Construction of Avian Hepadnavirus Variants with Enhanced Replication and Cytopathicity in Primary Hepatocytes

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Hepadnaviruses cause persistent noncytopathic infections of hepatocytes in humans and other animals. Virus replication depends on the pool of viral covalently closed circular DNA (cccDNA) molecules, which serve as transcriptional templates in the nuclei of infected cells. The size of this pool of cccDNA molecules is regulated by the ability of the large envelope protein of the virus to direct newly synthesized viral DNAs into a pathway for viral secretion and thereby inhibit their utilization for viral cccDNA synthesis. In this study, we showed that single amino acid changes in the large envelope protein could cause profound changes in cccDNA levels in transfected permissive cells or in infected cultured hepatocytes. While defects in cccDNA regulation were accompanied by a decrease of enveloped virus production in transfected cells, primary hepatocytes infected by such mutant viruses transiently produced wild-type or higher levels of enveloped virus. Moreover, high levels of cccDNA were always associated with cytopathic effects in the infected hepatocytes. The results demonstrate that the large envelope protein promotes persistent noncytopathic infection of hepatocytes by acting as an overall repressor of virus replication.

Duck hepatitis B virus (DHBV), an animal model for human hepatitis B virus (9), causes persistent noncytopathic infection of hepatocytes. We previously showed that viral DNA replication in infected cells is regulated by one of the two viral envelope proteins, p36. p36, in addition to carrying out a regulatory function, is required for the formation of enveloped virus particles and is also required for infectivity of enveloped virus particles (7, 14).

p36 carries out its regulatory function through its ability to inhibit amplification of the viral transcriptional template, covalently closed circular DNA (cccDNA). p36-mediated repression of cccDNA amplification separates hepadnavirus replication into two phases (16). The initial phase begins with the appearance in the nucleus of a single copy of cccDNA derived from an infecting virion. This molecule is transcribed to produce genomic RNAs (pregenomes), which are translated to produce the viral capsid protein (p35) and a multifunctional DNA polymerase, the P protein. Pregenomes, capsid proteins, and P proteins are assembled in the cytoplasm into nucleocapsids capable of viral DNA synthesis (13). A new copy of viral DNA is synthesized within each nucleocapsid by reverse transcription of the pregenome and synthesis of a complementary DNA strand to generate the final product, relaxed circular double-stranded DNA (13). Early during infection, newly synthesized DNA molecules are utilized through an intracellular pathway to produce additional molecules of cccDNA, resulting in amplification of the total copy number in the nucleus (14, 16, 17).

During the second phase of virus replication, viral envelope proteins accumulate in the infected cell, and one of these proteins, p36, inhibits further cccDNA amplification (7, 14, 15). Inhibition of cccDNA amplification is accompanied by the persistent release of enveloped virus from the infected cell. The function of p36 that is specifically responsible for inhibition of cccDNA amplification appears to be genetically indis-

tinguishable from one required for enveloped virus production, consistent with the model that p36 inhibits the utilization of cytoplasmic DNA-containing nucleocapsids for cccDNA synthesis by directing them into a pathway for virus assembly (7).

Virus replication therefore appears to be regulated by feedback inhibition of the synthesis of viral gene products by p36 through control of cccDNA synthesis. In this study, we investigated the consequences of missense mutations that diminished the ability of p36 to act as a feedback inhibitor of cccDNA synthesis. In spite of the fact that defects in this regulatory function of p36 produced accompanying defects in enveloped virus production when assayed in transfected cells, virus production was either unaffected or enhanced by such defects when assayed in primary hepatocytes. The results demonstrated that wild-type p36 acts as a regulator not only of cccDNA synthesis but also of enveloped virus production in hepatocytes, in which viral replication is entirely dependent on cccDNA levels. Moreover, such artificially constructed variants, in which repression of cccDNA levels and enveloped virus production by p36 was reduced, produced cytopathic infections in primary hepatocyte cultures. This result demonstrates the importance of viral regulation of replication to the ability of hepadnaviruses to infect hepatocytes persistently.

