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Bioluminescence imaging of hepatitis B virus enhancer and promoter activities in mice

Juan Du[a], Yong Zhou[a], Qiu-Xia Fu[a], Wei-Li Gong[b], Fang Zhao[a], Jian-Chun Peng[a], Lin-Sheng Zhan[a,*]

[a] *Laboratory of Blood-borne Virus, Beijing Institute of Transfusion Medicine, Tai Ping Road 27, Beijing 100850, China*
[b] *National Center of Biomedical Analysis, Tai Ping Road 27, Beijing 100850, China*

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Abstract **By bioluminescence imaging and hydrodynamic gene transfer technology, the activities of hepatitis B virus (HBV) promoters and the effects of HBV enhancers on these promoters in mice under true physiological conditions have been assessed. Our studies reveal that either of the two HBV enhancers can stimulate HBV major promoter activity in hepa 1–6 cells (in vitro) and in mouse liver (in vivo), and the enhancer effects on the three promoters (S1, S2 and X promoter) are markedly greater in vivo than in vitro. The two HBV enhancers have no cooperative action on HBV promoters in vitro or in vivo.**

Keywords: HBV; Promoter; Enhancer; Bioluminescence imaging

1. Introduction

Hepatitis B virus (HBV) is a human hepadnavirus that is known to cause persistent infection and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). The HBV genome is a circular, partially double-stranded DNA molecule containing four, partly overlapping, open reading frames (ORFs) [1]. Transcription is initiated by four promoters: S1 promoter, S2 promoter (also named preS promoter and S promoter), the core promoter and X promoter. The core promoter plays a central role in HBV replication and morphogenesis, directing the transcription of 3.5 kb mRNA. S1 and S2 promoter respectively direct the transcription of the 2.4 kb large surface protein (LHBsAg) mRNA and a group of 2.1 kb mRNAs with heterogeneous 5′ends by using different transcription start sites, and X promoter directs the synthesis of X mRNA. The activities of these four promoters are regulated by two enhancer elements: enhancer I (En I) and enhancer II (En II) [2–5]. Enhancer I spans a sequence of about 160 bp which is adjacent to the X promoter and has the classical ability to up-regulate transcription in an orientation independent manner. Enhancer II is 148 bp in length, and is located immediately upstream of the basal core promoter (BCP) on the HBV genome and is part of the core promoter. Both enhancers are liver-specific and enhance the activity of the four HBV promoters [6–9].

The activities of the individual (HBV) promoters and the effects of the HBV enhancer on these promoters in several human cell types have been compared by measuring the activity and RNA levels of the linked reporter gene (including chloramphenicol acetyltransferase and luciferase) [4,6–8]. Although these in vitro systems are available and easy to perform, they suffer from the common problem that many tissue specific transcription factors are lost when cells are kept in an artificial environment for an extended period of time. Now we have constructed transient mouse model by hydrodynamic gene transfer to examine the activity of promoters and the effect of the HBV enhancers on the activity of the promoters in whole animals under true physiological conditions by the IVIS camera, which provides quantitative bioluminescence imaging of live mice [10–12].

2. Materials and methods

2.1. Plasmid construction

All four HBV promoter sequences and two enhancer sequences were selected based on previously published gene sequence and synthesized by PCR using HBV DNA (ayw serotype, accession no. AY661792) as the template. The core promoter (1591–1822) was PCR amplified with primers 5′-CGAGCTCCAAGGTCTTACATAAG-3′/5′-CCAAG CTTTGGAGGCTTGAAC AGT-3′; the S1 promoter (2219–2780) with primers 5′-CGAGCTCTGTCTCACTTTTGGAAG-3′/5′-CCAAGCTTTTATATAATATACCCGCC-3′; the S2 promoter (2809–3152) with primers: 5′-CGAGCTCTTTGTGGGTCACCATA-3′/5′-CCAAGCTTCCTGACTGGCG ATTGGT-3′; the X promoter (1235–1374) with primers 5′-CGAGCTCTGCGTGGAACC TTTTC-3′/5′-CCAAGC TTGGAAACGATGTATATT-3′; the En I (1070–1234) with primers 5′-GGGTACCGTATTCAA TCTAAGCA-3′/5′-CGAGCTCTGCGCTGATGGC CCATGA-3′; the En II (1627–1774) with primers 5′-GGGGTACCCCCACCAAATATTGCC-3′/5′-CGAGCTCTAGTACAAAGACCTT T-3′ and 5′-CGGGATCCCC-CACCAA ATATTGCC-3′/5′-GCGTCGACTAGTACAAAGACCT TT-3′; the cytomegalovirus (CMV) Enhancer/Promote with primers 5′-GGGTACCTCAATATTGGCCATTA-3′/5′-CAAGCTTGATC TGACGGTTCACT-3′. All amplified promoter sequences were cloned into pGL3-basic vector (Promega, Madison, WI) by ligating the purified PCR fragments and linearized pGL3-basic vector after digestion. Each insertion in the plasmid construct was confirmed by restriction enzyme digestion and sequence analysis. Plasmids were transformed into *Escherichia coli* (strain DH-5a) and purified by the Qiagen®-plasmid purification kits (Hilden, Germany).

