Initiation of hepatitis B virus genome replication and production of infectious virus following delivery in HepG2 cells by novel recombinant baculovirus vector

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One of the major problems in gaining further insight into hepatitis B virus (HBV)/host-cell interactions is to improve the existing cellular models for the study of HBV replication. The first objective of this study was to improve the system based on transduction of HepG2 cells with a recombinant baculovirus to study HBV replication. A new HBV recombinant baculovirus, Bac-HBV-1.1, in which the synthesis of pre-genomic RNA is driven by a strong mammalian promoter, was generated. Transduction with this new recombinant baculovirus led to higher levels of HBV replication in HepG2 cells compared with levels obtained with previously described baculovirus vectors. The initiation of a complete HBV DNA replication cycle in Bac-HBV-1.1-transduced HepG2 cells was shown by the presence of HBV replicative intermediates, including covalently closed circular DNA (cccDNA). Only low levels of cccDNA were detected in the nucleus of infected cells. Data showed that cccDNA resulted from the recycling of newly synthesized nucleocapsids and was bound to acetylated histones in a chromatin-like structure. HBV particles released into the supernatant of transduced HepG2 cells were infectious in differentiated HepaRG cells. Several Bac-HBV-1.1 baculoviruses containing HBV strains carrying mutations conferring resistance to lamivudine and/or adefovir were constructed. Phenotypic analysis of these mutants confirmed the results obtained with the transfection procedures. In conclusion, an improved cell-culture system was established for the transduction of replication-competent HBV genomes. This will be useful for future studies of the fitness of HBV mutants.

INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem, with more than 350 million people chronically infected worldwide with a greater risk of developing severe liver disease, including decompensated cirrhosis and hepatocellular carcinoma. To stop or delay disease progression, several therapeutic options are possible based on several drugs approved by the US Food and Drug Administration, i.e. alpha interferon and four nucleos(t)ide analogues: lamivudine (LAM), adefovir dipivoxil (ADV), entecavir and telbivudine. Whilst alpha interferon has low efficacy and is associated with numerous side effects (Karayiannis, 2003), nucleos(t)ide analogues potently and specifically inhibit HBV replication by acting on its reverse transcriptase. The viral response induced by these drugs is associated with a significant improvement in biochemical and histological markers in most patients (Lau et al., 2000; Marcellin et al., 2003).

HBV is a small enveloped and partially double-stranded DNA virus. In the nucleus of infected cells, the 3.2 kb viral relaxed circular (RC) DNA is converted into covalently closed circular DNA (cccDNA) that serves as a transcription template for both viral mRNAs and 3.5 kb pre-genomic RNA (pgRNA) synthesis. Histone and
cccDNA is a major factor in virus persistence because of its long half-life (Zhu et al., 2001). To clear infection and cccDNA in chronically infected patients, long-term treatments are necessary. Unfortunately, long-term administration of nucleos(t)ide analogues favours the emergence of resistant strains bearing drug-specific mutations within the viral polymerase gene (Zoulim, 2004). Thus, mutations conferring resistance to LAM have been mapped within the C and B domains of the viral reverse transcriptase (rtM204I/V and rtL180M, respectively), whereas mutations conferring resistance to ADV have been mapped within the B and D domains (rtA181V and rtN236T, respectively) (Allen et al., 2004). The overall objective of this work was to improve and characterize this system to study HBV replication, with particular focus on cccDNA formation, HBV virion secretion and infectivity, and resistance to antiviral drugs. The improvement in the technique is illustrated by its usefulness in performing phenotypic studies with clinically relevant and low-level-replicating HBV mutant strains.

METHODS

Cell culture. HepG2 (ATCC), HepaRG (Gripon et al., 2002) and HepG2.2.15 (Sells et al., 1987) cells were maintained in William’s medium (Invitrogen) supplemented with 10 % fetal calf serum (FCS), 50 U penicillin/streptomycin ml⁻¹ (Invitrogen), 2 mM GlutaMAX (Invitrogen), 5 μg bovine insulin ml⁻¹ and 5 × 10⁻⁷ M hydrocortisone hemisuccinate (Roche Diagnostics) at 37 °C in humidified incubators at 5 % CO₂. For differentiation, HepaRG cells were maintained for 2 weeks in standard medium and then for at least a further 2 weeks in medium supplemented with 1.8 % DMSO (Sigma). Spodoptera frugiperda (Sf9) insect cells were cultured in suspension or as monolayers at 28 °C in SF-900 II medium (Invitrogen) containing 5 % (maintenance) or 10 % (infection) FCS (Perbio).