MATERIALS AND METHODS

Plasmids and mutations. The DHBV genome used in these studies was that sequenced by Mandart et al. (8). Site-directed mutagenesis was performed as described previously (6, 7), using an oligonucleotide which encoded a single nucleotide change in the second nucleotide of the codon for each of six amino acids 128 to 133 in the p36 open reading frame (7). These mutations were engineered so as not to alter the overlapping polymerase protein coding. A 900-nucleotide *Bgl*III-to-*Kpn*I restriction fragment of DNA containing the mutation was subcloned into an infectious viral genome-containing plasmid. This plasmid, pCMV.DHBV, consisted of the pregenome-encoding sequence positioned downstream of

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the immediate-early cytomegalovirus promoter, cloned in pUC119 (1). The mutations were designated by the one-letter code for the wild-type amino acid, the position in p36 of the substituted residue, and the substituted amino acid.

Genome 1165A contained a single nucleotide change which created a translational terminator in pre-S mRNA (14). Genome Δ DR1 (4) was used as a noninfectious helper plasmid in cotransfections to provide p36 envelope protein.

Cell culture and transfection. The avian hepatoma cell line LMH (5) was seeded at 3.5×10^6 cells per 60-mm-diameter dish in F12-Dulbecco modified medium (1:1) supplemented with 10% fetal bovine serum. Transfections were carried out by the calcium phosphate method (2, 3), using 10 μ g of DNA per dish. For cotransfections, 5 μ g of each plasmid was coprecipitated with calcium phosphate and added to the cells. The culture medium containing the calcium phosphate-DNA mixture was replaced with fresh medium after 18 h. At 2 and 3 days posttransfection, the medium was harvested for virus isolation. At 3 days posttransfection, the medium was removed, and the cells were washed once with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline and harvested for analysis of viral DNA.

Primary hepatocytes were prepared (17) from 3- to 7-day-old ducklings obtained from Metzger Farms (Redlands, Calif.) and cultured as previously described (11).

Assay of transfected cells for viral DNA. Isolation of replicative intermediates from transfected LMH cells has been previously described (15). cccDNA in transfected LMH cells was analyzed by the method of Yu and Summers (19). At 3 days posttransfection, the LMH cells were washed once with HEPES-buffered saline, and 1 ml of cccDNA isolation buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) was added to each plate. After incubation for 5 min at 37°C, KCl (0.25 ml of a 2.5 M solution) was added to the lysate, and the lysate was briefly vortexed and chilled on ice for 5 min. After removal of the detergent-protein precipitate by centrifugation, the supernatant was extracted with an equal volume of phenol and the nucleic acids were precipitated with ethanol. The dried pellet was dissolved in 40 μ l of Tris-EDTA (10:1).

To eliminate transfected plasmids that usually contaminated the viral cccDNA fraction, the nucleic acids were digested with *Dpn*I, which carries out methylation-dependent cleavage at many sites in the plasmid. Residual fragments of plasmid DNA were further digested with exonuclease III. Briefly, 10 μ l of the dissolved nucleic acid pellet was incubated with 5 U of *Dpn*I and 25 U of exonuclease III in restriction buffer containing 10 mM Tris (pH 7.5), 10 mM magnesium acetate, 50 mM NaCl, 1 mM dithiothreitol, and 0.01% Nonidet P-40 at 37°C for 3 h. One half of the viral cccDNA digest was assayed directly by agarose gel electrophoresis and Southern blot hybridization.

Concentration of enveloped virus from culture fluids. Culture fluids from transfected LMH cells were harvested at 2 and 3 days posttransfection, clarified by centrifugation at 10,000 rpm for 10 min, and stored at 4°C. Virus was precipitated by addition of polyethylene glycol to a final concentration of 10% and then incubated at 4°C for 1 h (15). The precipitates from 70 ml of medium were collected by centrifugation at 10,000 $\times$ g for 20 min and dissolved in 1.2 ml of hepatocyte culture medium containing 10 mM magnesium acetate. Residual DNA from the original transfection was removed by incubation with 100 μ g of DNase I (type II; Sigma) per ml at 37°C for 1 h. Twenty microliters of the suspension was saved for analysis of viral particles, and the remainder was used to infect eight 60-mm-diameter plates of primary hepatocytes.

