

In Vitro Study of the Effects of Precore and Lamivudine-Resistant Mutations on Hepatitis B Virus Replication[▽]

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Understanding the consequences of mutation in the hepatitis B virus (HBV) genome on HBV replication is critical for treating chronic HBV infection. In this study, HBV replication in HepG2 cells initiated by transduction with precore (PC), rtM204I, and wild-type (wt) HBV recombinant baculoviruses was compared. The pattern and magnitude of HBV replication initiated by the PC HBV recombinant baculovirus were similar to those observed for wt HBV throughout the time course examined. In contrast, when the rtM204I mutation was introduced into wt HBV, by day 10 postinfection the levels of intra- and extracellular HBV DNA were markedly reduced compared to those for wt HBV. Although the rtM204I mutation reduced the production of HBV replicative intermediates, no effect on the level of covalently closed circular DNA or HBV transcripts was observed at late time points. Coinfection studies with different ratios of wt and rtM204I baculoviruses showed that the rtM204I variant did not produce a product that inhibited HBV replication. However, the combination of the wt and rtM204I baculoviruses yielded HBV DNA levels at late time points that were greater than those for the wt alone, suggesting that wt polymerase may function in *trans* to boost rtM204I replication. We concluded that the rtM204I mutation generates a polymerase that is not only resistant to lamivudine but also replicates nucleic acids to lower levels in vitro.

Understanding the consequences of mutation in the hepatitis B virus (HBV) genome on HBV replication is critical for treating chronic hepatitis B (CHB). This study focuses on the precore G1896A stop codon (PC) mutation and the lamivudine-resistant rtM204I mutation.

The precore/core region of the HBV genome encodes the nucleocapsid protein HBeAg and HBsAg. HBeAg is a non-structural, secretory form of the nucleocapsid protein (38, 51, 53, 55) that is not required for viral replication in animal models (19) and humans (49, 50). The biological role of HBeAg in the life cycle of the virus remains to be elucidated. It has been suggested that HBeAg secretion may play a pivotal role in the persistence of HBV by inducing immunologic tolerance (20, 30, 31, 42). Certain precore mutants may have an inherent replicative advantage (5, 26), although this issue is controversial (12). It has been shown that mutations in the precore region of HBV serve to enhance the stability of the secondary structure of the pregenomic encapsidation signal (28, 34). The most common mutation in the precore region is a G-to-A mutation (TGG to TAG) at nucleotide 1896 (8, 9, 44). HBeAg-negative CHB is the most prevalent form of CHB in many parts of the world. Patients with HBeAg-negative CHB have a high risk of developing progressive chronic liver disease and cirrhosis (7, 32, 44).

Until recently, the only approved treatment for CHB was alpha

interferon (22, 48, 54). Unfortunately, only 30% to 40% of chronically infected individuals respond to interferon with sustained elimination of HBV (33). Interferon is not the ideal drug for HBV treatment because of its limited efficacy, parenteral administration, dose-limiting side effects, and high cost. Four additional antivirals, namely, lamivudine (3TC; Epivir-HBV), adefovir dipivoxil (Hepsera), entecavir (Baraclude), and telbivudine (Tyzeka), have been approved for HBV treatment.

Treatment with lamivudine is well tolerated and effective in reducing HBV DNA in the sera of chronically infected patients (16, 23, 45). The major clinical limitation of lamivudine is the emergence of drug-resistant mutants (3, 4, 15, 21, 27, 41). The incidence of lamivudine resistance is 14% to 32% after 1 year of treatment, 38% after 2 years, and 53% to 76% after 3 years (25). The most common mutations occur in the highly conserved YMDD motif in the catalytic domain of the HBV polymerase gene, resulting in the replacement of methionine with either valine or isoleucine at amino acid 204 (3, 35). Recently, an in vivo study of four patients was carried out to understand the HBV quasispecies variant dynamics at work during the emergence and amplification of lamivudine resistance (39). The results suggest that the rtM204I and rtM204V mutations are preexisting in HBV-infected patients and that virological breakthrough is preceded 2 to 4 months by the emergence of rtM204I and rtM204V variants. The emergence of lamivudine resistance is associated with progressive liver disease. Specifically, as the resistant mutants replace the drug-sensitive wild-type (wt) virus, the viral load increases, the serum alanine aminotransferase level increases, and liver histology worsens. Therefore, continued in vivo and in vitro studies of lamivudine-resistant mutants are needed.

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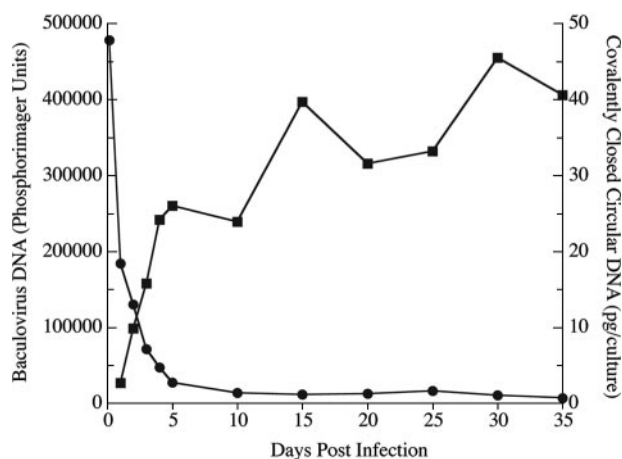


FIG. 1. Detection of baculovirus and HBV CCC DNAs with time. The figure shows the loss of baculovirus DNA with time (●) and the accumulation of HBV nuclear CCC DNA with time posttransduction with recombinant baculovirus (■). This is a graphic representation of previously reported data (2).

