Hepadnavirus Envelope Proteins Regulate Covalently Closed Circular DNA Amplification

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Primary duck hepatocytes were infected with a mutant duck hepatitis B virus defective in envelope protein but competent for viral DNA synthesis. Cells infected by this mutant accumulated higher levels of viral covalently closed, circular DNA (cccDNA) than those infected by wild-type virus. The accumulation of high levels of cccDNA was due to a failure of the mutant-infected cells to suppress de novo cccDNA synthesis compared with suppression by cells infected by the wild type. The envelope-defective virus failed to establish a persistent infection in vitro, possibly because of a virus-mediated cell death. Therefore, one or both viral envelope proteins are required for regulation of cccDNA synthesis and for maintenance of persistent infection in vitro.

The conversion of the viral relaxed, circular DNA genome into covalently closed, circular DNA (cccDNA) and its appearance in the nucleus is one of the earliest events in the replication of hepadnaviruses (4). cccDNA is thought to be the template for transcription of mRNAs and pregenome RNA, the template for synthesis of new DNA genomes by reverse transcription (10).

We have previously shown that within the first 4 days after infection of primary duck hepatocytes by the duck hepatitis B virus (DHBV), the amount of cccDNA in the infected cells increased to about 50-fold over the amount derived from the infecting virus (10). This cccDNA amplification was shown to occur through de novo synthesis by reverse transcription rather than through semiconservative replication. Circumstantial evidence suggested that cccDNA amplification occurred through an intracellular pathway prior to virus release rather than through reinfection of cells by progeny virus particles. Subsequent evidence showing that amplification is resistant to the presence in the medium of suramin, a drug that blocks DHBV infection (5), supports this view (13).

cccDNA levels in persistently infected hepatocytes in vivo have been estimated at various values, ranging from a low of 3 to a high of 50 copies per nucleus. These low copy numbers suggest that amplification of cccDNA in natural infections is confined to the early phase of the infection and that the size of the pool of cccDNA may be limited by specific control mechanisms. It seems likely that without controls to limit amplification of cccDNA in infected cells, continued accumulation of the viral transcriptional template would result in damage to the infected cells. Thus, control of cccDNA amplification is, theoretically, an important feature necessary for the ability of hepadnaviruses to infect cells persistently, without cytopathic effects.

Tuttleman et al. (11) postulated that amplification may be controlled through feedback inhibition of cccDNA synthesis by a viral gene product. We have therefore attempted to identify mutations in viral genes which may influence or destroy cccDNA copy number control. The only viral gene known not to be required for viral DNA synthesis is that encoding the viral envelope proteins, p36 and p17 (1), which

are encoded by the same open reading frame but are initiated from different AUGs (6, 9). We therefore tested, using site-directed mutagenesis, whether these two proteins are required for control of cccDNA amplification. The results demonstrate that the production of defective viral envelope proteins results in a greatly enhanced accumulation of cccDNA. This enhanced accumulation appears to be due to a failure to turn off cccDNA synthesis at early times after infection. The transient nature of the infection by the envelope-defective mutant suggests that failure to control cccDNA amplification may be cytotoxic.

MATERIALS AND METHODS

Cells and cell culture. Transfection of plasmid DNAs was carried out in the chicken hepatoma cell line LMH (2). LMH cells were maintained in F-12-Dulbecco modified Eagle medium (1:1) supplemented with 10% fetal bovine serum. Primary duck hepatocyte cultures were used for the growth of DHBV (12). Ducklings were obtained from Metzger Farms or Red Wing Hatcheries, and primary hepatocytes were prepared from 5- to 12-day-old ducklings by perfusion of the liver with collagenase as previously described (12). Cells were plated at confluence and maintained at 37°C in Leibowitz medium (L-15) supplemented with glucose (1 mg/ml), insulin (1 µg/ml), hydrocortisone hemisuccinate (10 µM), penicillin (100 µg/ml), streptomycin (100 µg/ml), and dimethyl sulfoxide (1%) (7).