Culture fluids from primary hepatocytes were harvested at 1,

2, 3, and 4 weeks postinfection and clarified as described above. Fetal bovine serum was added to a final concentration of 10% as a carrier, and the virus particles were precipitated with 10% polyethylene glycol. The DNase I digestion was omitted, and levels of virus were assayed as described below.

Assay of enveloped virus from transfected LMH cells or infected primary hepatocyte cultures. The enveloped virus particles precipitated from the culture fluids of transfected LMH cells or from infected hepatocytes were assayed for virus by selective protease digestion as described previously (7). Briefly, 5 μ l of the material saved for virus gel assay (see above) was added to 15 μ l of Tris-EDTA (10:1) containing 750 μ g of pronase per ml and incubated for 1 h at 37°C. Free viral DNA was then removed from the suspension by the addition of magnesium acetate to a final concentration of 10 mM and 2 μ l of DNase I (type II; 5 mg/ml; Sigma) and then incubated for 30 min at 37°C. The sample was subjected to electrophoresis through a 1% agarose gel, using DNA electrode buffer with buffer recirculation. Virus particles were transferred to a nylon filter by blotting, and viral DNA was detected by hybridization of the filter with a 32 P-labeled riboprobe specific for detection of viral minus strand (16).

The virus in the culture fluids of primary hepatocyte cultures was assayed by extraction of viral DNA from virus particles isolated by polyethylene glycol precipitation and pronase-DNase I digestion as described above. Viral DNA was extracted by the addition of SDS (0.5%) and pronase (500 μ g/ml) to the virus preparation after the DNase I digestion step and then subjected to phenol extraction, agarose gel electrophoresis, and Southern blot hybridization.

Infection of primary hepatocyte cultures. Culture supernatants from transfected LMH cells were concentrated as described above, and the pellets were dissolved in hepatocyte culture medium. Virus precipitated from 8 ml of LMH cell culture fluid was used to infect a 60-mm-diameter dish of duck hepatocytes. Infection of hepatocytes was carried out 2 days postplating by addition of the concentrated virus to the hepatocyte culture medium and incubation for 24 h at 37°C. Subsequently, the medium was changed daily for the course of the experiment.

Analysis of replicative intermediates and cccDNA in primary hepatocytes. Primary hepatocytes were collected at 1, 2, 3, and 4 weeks postinfection, and viral replicative intermediates and cccDNA were assayed. Cell layers were washed once with HEPES-buffered saline, and cells were lysed by the addition of 1 ml of lysis buffer (0.2% Nonidet P-40, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) to the plate. cccDNA was isolated directly from the nuclei by phenol extraction. Nuclei were pelleted by microcentrifuge centrifugation and resuspended in 1 ml of buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 1% SDS. Nucleic acids were purified by extraction with phenol followed by precipitation with ethanol.

Replicative DNA intermediates were isolated from the supernatant fraction by pronase digestion, phenol extraction, and ethanol precipitation. Typically 1/10 of the DNA extracted from one 60-mm-diameter plate was analyzed by electrophoresis through a 1% agarose gel, transferred to a nylon membrane, and hybridized with a 32 P-labeled RNA specific for detection of minus-strand nucleic acids.

Analysis of nucleocapsids in infected hepatocytes. Intracellular nucleocapsids and packaged RNA were assayed as previously described (1). Portions of the supernatant fraction described above were loaded on a 1% agarose gel and subjected to electrophoresis toward the anode. After electrophoresis, the capsids in the gel were transferred to a nitrocellulose filter (Hybond-C; Amersham Corp.) for detection of the