We previously reported an *in vitro* system for studying HBV replication by using an HBV recombinant baculovirus to efficiently deliver the HBV genome to HepG2 cells (1, 2, 13, 14). In HBV baculovirus-infected HepG2 cells, HBV replication levels can be obtained that are markedly higher than those from conventional transfection or from HBV-expressing cell lines. HBV transcripts, intracellular and secreted HBV antigens, HBV DNA replicative intermediates (RI), and covalently closed circular (CCC) HBV DNA are produced, and HBV virions are secreted. Replication remains at a high level for 30 to 35 days postinfection (p.i.) (1).

When HepG2 cells are transduced with a recombinant baculovirus containing a single gene under the control of a mammalian virus promoter, such as green fluorescent protein driven by cytomegalovirus, the expression of the gene product is transient, peaking at 48 to 72 h posttransduction and becoming undetectable by 5 to 6 days posttransduction. In contrast, when HepG2 cells are transduced with an HBV recombinant baculovirus containing more than one genome length of HBV DNA, the production of HBV proteins, transcripts, and intracellular and extracellular DNA is readily detected for 30 to 35 days posttransduction (2). Further analysis of the system showed that the input HBV recombinant baculovirus DNA markedly declines over the first few days posttransduction at the same time that HBV CCC DNA is accumulating (Fig. 1). We concluded from these findings that HBV transcription, including the production of pregenomic HBV RNA, occurs initially from the input recombinant HBV baculovirus DNA template. Under most conditions, such as transduction with a recombinant green fluorescent protein-expressing baculovirus, the transient expression of encoded gene products would cease in parallel with the loss of recombinant baculovirus DNA. However, expression and replication of HBV persist for at least an additional 3 to 4 weeks because HBV transcription occurs from HBV CCC DNA at these later time points. The length of time that robust levels of wt HBV replication occur in the HBV recombinant baculovirus system makes it possible to study the long-term effects of antivirals as well as the phenom-

enon of rebound after release from antiviral treatment (1, 2). Therefore, it would be predicted that the recombinant baculovirus system would be ideal for studying these same phenomena by using HBV mutant viruses. The goal of this study was to characterize HBV replication in HepG2 cells from PC and rtM204I HBV recombinant baculoviruses compared to replication from wt HBV recombinant baculovirus.

MATERIALS AND METHODS

Cell culture. The HepG2 cell line was maintained at 37°C in a humidified incubator at 5% CO₂ (24). HepG2 cells were fed minimal essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), designated MEM-FBS. *Spodoptera frugiperda* (Sf21) insect cells were maintained in Grace's insect medium supplemented with Yeastolate (Mediatech, Inc., Herndon, VA), lactalbumin hydrolysate (Mediatech), and 10% FBS in a nonhumidified incubator at 28°C without CO₂.

Recombinant HBV baculovirus amplification, purification, titration, and validation. The wt and rtM204I and PC mutant baculoviruses were generated as previously described (10, 13). Baculoviruses were amplified by infecting suspension cultures of Sf21 cells during the log phase of growth at a multiplicity of infection (MOI) of 0.2 PFU/cell. Infections were allowed to proceed until a majority of the cells in the flasks showed visible signs of infection (5 to 7 days). Virions were concentrated and purified from infected Sf21 medium as described previously (6, 37). Purified virus was titrated in Sf21 cells by plaque titration (37). Average titers were calculated from triplicate titrations.

To control for spontaneous deletion of HBV DNA, the HBV recombinant baculovirus was routinely plaque purified. In addition, every newly prepared HBV recombinant baculovirus stock was compared to a standard HBV recombinant baculovirus stock. The level of HBV replication in HepG2 cells routinely paralleled the MOI in insect cells. If we observed differences when a new stock was compared to the standard in a stock check, then the titration of the new stock was repeated. If a difference still remained, the stock was not used.

The medium collected from HepG2 cells infected with PC or wt HBV baculovirus at different times postinfection was analyzed for HBeAg levels by using a radioimmunoassay. As determined previously (2, 13, 14), infection with wt HBV recombinant baculovirus resulted in high levels of HBeAg production. In contrast, infection with PC HBV recombinant baculovirus did not yield measurable levels of HBeAg.

HBV baculovirus infection of HepG2 cells. The baculovirus infection procedure for HepG2 cells has been described (13). Briefly, HepG2 cells were seeded at approximately 20 to 40% confluence and allowed to attach and grow for 16 to 24 h prior to infection. On the day of infection, triplicate plates of cells were trypsinized, and the viable cell number was determined with a hemocytometer, using trypan blue exclusion. Average cell counts were calculated and used to determine the volume of high-titer viral stock necessary to infect cells at the indicated MOI. Baculovirus was diluted in MEM-FBS to achieve the appropriate MOI, using a volume of 0.5 ml to infect a 60-mm dish. Baculovirus was adsorbed to HepG2 cells for 1 h at 37°C, with gentle rocking every 15 min to ensure that the inoculum was evenly distributed. The inoculum was then aspirated, and HepG2 cells were washed two times with phosphate-buffered saline and refed MEM-FBS.

Baculovirus DNA extraction and normalization to gene transfer efficiency. To compare the levels of HBV replication initiated by wt, PC, and rtM204I recombinant baculoviruses, the relative efficiency of gene delivery by each of the recombinant baculoviruses was determined. At 4 h p.i. with HBV recombinant baculovirus, total DNA was harvested from HepG2 cells and analyzed for baculovirus DNA as described previously (2). A 332-bp fragment from pBlueBac 4.5 was used as the baculovirus probe during Southern blotting to measure the amount of each baculovirus DNA delivered when HepG2 cells were infected with the same multiplicity of infection for each recombinant baculovirus (2). The baculovirus DNA band was visualized using a PhosphorImager. The gene transfer efficiency for wt HBV recombinant baculovirus into HepG2 cells was set to 1.0, and the relative gene transfer efficiencies for PC and rtM204I recombinant baculoviruses were calculated. The relative gene transfer efficiencies ranged from 0.80 to 1.25. The values obtained from these analyses were used to normalize the production of extracellular HBV DNA, intracellular HBV RI, and nuclear HBV DNA to the input.

