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# Anti-sense expression of a metallopeptidase gene enhances nuclear entry of HBV-DNA

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**Abstract**

Although several putative hepatitis B virus (HBV) receptors have been identified, none of them is capable of initiating HBV replication in a non-permissive human cell line. Using an Epstein–Barr virus-based extrachromosomal replication system, we have screened through a human liver cDNA library and successfully identified a clone capable of facilitating nuclear transport of HBV-DNA during the early phase of HBV infection. This clone contained a cDNA encoding a metallopeptidase-like protein in anti-sense orientation. Pretreatment of naïve HepG2 cells with 1,10-phenanthroline, an inhibitor for liver metallopeptidases, led to nuclear entry of HBV-DNA after HBV infection. However, cccDNA was still undetectable in the nuclei, indicating other cellular factors required to complete the replication cycle were still missing. Our present data suggest that in the initial stage of HBV infection, liver metallopeptidase constitutes a barrier for effective nuclear entry of HBV genomic DNA. Attenuation of metallopeptidase activity may facilitate HBV infection.


*Keywords:* Hepatitis B virus; Metallopeptidase; Epstein–Barr virus; Nuclear entry; Anti-sense; Phenanthroline; Infection; Extrachromosomal replication; Permissive cell line; HepG2 cells

Although the molecular biology of hepatitis B virus (HBV) replication has been extensively studied, the mechanisms involving the first step of infection, i.e., the entry of the virions into hepatocytes, are poorly understood. It is believed that this initial event is mediated by the binding of viral envelopes to a putative viral receptor on the cell surface. Initially, it was reported that HBV surface antigen reacted with glutaraldehyde-polymerized human serum albumin, leading to the hypothesis that this molecule served as a bridge for the attachment of HBV to hepatocytes [1,2]. However, HBV surface antigen did not react with naturally occurring albumin polymers [2]. Subsequent experiments indicated that pre-S region was important for the attachment of HBV onto the cell membrane of hepatocytes [3,4]. Based on this finding, HBV receptor was vigorously searched. The presence of sequence homology between pre-S1 and the constant region of IgA led to the speculation that HBV attached to target cells through IgA receptor [5]. Evaluation of a large panel of CD antigens and cytokine receptors revealed that IL-6 contained recognition sites for the pre-S1 binding region [6]. Other molecules which were proposed capable of mediating membrane binding either directly or indirectly included fibronectin, endonexin II, annexin V, apolipoprotein H, transferrin receptor, and asialoglycoprotein receptor [7–11]. Although these molecules were all shown to mediate the binding of HBV envelope proteins to the cell membrane, none was proven to support functionally the initiation of HBV infection except for annexin V, which has been demonstrated to initiate HBV replication in a rat hepatoma cell line [12].

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The avian hepadenavirus infection appears to be an attractive model for pursuing the viral receptors. Similarly, the pre-S protein of duck HBV was shown to play an important role in interaction with the surface of target cells [13,14]. A carboxypeptidase, gp180, capable of mediating membrane binding of the pre-S protein, was proposed to be the authentic viral receptor [15,16]. However, no viral replication can be detected in immortalized non-permissive cells expressing this molecule.

Based on these reports, we speculate that other cellular factors are needed for effective cell entry and replication of HBV. In this study, we have developed a strategy to search for such factors.

## Materials and methods

*Cell lines, transfection, and establishment of transformants*. Human embryonic kidney cells constitutively expressing EBNA-1 protein from Epstein–Barr virus (293EBNA cells; Invitrogen, Carlsbad, CA) were maintained in Dulbecco's modified Eagle's media containing 10% fetal bovine serum and 250 μg/ml G418. HepG2 cells were maintained in minimal essential medium containing 10% fetal bovine serum. Huh-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. A human liver cDNA library (Clontech Laboratories, Palo Alto, CA) was constructed by inserting the cDNAs into a vector, pDR2, downstream of a Rous Sarcoma virus LTR promoter. This plasmid contains Epstain–Barr virus *OriP*, a gene for hygromycin B selection, and an ampicillin-resistant gene. The cDNA clones were first grouped into 300 sets with 50–100 cDNA clones per set. Plasmids were then extracted from each set of clones and transfected into 293EBNA cells using the standard $CaPO_4$ precipitation method. Transformants were selected by addition of 150 μg/ml of hygromycin B into the culture medium. Totally 300 transformants were established for the first round of HBV infection assay.