FIG. 1. Synthesis of virus, cccDNA, and replicative intermediates in transfected LMH cells by missense mutants. LMH cells were transfected with the indicated viral mutant DNAs, and after 4 days, the cells were assayed for relaxed circular (RC) and single-stranded (SS) DNA replicative intermediates and for cccDNA (CCC) by selective extraction and Southern blot hybridization. Enveloped virus produced by the same LMH cells transfected by the indicated mutant DNAs was analyzed by native agarose gel electrophoresis, transfer of the particles to a filter, denaturation, and hybridization. The amount of replicative intermediates and cccDNA loaded each represented 1/16 of a 60-mm-diameter plate, and the amount of virus loaded was 1/10 of that in a 24-h harvest of the medium from one plate. Exposure times for the replicative intermediates, cccDNA, and virus were 50 min, 5 h, and 24 h, respectively. WT, wild type.

capsid protein or to a nylon filter (Hybond-N; Amersham) for detection of total plus-strand nucleic acid. Capsid proteins and nucleic acids were detected as previously described (1).

Immunofluorescent staining of infected cells. Indirect immunofluorescence analysis was carried out on cells at 1, 2, 3 and 4 weeks postinfection. Cell layers were fixed at -20°C in 95% ethanol–5% acetic acid. The cells were then washed once with absolute ethanol at 22°C to extract intracellular lipid and dried. Fixed cells were incubated with rabbit anti-DHBV core antisera and then, after washing, with rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories).

RESULTS

Replication-competent DHBV genomes variant in cccDNA regulation and enveloped virus production were constructed by site-directed mutagenesis of the large envelope protein gene, p36, in a region previously shown by linker substitution scanning to produce coordinate defects in these two functions of p36 (7). Four such mutants defective to various degrees in these two properties were selected for the current study to determine the effect of such phenotypic variation on virus replication in primary hepatocyte cultures. When assayed in the LMH cell line, these variants produced levels of replicative viral DNA intermediates that were similar to those of the parental wild type, but virus production and cccDNA accumulation differed from that of the wild type (Fig. 1). As we reported previously, defects in enveloped virus production were always accompanied by defects in cccDNA regulation,

resulting in higher levels of cccDNA than those observed in wild-type-transfected cultures (7). In addition, amino acid substitutions diminished enveloped virus production to various degrees and resulted in corresponding increases in cccDNA. These defects were not due to an inability of the mutant proteins to be secreted from the cells (except for 1165A, which has a termination codon in the p36 open reading frame), since p36 was found in normal abundance in empty envelope particles in the culture medium of transfected cells (not shown). We concluded that the defects in these variant genomes were specifically in the ability of p36 to participate in whole virus assembly and regulation of cccDNA levels.

Virus replication in primary hepatocytes. We performed two independent studies of the effects of these mutations on the long-term infection of hepatocyte cultures, measuring the production of cccDNA, intracellular nucleocapsids, DNA replicative intermediates, extracellular virus, and cytopathic effects. In addition, we used a viral mutant in which p36 production was prevented by the presence of a stop codon created in the pre-S region of p36 by substitution of an adenylc acid residue at position 1165 (1165A [14]). Virus inocula were produced by transfection of LMH cells with cloned viral DNA containing the mutations. Wild-type p36 was provided by cotransfection of the LMH cells with a plasmid expression vector for viral proteins, ΔDR1 , in order to increase the yields of enveloped virus. Primary duck hepatocytes were infected with known amounts of DNA-containing particles for each of the variants and wild-type viruses and then incubated for 28 days. Cultures were harvested weekly for analysis. The results of these analyses, shown in Fig. 2 to 8, are normalized to the total viral products present in each culture or supernatant rather than to the number of infected cells.

