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Hepatitis B Virus X Protein Induces Expression of Fas Ligand Gene through Enhancing Transcriptional Activity of Early Growth Response Factor*

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FasL expressed in tumor cells plays an important role in the escape from immune surveillance by inducing apoptosis in T-cells bearing Fas. Since the Fas/FasL signaling pathway requires transcriptional induction of the FasL gene, elucidation of the precise mechanisms underlying regulation of FasL gene expression may provide useful molecular insights on tumor progression. We and others (Shin, E. C., Shin, J. S., Park, J. H., Kim, H., and Kim, S. J. (1999) *Int. J. Cancer* 82, 587–591; Lee, M. O., Kang, H. J., Cho, H., Shin, E. C., Park, J. H., and Kim, S. J. (2001) *Biochem. Biophys. Res. Commun.* 288, 1162–1168) have previously reported that hepatitis B virus X protein (HBx) plays a role in the induction of FasL expression in hepatitis B virus-associated hepatoma. In the present study, we analyzed the potential *cis*- and *trans*-acting factors that regulate FasL promoter. We found that HBx induced activity of the reporter containing FasL promoter through binding site for Egr but not through NFAT or SP-1, which are known as strong activators of the FasL promoter in T-cells. Transient expression of antisense Egr-2 and antisense Egr-3 abolished expression of FasL, which further confirmed the role of Egr in the HBx-mediated FasL expression. Also we observed that HBx increased the transcriptional activity of Egr-2 and Egr-3 by enhancing expression as well as the transactivation function of these proteins. HBx interacted with Egr-2 and Egr-3 *in vivo* and enhanced binding of Egr to the co-activator, cAMP-response element-binding protein-binding protein, which may explain the molecular mechanism by which HBx induced the transactivation function of Egr. Finally, we found that the carboxyl terminus of HBx was necessary and sufficient for FasL induction as well as activation of Egr. Taken together, our results show a novel mechanism by which HBx induces FasL gene expression that is mediated by enhancing transcriptional activity of Egr-2 and Egr-3.

Chronic infection with hepatitis B virus (HBV)[1] is a major risk factor for the development of hepatocellular carcinoma (HCC), but the mechanism by which HBV induces events leading to HCC is not clearly elucidated. The HBV genome consists of four overlapping open reading frames encoding DNA polymerase, HBV surface antigen, HBV core protein, and a regulatory X protein (HBx). Among these, HBx is considered one of the most important determinants in HBV-caused pathogenesis (reviewed in Refs. 1–3). HBx is distributed in cytoplasm but to some extent in nucleus, suggesting a dual function of the protein in modulating wide range of intracellular events of host cells (reviewed in Refs. 1–3). One function is related to its cytoplasmic localization that activates signal transduction cascades, including the Ras/Raf/mitogen-activated protein kinase, Src-dependent, and phosphatidylinositol-3 kinase pathways (reviewed in Refs. 1–3). Another function is related to the nucleus that modulates activity of the transcription apparatus including transcription factors such as activating protein 1 (AP-1), nuclear factor κB (NF-κB), tumor suppressor p53, and hypoxia-inducible factor-1 (3–5). Such pleiotropic effects of HBx may induce cell survival, proliferation, invasion/metastasis, and angiogenesis that contribute to the HBx-induced hepatocarcinogenesis.

The Fas receptor (Fas, Apo-1/CD95) and its ligand (FasL, CD95L) are transmembrane proteins of the tumor necrosis factor family of receptors and ligands. Engagement of Fas by FasL triggers a cascade of subcellular events that result in apoptosis, which has a fundamental role in normal biology as well as pathophysiology in human (6). FasL was initially thought to be expressed only in cells of the lymphoid/myeloid series including T-cells and natural killer cells. Recently, it has been shown that FasL is expressed by non-lymphoid cells, where it contributes to immune privilege by inducing apoptosis in infiltrating lymphocytes (7). FasL is also expressed in human cancer cells such as colorectal cancer, melanomas, and hepatocellular carcinomas (8–11). The functional activity of tumor-derived FasL has been shown by the ability of various cancer cell lines to induce apoptosis in Fas-sensitive lymphoid cells *in vitro*, and there is substantial evidence that tumor-derived FasL has a similar role *in vivo* (8–10). FasL was strongly expressed in hepatic metastatistic tumors of colonic adenocarcinoma, which may facilitate the establishment of tumor metastases as well as colonization in the liver, where the indigenous Fas-expressing normal cells are sensitive to FasL (12). Expression of FasL is controlled primarily by transcription factors that bind and transactivate FasL gene promoter (13). Therefore, elucidation of precise mechanisms underlying FasL gene expression may provide useful molecular insights on the human diseases associated with the Fas/FasL pathway.

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[1] The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; AP-1, activating protein 1; NF-κB, nuclear factor κB; FasL, Fas ligand; PMA, phorbol myristate acetate; Egr, early growth response factor; IRF, interferon regulatory factor; NFAT, nuclear factors for activated T-cells; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; GFP, green fluorescence protein; nt, nucleotide; aa, amino acid; AS, anti-sense; HA, hemagglutinin; Luc, luciferase.

We and others (14, 15) previously reported that HBx plays a role in the induction of FasL expression and apoptosis of Fas-bearing T-cells by HBV-associated hepatoma cells. Transcriptional activation of an orphan nuclear receptor, Nur77, which is an important regulator of T-cell apoptosis is involved in the course of FasL expression by HBx (15). Furthermore, HBx induces interleukin-18 gene expression, which enhances FasL expression by increasing interferon-γ in HBx-expressing hepatoma cells (16). However, the molecular details involved in the HBx-induced FasL expression have not been clearly illustrated. In the present investigation, therefore, we analyzed *cis*- and *trans*-acting factors that transactivate FasL promoter in response to HBx and demonstrated that Egr-2 and Egr-3 play a critical role in the up-regulation of FasL expression in liver cells. These results provide an important mechanism for the ability of HBx to escape from immune surveillance in development of the HBV-associated HCC.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Chang (ATCC CCL 13) and Chang X-34, in which HBx gene expression is under the control of a doxycycline-inducible promoter, were described previously (4, 15–17). A human hepatocellular carcinoma cell line, HepG2 (ATCC HB 8065), a human embryonal kidney cell line, 293 (ATCC CRL-1573), and a mouse fibroblast cell line, NIH-3T3 (ATCC CRL-1658), were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C in a humid atmosphere of 5% CO_2.