Baculovirus construction and transduction of mammalian cells. The recombinant transfer vector pTriEx-HBV-1.1 (Fig. 1a), which contains a 1.1 × unit-length HBV genome (genotype D, serotype ayw) and enables the synthesis of pgRNA under the control of the chicken b-actin promoter, was described previously (Durantel et al., 2004). The recombinant transfer vector pTriEx-HBV-1.3 was obtained by cloning a 1.3 × unit-length HBV genome (genotype D, serotype ayw) into pTriEx (Novagen). pTriEx-HBV-1.1 or pTriEx-HBV-1.3 was co-transfected with linearized baculovirus DNA (BacVector-1000 DNA kit; Novagen) into Sf9 cells using Cellfectin reagent according to the manufacturer’s instructions (Invitrogen). Recombinant viruses were isolated using standard procedures (King, 1992). Ten clones were picked for each construct, amplified and functionally screened for their ability to trigger HBV replication after transduction into hepatoma cells. Baculovirus stock production and plaque assays for virus titration were performed as described previously (King, 1992). Before use in transduction experiments, baculoviruses were concentrated 100-fold by ultracentrifugation. A baculovirus containing an HBV genome carrying a mutation (rtM204P) in the catalytic domain of the polymerase gene (Bac-HBV-1.3-YPDD) was obtained using the same procedure, starting from a transfer vector in which the mutation was inserted by mutagenesis using a commercially available kit (QuikChange Site-directed Mutagenesis kit; Stratagene) using forward primer 5′-TTTGGCGTTTCACGATCCGGATGATTTGGGGGCC-3′ (mutated nucleotides underlined) and the corresponding reverse primer. This amino acid exchange in the polymerase sequence (YMDD to YPDD) also resulted in an exchange in the surface protein (IWMM to IRMM). The previously described procedure for baculovirus transduction of HepG2 (Delaney & Isom, 1998) was modified slightly. Briefly, HepG2 cells were transduced at 80 % confluence (1 × 10⁵–2 × 10⁵ cells cm⁻²). Baculovirus was diluted in medium to achieve the appropriate m.o.i. and was adsorbed to HepG2 cells at 37 °C with gentle rocking every 15 min to ensure an even distribution. After 1 h, the inoculum was removed and the HepG2 cells were washed with PBS and cultured as described above.

Antiviral drugs and treatment. LAM and ADV (Gilead Sciences) were resuspended in sterile water to a final concentration of 10 mM, aliquoted and frozen at −20 °C to avoid repeated freezing and thawing. Drug dilutions were freshly prepared in medium at the time.

The most relevant cell-culture models to study HBV resistance to nucleoside analogues in phenotypic assays would be human primary hepatocytes and HepaRG cells because they support a complete HBV life cycle. However, both of these have limitations in terms of accessibility, reproducibility and yield of HBV replication (Gripon et al., 1988, 2002) because of the low percentage of infected cells and the large amounts of input virus that need to be used to infect a reasonable proportion of cells. Thus, transfection of hepatoma cell lines (HepG2 or Huh7) with plasmids containing the complete viral genome is usually used to study HBV resistance to drugs (Brunelle et al., 2004; Villeneuve et al., 2006). Moreover, complex polymerase gene mutants conferring multi-drug resistance have also been described (Villett et al., 2006, 2007).

Another possibility that can be used to trigger intracellular HBV replication is to use systems based on the transduction of HepG2 cells with recombinant adenovirus or baculovirus carrying the HBV genome (Delaney & Isom, 1998; Ren & Nasal, 2001). Transduction of hepatoma cells with HBV-loaded baculovirus has led to higher HBV replication levels compared with both transfection approaches and stable cell lines such as HepG2.2.15. Moreover, cccDNA was synthesized in transduced HepG2 cells, although the origin of its formation was not clearly explained (Delaney & Isom, 1998).
of the experiments. HepG2 cells were supplied with medium containing the indicated concentration of drug at the specified time after transduction with HBV baculovirus.