Analysis of intracellular HBV RI. Cytoplasmic preparations containing HBV core particles were isolated from HepG2 cells as described previously in detail (1,

2). RI were then analyzed by electrophoresis in 1% agarose gels, followed by Southern blotting as described previously (43). Nucleic acid hybridization was then performed. A full-length double-stranded (DS) HBV genome was used as a template to generate a ^{32}P -radiolabeled probe, using a Random Prime DNA labeling kit (Roche Diagnostics, Mannheim, Germany). HBV DNA bands were visualized and quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The relaxed circle (RC) and DS DNA bands, taken together, and the single-stranded (SS) DNA bands were selected for quantitation as RI.

Detection of extracellular HBV DNA. Conditioned medium was collected from HepG2 cells, centrifuged at $10,000 \times g$ for 10 min, and transferred to clean tubes to remove cellular debris. HBV particles were precipitated from medium samples, using polyethylene glycol 8000 (Sigma Chemical Co., St. Louis, MO) as described previously (52). Viral pellets were resuspended in phosphate-buffered saline, and DNAs were extracted as previously described (1, 2). Electrophoresis and Southern blotting were performed, and HBV DNA bands were visualized and quantitated using a PhosphorImager and ImageQuant software.

Detection of nuclear, non-protein-bound HBV DNA. Nuclear, non-protein-bound HBV DNA was extracted from HepG2 cells by using a modification of a previously described procedure (47). Nuclei were separated from the whole-cell lysate as described above for isolation of RI. The nuclear pellets were suspended in TE (50 mM Tris, 1 mM EDTA), and the sample was then lysed with 4% sodium dodecyl sulfate, followed by precipitation with 2.5 M KCl. The supernatants were phenol and chloroform extracted, and nucleic acids were recovered by isopropanol precipitation. Ten micrograms of tRNA was added as a carrier during precipitation. Extracted nucleic acids were resuspended in water and digested with 100 $\mu\text{g}/\text{ml}$ of RNase and 30 units of plasmid-safe ATP-dependent DNase (Epicenter Technologies, Madison, WI) for 15 min at 37°C , followed by a 30-min incubation at 65°C to inhibit the enzyme activity. The samples were analyzed by electrophoresis and Southern blotting as described above.

Analysis of RNA. Total RNA was isolated from HepG2 cells at the indicated time points by the single-step acid guanidinium method (11). Electrophoresis and Northern blot analysis were performed on 10 μg of total RNA. Both HBV- and glyceraldehyde-3-phosphate dehydrogenase-specific ^{32}P -radiolabeled probes were used, and the samples were visualized using a PhosphorImager.

RESULTS

Effects of PC and rtM204I mutations on production of extracellular HBV DNA and intracellular HBV RI. HepG2 cells were infected with 100 PFU/cell of PC, rtM204I, or wt HBV recombinant baculovirus. Three independent experiments were carried out, with cultures fed daily. On the indicated days up to 15 days p.i., the medium was collected for analysis of extracellular HBV DNA, and the cells were harvested for analysis of intracellular HBV RI.

Extracellular HBV DNA bands were visualized using a PhosphorImager, and a digital image of the Southern blot for a representative experiment is shown in Fig. 2A. HBV DNA standards were included in each gel subjected to Southern blot analysis, thereby making it possible to determine the amount of HBV DNA produced in pg/culture/24 h. HBV DNA bands were visualized and quantified using a PhosphorImager. HBV DNA levels were normalized for differences in gene transfer efficiency as described in Materials and Methods. The data for the three different experiments were then plotted as amounts of extracellular HBV DNA produced relative to days p.i. (Fig. 2B, C, and D). It is important that the HBV DNA levels represent the amounts of extracellular HBV DNA released into the medium over the 24-h period before collection.

Extracellular virus was not detected at day 1 p.i. when replication was initiated by any of the three recombinant baculoviruses. This was in agreement with what has been reported previously for experiments using wt HBV recombinant baculovirus (2, 13). High levels of extracellular HBV DNA were produced by day 2 p.i. for both mutants as well as for wt

recombinant baculovirus. The levels of extracellular HBV virions produced during the first 4 days p.i. did not differ significantly for all three recombinant baculoviruses.

Throughout the remaining time points of the experiment, no significant differences in production of extracellular HBV DNA were noted, whether HBV replication was initiated by PC or wt recombinant baculovirus. In one experiment (Fig. 2D), initiation of HBV replication with the PC baculovirus yielded slightly higher levels of extracellular HBV DNA than did replication initiated with wt HBV baculovirus. However, this difference was not seen in the other two experiments (Fig. 2B and C). In contrast, the introduction of the rtM204I mutation into wt HBV DNA had major effects on the production of HBV extracellular DNA by 10 days p.i. Specifically, at days 10 and 15 p.i., the levels of rtM204I HBV extracellular DNA were markedly reduced compared to the levels of wt or PC HBV extracellular DNA.