Plasmids and Transient Transfection—The luciferase reporter constructs containing a 2.3-kb fragment (nt −2365 to −2) and deleted fragments of genome region located 5′ upstream of the FasL translation initiation site have been reported previously (18). The 511mer-Luc and the m511mer-Luc containing a 4-bp substitution (GTGG → TGTT), the 16mer-Luc with a 16-bp FasL regulatory element (nt −220 to −205), and the m16mer-Luc containing a 4-bp substitution (GTGG → CACC) were described previously (19). The Gal4-*tk*-Luc containing a Gal4 binding site has been described elsewhere (4). The eukaryotic expression vector for wild-type full-length HBx (HpSVX) was described previously (4, 15). The Myc-tagged full-length HBx was described previously (4). The truncated constructs of HBx, HBx_{NT} (aa 1–57) and HBx_{CT} (aa 57–154) were constructed by inserting the corresponding PCR-amplified fragments into the EcoRI/NotI sites of pCMV-Myc (Clontech, Palo Alto, CA). The VP16-fused HBx construct was constructed by inserting PCR-amplified full-length HBx into EcoRI/XbaI sites of pVP16 (Clontech). The eukaryotic expression plasmids encoding each Egr-1, Egr-2, or Egr-3 were described previously (20). The antisense (AS)-Egr-1, AS-Egr-2, and AS-Egr-3 were constructed by inserting the restriction fragments in reverse orientation into the pcDNA3.0(+) or pcDNA3.1(−)/Myc-His(A) (Invitrogen, Carlsbad, CA), respectively. The yeast Gal4 DNA binding domain linked to the full-length coding region of Egr-1, Egr-2, and Egr-3 was constructed by inserting PCR-amplified full-length fragments into the pM (Clontech). The green fluorescence protein (GFP)-tagged full-length Egr-2 and Egr-3 were constructed by inserting PCR-amplified full-length fragments into the pEGFP-C2 (Clontech). The FLAG-tagged full-length Egr-1 and Egr-3 were constructed by inserting corresponding PCR-amplified fragments into the p3XFLAG^{TM}7.1 (Sigma). All of the new construction was confirmed by sequencing.

Western Blot Analysis and Immunoprecipitation—For transient transfection, Chang and Chang X-34 cells (8×10^5 cells/well in 6-well plate or 2×10^6 cells/60-cm^2 dish), NIH-3T3 cells (4×10^5 cells/well in 6-well plate or 8×10^5 cells/60-cm^2 dish), or 293 cells (1×10^6 cells/well in 6-well plate or 3×10^6 cells/100-cm^2 dish) were seeded and incubated overnight. The cells were transfected with 2–4 μg of plasmid DNA using Polyfect® (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Cells were lysed in a lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P-40, and protease inhibitors for 30 min on ice, and whole cell lysates were obtained by subsequent centrifugation. 50 μg of protein from whole cell lysates was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blocking was performed in 5% (w/v) nonfat dried milk in phosphate-buffered saline containing 0.1% Tween 20 and then incubated with specific antibodies against FasL, Egr-1, Egr-2, Egr-3, cAMP-response element (CREB)-binding protein (CBP), hemagglutinin (HA), Myc, GFP (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG (Sigma), or α-tubulin (Oncogene, Boston, MA). Secondary antibodies conjugated with horseradish peroxidase (Zymed Laboratory, South San Fransisco, CA) were used, and immunoreactive proteins were detected using the Super Signal (Pierce). The protein concentration was quantified by bicinchoninic acid assay (Pierce). For immunoprecipitation, 500 μg of whole cell lysates were incubated with 1 μg of anti-GFP, anti-FLAG, or anti-Myc antibody. The resulting immunecomplex was precipitated by adding 40 μl of protein A-agarose slurry. The resulting immunecomplex was washed five times with lysis buffer, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membrane was probed with either anti-Myc, anti-CBP, or anti-GFP antibody.

Reporter Gene Analysis—HepG2 cells (2×10^5 cells/well) were seeded in 12-well culture plates and transfected with reporter plasmid (0.3 or 0.6 μg) and β-galactosidase expression vector (0.2 μg) in the presence or absence of HBx expression vectors using LipofectAMINEPlus® (Invitrogen) as described previously (4). After 24 h of transfection, cells were lysed in the cell culture lysis buffer (Promega, Madison, WI). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity.

Reverse Transcriptase-PCR—Total RNA was prepared using RNeasy kit (Qiagen Inc.). PCR reaction was performed with specific primers for Egr-1 (forward, 5′-GATGATGCTGCTGAGCAACG-3′; reverse, 5′-GGCGGGTACTGGAGCGCTGT-3′), Egr-2 (forward, 5′-CCTTCACTTACATGGGCAAG-3′; reverse, 5′-ACGGATTGTAGAGAGTGGAG-3′), Egr-3 (forward, 5′-CGACTCGGTAGTCCATTAC-3′; reverse, 5′-GTCGTGGAAAGACACGGGCT-3′), HBx (forward, 5′-GCTCTAGAATGGCTGCTAGGCT-3′; reverse, 5′-CCCAAGCTTTTAGGCAGAGGTG-3′), and β-actin (forward, 5′-CGTGGGCCGCCCTAGGCACCA-3′; reverse, 5′-TTGGCTTAGGGTTCAGGGGGG-3′) as described previously (4). Genes were analyzed under the same conditions used to exponentially amplify the PCR products.

RESULTS

HBx Induces Expression of the FasL Gene—Since the role of HBx is implicated in gene expression of FasL in HBV-associated HCC, we examined the expression level of FasL protein in the Chang X-34 cell line, in which expression of the HBx gene is under the control of an inducible doxycycline promoter (4, 15–17). Consistent with previous observations, the expression of HBx as well as FasL protein was significantly induced when Chang X-34 cells were treated with doxycycline (Fig. 1*A*) (15, 16). Treatment with doxycycline alone did not induce expression of FasL in the parental Chang cells (data not shown). Since the induction of FasL was demonstrated to be achieved at the transcription level, we further characterized the FasL gene promoter, which controls FasL gene expression by distinct protein-DNA interactions (13). When a reporter gene containing a 2.3-kb fragment upstream of the FasL gene promoter was co-transfected with 10 ng of HBx expression vector into HepG2 cells, the FasL promoter reporter was activated about 3-fold, which was similar to that obtained by a treatment with phorbol myristate acetate (PMA) and ionomycin, known activators of the FasL promoter (Fig. 1*B*) (21). These results clearly showed that HBx induced FasL expression at the transcription level in liver cells.

