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Duck Hepatitis B Virus Infection of Hepatocytes Is Not Dependent on Low pH

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The pH dependency for initiation of infection by the hepadnavirus duck hepatitis B virus (DHBV) was investigated in primary duck hepatocytes. First, an infection assay was developed using a radioimmunoblot to measure DHBV e antigen secreted into tissue culture fluid from infected hepatocytes. The quantity of this viral marker was proportional to the duration of inoculation and the amount of DHBV used as inoculum. The role of pH in initiation of DHBV infection was investigated by using this assay, but no dependence on low pH was found. DHBV was able to infect hepatocytes in the presence of NH_4Cl and monensin, agents that raise the pH in intracellular vesicles and prevent penetration of viruses dependent on low pH in endosomes. In control experiments, infection by Semliki Forest virus, which is low pH dependent, was inhibited, whereas herpes simplex virus type 1 infection, which is pH independent, occurred. Attempts to trigger DHBV-cell fusion by exposure of DHBV prebound to hepatocytes to mildly acidic pH were unsuccessful. In these experiments, it was also observed that internalization of DHBV occurred only between pH 6.8 and 8.0. Additionally, in the absence of cells, infectivity of DHBV was stable at pH 4.6 to 4.8, which is lower than the pH encountered in endosomes (pH 5 to 6.6). Thus, no evidence for a role for mildly acidic pH in the initiation of DHBV infection was found. Therefore, we propose that the infection route followed by DHBV resembles that of the group of enveloped viruses, including herpesviruses, that fuse with their host cells at neutral pH.

To initiate infection, the genome and associated components of viral particles must gain entry to the cytoplasm. In enveloped viruses, this translocation is achieved when viral surface proteins mediate fusion between viral and cellular membranes. Two groups of enveloped viruses are distinguished by the pH at which fusion occurs (see references 5, 10, 25, and 29 for reviews). For one group, mildly acidic pH triggers rapid fusion by exposing a fusion peptide buried within the three-dimensional structure of a surface protein at neutral pH. The mechanism of this low-pH-dependent fusion is best understood for influenza virus (27), and other representatives include the alphavirus Semliki Forest virus (SFV), the rhabdovirus vesicular stomatitis virus, and flaviviruses. Internalization of these viruses is usually rapid. Conversion to the fusogenic form during infection occurs within the mildly acidic pH (pH 5 to 6.6) environment of endosomes, and consequently, agents that raise the pH in these vesicles inhibit penetration. In contrast, viruses belonging to the second group undergo fusion at neutral pH with relatively slow kinetics, and entry of these viruses is not inhibited by raising the pH of intracellular vesicles. These viruses have been called pH-independent viruses and include coronaviruses, herpesviruses, paramyxoviruses, and the retroviruses human immunodeficiency virus type 1 and Rous sarcoma virus (see reviews in references 5, 10, 25, and 29 and also see references 1, 4, 13, and 26).

The hepadnaviruses are a conserved group of animal viruses, for which human hepatitis B virus is the type member. Although the basic features of hepadnaviral replication are becoming understood in increasing detail, very little is known about the initiation of infection. This is in part because the full replication cycle occurs only in highly differentiated cells from specific hosts and no infectable cell lines have been established. Consequently, infection studies are only possible in primary hepatocytes. Duck hepatitis B virus (DHBV) provides the best model system, since primary duck hepatocytes are easily obtained and conditions for maintenance and infection have been described previously (2, 18, 22, 28). Nevertheless, the mechanism of initiation of infection by DHBV is not yet understood, and the cell receptor unit(s) remains elusive. Additionally, little is known about the role played by hepadnaviral surface proteins in attachment and entry into hepatocytes. In DHBV, there are two forms of surface protein, DHBV surface antigen (DHBsAg) and a larger DHBpre-S/S protein, consisting of an N-terminal extension, the pre-S region, in addition to the DHBsAg sequence. It is believed that the pre-S region carries the viral attachment site, but there is only scant evidence (8).

To begin to understand hepadnavirus infection processes, we determined the pH requirement for DHBV infection by performing several types of experiments. The results show that DHBV most likely initiates infection at neutral pH in a manner analogous to that of pH-independent viruses.

(The major findings of this work were presented at the meeting Molecular Biology of Hepatitis B Viruses held in San Diego, Calif., 5 to 9 August 1990.)

MATERIALS AND METHODS

Primary duck hepatocytes and DHBV. Primary duck hepatocytes were prepared and cultured essentially as described by Galle et al. (2), but triiodothyronine was omitted from maintenance medium. To seed cells, this medium was supplemented with 5% fetal calf serum, and the standard maintenance medium (called MM) lacked fetal calf serum but contained dimethyl sulfoxide (1.5%). Hepatocytes were seeded in 6-, 12-, or 24-well plates (Costar) at a density of 2.5 $\times 10^4$ to 10 $\times 10^4$ cells per cm^2, and the medium was changed

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to MM after 60 to 120 min at 37°C. Subsequently, the medium was changed every 1 to 2 days prior to inoculation to avoid acidification.