Intracellular HBV DNA was also analyzed. The DNA bands were visualized using a PhosphorImager, and a digital image of the Southern blot of HBV RI for a representative experiment is shown in Fig. 2E. HBV DNA standards were included in each gel subjected to Southern blot analysis, thereby making it possible to determine the amount of HBV RI produced in pg/culture. The RI levels were also normalized for differences in gene transfer efficiency. The data for the three different experiments were then plotted as amounts of HBV RI produced relative to days p.i. (Fig. 2F, G, and H).

When replication was initiated by any of the three recombinant baculoviruses, a band corresponding to HBV SS DNA was detectable at day 1 p.i., and HBV DS DNA became apparent by 2 days p.i. These findings were in agreement with what was previously observed for experiments using wt HBV recombinant baculovirus (2, 13). The levels of PC HBV RI were greater than wt RI levels throughout the course of the experiment. The rtM204I mutation had no major effects on the levels of HBV RI during the first 3 to 5 days p.i., but major differences, as observed for extracellular HBV DNA, became apparent by 10 days p.i. Specifically, the levels of rtM204I RI were markedly reduced compared to wt or PC HBV RI levels.

Effects of PC and rtM204I mutations on production of nuclear, non-protein-bound HBV DNA. HepG2 cells were seeded in 100-mm dishes at approximately 20% to 40% confluence, grown for 16 to 24 h, and then infected with 100 PFU/cell of PC, rtM204I, or wt HBV recombinant baculovirus. On the indicated days p.i., cells were harvested and analyzed for nuclear, non-protein-bound HBV DNA. The HBV DNA bands were visualized using a PhosphorImager, and the digital images are shown (Fig. 2I). Quantitative analysis of the normalized data indicated that the PC and rtM204I mutations had no effect on the production of nuclear CCC DNA compared to that for wt HBV (Fig. 2J). These studies showed that introduction of the rtM204I mutation reduced the production of RI and extracellular virus but had no effect on the CCC DNA level. rtM204I nuclear RC DNA levels were reduced compared to wt levels at days 10 and 15 p.i. We previously reported that nuclear RC DNA is less stable than CCC DNA (2, 14) and that the level of RC DNA is dependent upon recycling of RI. Therefore, it is not surprising that rtM204I RC DNA was decreased, thereby reflecting the reduced levels of RI being produced.