In both experiments, the kinetics of DNA accumulation of the mutant viruses in the infected cells differed from that of the wild type. In Fig. 2 and 5, the mutants are shown from left to right according to the severity of the defect in virus production in LMH cells, as demonstrated in Fig. 1. In hepatocytes infected with the mutant viruses, replicative intermediates reached maximum levels in the cultures and then decreased, while in hepatocytes infected with wild-type virus, viral DNA either continued to increase over the 4-week period of the experiments or reached stable levels. In addition, the level of replicative intermediates in cultures infected with envelope mutants with more severe defects reached a maximum and decreased at earlier times postinfection. For example, the mutant 1165A-infected cultures displayed peak levels of viral DNA at the earliest time point (1 week), while the cells infected with the missense mutants showed peak levels at 2 or 3 weeks postinfection. In contrast, cultures infected with wild-type parental virus continued to accumulate viral DNAs through week 3 (Fig. 2) or 4 (Fig. 5). The decrease of replicative intermediates in the mutant-infected cultures was largely due to selective killing of infected cells (see below). As expected from cell killing, the kinetics of cccDNA accumulation roughly paralleled that of replicative intermediates, except that the maximum levels of cccDNA were always much higher in the mutant-infected cultures than in the wild-type-infected cultures.

Virus production and release into the medium in both experiments also showed different kinetics in the mutant-infected cultures than in the wild-type-infected cultures. In particular, the amount of virus released into the medium was roughly proportional to the level of replicative intermediates in the mutant-infected cultures, rising and then falling at later times, except in the cultures infected with the 1165A mutant, which was unable to produce any virus because of the complete

FIG. 2. Synthesis of viral DNAs and enveloped virus particles by primary hepatocytes infected with missense mutants. Primary hepatocyte cultures were infected with DHBV particles produced by transfection of LMH cells with the indicated mutant viral DNAs. Culture media were harvested at 2, 3, and 4 weeks, and cells were harvested at 1, 2, 3, and 4 weeks postinfection. Panels represent the amounts of replicative intermediates, relaxed circular (RC) and single-stranded minus-strand (SS) DNAs, cccDNA (CCC), and enveloped virus (Virus) at the indicated weeks postinfection. The amounts of cccDNA and replicative intermediates were equivalent portions of a plate of infected hepatocytes and can be directly compared. WT, wild type.

absence of p36 in the infected cells. However, the levels of cccDNA required to support a given amount of enveloped virus production were always higher in the mutant-infected cultures than in the wild-type-infected cultures. In addition, in most cases, the ratio of cccDNA to virus appeared to increase according to the severity of the defect in enveloped virus production, as seen in Fig. 1 (1165A > L131H > D128V > G133E > wild type). This result suggested that the defects in mutant p36 function were compensated for by quantitative increases in mutant p36 production, mediated through increased amplification of cccDNA.

Intracellular nucleocapsids and the amount of packaged viral RNA were assayed for each time point (Fig. 3 and 6). The results of these assays differed somewhat between the two experiments. Capsids and packaged viral RNA appeared to be proportional to cccDNA levels rather than replicative intermediates at all time points in the experiment shown in Fig. 3, while nucleocapsid levels were proportional to cccDNA levels only at the early time points in the experiment shown in

Fig. 6. Overall, the results suggested that higher levels of cccDNA in the mutant-infected cultures resulted in increased synthesis and accumulation of viral RNA-containing nucleocapsids but that this increased synthesis was not necessarily reflected in increased viral DNA synthesis.

Cytopathic effects of p36 variants in primary hepatocytes. The difference in kinetics of viral DNA accumulation in the mutant- and wild-type-infected cultures was primarily due to the death of hepatocytes infected with mutant viruses and the survival of hepatocytes infected with the parental wild-type strain. Evidence for such cytopathic effects was seen in the results of immunostaining of the infected cultures at weekly intervals, as shown in Figs. 4 and 7. As judged by the frequency of core antigen-staining cells at 1 week postinfection, approximately equivalent numbers of cells were infected with the initial inoculum, except in the case of mutant L131H, which always yielded about 50% the level of virus particles even when complemented with wild-type p36. Cells infected with the mutant viruses stained distinctly brighter for the presence of

FIG. 3. Capsids and packaged RNA in primary hepatocytes infected with missense mutants. Nucleocapsids from infected primary hepatocytes in the experiment shown in Fig. 2 were assayed by nondenaturing agarose gel electrophoresis and capillary transferred to a nitrocellulose filter, and the capsid antigens (Cap) were detected by incubation with a rabbit anticapsid antiserum and 125 I-protein A. In a parallel gel, particles were transferred to a nylon filter, denatured, and probed for total plus-strand nucleic acids (RNA). WT, wild type.