DHBV-positive sera were obtained from 4- to 6-week-old ducks either congenitally or experimentally infected. Sera contained approximately 10^8 to 10^9 DNA genome equivalents per ml and were stored at −70°C. DHBV-containing sera were diluted at least 1/10 in the appropriate medium prior to inoculation. The volumes used to inoculate 6-, 12-, or 24-well plates were 0.6, 0.3, or 0.2 ml, respectively.

External virus was inactivated in some cases by treatment of cells with pH 2.2 buffer (50 mM glycine-HCl) at 4°C. After 20 to 30 s, the buffer was aspirated, and the cells were rinsed with phosphate-buffered saline (PBS) (140 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4) and incubated in MM.

Assay of DHBV infection. Routinely, DHBV e antigen (DHBeAg) in tissue culture fluids (TCF) was assayed by radioimmunoblot to measure infection of primary hepatocytes, and the validation of this assay is described in the Results. Hepatocytes were inoculated and washed at least twice to remove excess inoculum, and MM was changed at 1 and 2 days postinfection. TCF (0.25 ml) from hepatocytes 7 to 9 days postinfection was filtered by a dot-blot manifold onto nitrocellulose filters prewet with PBS. All subsequent incubations were performed in PBS–2% bovine serum albumin–0.01% sodium azide at room temperature. DHBeAg was detected with a titrated dilution of polyclonal rabbit anti-DHBc/eAg raised against denatured, bacterially produced DHBV core antigen (DHBcAg) (23). Bound immunoglobulin was detected with ^{125}I-labeled protein A (approximately 40 mCi/mg [Amersham] used at 0.03 μCi/ml) and exposure to X-ray film. Radioactivity was quantitated in a gamma counter after cutting out the individual dots. Values were corrected by subtracting the background from noninfected cells. Using denatured, bacterially produced DHBcAg as a standard, we were able to detect a minimum of approximately 5 ng of protein (10^{11} molecules).

DHBV DNA was assayed in total cellular DNA partially purified from hepatocytes by the method of Pugh and Summers (18). The DNA was denatured with NaOH (50 mM, 1 h, room temperature), neutralized with Tris-HCl, boiled, and applied to nitrocellulose. DHBV DNA was detected with DHBV plasmid DNA labeled by incorporation of [α-^{32}P] dATP by nick translation.

Low-pH treatment of DHBV prior to inoculation. A series of citrate buffers (0.1 M citric acid, 0.2 M disodium hydrogen phosphate) were prepared with pH values between pH 3.8 and pH 5.8. DHBV-containing serum or gradient-purified DHBV (40 μl) was diluted in buffer (160 μl) and incubated for 5 min at room temperature. A sample of the mixture (100 μl) was neutralized by the addition of the required volume of disodium hydrogen phosphate, diluted to 2 ml in MM, and inoculated onto hepatocytes (three wells per sample). After inoculation for 4 h at 37°C, cells were washed twice with MM and incubated. The pH of the remaining pH buffer-DHBV mixture (100 μl) was measured with a pH meter.

Exposure of DHBV at the cell surface to different pH values. For these experiments, William's medium E was replaced by Earle's salts solution (Serva) containing the same supplements as MM and buffered with 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 5.6 to 6.8) or HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.2 to 8.0) (ESM).

HSV-1 and SFV. To provide controls for the effects of ammonium chloride (NH_4Cl) and monensin, we used herpes simplex virus type 1 (HSV-1) and SFV. Propagation and infectivity determinations of HSV-1(17SYN) were performed in RC37 cells (kindly provided by G. Darai, University of Heidelberg). Purified SFV was a generous gift from Kai Simons, EMBL, Heidelberg, Germany. BHK cells were obtained from Mathias Falk, Zentrum für Molekulare Biologie (ZMBH), Heidelberg, Germany, and propagated in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. HSV-1 infection was followed by pulse-labeling hepatocytes with [^{35}S]methionine (>1,000 Ci/mmol [Amersham] at 5 μCi/ml in methionine-free Dulbecco's modified Eagle's medium) for 1 h at 6 to 8 h postinfection. Samples were loaded onto 10% polyacrylamide gels, dried, and autoradiographed. SFV infection was assayed by measuring incorporation of [5,6-^{3}H]uridine (35 to 50 Ci/mmol at 2 μCi/ml in MM) in the presence of AMD (modified from reference 6). The labeling period was extended to 5.5 h, and incorporation of [^{3}H]uridine into macromolecules was determined by trichloroacetic acid precipitation of lysed cells (1% Nonidet P-40, 10 mM Tris-HCl [pH 8], 1 mM EDTA) on filter paper discs. Treatment of NI cells with AMD reduced incorporation of label into macromolecules by at least 98%.

Inoculation in the presence of NH_4Cl or monensin. NH_4Cl and monensin were prepared freshly prior to use. Monensin (Sigma) was prepared as a 5% (wt/vol) solution in methanol, further diluted in water, and finally diluted 1/100 in MM. NH_4Cl was dissolved in water and then diluted 1/100 in MM. The same media were used for all incubations and washing in experiments with DHBV, HSV-1, SFV, hepatocytes, or BHK cells. Incubation at 37°C with these agents consisted of 1 h of preinoculation treatment, inoculation, two washes, and a postinoculation period, for the times given in the legends to Fig. 6, 7, and 8. After treatment, cells were washed twice in MM lacking the agents.