*Nuclear HBV-DNA detection assay*. An HBV-positive serum sample containing $10^9$ copies/ml of HBV-DNA, measured by Digiene HBV Test (Digene Corporation, MA), was used for HBV infection assay. The cells in a 60-mm Petri dish were incubated in medium containing 5 μl HBV-positive serum for 1 h. The cells were then washed on the dish for three times and incubated in fresh medium without HBV-positive serum. To detect HBV-DNA, cells were further washed on the dish for three times and trypsinized from the dish. The trypsinized cells were washed two more times with fresh medium by centrifugation. The supernatant of the first wash was collected (to detect HBV attached to the cell membrane) when necessary. The washed cells were then lysed in lysis buffer (10 mM Tris–hydrochloride [pH 7.0], 150 mM NaCl, and 0.5% Nonidet P-40) and centrifuged at 3000*g* for 1 min. The supernatant was collected (cytoplasmic fraction). The nuclear pellet was washed three times in lysis buffer. The third wash and nuclear fraction were both collected. The third wash was used as a contamination control. HBV-DNA was extracted from all fractions with the method of proteinase K digestion followed by phenol/chloroform extraction. HBV-DNA was detected by either Southern blot analysis or PCR as previously described [17]. As a control, β-actin gene or mRNA was detected in parallel when necessary. The primers used were A1, 5′-CACCAACTGGGACGACATGG-3′ (sense, nt. 301–320) and A2, 5′-AGGATCTTCATGAGGTAGTC-3′ (anti-sense, nt. 651–632).

*Detection of KIAA1226 RNA in sense or anti-sense orientation*. Total cellular RNA was extracted and equally divided into two aliquots for detection of KIAA1226 RNA and β-actin mRNA (as a control). The PCR primers used to detect KIAA1226 RNA were P14711L, 5′-GCCAGAAGTTAAAACCCTTG-3′ (sense, nt. 1093–1112) and P14711R, 5′-TAGGTGTTCAGCAAGCCTTC-3′ (anti-sense, nt. 1315–1296). To detect KIAA1226 RNA in sense orientation and β-actin mRNA, random primers were used for reverse transcription. To detect KIAA1226 RNA in anti-sense orientation, P14711L was used for reverse transcription. The procedure for specific detection of sense and anti-sense RNA was described elsewhere [18].

*Pretreatment of cells with 1,10-phenanthroline*. 293EBNA and HepG2 cells were incubated in medium containing 1 mM of 1,10-phenanthroline for 30 min. The cells were washed for two times with fresh medium and incubated in medium containing 5 μl HBV-positive serum for 1 h. The cells were then processed as described in the previous section.

## Results

### *Cellular uptake of HBV-DNA by 293EBNA and Huh-7 cells after infection*

As a pilot study, HBV-positive serum was used to infect 293EBNA and Huh-7 cells. HBV-DNA can be detected in both the trypsinized supernatant (containing HBV adhesive to the cell surface) and cytoplasmic fraction, but not the nuclear fraction at 1 h after infection (Fig. 1A, left panel). The HBV-DNA in the cytoplasmic fraction moved slightly faster in the gel during electrophoresis compared with those in the trypsinized supernatant and the serum sample, indicating that HBV-DNA extracted from the cytoplasmic fraction was not a result of contamination. Additionally, these data also suggested that HBV-DNA in the cytoplasmic fraction was molecularly altered (moving faster) compared with that of HBV adhesive to the cell surface. The molecular basis of this change was not clear at this time. The cytoplasmic HBV-DNA became undetectable by Southern blot 2 h after infection (see sections below), although it could still be detected by PCR. The

