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# The Action of Hepatitis B Virus Enhancer 2-Core Gene Promoter in Non-Viral and Retroviral Vectors for Hepatocyte-Specific Expression

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**Abstract :** Heptocyte-specific expression induced by Hepatitis B virus (HBV) enhancer 2-core gene promoter was examined in various hepatocyte and non-hepatocyte cell lines, using non-viral and retroviral vector systems in which chloramphenicol acetyltransferase (CAT) is used as a reporter. The non-viral plasmid containing the HBV enhancer 2-core promoter exhibited 22 and 66% of CAT activities in hepatoma cell lines, HepG2 and Hep3B, respectively when compared with CAT activity expressed by CMV promoter. The CAT activities, however, were found to be marginal in other tested hepatoma cell lines as well as mouse primary hepatocytes and non-hepatocytes. The HBV enhancer 2 located upstream the CMV promoter did not affect the CMV promoter activity nor provided hepatocyte-specific expression. Transfection of retroviral plasmid DNA containing the HBV enhancer 2-core promoter as an internal promoter exhibited high and specific CAT expression in HepG2 and Hep3B cell lines but the activity value was 5 to 10 fold lower than the non-viral plasmid with identical promoter. These results suggest that the usage of HBV enhancer 2-core promoter for liver specific expression is limited to certain vectors and hepatocyte cell lines.

**Key words :** HBV enhancer 2, Hepatitis B virus, HBV core gene promoter, liver-specific expression, retroviral vector

The liver plays an essential role in maintaining somatic homeostasis and providing most serum proteins (Starzl *et al.*, 1989). Many human metabolic diseases are caused by defects in genes that are normally expressed in the liver (Scriver *et al.*, 1989; Horwitz *et al.*, 1991). Although some genetic diseases can be treated by repeated infusion of the deficient protein to the liver (Scriver *et al.*, 1989), it is expensive, carries a risk of viral infection, and only temporarily reduces the disease symptoms. Liver transplantation can correct genetic deficiencies, but is limited by the availability of the organ and the need for an immunosuppression agent. The transfer of a functional gene into a genetically defective individual can correct the clinical manifestations.

One of the important aspects of liver-directed gene therapy is the specific expression of the target gene (Clayton *et al.*, 1985). To eliminate undesirable expression of the respective gene in other tissues, the use of liver-specific enhancers and promoters including the α1-antitrypsin gene (Kay *et al.*, 1992), albumin gene (Pinkest *et al.*, 1987), and phophoenolpyruvate carboxykinase gene (Hatzoglou *et al.*, 1990) have been considered in the development of *in vivo* targeting vectors.

Two regions of the Hepatitis B virus (HBV) genome have been shown to act as transcriptional enhancers (Honigwachs *et al.*, 1989; Trujillo *et al.*, 1991; Chen *et al.*, 1993). Enhancer 1 is active in most hepatoma lines as well as in some non-hepatocyte-derived cell lines. In contrast, enhancer 2 activity is restricted to hepatocytes. In this study, we examined the tissue-specificity of the HBV enhancer 2 and core gene promoter in various cell lines by using the chloramphenicol acetyltransferase (CAT) gene as a reporter. Also, the action of the HBV enhancer 2 in combination with the core gene promoter in a retroviral vector was examined.

## Materials and Methods

### Construction of recombinant plasmid vectors and retroviral vectors

Recombinant plasmid vectors expressing the CAT gene under the regulatory control of various promoters and

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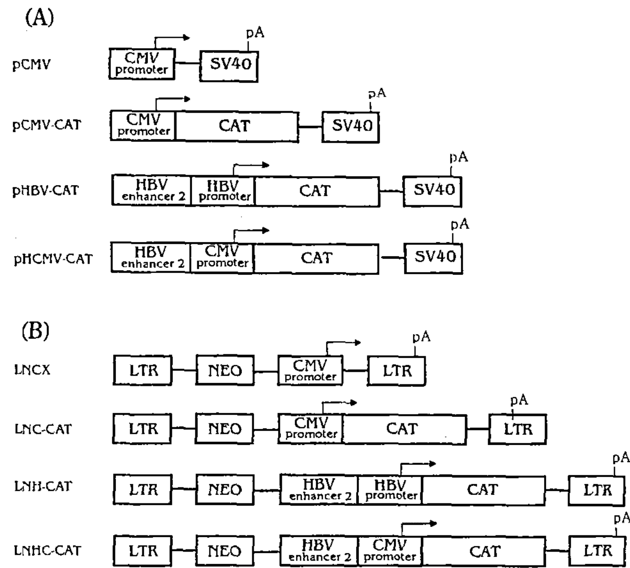