RESULTS

Radioimmunoblot of DHBeAg as an assay for DHBV infection. Infection assays for DHBV described to date involve the assay of viral DNA from infected cells by Southern or dot-blot hybridization (2, 18, 28). DHBV infection also leads to release of DHBeAg, the secretory form of the C-gene product (23), which can be detected in serum from infected ducks or in medium from infected cells. We exploited secretion of DHBeAg to develop a simple, quantitative dot-blot assay for infection in the tissue culture system.

The time course of appearance of DHBeAg in TCF and DHBV DNA in infected cells with time postinoculation was documented (Fig. 1). Presence of DHBeAg in TCF preceded the appearance of DHBV DNA in cells, as expected from in vivo studies of DHBV protein synthesis (33). Negligible amounts of DHBeAg were detectable in TCF collected 1 day after infection, and incubation of infected cells for increasing periods from 2 days postinfection resulted in accumulation of DHBeAg. In subsequent experiments, TCF was assayed at 7 to 9 days. DHBV DNA accumulated with time in infected cells, reaching detectable levels at 7 days postinfection in the experiment shown. This time point is the earliest at which release of infectious virus has been reported (28). We were unable to detect infectious DHBV in TCF collected 7 to 9 days postinoculation (data not shown). Therefore, the DHBeAg signals obtained are not influenced by reinfection, and detection of intracellular viral DNA does not indicate that progeny virus is being released.

To further validate the DHBeAg assay, we compared the quantities of viral markers from cells inoculated for different times or with various quantities of DHBV. In both cases, the

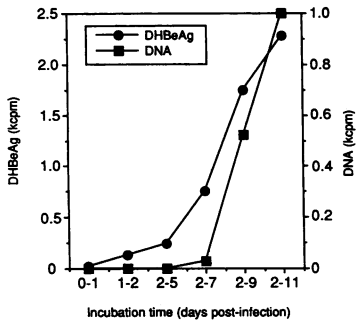

FIG. 1. Time course of production of DHBeAg and DHBV DNA after infection of hepatocytes. Hepatocytes in 24-well plates were inoculated with DHBV in MM (4 h, 37°C), washed twice with PBS, and further incubated. Medium was changed after 1 and 2 days, and subsequently, cells were incubated for up to 9 days without changing the medium (2 to 11 days). DHBeAg was assayed in samples from the TCF incubated with the cells for the times shown. DHBV DNA was assayed in DNA extracted from hepatocytes at the end of the incubation times shown, beginning 1 day postinfection. Average values from quadruplicate wells are plotted.

signals obtained with the DHBeAg radioimmunoblot were proportional to the amount of viral DNA detected in total DNA from infected cells. Inoculation of hepatocytes at 37°C with a constant amount of DHBV-containing serum, corresponding to approximately 100 DNA-containing particles per cell, yielded signals directly proportional to the duration of inoculation (Fig. 2A). Thus, the inoculation period is relatively long and no peak in the signals is evident, in agreement with other published observations showing that highly concentrated inocula of DHBV and/or long incubation times are required to achieve maximal infection (18). Consequently, the experiments described in the present report were all performed under subsaturating conditions.

Inoculation of cells at 37°C with different dilutions of DHBV serum gave rise to signals that were proportional to the log of the reciprocal dilution of DHBV-containing serum (Fig. 2B). Thus, the level of DHBeAg in TCF provides a simple, quantitative marker for DHBV infection, allowing rapid analysis of replicate samples. It would also allow assay of ongoing infection, since lysis of hepatocytes is not required, in contrast to the commonly used DNA assay.

Treatment of DHBV with low pH prior to infection. Fusion by low-pH-dependent enveloped viruses is triggered by exposure to acidic pH at or below the threshold for fusion (pH 5 to 6.6, depending on the virus and strain). This process is irreversible, and pretreatment of virus with suitably low pH in the absence of cells destroys infectivity. The pH values at which this occurs correspond to those in endosomes in which fusion is induced (29). Infectivity of enveloped viruses taken up in a pH-independent manner is often stable to acidic pH below that encountered in endosomes. We therefore determined the sensitivity of DHBV infectivity to acidic pH.

DHBV was diluted in buffer at different pH values, neutralized, and diluted in MM, and the residual infectivity was assayed in hepatocytes. Experiments performed over a wide pH range showed that DHBV remained infectious after treatment at pH 5.2 but was inactivated by exposure to pH 4.1 (data not shown). In some experiments, an increase in the level of infection was seen after treatment at pH 5.0 to 5.8. Further analysis showed that DHBV remained infectious after incubation at pH values down to pH 4.6 to 4.8 but was inactivated at pH 3.8 (Fig. 3). Thus, DHBV infectivity was sensitive to acidic pH, but below that normally encountered in endosomes. After treatment of DHBV at pH 3.7, DHBpre-S/sAg, the larger DHBV surface protein, could still be immunoprecipitated with four different anti-surface antigen antibodies (two monoclonal antibodies and one polyclonal antibody to the pre-S region and one polyclonal antibody raised against DHBV particles). However, viral DNA was no longer coprecipitated (data not shown), suggesting that inactivation by acidic pH was a consequence of disruption of virions, but was not accompanied by major irreversible changes in surface protein antigenicity.

