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Construction and Characterization of a Hepatitis B Virus Replicon*

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Abstract: To establish a replication cellular model of hepatitis B virus (HBV) and determine its application in antiviral drug evaluation, we constructed an expression plasmid which contained 1.3 copies of the HBV genome, and measured the level of viral replication after transient transfection in Huh7 cells. We then observed the effect of antiviral drug administration. 1.3 fold of the HBV(ayw) gene fragment was cloned into pCR2.1 by PCR and restriction endonuclease digestion. The recombinant plasmid was trans ient transfected into Huh7 cells, HBsAg, HBeAg and HBV DNA in supernatant of Huh7 cells were measured by ELISA and real-time PCR respectively; intracellular HBV replicative intermediates and intracellular HBV transcripts were detected by Southern blot and Northern blot respectively. The antiviral effect of adefovir, a novel anti-HBV nucleotide analogue, was evaluated in this cellular model system. The results indicated that a recombinant plasmid of HBV replicon was constructed successfully; the HBV genome carried in plasmid pHBV1.3 could efficiently replicate and be expressed in Huh 7 cells, adefovir could inhibit HBV replication in this cellular model, and the inhibition was dosage-dependent. The conclusion is HBV replicon, which can initiate viral replication efficiently in hepatoma cells, may be a useful tool in the study of HBV replication and antiviral drug.

Key words: Hepatitis B virus; Infectious replicon; Expression vector

Investigation of the expression and replication of the HBV genome as well as the full viral life cycle has been hampered by the lack of an *in vitro* tissue culture system in which HBV is propagated. The HepG2.2. 15 cell line is a putative HBV cellular model and it have been used for antiviral research for many years(9, 3, 7), however, HBV replication in HepG2.2.15 cells is very different from HBV natural replication *in vivo*. HBV covalently closed circular DNA(cccDNA), a key viral replicative intermediates in viral life cycle,is absent in this cell line. Here we constructed a HBV replicon containing a 1.3 × copy of the HBV genome, and established a transient

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transfection cellular model of HBV replication.

1. Materials and methods

1.1 Reagents

Restriction endonuclease *Aat* Ⅱ, *Nsi* Ⅰ, *Bgl* Ⅱ and *Bcl* Ⅰ were purchased from NEB Co., Pfu DNA polymerase, T4 DNA ligase were purchased from Promega Co., Minimal essential medium (MEM), Fetal bovine serum (FBS) and lipofectamine were purchased from Invitrogen Co.

1.2 Plasmids and bacteria

TA cloning vector pCR2.1, competent cells TOP10 and INV110 were purchased from Invitrogen Co. Plasmid pHBV-dimer, which harbors a head-to-tail dimer of the HBV(ayw) genome, was kindly provided by Prof. Mengji Lu(Institute of Virology, Duisburg-Essen University).

1.3 Cells culture

A human hepatoblastoma cell line, Huh7, were maintained in MEM medium supplement with 10% fetal bovine serum(FBS) at 37℃ in a moist atmosphere containing 5% CO_2 in air.

1.4 Primers design

Primers P1 and P2 were designed based on HBV gene sequence (GenBank accession number: AB042282), P1 (1070-1088): 5'-GTATACAAGCTAAGCAGGC-3' and P2(1984-1964):5'-TCGAATAGAAGGAAAGAAGTC-3' for amplifying HBV gene from nt 1070 to nt 1 984.

1.5 Construction of HBV replicon

A HBV replicon was constructed from pHBV-dimer. Firstly, a 915bp fragment (nt 1 070~1 984) of the HBV gene was amplified from pHBV-dimer, and cloned into pCR2.1.A plasmid containing a forward HBV gene fragment was identified and named as pHBV0.3. Secondly, a 2 852bp fragment of the HBV genome was digested from pHBV-dimer with *Aat* Ⅱ and *Nsi* Ⅰ, and ligated with pHBV0.3, which was digested with *Aat* Ⅱ and *Nsi* I; the recombinant plasmid was identified and named as pHBV1.0. Finally, a 921bp fragment of the HBV genome was digested from pHBV-dimer with *Nsi* Ⅰ and *Bgl* Ⅱ, and ligated with pHBV1.0, which was digested with *Nsi* Ⅰ and *Bcl* Ⅰ, the recombinant plasmid was identified and named as pHBV1.3.