Plasmids. Infectious DHBV DNA-containing plasmids consisted of *Eco*RI-linearized DHBV DNA cloned into the plasmid vector pSP65 (Promega Biotec) as a head-to-tail dimer. Mutants ΔDR1 (DNA⁻) and 1S (env⁻), previously described (1), were derived from the wild-type clone pSPD HBV 5.1 (2X) by precise deletion of both copies of DR1, a sequence required for correct minus- and plus-strand initiation (ΔDR1), or by single base substitutions (T→A at positions 1327, 1346, and 1349, numbered according to Mandart et al. [3]) to terminate translation of both envelope proteins p17 and p36 (1S). Plasmids were grown in *Escherichia coli* DH5α (Bethesda Research Laboratories) and purified through cesium chloride gradients containing ethidium bromide.

Transfections. Transfections were carried out as previ-

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ously described (8). Plasmid DNAs (3 µg total), either alone or in combination, were added as calcium phosphate precipitates directly to 60-mm dishes of LMH cells containing 3 ml of medium, and the plates were left undisturbed for 15 h. The medium was then removed and replaced with fresh medium. Growth medium was changed again in 2 days, and after 2 more days of incubation, the culture supernatants were removed and used as an inoculum for primary hepatocyte cultures.

Infections. Primary hepatocyte cultures were infected 1 to 3 days postplating. Culture supernatant from transfected LMH cells (0.3 ml) was added directly to the hepatocyte culture medium (4.0 ml), and the inoculated medium was allowed to remain on the cells for 24 h. The inoculum was removed and replaced with fresh medium (day 0). The medium was changed every day thereafter.

Isolation of viral DNA from infected hepatocytes. Infected hepatocytes in 60-mm dishes were lysed by the addition of 1 ml of lysis buffer (50 mM Tris hydrochloride [pH 8.0], 10 mM EDTA, 150 mM NaCl, 1% sodium dodecyl sulfate, 0.5 mg of pronase per ml) at 37°C for 60 min. The digested lysate was extracted one time with an equal volume of phenol, and nucleic acids were precipitated from the aqueous layer by the addition of 2 volumes of absolute ethanol. The pellet was collected by centrifugation, washed in 70% (vol/vol) ethanol and then absolute ethanol, and dried. Total nucleic acids were dissolved in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) and analyzed by gel electrophoresis and blot hybridization.

For the isolation of cccDNA and replicative intermediates, cells were lysed in lysis buffer without pronase, and protein-detergent complexes were precipitated by the addition of 0.25 ml of 2.5 M KCl. The lysates were mixed by shaking, and the insoluble material containing viral replicative intermediates (precipitated because they are covalently bound to protein) and most of the cellular DNA were removed by centrifugation. The supernatant, containing viral cccDNA, was extracted with an equal volume of phenol, and total nucleic acids were precipitated with 2 volumes of ethanol. Replicative intermediates were recovered from the detergent pellet by dissolving in 1 ml of TE containing 0.5 mg of pronase per ml. The pellet was dispersed by intermittent vortex mixing and incubation at 37°C. After 1 h, the digested pellet was extracted with phenol, and total nucleic acids were precipitated with 2 volumes of ethanol.

Analysis of viral DNA. Viral DNA was analyzed by electrophoresis through 1% agarose gels and transferred by blotting to nylon membranes (Hybond-N, Amersham Corp.). Hybridization was carried out at 50 to 52°C in hybridization medium containing 50% formamide as previously described (1). Hybridization probes were generated by *in vitro* transcription of pSP65 plasmids containing DHBV DNA cloned behind the SP6 promoter so that the radioactive transcript was complementary to the viral minus strand (11).

Cesium chloride gradient fractionation of bromodeoxyuridine (BUdR)-substituted DNA was carried out in a vTi80 rotor at 65,000 rpm for 15 h at 25°C. The starting density of cesium chloride was 1.75 g/cm³.

Immunostaining of infected cells. Indirect immunofluorescent analysis was carried out on cells that were fixed at -20°C in 95% ethanol-5% acetic acid. Fixed cells were incubated with rabbit antisera, kindly supplied by W. S. Mason, directed against either DHBV core protein or DHBV surface antigen. After washing, the cells were incubated with

rhodamine-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika).