Exposure of DHBV preattached to hepatocytes to different pHs. Enveloped viruses requiring low pH for entry can be fused to cytoplasmic membranes by allowing the virus to attach to the cell surface at low temperature and then exposing it to buffer at suitable pH. In some cases, this results in nonproductive infection, and for SFV, infection is initiated (30). Conversely, at mildly acidic pH, murine retroviruses and HSV-1 are immobilized at the cell surface and enter cells only after return to neutral pH (17, 21).

Before investigating the effects of exposure to low pH of DHBV prebound to the cell surface, we defined conditions at which only attachment occurs. Internalization of DHBV

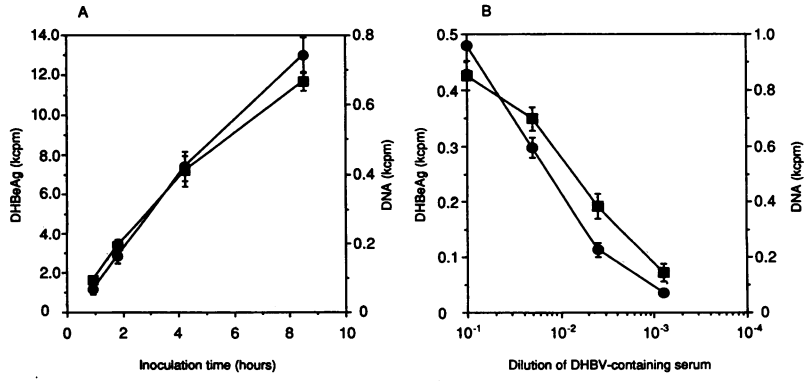

FIG. 2. Proportionality of the amount of DHBeAg in TCF and DHBV DNA in corresponding cells. Primary hepatocytes were inoculated at 37°C either for different times (A) or with serial dilutions of DHBV containing serum for 20 h (B). DHBeAg (●) was assayed by radioimmunoblot, and DHBV DNA (■) was assayed by dot-blot hybridization. Values are the corrected mean values for triplicate wells and a bar indicates the standard deviation.

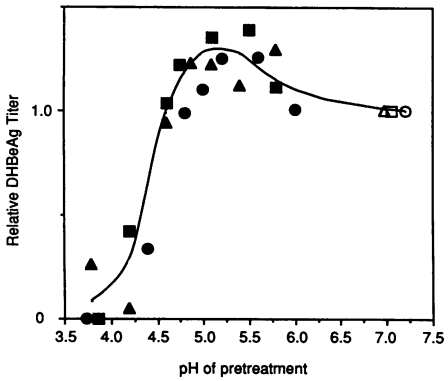

FIG. 3. Infection by DHBV after incubation at different pH values prior to inoculation. DHBeAg titers in TCF from hepatocytes inoculated with DHBV preincubated (see Materials and Methods) at the pH values shown (solid symbols). As a control, DHBV was incubated in buffer at pH 7.0 to 7.2, produced by neutralization of pH 3.8 buffer before addition of DHBV (open symbols). The results for three different DHBV preparations are shown relative to the DHBeAg titers obtained for the preneutralized samples (○ = 0.95; □ = 0.68; △ = 0.78 kcpm).

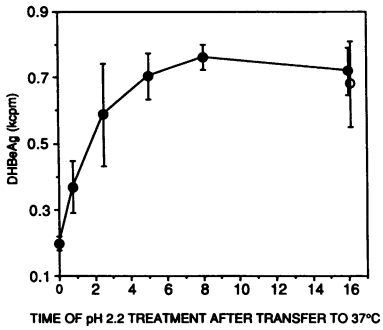

FIG. 4. Time course of internalization of DHBV bound to the surface of hepatocytes. DHBV diluted in MM was incubated with hepatocytes in 12-well plates for 3.5 h at 20°C. Cells were washed twice with PBS and transferred to 37°C. At the times indicated, external virus was inactivated with pH 2.2 buffer, as described in Materials and Methods. Untreated control samples (○) were washed twice with PBS after 16 h. The DHBeAg assay was performed 9 days after inoculation, and the standard deviation (three wells per time point) is indicated by bars.

from the cell surface was assayed by the same method as that of Pugh and Summers (18), inactivating external DHBV with pH 2.2 buffer. These workers showed that DHBV is internalized slowly after attachment to hepatocytes at 4°C. We extended these studies and quantitatively analyzed uptake. We found that DHBV also fails to enter during inoculation for up to 5 h at 15°C (data not shown) or 20°C (Fig. 4, zero time). DHBV bound to hepatocytes at low temperature could also be neutralized by the addition of antibody or inactivated by treatment with trypsin or chymotrypsin (data not shown). After transfer to 37°C, DHBV became increasingly resistant to inactivation by pH 2.2 buffer. Resistance was maximal after 8 h in the experiment shown (Fig. 4). In other experiments (not shown), maximal resistance to pH 2.2 inactivation was reached after 4 to 8 h at 37°C, regardless of whether prior attachment had been done at 4 or 20°C.

