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Development of cell cultures that express hepatitis B virus to high levels and accumulate cccDNA

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Abstract

Establishment of an infection with hepatitis B virus (HBV) requires synthesis and maintenance of a covalently closed circular DNA (cccDNA) form of the viral genome in the nucleus of host cells. To facilitate investigation of the synthesis of cccDNA, cell cultures were developed that express HBV to high levels. Cell lines derived from hepatoma cells Huh7 and HepG2 were created that express Epstein-Barr virus (EBV) nuclear antigen-1 and a fusion protein of the Tet repressor and Kox1 transcriptional repression domain stably. Transfection of these cell lines with an expression plasmid for HBV that contains the origin of plasmid replication of EBV (oriP) led to increases in the intracellular levels of HBV core protein (~8- to 51-fold) and encapsidated HBV DNA (~3- to 12-fold) in comparison to Huh7 and HepG2 cells. Virion production was also increased (~3- to 12-fold) in these cell cultures and an increase in the level of cccDNA (~3-fold) was observed in the Huh7-derived cell lines. In addition, these cell lines maintained the HBV expression plasmid upon selection and expressed HBV conditionally. Thus, these cell cultures exhibit several features that facilitate study of the synthesis of cccDNA and other aspects of replication of HBV.

Keywords

Hepatitis B virus; cccDNA; secretion; envelope proteins; intracellular amplification; EBNA-1

1. Introduction

HBV is a member of the family *Hepadnaviridae*, which use reverse transcription to replicate their genome. Unlike retroviruses, hepadnavirus replication does not require integration of the viral genome into the host cell genome. Instead, the viral genome is maintained in the nucleus of the infected cell as a covalently closed circular DNA (cccDNA) molecule (Miller and Robinson, 1984). Within the infectious particle, the HBV genome is a partially double-stranded DNA molecule with a relaxed-circular (RC) conformation (Summers et al., 1975). Upon infection, RC DNA is converted into cccDNA (Mason et al., 1983; Tuttleman et al., 1986). The cccDNA molecule is regarded as the template for viral transcription largely because it is the only form of the genome containing a circular minus-strand, which would be necessary for

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transcription of the greater-than-genome length pregenomic RNA (pgRNA). The pgRNA serves as an mRNA for the core protein, the structural subunit of the viral nucleocapsid, and the polymerase (P) protein. Several subgenomic RNAs are also transcribed from cccDNA and encode, in the case of HBV, the large (L), medium (M), and small (S) viral envelope proteins and the viral X protein. Genome replication begins when P and the pgRNA are encapsidated into the viral nucleocapsid. Reverse transcription and DNA synthesis result in the accumulation of nucleocapsids that contain RC DNA in the cytoplasm of infected cells (for a review of hepadnavirus replication see Fields et al., 2007). In the related duck hepatitis B virus, RC DNA-containing nucleocapsids produced within a cell as a result of viral replication can also contribute to synthesis of cccDNA via an intracellular amplification pathway (Wu et al., 1990). Conceptually, conversion of RC DNA to cccDNA through this pathway is the same as conversion of RC DNA to cccDNA during infection. This conversion includes completion of synthesis of plus-strand DNA, removal of P protein from the 5' end of minus-strand DNA, removal of the RNA primer used to initiate synthesis of plus-strand DNA synthesis, removal of one copy of a short terminal redundancy in minus-strand DNA, and ligation of each of the ends of the DNA strands (Gao and Hu, 2007). Recent evidence suggests that completion of the plus-strand DNA is required for removal of P protein from the minus-strand and that this step may occur within nucleocapsids in the cytoplasm (Gao and Hu, 2007; Guo et al., 2007; Guo et al., 2010). Otherwise, little is known about the process of cccDNA formation (reviewed in Nguyen et al., 2008).

Human hepatitis B virus (HBV) infects chronically an estimated 350 million people worldwide and these individuals are at an increased risk of developing liver cancer (WHO, 2001). The predominant drug therapy to treat those infected chronically with HBV are inhibitors of the reverse transcriptase (RT) activity of P, specifically nucleos(t)ide analogue chain-terminators. However, these drugs have not provided a cure. Reports indicate several of these drugs in clinical use and other inhibitors of the reverse transcriptase (RT) activity of P protein do not prevent the formation of cccDNA (Delmas et al., 2002; Kock et al., 2003; Kock and Schlicht, 1993). Additionally, hepadnaviral cccDNA is not eliminated from infected cells by treatment with inhibitors of the RT activity of P protein (Moraleda et al., 1997; Zhu et al., 2001). For this reason, it is believed that persistence of cccDNA over the course of treatment may be responsible for the recurrence of viremia in patients upon cessation of antiviral therapy (Dienstag et al., 1999). Preventing the synthesis of cccDNA and eliminating the existing pool of this molecule in infected cells is likely to be necessary to cure a chronic infection.

Several methods of expressing HBV in cell cultures have been used to study the synthesis and persistence of cccDNA. Infection of primary hepatocytes (human or tupia) and hepatocyte-like HepaRG cells (Gripon et al., 1988; Hantz et al., 2009; Kock et al., 2001), transduction of hepatoma cell lines (Delaney et al., 1999), and cell lines that express HBV stably (Gao and Hu, 2007; Guo et al., 2007) have been useful in studying certain aspects of cccDNA synthesis. However, primary hepatocytes and HepaRG cells are difficult to maintain in culture and producing virus for infection and transduction experiments requires a significant time investment. Additionally, stable cell lines are impractical for genetic analysis of viral contributions to cccDNA synthesis because the template for HBV expression cannot be altered readily. Therefore, existing systems for studying the synthesis of cccDNA could be improved.