**Analysis of viral DNA.** Purification of HBV DNA from intracellular core particles and analysis/quantification by Southern blotting with a radioactive probe were performed as described previously (Seigneres et al., 2002; Summers et al., 1990). HBV DNA was also quantified by real-time PCR using the primers 5' -GCTGACGGAAACCGCCACT-3' (forward) and 5' -AGGAGTTCCGAGTATGG-3' (reverse). An iCycler MyiQ thermocycler (96-well format; Bio-Rad) was used with an iQ SYBR Green Supermix kit.

**Isolation and detection of cccDNA.** Protein-free viral DNA (cccDNA and baculovirus DNA) was separated from protein-linked viral DNA (RC, linear and single-stranded intermediates) by KCl precipitation as described previously (Summers et al., 1990). To eliminate the baculovirus genome, enzyme digestion was performed with HindIII (New England Biolabs), which cuts several times in the baculovirus DNA, followed by a plasmid-safe DNase (Epipcentre) digestion performed by adding 2.5 mM ATP, 1 x reaction buffer and 10 U plasmid-safe DNase for 2 h at 37 °C. The DNase was inactivated at 70 °C for 30 min. The digested sample was used as the matrix for rolling-circle amplification (RCA) (Lasken & Egholm, 2003; Margeridon-Thermet et al., 2006). The RCA product (2 μl) was digested in a final volume of 10 μl with 3 U Spel (New England Biolabs), which cuts only once in the HBV genome. Digestion products were analysed by Southern blotting. Hybridization was performed with a specific S gene oligonucleotide probe labelled with [32P]dCTP by terminal deoxynucleotidyl transferase (Fermentas).

**Analysis of viral RNA.** Total RNA was extracted from cells using Extract All reagent according to the manufacturer’s instructions (EuroBio). Viral RNA was then analysed by electrophoresis in 1.2% agarose gels, followed by Northern blot analysis. Hybridization was performed using an HBV DNA-specific probe covering the whole genome.

**Analysis of secreted HBV antigens.** Detection of HBV surface antigen (HBsAg) and the amounts produced were analysed using a commercially available ELISA kit (MONOLISA Ag HBs PLUS; Bio-Rad) according to the manufacturer’s instructions. Supernatant collected from the cells was clarified by centrifugation at 10,000 g for 5 min and stored at −20 °C until analysed.

**Analysis of secreted HBV virions by transmission electron microscopy.** Concentrated supernatant from the cells was mounted onto Formvar-coated, carbon-stabilized copper grids (200 mesh). Grids were either stained directly with 1 % uranyl acetate or submitted to immunogold staining using a rabbit anti-HBs primary antibody and a goat anti-rabbit IgG conjugated to 5 nm gold particles (EMGAR5; British BioCell International) prior to uranyl acetate staining. Grids were observed under a 1200EX transmission electron microscope (JEOL).

**Infection of HepaRG cells.** Infection of differentiated HepaRG cells was performed in medium containing 4 % PEG 8000 (Sigma) for 8 h at 37 °C as described previously (Gripon et al., 2002). At the end of the incubation, cells were washed with medium and cultured as described above.

**Chromatin immunoprecipitation (ChiP) assays.** ChiP assays to detect cccDNA-bound acetylated histone H3 (acH3) and acetylated histone H4 (acH4) were performed as described previously (Policcino et al., 2006). Real-time ChiP results were expressed as the percentage of the ‘input’. ‘Input’ was the result of the real-time PCR performed with cccDNA-specific primers on the starting chromatin material from each experimental condition, including controls and samples, of the ChIP assay. In selected experiments, chromatin immunoprecipitates were digested with HindIII to eliminate the baculovirus genome prior to plasmid-safe DNase treatment for 1 h at 37 °C and then subjected to PCR analysis with cccDNA-specific primers or with primers designed to amplify the baculovirus vector DNA.

**RESULTS**

**Improvement of the recombinant HBV baculovirus system to initiate high levels of HBV genome replication**

Previous reports have described the use of a 1.3 x genome-length HBV recombinant baculovirus to trigger HBV replication in HepG2 cells. With this system, a higher level of HBV replication was obtained in HepG2 cells compared with that obtained in HepG2.2.15 cells (Delaney & Isom, 1998). Our objective was to improve the efficiency of such a system further, in particular to study poorly replicating mutant strains. To this end, a 1.1 x genome-length HBV recombinant baculovirus (Bac-HBV-1.1-WT) was generated. In this construct, expression of the pgRNA was placed under the control of a strong mammalian promoter, whereas in Bac-HBV-1.3-WT it was driven by the natural HBV promoter (Fig. 1a). As a control for direct comparison, Bac-HBV-1.3-WT was also constructed using the same HBV sequence.

