBx [Murakami et al., 1994a; Murakami et al., 1994b; Murakami et al., 1990], meaning that HBx expression is autoregulated.

The Enh1 consists of multiple protein binding sites. The Enh1 core sequence, overlapping with E-element [Faktor et al., 1990], harbors an AP-1 related site (cFAP-1) [Gutman et al., 1991] and a C-stretch sequence [Murakami et al., 1994a; Murakami et al., 1990], which has been reported to respond to various stimuli. Extracellular stimuli of inflammation and tumor-promotion, such as phorbol 12-myristate 13 acetate (TPA), has been shown to activate Enh1 through the cFAP-1 site [Murakami et al., 1994a]. Human interleukin-6 (IL-6) is one of the major acute inflammatory cytokines and has pleiotropic functions, including cell proliferation and induction of acute response genes, such as those of fibrinogen and C-reactive protein, in hepatocytes [Akira et al., 1990; Akira et al., 1992; Kishimoto et al., 1994]. Serum IL-6 levels have been reported to be elevated in patients infected with HBV and are believed to serve an important role in the progression of hepatitis [Devergne et al., 1991; Kakumu et al., 1992; Kakumu et al., 1991; Torre et al., 1994].

To elucidate the effect of IL-6 on HBV Enh1, we examined whether IL-6 and its transcription factor of NF-IL6 [Akira et al., 1990] affect the enhancer activity of the Enh1 core. It was found that the Enh1 enhancer activity was elevated by addition of IL-6, probably through activation of NF-IL6. These results suggest that serum IL-6 increases transcription of HBx gene by

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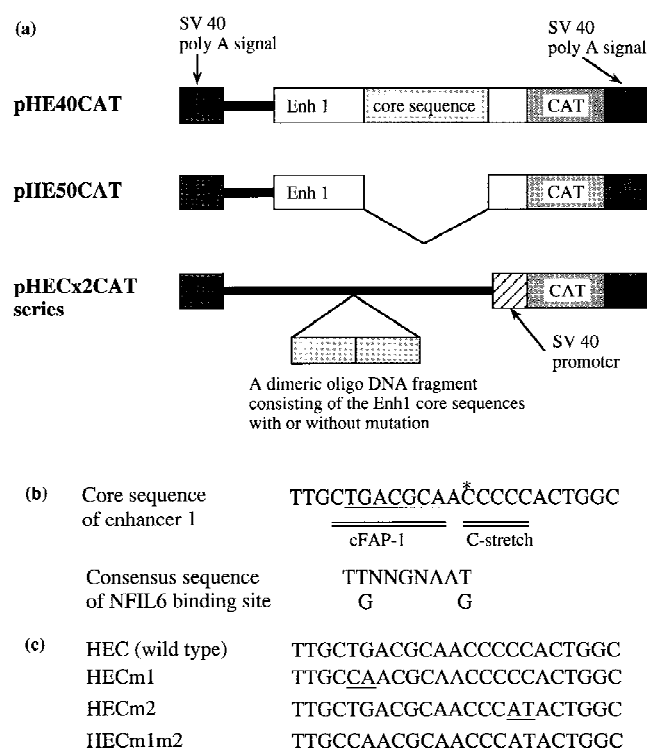


Fig. 1. (a): Illustrated are CAT reporter plasmid constructs. pHE50CAT and pHE40CAT consist of Enh1—X promoter (*DraI-NcoI* fragment), with or without internal deletion of a 51-bp region that includes 23 bp of the Enh1 core, respectively. pHECx2CAT consists of a dimeric oligo DNA fragment of Enh1 core and pUAP1CAT containing SV40 promoter. [Sequences of oligo DNA fragments are illustrated in Figure 1(c).] (b): A 23 bp of the Enh1 core has a sequence similar to a putative NF-IL6 binding site. Underlined letters indicate a sequence similar to a putative NF-IL6 binding site in the Enh1 core sequence, and double underlines indicate the cFAP-1 site and the C-stretch sequence. *indicates one base mismatch to the consensus sequence of the NF-IL6 binding site. (c): The oligo DNA sequences of the Enh1 core with or without substituted bases are underlined.

activating Enh1 and may stimulate HBV replication in the infected liver.

