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SHORT COMMUNICATION

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The replication cycle of the hepatitis B virus (HBV) is still incompletely understood. In particular, the early steps of the viral life cycle, such as absorption, penetration, uncoating, and nuclear translocation require further clarification. In this study we performed infection experiments with HBV in primary human hepatocyte cultures. To further elucidate the possible mechanism of virus uptake, infection experiments were performed at different pH levels, after pretreatment of viral particles with acidic buffers and in the presence of lysosomotropic agents (chloroquine and ammonium chloride, respectively). Using a selective PCR technique which discriminates between input virus DNA and the earliest replicative form, we could demonstrate viral replication 36 hr after inoculation. HBV was taken up most efficiently at a pH of 7.4. Infection was still successful after pretreatment of viral particles at low pH and was unaffected by the presence of lysosomotropic agents. In conclusion, this suggests HBV to be a pH-independent virus.

Hepatitis B virus (HBV) infection is a major global health problem with about 200 to 300 million chronic HBsAg (hepatitis B surface antigen) carriers and 1 million deaths annually (*1*). Unfortunately, *in vitro* systems for the investigation of HBV replication require well-differentiated primary human hepatocytes and thus are not readily available. Commonly used surrogate animal models are ducks and woodchucks, which have their own hepatitis viruses (DHBV and WHBV) (e.g., *2, 3*). Most recently, successful infections of tree shrews (*Tupaia belangeri*) with HBV were carried out by Walter *et al.* (*4*) which might present a new promising animal system. We have established an *in vitro* system for the propagation of HBV in primary human liver cells (*5*) which here was used to study the pH dependence of HBV infection.

Enveloped viruses enter the host cell either by direct fusion with the cell membrane or by receptor-mediated endocytosis (*6–12*). These two different forms of virus–host cell interaction can be distinguished by their pH requirements. Viruses with dependence on low pH such as influenza (*13–15*) and Semliki Forest virus (*16*) show sharp pH profiles regarding fusion with membranes of acidic endosomes; pH-independent viruses like herpes fuse with the plasma membrane and possibly also with the endosomes (*17–19*). In order to test the pH requirements of HBV, a set of different experiments was performed.

Viral particles were pretreated with buffers of low pH before infection. In the case of a pH-dependent virus this should result in inactivation of the virus, as membrane fusion is triggered off. In a second set of experiments we investigated the effect of lysosomotropic agents. Lysosomotropic agents, such as chloroquine or ammonium chloride, are weak bases which enter the lysosomes rapidly, become protonated, and increase the pH value within 1 to 2 min (*20–23*). Thus, they abolish intralysosomal acidification of the virus. The use of these agents in DHBV infection systems has resulted in conflicting reports. Offensperger *et al.* (*20*) demonstrated reduced infectivity of DHBV after treatment with chloroquine or ammonium chloride. Rigg and Schaller (*21*) showed undisturbed infection in the presence of ammonium chloride and monensin. Köck *et al.* (*22*) also found that lysosomotropic agents have no effect, suggesting that this virus is pH-independent.

In our study we used primary human liver cells prepared from healthy tissue obtained during hepatic surgery as previously described (*5*). The study was approved by the Ethics Committee of the Medizinische Gesamtfakultät der Universität Heidelberg.

To demonstrate successful viral infection we used a selective PCR technique which discriminates between total viral HBV DNA and double-stranded, repaired intracellular HBV DNA (CCC DNA). This technique has been previously introduced for DHBV (*24*) and was modified for HBV. PCR was performed with two different primer pairs which both bind to viral DNA, but only one of them selectively amplifies CCC DNA (*24*). As shown in Fig. 1

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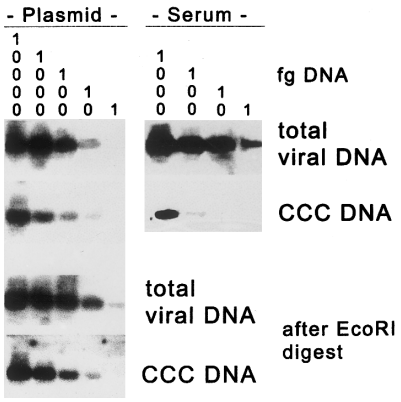

