Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells

O. Hantz,1,2 R. Parent,1,2 D. Durantel,1,2 P. Gripon,3 C. Guguen-Guillouzo3 and F. Zoulim1,2,4

1INSERM, U871, 69003 Lyon, France
2Université Lyon 1, IFR62 Lyon-Est, F-69008 Lyon, France
3INSERM, U522, Hôpital Pontchaillou, F-35033 Rennes, France
4Hospices civils de Lyon, Hôtel Dieu, Service d’Hépatologie, Lyon F-69002, France

The recently described hepatic cell line HepaRG is the sole hepatoma cell line susceptible to hepatitis B virus (HBV) infection. It provides a unique tool for investigating some unresolved issues of the virus’ biology, particularly the formation of the viral mini-chromosome believed to be responsible for the persistence of infection. In this study, we characterized the main features of HBV infection: it is restricted to a subpopulation of differentiated hepatocyte-like cells that express albumin as a functional marker and represents around 10% of all differentiated HepaRG cells. Infection may persist for more than 100 days in cells maintained at the differentiated state. Even though infected cells continued to produce infectious viral particles, very limited or no spreading of infection was observed. Low genetic variation was also observed in the viral DNA from viruses found in the supernatant of infected cells, although this cannot explain the lack of reinfection. HBV infection of HepaRG cells appears to be a very slow process: viral replication starts at around day 8 post-infection and reaches a maximum at day 13. Analysis of viral DNA showed slow and inefficient conversion of the input relaxed circular DNA into covalently closed circular (CCC) DNA, but no further amplification. Continuous lamivudine treatment inhibited viral replication, but neither prevented viral infection nor initial formation of CCC DNA. In conclusion, HBV infection in differentiated HepaRG cells is characterized by long-term persistence without a key feature of hepadnaviruses, the so-called ‘CCC DNA amplification’ described in the duck hepatitis B model.

INTRODUCTION

Hepatitis B virus (HBV) is a major cause of liver disease worldwide. Although the molecular biology of HBV has been extensively studied (Seeger & Mason, 2000), understanding the complete viral life cycle has somehow been hampered by the lack of a robust in vitro system. Due to the lack of cellular functions that may trigger early stages of the infection process, human hepatoma cell lines are usually refractory to viral infection. Until recently, only primary cultures of human (and Tupaia belangeri) hepatocytes could be efficiently infected to support a complete cycle of viral replication (Galle et al., 1994; Gripon et al., 1988a; Köck et al., 2001). Unfortunately, the scarcity of human liver cells limits the use of this model. Alternatively, the main mechanisms of HBV replication have been elucidated in the duck hepatitis B virus (DHBV) animal model (Mason et al., 1980; Tuttleman et al., 1986a) using duck hepatocytes in primary cultures. However, infection with avian hepadnaviruses is generally not associated with liver disease and hepatocellular carcinoma. Moreover, DHBV clearly differs from mammalian hepadnaviruses: the DHBV genome is shorter and lacks the regulatory X gene (Bouchard & Schneider, 2004) that is crucial for replication of the Woodchuck hepatitis B virus (Zoulim et al., 1994). The organization of the gene coding for the envelope differs by two proteins (S and L) for DHBV versus three (S, M and L) for the mammalian viruses. Finally, transcriptional regulation of avian and mammalian hepadnaviruses is also distinct (Beckel-Mitchener & Summers, 1997; Tang et al., 2001; Tang & McLachlan, 2002).

Despite a similar overall replication strategy, HBV and DHBV may differ in several aspects and our understanding...
of the complete HBV life cycle remains to be completed. Gripon et al. (2002) clearly demonstrated that HepaRG cells constitute the first human hepatoma cell line susceptible to HBV and may provide a new relevant in vitro model of HBV infection. However, initial stages of viral replication including the formation of the CCC DNA pool and further production of viral particles in this cellular system remain to be elucidated. The goals of the current study were: to establish standardized and reproducible conditions for infection of HepaRG cells, to characterize their viral progeny, and to analyse the formation and fate of viral CCC DNA in infected cells.