MATERIALS AND METHODS

Plasmids Constructions for Mammalian Expression Vectors

Plasmids, pSGHX-1 [Mahe et al., 1991; Murakami et al., 1994a; Murakami et al., 1990], and CMVNF-IL6 [Akira et al., 1990] were used for HBV X and human NF-IL6 gene expression vectors, respectively. A DNA fragment encompassing HBV Enh1 and the X promoter was inserted into pUAP1CAT [Murakami et al., 1994a] and designated as pHE40CAT (Fig. 1). pHE50CAT is deleted of a 51 bp-DNA fragment of the Enh1 core sequence from pHE40CAT [Murakami et al., 1994a]. A series of pHECx2CAT plasmids were made by inserting a dimerized 23 bp-DNA fragment in the center of the enhancer region with or without substituted bases (Fig. 1) [Murakami et al., 1994a]. pHECx2CAT had wild type sequences of the Enh1 core.

DNA Transfection and CAT Assay

Transient transfection of plasmids and successive CAT assay were performed as described previously

[Gorman et al., 1982; Mahe et al., 1991]. HepG2 cells were maintained in modified Dulbecco medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% fetal calf serum (GIBCO BRL, Life Technologies Inc., NY, USA) in a 60 mm culture dish (Corning Coaster Co., Ltd. Cambridge, MA). The transfected cells were treated without or with phorbol 12-myristate 13 acetate (TPA) (Sigma Chemical Company, St. Louis, MO: 50 ng/ml) or human recombinant interleukin-6 (hIL-6) (kindly given by Kouji Matsushima, Kanazawa University, Japan) for 8 hr before cell harvesting. The CAT assay was performed by 30 min incubation at 37°C using 20 µg protein of cell lysate. CAT activities were measured from the conversion rate of ¹⁴C-chloramphenicol to acetylated forms with a Bio-image analyzer BA1000 MacBas (Fuji photo Film Co., Ltd., Tokyo, Japan) [Murakami et al., 1994a]. Transfection and CAT assays were repeated at least 3 times.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts of HepG2 cells at the sub-confluent stage were prepared according to previous reports [Digman et al., 1983; Murakami et al., 1990]. The concentration of protein was determined by the method of Bradford [1976]. Electrophoretic mobility shift assays (EMSA) were carried out as described previously [Murakami et al., 1990]. A dimerized DNA fragment encoding the Enh1 core (5'-TTGCTGACGCAACCCCCACTGGC-3') was 5'-³²P-labeled with T4 polynucleotide kinase and used as the probe. Binding reactions were carried out in 10 µl volume for 30 min at 30°C. Competitor oligo DNA fragments were added to the reaction mixture 15 min before loading. Dimerized oligo competitor DNA fragments were made for the nuclear factor for IL-6 (NF-IL6) binding site (NF-IL6BS: 5'-AGATTGTGCAATCT-3') [Akira et al., 1990] and hepatocyte nuclear factor 1 binding site (HNF-1BS: 5'-GAAGGTTACTAGTTAACA-3'). The reaction mixtures contained 5 fmole of 5' end-labeled probe (1 × 10⁴ cpm), 1 µg of poly (dI-dC) double-stranded heteropolymer (Pharmacia Pharmaceuticals Uppsala, Uppsala, Sweden) in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9), 50 mM KCl, 7 mM MgCl₂, 2 mM dithiothreitol, and 10% glycerol. Protein-DNA complex formations were resolved on 6% (29:1 cross-linked) native gel in x ¼ TBE at 4°C. After electrophoresis, gels were dried and analyzed using a Bio-image analyzer BA1000 MacBas (Fuji photo Film Co., Ltd., Tokyo, Japan) [Murakami et al., 1990].