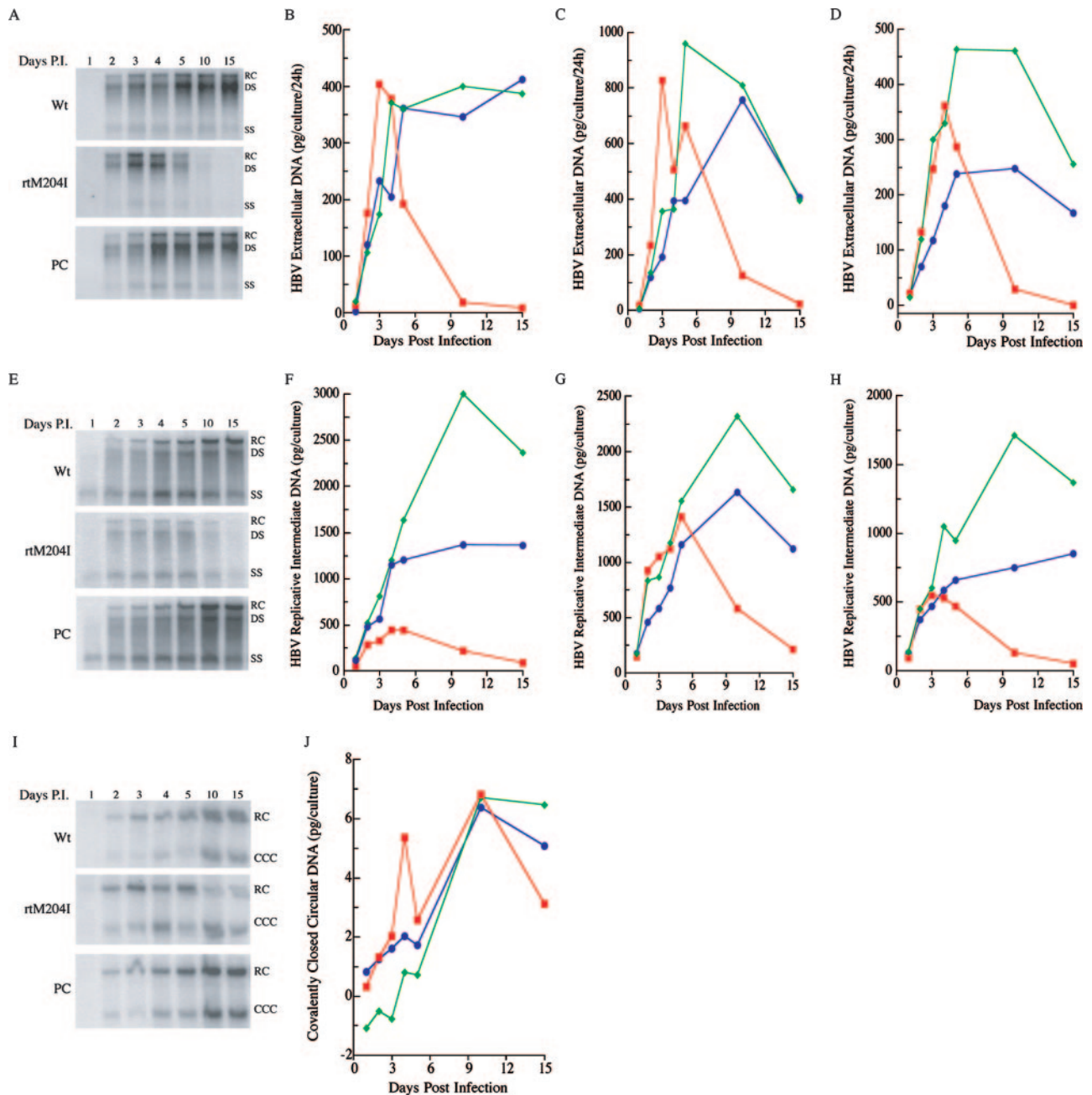


FIG. 2. Effects of PC and rtM204I mutations on extracellular HBV DNA production, intracellular HBV RI, and nuclear, non-protein-bound HBV DNA. HepG2 cells were seeded in 60-mm dishes at approximately 20% to 40% confluence, grown for 16 to 24 h, and then infected with 100 PFU/cell of either PC, rtM204I, or wt HBV recombinant baculovirus. Cells were fed daily. On the indicated days p.i., medium was collected for extracellular HBV DNA analysis, and cells were harvested for HBV RI DNA (cytoplasm) and nuclear, non-protein-bound HBV DNA (nucleus) analysis. HBV DNAs were detected in all samples by Southern blot analysis. HBV DNA standards were included in each gel, thereby making it possible to determine the amount of HBV DNA produced in pg/culture/24 h (extracellular HBV DNA) or in pg/culture (HBV RI and nuclear, non-protein-bound HBV DNA). The HBV DNA bands were visualized using a PhosphorImager and quantitated using ImageQuant software. All data were normalized for differences in gene transfer efficiency and plotted as the amount of HBV DNA produced relative to days p.i. The digital images from a representative experiment for extracellular HBV DNA are shown in panel A, followed by the quantitation of three independent experiments (B, C, and D). The digital images for intracellular HBV RI are shown in panel E, and the quantitation of three independent experiments is shown in panels F, G, and H. The digital images for nuclear, non-protein-bound HBV DNA are shown in panel I, and the quantitation of the images is represented in panel J. RC, relaxed circular DNA; DS, double-stranded DNA; SS, single-stranded DNA; CCC, covalently closed circular DNA. Lines: blue, wt (●); green, PC mutant (◆); red, rtM204I mutant (■).

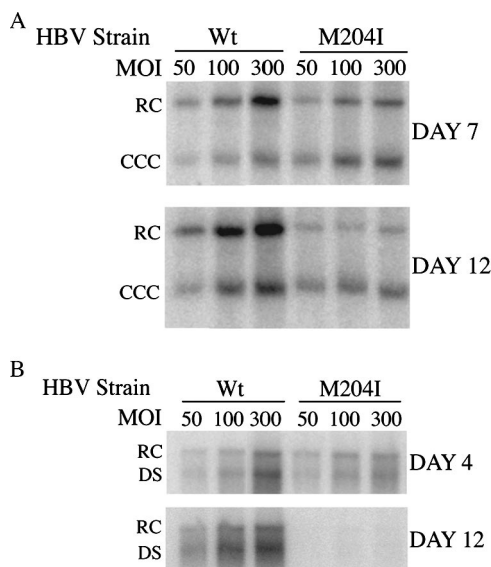


FIG. 3. Lack of effect of rtM204I mutation on CCC DNA level. HepG2 cells were infected with 50, 100, or 300 PFU/cell of either rtM204I or wt HBV recombinant baculovirus. Cultures were fed daily. On the indicated days p.i., cells were harvested and analyzed for nuclear, non-protein-bound HBV CCC DNA, using Southern blotting (A). The medium was collected and also analyzed for extracellular HBV DNA, using Southern blotting (B).

To further evaluate this finding, the studies were repeated using different levels of input recombinant baculovirus. HepG2 cells were infected with 50, 100, or 300 PFU of rtM204I or wt recombinant baculovirus. Cultures were fed daily. On the indicated days p.i., medium was collected, and cells were harvested and analyzed for nuclear CCC DNA. At both 7 and 12 days p.i., the levels of CCC DNA were similar whether infection was initiated with rtM204I or wt recombinant baculovirus (Fig. 3A). When medium harvested from the same cultures was analyzed, we observed that no extracellular HBV DNA was detected by 12 days p.i. with the rtM204I recombinant bacu-

lovirus (Fig. 3B), thereby repeating the previously observed results.

Effects of rtM204I mutation on levels of HBV transcripts. Since the rtM204I mutation had a significant effect on the production of extracellular HBV DNA and intracellular DNA by 10 days p.i. with HBV recombinant baculovirus, but not on the HBV CCC DNA level, we wanted to determine the effect of this drug resistance mutation on the production of HBV transcripts. HepG2 cells were infected with 100 PFU/cell of rtM204I or wt HBV recombinant baculovirus. Cultures were fed daily. On the indicated days p.i., medium was collected, and RNAs were isolated from the cells. Northern blot analysis was carried out using an HBV-specific probe and a glyceraldehyde-3-phosphate dehydrogenase probe to control for RNA loading. Analysis of the data indicated that the levels of the 3.5-, 2.4-, and 2.1-kb HBV RNA transcripts remained high throughout 25 days p.i., whether infection was initiated with rtM204I or wt recombinant baculovirus (Fig. 4). Southern blot analysis of the medium for extracellular HBV DNA showed the same findings as those observed previously; specifically, the rtM204I mutation had major effects on the production of HBV extracellular DNA by 10 days p.i.