To compare the efficiency of both constructs to initiate an HBV replication cycle, HepG2 cells were transduced at an m.o.i. of 100 with each baculovirus vector. Encapsidated viral DNA and viral RNA were isolated at various time points post-transduction (p.t.), whilst culture medium was collected to analyse HBsAg production. In HepG2 cells transduced with Bac-HBV-1.1-WT, the amount of HBV transcripts was maximal at day 1 p.t. and declined afterwards until the last day of analysis (day 12 p.t.; Fig. 1b). In HepG2 cells transduced with Bac-HBV-1.3-WT, HBV transcripts were barely detectable at day 1 p.t. by Northern blotting, reached a peak level between days 3 and 6 p.t. and declined thereafter (Fig. 1b). As synthesis of HBV transcripts was higher in Bac-HBV-1.1-WT-transduced HepG2 cells, the subsequent amounts of encapsidated replicative intermediates and secreted HBsAg were also higher in Bac-HBV-1.1-WT-transduced HepG2 cells compared with those observed in Bac-HBV-1.3-WT-transduced HepG2 cells (Fig. 1c and d). HBV encapsidated replicative intermediates and secreted HBsAg remained detectable after day 12 p.t. for up to 1 month in HepG2 cells that were not split (data not shown). The amount of intracellular baculovirus genome was maximal at day 1 p.t. and decreased thereafter, with kinetics mirroring those of pgRNA, but was still detectable by Southern blotting at day 27 p.t. (data not shown). The differences in kinetics of accumulation and clearance of both baculoviral and HBV nucleic acids suggested that the half-life of encapsidated HBV DNA was longer than that of pgRNA or the baculoviral genome. It was noteworthy that clearance of
encapsidated HBV DNA was faster when transduced cells were split every 3–4 days (data not shown). Finally, it is important to note that, despite the high level of HBV replication, no significant cytotoxicity was observed with either baculovirus vector (Fig. 1e), thus confirming that differences observed in replication kinetics after transduction with these baculoviruses vectors were not due to differences in toxicity.

Collectively, these results demonstrated the superiority of Bac-HBV-1.1-WT over Bac-HBV-1.3-WT in initiation of HBV genome replication in HepG2 cells.

**Initiation of HBV genome replication is followed by the formation of transcriptionally active cccDNA in transduced HepG2 cells**

Delaney & Isom (1998) reported that cccDNA formation takes place in Bac-HBV-1.3-transduced HepG2 cells. Similar experiments were performed with HepG2 cells obtained from ATCC. To detect low levels of cccDNA, a sensitive method based on RCA was used (Margeridon-Thermet et al., 2006). HepG2 cells were transduced and cccDNA was isolated at various times p.t. and amplified by RCA after complete elimination of baculovirus genomes by HindIII and plasmid-safe DNase digestions. cccDNA was detected at day 1 p.t. and remained detectable up to the last day of the assay (day 12 p.t.; Fig. 2a). To demonstrate the specificity of the method for cccDNA detection, RCA was performed on samples digested by EcoRI (which cuts once in the HBV genome and cccDNA) or HindIII (no digestion sites in the HBV genome). As expected, the cccDNA signal was lost when viral DNA was linearized by EcoRI prior to RCA (Fig. 2b).