RESULTS

Enhancer Activity of Enh1 and hIL-6

IL-6 is known as one of the most important cytokines in the immune response and is increased in several physiological responses, such as the acute-phase response in hepatocytes, through transcriptional activation of the genes [Akira et al., 1992; Kishimoto et al., 1994]. We analyzed whether hIL-6 controls the enhancer activity of HBV Enh1. The number of cells did

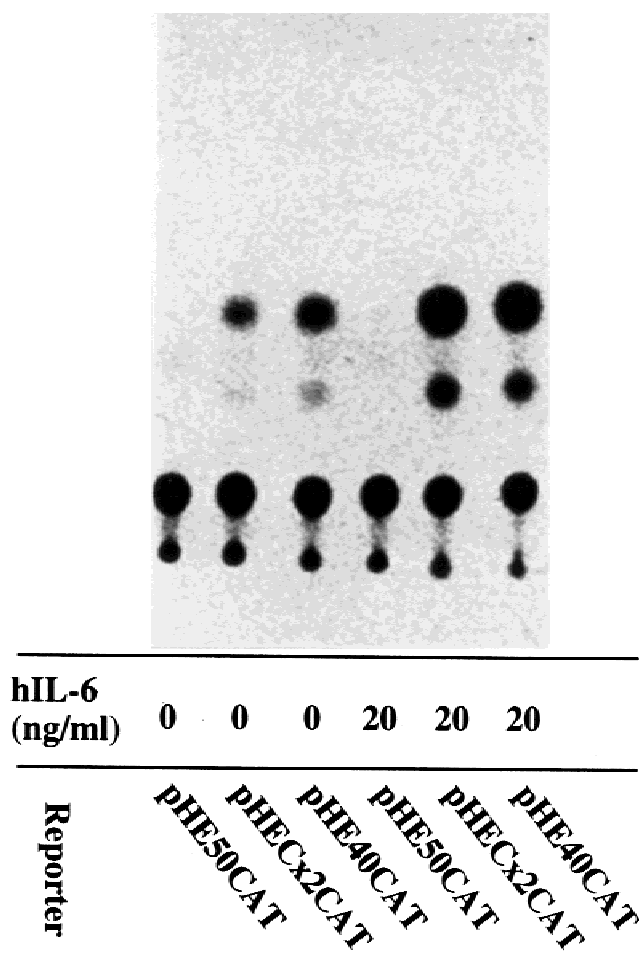


Fig. 2. IL-6 activates enhancer activities of HBV Enh1 and Enh1 core. HepG2 cells were transfected with the CAT reporters (5 μ g/plate) as indicated and were either not treated or stimulated by recombinant human IL-6 (hIL-6 (20 ng/ml)) for 8 hours before cells were harvested.

not differ significantly with IL-6 treatment for 8 hours. HepG2 cells were transfected with the CAT reporter plasmid of pHE40CAT, containing a fragment from Enh1 to the X promoter region of HBV with or without hIL-6 stimulation. CAT activities in hIL-6 treated HepG2 cells were 4 times stronger than in non-stimulated cells (Fig. 2). hIL-2 also increased enhancer activity by 6.3 times the enhancer activity when HepG2 cells were transfected with pHECxCAT having a dimer of the 23 bp of the Enh1 core sequence. hIL-6 did not stimulate the enhancer activity of pHE50CAT which does not contain the core sequence (Fig. 2). Thus hIL-6 increases the enhancer activity of Enh1 through the core sequence.

cFAP-1 Site and C-Stretch Sequence and hIL-6 Response

The core sequence of HBV Enh1 includes a cFAP-1 site and a C-stretch sequence [Murakami et al., 1990]. The cFAP-1 site is demonstrated to be essential for

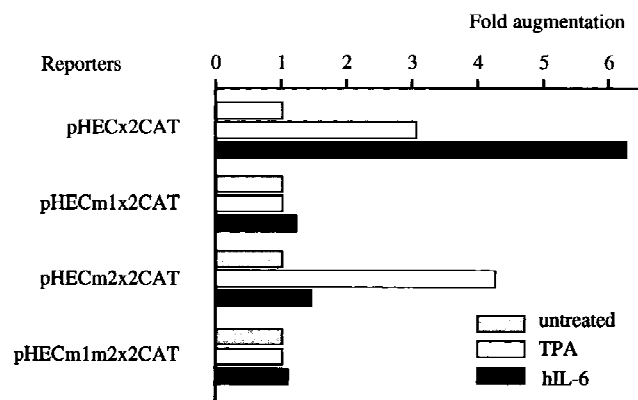


Fig. 3. Both the cFAP-1 site and the C-stretch sequence in the Enh1 core were necessary for IL-6 augmentation. HepG2 cells were transfected with CAT reporters (5 μ g) having 23 bp of the Enh1 core with or without base substitutions. The transfected cells were stimulated by either recombinant human IL-6 (hIL-6 (20 ng/ml)) or TPA (50 ng/ml) for 8 hours as indicated in the figure. CAT assays were performed with HepG2 cell lysate as described in Materials and Methods. Plotted are relative fold augmentation of CAT activities in stimulated cells compared to that in unstimulated cells.