*Corresponding author. Fax: +86 10 66931292.
E-mail address: lszhan91@yahoo.com (L.-S. Zhan).

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ORFs, open reading frames; LHBsAg, large surface protein; En I, enhancer I; En II, enhancer II; CMV, cytomegalovirus; BCP, basal core promoter.

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2.2. Cell culture and transfections

Hepa 1–6 cells from mouse hepatocellular carcinomas were purchased from ATCC (Manassas, VA) and cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (HyClone, South Logan, UT); and were kept in a 5% CO_2 incubator at 37 °C. The transfection experiments were conducted by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. In brief, 1 μg of a plasmid DNA mixture was used for each transfection reaction for one well of a twenty-four-well plate, containing 4.0×10^5 Hepa 1–6 cells.

2.3. Mice

Balb/c mice (male, 18–20 g) were maintained at the animal facility of Academy of MilitaryMedical Sciences in a specific pathogen-free unit, under a 12 h light/dark cycle, and were provided with food and water.

2.4. Hydrodynamic tail-vein injections

Balb/c mice were rapidly injected via the tail vein with 1.6 ml saline containing 20 μg of plasmid DNA. The total volume was injected rapidly, over 5–8 s, into the tail-vein of a mouse.

2.5. In vivo and in vitro luciferase activity monitoring by the IVIS camera

For in vitro bioluminescence imaging, the cells were harvested and resuspended in 100 μl PBS, 8 min before monitoring light emission, 100 μl (1 mg/ml in PBS) of D-luciferin was added to the cell suspension. Cells were then scanned for 30 s using the Xenogen IVIS-50 optical imaging system (Xenogen Corporation, Alameda, CA). For in vivo bioluminescence imaging, mice which were transfected with fly luciferase reporter gene were anesthetized, and 8 min before monitoring light emission, the animals were injected with 100 μl (15 mg/ml in PBS) of D-luciferin i.p. Mice were then scanned for 1 min using the Xenogen IVIS-50 optical imaging system. Regions of interest (ROI) were drawn by the instrument automatically, resulting that 90% of the bioluminescence signal is contained inside ROI. The quantification is reported as the maximum photon flux within ROI. The bioluminescence signal is represented as photons/s/cm^2/sr.

2.6. Immunohistochemistry

Mice were killed at 24 h after the hydrodynamic injection. Liver tissues were fixed in 10% formaldehyde, embedded with paraffin and cut into 4 μm thick sections. For luciferase detection, the sections were incubated with goat anti-luciferase antibody (Rockland Immunochemicals Inc.), and were detected by DAB staining. The liver sections were also stained with hematoxylin.

2.7. Statistics

Statistical analysis was performed with Student's *t*-test for quantitative variables. Quantitative data are expressed as the means ± S.D. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Comparison of HBV promoters activity in vitro and in vivo

