Kinetics of early molecular events in duck hepatitis B virus replication in primary duck hepatocytes

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This paper describes the use of one-step growth conditions to study the kinetics of duck hepatitis B virus (DHBV) replication in primary duck hepatocytes. Synchronized infection was achieved using partially purified DHBV virions at an m.o.i. of 640 DHBV DNA-containing virions per cell, and these conditions were shown to produce a single cycle of infection. In this model, input purified DHBV DNA was rapidly internalized by cells at $0-5$ h, and localized to the nucleus by 4 h, but both covalently closed circular (CCC) DNA and single-stranded DNA were not detected until 48 h post-inoculation (p.i.), suggesting that there was a $\geq 40$ h delay between DHBV localization to the nucleus and formation of CCC DNA. In contrast, CCC DNA can be first detected in hepatocytes at 6 h p.i. in in vivo infection of ducks with the same DHBV strain. In an analysis of the nuclear transport of the DHBV genome, release of nuclear viral DNA from a particulate form to a soluble nucleoplasmic form was only 50% complete by 48 h p.i. However, this process occurred simultaneously with genome uncoating since all soluble nucleoplasmic DHBV DNA was free of nucleocapsid material; this suggests that nucleocapsid disassembly and genome uncoating may occur at the nuclear membrane and not within the nucleus. Quantitative analysis demonstrated inefficiency in a number of steps including virus uptake and internalization, translocation of nucleocapsid across the nuclear membrane and antigen expression from intranuclear viral DNA.

Introduction

Despite considerable knowledge of the later events in hepadnavirus replication from viral genome replication to virion release, little is known about the early molecular events including the nature of the virus–receptor interaction, virus uptake and uncoating. A major limitation to such studies is the lack of suitable susceptible cell lines for infection.

Early steps in the hepadnavirus life-cycle include species-specific attachment of the virus to hepatocytes (Neurath et al., 1985; Qiao et al., 1992). The exact nature of the hepatitis B virus (HBV) receptor molecule is still unknown, although the pre-S domain of the HBV large surface protein including amino acids 21–47 was shown to be essential for specific attachment of HBV to cultured hepatoma-derived cells (HepG2, Neurath et al., 1986) and to human liver plasma membrane (Pontisso et al., 1989). However, this interaction was not sufficient for infection of HepG2 cells (Neurath et al., 1985; Qiao et al., 1994), suggesting that the hepadnavirus–receptor(s) interaction may be a complex process involving more than one receptor molecule, or that early steps in replication were restricted in these cells.

Similarly, Klingmüller & Schaller (1993) have proposed that duck hepatitis B virus (DHBV) infection of hepatocytes was initiated by attachment of the pre-S domain of the DHBV envelope to a limited number of specific binding sites, while reversible less specific, non-saturable binding to a range of cell types was also described. Subsequently, Kuroki et al. (1995) and Tong et al. (1995) have described a host cell glycoprotein (gp) 180/gp170 as a high affinity receptor and identified this as a novel member of the basic carboxypeptidase gene family. Furthermore, Li et al. (1996) described a 120 kDa (p120) pre-S-binding protein that may be part of the DHBV receptor complex which was activated after virus binding to gp180. In addition, a 55 kDa protein expressed in duck liver and several duck tissues was thought to be involved in some early steps in the infectious pathway, including adsorption and uptake of DHBV; however, this protein was unlikely to be one component of a receptor complex (Guo & Pugh, 1997).
Our previous investigation (Qiao et al., 1994) supported the view that despite specific interaction between HBV and HepG2 cells, HBV nucleocapsid particles failed to be transported into the nucleus and thus infection could not be initiated. Recently, Lu et al. (1996) reported that HBV was more readily taken up by HepG2 cells after proteolytic cleavage of the pre-S domain by V8 protease or chymotrypsin and incubation at pH 5.5, suggesting that proteolysis-dependent exposure of a fusion domain within the envelope protein of HBV was necessary during natural infection. This implied that HepG2 cells might not provide a sufficiently active protease for cleavage of the HBV middle and large surface proteins.