The next objective was to gain insight into the origin of cccDNA formation. We tested two main hypotheses. First, we tested whether cccDNA could be formed by nucleocapsid recycling back towards the nucleus, as described previously in the duck HBV model (Tuttleman et al., 1986). Secondly, we tested whether cccDNA could be formed by recombination from the baculovirus vector between the repeated sequences of HBV (Fig. 1a). HepG2 cells transduced with Bac-HBV-1.1-WT were treated with high concentrations of LAM. If cccDNA is formed by nucleocapsid recycling, LAM administration would also prevent cccDNA formation. Our results showed that high concentrations of LAM, which completely eliminates
observation that baculovirus- and cccDNA-bound H4 acetylation and the HepG2 cells was supported by the different kinetics of assay in the context of Bac-HBV-1.1. Next, we asked whether cccDNA formed in Bac-HBV-1.1-WT-transduced HepG2 cells could be used as a template for HBV RNA synthesis. The acetylation status of cccDNA-bound histones H3 and H4 was analysed. HepG2 cells were transduced and formaldehyde-cross-linked nuclear chromatin was immunoprecipitated with specific anti-AcH3 and anti-AcH4 antibodies. The specificity of the cccDNA ChIP assay in the context of Bac-HBV-1.1-WT-transduced HepG2 cells was supported by the different kinetics of baculovirus- and cccDNA-bound H4 acetylation and the observation that HindIII digestion of anti-AcH4 chromatin immunoprecipitates eliminated the baculovirus-specific PCR signal without affecting the cccDNA-specific PCR signal (Fig. 3b). A cccDNA-specific real-time PCR assay (Werle-Lapostolle et al., 2004) was then performed to quantify cccDNA in the anti-AcH3 histone and anti-AcH4 histone ChIPed material. At day 1 p.t., approximately 30 and 8 % of the cccDNA formed contained AcH3 and AcH4 histones, respectively (Fig. 3c). The acetylation of bound H3 and H4 histones remained detectable at day 3 p.t. but disappeared at day 6 p.t. Moreover, no significant acetylation of bound H4 histones was observed in Bac-HBV-1.1-WT-transduced HepG2 cells treated with LAM (Fig. 3d), consistent with the observation that cccDNA is not formed in LAM-treated cells. These results suggested that the cccDNA formed in HepG2 cells transduced with Bac-HBV-1.1-WT was probably transcriptionally active and used as a template for HBV RNA synthesis.

**HepG2 cells transduced with recombinant baculovirus vectors produce infectious HBV virions**

It has been shown previously that HepG2 cells transduced with Bac-HBV-1.3 produced HBV particles that peaked at 1.21 g cm⁻³ in isopycnic density gradient analysis (Delaney & Isom, 1998). Supernatant from HepG2 cells transduced with Bac-HBV-1.1-WT was characterized by electron microscopy analyses and immunogold staining in comparison with that produced in stably transfected HepG2.2.15 cells (Sells et al., 1987). The results revealed the presence of Dane particles, spheres and filamentous particles in concentrated supernatant from HepG2 cells transduced with Bac-HBV-1.1-WT (Fig. 4a), as well as in supernatant from HepG2.2.15 cells. Of note with regard to the infectivity experiments described below, no baculoviruses were observed in the same concentrated supernatants.

To investigate whether HBV virions produced in HepG2 cells transduced with Bac-HBV-1.1-WT were infectious, HepaRG cells were inoculated with the corresponding concentrated supernatant. As a positive control, supernatant from HepG2.2.15 cells was also used. In addition, we ruled out the presence of residual baculoviruses that could have been responsible for HepaRG transduction using microscopy and functional assays (data not shown). The amount of HBsAg production in the supernatant of inoculated HepaRG cells was followed for 12 days and cells were lysed to analyse HBV RNA by Northern blotting 12 days post-inoculation. HBsAg and HBV RNA synthesis were detectable not only in HepaRG cells infected by supernatant from HepG2.2.15 cells, but also in cells infected by supernatant from HepG2 cells transduced with Bac-HBV-1.1-WT (Fig. 4b). These results indicated that HBV virions produced from HepG2 cells transduced with Bac-HBV-1.1-WT were infectious.