In another set of experiments, we attempted to trigger fusion of DHBV to the cell surface by exposure to low pH. DHBV was bound to hepatocytes at 20°C and exposed to buffers with pH values between 5.6 and 8.0 for 2 h at 37°C, and the external virus was subsequently inactivated by washing with pH 2.2 buffer. Successful infection occurred at pH 7.2 to 8.0, but at pH values of 5.6 to 6.4, attached DHBV remained susceptible to inactivation (Fig. 5A). This result suggests either that DHBV had fused because of exposure to mildly acidic pH resulting in nonproductive infection or that the virus simply remained at the cell surface. To distinguish between these possibilities, after incubation at the given pH values, parallel dishes of cells were further incubated in standard medium before inactivation of virus that had failed to enter. Returning cultures previously incubated at pH 5.6 to 6.4 to neutral pH resulted in infection (Fig. 5B). Thus, at pH values of 5.6 to 6.4, no DHBV-cell fusion was induced; rather, DHBV remained at a site where it could be inactivated by washing with pH 2.2 buffer and from which infection could be initiated on return to neutral pH. Hepatocytes did not recover from incubation for long periods at 37°C in buffers of pH 5.2 or lower, precluding attempts to fuse hepatocytes at the pH that inactivates DHBV infectivity in the absence of cells.

Effect of raising endosomal pH on initiation of infection by DHBV. Fusion of viruses reliant on mildly acidic pH for entry is triggered at mildly acidic pH in endosomes. Raising the pH in endosomes, and other intracellular vesicles, with agents such as NH_4Cl or monensin thus inhibits infection by low-pH-dependent viruses, for example, SFV. Infection by viruses, such as HSV-1 (31), that fuse at neutral pH is not inhibited by this type of agent. Therefore, in experiments to test the influence of NH_4Cl and monensin on initiation of DHBV infection in hepatocytes, we utilized HSV-1 and SFV as controls.

We first established conditions for infection of primary duck hepatocytes by HSV-1 or SFV, using BHK cells as a comparison, since these cells are known to be permissive for these viruses. Infection of hepatocytes with HSV-1 or SFV resulted in a cytopathic effect 8 to 12 h postinoculation, but no infectious progeny could be detected. We therefore monitored HSV-1 infection by pulse-labeling with [^{35}S]methionine. A multiplicity of infection of 1 or higher resulted in a characteristic polypeptide profile (see Fig. 8C and D, lanes 1 compared with lanes 5). Radiolabeled high-molecular-weight proteins were virus specific, since they were absent in noninfected cells and could be specifically immunoprecipitated from infected cells with an anti-HSV-1 antiserum (provided by G. Darai, University of Heidelberg) (data not shown). HSV-1 infection of hepatocytes was also inhibited by heparin (data not shown), as described for undifferentiated cells (32). SFV infection was monitored by measuring incorporation of [^{3}H]uridine into RNA as described in Materials and Methods. A multiplicity of infection of 0.1 or above yielded detectable signals in hepatocytes and BHK cells (data not shown) and a multiplicity of infection of 1 to 10 was used in subsequent experiments. Having established assays for replication of HSV-1 and SFV in duck hepatocytes, we determined the susceptibility of these viruses to raising the pH of intracellular vesicles. SFV infection was inhibited in hepatocytes as effectively as in the BHK control, for a particular concentration of NH_4Cl (Fig. 6), at concentrations corresponding to those reported by others (reference 6, for example). Accordingly, HSV-1 infection was not affected by the presence of NH_4Cl (20 mM) from 1 h prior to infection until pulse-labeling (data not shown). Thus, SFV and HSV-1 responded to NH_4Cl in duck hepatocytes as

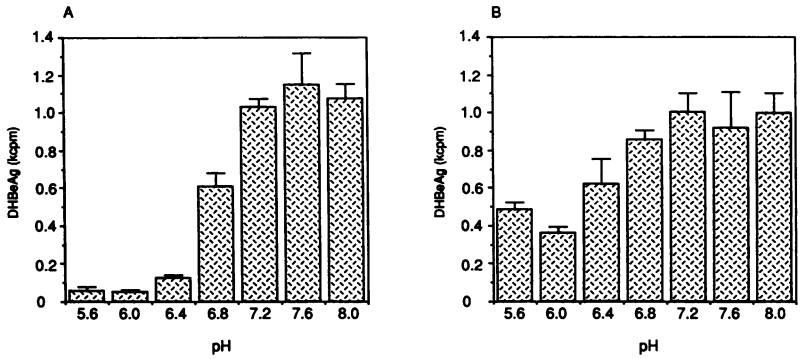

FIG. 5. Effect on infection of exposure of DHBV preattached to hepatocytes to different pH values. Hepatocytes in 12-well plates were inoculated for 5 h at 20°C with DHBV serum in MM. Unbound virus was removed by washing twice with PBS, and the cells were incubated at 37°C in ESM at the pH values shown. (A) Plates were incubated for 2 h and then treated with pH 2.2 buffer; (B) plates were incubated for 1 h and then incubated for 1.6 h in MM before pH 2.2 treatment. DHBeAg was determined 7 days after inoculation. Values are the means from triplicate wells, and the standard deviation is indicated.

expected from the routes of infection established in other cell types.