It is also not clear how and in what form the viral genome is translocated into the nucleus of the cell. Since the nucleocapsid protein of HBV contains a nuclear localization signal in the C terminus (Ou et al., 1989; Yeh et al., 1990) and the diameter of the nucleocapsid particle (25–27 nm) is similar to the maximal nuclear pore size (Forbes, 1992), it is possible that translocation of the nucleocapsid particle itself into the nucleus may occur (Ganem, 1996). However, this seemed unlikely because HBV nucleocapsid particles did not cross the nuclear membrane in either direction in a study of a transgenic mouse model system (Guidotti et al., 1994). Alternatively, nucleocapsid particles might bind to the nuclear membrane and release viral DNA into the nucleus as part of the uncoating process. Thirdly, uncoating might take place within the cytoplasm itself. A recent study of an in vitro model of woodchuck hepatitis virus (WHV) by Kann et al. (1997) has shown that nuclear transport of viral DNA occurred most probably by disassembly of the core particles which were bound to the nuclear membrane, leading to release of the viral genome and its transport into the nucleus.

In order to further examine these early molecular events of hepadnavirus replication, we describe in this paper the validation of a one-step growth conditions of DHBV in primary duck hepatocytes (PDH) that was achieved by simultaneous infection of all susceptible cells during a defined adsorption period. This approach provided a model system to study the fate of individual DHBV-infected PDH, and was used to examine details of some early molecular events in hepadnavirus replication.

Methods

- **Preparation of PDH.** Hepatocytes were isolated from uninfected 2–4 week old Pekin-Aylesbury ducks hatched from DHBV-free eggs. The methods of hepatocyte preparation and culture were as described by Tuttleman et al. (1986a) with minor modifications. Briefly, ducks were anaesthetized with 0.4–0.8 ml pentobarbital sodium (Nembutal) by intravenous injection and the liver was perfused via the right atrium of the heart with 600–800 ml of S-MEM medium (GIBCO) which contained 0.5 mM EGTA in HBS (20 mM HEPES, 0.15 M NaCl, pH 7.4, 0.1% glucose) containing 2 mg/ml penicillin and 10 mg/ml gentamicyn. After the liver was well blanched, perfusion was continued with a further 200 ml S-MEM medium containing 100 mg collagenase type 1 (Sigma), 5 mg trypsin inhibitor (Sigma) and 5 mg aprotinin (Sigma) dissolved in 2.5 mM CaCl2. After perfusion, the liver was removed and the cells were dispersed in L-15 medium (GIBCO), pH 7.4, containing 5% foetal bovine serum (FBS; GIBCO), filtered through six layers of gauze, and further purified by a percoll method (Kreamer et al., 1986). The cells were seeded at a density of 1.5 × 10^6/cm² in six-well plates (10 cm² per well; Falcon) in L-15 medium supplemented with FBS, and the cultures were maintained in a 37 °C incubator without CO2. From the day after plating, the cells were cultured in L-15 medium without FBS and DMSO, and the medium was changed daily for the first 7 days and then every 2 days for the next few weeks, if necessary.

- **Partial purification of DHBV virions.** DHBV virions were partially purified from a pool of the Australian strain of DHBV (9.5 × 10⁹ viral genome equivalents (vge) DHBV DNA per ml) by two-step sucrose gradient centrifugation as described by Klingmuller & Schaller (1993) with some modifications. Seven ml of the pooled serum was loaded on 3 ml of 20% and 2 ml of 70% (w/v) sucrose in PBSE (PBS, 1 mM EDTA), and centrifuged in an SW41 rotor (Beckman) at 170,000 g for 2.5 h at 4 °C. Six hundred µl fractions were collected from the bottom of the tube and the peak DHBV DNA-positive fractions were pooled. BSA was added to a final concentration of 0.5% and dialysed against PBS. The preparation was then concentrated to 0.5 ml using polyethylene glycol 6000 powder sprinkled on the outside of the dialysis tubing. This sample was overlaid on a continuous gradient of 2 ml of each of 20%, 30%, 40%, 50%, 60% and 1 ml of 70% sucrose in PBSE, centrifuged in an SW41 rotor (Beckman) at 170,000 g for 4 h at 4 °C. Again, 600 µl fractions were collected and examined for sucrose density (calculated from the refractive index; Griffith, 1979), for viral proteins by SDS–PAGE and for DHBV DNA content by dot blot hybridization. Fractions containing DHBV virions were pooled, dialysed and then stored in small aliquots at −70 °C prior to use in infection experiments (see below).