1.6 Transient transfection of pHBV1.3

Huh7 cells were seeded at 5×10^5 cells/well in 6-well plates. 24 h later, 5 μg pHBV1.3 plasmids were transfected with 5 μL of lipofectamine reagent according to the manufacturer's instructions. Fresh media were added and maintained for 96h. HBsAg, HBeAg, HBV DNA, and HBV replicative intermediates and transcripts were measured everyday.

1.7 Persistence of HBV replicon

Huh7 cells were transfected with pHBV1.3 as described above; cells were passaged at 4 day intervals. Intracellular HBV replicative intermediates were detected at day 4, 8, 12 and 16.

1.8 HBV replication and expression

HBsAg,HBeAg in supernatant were detected by ELISA according to the manufacturer's instructions. HBV DNA was quantified by real-time PCR. In brief, 200 μL supernatant were treated with 50 μg/mL of DNase Ⅰ for 30min at 37℃, and total DNA was extracted and measured by real-time PCR. Intracellular HBV replicative intermediates and transcripts were measured by Southern blot and Northern blot respectively. In brief, total cellular DNA and RNA were isolated, 5 μg of total DNA and 20 μg of RNA were loaded on a 1.5% agarose gel and formaldehyde agarose gel respectively, and transferred to a nylon membrane, then hybridized with a ^{32}P-labeled HBV DNA probe and analyzed with

storage phosphor system (Cyclone , Packard Co.)

1.9 Antiviral test in Huh7 cells transfected with pHBV1.3

Huh7 cells were transfected with pHBV1.3 as described above. Fresh medium containing 0.01, 0.1, 1 and 10 μmol/L adefovir (Gilead Sciences, USA) were added 24h later, and maintained for 72 h. Total cellular DNA were isolated, intracellular HBV replicative intermediates were measured by Southern blot as described above.

2. Results

2.1 Construction of plasmid pHBV1.3

A 915 bp fragment was obtained by PCR. This fragment was inserted to pCR2.1 vector. Plasmids containing forward fragment were identified by restriction endonuclease analysis and named as pHBV0.3, sequence analysis showed that there was no mutation in the inserted fragment. A 2852 bp fragment was obtained from pHBV-dimer by digestion with *Aat* Ⅱ and *Nsi* Ⅰ, and inserted into pHBV0.3, plasmids containing 1.0 fold of HBV genome was constructed and named as pHBV1.0. A 921 bp fragment was obtained from pHBV-dimer by digestion with *Nsi* Ⅰ and *Bgl* Ⅱ,and inserted into pHBV1.0; plasmid pHBV1.3 was identified by restriction endonuclease analysis. After digestion with *Aat* Ⅱ, There were two fragments of 3 200 bp and 3 856 bp (Fig.1).

2.2 Characterization of HBV replicon in vitro

Plasmids were transfected into Huh7 cells and allowed to replicate for 96 h.Quantities of HBsAg and HBeAg present in the supernatant were measured by ELISA; quantity of HBV DNA in supernatant was determined by real-time PCR; intracellular replicative intermediates and transcripts were analyzed by Southern blot and

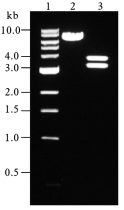

Fig.1. Restriction endonuclease analysis of plasmid pHBV1.3. pHBV1.3 was digested with restriction endonuclease *Aat* Ⅱ, the fragments were separated by electrophoresis in a 1% agarose gel. The fragments are 3 200 bp and 3 856 bp (lane3). Plasmid p HBV1.3 is 7 056 bp(lane2). The molecular weight standard is 1 kb DNA ladder (lane1).