Next, the effect of raising the pH of intracellular vesicles on initiation of infection by DHBV was investigated, using SFV and HSV-1 in parallel controls. Hepatocytes were incubated with NH_4Cl (5, 10, or 20 mM), monensin (10 μM), or MM for 1 h prior to inoculation and during inoculation with DHBV. After inoculation, hepatocytes were washed and incubated in the respective media for 4 h. No inhibition of DHBV infection was seen in hepatocytes treated with NH_4Cl or monensin (Fig. 7A), demonstrating that the presence of these agents is not inhibitory during the initial stages of interaction of DHBV with host cells. In parallel, SFV infection of hepatocytes or BHK cells was abrogated by treatment of cells with 20 mM NH_4Cl or 10 μM monensin (Fig. 8A and B). Thus, the concentration of these agents was sufficient to raise the pH in endosomes above pH 6, the pH at which SFV undergoes fusion. Initiation of infection by HSV-1 in hepatocytes or BHK cells was unaffected by the presence of either NH_4Cl or monensin (Fig. 8C and D).

The effect of NH_4Cl and monensin on pH-dependent viruses is reversible during internalization, but infection by influenza virus or vesicular stomatitis virus is prevented by incubation with NH_4Cl for 5 h after inoculation (4, 26). To take account of the slow internalization of DHBV (Fig. 4), we also investigated the effects of NH_4Cl on infection by DHBV in experiments in which (i) hepatocytes were washed with pH 2.2 buffer at the end of inoculation to prevent further internalization of virus; and (ii) inoculated cells were incubated with this agent for 16 h after inoculation, that is, longer than the 4 to 8 h required for complete internalization of DHBV. Even under these conditions, no reduction of DHBV infection was observed in the presence of NH_4Cl (Fig. 7B). In some cases, inoculating DHBV onto hepatocytes treated with NH_4Cl or monensin resulted in signals in the DHBeAg assay up to twofold higher than those obtained with untreated cells (data not shown).

DISCUSSION

DHBV, an animal model for human hepatitis B virus, infects cultured primary duck hepatocytes, allowing the study of infection events for a hepadnavirus in vitro. While much is known about the mechanism of hepadnavirus replication, this is one of the first studies to address the mechanism of virus uptake. We investigated the role of pH in the initiation of infection by DHBV, one of the fundamental parameters governing viral penetration.

First, we developed a quantitative immunoblot assay for DHBV infection, measuring DHBeAg secreted into TCF. The signals obtained with this assay reflect the duration of inoculation and concentration of DHBV-containing serum used as inoculum. Additionally, the signals obtained were proportional to the quantities of viral DNA in total cellular DNA extracted from the corresponding cells, the viral marker formerly exploited to assay infection. This pattern was consistently observed in vitro and appears to parallel core protein synthesis in livers of infected ducks (33), while not being subject to host factors that may influence the level of DHBeAg in serum during DHBV infection in vivo (24).

Important for our studies was the identification of conditions that allowed attachment to, but not entry of, DHBV into hepatocytes. Classically, viruses are incubated with cells at 0 to 4°C to limit penetration. DHBV prebound to hepatocytes in this way can be inactivated by washing with pH 2.2 buffer (18), presumably because the virus remains localized at the cell surface. We extended these observations

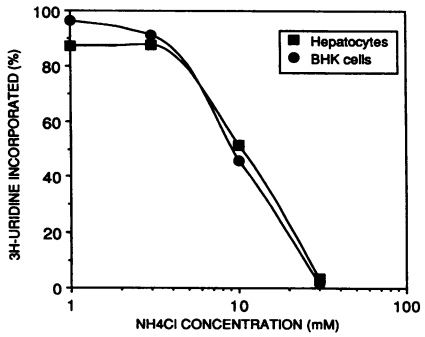

FIG. 6. Inhibition of SFV infection by NH_4Cl in duck hepatocytes or BHK cells. Hepatocytes or BHK cells incubated with NH_4Cl at the concentrations shown (four wells per concentration) were inoculated with SFV. NH_4Cl was present for 1 h prior to inoculation, during the 2-h inoculation, and during the 5.5-h labeling period. Incorporation of [^{3}H]uridine was calculated as a percentage of that in the absence of NH_4Cl (12.8 kcpm for hepatocytes; 128.9 kcpm for BHK cells).

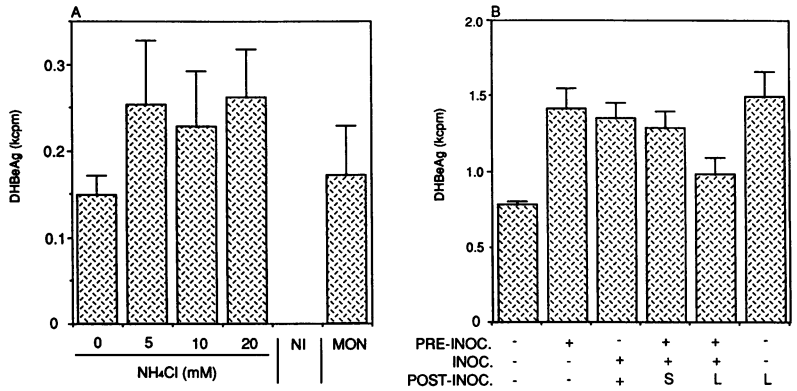

FIG. 7. DHBV infection of hepatocytes in the presence of NH_4Cl or monensin. Hepatocytes were inoculated, and DHBeAg in samples from replicate wells was assayed after 7 days. Standard deviation is indicated by a bar. (A) Hepatocytes in 24-well plates were treated with monensin (MON) (10 μM) or NH_4Cl at the concentrations shown (four wells per concentration) prior to infection, during a 2-h inoculation period, and for 4 h subsequently. (B) Triplicate wells of hepatocytes were incubated in the presence (+) or absence (−) of NH_4Cl (30 mM) at the stages shown. Incubation prior to inoculation (PRE-INOC.) was for 1 h. At the end of inoculation (INOC.) (1.5 h), cells were washed with pH 2.2 buffer, as described in the Materials and Methods. Postinoculation (POST-INOC.) incubation was for 2.5 h (S) or 16 h (L).