- **Quantification of virus particles.** Viral protein content was quantified using the Analytical Imaging Station and low light imaging system analysis of SDS–PAGE by comparison with a range of concentrations (20, 30, 50, 100 ng) of the protein standards carbonic anhydrase and trypsin inhibitor. To estimate virus particle numbers, we used the figures 7 × 10⁶ particles per ng of pre-S/S protein (Klingmüller & Schaller, 1993) and 3 × 10⁶ genomes per pg of DHBV DNA.

- **Infection of PDH.** Cell monolayers were infected at 2 days after plating. After removal of the residual culture medium by aspiration, 1 ml of the purified virus inoculum diluted in L-15 medium without FBS was added to each well (in six-well plates, 10 cm²). Adsorption was performed at ≤10 °C for 6 h, taking care to prevent the plate from drying out. At the end of the adsorption period, the residual inoculum was removed without washing, 3 ml of fresh L-15 medium was added per well and the culture was incubated at 37 °C for up to 7 days post-inoculation (p.i.).

- **Detection of DHBV internalization.** At different time intervals after 37 °C incubation, the culture medium was removed and the cell monolayer treated with glycine solution (50 mM glycine pH 2.2, 150 mM NaCl) for 2 min to remove extracellular viruses (Pugh & Summers 1989). The cell monolayer was rinsed three times with PBS. To separate cytoplasmic and nuclear fractions, glycine-treated monolayers were treated with 300 µl of 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 10 mM Tris–HCl pH 7.6, mixed gently and kept on ice for 5 min then centrifuged at 12,000 r.p.m. in a microcentrifuge for 20 s at 4 °C (Qiao et al., 1994). The supernatant (cytoplasm) was transferred into a fresh tube and the pellet (nuclei) was washed three times with PBS.

The extent of cell disruption and the integrity of free nuclei after this treatment were examined by light microscopy after trypan blue staining.
These samples were then proteinase K-digested and the DNA was extracted and redissolved in TE-8 (10 mM Tris–HCl pH 8.0, 1 mM EDTA) as described previously (Qiao et al., 1994).

- **Nucleic acid hybridization.** In some experiments, DHBV DNA was detected and quantified by spot blot hybridization and Southern blot hybridization. For spot blot hybridization, 5 µl of the above extracted DNA samples was denatured by boiling for 5 min and quenched on ice, and spotted onto a 10 × SSC-treated nitrocellulose membrane (Hybond-C-extra, Amersham). The membrane was then baked at 80 °C for 2 h and hybridized using a random prime 32P-labelled full-length DHBV probe (pSPDHBV 5.1, a gift from J. Pugh) as described (Qiao et al., 1990). The intensity of the signals was quantified by phosphorimager analysis by comparing with a DHBV DNA standard that had been extracted from DHBV-containing serum (see above) in the same experiment and quantified against known amounts of plasmid DHBV DNA. Detection of total DHBV DNA and covalently closed circular (CCC) DNA was done by Southern blot hybridization as described by Tuttleman et al. (1986) and each species of DNA was identified by reference to DNA standards seen in the same Southern blot hybridization and quantified by phosphorimager against the DHBV DNA standards.

- **Immunoprecipitation.** In some experiments, the cytoplasmic and nuclear fractions from infected hepatocytes at different times p.i. were subjected to immunoprecipitation followed by DHBV DNA detection as described (Qiao et al., 1994). The nuclei were disrupted by four freeze–thaw cycles prior to immunoprecipitation. A sample (150 µl) of cytoplasmic or nuclear cell extract was cleared by the addition of 10 µl normal rabbit serum (NRS) and 30 µl protein A–Sepharose 6MB (Pharmacia) and incubated for 1 h at 4 °C with gentle mixing, followed by centrifugation at 12,000 r.p.m. for 5 min. The pellet was discarded, and 10 µl rabbit antisera to recombinant DHBV core antigen (anti-rDHBcAg; Jilbert et al., 1992) and 30 µl protein A–Sepharose were added to the supernatant and allowed to precipitate for 16 h at 4 °C with gentle agitation. The sample was then centrifuged as above, the supernatant was transferred into a fresh Eppendorf tube and the pellet was resuspended in 150 µl PBS. Both supernatant and pellet samples were then proteinase K-digested, and the DNA was extracted and redissolved in 10 µl TE-8. The DHBV DNA content was then determined by spot blot hybridization.