the response to TPA stimulation [Murakami et al., 1994a]. We investigated, therefore, the role of both the cFAP-1 site and the C-stretch sequence in hIL-6 stimulation, using a series of pHECxCAT plasmids. pHEC1x2CAT, pHEC2x2CAT and pHEC1m2x2CAT have mutated sequences in their cFAP-1 site, the C-stretch sequence and in both elements, respectively (Fig. 1). Compared to pHECxCAT, CAT activities were not augmented by pHEC1x2CAT, pHEC2x2CAT or pHEC1m2x2CAT. Only pHECxCAT harboring the intact cFAP-1 site and the intact C-stretch sequence responded to hIL-6 stimulation. In the case of TPA stimulation, however, pHECxCAT and pHEC2x2CAT responded, and both CAT activities increased to more than 3 times their basal level without TPA stimulation as reported previously (Fig. 3) [Murakami et al., 1994a].

Enhancer Activity of Enh1 Core Sequence and NF-IL6

A transcription factor for IL-6 (NF-IL6) is one of major transcription factors for IL-6 [Akira et al., 1990]. Therefore, functional interaction between the Enh1 core and NF-IL6 was confirmed by co-transfection using pHECxCAT and a mammalian NF-IL6 expression vector plasmids of CMVNF-IL6 [Akira et al., 1990]. Co-transfection with CMVNF-IL6 resulted in 14-fold higher levels of gene expression than transfection with control vector plasmid alone. The transactivation effect of NF-IL6 on the Enh1 core was comparable to that of HBx (Fig. 4) [Murakami et al., 1994a].

Next we addressed whether the NF-IL6 required the same *cis*-elements of the IL-6 responsiveness in the Enh1 core. Reporters with mutated sequences were not able to respond efficiently to either NF-IL6 or to HBx (Fig. 4). Induced CAT activity of these reporters in CMVNF-IL6 transfected cells was less than 2-fold

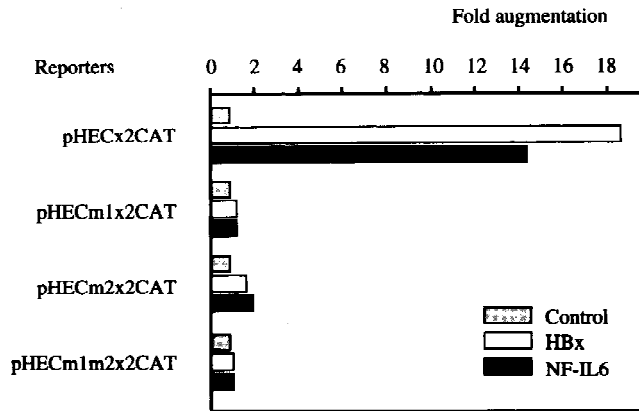


Fig. 4. Both the cFAP-1 site and C-stretch sequence in the Enh1 core were necessary for NF-IL6 augmentation. HepG2 cells were co-transfected with CAT reporters (5 μ g) that have 23 bp of the Enh1 core with or without base substitutions, and either the HBx expression vector (pSGHX-1 (1 μ g) and pSG5UTPL (4 μ g), NF-IL6 expression vector (CMVNF-IL6 (1 μ g) and pSG5UTPL (4 μ g), or control vector plasmid (pSG5UTPL (5 μ g)). CAT assays were performed with HepG2 cell lysate as described in Materials and Methods. Plotted are relative fold augmentation of CAT activities in CMVNF-IL6 or pSGHX-1 transfected cells compared to that in control vector-transfected cells. Transfection of expression vectors was confirmed by Western blotting with specific antibodies to NF-IL6 and HBx proteins using transfected cell lysates (data not shown).

higher than that in control cells. Therefore, NF-IL6-responsive elements in the Enh1 core were identical to the IL-6 responsive elements (Figs. 3 and 4), suggesting that NF-IL6 may involved in the IL-6 signaling which transactivates the Enh1 core.