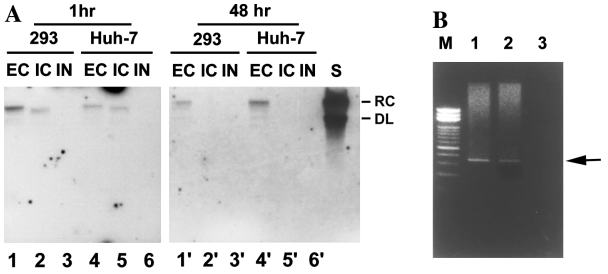


Fig. 1. Cellular uptake of HBV-DNA by 293EBNA and Huh-7 cells after HBV infection. (A) 293EBNA and Huh-7 cells were incubated with medium containing 5 μl HBV-positive serum for 1 h. The cells were then washed on the dish and incubated in fresh medium without HBV-positive serum. HBV-DNA in the extracellular fractions (EC, for HBV adhesive to cell surface), the intracellular/cytoplasmic fractions (IC), and the intranuclear fractions (IN) was detected by Southern blot at 1 h (left panel; lanes 1–6) and 48 h (right panel; lanes 1′–6′) after infection. HBV-DNA extracted from 5 μl of the HBV-positive serum (S) was included for comparison. (B) HBV-DNA in the extracellular (lane 1), cytoplasmic (lane 2), and intranuclear (lane 3) fractions derived from infected 293EBNA cells was detected using PCR. Arrow, PCR product of HBV-DNA; RC, relaxed-circular form; and DL, double-stranded linear form.

HBV adhesive to the cell surface remained detectable 48 h after infection (Fig. 1A, right panel). No HBV-DNA can be detected in the nuclei using either Southern blot analysis or PCR detection in 293EBNA cells (Fig. 1B). These data indicated that HBV could adhere and enter the cells accompanied by unknown molecular changes of HBV-DNA. After entering the cells, HBV-DNA cannot reach the nuclei and was lost/degraded in the cytoplasm within 2 h.

*Cloning strategy*

Based on our pilot study, we have developed a strategy to identify cellular factors necessary for nuclear entry of HBV genome. Expression of Epstein–Barr nuclear antigen-1 (EBNA-1) in cells allows extrachromosomal replication of plasmids carrying Epstein–Barr virus replication origin region (*OriP*). A cDNA library constructed using this system theoretically expresses a high level of transcripts in EBNA-1 expressing cells [19,20]. A liver cDNA library equipped with the Epstein–Barr virus-based system was first grouped into 300 sets with 50–100 clones per set (Fig. 2A). Three hundred sets of 293EBNA transformants each transfected by one set of mixed cDNA clones were thus established. HBV infection assay was performed for all 300 sets of transformants to search for those with positive nuclear HBV-DNA. For all tests, the nuclear pellets of infected transformants were washed three times with lysis buffer, which induced damage to the microsomal membrane while the inner nuclear membrane was largely intact [21,22]. The third wash was thus used as a contamination control. Two methods were used to detect HBV-DNA. First, 1/500 of the extracted cellular DNA was used for PCR assay (Fig. 2B, upper panel). Alternatively, 1/10 of the extracted cellular DNA was used for PCR assay (Fig. 2B, lower panel). The latter method resulted in a high background (smears) because of a very high amount of cellular DNA in the PCR mixture. Nevertheless, two sets of transformants were tested positive for nuclear HBV-DNA (set 43 and set 147) using the latter method, while no transformant was tested positive using the first method. The assay was repeated if a positive signal appeared in the contamination control. For example, the experiments for sets 79 and 60 (Fig. 2B, upper panel) and for set 13 (Fig. 2B, lower panel) were repeated. The clones in set 43 and set 147 were subsequently sub-grouped into 10 clones per set and the assays were repeated. One sub-set of the transformants derived from set 147 were tested positive for the assay, whereas none of the transformants derived from set 43 was tested positive. Finally, cDNA clones from the positive sub-set of set 147 was tested individually and a single clone, 147-11, was found to be positive for this assay.