RESULTS

We have previously described a system for the production of infection-competent DHBV particles containing mutant viral genomes (1). This system employs cotransfection of a human hepatoma cell line, Huh-7, with a plasmid containing a mutated DHBV genome to be packaged, and a plasmid which provides viral proteins for packaging *in trans* but which cannot synthesize infectious virus because of a deletion of both copies of DR1 (ΔDR1). Since the function of DR1 acts only in *cis*, viral particles produced are free of DNA-containing helper virus and contain only the DNA genome of the mutant. We have adapted this system to transfection of the chicken hepatoma cell line LMH (2), which has been reported to produce at least 10-fold higher yields of infectious virus than do Huh-7 cells following transfection (L. D. Condreay, C. E. Aldrich, L. Coates, W. S. Mason, and T.-T. Wu, *J. Virol.*, in press).

Envelope proteins are not required for cccDNA amplification. We utilized this cotransfection system to produce infection-competent particles that contain an envelope-defective, but DNA synthesis-competent, viral genome in order to determine whether envelope expression is necessary for either the amplification or the copy number control of viral cccDNA in infected hepatocytes. To determine whether envelope function is required for cccDNA amplification, we carried out an infection of primary hepatocytes with supernatant fluids from LMH cells that had been cotransfected with ΔDR1 and the envelope mutant, 1S. BUdR (10 µg/ml) was added at the time of addition of the inoculum in order to density label all cccDNA that was synthesized *de novo*, i.e., amplified. After 4 days, cccDNA was extracted from the infected cells, fractionated by isopycnic centrifugation in a cesium chloride gradient, and analyzed by gel electrophoresis and blot hybridization. The results of this experiment (Fig. 1) indicate that at least 90% of all cccDNA present in the mutant-infected cells at 4 days postinfection was density labeled and therefore not derived from the infecting virus. Since the mutant virus cannot produce infectious particles, this result indicates that amplification can occur through an intracellular pathway.

Envelope proteins regulate cccDNA amplification. We compared the accumulation and rate of synthesis of viral cccDNA in hepatocytes infected with the envelope-defective virus and wild-type virus. In order to prevent initiation of new rounds of infection in the wild-type-infected cultures, we added suramin to the medium of all cultures upon removal of the initial inoculum. Infected cells were exposed to BUdR for periods of 3 days at different times after infection, and intracellular viral DNA was extracted immediately after the pulse. In comparing the amounts of cccDNA (Fig. 2A) or replicative forms (Fig. 2B) that had accumulated in the cells by 4, 7, and 10 days postinfection, it was seen that accumulation of replicative forms was approximately equal in the wild-type- and mutant-infected cultures. In contrast, accumulation of cccDNA by the 1S mutant greatly surpassed that of the wild type at 7 and 10 days. In fact, it appeared that cells infected by wild-type virus did not further accumulate cccDNA after 4 days, while the mutant-infected cultures continued to amplify cccDNA at an accelerated rate through day 10.

In order to measure the relative rates of cccDNA synthesis during the BUdR labeling periods, we fractionated the

FIG. 1. Cesium chloride fractionation of BUdR-labeled cccDNA from primary hepatocytes infected with an envelope-defective mutant. Primary hepatocytes were infected with the supernatant fluid of LMH cells that had been transfected with Δ DR1- and 1S-containing plasmid DNAs, as described in Materials and Methods. BUdR (10 μ g/ml) was added at the time of addition of the inoculum, and after 4 days, cccDNA was extracted and fractionated in isopycnic cesium chloride density gradients. Fractions were analyzed by electrophoresis through a 1% agarose gel and blot hybridization. The buoyant densities of fully substituted (H/H) and unsubstituted DNAs (L/L) and the gel migration positions of cccDNAs (CCC) and relaxed circular DNAs (RC) are indicated. Molecular weight markers were run in lane St. Sizes are indicated on the right in kilobase pairs (kbp).