FIG. 1. Differential amplification of virus- or plasmid-derived HBV DNA. To differentiate between encapsulated viral DNA (RC DNA) and complete double-stranded forms (CCC DNA/plasmid DNA) two pairs of primers were used for amplification of serial dilutions of serum-derived virus (after digestion with 0.1 mg/ml proteinase K at 37° for 5 hr) or of plasmid-derived HBV DNA (pd4aI, Ref. *5*): HBV 1966+, 5′TCG CAT TTG TTC AGT GGT TCG TAG GGC 3′ and HBV 2830−, 5′CCG GCA GAT GAG AAG GCA CAG ACG 3′ for the amplification of total viral DNA; HBV 2537+, 5′CCT CTG CCG ATC CAT GCG GAA 3′ and HBV 470−, 5′CTG CGA GGC GAG GGA GTT CTT CTT 3′ for the preferential amplification of double-stranded viral CCC DNA (*24*). A PCR sample of 100 μl contained 10 μl 10× *Taq* buffer, 2.5 U *Taq* polymerase (Stratagene, Heidelberg, Germany), nucleotide mix of 20 n*M* each and 2 ng of each primer. Distilled, sterile water was added up to 90 μl. Amplification was performed using a thermal cycler C100 (Perkin–Elmer, Norwalk, CT): one denaturation step of 94° for 3 min was followed by 30 cycles of 94° for 1 min, 58° for 1 min, and 72° for 2 min. 10 μl of the PCR products was separated on 1% agarose and transferred to a nylon membrane (Pall, Langen, Germany) by a pressure-blotting system (Stratagene). Southern blotting was performed with the DIG system (Boehringer, Mannheim, Germany) according to the protocol of the manufacturer, using CSPD (Tropix, MA) as substrate and random-labeled plasmid pd4aI as probe. The X-ray film was exposed for about 30 min.

(top) both primer pairs amplify plasmid-derived continous HBV DNA (pd4aI; Ref. *5*) at comparable levels. In contrast, discontinuous input viral DNA was detected with about 1000-fold lower efficiency by the primer pair for CCC DNA than by the primer pair for total viral DNA, demonstrating the potential of this method to differentiate between the two DNA species. Since denaturation of CCC DNA depends on strand scission we performed a control PCR after enzymatic digest of plasmid DNA with *Eco*RI (Fig. 1, bottom). This resulted in an insignificant increase in sensitivity, proving that CCC DNA is efficiently denatured during the PCR reaction.

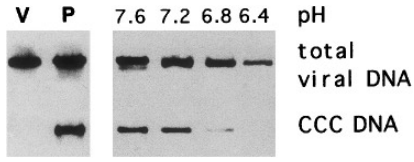

FIG. 3. PH optimum of viral uptake. Inoculation was performed for 1 hr in Earle's salt solution (Serva, Heidelberg, Germany) containing supplements identical to those in the culture medium. It was buffered to pH values 6.4 and 6.8 with piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) and to pH values 7.6 and 7.2 with *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid. Cells were harvested after 48 hr and analyzed for total and replicative viral CCC DNA. Experiments were performed independently three times. V, virus control; P, plasmid control.

By means of this technique successful infection was shown 36 hr postinoculation by the appearance of CCC DNA (Fig. 2a), clearly earlier than can be achieved by conventional Southern blotting (*5*). Due to the high sensitivity of this technique successful infection as demonstrated by the appearance of CCC DNA 48 hr after inoculation could be achieved down to a multiplicity of infection (m.o.i.) of 5×10^5 to 1×10^5 virions per assay (Fig. 2b).

This system was used to further study pH dependence of HBV entry and replication in host cells. To define the pH optimum of viral uptake infection, experiments were performed at different pH and analyzed for the appearance of CCC DNA 48 hr after infection. Strongest signals for CCC DNA were obtained at physiological pH (Fig. 3). Acidic pH progressively prevented the infection of hepatocytes with HBV, as demonstrated by the loss of the CCC DNA signal with increased acidification of the culture medium.