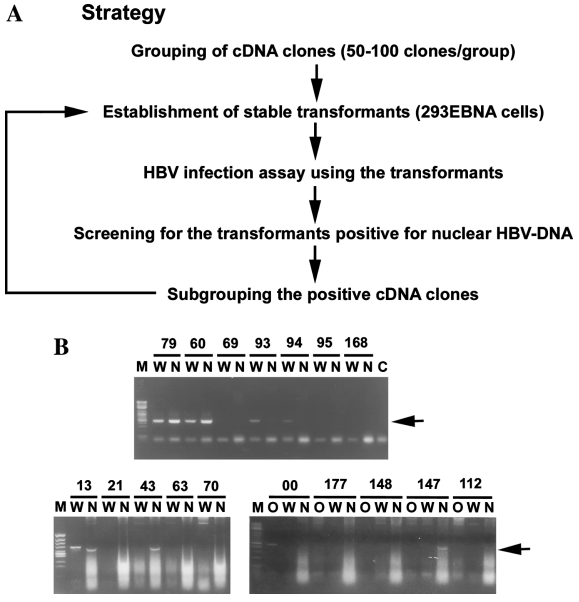


Fig. 2. Strategy to clone a cDNA capable of facilitating nuclear transport of HBV-DNA. (A) Flowchart of the cloning strategy. (B) Two assays were used to detect nuclear HBV-DNA: 1/500 of the extracted nuclear DNA was used for PCR assay (upper panel) or 1/10 of the extracted nuclear DNA was used for PCR assay (lower panel). Before extracting nuclear DNA, the nuclear fractions (N) were washed three times with lysis buffer and the third aliquots of wash solution (W) were used as contamination control. In some of our experiments, the fresh medium (O) used to wash the trypsinized cells was also included for HBV-DNA detection (lower panel, to the right). DNA extracted from un-infected 293EBNA cells was used as a negative control (C) for each batch of PCR assay. Arrow, PCR product of HBV-DNA.

*Clone 147-11 facilitates nuclear entry of HBV-DNA in 293EBNA cells*

Sequence analysis of clone 147-11 revealed a cDNA encoding a fragment of a published mRNA but in anti-sense orientation (GenBank Accession No. NM_020726, nt. 2078–1079). To further verify our result, the nuclear HBV-DNA assay was performed in naïve 293EBNA cells, cells stably transfected with clone 147-11, and cells stably transfected with an engineered clone containing the same cDNA fragment but in sense orientation (Figs. 3A and B). Only clone 147-11 was capable of facilitating nuclear entry of HBV genome (Fig. 4A). To understand whether HBV can replicate in this cell line after successful transport of HBV genome into the nuclei, HBV replication intermediates were detected in all three cells 72 h after HBV infection. No HBV replication intermediates could be found (Fig. 4B). In contrast, transfection of a plasmid, pSV2a-neo-HBV2, containing a head to tail dimer, into 293EBNA cells resulted in efficient HBV replication.

*Pretreatment of culture cells with 1,10-phenanthroline facilitates nuclear entry of HBV-DNA*

Because clone 147-11 contained a cDNA encoding a metallopeptidase-like protein in anti-sense orientation,

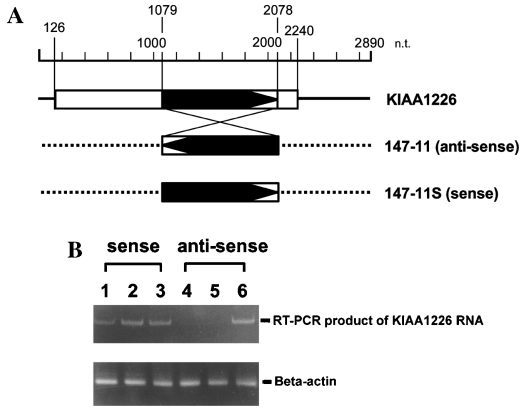


Fig. 3. Sense and anti-sense specific detection of KIAA1226 RNA in 293 cells. (A) Clone 147–11 encoded a fragment of KIAA1226 mRNA in anti-sense orientation. A plasmid, pDR147-11S, containing the same DNA fragment but in sense orientation was constructed. Solid line, the untranslated regions of KIAA1226 mRNA; dotted line, the pDR2 vector. (B) Sense (lanes 1–3) and anti-sense (lanes 4–6) KIAA1226 RNAs were detected using sense and anti-sense specific RT-PCR. Lanes: 1 and 4, naïve 293EBNA cells; 2 and 5, cells transfected with pDR147-11S; and 3 and 6, cells transfected with clone 147-11. As a control, β-actin mRNA was detected in parallel.