It would be desirable to study the synthesis of cccDNA with cell lines that can be cultured easily, accumulate cccDNA to levels that are detected easily, and are able to express genetic variants of HBV. Therefore, cell lines derived from Huh7 and HepG2 hepatoma cells were developed, which can be propagated in culture and are transfected readily. These cell lines express Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) and a second protein, a fusion of the Tet repressor (TetR) and the Kox1 transcriptional repression domain (KRAB) stably. They can be transfected with a plasmid that expresses the HBV pgRNA from the

cytomegalovirus immediate-early (CMV) promoter and contains the origin of plasmid replication of EBV (oriP). Expression of EBNA-1 has been shown to lead to increased gene expression from several viral promoters, including the CMV promoter, when the promoters are located on plasmids containing oriP (Langle-Rouault et al., 1998). Additionally, EBNA-1 is sufficient to facilitate replication of plasmid DNAs containing oriP in eukaryotic cells (Yates et al., 1985). It was reasoned that this plasmid would engender high levels of HBV replication when transfected into these cell lines and could be modified to express genetic variants of HBV. It was found that transfection of these cell lines with versions of this plasmid expressing either wild type or an envelope deficient variant of HBV resulted in increased levels of viral DNA synthesis, including cccDNA, and virion secretion, as compared with Huh7 or HepG2 cells transfected similarly. These oriP-containing plasmids also contain seven tandem copies of the Tet operator sequence (7xTetO) positioned adjacent to the CMV promoter, which directs expression of the viral pgRNA. TetR protein binds to the Tet operator sequence with high affinity in a tetracycline (Tet) responsive manner (Hillen et al., 1982). The TetR-KRAB fusion protein can repress gene expression from the CMV promoter when the TetO sequence is positioned in cis to the promoter (Deuschle et al., 1995). Upon transfection of cells that express EBNA-1 and TetR-KRAB with these oriP plasmids and growth in selection media, cell lines were generated that carry the oriP plasmid and express HBV conditionally.

2. Materials and Methods

2.1 Molecular clones and plasmids

All molecular clones of HBV were derived from the sequence found at GenBank accession number V01460 (subtype ayw; genotype D). The C of the unique EcoRI site (GAATTC) was designated nucleotide position 1.

The plasmid TL1 was derived from the plasmid LJ144 (Haines and Loeb, 2007). Plasmid TL1 has the sequence of 7xTetO positioned immediately upstream of the CMV promoter (GenBank accession number GU937742.1; coordinates 174 669 - 175 249), which directs HBV pgRNA expression. The HBV sequence of this plasmid contains several base substitutions to prevent expression of the viral envelope proteins due to introduction of a stop codon. These mutations are silent in the overlapping P gene. To construct plasmid TL1, a DNA fragment containing 7xTetO (Gossen and Bujard, 1992) was amplified via PCR and cloned into the unique SalI site of plasmid LJ144.

The plasmid TL7 contains the same 7xTetO-CMV-HBV sequence described for plasmid TL1 and also the oriP sequence of EBV and expresses the gene for puromycin resistance. To construct plasmid TL7, a DNA fragment containing EBV oriP (GenBank accession number V01555; coordinates 7 337 - 9 516) was cloned into plasmid LJ144. The 7xTetO sequence was then inserted into the unique SalI site of this plasmid, as described for plasmid TL1. Finally, a DNA fragment containing the SV40 promoter (GenBank accession number EF579804; coordinates 5 235 - 5 243/1 - 291) directing expression of the puromycin resistance gene was cloned into unique NheI and SfoI sites to produce plasmid TL7. The plasmid TL25 is derived from plasmid TL7 and has expression of HBV envelope proteins restored. To generate plasmid TL25, the wild type (wt) HBV envelope gene sequence from pCH-9/3091 (Nassal, 1992) was cloned into plasmid TL7.

The plasmid 2547 has been described (Lam et al., 2004). Briefly, plasmid 2547 encodes EBNA-1 and the G418 resistance gene on a bicistronic mRNA, EBNA-1-IRES-G418R, expressed from the phosphoglucokinase promoter. It also encodes the TetR-KRAB fusion protein (Deuschle et al., 1995) on a second mRNA expressed from the CMV promoter.

2.2 Cell Cultures, transfection, and selection of stable cell lines

Human hepatoma cell lines Huh7 and HepG2 were cultured and transfected using the calcium phosphate precipitation method described previously (for HepG2 see Lewellyn and Loeb, 2007; for Huh7 see Liu et al., 2004) with the exception that Huh7 cultures were supplemented with 5% FBS instead of 10% in some experiments. For transfection, 10^6 cells were seeded to 60 mm plates and grown to ~75% confluence. A precipitate consisting of 9 μ g of HBV expression plasmid and 1 μ g of a plasmid that expresses GFP (provided by Bill Sugden, University of Wisconsin - Madison, USA) was added to 5 ml DMEM/F12 media and placed onto cells. Medium was replaced ~16-20 hrs after transfection, DMEM/F12 media for Huh7 cells and RPMI media for HepG2 cells, and changed as necessary over 4 days when cells were collected. Transfection of the cell line HepG2 was carried out in DMEM/F12 media because RPMI media was incompatible with the calcium phosphate precipitation method of transfection (data not shown).

The cell line HepAD38 (Ladner et al., 1997) was cultured in RPMI media containing G418 (Invitrogen, Carlsbad, USA) at a concentration of 400 μ g/ml and Tet at a concentration of 300 ng/ml (repressed). To express HBV, 10^6 cells were seeded to 60 mm plates and after 48 hrs, ~50% confluence, media lacking Tet was changed onto the cells. Media was changed as necessary over 8 days, when cells were collected.