to show that DHBV fails to enter when incubated with hepatocytes for up to 5 h at 20°C. This is intriguing, first because at 20°C endocytosis occurs and SFV and influenza virus are able to enter host cells (9, 24), suggesting that endocytosis alone, if indeed required, is insufficient for efficient internalization of DHBV. Second, while low-pH-dependent viruses fuse efficiently at low temperature, viruses fusing at neutral pH appear not to undergo fusion at or below 20°C (29).

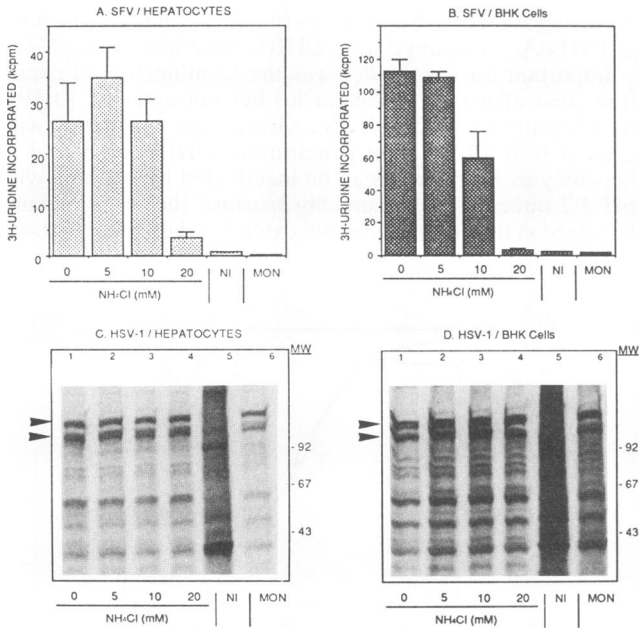

FIG. 8. Infection by HSV-1 or SFV in the presence of monensin or NH_4Cl. In parallel to the experiment shown for DHBV (Fig. 7A), primary hepatocytes or BHK cells were treated with monensin or NH_4Cl as shown and inoculated with HSV-1 or SFV. HSV-1 was inoculated for 1 h, and postinoculation incubation with agents lasted 1 h. Arrowheads indicate proteins with mobility similar to that of proteins that could be immunoprecipitated with an anti-HSV-1 antiserum. Conditions for SFV are as described in the legend to Fig. 6.

Using pH 2.2 buffer to inactivate external DHBV, we quantitatively examined the internalization of DHBV prebound to hepatocytes at 20°C (or 4°C). Uptake occurred over 4 to 8 h after transfer to 37°C. This is consistent with and extends a previous analysis of internalization of DHBV by hepatocytes (18). Our determination of the time required for internalization is shorter than the 16 h estimated by these researchers for internalization after attachment at 4°C. The slow internalization of DHBV appears to parallel the fusion kinetics of paramyxoviruses and Rous sarcoma virus, both of which fuse at neutral pH, and contrasts with rapid uptake of low-pH-dependent viruses (4, 10). Thus, internalization of DHBV is a relatively slow process, and the temperature dependence for initiation of infection suggests that endocytosis is not involved.

In the main part of this study, we investigated whether low pH was involved in initiation of DHBV infection. Hepatocytes were inoculated with DHBV in the presence of the weak base NH_4Cl or the carboxylic ionophore monensin, which raise the pH of intracellular vesicles by different mechanisms. These types of agents inhibit infection by viruses that require low pH for fusion, for example, SFV, but do not inhibit infection by viruses that initiate infection at neutral pH, for example, HSV-1 (10, 31). We found that DHBV was able to initiate infection in the presence or absence of NH_4Cl or monensin. We conclude, therefore, that the low pH of intracellular vesicles is not important for entry of DHBV. This conclusion is supported by results of parallel control experiments in which primary duck hepatocytes or BHK cells were inoculated with SFV or HSV-1 in the presence or absence of NH_4Cl or monensin. In both cell types, SFV infection was inhibited by these agents, whereas infection by HSV-1 was unaffected. Thus, initiation of infection by DHBV resembles that by HSV-1 in that it proceeds when the pH of intracellular vacuoles is raised sufficiently to preclude SFV infection.