**Use of the improved recombinant HBV baculovirus system to study a complex mutant with low replication capacity**

It has been shown previously that some complex and clinically relevant HBV mutants have significantly lower replication capacity levels (Brunelle et al., 2005; Villeneuve et al., 2003; Villet et al., 2006, 2007). In these cases, phenotyping analyses are difficult to perform with a transfection system. Thus, the baculovirus system could be very useful, as levels of replication are higher. To test this hypothesis, mutations conferring resistance to LAM...
(rtL180M+M204V), ADV (rtN236T) and to both drugs (rtL180M+M204V+N236T) that were engineered previously into the sequence used for the construction of wild-type baculoviruses (Brunelle et al., 2005) were used to derive corresponding baculovirus vectors, i.e. Bac-HBV-1.1-NT (containing the rtN236T substitution), Bac-HBV-1.1-LMMV (containing the rtL180M+M204V substitutions) and Bac-HBV-1.1-LMMVNT (containing the rtL180M+M204V+N236T substitutions). First, HepG2 cells were transduced with these baculoviruses to study the replication ability of these HBV mutant strains. DNA from intracellular core particles was extracted at 2 days p.t. and submitted to Southern blot and real-time PCR analysis (Fig. 5a). As reported previously by transfection of plasmids (Brunelle et al., 2005), the replication rate of the mutants was reduced in the following order: WT (100 %)>rtN236T (43 %)>rtL180M+rtM204V (30 %)>rtL180M+rtM204V+rtN236T (7 %). Secondly, the mutant HBV/baculovirus vectors were used to study the susceptibility of mutants to LAM and ADV. As expected, the rtL180M+M204V and rtN236T strains were susceptible to ADV and LAM, respectively, whereas the WT strain was susceptible to both drugs. Both the rtN236T and rtL180M+M204V+N236T strains were less susceptible to ADV, with 1.7- and 7.8-fold resistance, respectively, and both the rtL180M+M204V and rtL180M+M204V+N236T strains were resistant to LAM, with >33-fold resistance (Fig. 5b). Interestingly, cccDNA was detected by RCA, performed at 48 h p.t., for each mutant strain, even with the low-level-replicating rtL180M+rtM204V+rtN236T mutant. As expected, LAM administration prevented the formation of cccDNA in HepG2 cells.
transduced with baculovirus loaded with WT or rtN236T strains, but not in cells transduced with baculovirus loaded with LAM-resistant strains (rtL180M + M204V and rtL180M + M204V + N236T) (Fig. 5c).

**DISCUSSION**

Stably transfected hepatocyte-derived cell lines such as HepG2.2.15 (Sells et al., 1987) or transient transfection of plasmid containing the HBV genome in HepG2 or HuH7 cells are the models currently used for in vitro studies of HBV replication (Brunelle et al., 2005; Durantel et al., 2005). It has been demonstrated that HBV recombinant baculovirus vectors can also be used to transduce HepG2 cells in order to initiate high levels of HBV replication and study HBV resistance to nucleoside analogues (Delaney & Isom, 1998; Delaney et al., 1999, 2003).

To study virus replication and HBV mutants with a low replication capacity, we designed a new HBV recombinant baculovirus vector (Bac-HBV-1.1-WT) in which HBV pgRNA synthesis is driven by a strong mammalian promoter. This vector differed from the previously described Bac-HVB-1.3-WT that contained 1.3 unit-length HBV genomes and in which the pgRNA was driven by the endogenous HBV promoter (Delaney & Isom, 1998). Another advantage of our vector was that the rate of pgRNA synthesis was identical, regardless of the HBV genome cloned, as shown previously (Durantel et al., 2004). It is noteworthy that the construction of transfer vectors used for the production of a given baculovirus has been optimized previously, thus facilitating the generation of novel recombinant vectors harbouring relevant HBV genomes (Durantel et al., 2004). HBV replication was found to be higher in HepG2 cells transduced with Bac-HBV-1.1-WT compared with cells transduced with Bac-HVB-1.3-WT. This improved viral genome replication was most likely due to increased synthesis of pgRNA. Despite a higher replication rate, it was not possible to produce a persistent infection in HepG2 cells.

Delaney & Isom (1998) studied HBV virion secretion in HepG2 cells transduced with their HBV recombinant baculovirus by isopycnic centrifugation in a CsCl gradient and fractionation. They showed that HBV DNA and HBsAg co-migrated at a density that has been reported for Dane particles. The results of our experiments using electron microscopy analysis showed that HepG2 cells transduced with Bac-HBV-1.1-WT produced Dane particles, spheres and filamentous particles usually found in the sera of patients (Ganem, 1991). Furthermore, we provided new data showing that the supernatant from HepG2 cells transduced with Bac-HBV-1.1-WT contained HBV particles that were infectious and could initiate HBV replication in HepaRG cells. Therefore, this system may prove useful for studies of the infectivity and fitness of clinically relevant HBV mutants.