To produce cell lines that express EBNA-1 and TetR-KRAB stably, Huh7 and HepG2 cells were grown on 100 mm plates to ~75% confluence and transfected with concatenated 2547 DNA. Concatenated DNA was created by digestion of 2547 plasmid DNA with PvuI and ligation at a concentration of ~0.5 μ g/ μ l, to promote intermolecular ligation. After ~48 hrs, cells were suspended and ~10 000 transfected cells were seeded onto a 100 mm plate in the absence of drug. After another ~48 hrs, media containing G418 at a concentration of 600 μ g/ml was added to the cells. Three weeks later, resistant colonies were cloned and the resulting cell lines were maintained in media containing 600 μ g/ml of G418. Each cell line was given a name composed of the name of the parental cell line followed by a unique two character identifier (e.g. Huh7-C3). Cell lines were transfected as described for Huh7 and HepG2 cells with the exception that transfection media was replaced with media containing G418 and Tet at 100 ng/ml (de-repressed).

Huh7-C3 cell lines that carry plasmid TL7 were created using the same method described for creation of Huh7-C3 cells, with the following changes. Huh7-C3 cells were transfected with TL7 plasmid DNA and selected for maintenance of the oriP plasmid with 0.5 μ g/ml of puromycin (Sigma-Aldrich, St. Louis, USA). The resulting cell lines were cultured in the presence of 0.5 μ g/ml of puromycin and induced (de-repressed) with Tet at 100 ng/ml as indicated. Each cell line was given a name composed of the name of the parental cell line followed by the name of the oriP plasmid transfected and a unique single character identifier (e.g. Huh7-C3 TL7 G).

2.3 Isolation of viral DNA and Southern blotting

Upon collection, plates containing cells were washed with HBS-EGTA (2 mM HEPES, 150 mM NaCl, 0.5 mM EGTA) and frozen at -70°C . After being thawed to room temperature, cells were lysed on the plate in 10 mM Tris, 1 mM EDTA, 0.2% NP-40, pH 8.1. The resulting lysate was split for isolation of intracellular encapsidated viral DNA (vDNA), cccDNA, and protein. One portion was adjusted to 50 mM Tris, 1 mM EDTA, 0.2% NP-40 and vDNA was isolated and analyzed by Southern blotting as described (Lewellyn and Loeb, 2007). Briefly, nuclei were pelleted at $16\,000 \times g$ for 3 min at 4°C . Supernatants received CaCl_2 to 2 mM and were incubated with 30 units of micrococcal nuclease (Worthington, Lakewood, USA) for 1.5 hr at 37°C . Samples were then brought to 10 mM EDTA, 0.4% SDS, 100 mM NaCl, 0.4 mg/

ml Pronase (Roche, Basel, Switzerland) and incubated at 37°C for 2 hr. After extraction with phenol, the DNA was precipitated in ethanol. For Southern blotting, DNA was resolved by electrophoresis through a 1.25% agarose gel buffered with 1x TBE (90 mM Tris-borate, 2.5 mM EDTA, pH 8.5), transferred to Hybond-N membrane, and hybridized with a ribonucleotide probe of 351 nucleotides specific for HBV minus-strand DNA with coordinates 3 096 - 3 182/1 - 264. This probe does not detect DNA produced by reverse transcription of most spliced pgRNA molecules, which may be present in these cultures (Abraham et al., 2008). Molecules containing a full-length minus-strand, defined as those migrating from the position of single-stranded (SS) intermediate of HBV DNA synthesis up to the position of RC DNA, were quantified using the Typhoon 8600 Imager and ImageQuant 5.2 software (GE Healthcare, Fairfield, USA). The mass of full-length minus-strand DNA was determined by comparison to known masses of a linear double-stranded fragment of the HBV genome.

For isolation of cccDNA, an alkaline lysis procedure was used (Yang et al., 1996). A second portion of the cell lysate was mixed with an equal volume of a solution of 6% SDS, 0.15 M NaOH and incubated at 37°C for 15 min. The sample was then brought to 0.6 M KOAc, pH 5.0 and centrifuged at $16,000 \times g$ for 3 min at 4°C. The supernatant was extracted with phenol, which results in partitioning of single-stranded DNA into the organic phase at this pH according to Yang et al. (1996), and the DNA in the aqueous phase was precipitated with ethanol. For Southern blotting of cccDNA, samples were treated with 10 ug/ml of RNase A for 10 min at 37°C and electrophoresed in the presence of 0.5 ug/ml of EtBr through a 1.25% agarose gel buffered with TBE. After electrophoresis, the gel was treated with 0.025 N HCl / 1.5 M NaCl to depurinate plasmid DNA and cccDNA prior to Southern blotting and transfer to Hybond-N membrane. HBV DNA was detected and quantified as described for vDNA above. The mass of cccDNA was determined by comparison to known masses of a plasmid containing HBV sequence. In transfected cell cultures, the number of molecules per cell was calculated based on an estimate of $\sim 1.25 \times 10^7$ cells per 60mm plate at the time of collection and transfection of $\sim 20\%$ of the cells. Transfection efficiency was estimated from visualization of GFP positive cells by fluorescent microscopy. In Huh7-C3 TL7 G cell cultures, the number of molecules per cell was calculated based on an estimate of 2.5×10^7 cells per 60mm plate at the time of collection. These estimates for cell numbers per 60mm plate were derived from cell counts performed in several experiments.