Delineation of HBx-responsive cis-Acting Elements in the FasL Promoter—The FasL promoter is transactivated by several *cis*-acting regulatory elements, such as binding sites for nuclear factors of activated T-cells (NFAT), SP-1, interferon response factor (IRF), and Egr as shown in Fig. 2*A* (19, 21–34). To delineate the HBx-responsive *cis*-acting element in the FasL gene promoter, we employed reporters containing serially deleted FasL promoter (Fig. 2*A*) (18). All of the reporters except one containing the fragment of nt −205 to −2 were activated by HBx as strongly as reporter containing full-length promoter (Fig. 2*B*). The results suggested that the putative HBx-responsive element located within the nt −271 to −205 region of the FasL promoter, which contained binding sites for NFAT, IRF, and Egr (19, 24, 29–32). The binding of Egr-2 and Egr-3 to the

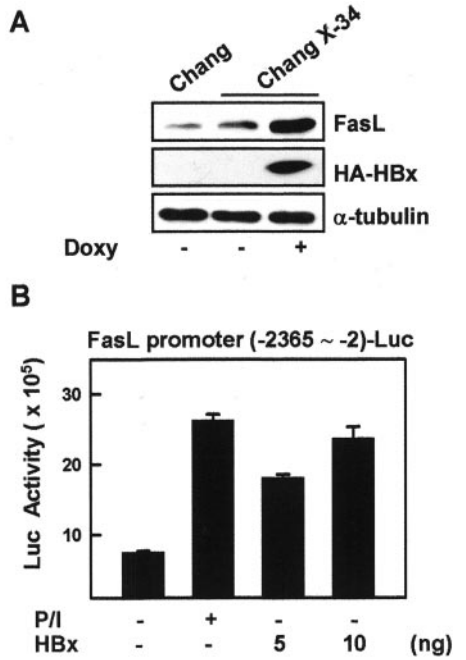

FIG. 1. **HBx increases the expression of FasL.** *A*, Chang and Chang X-34 cells (2×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were treated with vehicle or 2 μg/ml doxycycline (*Doxy*) for 24 h. 50 μg of whole cell lysates were analyzed for the expression of FasL, HA-HBx, and α-tubulin using specific antibodies by Western blot analysis as described under "Experimental Procedures." *B*, the FasL promoter (-2365 to -2) reporter (0.6 μg) was co-transfected with the indicated amount of expression plasmid for HBx (HpSVX) into HepG2 cells. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. *P/I* represents a treatment with PMA (50 ng/ml) and ionomycin (0.5 μM) for 6 h, which was shown as a positive control. Data represent the mean ± S.D. of three independent experiments.

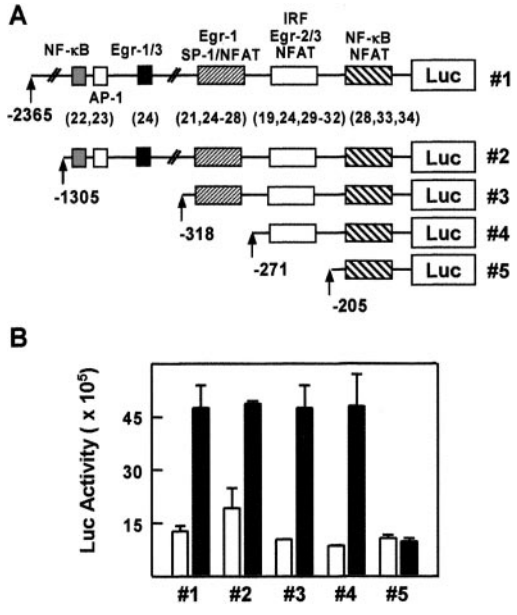

FIG. 2. **Delineation of the HBx-responsive region in the FasL promoter.** *A*, a schematic representation of the FasL promoter cloned upstream of luciferase reporter genes containing transcription factor binding sequences. References are shown in *parentheses*. Numbering is based on the translational initiation codon of the FasL gene. *B*, each reporter construct (0.6 μg) was co-transfected with (*filled bar*) or without (*empty bar*) 5 ng of HpSVX into HepG2 cells. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments.

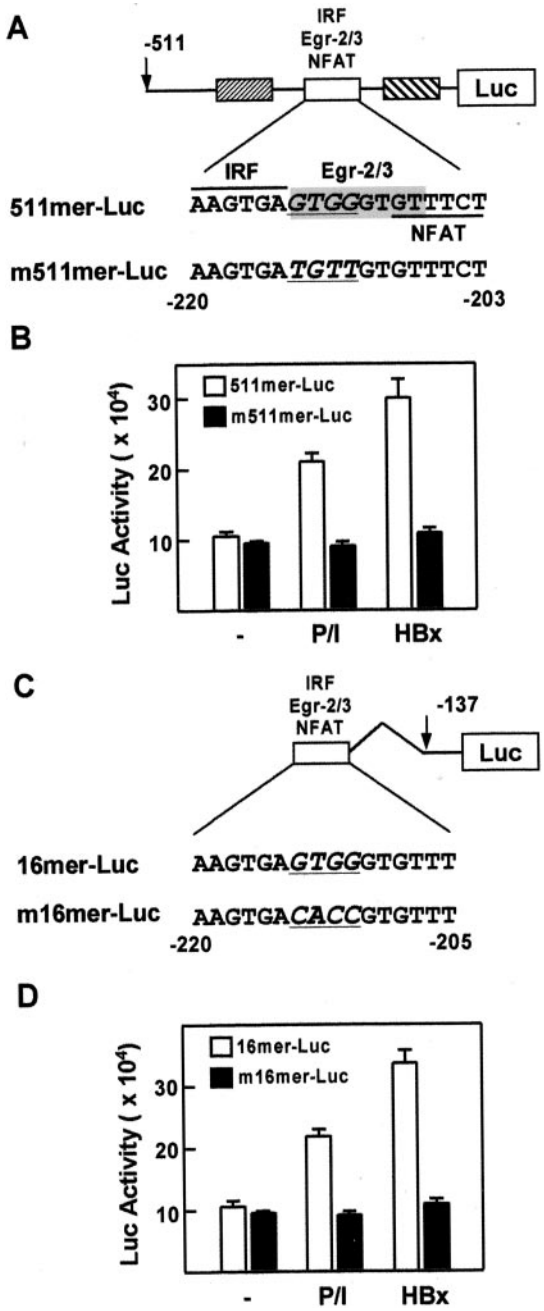

FIG. 3. **HBx enhances transcriptional activity of the FasL promoter through binding site for Egr.** *A*, a schematic representation of the FasL promoter (nt -511 to -2)-Luc, 511mer-Luc, and m511mer-Luc along with the Egr binding site. The nucleotide sequences of the Egr binding site and the mutated site are shown. *B*, each reporter construct (0.3 μg) shown in *A* was transiently transfected into HepG2 cells together with 10 ng of HpSVX. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. *P/I* represents a treatment with PMA (50 ng/ml) and ionomycin (0.5 μM) for 6 h, which was shown as a positive control. Data represent the mean ± S.D. of three independent experiments. *C*, a schematic representation of 16mer-Luc and m16mer-Luc reporter constructs containing the Egr binding site and its mutant, respectively. *D*, each reporter construct (0.3 μg) was transiently transfected into HepG2 cells together with 10 ng of HpSVX. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. *P/I* represents a treatment with PMA (50 ng/ml) plus ionomycin (0.5 μM) for 6 h, which was shown as a positive control. Data represent the mean ± S.D. of three independent experiments.