**METHODS**

**Cell culture and HBV infection.** HepaRG cells were cultured as described previously by Gripon et al. (2002) in Williams E medium supplemented with 10% fetal calf serum, 50 U penicillin/streptomycin (Gibco-Invitrogen) ml⁻¹, 5 µg bovine insulin ml⁻¹ and 5×10⁻⁷ M hydrocortisone hemisuccinate (Roche Diagnostics and Boehringer Mannheim, respectively). The cell cultures were maintained in a 5% CO₂ atmosphere at 37 °C. During the proliferation phase, culture medium was renewed every 2 days. For differentiation and infection, cells were maintained for 2 weeks in standard medium and then for at least 2 more weeks in medium supplemented with 1.8–2.0% DMSO (Sigma). Growth factors (human, recombinant) epidermal growth factor (EGF) (5–25 ng ml⁻¹; Peprotech Inc), hepatocyte growth factor (HGF) (25 ng ml⁻¹; Sigma), tumour growth factor alpha (TGF-α) 20 ng ml⁻¹ and tumour growth factor beta (TGF-β) (2 ng ml⁻¹) (Gibco-Invitrogen) were added to the Williams E medium in combination with 20 mM sodium pyruvate (Talarmin et al., 1999) where indicated. Oncostatin M and retinoic acid were from Sigma.

HBV inoculum was prepared from freshly collected supernatants of the HBV stably transfected HepG2 cell line, clone 2.2.15 (Sells et al., 1987). Viral particles were concentrated from supernatant by ultracentrifugation in Ti 50.2 Beckman rotor at 45 000 r.p.m. for 1 h at 4 °C. The pellet was resuspended in Williams E medium at a concentration of 10¹⁰ genome equivalents ml⁻¹. Inoculation of cells was performed with 100–200 genome equivalents per cell in culture medium containing 4% PEG 8000 (Sigma) for 16 h at 37 °C or six-well plates. At the end of the incubation period, cells were briefly washed once with trypsin, then three times with medium and cultured in Williams E medium containing DMSO as indicated. Alternatively, cells in 75 or 175 cm² flasks were infected as described above and then trypsinized, washed by two successive centrifugations and replated in 12-well collagen I-coated plates (BD Biocoat; Becton Dickinson) at a density of 1×10⁶ cells per well.

Cell viability was measured by both neutral red and MTS (CellTiter 96 Aqueous One Solution Cell proliferation assay; Promega) tests.

**Detection of HBs and HBe antigen (Ag).** Hepatitis B surface (HBsAg) and e (HBeAg) Ags were detected in the culture medium using ELISA kits and following instructions obtained from Bio-Rad.

**Analysis of viral DNA.** HBV DNA from culture supernatants was detected by a specific dot-blot hybridization assay with a full-length HBV genomic DNA probe labelled with [α-³²P]CTP as described previously (Lambert et al., 1990). Quantitative analyses were carried out using a PhosphorImager SI system. The limit of detection of viral DNA by this assay is 100 pg ml⁻¹ (equivalent to 5 pg HBV DNA per 50 µl sample on a dot-blot point) as confirmed by Amplipcr HBV Monitor assay (Roche Diagnostic). For PCR analysis and sequencing, DNA was extracted from 400 µl clarified supernatant using the MasterPure Complete DNA purification kit from Epicentre.

For intracellular DNA analysis, cells were trypsinized, washed once with medium, then with PBS, and lysed in 10 mM Tris buffer pH 8 containing 10 mM EDTA and 0.5% SDS. Total DNA was extracted by phenol extraction after proteinase K digestion as described previously (Borel et al., 2001). Alternatively, protein-free CCC DNA was separated from protein-linked viral DNA [relaxed circular (RC), linear (L) and single-strand (SS) intermediates] by KCl precipitation (Summers et al., 1990) as modified by Guo & Hu (2007). Genomic DNA or CCC DNA preparations (10 µg) as well as an equivalent volume of the corresponding replicative intermediate DNA preparations were analysed by electrophoresis on a 1.1% agarose gel, followed by Southern blot analysis (Borel et al., 2001). Linear HBV DNA (25 ng) obtained by EcoRI digestion of plasmid and DNA extracted from the stably transfected HepG2.2.15 cell line were used as standards.

Membranes were analysed by autoradiography and scanned with the PhosphorImager System SI. Quantitative image analysis was performed with ImageQuant software (Molecular Dynamics).

**Analysis of viral RNA.** Total RNA was isolated by using the Trizol reagent (Gibco-Invitrogen) and treated with RNase-free DNase I. Total RNA (10 µg per sample) was glyoxalated and separated on 1% agarose gels, using NorthernMax-Gly kit (Ambion). After capillary transfer to Hybond-N⁺ membranes (Amersham), RNA was fixed by UV cross-linking and hybridized with a 32P-labelled genomic HBV probe as above. Hybridizations were carried out at 42 °C and washed at 65 °C with 2×, 1× and 0.5× saline sodium citrate buffer supplemented with 0.1% SDS, respectively.