2.4 Screening of cell lines for expression of EBNA-1 and TetR-KRAB

To evaluate the expression of EBNA-1, cells were mixed 1:1 with 2x sample buffer (5% SDS, 50 mM Tris, 5% glycerol, 0.04% Bromophenol blue, 2.5% 2-Mercaptoethanol, pH 6.8) and sonicated to produce a total cell lysate. Total cell lysates were electrophoresed through a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Hybond-ECL; Amersham, Buckinghamshire, UK). EBNA-1 protein was detected with mouse anti-EBNA-1 antibodies 3EB7, 3EB9, and 1EB12 (Duellman and Burgess, 2009) and goat anti-mouse antibody conjugated to alkaline phosphatase (Promega, Madison, USA). Bands were visualized by using 5-Bromo-4-Chloro-3-Indoyl Phosphate, p-Toluidine salt (EMD, Gibbstown, USA) and Nitro BT (Promega, Madison, USA).

To determine TetR-KRAB activity, cells were transfected with plasmid TL1 (Fig. 1) and cultured in the absence (pgRNA transcription repressed) or presence (pgRNA transcription de-repressed) of Tet. Four days after transfection, cells were lysed in 0.2% NP-40, 50 mM Tris, 1 mM EDTA, pH 8.3 and vDNA was isolated as described in Section 2.3. Samples were spotted to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, UK) which was submerged in 0.5 M NaOH, 1.5 M NaCl for 2 min, 1 M Tris, 1.5 M NaCl, pH 7.4 for 5 min, and H₂O for 5 min and then dried with a heat lamp. HBV DNA was detected by hybridization with a genome-length riboprobe specific for HBV minus-strand DNA.

2.5 Immunoblotting

To measure the levels of core and GFP protein in transfected cells, a third portion of the cell lysate described in Section 2.3 was mixed with 2x Laemmli buffer (4% SDS, 20% Glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl, pH 7), sonicated briefly, separated by SDS-PAGE through a 15% gel, and transferred to Hybond-ECL membrane. Blocking and antibody binding were performed in SEA BLOCK (Thermo Scientific, Waltham, USA) diluted 1:1 in PBS. Protein was detected with primary antibodies rabbit anti-core (Dako, Glostrup, Denmark) and mouse anti-GFP (Santa Cruz, Santa Cruz, USA) and secondary antibodies goat anti-mouse IRDye 800CW and goat anti-rabbit IRDye 680 (LI-COR, Lincoln, USA). Blots were imaged on a LI-COR Odyssey Imager. Quantification of images was done using LI-COR Odyssey software. Linearity of detection of signal was established in each analysis with a dilution series of one sample.

2.6 Native particle blotting

The level of virions secreted into the culture medium was measured by electrophoresis of concentrated medium through a native agarose gel, blotting to a membrane, and detection of viral DNA as described (Lenhoff and Summers, 1994), with the following changes. Culture media was clarified and then concentrated to ~20 ul either by centrifugation at $14\,000 \times g$ with a 100 kDa Microcon filter device (Millipore, Bedford, USA) or addition of PEG-8000 to 15% (w/v), pelleting by centrifugation and suspension in TE buffer. Samples were then electrophoresed through a 1% agarose gel buffered with TAE (40 mM Tris-acetate, 0.1 mM EDTA, pH 8.4) and transferred to Hybond-N using TNE buffer (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.2). Subsequently, the membrane was washed for 2 min in 0.5 M NaOH, 1.5 M NaCl, for 5 min in 1 M Tris, 1.5 M NaCl, pH 7.4, and for 5 min H₂O. After being dried under a heat lamp, the membrane was hybridized with a riboprobe specific for viral minus-strand DNA as described in Section 2.3.

2.7 Statistical analysis

Statistical comparisons were made using the Wilcoxon signed-rank test between paired data samples. Paired data samples were defined as plates transfected, isolated and analyzed at the same time.

3. Results

3.1 Creation of cell lines that express EBNA-1 and TetR-KRAB stably

To create cell lines that express EBNA-1 and TetR-KRAB stably, Huh7 and HepG2 cells were transfected with plasmid 2547 (Fig. 1) and selected for stable integration in media containing 600 ug/ml of G418. Colonies of resistant cells were observed after three weeks of selection and were expanded to produce cell lines which were considered to be clonal. Total cell lysates were prepared from these cell lines and analyzed for expression of EBNA-1 protein by immunoblotting (Supplemental Fig. 1A). EBNA-1 protein was detectable in 19 of the 26 cell lines derived from HepG2 cells and 12 of the 14 cell lines derived from Huh7 cells. Cell lines expressing EBNA-1 protein were subsequently evaluated for TetR-KRAB activity (Supplemental Fig. 1B). To evaluate TetR-KRAB activity, cells were transfected with either plasmid LJ144 or plasmid TL1. Plasmid LJ144 expresses HBV from the CMV promoter and does not contain the Tet operator sequence. Plasmid TL1 is derived from plasmid LJ144 and contains 7xTetO positioned adjacent to the CMV promoter. Expression of HBV from plasmid TL1 should be repressed in cell lines that express TetR-KRAB when Tet is not present. Cells were transfected and cultured with and without Tet. After four days, HBV vDNA was isolated from cells and the level of HBV minus-strand DNA was determined by dot blot hybridization. Four lines derived from Huh7 cells exhibited reduced levels of HBV minus-strand DNA when

transfected with plasmid TL1 in comparison to plasmid LJ144. This difference was not seen when Tet was added to the culture medium. Two of these cell lines, designated Huh7-H1 and Huh7-C3, and one of the HepG2 cell lines, designated HepG2-K1, were evaluated further for their ability to express HBV from a plasmid that contains oriP of EBV.