FIG. 4. Cytopathic effects in primary hepatocytes infected with missense mutants. Primary hepatocyte cultures from the experiment shown in Fig. 2 were fixed at weeks 1, 2, 3, and 4 postinfection and stained for the presence of cells containing viral capsid antigen. The appearance of amorphous staining debris was seen only in infected cultures and was considered to represent the death of infected cells. WT, wild type.

the capsid protein, adding further support to the notion that these cells produced higher levels of capsid protein, probably as a result of higher levels of cccDNA. However, mutant-infected cultures accumulated increasing amounts of speci-

cally staining amorphous material which appeared to represent the remnants of injured and dead cells. The disappearance of morphologically normal staining cells and the appearance of core antigen-positive cellular debris paralleled the decreases in

FIG. 5. Synthesis of viral DNAs and enveloped virus particles by primary hepatocytes infected with missense mutants. Shown is analysis as in Fig. 2 of a second long-term infection of primary hepatocyte cultures. Designations are as in Fig. 2.

FIG. 6. Capsids and packaged RNA in primary hepatocytes infected with missense mutants. Shown is analysis as in Fig. 3 of a second long-term infection of primary hepatocyte cultures. WT, wild type.

viral replication seen in the cultures. In addition, mutant-infected cultures released into the medium large amounts of free nucleocapsids, coinciding with the appearance of cytopathic effects (data not shown). The apparent survival time of cells infected with the viral mutants appeared to be related to the ability of their respective p36 envelope proteins to support virus production and to regulate cccDNA synthesis (wild type > G133E > D128V > L131H > 1165A).

Production of infectious virus. Figure 8 shows that the excess production of enveloped virus particles by the G133E mutant-infected cultures was accompanied by high levels of infectivity in the culture fluids. Virus particles from culture media removed at 3 and 4 weeks postinfection were used to

inoculate fresh primary hepatocyte cultures. The infected cultures were harvested at 1 and 2 weeks postinfection, and the relative levels of infection were determined from the levels of replicative intermediates. In addition, the levels of cccDNA were measured to ascertain that virus growing in the cultures remained phenotypically true. In both cases, the infectivity in the culture media was roughly proportional to the amount of DNA-containing enveloped particles (Fig. 2 and 5). Moreover, infectious virus in the culture medium displayed the same phenotype for cccDNA accumulation as the original inoculum, i.e., increasing through 2 weeks postinfection in the G133E-infected cells but remaining relatively constant in the wild-type-infected cells.

FIG. 7. Cytopathic effects in primary hepatocytes infected with missense mutants. Shown is analysis as in Fig. 4 of a second long-term infection of primary hepatocyte cultures. WT, wild type.

FIG. 8. Infectious virus assay of culture fluids of primary hepatocytes. Virus particles harvested at 3 and 4 weeks postinfection (as indicated above the lanes) from primary hepatocytes infected with mutant G133E or with wild-type (WT) virus were used to infect fresh cultures of primary hepatocytes. The secondary infected cultures were harvested at 1 and 2 weeks postinfection (as indicated below the lanes) and assayed for replicative intermediates (relaxed circular [RC] and single stranded [SS]) and for cccDNA (CCC). The upper and lower panels can be directly compared as in Fig. 2 and 5.

DISCUSSION

We have shown that infectious DHBV variants with small alterations in the p36 envelope protein can display characteristics of virus production and persistence that are quite different from those of the wild-type strain found in most naturally occurring infections. In particular, missense mutations that disturbed the regulation of cccDNA amplification produced virus strains that were infectious but, unlike wild-type strains, were cytopathic. Moreover, these strains were capable of transiently producing virus yields that equaled or exceeded wild-type levels in primary hepatocyte cultures. Whether such simple variants could be transmitted naturally or cause virus-mediated hepatic damage *in vivo* is not known.