Effect of coinfection with rtM204I and wt recombinant baculoviruses on wt HBV replication. The next question addressed was whether the effect of the rtM204I mutant on its own production of RI and virus at later time points would function in *trans*. Introduction of the rtM204I mutation not only alters HBV polymerase but also affects HBsAg. The rtM204I codon alteration in the mutant used in this study generates the W196S mutation in HBsAg. If the mutation leads to the production of an aberrant HBV gene product (polymerase, surface antigen, etc.) that blocks replication, then we would predict that infection with the rtM204I mutant should alter wt HBV replication at later time points. We know from previous studies that when HepG2 cells are infected with multiple recombinant baculoviruses expressing different reporter genes, the same cell can be coinfecting (H. C. Isom, unpublished data). HepG2 cells were coinfecting with wt and rtM204I recombinant baculoviruses and with baculovirus (empty vector)

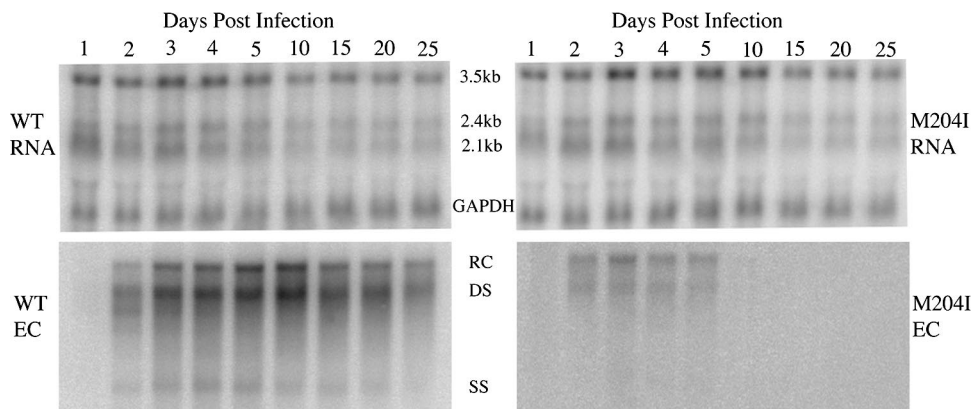


FIG. 4. Lack of effect of the rtM204I mutation on the levels of HBV transcripts. HepG2 cells were seeded in 60-mm dishes at approximately 20% to 40% confluence, grown for 16 to 24 h, and then infected with 100 PFU/cell of either rtM204I or wt HBV recombinant baculovirus. On the indicated days p.i., RNAs were isolated from cells, and medium was collected for extracellular HBV DNA analysis as described in Materials and Methods. Northern blot and Southern blot analyses were carried out, and digital images are shown. RC, relaxed circular DNA; DS, double-stranded DNA; SS, single-stranded DNA; EC, extracellular DNA.

under the following three conditions: rtM204I virus at 100 PFU/cell plus wt virus at 25 PFU/cell, wt virus at 25 PFU/cell plus 100 PFU/cell baculovirus, and rtM204I virus at 100 PFU/cell plus 25 PFU/cell baculovirus. Baculovirus containing no HBV was used as a control to maintain the multiplicity of infection at a constant value of 125 PFU/cell. A low MOI for wt HBV recombinant baculovirus (25 PFU/cell) and a high MOI for the rtM204I virus (100 PFU/cell) were used to maximize the detection of inhibitory effects of the rtM204I mutation on wt replication. On the indicated days, the medium was collected and analyzed for extracellular HBV DNA (data not shown), and cells were harvested for intracellular HBV DNA analysis (Fig. 5). Coinfection of wt HBV with rtM204I virus did not decrease the wt viral replication pattern, indicating that the failure of the rtM204I virus to replicate at late time points is not caused by the production of a product that can function *in trans* to inhibit wt HBV replication.

The fact that wt virus at 25 PFU/cell plus rtM204I virus at 100 PFU/cell generated higher levels of HBV replication (two-fold) than did wt virus at 25 PFU/cell plus control baculovirus on days 10 to 25 p.i. was unexpected. We anticipated no difference between wt virus at 25 PFU/cell and wt virus at 25 PFU/cell plus rtM204I virus at 100 PFU/cell because rtM204I virus at 100 PFU/cell is only barely detectable at days 10 to 25 p.i. Levels of HBV replication are linear when HepG2 cells are transduced with HBV recombinant baculovirus at multiplicities ranging from 25 to 200 PFU/cell (E. Chiari and H. C. Isom, unpublished data). If the rtM204I virus were fully reconstituted, then the levels of HBV replication from coinfection of wt virus at 25 PFU/cell and rtM204I virus at 100 PFU/cell would be increased fivefold instead of twofold. Therefore, whatever function is being provided *in trans* by wt HBV is only partially effective.

DISCUSSION

One of the initial goals of generating and characterizing HBV replication in HepG2 cells by using PC and rtM204I recombinant baculoviruses was to determine whether the HBV recombinant baculovirus/HepG2 system could be used to evaluate the effects of long-term antiviral treatment on HBV replication and the phenomenon of rebound after release from antiviral treatment for HBV mutants, as our laboratory has previously reported for wt HBV (1, 2, 13, 14). The mutants that were selected for this study were the PC mutation and the lamivudine resistance mutation in the C domain of the HBV polymerase protein causing the methionine residue at amino acid 204 to be replaced with isoleucine. HBeAg-negative CHB related to the PC mutation G1896A is seen in many parts of the world. Lamivudine, which is one of the approved therapies for treatment of CHB, is regarded as effective and safe, but the frequency of antiviral drug resistance mutations increases with the duration of therapy and has been associated with disease progression (21, 25).