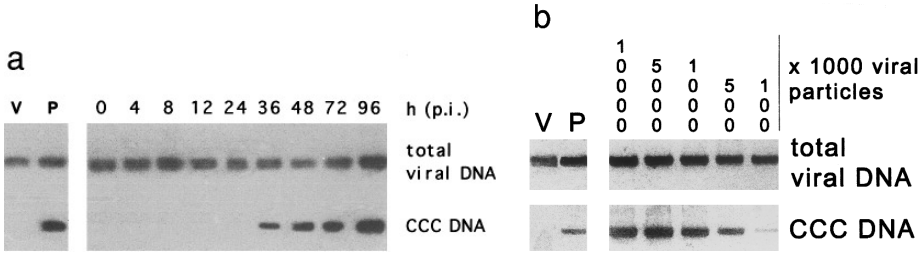

FIG. 2. Detection of total and replicative viral CCC DNA. (a) Cells were prepared and cultured as previously described (*5*). Infection was performed by incubation with 100 μl HBV-containing serum (about 1×10^8 viral particles/ml) for 1 hr in 12-well dishes. Cells were harvested at indicated times and digested with lysis buffer (50 m*M* KCl, 10 m*M* Tris, 0.45% Tween 20, 0.45% Nonidet-P40, 0.1 mg/ml proteinase K) at 56° for 2 hr. Proteinase K was inactivated at 95° for 10 min (*24*). 10 μl was used for PCR as described for Fig. 1. Similar results were obtained in several independent infection experiments. To control differential amplification of RC vs CCC DNA serum-derived viral DNA (V) and plasmid DNA (P) were used as controls. (b) Different m.o.i.s were used for infection experiments as indicated and a PCR reaction was performed 48 hr after inoculation.

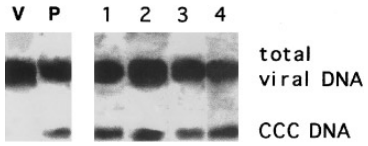

FIG. 4. Acidic pretreatment of viral particles and effect of lysosomotropic agents. Lane 1, control infection for 1 hr with HBV. Cells were washed with glycine buffer (50 m*M* glycine, 150 m*M* NaCl, pH 2.2) afterward to inactivate noninternalized virus. Lane 2, viral particles were pretreated with citrate buffer, pH 4.3 (100 m*M* citric acid, 200 m*M* Na_2HPO_4) for 10 min and afterward neutralized with 0.5 *M* Na_2HPO_4. Lane 3, preincubation for 1 hr with and infection for 1 hr in the presence of 50 μM chloroquine or in the presence of 30 m*M* ammonium chloride (lane 4). Noninternalized virus was inactivated as described above. Cells were harvested after 5 days and analyzed for total and replicative viral CCC DNA. Experiments were performed independently three times. V, virus control; P, plasmid control.

Pretreatment of viral particles with acidic pH is expected to prevent successful infection in the case of a pH-dependent virus. However, pretreatment of HBV with acidic buffers down to pH 4.3 did not prevent infection, as is exemplified for pH 4.3 (Fig. 4, lane 2). In contrast, pretreatment of HBV with glycine buffer at pH 2.2, known to inactivate the virus, resulted in complete loss of the CCC DNA signal (not shown). Furthermore, the requirement for low intralysosomal pH was investigated using lysosomotropic agents during virus inoculation. Neither chloroquine (Fig. 4, lane 3) nor ammonium chloride (Fig. 4, lane 4) significantly reduced the amount of CCC DNA compared to untreated controls (Fig. 4, lane 1). Similar results were obtained for treatment periods between 1 and 17 hr and with a 10-fold lower virus concentration (not shown).

The above experiments confirm that this selective PCR technique can be successfully used for analysis of the early stages of the hepadna viral life cycle and extend its usage to HBV. The results suggest that uptake of HBV is independent of low pH. Our data are in agreement with experiments performed with DHBV by Rigg and Schaller (*21*). In contrast, Offensperger *et al.* (*20*) observed inhibition of infection for ammonium chloride and chloroquine. This discrepancy might be explained by the use of long-term treatment (2 weeks) with lysosomotropic agents which might significantly affect the functional integrity of hepatocytes.

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