To determine the relative strengths of the four HBV promoters, we constructed plasmids containing the fly luciferase gene linked to the four HBV promoters respectively, pS1-Luc, pS2-Luc, pC-Luc, and pX-Luc. These plasmids were transfected into the liver cell line Hepa 1–6 and mouse liver. The relative transcriptional activities of the four HBV promoters in vivo and in vitro were screened by the IVIS camera and compared (Fig. 1A and B). Immunohistochemical staining was performed to examine luciferase expression in the liver. As illustrated in Fig. 1C, in the animals injected with pCMV-Luc, the liver cells were stained positive by anti-luciferase antibody. Fig. 1D shows in vitro, that among the five plasmids evaluated, the CMV promoter provides the highest luciferase gene expression, and the S1, S2 promoter exhibit the lowest level activity, approximately two orders of magnitude lower than that of CMV. The promoter strength for core promoter (including enhancer II) and X promoter is one order of magnitude lower than that of CMV. The overall order of activity based on the luciferase activity is as follows: CMV > C, X > S1, S2. In vivo, the CMV promoter provides the highest luciferase expression also, and the S1, X promoter exhibit the lowest level of luciferase activity, approximately three orders of magnitude lower than that of CMV. The S2 promoter exhibits lower activities, approximately two orders of magnitude lower than that of CMV. The promoter strength for core promoter (including enhancer II) is at the same order of magnitude of CMV. The overall order of activity based on the luciferase activity is as follows: CMV > C > S2 > S1, X.

3.2. Enhancer regulation of HBV S1 and S2 promoter activities in vitro and in vivo

To determine how the two enhancers affect HBV S1 promoter activity and whether the two enhancers interact cooperatively to modulate HBV gene expression, we have constructed plasmids containing the fly luciferase gene linked to the HBV S1 promoters with one or two HBV enhancers respectively. We transfected these plasmids into the mouse liver and cell line Hepa 1–6, respectively, and luciferase activity was determined by the IVIS camera and normalized to that of pS1-Luc with no enhancer (Fig. 2). In Hepa 1–6 cells, En I in pEIS1-Luc stimulates luciferase expression ≈3-fold, whereas pEIIS1-Luc generates levels of luciferase activity similar to that of pS1-Luc. pES1-Luc which has two enhancers fails to produce higher levels of luciferase activity than pEIS1-Luc, suggesting that En II has no activity and En I alone stimulates the promoter activity in Hepa 1–6 cells. In vivo, S1 promoter activity is stimulated >60-fold by En I, and it is stimulated 34-fold by En II. Luciferase activity produced by pES1-Luc with both enhancers is comparable to that of pEIS1-Luc, suggesting that En II is dispensable for S1 promoter activity in mouse liver. As shown in Fig. 2, the stimulatory effect of En I on S1 promoter is stronger than that of En II in vitro and in vivo.

Similar plasmids using the S2 promoter instead of the S1 promoter to regulate luciferase gene expression were constructed, and the results of transfection with these plasmids are shown in Fig. 3. In Hepa 1–6 cells, S2 promoter activity is stimulated 6-fold by En I (pEIS2-Luc), 2-fold by En II (pEIIS2-Luc) and 5-fold by two enhancers (pES2-Luc), suggesting that luciferase expression from S2 promoter is solely depend on En I, just like the S1 promoter. In mouse liver, S2 promoter activity is stimulated 17-fold by En I and 14-fold by En II. pES2-Luc which have two enhancers generates levels of luciferase activity similar to that of pEIIS2-Luc, suggesting that both En I and En II are important to stimulates transcriptional activity of S2 promoter in vivo. As shown in Fig. 3, the stimulatory effect of En I on S2 promoter is stronger than that of En II in vitro, but is similar to En II in vivo.

3.3. Enhancer regulation of HBV X promoter activities in vitro and in vivo

Similar plasmids using the X promoter instead of the S1 promoter to regulate luciferase gene expression were constructed, and the results of transfection with these plasmids are shown in Fig. 4. X promoter activity is stimulated to the maximum level by En I alone, suggesting that En I is indispensable for X promoter activity. In mouse liver, X promoter activity