- **Immunofluorescent (IF) staining of DHBV-infected PDH.** Cell monolayers in six-well plates were washed three times with cold PBS (4 °C) and fixed directly by ethanol–glacial acetic acid (95:5) at −20 °C for ≥1 day. The cells were then washed twice with PBS and pre-incubated with a 1:50 dilution of normal duck serum (NDS) in PBS for 5 min at room temperature. This was then followed by incubation at 37 °C for 60 min with a 1:50 dilution of both rabbit anti-rDHBcAg and NDS in PBS (for negative control, the primary antibody was substituted with 1:50 dilution of NRS). The cells were then washed twice with PBS for 5 min each, incubated with a 1:50 dilution of FITC-conjugated sheep anti-rabbit IgG (Silenus) for 30 min at 37 °C. The cells were again washed twice for 5 min with PBS, mounted in 90% glycerol in PBS plus 50 mM Tris–HCl pH 8.6 and viewed under an inverted UV microscope.

### Results

#### Establishment of one-step growth conditions in PDH

In order to achieve one-step growth conditions, we used an inoculum enriched in virions to reduce possible competition of receptor binding sites by subviral particles (SVP). DHBV virions were partially purified from the pooled serum by pelleting through 20% sucrose onto a 70% sucrose cushion, followed by rate zonal sucrose gradient centrifugation. The ratio of SVP to DHBV DNA-containing virions was reduced by 94% from 500:1 to 30:1 after the two-step centrifugation (data not shown), indicating that considerable enrichment of DHBV DNA-containing virions had been achieved. Initially, the inoculum was titrated to achieve a calculated m.o.i. of 40:1, 160:1 and 640:1 and 2580:1 based on the DHBV DNA content. To attempt to infect all cells simultaneously, we performed the adsorption step at ≤10 °C to allow virus adsorption but not penetration. This was then followed by a 37 °C incubation to allow virus penetration in a synchronized fashion. The percentage of cells infected each day was determined by IF staining for DHBcAg. Our results showed that both an m.o.i. of 640:1 and 2580:1 resulted in greater than 60% of cells expressing DHBcAg by 4 days p.i., followed by a plateau of ≥90% cells DHBcAg-positive by 5 days p.i. (Fig. 1). In contrast, lower m.o.i. of 40:1 and 160:1 produced a slower increase in the number of infected cells, and a maximum of only 10% and 40% infected cells, respectively, was not reached until 7 days p.i. (Fig. 1). The rapid increase in infected cells between day 3 and day 5 p.i. with m.o.i. of 640:1 and 2580:1 suggested that the infection in the majority of cells was synchronized under these experimental conditions and the first 4–5 days p.i. may represent a one-step growth curve in PDH (Fig. 1). However, for practical reasons we chose to use an m.o.i. of 640:1 in the subsequent infection experiments. Our attempt to enhance virus infection by incubation with 1:5% DMSO, 5% FBS or at 41 °C (the physiological temperature of the duck) did not lead to more rapid kinetics or increased yields.

#### Demonstration of a single cycle of infection

To formally examine whether the above infection kinetics at an m.o.i. of 640:1 resulted from a single cycle or secondary cycles of infection, we then performed infection experiments in

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**Fig. 1.** Titration of the purified DHBV inoculum at the indicated m.o.i. in PDH culture. The adsorption was carried out at ≤10 °C for 6 h and cells were incubated for up to 7 days p.i. Percentage cell infection was determined by IF staining of DHBcAg in infected cells.
which either a 1/100 dilution of rabbit anti-DHBV antibody shown to neutralize DHBV in vivo (Qiao et al., 1990) or 100 µg/ml suramin was added to the culture medium at day 1 p.i. and maintained throughout the 7 day culture period. Suramin has been shown to effectively block DHBV uptake and thus prevent spread of virus to new cells (Petcu et al., 1988). Total DHBV DNA extracted from cells incubated in the presence of suramin (Fig. 2B) or rabbit anti-DHBV antibody (Fig. 2C) or without either agent (Fig. 2A) was compared by Southern blot hybridization. The first appearance and the levels of CCC DNA and single-stranded (SS) DNA were identical regardless of the presence or absence of suramin or anti-DHBV antibody. In addition, the level of total DHBV DNA synthesized (Fig. 2) or the percentage of DHBcAg-positive cells (data not shown) over the first 4–5 days p.i. was not significantly reduced by suramin or anti-DHBV antibody. These results suggested that the replication kinetics with an m.o.i. of 640:1 as shown above resulted from a single cycle of infection initiated prior to day 1 p.i.