It is likely that there is a strong natural selection against DHBV variants that produce hepatic damage since this virus is normally transmitted *in ovo* by persistently viremic, healthy laying hens (9, 10). Mammalian hepadnaviruses, including human hepatitis B virus, depend to a large extent on perinatal vertical transmission from persistently infected mothers for maintenance of the viral reservoir in a population of asymptomatic carriers (12), and therefore a similar selection against cytopathic viruses by this transmission route could be expected. However, cytopathic hepadnavirus strains might be propagated under special conditions of horizontal transmission that do not require the health or long-term survival of the infected host.

The kinetics of virus replication in these experiments is the product of many factors whose contributions we have not quantitatively resolved. These factors include (i) the rate of production of viral DNA and gene products in each cell, (ii) the duration of the infection in any one cell, (iii) the effect of cytopathic effects on the rates of various intracellular steps of virus replication, (iv) the rate of disappearance of viral DNA and viral gene products from the cultures as a result of cell

death, and (v) the contribution of second rounds of infection. The interplay of these and other factors can be used to explain the seemingly paradoxical result that progressive decreases in the ability of p36 to suppress cccDNA synthesis led to lower rather than higher recoveries of intracellular viral DNA compared with the wild type, particularly at later times (1165A < L131H < D128V < G133E). First, our assays roughly measured the average amount of replicative intermediates and cccDNA per infected cell, but the higher levels that result in cytopathic effect may occur only transiently before cell death and thus be seen only in a small fraction of the population at any one time. Second, the amount of viral gene products produced by an infected cell is the integral with time of the rate of production (e.g., cccDNA levels) over the duration of the infectious cycle. If defects in p36 shortened the time required to achieve a cytopathic level of cccDNA, the total accumulation of viral products in the cell would be decreased as well. Third, the loss of viral DNA and viral gene products from the cultures due to cell lysis may have contributed to the lower levels of cell-associated virus replicative intermediates seen in the mutant p36-infected cultures. Finally, second cycles of infection would tend to prolong the time during which cell-associated viral DNA could be observed in the cultures.

Our results also raise the question of the mechanism of cell damage resulting from the deregulation of cccDNA amplification. We have shown previously that high levels of cccDNA resulted in high levels of viral RNA (15), and the experiments reported here suggested that higher levels of capsid and envelope proteins accumulated in these infected cells, although these higher levels were not reflected in the amount of DNA replicative intermediates produced. Which of these viral products, i.e., cccDNA, RNA, or proteins, might have been responsible for damage to the cell, or even whether cell damage was a direct toxic effect of viral genes or gene products is unclear. The failure of cultures that accumulated high levels of RNA-containing nucleocapsids to produce equivalent high levels of viral replicative DNA intermediates may have been due to impairment of viral DNA synthesis due to cell damage induced by one of these viral products.

Comparison of mutants with defects of different severity in p36 suggests that the most severe defects resulted in the most rapid death of infected hepatocytes. Moreover, delayed onset of cytopathic effect seemed to be correlated with the accumulation of higher levels of viral DNA in the cultures and correspondingly higher levels of enveloped virus production, even exceeding that observed in wild-type-infected cells (G133E; Fig. 2 and 5). This increase in viral DNA and enveloped virus may have been due to the extended period during which G133E-infected cells were accumulating and expressing abnormal levels of cccDNA prior to their death. It is possible that defects in p36 less severe than those reported here could produce more profound increases in virus replication levels in infected cultures if these defects allowed prolonged survival of the infected cells.

In mutant G133E, a decrease in the ability of p36 to participate in enveloped virus production in transfected LMH cells (Fig. 1) resulted in a significantly enhanced production of enveloped virus in primary hepatocytes (Fig. 2 and 5). This effect demonstrates that the large envelope protein of DHBV, p36, functions as a net regulator of virus production according to its ability to control the cccDNA pool size. Moreover, seemingly modest defects in p36 function resulted in cytopathic effects. We conclude that profound variation in the growth and cytopathic properties of hepadnaviruses can occur through simple amino acid substitutions in p36; however, the requirements of viral persistence and transmission can explain

why this repressor activity of p36 has been maintained in wild-type hepadnavirus strains.

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