**Fig. 1.** Physical structures of non-viral (A) and retroviral (B) plasmid DNAs. Abbreviations are: CMV, cytomegalovirus immediate early gene enhancer/promoter; SV40, simian virus 40 promoter; pA, poly A tail; CAT,chloramphenicol acetyl transferase; LTR, long terminal repeat; NEO, neomycin resistant gene.

enhancer were constructed. pCMV-CAT was made by insertion of the 792bp HindIII fragment containing the CAT cassette (Pharmarcia, Pscataway, USA) into the HindIII site of pCMV plasmid DNA (Fig. 1A). The 345p AvaI-HindIII fragment containing HBV enhancer 2-core gene promoter of plasmid pCEPΔ2 was filled with the Klenow fragment, and replaced the CMV promoter of pCMV-CAT to yield pHBV-CAT. Plasmid pH-CMV-CAT, which the contains HBV enhancer 2 and CMV promoter, was constructed by insertion of the HBV enhancer 2 containing the AvaI-DdeI fragment into the end-filled BamHI site of pCMV-CAT.

LNC-CAT was constructed by cloning the 792bp HindIII fragment containing the CAT cassette into the HindIII site of the LNCX retroviral vector, the end-filled AvaI-HindIII fragment of plasmid CEPΔ2 containing the HBV enhancer 2 core gene promoter was ligated to the 6.1 kb end-filled fragment, which was generated by cleavage of the BamHI and HindIII enzymes of LNCX. For the construction of LNH-CAT, the HindIII fragment of the CAT gene was blunt-ended and inserted into the HpaI site of LNHX. To create LNHC-CAT, HBV enhancer was cut from the AvaI-DdeI region of CEPΔ2 containing HBV enhancer 2, filled with the Klenow fragment, and inserted into the filled BamHI site of LNC-CAT.

### Transient Transfection

Cells were cultured in Dulbecco's modified Eagle minimal essential medium (Gibco Laboratories, Gland Island, USA) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), supplemented with 10% fetal bovine serum for MCF-7 (human breasted adenocarcinoma cell line, ATCC HTB22), HepG2 (human hepatocellular carcinoma cell line, ATCC HB 8065), Hep3B (human hepatocellular carcinoma cell line, ATCC HB 8064), 293 (human transformed primary embryonic kidney cell line, ATCC CRL 1573), Hepa 1-6 (mouse hepatoma cell line, ATCC CRL 1830) cells or with 10% calf serum for NIH3T3 (NIH Swiss mouse embryo cell line, ATCC CRL 1658) cell. For Chang liver (human liver cell line, ATCC CCL 13) cells, Basal Medium Essential (GIBCO Laboratories) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum was used. Mouse primary hepatocyte was isolated by the collagenase perfusion method and cultured as previously described (Ponder et al., 1991).

The cells were seeded 1 to 3 days prior to transfection. At the time of transfection, the cells were 40 to 80% confluent. Transfection was performed by calcium phosphate coprecipitation with 10 μg of the plasmid DNAs. After 40 h, cells were washed once with phosphate buffered saline and harvested in 200 μl of 250 mM Tris (pH 8).

Extracts were prepared by four cycles of freezing and thawing, and heated at 65°C for 10 min to inactivate deacetylase activity. CAT assays were performed at 37°C in a total volume of 150 μl with 1 M Tris (pH 7.8)-2 μl[$^{14}$C] chloramphenical (0.1 mCi/ml)-2 μl acetyl coenzyme A (30 mg/ml). Reaction products were dissolved in ethylacetate and used to analyze CAT activity by thin layer chromatography on silica gels. The silica gels were exposed to X-ray film. To quantify CAT activity, the radioactive spots were cut from the TLC plate and radioactivities were measured in a liquid scintillation counter.

### Creation of retroviral producer cell lines and *in vitro* transduction

The amphotropic packaging cell line ΨCRIP (Danos and Mulligan, 1988) was maintained in Dulbecco's modified Eagle minimal essential medium (GIBCO Laboratories) containing penicillin (100 U/ml) and streptomycin (100 μg/ml), and 10% calf serum. After transfection with retroviral vector DNAs by the calcium phosphate method, cells were cultured for 10 days in 400 μg/ml of G418 supplemented with DMEM and 10% calf serum. Supernatant from G418 resistant clones was filtered (0.45 μm), spiked with DEAE dextran to a final concentration of 10 μg/ml, and added to packaging cell line. After four hours, the medium was exchanged with fresh medium. Forty hours later, the transduced

cells were harvested and CAT assay was performed as previously described (Sambrook *et al.*, 1989).