Egr binding site in this region was shown previously to be important for FasL gene expression in T-cell receptor triggering by anti-CD3 antibody (19, 31, 32). Therefore, we employed

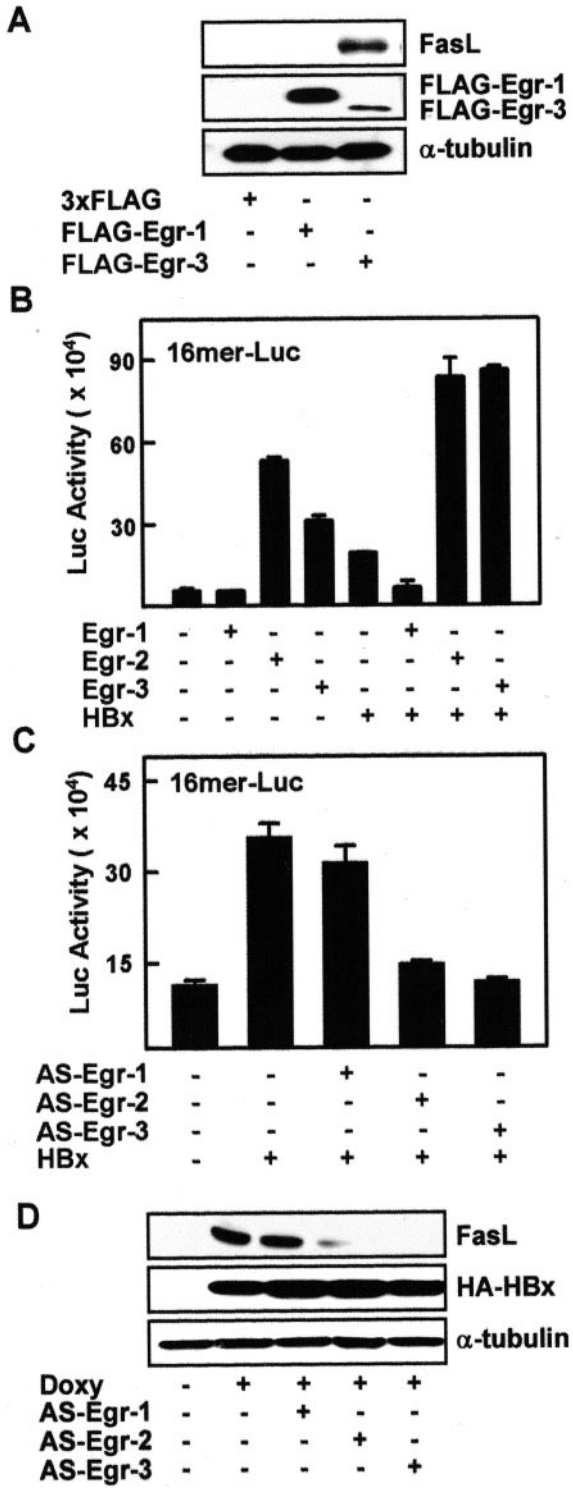

FIG. 4. **Egr-2 and Egr-3 mediate induction of FasL expression by HBx.** *A*, Egr-3 increases the expression of FasL. NIH-3T3 cells (4×10^5 cells/well) were seeded in 6-well plates and incubated overnight. The cells were transfected with 3 μg of p3XFLAG^{TM}7.1-Egr-1 and p3XFLAG^{TM}7.1-Egr-3. 50 μg of whole cell lysates were analyzed for the expression of FasL, FLAG-Egr-1, FLAG-Egr-3, and α-tubulin using specific antibodies by Western blot analysis. *B*, Egr-2 and Egr-3 enhance the transcriptional activity of the 16mer-Luc. The 16mer-Luc reporter gene (0.3 μg) was co-transfected with or without 100 ng of expression vector for Egr and/or 10 ng of HpSVX into HepG2 cells as indicated. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments. *C*, blocking of the Egr function decreases the transcriptional activity of the 16mer-Luc. The 16mer-Luc reporter gene (0.3 μg) was co-transfected with or without 200 ng of expression vector for AS-Egr and/or 10 ng of HpSVX into HepG2 cells as indicated. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments. *D*, blocking of Egr function represses the expression of FasL protein. Chang X-34 cells (1×10^6 cells/well) were seeded in 6-well plates and incubated overnight. The cells were transfected with 4 μg of eukaryotic expression vector encoding AS-Egr-1, AS-Egr-2, or AS-Egr-3. After 1 h of transfection, the cells were treated with vehicle or doxycycline (*Doxy*) for 24 h. At the end of incubation, cells were lysed, and 50 μg of whole cell lysates were analyzed for expression of FasL, HA-HBx, and α-tubulin protein. Representative figures of at least three independent experiments with similar results are shown.

a reporter-containing mutation on this site to examine involvement of Egr in the FasL promoter activation (Fig. 3*A*) (19). The reporter containing the fragment of nt −511 to −2 of FasL promoter, the 511mer-Luc, was activated by HBx, while the reporter containing the mutation on the Egr binding site, m511mer-Luc, completely lost the activity. To further confirm the involvement of Egr, we tested the HBx responsiveness of the reporter containing only 16 nt of the Egr binding site (nt −220 to −205) in the FasL promoter, the 16mer-Luc (Fig. 3*C*) (19). The 16mer-Luc was activated by HBx with the same degree of the full-length FasL promoter, whereas the reporter-containing mutation on this Egr binding site, m16mer-Luc, was not (Fig. 3*D*). These results clearly showed that the Egr binding site located between nt −220 and −205 in the FasL promoter responded to HBx.