**Kinetics of penetration and nuclear translocation**

Using the one-step growth conditions as a model, we next examined events after adsorption, namely virus penetration and nuclear translocation. Purified virus was adsorbed at \(\leq 10^\circ\text{C}\) for 6 h at an m.o.i. of 640:1, the culture was then incubated at 37 °C for varying lengths of time to allow bound virus to internalize synchronously, and the extracellular (cell membrane-bound) DHBV was removed by low pH treatment (50 mM glycine pH 2-2, 150 mM NaCl; Pugh & Summers, 1989). Cell fractionation was then performed to examine the intracellular localization of the DHBV DNA. The separation of the cytoplasmic and nuclear fractions was achieved by 0-5% NP-40 treatment followed by centrifugation. The integrity and the numbers of free nuclei were examined by light microscopy after trypan blue staining. Both fractions were then subjected to DNA extraction, and DHBV DNA was detected by Southern blot hybridization and quantified by phosphor-imager analysis.

The results of this experiment are shown in Fig. 3. DHBV DNA was undetectable in the cytoplasmic fraction at 0 h (without 37 °C incubation; Fig. 3, track 6); this provided a negative control showing that the cell membrane-bound virus (Fig. 3, track 4) was completely removed by low pH glycine treatment and that internalization did not occur at detectable levels at \(\leq 10^\circ\text{C}\). In contrast, DHBV DNA was first detected in the cytoplasmic fractions from 1 h post-incubation at 37 °C (Fig. 3, track 8) and at 0-5 h in other experiments (data not shown), whereas DHBV DNA was first detected in the nuclear fractions at 4 h post-incubation at 37 °C (Fig. 3, track 11), suggesting that intranuclear translocation had occurred by then. Samples collected 2 h post-incubation at 37 °C were negative for nuclear DHBV DNA (data not shown).

Viral DNA present in the nuclear fractions consisted of small quantities of relaxed circular (RC) DNA and double-stranded linear DNA from 4 h post-incubation at 37 °C (Fig. 3, tracks 11, 13, 15), while CCC DNA was not detected in the nuclear fractions until 48 h by the CCC DNA enrichment method (data not shown) and 72 h by the total DHBV DNA detection method (Fig. 3, track 19). In contrast, no detectable CCC DNA was present in the cytoplasmic fractions at any time-point. This result suggested that it required \(\geq 40\) h for CCC DNA to form despite RC DNA being already present in the nuclear fraction from 4 h post-incubation at 37 °C. Further attempts were made to detect low levels of CCC DNA by PCR (Kock & Schlicht, 1993), but no detectable CCC DNA-specific signals were detected prior to 48 h p.i. (data not shown). However, SS DNA was also detected in the cytoplasm from 48 h post-incubation at 37 °C, suggesting that SS DNA synthesis was initiated rapidly after CCC DNA formation.

The kinetics of early DHBV replication in PDH based on the quantification of DHBV DNA in the cytoplasmic and nuclear fractions are summarized in Fig. 4. It shows that under standard adsorption conditions (\(\leq 10^\circ\text{C}, 6\) h) at an m.o.i. of...
DHBV replication in primary duck hepatocytes