3.2 High levels of HBV replication in Huh7-H1, Huh7-C3 and HepG2-K1 cell lines transfected with an expression plasmid containing oriP of EBV

The cell lines Huh7-H1, Huh7-C3 and HepG2-K1 were used in combination with an HBV expression plasmid containing oriP to determine whether high levels of HBV expression were engendered. To this end, Huh7, HepG2, and the derivative cell lines Huh7-H1, Huh7-C3, and HepG2-K1 were transfected with either a plasmid that contained oriP (TL7) or did not contain oriP (TL1)(Fig. 1). The plasmids TL1 and TL7 contain 7xTetO immediately upstream of the CMV promoter, which directs transcription of HBV pgRNA. In addition these plasmids do not express the viral envelope proteins due to a stop codon within the S portion of the envelope gene (Liu et al., 2004). This mutation was introduced to improve detection of cccDNA because the viral envelope proteins have been shown to reduce the accumulation of this molecule during hepadnavirus replication (Gao and Hu, 2007;Guo et al., 2007;Summers et al., 1990). Plasmid TL7 also contains the oriP sequence of EBV. A GFP expression plasmid was co-transfected as a way to evaluate the efficiency of transfection. After transfection, cells were cultured in the presence of 100 ng/ml Tet (de-repressed) for four days and then analyzed for HBV expression. First, the levels of intracellular core protein were determined by immunoblotting and are reported relative to the level in the parent cell line (Fig. 2A). Core protein levels were normalized to GFP protein levels to account for variations in transfection efficiency and sample manipulation. Core protein levels were increased in Huh7-C3 (~8-fold; $p < .004$) and Huh7-H1 (~10-fold; $p < .004$) cell lines compared with Huh7 cells and in the HepG2-K1 cell line (~36-fold; $p < .004$) over HepG2 cells when cells were transfected with plasmid TL7, which contains oriP. When transfected with plasmid TL1, which does not contain oriP, there was no difference between core protein levels in Huh7-C3 and Huh7-H1 cell lines versus Huh7 cells and a relatively small increase in the HepG2-K1 (~3-fold; $p < .016$) cell line compared with HepG2 cells. These results indicated that in cells containing EBNA-1, HBV core protein levels were higher when the expression plasmid contained oriP.

Next, the level of HBV DNA in these cell lines was determined. Intracellular vDNA was isolated and the levels were measured by Southern blotting (Fig. 2B). Again, values were normalized to GFP protein levels and measurements are reported relative to the level in the parent cell line. In Huh7-C3 (~3-fold; $p < .008$) and Huh7-H1 (~3-fold; $p < .004$) cell lines an increase in the level of vDNA over Huh7 cells was observed when cells were transfected with plasmid TL7. An increase in the level of vDNA was also observed in the HepG2-K1 (~9-fold; $p < .008$) cell line compared to HepG2 cells when transfected with plasmid TL7. The total mass of HBV full-length minus-strand DNA isolated from a 60 mm plate of cells was, on average, $\sim 22 \pm 13$ ng and $\sim 37 \pm 26$ ng in Huh7-C3 and Huh7-H1 cultures, respectively, and $\sim 48 \pm 27$ ng in HepG2-K1 cultures. As seen with core protein, the increase in vDNA levels depended on the presence of oriP in the expression plasmid. When transfected with plasmid TL1, which does not contain oriP, the level of vDNA decreased in Huh7-C3 (<2-fold; $p < .032$) and Huh7-H1 (<2-fold; $p < .016$) cell lines and did not change in the HepG2-K1 cell line when compared with Huh7 and HepG2 cells, respectively.

Having found that HBV core protein and vDNA levels were increased in the cell lines expressing EBNA-1, it was of interest to know whether cccDNA was also increased. To determine whether this was the case, cccDNA was isolated and its level was measured by Southern blotting (Fig. 2C). Levels of cccDNA were normalized to the level of GFP protein and are reported relative to the level in the parent cell line. In both Huh7-C3 (~3-fold; $p < .016$)

and Huh7-H1 (~3-fold; $p < .016$) cell lines, cccDNA was increased compared to the level in Huh7 cells when transfected with plasmid TL7, corresponding to 113 ± 23 pg and 239 ± 100 pg per 60 mm plate, respectively, or ~13 and ~27 molecules per cell. This difference in absolute levels reflects a ~2-fold difference in the efficiency of transfection between Huh7-C3 and Huh7-H1. Using this isolation procedure (see Section 2.3), cccDNA was not detected in HepG2 or HepG2-K1 cells. The inability to detect cccDNA in HepG2 cells by Southern blotting was due to high background that obscured visualization of specific bands and may not reflect the absence of this molecule in these cells.

3.3 High levels of virions are produced by Huh7-H1, Huh7-C3 and HepG2-K1 cell lines transfected with an oriP plasmid

To measure the level of virions produced by these cultures, plasmid TL25 was made (Fig. 1). Plasmid TL25 is identical to plasmid TL7, but does not contain a premature stop codon in the envelope gene and thus expresses the envelope proteins. Cells were co-transfected with plasmid TL25 and a plasmid that expresses GFP and cultured in the presence (de-repressed) of Tet for four days at which point they were analyzed. The levels of core protein and vDNA were measured as described (see Section 3.2). Similar to transfection with plasmid TL7, the levels of core protein and vDNA were higher in cell lines expressing EBNA-1 (Fig. 3A and B). Core protein levels were higher in Huh7-C3 (~11-fold; $p < .016$) and Huh7-H1 (~11-fold; $p < .032$) cell lines than in Huh7 cells and higher in the HepG2-K1 (~51-fold; $p < .016$) cell line than in HepG2 cells. Levels of vDNA were increased in Huh7-C3 (~4-fold to 38 ± 7 ng; $p < .016$) and Huh7-H1 (~4-fold to 57 ± 21 ng; $p < .032$) and in HepG2-K1 (~12-fold to 80 ± 31 ng; $p < .032$) relative to the levels in Huh7 and HepG2 cells, respectively.