## Results

### Hepatocyte-specific expression by HBV enhancer 2-core gene promoter

To develop a gene transfer system which drives a specific expression of delivered gene in liver cells, the possible usage of the enhancer 2 and core gene promoter of hepatitis B virus (HBV) were examined in non-viral and retroviral vector systems. A reporter gene, chloramphenicol acetyltransferase (CAT), was linked to the indicated promoters (Fig. 1), then the activities of the promoters were compared by the CAT assay. The HBV enhancer 2, the promoter of CMV or HBV were placed and the CAT gene was located upstream SV40 transcriptional terminator of a non-retroviral vector pCMV (Fig. 1A). In parallel, a series of retroviral vectors were constructed in such a way that the enhancer and the promoters were placed in between the neomycin resistant gene (NEO) and LTR of the LNCX vector (Fig. 1B).

The hepatocyte specificity of the HBV enhancer cell lines (Chang liver, HepG2, Hep3B, mouse primary hepatocytes and Hepa 1-6) and non-hepatocyte cell lines (MCF7, 293T, and NIH3T3) (Table 1). Each cell line was transfected with the indicated plasmid DNAs by the calcium phosphate method and the CAT activities expressed by pCMV, pHBV-CAT and pHCMV-CAT were presented relative to the CAT activity of pCMV-CAT. pCMV, which does not possess an enhancer or a promoter also exhibited residual levels of CAT activities. The CAT activities of pHBV-CAT, which contains the HBV enhancer 2-core gene promoter, were highest in the human hepatoma cell lines, HepG2 and Hep3B, but found to be marginal in other hepatocyte cell lines such as Chang liver and Hepa 1-6, or mouse primary hepatocyte as well as nonhepatocytes. The presence of HBV enhancer 2 upstream from the CMV promoter in pHCMV-CAT was not able to enhance the rate of expression nor the specific expression in the hepatocytes. The results might be caused by the domination of the strong influence of CMV promoter in various cell lines.

### HBV enhancer 2-core gene promoter in the retroviral vector

In order to investigate the effect of internally located HBV enhancer 2-core gene promoter in a retroviral vector, the enhancer and the promoters fused to the CAT gene were inserted between the neomycin resistant gene and the LTR of the viral vector, LNCX (Fig. 1B). Following transfection of each viral constructs the CAT activity expressed in the transiently transfected cell lines were determined. These values are presented relative to the activity of LNC-CAT which contains only the CMV promoter (Table 2). Among the tested cell lines, LNH-CAT vector containing the HBV enhancer 2-core gene promoter exhibited the highest activity in HepG2 and Hep3B. However, the CAT activities of LNH-CAT expressed in HepG2 and Hep3B were 5 to 10 fold lower than those of pHBV-CAT, the nonviral vector which contains an identical enhancer and promoter (Table 1 and 2). As seen in the LNH-CMV-CAT transfected cells, the HBV enhancer when fused to the CMV promoter did not influence hepatocyte-specificity nor promoter strength.

Retrovirus was produced by using an amphotrophic packaging cell line, ψCRIP. Culture supernatants containing each viral particle were infected to non-hepatic (MCF-7) and hepatic cell line(HepG2) and the cells were subjected to CAT assay (Fig. 2). The activities of CMV promoter in retrovirus LNC-CAT and LNHC-CAT were similar in both MCF7 and HepG2 cell lines. In contrast, although the CAT expression from the HBV enhancer-core gene promoter containing virus LNH-CAT was much lower than those from the retrovirus containing CMV promoter, the level of CAT expression exerted by the HBV enhancer 2-core gene promoter was significantly higher in hepatic cell line HepG2 than non-hepatic cell line MCF7.

## Discussion

The expression of genes containing HBV enhancer 2-core promoter has been shown to be restricted to liver cells (Yee, 1989; Su *et al.*, 1992). In this study, various cell lines including primary mouse hepatocyte, hepatocarcinoma and non-hepatocytes were employed to

**Table 1.** Specific expression of CAT by HBV enhancer 2-core promoter in hepatoma cell lines, HepG2 and Hep3B. Each cell line was transfected with the indicated plasmid DNAs, then CAT activities were analyzed as described in the "Materials and Methods". The CAT activities are presented relative to the activity of pCMV-CAT.