**PCR amplification of HBV DNA and direct sequencing.** Cells and supernatants were collected and DNA extracted as described above. One microgram total cellular nucleic acid or the extract corresponding to 200 µl cell supernatant was used as DNA template for PCR. A 1325 nt fragment (nt 2269–400) on HBV genome GenBank accession no. U95551) starting from the terminal protein domain of the polymerase to the middle of the S gene (hbvtp2: 3'-GGAGTGTGGATTCGCACTCCT-5' and hbwp2: 3'-TATGATAAAAAAGCCGCAGACAT-CCA-5') was amplified using the Expand High Fidelity PCR system (Roche) as described previously (Durantel et al., 2004). After amplification, 15 µl each sample was separated on a 1% agarose gel and visualized by ethidium bromide staining. Direct sequencing of PCR products was performed using the same primers. Real-time PCR for total HBV DNA and CCC DNA was performed as described previously (Werle-Lapostolle et al., 2004).

**Sucrose-gradient density centrifugation and electron microscopy.** Culture medium was centrifuged to remove cellular debris. Afterwards, the supernatant underwent ultracentrifugation at 25 000 r.p.m. in a SW28 rotor (Beckman Coulter) for 16 h at 4 °C. The resulting pellet was resuspended in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 150 mM NaCl, the suspension was then added to SW41 tubes (Beckman Coulter) containing 10 ml 10–20% sucrose gradient, the samples were then ultracentrifuged at 35 000 r.p.m. for 16 h at 4 °C. After sucrose-gradient density centrifugation, the resulting pellet was resuspended in H₂O and the suspension was deposited onto Formvar-coated, carbon-stabilized copper grids (200 mesh), negatively stained with 2% sodium phosphotungstate (pH 7.0) then observed under a transmission electron microscope 1200EX (JEOL).

**Immunofluorescence studies.** For the co-detection of albumin and HBV proteins, cells were cultured on collagen I-coated glass slides (BD Biocoat; Becton Dickinson), fixed with 2% paraformaldehyde in PBS and permeabilized with 0.1% saponin in PBS. The cells were stained with rabbit anti-albumin (Dako) and mouse monoclonal anti-preS (Chemin et al., 1991) and anti-HBs (a gift from M. A. Petit, INSERM.
U871, Dardilly, France). Antibodies were diluted in PBS containing 0.1% saponin and 3% BSA. After washing in the same buffer, bound antibodies were labelled with secondary antibodies, Alexa Fluor 546 nm goat anti-mouse and Alexa Fluor 488 nm goat anti-rabbit (Molecular Probes). Following several additional washes, slides were mounted with fluorescence mounting medium (Dako) and observed under a Nikon Inverted IF microscope (ECLIPSE TE 200-E).

RESULTS

HBV infection of HepaRG cells

As reported by Gripon et al. (2002), HBV susceptibility is strongly dependent on HepaRG cells’ differentiation status: only differentiated cells obtained by treatment of confluent cultures with 2% DMSO for at least 15 days can be efficiently infected. However, in our experience, the use of 2% DMSO treatment during the differentiation phase was associated with variable cell death, leading to great variations in HBV replication levels from one experiment to another. In preliminary experiments, confluent cells were treated with other compounds known to induce hepatocyte differentiation such as EGF (20 ng ml$^{-1}$) or HGF (10 ng ml$^{-1}$), TGF-$\alpha$ (20 ng ml$^{-1}$), oncostatin M (10 ng ml$^{-1}$), retinoic acid (5 $\mu$M) alone or in combination. No clear changes could be observed regarding the cellular morphology of HepaRG cells cultured in these different conditions, but only DMSO-treated HepaRG cells were found susceptible to HBV infection, while other compounds alone did not induce susceptibility to viral infection. Combination of EGF, HGF or TGF-$\alpha$ with different concentrations of DMSO ranging from 1.0 to 2.6% during the 2 weeks of differentiation process, and/or after HBV infection, were also tested. As shown in Fig. 1, DMSO concentration ranging from 1.5 to 2.5% increased cell susceptibility but was associated with a dose-dependent variable toxic side effect despite the addition of EGF and very variable results from one experiment to another. Among the various conditions tested, addition of 1.8% DMSO and EGF (10 ng ml$^{-1}$) to the culture medium during the differentiation phase gave the best results in terms of cell viability, culture stability and reproducibility of HBV infection.