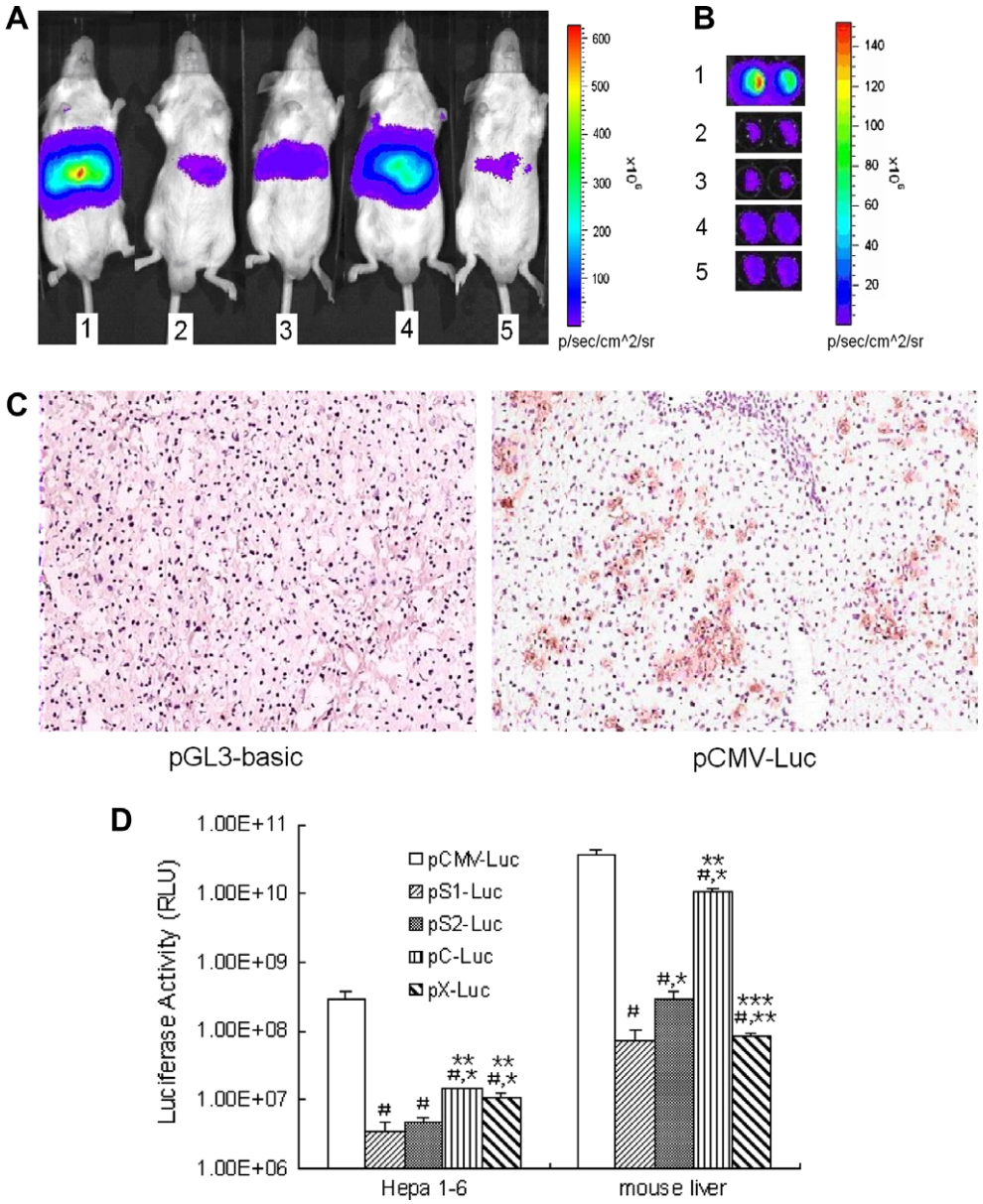

Fig. 1. (A, B) Bioluminescence imaging luciferase expression in mice and in cell line Hepa 1–6: they were transfected with five plasmids pCMV-Luc(1), pS1-Luc(2), pS2-Luc(3), pC-Luc(4) and pX-Luc(5). (C) Immunohistochemical analysis of luciferase expression in liver tissues of mice. Animals were hydrodynamically transfected with pCMV-Luc or pGL3-basic plasmid, and the expression of luciferase in liver was detected by immunohistochemical staining at 24 h after the hydrodynamic injection. Magnification 100×. (D) Evaluation of promoters for driving luciferase expression in vivo and in vitro. Luciferase activity was analyzed 24 h after transfection. Values represent means ± S.D. ($n = 3$). $^{\#}P < 0.05$ compared with pCMV-Luc group; $^{*}P < 0.05$ compared with pS1-Luc group; $^{**}P < 0.05$ compared with pS2-Luc group; $^{***}P < 0.05$ compared with pC-Luc group.

is stimulated >150-fold by En I and >100-fold by En II. pEX-Luc with two enhancers generates levels of luciferase activity similar to that of pEIIX-Luc. Different from that in Hepa 1–6 cells, either En I or En II is able to stimulate X promoter activity to the high level in vivo. As shown in Fig. 4, the stimulatory effect of En I on X promoter is stronger than that of En II in Hepa 1–6 cells, but is similar to En II in vivo, just like S2 promoter.