Fig. 3. Kinetics of DHBV DNA internalization and initiation of infection. After virus adsorption at \( \leq 10^\circ \text{C} \) for 6 h and subsequent 37 \( ^\circ \text{C} \) incubation for various lengths of time, the cells were treated with glycine (pH 2.2), and nuclear and cytoplasmic extracts were examined separately for DHBV DNA by Southern blot hybridization. Tracks 1 and 2, 50 pg full-length DHBV plasmid DNA pSPDHBV 5.1 (3 kb) and 200 pg DHBV DNA extracted from DHBV-containing serum, respectively; 3, uninfected PDH control; 4–23, infected cell cytoplasmic (C) and nuclear (N) fractions harvested at different times post-incubation at 37 \( ^\circ \text{C} \). Tracks 4 and 5, T0 (no 37 \( ^\circ \text{C} \) incubation) and no low pH glycine treatment; 6 and 7, T0 (no 37 \( ^\circ \text{C} \) incubation) but with low pH glycine treatment. Tracks 8 and 9, 10 and 11, 12 and 13, 14 and 15, 16 and 17, 18 and 19, 20 and 21, 22 and 23 represent 1 h, 4 h, 6 h and 1, 2, 3, 4, 5 days post 37 \( ^\circ \text{C} \) incubation, respectively, and all had been low pH glycine-treated at the end of each 37 \( ^\circ \text{C} \) incubation. The positions of RC, double-stranded linear (DL), CCC and SS DNA are indicated.

64%: 1, 67.5% (approx. 430 vge per cell) of the total inoculated DHBV bound to the cell membrane, and about 80% of this cell membrane-bound DHBV (approx. 345 vge per cell) became internalized between 2 and 6 h post-incubation at 37 \( ^\circ \text{C} \) (Fig. 4A). Although the cytoplasmic RC DNA copy number reached approx. 280 vge per cell at 2 h post-incubation at 37 \( ^\circ \text{C} \), only approx. 60 vge per cell was subsequently detected in the nucleus (Fig. 4B). This suggests that intranuclear translocation was an inefficient process, since only approx. 10% of the input DHBV DNA was transported into the nucleus. Extracellular DHBV DNA was first detected in the culture supernatant on day 4 p.i. (Fig. 4A) and levels rose subsequently. As there was no morphological evidence for cell damage during the course of infection, virus release was unlikely to be due to breakdown of infected cells.

Analysis of nuclear transport of DHBV DNA

To determine how and in what form the viral genome is translocated into the nucleus of the cell, we performed a detailed analysis of nuclear DHBV DNA. Nuclei collected at 4, 24 and 48 h post-incubation at 37 \( ^\circ \text{C} \) were washed and treated with either glycine, trypsin or freeze–thawing (Qiao et al., 1994), centrifuged for 5 min at 12000 r.p.m., and the pellet and supernatant were examined separately for DHBV DNA. Treatment by low pH glycine or trypsin should be expected to remove any virus bound to the cytoplasmic face of the nuclear membrane, allowing it to be detected in the supernatant after centrifugation. DHBV DNA detected in the pellet, however, would represent virus bound to the nucleoplasmic face of the nuclear membrane or in the nucleoplasm and thus protected from these treatments. In contrast, freeze–thawing disrupts nuclear membranes and thus would release nucleoplasm into the supernatant, while nuclear membranes remain in the pellet after centrifugation.

In low pH glycine-treated nuclei (Fig. 5A), approx. 75% of the nuclear-associated DHBV DNA was detected in the pellet and only 25% in the supernatant at 4 h post-incubation at 37 \( ^\circ \text{C} \), indicating that the majority of the nuclear DHBV was not dissociated from nuclei by this treatment. The 25% of DHBV DNA that was dissociated from nuclei by this treatment was likely to represent DHBV bound to the cytoplasmic face of the nuclear membrane; DHBV DNA bound to this location was reduced to approx. 5% and 2% by 24 h and 48 h post-incubation at 37 \( ^\circ \text{C} \), respectively. Similarly, trypsin treatment (Fig. 5B) revealed that 40% of DHBV DNA was released to the supernatant at 4 h, 10% at 24 h and 5% at 48 h. Because the nuclei were shown to remain morphologically intact after this treatment, it was unlikely that the released DHBV DNA was derived from disruption of the nuclei. In freeze–thaw-treated nuclei, however, > 95% of DHBV DNA was detected in the pellet at 4 h post-incubation at 37 \( ^\circ \text{C} \) (Fig. 5C), indicating that at this stage most of the nuclear DHBV DNA was still associated with particulate material, possibly nuclear membrane, and not free in the nucleoplasm. However, by 24 h and 48 h post-incubation at 37 \( ^\circ \text{C} \), respectively, approx. 20% and 50% of the DHBV DNA was detected in the supernatant, indicating release of DHBV DNA to the nucleoplasm.