Induction of FasL by HBx Is Mediated by Egr-2 and Egr-3—Next we examined whether Egr itself induced FasL expression. As shown in Fig. 4*A*, FasL protein expression was largely increased when Egr-3 was overexpressed. However, Egr-1 did not affect the FasL expression (Fig. 4*A*). Consistently, we observed that the 16mer-Luc was activated when Egr-2 or Egr-3, but not Egr-1, was transfected (Fig. 4*B*). The results were similar to the previous report that Egr-2 and Egr-3, but not Egr-1, induced reporter containing the FasL promoter following anti-CD3 stimulation (31, 32). Expression of HBx alone activated the reporter about 3-fold; however, together with Egr-2 or Egr-3, it largely enhanced the reporter activity. This synergistic activation may suggest a strong cooperation between Egr and HBx for maximal expression of FasL. To examine whether the HBx-induced FasL expression was mediated by Egr-2 and Egr-3, we tested whether AS-Egr blocked the FasL gene expression in HepG2 cells. The 16mer-Luc activity was largely decreased when AS-Egr-2 or AS-Egr-3 was co-transfected, which is in contrast to the result obtained with AS-Egr-1 (Fig. 4*C*). Transfection of AS-Egr-2 or AS-Egr-3 dramatically decreased the expression of FasL protein in Chang X-34 cells whereas that of AS-Egr-1 did not. These data suggest that Egr-2 and Egr-3, but not Egr-1, play a critical role in the HBx-induced FasL expression.

HBx Induces Expression as Well as Transactivation Function of Egr-2 and Egr-3—To understand the molecular mechanism by which HBx enhanced the transcriptional activity of Egr, we further examined whether HBx increased the expression of Egr. The expression of all three Egr family members was largely enhanced in Chang X-34 when treated with doxycycline (Fig. 5*A*). The induction was achieved at the transcription level, since the mRNA level of Egr was remarkably increased when HBx was present. The increases were completely abolished in the presence of cyclosporin A, a strong inhibitor of Egr expression (Fig. 5*B*) (31, 32).

Transcriptional activity of Egr could also be obtained by increasing the transactivation function by HBx. To check this possibility, we first tested whether HBx was physically associated with Egr at protein level. For the tests, we performed a mammalian two-hybrid assay that reproduced the heterodimeric protein-protein interactions. As shown in Fig. 6*A*,

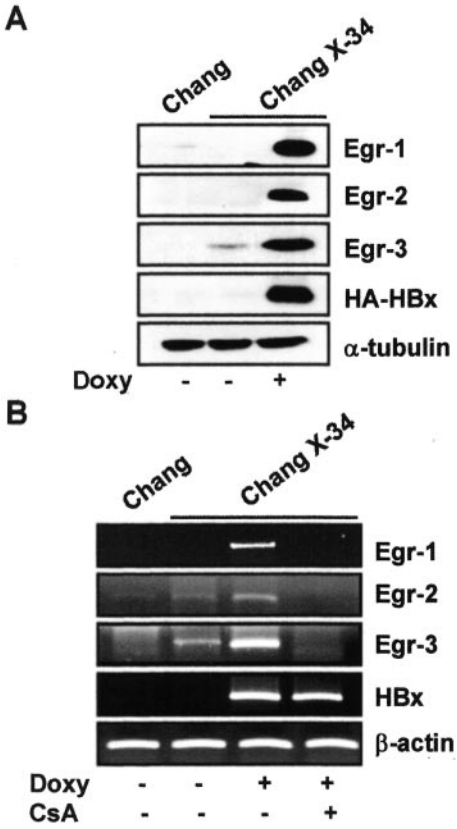

FIG. 5. **HBx induces expression of Egr at transcription-level.** *A*, HBx induces expression of Egr protein. Chang and Chang X-34 cells (2×10^6 cells/dish) were seeded into 100-cm^2 dishes and incubated overnight. The cells were treated with 2 μg/ml doxycycline (*Doxy*) for 24 h. 50 μg of whole cell lysates were analyzed for the expression of the indicated proteins by Western blot analysis. *B*, HBx induces transcripts of Egr. Chang and Chang X-34 cells (2×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were treated with vehicle or 2 μg/ml doxycycline (*Doxy*) for 24 h. Cyclosporin A (*CsA*, 2 μg/ml) was pretreated at 2 h before doxycycline treatment and continued for next 24 h. Total RNA was prepared and analyzed for expression of the indicated transcripts by reverse transcriptase-PCR using specific primers. The expression of β-actin was monitored as control. One representative of at least three independent experiments with similar results is shown.