3.4. Enhancer regulation of HBV core promoter activities in vitro and in vivo

Because En II is part of the core promoter, we cannot separate En II from the core promoter, the plasmids with En II and with two enhancers were constructed, and the results of transfection with these two plasmids are shown in Fig. 5.

The luciferase activity of pEC-Luc with both enhancers is approximately 6-fold higher than the activity of pEIIC-Luc with En II alone, suggesting that the activity of En I is important for regulating core gene expression in this cell line. In vivo, the luciferase activity of pEC-Luc with both enhancers is similar to that of pEIIC-Luc with En II alone, indicating that En I is dispensable for core promoter activity in mouse liver. Because we have not yet been able to separate En II activity from the core promoter activity, assessment of the effect of En I alone on core promoter activity is not possible.

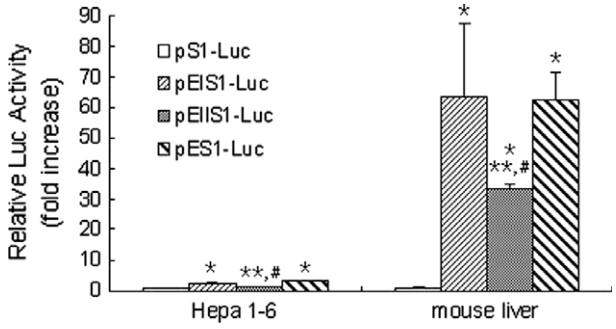

Fig. 2. Activation of HBV S1 promoter activity by two HBV enhancers in cell line Hepa 1–6 and in mouse liver. Luciferase activity was analyzed 24 h after transfection. Luciferase activity of plasmid pS1-Luc in vivo and in vitro was defined as 1. Values represent means ± S.D. ($n = 3$). $^{*}P < 0.05$ compared with pS1-Luc group; $^{**}P < 0.05$ compared with pEIS1-Luc group; $^{\#}P < 0.05$ compared with pES1-Luc group.

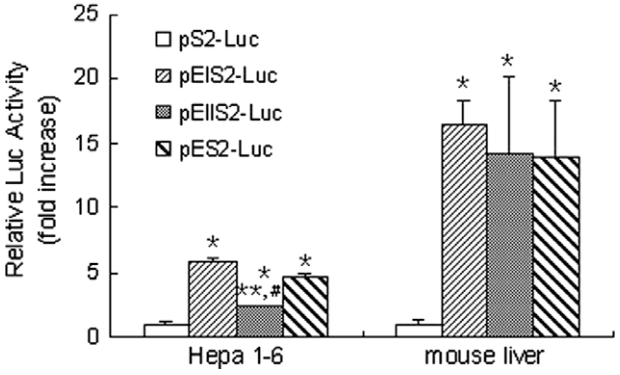

Fig. 3. Activation of HBV S2 promoter activity by two HBV enhancers in cell line Hepa 1–6 and in mouse liver. Luciferase activity was analyzed 24 h after transfection. Luciferase activity of plasmid pS2-Luc in vivo and in vitro was defined as 1.Values represent means ± S.D. ($n = 3$). $^{*}P < 0.05$ compared with pS2-Luc group; $^{**}P < 0.05$ compared with pEIS2-Luc group; $^{\#}P < 0.05$ compared with pES2-Luc group.

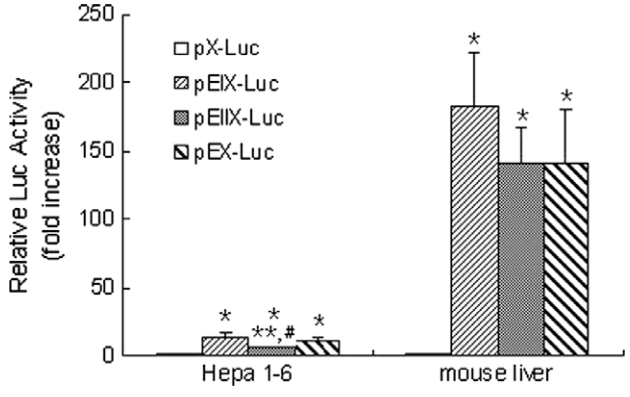

Fig. 4. Activation of HBV X promoter activity by two HBV enhancers in cell line Hepa 1–6 and in mouse liver. Luciferase activity was analyzed 24 h after transfection. Luciferase activity of plasmid pX-Luc in vivo and in vitro was defined as 1.Values represent means ± S.D. ($n = 3$). $^{*}P < 0.05$ compared with pX-Luc group; $^{**}P < 0.05$ compared with pEIX-Luc group; $^{\#}P < 0.05$ compared with pEX-Luc group.