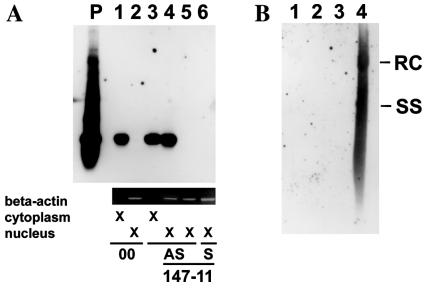


Fig. 4. Detection of nuclear HBV-DNA in HBV-infected 293EBNA cells transfected with clone 147-11. (A) Naïve 293EBNA cells (lanes 1 and 2), cells transfected with clone 147-11 (lanes 3 and 5), and cells transfected with pDR147-11S (lane 6) were subjected for HBV infection assay. HBV-DNA in the nuclear fractions (lanes 2, 4, and 6) was detected by PCR followed by Southern analysis. HBV-DNA in the cytoplasmic fractions (lanes 1 and 3) was also assayed. The nuclear HBV-DNA in cells transfected with 147-11 became undetectable 24 h later (lane 5). As a control, β-actin gene was detected in parallel (lower panel). (B) Detection of HBV replication intermediates in naïve 293EBNA cells (lane 1), cells transfected with clone 147-11 (lanes 2), and cells transfected with pDR147-11S (lane 3) at 72 h after HBV infection. HBV replication intermediates were detected in 293EBNA/147-11 cells 72 h after transfection with pSV2a-neo-HBV2 (lane 4). RC, relaxed-circular HBV-DNA; SS, single-stranded HBV-DNA.

its effect of enhancing nuclear transport of HBV-DNA was presumably a result of decreased metallopeptidase activity. To test this hypothesis, 293EBNA cells were pretreated with 1,10-phenanthroline, a metallopeptidase inhibitor, before inoculation of HBV (Fig. 5A) [25]. It was found that pretreatment of the cells with this drug facilitated nuclear entry of HBV-DNA (Fig. 5A, lane 2). To understand whether nuclear HBV-DNA established in this way was capable of initiating viral replication,

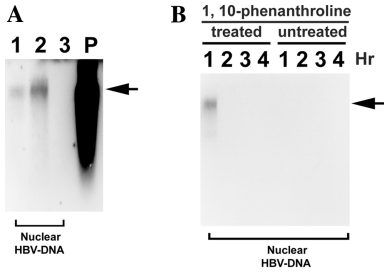


Fig. 5. Nuclear transport of HBV-DNA in the culture cells pretreated with 1,10-phenanthroline. (A) Naïve 293EBNA cells were either pretreated with 1,10-phenanthroline for 30 min (lanes 1 and 2) or untreated (lane 3) before performing HBV infection assay. Nuclear HBV-DNA was detected by Southern analysis at 15 min (lane 1) or 1 h (lane 2) after HBV infection for pretreated cells. HBV-DNA extracted from the same amount (5 μl) of HBV-positive serum used for infection assay was loaded on lane 4. (B) HepG2 cells were pretreated with 1,10-phenanthroline for 30 min before performing HBV infection assay. Intracellular HBV-DNA was detected 1 to 4 h after HBV infection. A small amount of intracellular HBV-DNA can be detected at 1 h after infection in pretreated cells.

HepG2 cells were pretreated with 1,10-phenanthroline before inoculation of HBV. After infection, the cells were incubated for 1–4 h (Fig. 5B). It was found that nuclear HBV-DNA was detectable in pretreated HepG2 cells but not in the untreated cells. Nuclear HBV-DNA became undetectable 2 h after inoculation.