An important issue in HBV infection concerns the synthesis of cccDNA, as this is the template for viral RNA synthesis in vivo and plays a crucial role in virus persistence. Delaney & Isom (1998) demonstrated that cccDNA could be formed after transduction of HepG2 with HBV recombinant baculovirus. However, two main questions remained largely unanswered: what was the
Fig. 5. Analysis of HBV mutant strains using the improved baculovirus system. HepG2 cells were transduced at an m.o.i. of 100 with Bac-HBV-1.1-WT, Bac-HBV-1.1-NT, Bac-HBV-1.1-LMMV or Bac-HBV-1.1-LMMVNT. (a) At 48 h p.t., cells were lysed and encapsidated DNA was isolated and analysed by Southern blotting (left) or real-time PCR (right). The values obtained for each mutant were divided by the WT value, which was assumed to represent 100% virus replication. Each value is the mean ± SD of at least three independent experiments. (b) HepG2 cells were treated immediately after transduction with six different concentrations of LAM or ADV. The medium was removed and replaced by fresh medium containing the drug at different concentrations every day for 3 days. DNA from intracellular core particles was extracted and submitted to Southern blotting. The panels represent different autoradiograms with different exposure times. The HBV DNA hybridization signal was quantified by PhosphorImager and ImageQuant software and the results are shown in (b) as EC_{50} (drug concentration for which HBV replication has decreased by 50%) and fold resistance (mutant EC_{50}/WT EC_{50}). Values are means ± SD of three independent experiments, each performed in duplicate. (c) At 48 h p.t., cells were lysed and cccDNA was isolated at various time p.t. and submitted to RCA. Mock, non-transduced cells; NT, Bac-HBV-1.1-NT; LMMV, Bac-HBV-1.1-LMMV; LMMVNT, Bac-HBV-1.1-LMMVNT.
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origin of the cccDNA synthesized, and was this cccDNA transcriptionally active and therefore responsible for the synthesis of viral RNAs? Our results showed that low levels of cccDNA could be detected after transduction with baculoviruses loaded with WT or mutant HBV strains. The overall amount of cccDNA detected in both cases was lower than that detected by Delaney & Isom (1998), as it was not detectable by Southern blot analysis. One may hypothesize that differences in cell-line batches and experimental conditions may explain this discrepancy. The results of experiments with LAM treatment suggested that cccDNA is most probably formed by nucleocapsid recycling to the nucleus. This was consistent with the results of experiments using constructs expressing a polymerase-inactive mutant, which ruled out the possibility that cccDNA could be formed by recombination from the baculovirus vector.

It has been shown that cccDNA can persist in cell nuclei as a stable chromatin-like episome associated with \(^{\alpha H}3\) and \(^{\alpha H}4\) histones when replication is active (Pollinico et al., 2006). Using a ChIP/cccDNA assay described recently (Pollinico et al., 2006), we found that H3 and H4 histones bound to cccDNA were acetylated, suggesting that cccDNA formed in HepG2 cells transduced with Bac-HBV-1.1-WT may be transcriptionally active. However, the acetylation of cccDNA-bound H3 and H4 histones was only transient in transduced HepG2 cells. This may explain why a persistent HBV replication could not be produced in transduced HepG2 cells, as well as the absence of selection of HBV resistant mutants when suboptimal doses of drugs were administrated to transduced HepG2 cells (data not shown).

With the generation of additional 1.1 x genome-length HBV recombinant baculovirus vectors carrying mutations conferring resistance to LAM, ADV or both, we demonstrated that this technology is also relevant for studying virus resistance to nucleoside analogues in so-called phenotypic studies. Thus, the strong mammalian promoter in the Bac-HBV-1.1 vector led to a higher level of HBV genome replication than that obtained with the Bac-HBV-1.3 vector in which the pgRNA synthesis is driven by the endogenous HBV promoter, without affecting the order of replication levels of the HBV mutants and their susceptibility to nucleosides analogues (Brunelle et al., 2005). Moreover, cccDNA formation occurred after transduction of HepG2 cells with each HBV mutant baculovirus, including the low-level-replicating rtL180M + rtM204V + rtN236T strain. Taken together, these results demonstrate the usefulness of baculovirus transduction for the study of low-level-replicating HBV mutant strains. This approach may also prove useful for the production of HBV mutant particles in vitro to perform proper fitness studies both in vitro and in vivo.

In conclusion, the results of our study show that a recombinant HBV baculovirus vector encoding a 1.1 x unit-length HBV genome may initiate high levels of HBV replication following transduction of HepG2 cells, including the formation of transcriptionally active cccDNA and the production of infectious HBV particles. This system may be of interest for in vitro studies of the fitness of antiviral-resistant mutants.

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REFERENCES