| | pCMV-CAT | pCMV | pHBV-CAT | pHCMV-CAT |
|---|---|---|---|---|
| MCF-7 | 100 | 0.5 | 0.7 | 101.3 |
| 293 | 100 | 0.2 | 0.3 | 100.1 |
| NIH3T3 | 100 | 0.5 | 0.5 | 101.1 |
| Chang liver | 100 | 0.6 | 0.9 | 101.7 |
| HepG2 | 100 | 0.5 | 66.5 | 100.3 |
| Hep3B | 100 | 0.5 | 22.1 | 99.8 |
| Mouse primary | 100 | 0.4 | 0.6 | 100.9 |
| Hepa 1-6 | 100 | 0.7 | 1.3 | 99.7 |

**Table 2.** Expression of the HBV enhancer 2-core gene promoter in a retroviral vector. Transfection and the description of CAT activity were as described in Table 1

| | LNC-CAT | LNCX | LNH-CAT | LNHC-CAT |
|---|---|---|---|---|
| MCF-7 | 100 | 0.5 | 0.7 | 103.2 |
| 293 | 100 | 0.5 | 0.3 | 100.6 |
| NIH3T3 | 100 | 0.5 | 0.5 | 100.9 |
| Chang liver | 100 | 0.7 | 0.9 | 99.9 |
| HepG2 | 100 | 0.6 | 6.2 | 98.6 |
| Hep3B | 100 | 0.2 | 4.4 | 100.2 |
| Mouse primary | 100 | 0.5 | 0.6 | 102.4 |
| Hepa 1-6 | 100 | 0.6 | 0.8 | 101.4 |

examine the possibility of using HBV enhancer 2-core gene promoter for liver-specific expression. Among the tested cell lines, the expression of CAT gene linked to the HBV enhancer 2-core gene promoter was highest in hepatoma cell lines (Table 1 and 2).

The activity of HBV enhancer 2-core gene promoter in non-retroviral vector was about 22 and 66% of CMV promoter when transfected to Hep3B and HepG2, respectively. HepG2 cell is highly differentiated (Nakabayashi *et al.*, 1982) and Hep3B cell contains an integrated part of the HBV genome (Hsu *et al.*, 1993). Relatively high activities of HBV enhancer 2-core promoter in HepG2 and Hep3B compared to those in primary hepatocyte and other hepatoma cell lines suggested the limited usage of HBV enhancer 2-core promoter for the specific gene expression in the liver.

When the HBV enhancer 2-core promoter was used as an internal promoter in retroviral plasmid DNA, the promoter activities in HepG2 and Hep3B cells were 5 to 10 fold reduced compared to activities of the promoter in non-retroviral DNA (Table 1 and 2). This result can be explained by the previous observation that a high level of expression from the LTR interferes with the expression from an internal promoter (Soriano *et al.*, 1991). However, CMV promoter used as an internal promoter in LNC-CAT was as active as the CMV promoter in pCMV-CAT (Fig. 1; Table 1 and 2).

Organ-specific expression vectors for *ex vivo* gene therapy directed to liver have been developed (Grossman *et al.*, 1994). Combined use of liposome and a receptor-mediated gene transfer system is being attempted for *in vivo* hepatic gene therapy. Thus, development of strong promoters which confer tissue specificity will be critical for liver gene therapy. As the promoter strength of the HBV enhancer 2-core gene promoter depends on the hepatoma cell lines, the promoter containing vectors developed during the course of this study might be useful for transferring the genes into certain hepatoma cell types.

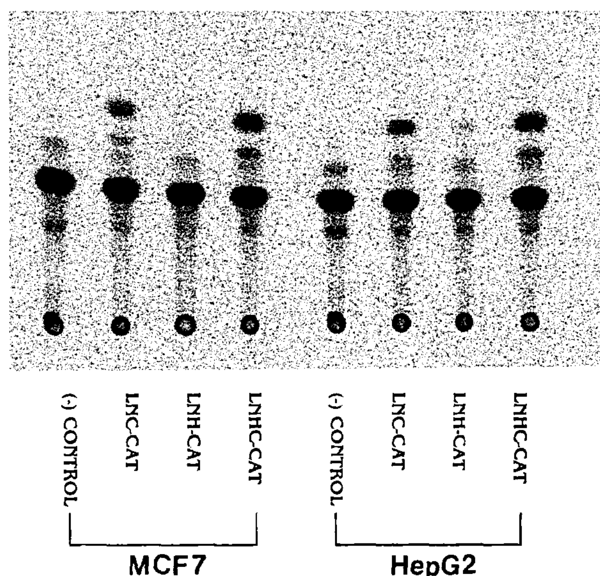


**Fig. 2.** Effect of the HBV enhancer 2-core gene promoter in retrovirus. The indicated DNAs were transfected to ΨCRIP, then each retrovirus was obtained as described in the "Materials and Methods". Transduction and CAT assays are described in the "Materials and Methods".

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