To further determine the site and the timing of genome uncoating, we then performed immunoprecipitation on the
supernatant (nucleoplasmic fraction) of freeze–thaw-treated nuclei using anti-rDHBcAg to differentiate whether DHBV DNA was nucleocapsid-associated or free. The procedure has been successfully used in a similar study for HBV (Qiao et al., 1994). Firstly, the specificity of the antiserum was tested by using purified DHBV virions pretreated with 1% NP-40 and 0.3% β-mercaptoethanol to disrupt the envelope of the virion and to release nucleocapsids. It showed that anti-rDHBcAg antibody specifically precipitated nucleocapsid-associated DHBV DNA since only anti-rDHBcAg- but not anti-DHBsAg-precipitated material contained DHBV DNA (Fig. 5E). When anti-rDHBcAg antibody was incubated with the supernatant (nucleoplasmic fraction) of freeze–thaw-treated nuclei, DHBV DNA was only detected in the supernatant but not in the pellet at both 24 h and 48 h post-incubation at 37 °C; as noted above, the earliest detection of DHBV DNA in the nucleoplasm was 24 h post-incubation at 37 °C (Fig. 5D). These results indicated that DHBV DNA detected in the nucleoplasm had already been disassociated from nucleocapsid proteins. Therefore, it was likely that nuclear translocation of DHBV occurred concurrently with genome uncoating, and that this may have been achieved by disassembly of the core particles while bound to the nuclear membrane and release of the viral genome into the nucleoplasm as described for WHV by Kann et al. (1997).

Taken together, our investigation of the viral DNA associated with the nucleus (Fig. 5) demonstrated that, even by 4 h post-incubation at 37 °C, 60–75% of the nuclear DHBV DNA was not only bound to the nuclear membrane but also protected from low pH and trypsin dissociation from nuclei, suggesting that some irreversible progression or internalization may have occurred. However, > 95% of the viral DNA was still associated with the particulate nuclear membrane fraction at this time-point. The release of viral DNA into the nucleoplasm from the particulate to the soluble nuclear fraction occurred at ≥ 24 h post-incubation at 37 °C; however, the efficiency of this process was only 50% by 48 h post-incubation at 37 °C. We then showed that nucleoplasmic DHBV DNA was free from nucleocapsids (non-precipitable by
anti-DHBc), suggesting that uncoating of the viral genome may have occurred at the nuclear membrane level.

Discussion

In this paper, we have characterized one-step growth conditions for DHBV replication in PDH and successfully used this model to study the early molecular events of hepadnavirus replication. The evidence that one-step growth conditions had been achieved came from (i) the relatively synchronized appearance of DHBcAg in 60–80% of hepatocytes between days 3 and 4; (ii) the finding that the infection kinetics were unchanged when cultures were treated from day 1 onwards with agents (suramin and anti-DHBV antibody) designed to prevent secondary spread.

The kinetic analysis of early events showed that approx. 44% of the virus inoculum (280 vge per cell) was internalized by 2 h post-incubation at 37 °C, but only 10% of the inoculum (approx. 60 vge per cell) reached the nuclear fraction at the peak time-point of 4 h post-incubation at 37 °C. Furthermore, input virus DNA was internalized and migrated to the nucleus very rapidly (≥ 4 h), but a considerable delay then ensued until CCC DNA was first detected at 48 h. The appearance of SS DNA at the same time (48 h p.i.) indicated that once CCC DNA was formed, transcription and cytoplasmic reverse transcription of pregenomic RNA occurred rapidly, while translation of DHBcAg to levels sufficient for detection by IF required a further 24–48 h. Our first detection of CCC DNA and SS DNA at day 2 p.i., and DHBV protein expression at days 3–4 p.i. was in agreement with some previous reports of DHBV replication in PDH (Tuttleman et al., 1986a; Uchida et al., 1988) but slower than reported by Kock & Schlicht (1993). The efficiency of virus replication was not enhanced by the addition of DMSO to the culture medium, used by some workers to suppress possible dedifferentiation of cultured hepatocytes or by raising the culture temperature to 41 °C (the physiological temperature of the duck).