Table 1. HBV products in culture supernatant after transfection with HBV replicon($\bar{X}\pm S$)

Item	24h	48h	72h	96h
HBsAg	0.336±0.048	0.336±0.048	0.336±0.048	0.336±0.048
HBsAg	0.217±0.028	0.217±0.028	0.217±0.028	1.189±0.305
HBV NDA	6.5×10^2	1.2×10^3	2.5×10^3	2.7×10^3

Northern blot respectively. The levels of HBsAg and HBeAg reached a plateau within 72 h. Further incubation did not increase the concentration of HBsAg and HBeAg in the culture supernatant. There was a well consistency of the levels between HBsAg and HBeAg in supernatants (Table 1.). Meanwhile, the levels of HBV DNA in the culture supernatant were 6.5×10^2 copies/mL, 1.2×10^3 copies/mL, 2.5×10^5 copies/mL and 2.7×10^5 copies/mL at 24 h, 48 h, 72 h, and 96 h respectively. Furthermore, the intensity of the hybridization signal, for intracellular replicative intermediates and int-racellular transcripts also reached a maximum within 72 h(Fig.2). The results indicated that the HBV replicon could effectively initiate HBV replication and

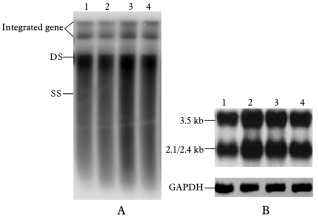

Fig.2. Intracellular HBV replicative intermediates (A) and intracellular HBV transcripts RNA (B) in Huh7 cells transfected with a HBV replicon. A: Southern blot analysis. 5 μg of total DNA was extracted from Huh7 cells transfected with pHBV1.3, and probed by a ^{32}P-labeled HBV-specific DNA probe. Bands corresponding to the expected size of double-stranded (DS) and single-stranded (SS) HBV DNAs are indicated. B: Northern blot analysis. 20 μg of total RNA was extracted from transfected cells, and hybridized with ^{32}P-labeled HBV-and GAPDH-specific probe. Bands corresponding to the expected sizes of 3.5 kb and 2.1/2.4 kb HBV transcripts are indicated. Lane1-4: 24 h, 48 h, 72 h and 96 h post transfection respectively.

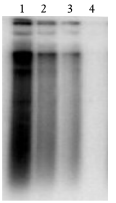

Fig.3. Antiviral test of *in vitro* system transfected with HBV replicon. Huh7 cells were transfected with plasmid pHBV1.3, and then treated with 0.01,0.1, 1 and 10 μmol/L of adefovir for 72h. At the end of the treatment, total DNA was isolated and analyzed by Southern blot. Lane1-4: total DNA from Huh7 cells treated with 0.01, 0.1, 1 and 10 μmol/L of adefovir respectively.

expression of HBV proteins.

2.3 Persistence of HBV replicon in Huh7 cells

Huh7 cells transfected with pHBV1.3 were passaged at an interval of 4 days. Southern blot analysis shown that intracellular HBV DNA replicative intermediates could be found for at least twelve days. The results indicate that the HBV replicon can persist for an extended time, even after cell division.

2.4 Adefovir activity analysis in Huh7 transfected with HBV replicon

A cell-based antiviral assay was used to test the adefovir activity. Huh7 cells were transfected with pHBV1.3, and treated with different concentrations of adefovir on the next day. Southern blot analysis indicated 10 μmol/L of adefovir could completely inhibit HBV replication in Huh7 cells transfected with plasmid pHBV1.3, and the inhibition is dose-dependent(Fig.3).

3. Discussion

The hepatitis B virus(HBV) contains a 3.2-kb, circular, double-standed DNA genome and causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma(5, 10). Because of the limited host range of HBV and the inability to grow it *in vitro*, much of what is known about the HBV life cycle has come from studies of other members of the hepadnaviridae family,especially the woodchuck hepatitis virus (WHV) and the duck hepatitis B virus(DHBV), which infect their native species and, in the case of the DHBV, can be grown in tissue culture(1,11,8).