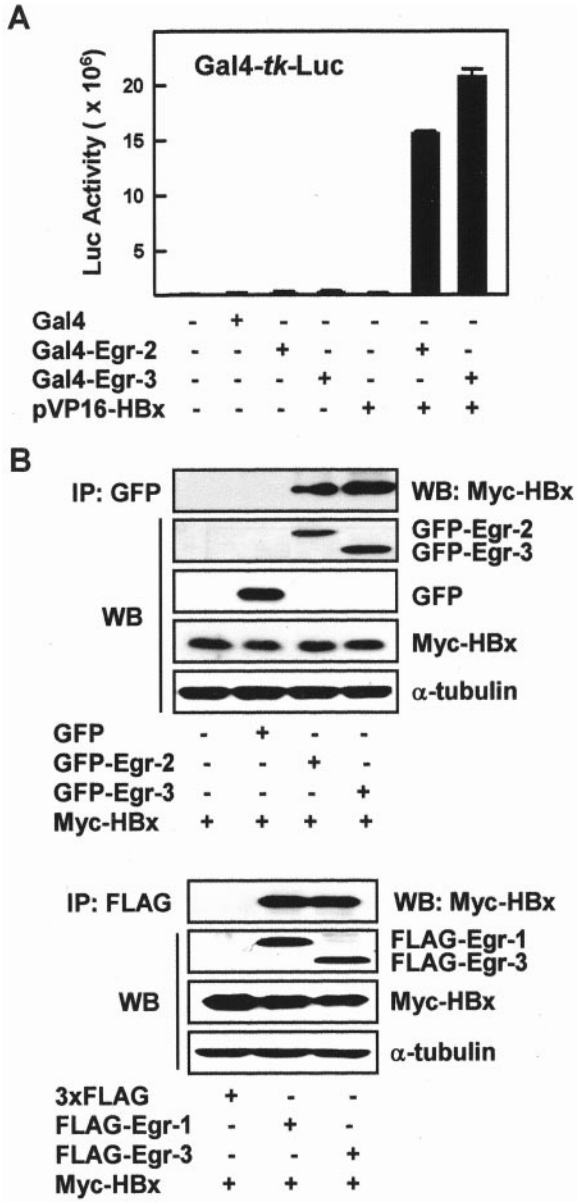

FIG. 6. **HBx interacts with Egr *in vivo*.** *A*, HBx binds Egr-2 or Egr-3 in mammalian two-hybrid system. The Gal4-*tk*-Luc (0.2 μg) reporter, 100 ng each of pGal4-Egr-1, pGal4-Egr-2, and pGal4-Egr-3, and 10 ng of pVP16-HBx were co-transfected as indicated into HepG2 cells. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments. *B*, HBx is co-immunoprecipitated with Egr. NIH-3T3 cells (8×10^5 cells/60-cm^2 dish) were seeded in 60-cm^2 dishes and incubated overnight. The cells were transfected with the indicated combination of 3 μg of pEGFP, pEGFP-Egr-2, pEGFP-Egr-3, and pCMV-Myc-HBx (*upper panel*), or 3 μg of p3XFLAG^{TM}7.1-Egr-1, p3XFLAG^{TM}7.1-Egr-3, and 3 μg pCMV-Myc-HBx (*lower panel*). 500 μg of whole cell lysates were immunoprecipitated with anti-GFP or anti-FLAG antibody and then analyzed using anti-Myc antibody. 50 μg of whole cell lysates were analyzed for the expression of the indicated proteins. Representative figures of at least three independent experiments with similar results were shown.

co-transfection of pVP16-HBx together with Gal4-Egr-2 or Gal4-Egr-3 activated the Gal4-*tk*-Luc reporter gene, whereas pVP16-HBx, Gal4-Egr-2, or Gal4-Egr-3 alone did not. Consistent with the results, HBx protein was co-immunoprecipitated with GFP-Egr-2 and GFP-Egr-3 but not with GFP. FLAG-tagged Egr-1 also bound to HBx as strong as FLAG-tagged Egr-3 (Fig. 6*B*). To assess the functional consequences of these interactions, we examined whether HBx enhanced the transactivation function of Egr. As shown in Fig. 7*A*, Gal4-Egr alone did not activate the Gal4-*tk*-Luc reporter; however, together with HBx, the reporter activity was greatly increased, suggesting that HBx endows Egr with transactivation function. Among the Egr family members, Egr-3 was the most potent, whereas Egr-1 was the least potent in HBx-induced transactivation. In the presence of HBx, binding of Egr-3 to the co-activator, CBP, was dramatically increased. In contrast, the binding was much weaker when Egr-1 was examined (Fig. 7*B*). Together, these results suggest that HBx physically interacts with all three Egr family members but induces strong transactivation function of Egr-2 and Egr-3, but not of Egr-1, by efficiently recruiting co-activator CBP.

The COOH-terminal Region of HBx Is Sufficient to Induce Transcriptional Activity of Egr—HBx has two defined structural domains that are important for the function of the protein. While the first 50 aa in the amino terminus are known to be sufficient to transform infected host cells, they have no transactivating potential. In contrast, the HBx mutant lacking the 50 amino-terminal aa has a very low transforming capability but retains an intact transactivating potential (35). The transactivation function is located in the central region (aa 67–69) and in the carboxyl terminus (aa 110–139) of HBx (Fig.

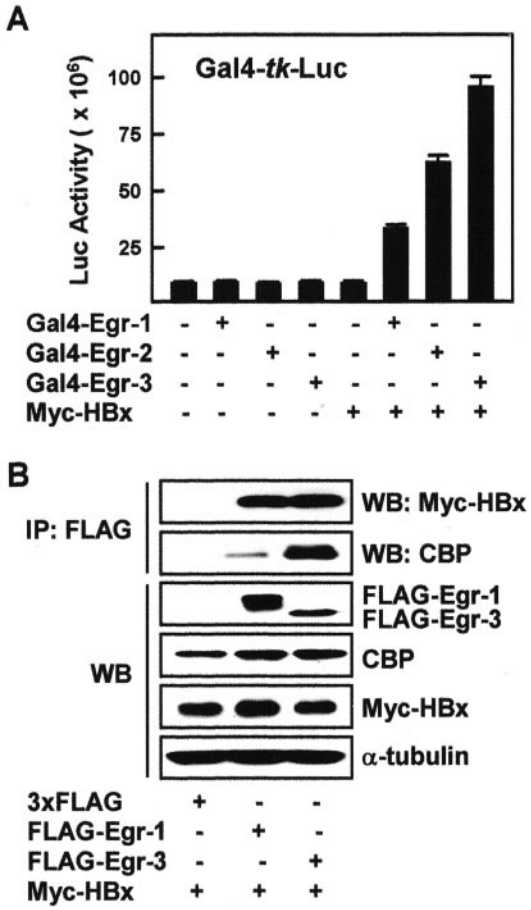

FIG. 7. **HBx increases transactivation function of Egr by recruiting CBP.** *A*, HBx increases transactivation function of Egr. The Gal4-*tk*-Luc (0.3 μg) reporter, 100 ng each of pGal4-Egr-1, pGal4-Egr-2, and pGal4-Egr-3, and 1 ng of pCMV-Myc-HBx were co-transfected as indicated into HepG2 cells. After 24 h of transfection, the cells were harvested, and the cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Data represent the mean $\pm$ S.D. of three independent experiments. *B*, HBx enhances binding of Egr with CBP. NIH-3T3 cells (1×10^6 cells) were seeded in 6-well plates and incubated overnight. 3 μg each FLAG-Egr-1 or FLAG-Egr-3 was co-transfected with either 3 μg of pCMV-Myc or 3 μg of pCMV-Myc-HBx. 500 μg of whole cell lysates were immunoprecipitated with anti-GFP antibody and then probed with either anti-Myc and anti-CBP antibody. 50 μg of whole cell lysates were analyzed for the expression of the indicated proteins. Representative figures of at least three independent experiments with similar results were shown.

8*A*). To study the functional domain of HBx that induces Egr activation, we constructed two truncated mutants, HBx_{CT} and HBx_{NT} (Fig. 8*A*). While the HBx_{NT} did not induce transactivation of the 16mer-Luc, the HBx_{CT}, in which 57 aa were deleted from the amino terminus of HBx protein, still activated the reporter as strongly as the wild-type HBx (Fig. 8*B*). Our observation was similar to the previous results that the carboxyl terminus of HBx was essential for maintaining the transactivation function of AP-1 *in vivo* (35). The expression of Egr-3 was increased in the presence of wild-type HBx as well as HBx_{CT} but not HBx_{NT} (Fig. 8*C*). The HBx_{CT} interacted with Egr-3, and it increased the binding of Egr to co-activator CBP (Fig. 8*D*). Consistently, the HBx_{CT}, but not HBx_{NT}, highly induced the transactivation function of all three Gal4-Egrs in the Gal4-*tk*-Luc reporter, although the reporter activity was much higher for Gal4-Egr-2 or Gal4-Egr-3 to compare with Gal4-Egr-1 (Fig. 8*E*). Together, these results showed that the carboxyl terminus of HBx was necessary and sufficient for the transcription as well as transactivation of Egr.