HBV infection was performed with a standardized inoculum obtained from supernatants of HepG2.2.15 cells in the presence of 4% PEG. Results of a typical infection, representative of several experiments, are shown in Fig. 2. Active viral replication was evidenced by secretion of HBsAg and HBeAg as well as virion-associated HBV DNA in culture supernatant (Fig. 2a). Intracellular viral DNA was detected by Southern blot analysis (Fig. 2b): the Hirt extraction procedure was used to selectively isolate protein-linked viral replicative DNA and protein-free viral DNA. Active viral replication was evidenced by a dramatic increase of viral replicative forms including RC, L and SS DNA from days 3 to 13 post-infection. In protein-free DNA fractions, progressive accumulation of CCC DNA is observed along with a second species likely to correspond to the deproteinized RC DNA as described by Gao & Hu (2007). Quantitative analysis of total and closed circular HBV DNA by real-time PCR performed on five independent experiments showed that only 1–5% of the RC DNA internalized at day 1 post-infection entered the nucleus and was converted into deproteinized RC and CCC DNA. Two to five copies of CCC DNA per infected cell were detected at day 14 post-infection (Table 1). In long-term experiments, one to two copies of CCC DNA may persist in infected cells for more than 50 days. Viral RNA (Fig. 2c) appearing at day 3 post-infection, increased until day 6 then remained constant or fluctuated, paralleling the levels of CCC DNA present in infected cells.

Virus purified from HepG2.2.15 cells served as both unlimited and standardized source of inoculum. High m.o.i. [100 virus genome equivalents (vge) per cell] in the presence of 4% PEG was required to obtain high levels of infection. Fig. 3 shows that increasing the amount of virus resulted in higher levels of HBsAg production in cell supernatants (Fig. 3a) and in greater numbers of HBsAg-expressing cells (Fig. 3b). However, a maximum of 20% of cells can be infected regardless of the amount of virus used (>200 vge per cell). Infection was restricted to a subfraction (no more than 40%) of the hepatocyte-like cells expressing albumin as demonstrated by the co-detection of albumin and HBsAg (Fig. 3c) and was never detected in larger epithelial cells. During long-term cultures (30–100 days), the percentage of infected cells either slowly decreased or remained constant. This result

![Fig. 1. Infection of HepaRG cells is strongly dependent on the DMSO concentration in culture medium and is improved by the addition of EGF. Cells at confluence were differentiated for 2 weeks by the addition of EGF (10 ng ml$^{-1}$) and DMSO at different concentrations as indicated. HepaRG cells were then infected in the same conditions with a standard inoculum and then cultured in the same condition for 10 days. HBV DNA excreted into the culture supernatant was quantified by dot-blot hybridization. Relative infection of HepaRG cells was assessed by measuring the ratio of HBV DNA in infected culture conditions to that obtained in the presence of 2% DMSO without EGF as described previously by Gripon et al. (2002). Cell viability (●) was assessed by a neutral red assay (arbitrary units). Values are the means of four different independent experiments. Bars represent SEM.](http://vir.sgmjournals.org)
confirmed that only a limited proportion of HepaRG cells can be infected and can support complete viral replication. Super infection of the cells 2 weeks after the initial infection failed to increase the number of infected cells (data not shown), confirming that, in these culture conditions, no viral spread occurred.

**Infected HepaRG cells produced complete infectious viral particles with stable genetic material**

HepaRG cells were infected as described above and maintained for more than 2 months without splitting. Cell supernatants were collected and analysed for the presence of HBsAg and HBV DNA. As shown in Fig. 2, production of both HBsAg and HBV DNA reached a maximum between days 9 and 12 post-infection then decreased and fluctuated, but remained detectable for 100 days post-infection. To confirm that virions produced by HepaRG cells were infectious, supernatants collected between days 10 and 30 post-infection were pooled and concentrated by ultracentrifugation. An aliquot of this pool was further purified by sucrose-gradient centrifugation and examined by negative-staining electron microscopy. In this sample preparation, few 22 nm particles, tubules and 42 nm Dane particles could be observed (Fig. 4a).