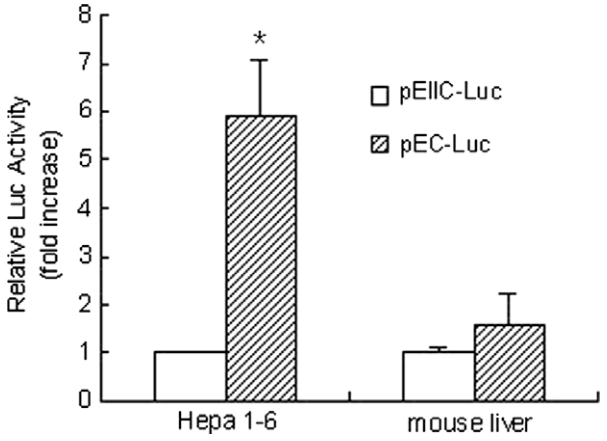

Fig. 5. Activation of HBV core promoter activity by two HBV enhancers in cell line Hepa 1–6 and in mouse liver. Luciferase activity was analyzed 24 h after transfection. Luciferase activity of plasmid pC-Luc in vivo and in vitro was defined as 1. Values represent means ± S.D. ($n = 3$). $^{*}P < 0.05$ compared with pEIIC-Luc group.

The effect of the two HBV enhancers on different promoters in vivo and in vitro is summarized in Table 1.

4. Discussion

Now we examine the activity of HBV four promoters in mice and in Hepa 1–6 cells by bioluminescence imaging. The order in Hepa 1–6 cells is C, X > S1, S2 which is consistent with the previous observation in human cell lines such as HepG2, HeLa and HS27 cells [7,8]. This order in vivo is C > S2 > S1, X, and is different from that in vitro. This suggests that the promoter activity in whole animals under true physiological conditions is different from the activity in vitro.

HBV enhancer I has previously been shown to contain overlapping binding sites for multiple transcription factors including HNF3, HNF4, C/EBP, AP-1, CREB, and ATF [13–15]. The ubiquitous distribution of some of these transcriptional factors may account for this enhancer functions in many kinds of cell lines such as human hepatoma cells (PLC/PRF/5, Hep3B, HepG2,Huh7, and Huh6 cells) [3,7,16], non-liver cells (HeLa, HS27 cells) [8]. In contrast, enhancer II activity is strictly liver-specific, and its activity is highly variable in the different hepatoma lines used [5,17]. These may explain our results that either of the two HBV enhancers can stimulate S1, S2 and X promoter activity in Hepa 1–6 cells, but the stimulatory effect of En I is much stronger than that of En II. Although the hepatitis B viral enhancer behaves as a classic cis activator in the sense that it is active in both orientations, its activity is strongly influenced by the nature of the cell in which it operates. In the Hepa 1–6 cells tested, the HBV enhancers modestly stimulate three HBV promoters (S1, S2 and X promoter) activity (2–13-fold by En I and 2–7-fold by En II). In contrast, the HBV enhancers stimulate the three promoters activity dramatically (17–180-fold by En I and 14–140-fold by En II) in the mouse liver. The stimulatory effects of the two enhancers on the three promoters are much stronger in vivo than that in vitro. Our studies also show that in Hepa 1–6 cells, the stimulatory effect of En I on S1, S2 and X promoter is stronger than that of En II, but in vivo, the effect of En I on S2 and X promoter is similar to that of En II.

Table 1
HBV enhancer activity on different promoters in vitro and in vivo

Construction	HBV enhancer activity, -fold induction			
	Without En	With En I	With En II	With En I + II
In Hepa 1–6 cells[a]				
S1-Luc	1.0[b]	2.7	1.4	3.3
S2-Luc	1.3	7.3	3.0	5.9
C-Luc	NA[c]	NA	4.1	24
X-Luc	2.9	38	20	34
In mouse liver[d]				
S1-Luc	1.0[b]	64	34	63
S2-Luc	4.1	68	59	57
C-Luc	NA	NA	150	240
X-Luc	1.2	221	170	171

[a]Values for the Hepa 1–6 cell line are normalized to luciferase activity of pS1-Luc with no enhancer in Hepa 1–6 cells.
[b]The actual value represented by 1 in the mouse liver is 20 times greater than the values represented by 1 Hepa 1–6 cell line.
[c]NA ËConstruct was not available.
[d]Values for the mouse liver are normalized to luciferase activity of pS1-Luc with no enhancer in mouse liver.