DISCUSSION

Certain tumor cells, including those of non-lymphoid origin such as melanoma and hepatoma, constitutively express FasL, which contributes to the immune privilege of tumors (8–11). Because the Fas/FasL signaling pathway, which results in apoptosis of Fas-bearing cells, requires transcriptional induction of the FasL gene, elucidation of the precise mechanism underlying regulation of FasL gene expression may provide useful molecular insights on immune evasion of malignant tumors. We and others (14–16) reported previously that HBx plays a role in the induction of FasL expression and thereby in apoptosis of T-cells induced by HBV-associated hepatoma cells. In this report, we show that Egr-2 and Egr-3 play a critical role in the up-regulation of FasL expression in liver cells and that HBx induces the expression as well as the transcriptional function of these transcription factors.

Many transcription factors have been reported to regulate the FasL promoter by DNA-protein interaction upon diverse biological signals in different cells and tissues. In the process of activation-induced cell death in mature T-lymphocytes, NFAT binding sequences in the FasL promoter provide the induction of FasL gene expression (21, 26–29). The lack of FasL expression in NFAT-deficient mice further supports the role of NFAT in FasL regulation (36). Egr-2 and Egr-3, but not Egr-1, directly up-regulate FasL transcription through an Egr binding site in the FasL promoter in T-lymphocytes (31, 32). Transcriptionally active NFAT binding sites are present in the promoter of Egr, and the NFAT-mediated induction of Egr-2 and Egr-3 is essential for optimal FasL expression (36, 37). In addition, NF-κB and the IRF family participate in regulation of FasL gene expression during T-cell activation (22, 23, 30, 33, 34). DNA-damaging agents also induce FasL expression in T-cells through activation of NF-κB and AP-1, while inhibition of NF-κB and AP-1 blocks expression of FasL as well as apoptosis of T-cells (23). SP-1, but not NFAT, is important in the regulation of constitutive expression of FasL in Sertoli cells (27, 28). We have shown previously that orphan nuclear receptor Nur77 regulates FasL expression in the presence of HBx despite the absence of recognizable responsive elements in the FasL promoter (15). These observations may suggest that the relative importance of each transcriptional factor-regulating FasL promoter may depend on activating signals as well as cell types. In the present study, we show that Egr-2 and Egr-3 directly induce transcription of FasL when HBx is present in liver cells (Figs. 3 and 4). However, the fact that HBx activates NF-κB and NFAT raises the possibility that these factors may also contribute in part to the Egr-mediated FasL induction in a binding site-independent manner (1–3, 38).

The Egr family of zinc finger transcription factors is expressed in a wide range of cell types including hepatocytes (39). The family includes Egr-1 (also called Krox-24, zif/268, NGFI-A, and TIS8), Egr-2 (Krox-20), Egr-3 (PILOT), and Egr-4 (NGFI-C). The family members share a highly homologous DNA binding domain that recognizes an identical DNA response element, although their flanking regions are much less conserved (39). Among the family members, the best characterized is Egr-1, which is implicated in the regulation of immune effectors such as interleukin-2, ICAM-1, and CD44 in the immune system (40). However, less is known about the regulation and function of Egr-2 and Egr-3. On the basis of this study, we suggest a new role for Egr-2 and Egr-3 in the HBV pathogenesis. Although Egr-1 forms a complex with HBx, it does not appear to have a major role in the HBx-induced up-regulation of FasL expression (Fig. 4). Inefficient recruitment of co-activator by Egr-1 on the FasL promoter may be an important reason why it fails in the induction of FasL expres-