cccDNA samples in cesium chloride density gradients. Figure 3 shows the results of agarose gel electrophoresis of the gradient fractions. As previously observed (11), amplification of cccDNA by wild-type-infected cells occurred between 1 and 4 days postinfection, as judged by the amount of density-labeled cccDNA that was present. Between 4 and 7 days and 7 and 10 days postinfection, decreasing amounts of cccDNA were produced *de novo*, corresponding to the lack of accumulation seen in the unfractionated sample. In mutant-infected cells, density-labeled cccDNA appeared in increasing amounts in the sequential labeling periods, indicating an accelerating rate of synthesis. This increasing rate of synthesis was reflected in a striking accumulation of cccDNA in the infected cells which was clearly different from that seen in the wild-type infection.

Curiously, a large amount of relaxed circular viral DNA was seen in the cccDNA fraction from wild-type-infected cells at 10 days postinfection. The presence of relaxed, circular viral DNA in this fraction suggests that, unlike relaxed, circular DNA from viral cores, it was not covalently linked to protein. This DNA was not derived from nicking of cccDNA during its isolation, since its density distribution in CsCl gradients was unlike that of authentic cccDNA from the same sample. Since the density labeling characteristics of this species resembled those of cccDNA at 10 days in cells infected with the 1S mutant, it is possible that this DNA represents an immediate precursor to cccDNA whose conversion to cccDNA was blocked by envelope expression in the wild-type infection. However, other explanations are also possible.

In three separate experiments, accumulation of non-protein-bound, relaxed, circular DNA after 10 days postinfection has been observed in wild-type infections incubated in the presence of suramin but not in cultures incubated in the absence of suramin. Accumulation of this species has never been observed in cultures infected with envelope mutants,

FIG. 2. Accumulation of cccDNA and replicative intermediates in primary hepatocytes infected with wild-type and envelope-defective viral genomes. cccDNA or replicative intermediates were harvested from infected cell layers at 4, 7, and 10 days postinfection. Each culture was labeled with BUdR for 3 days prior to harvest, and a portion of each sample equivalent to one-fifth of a 60-mm plate was electrophoresed through a 1% agarose gel and analyzed by blot hybridization. Replicative intermediates are shown in panel A, and cccDNAs are in panel B. Gel migration positions of relaxed circular (rc), covalently closed circular (ccc), and single-stranded (ss) forms of viral DNA are indicated.

incubated either with or without suramin. This dependence on both suramin and functional envelope proteins and the late appearance of this species (compare 7 and 10 days, Figure 2B) are also consistent with a defect in initiation of a second round of infection in the presence of suramin. We do not know, at this time, the structure of this species of viral DNA, nor do we know whether its accumulation occurs in the initially infected cells or in the uninfected cell population which has been exposed to progeny virus.

Envelope proteins are required to establish a persistent infection. The long-term consequences of a lack of regulation of cccDNA synthesis were examined in infected hepatocytes by observing the fate of wild-type- or mutant-infected cells for 20 days postinfection. Total DNA was extracted from the cell layer at various times after infection and analyzed by Southern blot hybridization (Fig. 4). Viral DNA in wild-type-infected cultures continued to increase throughout the experimental period, while the level of viral DNA in mutant-infected cultures declined rapidly after 12 days postinfection and by 20 days was less than 1/10 the maximum level achieved in the culture. The accumulation of cccDNA by the mutant was readily apparent at 4 and 8 days postinfection, and by that time, cccDNA constituted a major species of viral DNA in the cell. In contrast, cccDNA in the wild-type-infected cells was barely discernible among the predominant replicative intermediates in all samples.

FIG. 3. Cesium chloride density gradient fractionation of cccDNA labeled with BUdR at various times postinfection. cccDNA samples shown in Fig. 2 were fractionated by buoyant density in isopycnic cesium chloride gradients, and the fractions were analyzed by electrophoresis through a 1% agarose gel and blot hybridization. The buoyant densities corresponding to fully substituted (h/h) and unsubstituted (I/I) DNA and the migration positions of relaxed circular DNA (rc) and cccDNA (ccc) are indicated.