Previous observation indicated that the two HBV enhancers interact cooperatively to stimulate HBV S (S2) promoter activity in Huh7 and HepG2 cells and to stimulate HBV preS (S1) promoter activity in Huh6 cells [7,8]. But in our test, the most efficient luciferase expression comes from pEIS1-Luc, pEIS2-Luc and pEIX-Luc which have En I alone, indicating that the two HBV enhancers cannot interact cooperatively to stimulate HBV promoters activity in vivo and in vitro.

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References

[1] Ganem, D. and Varmus, H.E. (1987) The molecular biology of the hepatitis B viruses. Ann. Rev. Biochem. 56, 651–693.
[2] Johnson, P.F., Landschulz, W.H., Graves, B.J. and Mc-Knight, S.L. (1987) Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Gene Dev. 1, 133–146.
[3] Doitsh, G. and Shaul, Y. (2004) Enhancer I predominance in hepatitis B virus gene expression. Mol. Cell. Biol. 24, 1799–1808.
[4] Bock, C.T., Kubicka, S., Kubicka, M.P. and Trautwein, C. (1999) Two control elements in the hepatitis B virus S-promoter are important for full promoter activity mediated by CCAAT-binding factor. Hepatology 29, 1236–1247.
[5] Yee, J.K. (1989) A liver-specific enhancer in the core promoter region of human hepatitis B virus. Science 246, 658–661.
[6] Miguel, A.T., John, L., Hugh, F.M., Manuel, L. and Aleem, S. (1991) Functional analysis of a liver-specific enhancer of the hepatitis B virus. Proc. Natl. Acad. Sci. USA 88, 3797–3801.
[7] Henry, S. and Yee, J. (1992) Regulation of hepatitis B virus gene expression by its two enhancers. Proc. Natl. Acad. Sci. USA 89, 2708–2712.
[8] Tammy, K.A. and William, J.R. (1989) Hepatitis B Virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. J. Virol. 63, 579–583.
[9] Honigwachs, J., Faktor, O., Dikstein, R., Shaul, Y. and Laub, O. (1989) Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and the enhancer. J. Virol. 63, 919–924.
[10] Wang, Y., Iyer, M., Annala, A.J., Chappell, S., Mauro, V. and Gambhir, S.S. (2005) Noninvasive monitoring of target gene expression by imaging reporter gene expression in living animals using improved bicistronic vectors. J. Nucl. Med. 46, 667–674.
[11] Deroose, C.M., Reumers, V., Gijsbers, R., Bormans, G., Debyser, Z., Mortelmans, L. and Baekelandt, V. (2006) Noninvasive monitoring of long-term lentiviral vector-mediated gene expression in rodent brain with bioluminescence imaging. Mol. Ther. 14, 423–431.
[12] Klopstock, N., Levy, C., Olam, D., Galun, E. and Goldenberg, D. (2007) Testing transgenic regulatory elements through live mouse imaging. FEBS Lett. 581, 3986–3990.
[13] Chen, M., Hieng, S., Qian, X., Costa, R. and Ou, J.H. (1994) Regulation of hepatitis B virus ENI enhancer activity by hepatocyte-enriched transcription factor HNF3. Virology 205, 127–132.
[14] Faktor, O., Budlovsky, S., Ben-Levy, R. and Shaul, Y. (1990) A single element within the hepatitis B virus enhancer binds multiple proteins and responds to multiple stimuli. J. Virol. 64, 1861–1863.
[15] Zheng, Y.Y. (2005) Regulation of hepatitis B virus gene expression and replication. UMI Microform, 3180394.
[16] Bock, C.T., Malek, N.P., Tillmann, H.L., Manns, M.P. and Trautwein, C. (2000) The enhancer I core region contributes to the replication level of Hepatitis B virus in vivo and in vitro. J. Virol. 74, 2193–2202.
[17] Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M. and Hayashi, N. (2000) Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. J. Virol. 74, 1241–1251.