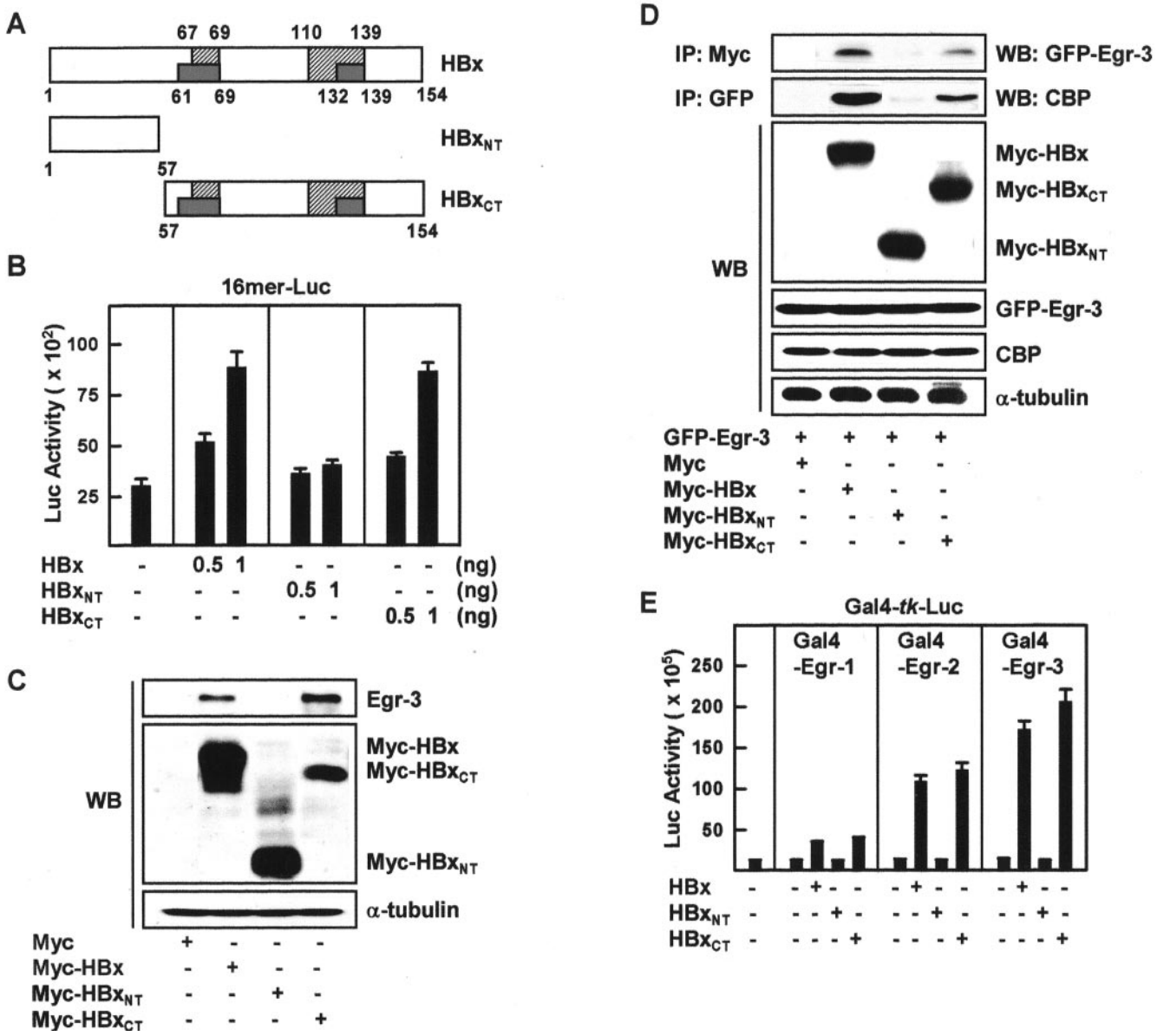

FIG. 8. **The COOH-terminal of HBx is essential for enhancing transcriptional activity of Egr.** *A*, a schematic representation of the wild-type HBx and the HBx truncated mutant constructs. The *shaded* and *striped regions* represent Kunitz domain homologous region and essential transactivation domain, respectively (35). *B*, HBx$_{CT}$ increases transcriptional activity of the 16mer-Luc. The 16mer-Luc reporter gene (0.3 μg) was co-transfected with the indicated amount of the expression plasmid for Myc-HBx, Myc-HBx$_{NT}$, or Myc-HBx$_{CT}$ into HepG2 cells. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments. *C*, HBx$_{CT}$ enhances expression of Egr-3. 293 cells (1×10^6 cells/dish) were seeded in 6-well plates and incubated overnight. The cells were transfected with 3 μg of the expression vector encoding each Myc-HBx$_{NT}$, Myc-HBx$_{CT}$, or Myc-HBx. 50 μg of whole cell lysates were analyzed for the expression of the indicated proteins. One representative of at least three independent experiments with similar results is shown. *D*, HBx$_{CT}$ interacts with Egr-3 and enhances binding of Egr-3 to CBP. 293 cells (3×10^6 cells/dish) were seeded in 100-cm^2 dishes and incubated overnight. The cells were transfected with 3 μg of the expression vectors for pEGFP-Egr-3, Myc-HBx$_{NT}$, Myc-HBx$_{CT}$, and Myc-HBx as indicated. After 24 h of transfection, 500 μg of whole cell lysates were immunoprecipitated with anti-Myc or anti-GFP antibody and then analyzed using either anti-GFP or anti-CBP antibody. 50 μg of whole cell lysates were analyzed for the expression of the indicated proteins. Representative figures of at least three independent experiments with similar results are shown. *E*, HBx$_{CT}$ increases transactivation function of Egr. Gal4-*tk*-Luc (0.3 μg) reporter together with 100 ng each of pGal4-Egr-1, pGal4-Egr-2, or pGal4-Egr-3 was co-transfected with or without 1 ng of the expression plasmid for Myc-HBx, Myc-HBx$_{NT}$, or Myc-HBx$_{CT}$ into HepG2 cells as indicated. After 24 h of transfection, cell lysates were obtained and assayed for luciferase activity. Data represent the mean ± S.D. of three independent experiments.

sion (Fig. 6). However, Yoo *et al.* (41) reported that HBx induces transcription of Egr-1 and thereby enhances the expression of tumor growth factor-β1, a cytokine that has been implicated in the pathogenesis of liver disease. Although the mechanism of how HBx differentially regulates transcription of the Egr-1 target genes is not known yet, our observation together with others suggest a cooperative network of Egr family members during the course of HBV-associated hepatocarcinogenesis and also suggest that the Egr family members are a potential therapeutic target to control HBV-induced acute and chronic liver diseases.

Although HBx is known to be a transcriptional co-activator that plays a significant role in the regulation of many cellular transcription factors, the molecular details for the transcription activation function have not been clearly elucidated. HBx may promote assembly of the transcriptional preinitiation complex by directly interacting with basal transcriptional machinery such as RNA polymerase subunit RPB5, transcription factor IIB, and TATA-binding protein (42–44). HBx may modify the DNA binding ability of transcription factors such as CREB and activating transcription factor 2 by forming protein-protein

complexes (45). Recently, it was reported that HBx induces stabilization of a transcriptional factor such as hypoxia-inducible factor-1α, which has a role in new vessel formation (4, 5). Kong *et al.* (46) reported that HBx stabilizes activating signal co-integrator 2, which mediates the transactivation of mitogenic transcription factors such as AP-1 and NF-κB through direct protein-protein interaction. In this report, we show for the first time that HBx is physically associated with Egr, and it transactivates these transcription factors by enhancing binding to co-activator CBP, which coordinates a variety of transcriptional pathways with potent histone acetylase activity (47). Therefore, we suggest a novel mechanism of HBx-induced transactivation that HBx may induce favorable chromatin remodeling for transcriptional activation of transcription factors by recruiting co-activators.

Expression of Fas and FasL can be modulated by some viral proteins as a strategy to evade immune surveillance, which results in persistent infection. For instance, the immediate-early gene product 2 of human cytomegalovirus up-regulates FasL in infected retinal epithelial cells, which may be a potential mechanism for the pathogenesis of cytomegalovirus-induced retinitis (48). Tax, the human T-cell leukemia virus type-1 viral transactivator, activates the NFAT binding site in the FasL promoter, and thus FasL is constitutively expressed in T-cells infected with human T-cell leukemia virus type-1 (26). Similarly, Nef, a viral protein of the human immunodeficiency virus up-regulates FasL expression, accompanied by extensive apoptosis of the virus-specific cytotoxic T-cells (49). The human immunodeficiency virus Tat protein also induces FasL on the surface of the infected macrophages, and NF-κB binding sites are required for the Tat-mediated activation of FasL promoter (33). Similar to our observations, Yang *et al.* (31) reported that activation of Egr-2 and Egr-3 mediates the Tat-enhanced FasL induction. In the case of viral hepatitis, the core protein encoded by hepatitis C virus induces the FasL expression (50). The similarities between these diverse viral transactivators indicate that the Fas/FasL pathway may be involved in a wide spectrum of viral pathogenesis. Therefore, the Fas/FasL pathway, including transcription factors such as Egr-2 and Egr-3, is an important determinant in the pathogenesis of acute and chronic viral diseases, and better understanding of this pathway may provide valuable tools to control viral infectious diseases.

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