Complex Formation Between Nuclear Proteins and DNA Fragment of Enh1 Upon hIL-6 Stimulation and NF-IL6 Transactivation

To examine the effect of IL-6 signal transduction on nuclear proteins which can bind the Enh1 core, electrophoresis mobility shift assays (EMSA) were carried out using the Enh1 core as a DNA probe. Three DNA-protein complexes (bands a, b, and c) associated with the Enh1 core were resolved using HepG2 nuclear extracts. The intensity of band a was 3.3- or 2.2-fold greater in the lysates of cells treated with hIL-6 or treated with CMVNF-IL6 transfection compared to non-treated lysates (Fig. 5a, lanes 2–4). Further, we analyzed complex formation using nuclear extracts from hIL-6 stimulated cells. In competition assays, only band a was competed out by a 50-fold excess of the non-labeled homologous oligo DNA (Fig. 5b, lanes 1–4). Therefore, band a was considered a specific complex with the Enh1 core. We performed a competition assay using an oligo DNA fragment harboring the NF-IL6 binding site to determine whether band a contains NF-IL6. The competitor DNA fragment of the NF-IL6 binding site weakly reduced band a complex formation; however, one-half the amount of band a remained in the presence of 500-times excess competitor DNA fragment (Fig. 5b, lanes 1 and 5–7). The HNF-1 binding site, having no homology to the Enh1 core, did not affect band a formation (Fig. 5b, lanes 1 and 8–10).

DISCUSSION

Addition of IL-6 increased the enhancer activity of HBV Enh1 through the Enh1 core region (Fig. 2). One of the main transcription factors of IL-6, NF-IL6, increased similarly the enhancer activity using the same Enh1 core sequence (Figs. 3 and 4). Thus IL-6 may affect the enhancer activity of HBV Enh1 through NF-IL6, although many transcription factors have been reported to participate in IL-6 signal transduction pathways. Our results are similar to the finding that NF-IL6 is induced by IL-6 and binds to the promoter regions of acute phase response genes such as C-reactive protein and fibrinogen [Akira et al., 1992; Kishimoto et al., 1994]. The specific complex formation of nuclear protein with the Enh1 core, band a, was detected and augmented in the lysates of cells treated with IL-6 or NF-IL6 transfection (Fig. 5). The Enh1 core has a sequence similar to the reported NF-IL6 binding site [Akira et al., 1990]; however, complex formation was not efficiently competed with NF-IL6 binding sites. Less than 10% of the complex was super-shifted by addition of specific antibodies to NF-IL6 (data not shown). Therefore NF-IL6 may indirectly affect the Enh1 core. However, the precise mechanism by which NF-IL6 acts on the Enh1 core was unclear.

We reported that TPA stimulation activated the enhancer activity of Enh1 (using the cFAP-1 site of Enh1 through the protein kinase C pathway). TPA activation was not influenced by mutation of the C-stretch sequence in the Enh1 core. However, both the cFAP-1 site and the C-stretch sequence in the Enh1 core were necessary for the response to IL-6 stimulation or NF-IL6 transactivation, which is similar to the response against HBx transactivation [Murakami et al., 1994a] (Fig. 4). NF-IL6 activation by IL-6 is caused through Ras, Raf, and mitogen-activated protein (MAP) kinase [Kishimoto et al., 1994], and HBx is also reported to use Ras, Raf, and the MAP kinase cascade for the transactivation [Benn et al., 1994]. It is possible that the signal transduction pathway is shared by HBx and IL-6. However, the precise mechanism whereby IL-6 and/or NF-IL6 modulate the HBV enhancer 1 has not been established.