We were mindful that elevation of the pH of intracellular vesicles is inhibitory only during initiation of infection by low-pH-dependent viruses and is reversible in the early stages of infection. For example, pretreatment alone of BHK

cells with NH_4Cl does not inhibit SFV infection (6), and addition of NH_4Cl after inoculation does not completely inhibit SFV or influenza virus (6, 34). It is recommended that an event early after infection is assayed in studies with agents that raise the pH in intracellular vesicles (10). Alternatively, as in this study, inoculated cells can be maintained in these agents for a period after inoculation. Incubation of cells inoculated with influenza virus or vesicular stomatitis virus, both dependent on low pH, with NH_4Cl for 5 h after inoculation inhibits infection judged by assays performed 24 or 18 h after inoculation, respectively (4, 26). Under these conditions, the influenza virus nucleocapsid fails to enter the cytoplasm, is transported further to lysosomes, and is degraded (11). Similar treatment of cells inoculated with human immunodeficiency virus type 1, for which initiation of infection is pH independent, results in no reduction in virus titer, although NH_4Cl inhibits maturation of human immunodeficiency virus type 1 and HSV-1 (7, 12). Having documented the slow internalization of DHBV, we therefore performed experiments in which hepatocytes were inoculated with DHBV, washed with pH 2.2 buffer to inactivate DHBV not yet internalized, and treated with NH_4Cl for long periods after inoculation. However, the continuous presence of NH_4Cl for 1 h prior to and during inoculation and for 16 h after inoculation resulted in no reduction in DHBV infection. The possibility that the failure to detect inhibition of DHBV infection by these agents is a result of assaying infection with the DHBeAg assay 7 to 9 days after inoculation seems unlikely, since reduction in DHBeAg was measurable in similar experiments with Suramin (19a), known to inhibit attachment and uptake of DHBV (16).

Exposure of low-pH-dependent viruses prebound to the cell membrane to suitable pH induces fusion. Attempts to fuse DHBV to hepatocytes in this way failed. Indeed, at pH 5.6 to 6.4, the virus remained immobilized at the surface and capable of initiating infection upon return to neutral pH. Because the hepatocytes were sensitive to lower pH, we were unable to test the effect of buffer at pH 3.8, the pH at which infectivity of DHBV is inactivated in the absence of cells. We conclude that mildly acidic pH does not trigger fusion of DHBV or damage infectivity and suggest that DHBV fuses only at approximately neutral pH. In this respect, DHBV again closely resembles HSV-1, which also initiates infection at neutral pH (21). Our results with DHBV and similar studies with HSV-1 (21) show that initiation of infection by these viruses is not strictly independent of pH, since moderately acidic conditions are inhibitory. Intriguingly, both of these viruses assemble by budding intracellularly (3, 20) early in the secretory pathway, in which at least some of the vesicles are acidic (14). Fusion induced by low pH would in this case be detrimental. Inhibition of a postadsorption step by acidic pH has also been reported for retroviruses (17). While endocytosis of some markers is inhibited at acidic pH, it is also speculated that protease cleavage of surface proteins, required for initiation of infection, is inhibited at acidic pH (13). In pursuit of this idea, DHBV attached to hepatocytes was treated with exogenous protease, but only inactivation of infectivity was observed (19a).

The pH sensitivity of DHBV was determined by pretreating virus with buffers at various pH values and measuring residual infectivity. Generally, the infectivity of viruses dependent on mildly acidic pH for entry is inactivated by exposure to pH 5 to 6.6. This is virus and strain dependent, and there is one exception (19). Viruses that fuse at neutral pH appear to be stable to lower pH, e.g., human immunodeficiency virus type 1 (pH 4.2 [12]) and Rous sarcoma virus (pH 4.8 [4]). DHBV was stable to pH 4.6 to 4.8, but treatment at pH 3.8 or lower inactivated infectivity, and this inactivation was associated with disruption of virions. The pH sufficient for inactivation is below that normally encountered in endosomes. Therefore, if DHBV were to be taken up by endocytosis, this would imply that fusion of DHBV occurs late after internalization, perhaps in lysosomes, and would further imply that entry of DHBV would be sensitive to relatively low concentrations of agents that raise the pH of intracellular vesicles. This is not the case, since the concentrations of NH_4Cl and monensin used in this study raised the pH sufficiently to prevent infection by SFV, which fuses at approximately pH 6. An increase in the levels of infection obtained after pretreatment of DHBV at pH 5.0 to 5.8 was not noticeable in the experiments in which DHBV attached to hepatocytes was exposed to mildly acidic pH.

Thus, using different experimental approaches, we found no evidence for a role for low pH in initiation of infection by DHBV. On the contrary, we found that DHBV resembles viruses belonging to the group that fuse at neutral pH which have been termed pH-independent viruses. Since the pH requirement is often common to a family of viruses, it will be interesting to see whether this is true for other hepadnaviruses.

During preparation of this report, results of experiments to determine the effects of NH_4Cl and chloroquine on DHBV infection were published by Offensperger and coworkers (15). These investigators report inhibition of DHBV infection by these agents, in contrast to our results with NH_4Cl and monensin. However, the conditions used by these researchers differed from ours in that they maintained cells treated with NH_4Cl or chloroquine in these agents for 2 weeks between inoculation and assay of infection. We used SFV and HSV-1 as functional controls for the effect of NH_4Cl and monensin in hepatocytes, and treatments were limited to 18.5 h maximum, to allow internalization of DHBV, while taking into account the fact that long-term incubation in these agents can affect other stages of virus replication (7, 11).

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