A number of formal possibilities exist to explain the delay in formation of CCC DNA following localization of DHBV DNA to the nucleus, including (1) delays in nuclear transport of the nucleocapsid particles; (2) delays in viral genome uncoating; (3) delays in DNA strand repair and supercoiling. In order to examine the role of nuclear transport and uncoating on the delay in CCC DNA formation, we carried out a more detailed analysis of the viral DNA associated with the nucleus. Release of viral DNA from its association with particulate nuclear material into the soluble nuclear fraction was slow and only 50% complete by 48 h post-incubation at 37 °C, although all soluble nucleoplastic DHBV DNA present was free from nucleocapsid proteins. These findings suggest that a slow rate of either translocation of nucleocapsids across the nuclear membrane, and/or release of DHBV DNA from nucleocapsids, may have been responsible for the delay in replication in PDH culture.

Similar results were described in a separate study by Kann et al. (1997) using whole core particles of WHV and digitonin-permeabilized, cytosol-reconstituted HuH7 cells. This showed that the majority of encapsidated WHV genomes remained in the cytoplasm, while a minor fraction bound to the nucleus. Genomes present within nucleocapsids were shown to be transported into the nucleus with low efficiency, while soluble viral polymerase-DNA complexes were rapidly transported, suggesting that core particles are only slowly degraded. This lack of efficiency of nuclear transport is also consistent with our previous finding that intracellular accumulation and nuclear localization of HBV core particles occurred after abortive infection of HepG2 cells, but without delivery of HBV DNA into the nucleus (Qiao et al., 1994). In contrast to the previous reports using abortive or artificial conditions, the present study confirms in a fully productive optimized cell culture system that a major delay in hepadnavirus replication occurs at the stage of viral DNA release from nucleocapsid bound to nuclear membranes.

It is also possible that only a very small quantity of CCC DNA was produced in the first round of replication (prior to 48 h post-incubation at 37 °C) which was below the detectable level by Southern blot hybridization and PCR. In this scenario, CCC DNA pool expansion by recycling progeny genomes might be necessary for accumulation of CCC DNA in the same infected cells. Such a process would not be blocked by suramin or anti-DHBV antibody. However, the PCR used would have detected one copy of CCC DNA in 3 × 10^2 cells, and it would be most unlikely if recycling progeny genomes was the process responsible for expansion from this level of infection to 60 genomes per nucleus between days 2 and 4.

Bruns et al. (1998) have recently shown that the pre-S protein in SVP can enhance replication in vitro when using low multiplicity of virus (0·01 or 0·1 virions per cell). With our particle purified inoculum, a virion m.o.i. of 640 would represent approx. 2 × 10^4 SVP per cell, similar to the figure reported by Bruns for significant enhancement. However, Bruns et al. (1998) did not describe achieving synchronous infection at low virus multiplicity. When we inoculated the same virus stock (without purification) as a large inoculum (1·9 × 10^11 vge) into adult ducks of the same genetic background as those used to prepare PDH cell cultures, CCC DNA was first detected at 6 h p.i. and SS DNA and viral antigens at 18 h and 30 h p.i., respectively, in the infected liver tissue, i.e. full virus replication progressed more rapidly in vivo (A. R. Jilbert, personal communications). Furthermore, in this in vivo system, 1 ID_{50} corresponds to approx. one virus particle (Jilbert et al., 1996). Thus, the in vivo system exhibits inefficiency, because (i) only 10% of inoculated virion DNA reached the nucleus; (ii) approximately 750 virions are required for 1 TCID_{50}; and (iii) temporal delay occurred in production of CCC DNA in comparison to the in vitro system. Although significant differences may exist between the in vitro and in vivo anatomic routes by which virus gains access to hepatocytes,
these are not likely to be the reasons for delayed replication in vitro because, as demonstrated above, replication in vitro rapidly proceeds to the point of nuclear localization of DHBV DNA. The low efficiency of the nuclear transport may be caused by changes in cell metabolism or differentiation resulting from establishment of cell monolayers (e.g. manipulations, changes in extracellular milieu, changes in cell–cell relationships). In summary, this paper documents a cell culture model system for the study of hepadnavirus replication and demonstrates that in a fully productive hepadnavirus replication cycle in cell culture, a major delay occurs at the stage of release of incoming virus DNA from nucleocapsid bound to nuclear membranes similar to what has previously been suggested from cell reconstitution models.

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