The results for the PC mutant relative to the wt were straightforward and showed overall that the pattern and magnitude of HBV replication, when initiated by a PC HBV recombinant baculovirus, were similar to those observed when replication was initiated by a wt HBV recombinant baculovirus. One advantage of the baculovirus system is that the level

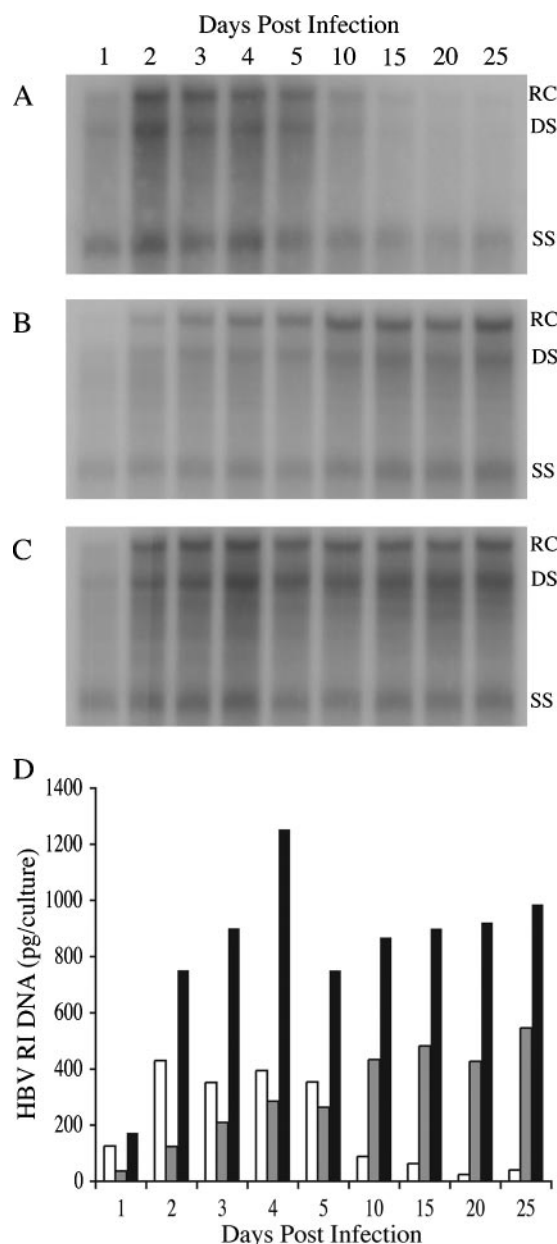


FIG. 5. Effect of coinfection with rtM204I and wt recombinant baculoviruses on wt HBV replication. HepG2 cells were seeded in 60-mm dishes at approximately 20% to 40% confluence, grown for 16 to 24 h, and then infected with either rtM204I mutant baculovirus (MOI = 100) plus baculovirus (MOI = 25) (A), wt virus (MOI = 25) plus baculovirus (MOI = 100) (B), or wt virus (MOI = 25) plus rtM204I mutant virus (MOI = 100) (C). On the indicated days p.i., cells were harvested and analyzed for HBV RI DNA by Southern blotting. The HBV DNA bands were visualized using a PhosphorImager and quantitated using ImageQuant software. The quantitation of the images is shown in panel D. RC, relaxed circular DNA; DS, double-stranded DNA; SS, single-stranded DNA. White bars, rtM204I mutant (MOI = 100) plus baculovirus (MOI = 25); gray bars, wt (MOI = 25) plus baculovirus (MOI = 100); black bars, wt (MOI = 25) plus rtM204I mutant (MOI = 100).

of replication is sufficiently high that Southern blot analysis can be carried out on a 24-hour medium sample to measure extracellular HBV production. We observed that the levels of intracellular RI were higher for the PC mutant than the wt in all

three experiments. However, the levels of extracellular virus were higher for the PC mutant in only one of the three experiments. It is important in making this comparison that for each time point in each experiment, the extracellular DNA and RI were harvested from the same culture plate. We concluded from these studies that the PC G1896A recombinant baculovirus demonstrates a long plateau of virus replication similar to that observed for wt recombinant baculovirus in HepG2 cells, and as such, that the HBV recombinant baculovirus/HepG2 system can be used to study the effects of antivirals on PC virus replication and also to analyze the phenomenon of rebound after cessation of antiviral therapy.

The results obtained for the rtM204I mutant were quite different. Infection with an HBV recombinant baculovirus carrying the rtM204I mutation resulted in the transient production of levels of intracellular HBV RI and extracellular HBV DNA similar to those seen for wt HBV and the PC mutant at early time points p.i. However, in contrast to what was observed for infection initiated by wt or PC recombinant baculovirus, the level of rtM204I intracellular RI or extracellular DNA did not remain high after day 5 p.i. However, the levels of CCC DNA and HBV transcripts produced by the rtM204I virus paralleled those for the wt at late as well as early time points. We concluded that at early time points posttransduction, there is little or no difference in replication of rtM204I virus compared to the wt.

Several previous studies have reported that the rtM204I mutant replicates *in vitro* with a lower efficiency than that of wt HBV (17, 18, 29, 36). The data we report here disagree with these findings for early time points after replication is initiated by HBV recombinant baculovirus and agree with these findings for late time points. Differences exist between the experimental systems used with regard to cell type, HBV strain, method used to initiate HBV replication, time points evaluated, etc. The data reported in our study make use of at least seven time points over at least a 15-day period. Fu and Cheng (17) showed that extracellular and intracellular HBV DNA levels were 100-fold less for the rtM204I mutant compared to the wt. In these studies, HepG2 cells were transfected using calcium precipitation, and cells were harvested at 3, 6, and 9 days posttransfection. Transfection efficiencies were not determined, and the amount of HBV DNA produced was not quantitated, making it difficult to compare to our studies. In addition, one potentially important difference is that the rtM204I mutation was in the HBV *adr* strain in the Fu and Cheng study and in the *ayw* strain in our study. In a second study, in which the rtM204I mutation was also analyzed in an HBV *adr* background, using Huh7 cells and harvesting at day 3 posttransfection, the levels of rtM204I DNA from intracellular core particles were approximately 10-fold less than the wt level (36). Levels of HBV DNA were reported relative to wt levels, and no absolute values were determined (36). In a study in which the rtM204I mutation was in the *ayw* background, RI were markedly reduced when hepatocellular carcinoma cells were transfected with plasmids containing the rtM204I mutant compared to transfection with wt HBV DNA (29). It is not clear whether HepG2 or Huh7 cells were used for these studies, and the time posttransfection was not provided. In a more recent study using transfection of HepG2 cells, intracellular rtM204I DNA was reduced 7-fold compared to wt DNA, and extracellular rtM204I DNA was