FIG. 4. Accumulation of DHBV DNA in primary hepatocytes infected with wild-type and envelope-defective genomes. Primary hepatocyte cultures were infected with virions containing wild-type and 1S mutant genomes. Total DNA was harvested at 4, 8, 12, 16, and 20 days postinfection, and virus-specific sequences were analyzed by electrophoresis through 1% agarose and blot hybridization. The positions of viral DNA forms are indicated as in previous figures. Molecular weight markers (as in Fig. 1) are in the left lanes of each panel.

Cultures were fixed and stained for viral antigens by immunofluorescence (Fig. 5). Single cells stained for both capsid antigens and envelope antigens were seen at day 6 in wild-type-infected cultures, but only cells stained for capsid antigens were seen in the mutant-infected cultures. At 20 days postinfection, the spread of wild-type virus in the cultures was evidenced by the greatly increased number of capsid antigen-stained cells. In the mutant-infected cultures, few cells stained for capsid antigens were seen after 20 days. The scarcity of infected cells corresponded to the disappearance of viral replicative DNA from the mutant-infected cultures.

A transient infection, similar to that seen in cultures infected by the mutant virus, could not have been observed in the cultures infected by wild-type virus, since the spread of progeny virus would have masked any disappearance of replicative forms in the initial infected cells. Therefore, we carried out an infection of primary hepatocytes with mutant and wild-type virus and incubated the infected cells in the presence of suramin to inhibit virus spread (5) (Fig. 6). Suramin added to the culture medium immediately after removal of the inoculum did not inhibit the accumulation of replicative intermediates in either infected culture, and at times at which viral DNA had significantly diminished in the mutant-infected cells, cells infected by wild-type virus were still accumulating viral DNA. This result indicates that the envelope mutation destroyed the ability of the mutant genome, compared with that of the wild type, to establish a persistent infection in cultured hepatocytes.

DISCUSSION

The results of this study indicate that (i) neither of the viral envelope proteins is required for amplification of cccDNA during the early phase of hepadnaviral infection, but one or both of the viral envelope proteins are necessary for eventual suppression of amplification, and (ii) amplification is suppressed at the level of DNA synthesis. The mechanism

FIG. 5. Immunostaining of primary duck hepatocytes for DHBV capsid and envelope proteins. Primary hepatocyte cultures infected with virions containing wild-type and 1S mutant genomes as in Fig. 4 were fixed at either 6 or 20 days postinfection and stained for viral capsid (α -core) or envelope (α -surface) proteins as indicated. Each field contains approximately 500 cells.

FIG. 6. Accumulation of viral DNA in primary hepatocytes maintained in the presence of suramin. Primary hepatocytes were infected with virions containing wild-type or 1S mutant genomes. Upon removal of the inoculum after 24 h, the medium was replaced with hepatocyte medium containing suramin (100 μ g/ml). The infected cells were maintained in the presence of suramin until they were harvested at the times indicated. Total DNA was extracted, and viral specific DNA was analyzed by electrophoresis through 1% agarose and blot hybridization. The positions of viral DNA forms are indicated as in previous figures. Molecular weight markers (as in Fig. 1) are in the right lanes of each panel.

by which this suppression of cccDNA synthesis occurs was not elucidated by these experiments.

Tuttleman et al. (11) showed that cccDNA amplification involves the conversion of cytoplasmic relaxed circular viral DNA to nuclear cccDNA. Virtually all cytoplasmic, relaxed, circular DNA is synthesized inside, and contained within, viral nucleocapsids. In a wild-type infection, conversion of a small fraction of this DNA to nuclear cccDNA must involve several steps: nuclear transport, unpackaging, and DNA repair and ligation. The order in which these processes occur is not known.