**Table 1. Intracellular CCC DNA and extracellular HBsAg and HBV DNA levels in HepaRG-infected cells**

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Treatment</th>
<th>CCC DNA/infected cell*</th>
<th>HBV DNA (vge×10⁶ ml⁻¹)</th>
<th>HBsAg (ng ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>None</td>
<td>2.38 (± 0.6)</td>
<td>7 (± 0.8)</td>
<td>58 (± 12)</td>
</tr>
<tr>
<td>14</td>
<td>Lamivudine</td>
<td>2.8 (± 0.6)</td>
<td>0.8 (± 0.08)</td>
<td>52 (± 12)</td>
</tr>
<tr>
<td>25</td>
<td>None</td>
<td>0.97 (± 0.3)</td>
<td>3 (± 0.3)</td>
<td>31 (± 10)</td>
</tr>
<tr>
<td>25</td>
<td>Lamivudine</td>
<td>1.01 (± 0.3)</td>
<td>0.2 (± 0.04)</td>
<td>27 (± 10)</td>
</tr>
</tbody>
</table>

*CCC DNA/infected cell was calculated by the quantification of CCC DNA by real-time PCR in the whole cell population and estimation of the numbers of infected cells using immunofluorescence detection of HBsAg.
Southern blot analysis of viral DNAs corresponding to this purified virus showed the presence of RC DNA and linear DNA in proportions very similar to those found in HepG2.2.15-derived virions (Fig. 4b). The pool of concentrated particles was used to infect HepaRG cells in standard conditions, but with a lower m.o.i. (10–20 vge per cell), and HBV replication was detected by the analysis of viral RNA in infected cells. As shown in Fig. 4(c), viral RNA was detected 13 days after infection with HBV virions derived from HepaRG cells. To check if genome variation occurred during viral replication, fragments of 1325 nt corresponding to the preS/S regions were PCR amplified and directly sequenced from viral DNA in the cell supernatants taken at days 10, 15, 20 and 35 post-infection. All amplified sequences were similar to that of the original HepG2.2.15-derived HBV inoculum (HBV subtype ayw; GenBank accession no. U95551) with minor genetic variations (Table 2).

**CCC DNA is synthesized from input viral RC DNA**

A key step in the HBV infection process is the formation of CCC DNA from the virus-associated RC DNA. Thus, we performed a time-course experiment to follow the conversion of the input viral DNA into CCC DNA. To exclude *de novo* CCC DNA synthesis by nucleocapsid recycling to the nucleus after reverse transcription of the pre-genomic RNA, the study was performed in the absence or presence of lamivudine, a potent inhibitor of HBV reverse transcriptase. Our conditions were a high concentration of lamivudine (25 μM) that was added continuously, starting on the day of infection. It almost completely inhibited viral replication as measured by the amount of excreted HBV DNA (Fig. 5a) without affecting the expression of HBsAg and the percentage of infected cells (Fig. 5b). Southern blot analysis of protein-linked viral DNA (Fig. 5c) showed that RC and L DNA species, likely corresponding to the input viral DNA, were progressively...
eliminated when viral replication was inhibited by lamivudine, while they increase in normal conditions. In the protein-free viral DNA fraction (Fig. 5d), deproteinized RC and CCC DNA forms were detected as described in transfected hepatoma cell lines (Gao & Hu, 2007; Guo et al., 2007). Both forms are present at similar ratios, decreased after the peak of infection at day 11 but persisted for more than 30 days. Remarkably, the levels of these RC and CCC DNA species were minimally affected by the presence of lamivudine, indicating that they are likely to originate from input viral DNA and may persist independently of active viral replication. Therefore, active replication and de novo synthesis of cytoplasmic RC DNA was not associated with further increases in the amount of CCC DNA. Northern blot analysis showed that the levels of viral RNA (reflecting those of the transcriptional template) were almost similar in both control and lamivudine-treated cells until day 28 after infection (data not shown). Moreover, withdrawal of lamivudine at days 13, 25, 29 or 33 after infection was associated with a reappearance of viral DNA in cell supernatant as shown in Fig. 5(a), indicating that at least a proportion of CCC DNA molecules in infected cells still remained functional despite the absence of active viral replication. Real-time PCR quantification of both total HBV DNA and CCC DNA confirmed that CCC DNA levels at days 14 and 25 post-infection were very similar in both control and lamivudine-treated cultures (Table 1). Similarly, HBsAg levels measured in cell supernatants were the same in both conditions, while HBV DNA excreted in supernatants was clearly inhibited by lamivudine treatment.