## Discussion

Although many molecules located on the cell membrane have been shown to bind specifically either to the virions or pre-S/S proteins of HBV, successful binding by itself did not lead to viral replication [23]. Presumably, other cellular factors are required to complete the replication cycle. Our initial attempt in this study was to search for such a factor. Unfortunately, after several years of intensive search, we failed to identify a cDNA capable of reconstituting HBV replication cycle in a non-permissive cell line (data not shown). The aim of this study was then changed to pursue a cellular factor capable of bringing HBV genomic DNA into the nuclei, which is theoretically the next step following cell entry of the viruses. Instead of obtaining a cDNA encoding such a factor, we identified a cDNA encoding a metallopeptidase-like protein in anti-sense orientation using this strategy. This anti-sense DNA fragment corresponds to a region of human gene for KIAA1226 protein. Although the function of this protein has not been well characterized, its porcine homolog was known to be a liver metallopeptidase, named endopeptidase 24.16. Several properties of this enzyme were reported, including its hydrolyzing activity of Pz-peptide, angiotensin-binding activity, processing activity of γ-carboxyglutamic acid-containing blood

coagulation factors, and inactivating activity of neurotensin [24–26]. This enzyme was classified as a member of the M3 family of metallopeptidase [27]. Interestingly, a member of this family purified from the microsomal fraction of rabbit liver is capable of hydrolyzing the carboxyl side of paired arginine–arginine residues [25,28]. On the other hand, the porcine endopeptidase 24.16 could target to different subcellular compartments including cytosol and mitochondria by alternative promoter usage [29]. Thus, this human metallopeptidase is potentially capable of hydrolyzing HBV viral proteins such as core protein at either the arginine-rich tail or other sites during its travel from the cell membrane to the nuclei.

In this study, we discovered that expression of the anti-sense RNA of this metallopeptidase-like gene was capable of facilitating nuclear transport of HBV genomic DNA. Consistent with this result, the nuclear transport of HBV genomic DNA can be enhanced by pre-treating the cells with an inhibitor of metallopeptidase. Based on these findings, we speculate that the liver metallopeptidase possibly functions as a barrier preventing HBV genomic DNA from reaching the cell nuclei. This effect could be achieved by over-degradation of HBV core protein. This speculation is supported by the observation that HBV core protein could carry the genomic DNA to dock on the nuclear membrane and thus facilitate its nuclear entry [30]. Other reports supporting the view that attenuation of endopeptidase activity is beneficial to HBV replication came from the studies of HBV X protein. Several groups have discovered that HBV X protein colocalized with cellular proteasome and inhibit the proteasome activity [31–33]. Presumably, attenuation of proteasome activity is one of the ways X protein used to help HBV replication. Recently, it was further demonstrated that inhibition of HBV replication by interferon required proteasome activity, suggesting that proteasome defended the cells from HBV infection [34]. It is not clear at this moment whether the metallopeptidase activity is associated with the cellular proteasome activity. They could be two different components of the same protein degradation pathway. For example, during degradation of HBV viral proteins, the protease activity of metallopeptidase could either be upstream or downstream of that of the cellular proteasome. Alternatively, these two enzymes may belong to two independent protein degradation pathways but both serve to digest HBV viral proteins. Attenuation of these activities, through the interaction of X protein or other unknown mechanisms, helps HBV infection and replication.

After nuclear entry of the genomic DNA, the virus was still unable to replicate, indicating that other cellular factors were still missing. The fact that a head-to-tail dimmer could initiate HBV replication in 293EBNA cells indicated that this cell line harbored most of the factors needed for HBV replication except for those required for transformation from relaxed-circular HBV-DNA to cccDNA. It is pivotal to identify these factors.

In summary, we have identified a cDNA encoding a metallopeptidase-like protein in anti-sense orientation. Expression of the anti-sense RNA facilitates nuclear entry of HBV genomic DNA. We speculate that liver metallopeptidase forms a barrier to prevent nuclear entry of HBV genomic DNA.

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