The production of virions by these cultures was evaluated using native particle electrophoresis and blotting. In this procedure, culture media was concentrated, electrophoresed through a native agarose gel, and transferred to nylon membrane. In these gels, HBV virions have a characteristic mobility and the DNA they contain can be detected by nucleic acid hybridization. Unenveloped nucleocapsids (naked capsids) are also present in the culture medium. Naked capsids have a faster mobility than virions during electrophoresis and these two entities are easily distinguished. The identity of both virion and naked capsids was verified by immunoblotting and immunoprecipitation methods (data not shown). Virion DNA was detected by hybridization with the same riboprobe used to detect vDNA, which is specific for HBV minus-strand DNA (Fig. 3C). An increase in virion production was observed in Huh7-C3 (~12-fold; $p < .016$) and Huh7-H1 (~4-fold; $p < .032$) cell lines, respectively, when compared with Huh7 cells. An increase was also observed in the HepG2-K1 (~8-fold; $p < .016$) cell line compared to HepG2 cells.

In summary, it was found that transfection of the Huh7-C3, Huh7-H1, and HepG2-K1 cell lines with an HBV expression plasmid containing oriP resulted in increased expression of HBV core protein, synthesis of vDNA, and secretion of virions. Additionally, the levels of vDNA that accumulated in these cell lines upon transfection with an oriP plasmid (TL7 or TL25) were similar to or greater than the level observed in the cell line HepAD38, which expresses HBV conditionally from an integrated template (Ladner et al., 1997), after induction for 8 days (24 ± 5 ng; Supplemental Table 1). Thus, these cultures will be useful for studying mechanisms of HBV replication, especially applications requiring high levels of HBV expression.

3.4 Generation of cell lines that conditionally express HBV

Another reason for creating Huh7-C3 and Huh7-H1 cells, which express EBNA-1 and TetR-KRAB stably, was to generate cell lines that replicate and maintain an oriP plasmid and express HBV conditionally. EBNA-1 can facilitate replication and partitioning of plasmids that contain oriP in eukaryotic cells (Yates et al., 1985). TetR-KRAB binds the TetO sequence and can

repress transcription from adjacent promoters (Deuschle et al., 1995). The Huh7-C3 cell line expressed EBNA-1 and exhibited the highest level of TetR-KRAB activity among cell lines tested (Supplemental Fig. 1A and B). For these reasons, the Huh7-C3 cell line was used to determine whether cell lines could be established that carry the plasmid TL7 and express HBV conditionally. Cells were transfected with plasmid TL7 and cultured in the presence of 0.5 μ g/ml of puromycin (HBV expression repressed in the absence of Tet). Five cell lines, Huh7-C3 TL7 B, C, D, G and H, were cloned and examined for the ability to express HBV. In this analysis, cells were cultured for 6 or 8 days, as indicated, in the absence (repressed) or presence (de-repressed) of Tet. To determine whether these cell lines expressed HBV conditionally, vDNA was isolated and analyzed (Fig. 4). In the absence of Tet (repressed), vDNA was not detected. Addition of Tet (100 ng/ml) to the culture medium led to accumulation of vDNA in all five cell lines. The cell line Huh7-C3 TL7 G exhibited a high level of HBV expression and was used in further analyses. TL7 plasmid DNA was detectable in all cell lines, and ranged from <1 to ~5 copies per cell.

3.5 Characterization of conditional HBV replication in the Huh7-C3 TL7 G cell line

To know if the Huh7-C3 cell lines that carried the plasmid TL7 accumulated cccDNA, cells were cultured in the presence (de-repressed) or absence (repressed) of Tet (100 ng/ml) for 12 days at which point vDNA and cccDNA were isolated and analyzed (Fig. 5A). As seen previously, vDNA was detectable only in the presence of Tet. However, cccDNA was detected as early as day 6 and accumulated to an estimated ~140 pg per 60 mm plate or ~two molecules per cell by day 12. This level per cell is lower than that observed in TL7 transfected Huh7-C3 and Huh7-H1 cells (see Section 3.2), but the total mass of cccDNA that accumulated per plate was comparable.

To determine whether HBV expression in Huh7-C3 TL7 G cells could be modulated with Tet, a time course was carried out in which a set of plates was cultured in medium containing Tet (de-repressed) for 12 days. A second set of plates was cultured in the presence of Tet for six days (de-repressed) then switched to medium without Tet (repressed) for an additional six days. Plates were collected daily. After the last time point, vDNA was isolated from all plates and analyzed by Southern blotting (Fig. 5B). In cells cultured in the presence of Tet (de-repressed) for 12 days, the level of vDNA continued to increase until day 12 (reaching ~43 ng per 60 mm plate). The level of vDNA that accumulated in this cell line after 8 days induction (~27 ng) is similar to the level produced in HepAD38 cells after 8 days induction (~24 $\pm$ 5 ng; Supplemental Table 1). In contrast, removal of Tet from the media led to a steady decrease in vDNA levels from ~25 ng on day 7 to ~7 ng on day 12. A similar result was obtained in the cell line Huh7-C3 TL7 D (data not shown).