reduced 10-fold compared to wt DNA (18). Indeed, these findings may be very similar to ours. We did not have the same time point of 7 days after the introduction of baculovirus; however, in our system, levels of rtM204I mutant intracellular and extracellular DNA dropped markedly between 5 and 10 days p.i. Since only one time point was taken in the previous study (18), it is not possible to further compare the data. The data reported here are the first to show a condition under which the rtM204I mutant replicates to wt levels, as well as the converse, a condition under which rtM204I mutant replication is markedly impaired. This most likely stems from the fact that rtM204I and wt HBV replication was quantitated over a 15-day period, using multiple time points. In addition, recombinant baculovirus technology is highly efficient in transducing the majority of the population of cells, and this is the first *in vitro* model in which replication from input HBV DNA can be compared to replication from accumulated HBV CCC DNA. Further understanding the replication of the rtM204I mutant is critical because it can replicate to titers of 7 to 9 logs and be highly pathogenic in patients (40, 46).

The question that arises from the data presented in this study is as follows: why does the production of intra- and extracellular HBV DNA resulting from initiation of replication from rtM204I recombinant baculovirus occur at wt levels at early but not late times posttransduction? The rtM204I mutation, which results in lamivudine resistance, clearly also reduces HBV replication *in vitro*. These defects or differences are apparent only when HBV replication in the HBV recombinant baculovirus/HepG2 system is being driven from rtM204I CCC DNA, not recombinant baculovirus DNA. Levels of CCC DNA and HBV transcripts are the same for the rtM204I mutant compared to the wt at late time points. Possible defects include alterations in RNA encapsidation and/or decreased enzymatic activity of the polymerase during reverse transcription or second-strand DNA synthesis. Invoking these explanations also implies that at early time points, the defects can be overcome because more polymerase is synthesized, the polymerase that is synthesized has greater stability, or some other reason. HBV polymerase protein cannot be detected using the current HBV recombinant baculovirus/HepG2 system, and hence, future studies are needed to specifically address these questions.

Kinetic analyses have shown that YMDD mutant polymerases have lower affinities than wt polymerases for the natural deoxynucleoside triphosphate (dNTP) substrates (18). However, little is known about the functions of rtM204I mutant compared to wt polymerase expressed in cells of hepatic origin and/or in the context of HBV replication in cell culture. We cannot rule out the possibility that dNTP concentrations may play a role in the differences we observe in rtM204I mutant replication from input baculovirus compared to that from HBV CCC DNA. However, it is unlikely for two reasons. First, "infection" (transduction) with baculovirus yields far more robust levels of HBV replication than transfection. If dNTP pools are a limiting factor, it is surprising that the rtM204I mutant can achieve wild-type levels of replication during the first few days postinfection. Second, when HepG2 cells infected with rtM204I or wt recombinant baculovirus are subcultured 1:4 at 10 days p.i. and are rapidly growing, levels of rtM204I RI and extracellular virus remain low, while levels of

wt RI and extracellular virus remain high (Heipertz and Isom, unpublished data). It has been reported that rapidly dividing cells have higher concentrations of dNTPs than confluent or slower-growing cells (29). If dNTP pools were affecting rtM204I mutant replication, then we would have anticipated that subculture would have favored enhanced rtM204I HBV replication, as observed at early time points.

The data from this study have provided further information about using HBV recombinant baculovirus as a tool for studying HBV. Specifically, at early time points after transduction, the system is highly efficient at DNA transfection in that transcription is driven from the input DNA, whereas at later time points (7 to 10 days and beyond) transcription is driven from CCC DNA that has accumulated in the nuclei of HepG2 cells. Therefore, it is possible to use the baculovirus system by using the same cells in one experiment to compare transcription and subsequent replication from input recombinant DNA (at early time points) with transcription from CCC DNA (at late time points), the more natural template seen in the livers of patients with CHB. The data reported in this study also raise questions concerning the best methods to use to understand the replication of HBV drug-resistant viruses, in this case the rtM204I mutant. If the study described above had been limited to early time points posttransduction with each of the recombinant baculoviruses (3 to 5 days p.i.), differences in the production of RI or extracellular virus would not have been observed. Indeed, many studies of HBV are carried out using transient transfection with plasmid DNA and are restricted to several days.

We conclude that long-term effects of antivirals as well as the phenomenon of rebound after release from antiviral treatment can be studied using the recombinant baculovirus/HepG2 system for HBVs containing the G1896A PC mutation but not for viruses containing the rtM204I mutation. Short-term studies with antivirals can be carried out using rtM204I recombinant baculoviruses as long as it is realized that transcription is being driven from input baculovirus DNA, not CCC DNA. These studies have revealed two additional findings that require further investigation. First, the rtM204I mutation may cause differences in polymerase function. Second, in HepG2 cells at times when rtM204I HBV DNA production is significantly reduced compared to that of the wt, the rtM204I virus may be able to synergize with coinfecting wt virus as a result of functional wt polymerase being provided in *trans*.

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