The HBV cellular model system has been the subject of many studies in the last four decades, unfortunately, so far no group has reported the identification of an appropriate tissue culture system to propagate HBV. This has greatly hampered the development of HBV studies in many fields, such as virology, molecular biology, immunology and antiviral therapy. In an

attempt to overcome this obstacle, several groups transferred the HBV genome into human hepatocytes via plasmids, in which the HBV gene could replicate, express, and even assemble infectious virions (9,12). Because the HBV genome is very compact and all genes are over-lapped with each other, HBV genome were inserted into plasmids in a head-to-hail linkage manner. Sells *et al*(1987) transfected plasmids containing two head-to-tail copies of the HBV genome into the hepto-blastoma cell line HepG2 to obtain the HepG2.2.15 cell line. This popular cell line has been widely used in antiviral studies as a putative HBV cellular model. However, HBV replication in this cellular system is very different from natural HBV replication *in vivo* because the HBV genome is integrated with a cellular chromosome and the covalently closed circular DNA (cccDNA) is absent. Its use is therefore limited to anti-viral and replication studies of HBV.

Recently some group have transfected an overlength HBV genome into hepatocytes for HBV replication and anti-HBV studies. It is well known that the longest HBV transcript is a 3.5 kb mRNA; therefore, several plasmids carrying multiple HBV genomes were constructed , such as 2.0 copies, 1.3 copies, 1.2 copies and 1.1 copies. The 1.3- fold HBV genome contains complete HBV replication units (such as *Enh* Ⅰ, *Enh* Ⅱ, DR1 , DR2, the transcription origin site of the viral pre-genome, promoter and ORFs), and its replication efficiency is markedly higher than that of others(2, 13), therefore, a plasmid vector containing a 1.3-fold HBV genome was preferentially considered. We constructed a HBV replicon based on the plasmid pHBV-dimer which carried two head-to-tail copies of the HBV genome. The HBV 1.3-fold genome was cloned into the pCR2.1 vector by combined PCR and restriction endonuclease digestion. The same enzyme sites for the HBV genome and plasmid pCR 2.1 vector were used, such as *Nsi* Ⅰ and *Bgl* Ⅱ/*Bcl* Ⅰ. The restriction endonuclease *Bgl* Ⅱ and *Bcl* Ⅰ used for inserting the 921 bp fragment into pHBV1.0 have similar function, *Bgl* Ⅱ recognition sequence is A↓GATCT while *Bcl* Ⅰ recognition sequence is T↓GATCA (arrow shown cleavage site), so DNA sequence from pHBV-dimer with *Bgl* Ⅱ digestion could be inserted to linear pHBV1.0 that digested with *Bcl* Ⅰ digestion. But *Bcl* Ⅰ was sensitive to dam methylase, and general bacteria but not INV 110 possess dam methylase, with which the sequence GATC will be methylate sequence G^{m}ATC, so INV 110 were selected to serve as competent cells.

After HBV replicon was transfected into Huh7 cells, HBV genome could effectively replicate and express. There were high levels of HBsAg, HBeAg and HBV DNA in cultures supernatants and high levels of intracellular HBV replicative intermediates and intracellular HBV transcripts. Comparing with HBV replication in HepG2.2.15 cell line, it was much more similar to natural status of HBV infection. Furthermore, HBV replicon was able to replicate in many kind of hepatocytes, such as Huh7 cells, HepG2 cells (data not shown) and primary human liver cells. Therefore, this cellular system has much more advantage in study of antiviral agents and viral replication than that of HBV genome integrated hepatocyte system. The intracellular HBV replicative intermediates could be probed after transfected cells were passaged for three times (12 days), this result indicated HBV replicon could persist for a long time even after cell division. But HBV genome will be progressively lost because of continuous cell division. Furthermore, adefovir, a novel antiviral drug (6, 4) , reduced replication of HBV in this cellular model, it

was shown that this cellular model system might be used for study of anti-HBV agents in future.

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