**DISCUSSION**

Several key issues concerning the biology of HBV remain to be elucidated, including the identification of the cellular receptors, the role of the X gene and the mechanism of formation of the viral mini-chromosome. HepaRG cells are the first hepatoma cell line susceptible to HBV and the associated hepatitis delta virus. Characterization of the mechanism of HBV entry in these cells is actively studied (Abou-Jaoude & Sureau, 2007; Blanchet & Sureau, 2006; Engelke et al., 2006; Schulze et al., 2007), but other steps of HBV infection in this system have been poorly investigated. In this study, we described experimental conditions allowing reproducible infection of HepaRG cells and higher levels of viral replication to better analyse the mechanisms leading to the formation of the viral mini-chromosome. Our results indicate that the susceptibility of HepaRG cells to HBV is strongly dependent on the differentiation state induced by DMSO treatment as described by Gripon et al. (2002). Among various compounds known to induce hepatocyte differentiation such as EGF, HGF, oncostatin M or retinoic acid (Lee et al., 2004), only DMSO induced efficient cell susceptibility to HBV infection. Similarly, DMSO is able to maintain the susceptibility of primary HepaRG cells to HBV infection. Therefore, active replication and de novo synthesis of cytoplasmic RC DNA was not associated with further increases in the amount of CCC DNA. Northern blot analysis showed that the levels of viral RNA (reflecting those of the transcriptional template) were almost similar in both control and lamivudine-treated cells until day 28 after infection (data not shown). Moreover, withdrawal of lamivudine at days 13, 25, 29 or 33 after infection was associated with a reappearance of viral DNA in cell supernatant as shown in Fig. 5(a), indicating that at least a proportion of CCC DNA molecules in infected cells still remained functional despite the absence of active viral replication. Real-time PCR quantification of both total HBV DNA and CCC DNA confirmed that CCC DNA levels at days 14 and 25 post-infection were very similar in both control and lamivudine-treated cultures (Table 1). Similarly, HBsAg levels measured in cell supernatants were the same in both conditions, while HBV DNA excreted in supernatants was clearly inhibited by lamivudine treatment.

**DISCUSSION**

Several key issues concerning the biology of HBV remain to be elucidated, including the identification of the cellular receptors, the role of the X gene and the mechanism of formation of the viral mini-chromosome. HepaRG cells are the first hepatoma cell line susceptible to HBV and the associated hepatitis delta virus. Characterization of the mechanism of HBV entry in these cells is actively studied (Abou-Jaoude & Sureau, 2007; Blanchet & Sureau, 2006; Engelke et al., 2006; Schulze et al., 2007), but other steps of HBV infection in this system have been poorly investigated. In this study, we described experimental conditions allowing reproducible infection of HepaRG cells and higher levels of viral replication to better analyse the mechanisms leading to the formation of the viral mini-chromosome. Our results indicate that the susceptibility of HepaRG cells to HBV is strongly dependent on the differentiation state induced by DMSO treatment as described by Gripon et al. (2002). Among various compounds known to induce hepatocyte differentiation such as EGF, HGF, oncostatin M or retinoic acid (Lee et al., 2004), only DMSO induced efficient cell susceptibility to HBV infection. Similarly, DMSO is able to maintain the susceptibility of primary HepaRG cells to HBV infection.
duck hepatocytes to DHBV infection (Galle et al., 1989; Pugh & Summers, 1989) and to enhance the infection process of primary human hepatocytes by HBV (Gripon et al., 1988b). Thus, it is likely that DMSO specifically induces, at the transcriptional or translational level (Aninat et al., 2006; Parent & Beretta, 2008), unknown cellular factors triggering susceptibility to infection in addition to liver-enriched transcription factors (Mizuguchi et al., 1998) that stimulate viral transcription (Tang & McLachlan, 2001, 2002). However, in our experience, treatment of HepaRG cells with 2% DMSO was associated with variable cell toxicity leading to irreproducible results. Addition of EGF and reduction of DMSO concentration during the differentiation phase allowed the limiting of this toxic effect, thereby obtaining reproducible infection of HepaRG cells. Productive infection was restricted to the hepatocyte-like subpopulation of cells. Infected cells produced complete infectious virions but the percentage of infected cells remained constant until 90 days after infection, indicating the absence of viral spread throughout the culture. It is conceivable that all permissive cells have been infected during the first initial round of infection and that the other cells remain intrinsically non-permissive.