Serum levels of IL-6 have been reported to increase during acute and chronic HBV infection [Kakumu et al., 1992; Kakumu et al., 1991; Sun et al., 1992; Torre et al., 1994]. The levels correlate with the serum levels of aminotransferase [Kakumu et al., 1991] and reach a maximum at the peak of hepatitis in some patients with chronic hepatitis B. The liver is one of the major sources of IL-6 production, and hepatitis may increase IL-6 production in the liver. In fact, Kakumu et al. [1992] demonstrated enhanced production of IL-6 by hepatic endothelial cells of the liver from patients with both acute and chronic hepatitis. The effect of elevated IL-6 on hepatitis is not clear. However, it is speculated that IL-6 is involved in regulating inflammatory and immunological responses in the liver through cytokines and cellular factors [Andus et al., 1991]. In the present

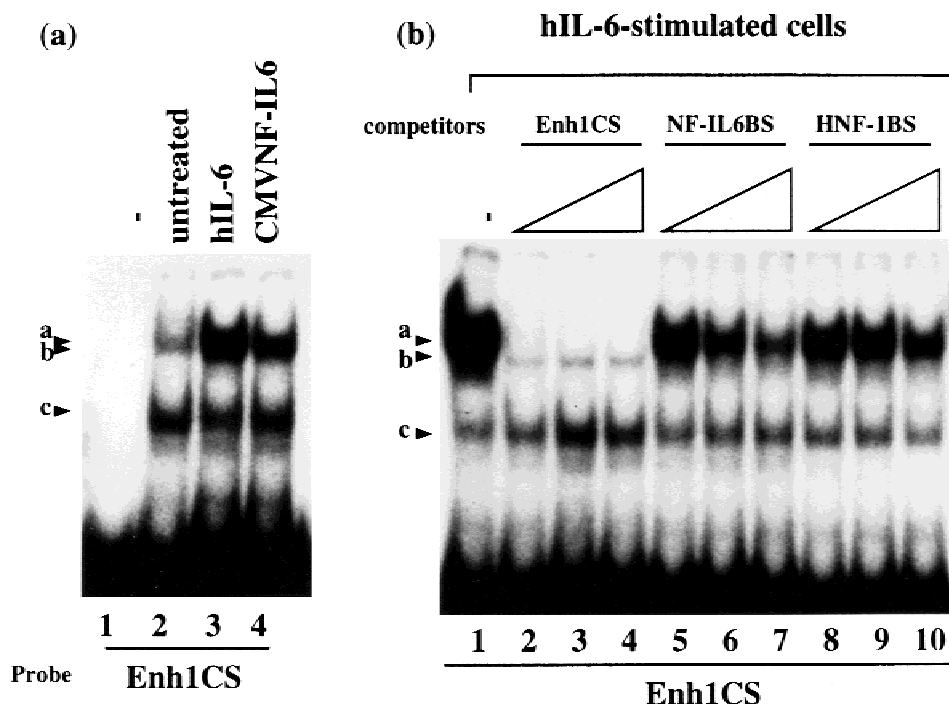


Fig. 5. IL-6- or NF-IL6-inducible complex forming proteins in HepG2 cells were identified by electrophoretic mobility assays (EMSA) using the Enh1 core as probes. EMSA were performed using the 32 P-labeled probes of the 23 bp of Enh1 core sequence (Enh1CS). (a): Three μ g of crude nuclear extracts from HepG2 cells that were untreated (lane 2), recombinant IL-6 stimulated [hIL-6 (20 ng/ml, 4.5 hours) (lane 3)], and 1 μ g/plate of CMVNF-IL6 transfected (lane 4) were incubated with the labeled probe. The formed complexes with

the Enh1 core (a, b, and c) are shown by arrows. (b): Competition assays were performed using the Enh1 core probe and 3 μ g of crude nuclear extracts from the hIL-6-stimulated HepG2 cells (lanes 1–10). Illustrated are non-labeled competitors: without competitor (lane 1), Enh1 core (lanes 2–4), NF-IL6 binding site (NF-IL6BS) (lanes 5–7), and HNF-1 binding site (HNF-1BS) (lanes 8–10). Relative to the probe, the amounts of competitors are 50 times (lanes 2, 5, and 8), 200 times (lanes 3, 6, and 9) or 500 times (lanes 4, 7, and 10).

report, it is demonstrated that IL-6 regulated the enhancer activity of HBV Enh1. Thus it is speculated that the elevated IL-6 level seen in hepatitis increases HBx expression and HBV replication through HBV Enh1 and modulates the pathological responses in the HBV-infected liver through other IL-6 cascades.

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