Lastly, the Huh7-C3 TL7 G cell line was evaluated for maintenance of the oriP plasmid. Cells were cultured for 10 passages (~40 cell generations), during which the level of TL7 plasmid DNA was measured by Southern blotting after each passage. TL7 plasmid was detectable after each passage. However, the level of TL7 plasmid DNA decreased steadily during this time (data not shown). Although this result demonstrated that the Huh7-C3 TL7 G cell line replicated and maintained the oriP plasmid, which would otherwise be lost over ~40 cell generations, it also revealed that the copy number per cell of the plasmid changed with time. It is worth noting that this cell line retained the ability to express HBV conditionally from the oriP plasmid after passage 10, but that the level of HBV DNA that accumulated upon de-repression was reduced when compared to earlier passages (data not shown).

4. Discussion

The mechanism of HBV cccDNA synthesis is not well understood. This lack of understanding is, in part, due to the low number of copies per cell of cccDNA, making it difficult to detect

by methods such as Southern blotting. Several methods based on PCR have been used to detect cccDNA. These methods amplify a region of the HBV genome that is contiguous in the cccDNA form but not other forms of HBV DNA (i.e. RC DNA), which contain a gap in plus- and minus-strand DNA (Addison et al., 2000; Jun-Bin et al., 2003; Kock et al., 1996; Werle-Lapostolle et al., 2004). However, in these procedures amplification from RC DNA can occur and the selectivity of this procedure for cccDNA is lost when the amount of RC DNA in the sample exceeds ~1 pg (~300 000 molecules) (Kock et al., 1996). One goal of this study was to develop cell cultures in which 1) the level of cccDNA was easily detectable by Southern blotting, and 2) can express variants of HBV, in order to use genetic approaches to study the synthesis of cccDNA. Transfection of Huh7 and HepG2 hepatoma cells is used routinely to express genetic variants of HBV. Because cccDNA can be synthesized from RC DNA via an intracellular amplification pathway (Wu et al., 1990), increasing the level of HBV replication in cultures could lead to the accumulation of increased levels of cccDNA.

To increase the level of HBV replication, the cell lines Huh7-C3, Huh7-H1, and HepG2-K1 were created. These cell lines express EBV EBNA-1 and the transcriptional repressor TetR-KRAB stably. EBNA-1 has been shown to increase expression of a reporter gene from plasmids containing oriP by increasing transport of the oriP plasmid to the nucleus of cells and by transcriptional trans-activation of promoters, including the CMV promoter (Kennedy and Sugden, 2003; Langle-Rouault et al., 1998). Additionally, de novo replication and partitioning of oriP plasmids in these cell lines due to the presence of EBNA-1 could contribute to the level of gene expression. Transfection of the cell lines Huh7-C3, Huh7-H1, and HepG2-K1 with a plasmid that contains oriP and expresses an envelope-deficient variant of HBV (TL7) led to increased levels of HBV core protein and vDNA when compared to Huh7 and HepG2 cells transfected in a similar manner (Fig. 2A and B). A similar result was also seen when cells were transfected with an oriP plasmid that expresses wild-type HBV (TL25), in which case virion production was increased as well (Fig. 3). In contrast, no difference in the levels of expression of HBV was observed when cells were transfected with a similar plasmid that did not contain oriP (TL1). These results indicate that EBNA-1 positively affects expression of HBV in these cell cultures in a manner that depends on the presence of oriP on the plasmid.

Coincident with the increase in levels of core protein and vDNA, cccDNA was also increased in Huh7-C3 and Huh7-H1 cells when transfected with the plasmid TL7 (Fig. 2C). One possible explanation for this observation is that a higher level of HBV expression is leading to increased cccDNA synthesis. In contrast to Huh7 cultures, cccDNA could not be detected in HepG2-K1 or HepG2 cells due to a high signal background during Southern blotting (see Section 3.2). The reason for this signal background is unknown but it did not appear when cccDNA was isolated from HepAD38 cells, a cell line derived from HepG2 cells which expresses HBV conditionally from an integrated genome (Supplemental Table 1). This suggests that the signal background is specific to transfected HepG2 cultures and may represent products from degradation of the transfected plasmid.

In addition to producing higher levels of HBV replication after transfection, the cell lines Huh7-C3 and Huh7-H1 are able to maintain an oriP plasmid and express HBV conditionally. This was demonstrated by generation of cell lines derived from Huh7-C3 cells that carried the oriP plasmid, TL7, and expressed HBV DNA upon de-repression with Tet (Fig. 4). In one of these cell lines, Huh7-C3 TL7 G, the level of vDNA continued to increase for 12 days to ~43 ng per 60 mm plate and cccDNA accumulated to ~2 molecules per cell upon induction with Tet (Fig. 5). Although these levels are not as high as observed in analyses using transient transfection, such cell lines could be useful for studying the kinetics of HBV replication, in spite of the observation that the copy number per cell of plasmid TL7 was not stable over ~40 generations. It is not known why the copy number per cell of the plasmid TL7 decreases with passaging. One possible explanation is that there is a negative selective pressure against a gene product

expressed from plasmid TL7. Thus, in the absence of absolute repression of transcription from the plasmid, cells with fewer copies of the plasmid may have a growth advantage, leading to a reduction in the copy number per cell with time. Several reports have described the use of stable cell lines that conditionally express HBV to investigate cccDNA synthesis (Gao and Hu, 2007; Guo et al., 2007). In these studies the template for expression of HBV was integrated into the host cell genome. An advantage of the cell cultures reported here is the relative ease of establishing cell lines that express HBV conditionally; 5 of 5 Huh7-C3 TL7 cell lines that could be expanded under puromycin selection carried the oriP plasmid and expressed HBV conditionally in contrast to 1/118 cell lines for HepAD38, one of the cell lines used in the studies described above (Ladner et al., 1997).