In this study, HBV replication was associated with low genetic variability after more than 1 month of fully active viral replication, at least in the major population of the viral quasi-species. This mutation rate is higher than that observed in long-term longitudinal studies of HBV chronically infected patients (Hannoun et al., 2000; Osiowy et al., 2006), but remained to be confirmed on the full-length HBV genome and quasi-species analysis. In addition, long-term treatment with lamivudine does not result in the emergence of resistant mutants. The absence of reinfection and the lack of CCC DNA amplification (see below) may explain this finding.
Compared to infection of primary duck hepatocytes by DHBV (Borel et al., 2001; Turin et al., 1996; Tuttleman et al., 1986b), HBV infection of HepaRG cells is a slow process characterized by a progressive conversion of input RC DNA into CCC DNA during the first 4 days post-infection. A striking difference of HBV infection is the absence of CCC DNA amplification that occurs in DHBV-infected hepatocytes by nuclear recycling of de novo synthesized nucleocapsids (Tuttleman et al., 1986a). In HepaRG cells, CCC DNA molecules are likely to be formed exclusively from input virions-associated RC DNA. After the initiation of replication, all formed nucleocapsids are probably exported, either poorly or not recycled to the nucleus to increase the pool of CCC DNA. Our study with lamivudine, an efficient inhibitor of the HBV reverse transcriptase, shows that the amount of CCC DNA is independent of active viral replication and confirms that the initial formation of CCC DNA depends mainly on cellular enzymes and not on activity of the viral polymerase. In addition, rebound of viral replication was observed after lamivudine withdrawal as reported in other experiments using primary duck or Tupaia hepatocytes (Delmas et al., 2002; Köck et al., 2003) following long-term treatment, thereby suggesting the stability of CCC DNA in HepaRG cells. This so-called ‘CCC DNA amplification’ phenomenon is one of the most remarkable features of hepadnavirus replication and is believed to play a fundamental role in viral persistence even though it has only been observed on DHBV. The absence of this phenomenon for HBV in the HepaRG cells is therefore very striking but can also be explained by the experimental system used and needs to be confirmed in primary human hepatocytes. Primary human hepatocytes are flawed with several limitations, including scarce availability and unpredictable variability but remain the gold standard for in vitro study of liver functions, including viral infections. Differentiated HepaRG resemble primary human hepatocytes in many aspects of these functions (Guillouzo et al., 2007) and, likely, of HBV infection (Gripon et al., 1993; Rumin et al., 1996). Since CCC DNA is the main cause for replication recurrence after antiviral removal in patients, it is of utmost importance to confirm that HepaRG cells can also constitute a relevant tool for deciphering the molecular basis for the persistence of HBV CCC DNA in the liver.

In the DHBV model, CCC DNA amplification is regulated by cell cycle progression and clearly inhibited by DMSO, which induces cell cycle arrest of duck primary hepatocytes in the G1 phase (Borel et al., 2001; Schorr et al., 2006; Turin et al., 1996). It is possible that in the HepaRG cell system, DMSO is detrimental for the recycling of nucleocapsids and further de novo synthesis of CCC DNA. In preliminary experiments, we found that subculturing infected HepaRG cells in the absence of DMSO is associated with a clear decrease of viral replication but a persistence of HBsAg production for three weekly passages. However, it is difficult to draw any definitive conclusion from this experiment since, as stated above, withdrawal of DMSO from the medium of infected cells is also associated with a decrease in viral transcription. In these conditions, the synthesis of RC DNA-containing nucleocapsids become very low and the fate of these capsids also becomes very difficult to determine. Other experimental conditions, e.g. induction of cell cycle without decreasing viral transcription and studies performed in primary human hepatocytes that generally support higher viral replication levels and could allow DMSO withdrawal are needed to resolve these issues.

ACKNOWLEDGEMENTS

This study was supported by grants from the ANRS (Agence Nationale pour la Recherche sur le Sida et les hépatites B et C-France) and from La Ligue Regionale Contre le Cancer (Rhône-Alpes, France). This work is part of the VIRGIL European Network of Excellence on Antiviral Drug Resistance and supported by a grant (LSHM-CT-2004-503359) from the Priority 1 ‘Life Sciences, Genomics and Biotechnology for Health’ programme in the sixth Framework Programme of the EU.

REFERENCES