The majority of cytoplasmic nucleocapsids containing relaxed circular DNA are ultimately secreted from the cell as mature virions, enveloped in lipid-containing membranes containing the viral envelope proteins. Therefore, there are at least two alternative pathways for determining the fate of cytoplasmic DNA; one of these pathways depends on viral envelope production. The suppression of cccDNA synthesis by envelope proteins might be explained as a selection of all capsids for the secretory pathway to the exclusion of the pathway for conversion to cccDNA. For example, the interaction of a nucleocapsid with one or both envelope proteins may render that capsid unavailable for cccDNA synthesis. Lack of such an interaction may result in efficient utilization of the nucleocapsid for cccDNA synthesis. The specific mechanisms by which nucleocapsids are selected for either pathway are not known.

For such a model to account for the suppression of amplification, it is necessary to explain how cccDNA synthesis could occur at early times after infection but be suppressed at later times. This temporal change in regulation could occur through at least two mechanisms. First, envelope proteins may act cooperatively in their reaction with nucleocapsids. If this were the case, inhibition of cccDNA synthesis would increase exponentially with envelope protein concentration in the cell. Secondly, since envelope genes are translated from different mRNAs which are, in

turn, transcribed from different promoters, they may be differentially expressed during the infection. Thus, differential expression of envelope proteins could account for changes in the inhibition of cccDNA synthesis during the course of infection.

As an alternative model, envelope protein(s) may inhibit cellular factors required for cccDNA synthesis. In this case, cccDNA synthesis could occur early after infection, before sufficient envelope protein is accumulated to inhibit the cellular factor(s). In either case, it seems likely that suppression of cccDNA synthesis would depend directly on the concentration (or rate of synthesis) of one or both envelope proteins in the cell.

Direct evidence that the rate of production of envelope proteins in infected hepatocytes is proportional to the copy number of cccDNA in the nucleus is lacking. Tuttleman et al. (11) observed that viral DNA synthesis by hepatocytes infected *in vitro* was sensitive to the incorporation of BUdR during cccDNA amplification, suggesting a crucial role for amplification in subsequent virus production. They speculated that the effect of cccDNA amplification was to increase the level of expression of viral RNA through increasing the number of DNA molecules that could serve as templates for transcription. In agreement with this hypothesis, we have been unable to detect any envelope or capsid protein expression in hepatocytes infected by mutants defective in DNA amplification (unpublished observations), further suggesting that amplification is necessary for the expression of antigen at detectable levels. If increases in gene dosage do, in fact, increase expression of viral envelope proteins, then inhibition of cccDNA synthesis by envelope proteins would constitute a feedback control. The effect of this control would be to regulate the rate of production of envelope proteins, as well as other viral gene products, by controlling the number of DNA template molecules available for transcription.

This regulation would appear to be required for maintenance of a persistent infection *in vitro* and possibly *in vivo*. One can imagine that high levels of virus replication combined with low levels of secretion could be cytotoxic, and this effect would account for the failure of the envelope-defective mutant genome to establish a persistent infection *in vitro*. However, no direct evidence of virus-specific cell death in the mutant-infected cultures was obtained, since the numbers of infected hepatocytes were too low to allow a distinction of virus-induced cell death from normal attrition in primary cultures. Both mutant- and wild-type-infected cell layers remained healthy during long-term infection, although a large proportion of the cells was infected by day 20 in the wild-type-infected cultures. This observation and the persistence of viral DNA replicative intermediates in the presence of suramin strongly suggest that wild-type viral infections are persistent and noncytotoxic *in vitro*. Careful analysis of supernatant fluids of wild-type- and mutant-infected cultures for products of specific lysis of infected cells will be necessary to establish whether a cytotoxic effect is the basis for the transient nature of the infection by the envelope-defective virus.

Recently, we have found that mutation of the pre-S envelope protein alone is sufficient to destroy both control of

cccDNA amplification and the secretion of DNA-containing particles. This finding suggests that the pre-S protein may physically bind to the viral nucleocapsid to promote packaging and that such a physical interaction may also be responsible for the control of cccDNA synthesis. Mutational mapping of the pre-S domains required for packaging or control of cccDNA amplification or both may help to resolve whether these domains actually recognize binding receptors on the surface of the nucleocapsid.

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