Thus, the high levels of HBV expression and the ability to express genetic variants make these cell cultures useful for studying mechanisms of HBV replication, including cccDNA synthesis. Additionally, these cultures pose an efficient means to generate cell lines that carry an oriP plasmid and express genetic variants of HBV conditionally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Linear representation of plasmids. The plasmid 2547 expresses the TetR-KRAB fusion protein from the human cytomegalovirus immediate-early (CMV) promoter as well as EBNA-1 and the G418 resistance gene (Neo) on a bicistronic mRNA from the phosphoglucokinase (PGK) promoter. Plasmid TL1 expresses an envelope deficient variant of HBV (due to a stop codon in the envelope gene; gray line) from the CMV promoter and contains seven tandem copies of the tetO sequence (7xtetO). Plasmids TL7 and TL25 contain the genetic elements found in TL1 as well as the EBV origin of plasmid replication (oriP) and they express the puromycin resistance gene (Puro) from the SV40 promoter. Plasmid TL7 expresses the same envelope deficient variant expressed from TL1. Plasmid TL25 is derived from TL7 and expresses wt HBV. IRES, internal ribosome entry site. Stop, indicates position of stop codon introduced into HBV envelope gene.

Fig. 2.

Accumulation of HBV core protein, vDNA and cccDNA in transfected cell lines. Huh7, HepG2 and derived cell lines, Huh7-C3, Huh7-H1, and HepG2-K1, were transfected with either plasmid TL1 or TL7 and analyzed for expression of HBV. (A) Immunoblotting of total cell lysates for HBV core and GFP proteins. Rabbit anti-HBcAg (Dako) and mouse anti-GFP (Santa Cruz) antibodies were used to detect HBV core and GFP proteins, respectively. Both proteins were visualized and quantified on the same membrane using the LI-COR Odyssey. (B) Southern blot analysis of vDNA. HBV DNA was detected using a probe specific for nucleotide coordinates 3096-3182/1-264 of the minus-strand. Molecules containing full-length minus-strand DNA [fl (-) vDNA], defined as those migrating from SS DNA to RC DNA (indicated by bracket), were measured for comparison. (C) Southern blot analysis of HBV cccDNA. The same probe described for vDNA was used to detect cccDNA. A marker for cccDNA was generated by ligating linear monomers of the HBV genome (marker). vDNA isolated from Huh7-H1 cells transfected with plasmid TL7 also was included (TL7 vDNA). Lanes containing 'marker' and 'TL7 vDNA' samples are shown at a different grayscale than lanes containing cccDNA isolated from transfected cells. Histograms show the levels of HBV core protein (A), vDNA (B), and cccDNA (C) in each of the cell lines as fold of the level in the parental cell line (hashed gray line) after normalization to GFP protein. Error bars represent one standard deviation.

Fig. 3.

Virions produced by transfected cell lines. Huh7, HepG2 and derived cell lines, Huh7-C3, Huh7-H1, and HepG2-K1, were transfected with plasmid TL25 and analyzed for expression of HBV. HBV core protein (A) and vDNA (B) were analyzed as described in Figure 2. (C) Native particle blot analysis of virions secreted into the culture medium. Culture medium was concentrated, electrophoresed through a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a probe specific for viral minus-strand DNA, used to detect vDNA in (B). This method detects HBV DNA associated with virions and naked capsid particles (Capsids), which are also released into the culture medium. Histograms show the levels of HBV core protein (A), vDNA (B), and secreted virions (C) in each of the cell lines as fold of the level in the parental cell line (hashed gray line) after normalization to GFP protein. Error bars represent one standard deviation.

Fig. 4.

Conditional HBV replication in Huh7-C3 TL7 cell lines. Huh7-C3 cells were transfected with plasmid TL7 and cultured under selection with puromycin. Drug resistant cell lines Huh7-C3 TL7 B, C, D, G and H were screened to determine if they carried TL7 plasmid DNA and if they accumulate HBV DNA. Cells were cultured either in the presence (+) or absence (-) of Tet for the indicated number of days, at which point cells were collected and analyzed. Plasmid DNA (top panel) and vDNA (bottom panel) were detected by Southern blotting. TL7 plasmid DNA prepared from *E. coli* (marker) was included for comparison.

Fig. 5.

Characterization of HBV DNA synthesis in Huh7-C3 TL7 G cells. The cell line Huh7-C3 TL7 G was investigated for accumulation cccDNA and the ability to modulate HBV DNA synthesis with Tet. (A) Southern blot analysis of vDNA (left panel) and cccDNA (right panel) isolated from Huh7-C3 TL7 G cells cultured for 12 days in the presence (+) or absence (-) of Tet, as indicated. Circular monomers of the HBV genome were included for comparison (marker). (B) Southern blot analysis of vDNA in Huh7-C3 TL7 G cells. To investigate modulation of viral DNA synthesis with Tet, duplicate sets of plates containing Huh7-C3 TL7 G cells were generated. One set was cultured in the presence of Tet for 12 days (left panel; black diamonds). The second set of plates was cultured in the presence of Tet for six days at which point Tet was removed from the culture medium and cells were cultured for an additional six days (right panel; gray triangles). Plates were collected daily and vDNA was analyzed by Southern blotting. The line graph shows the total mass of vDNA (ng) isolated from a 60 mm plate over the 12-day time course.