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Hepadnavirus Infection Requires Interaction between the Viral Pre-S Domain and a Specific Hepatocellular Receptor

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To better define the molecules involved in the initial interaction between hepadnaviruses and hepatocytes, we performed binding and infectivity studies with the duck hepatitis B virus (DHBV) and cultured primary duck hepatocytes. In competition experiments with naturally occurring subviral particles containing DHBV surface proteins, these DNA-free particles were found to interfere with viral infectivity if used at sufficiently high concentrations. In direct binding saturation experiments with radiolabelled subviral particles, a biphasic titration curve containing a saturable component was obtained. Quantitative evaluation of both the binding and the infectivity data indicates that the duck hepatocyte presents about 10^4 high-affinity binding sites for viral and subviral particles. Binding to these productive sites may be preceded by reversible virus attachment to a large number of less specific, nonsaturable primary binding sites. To identify which of the viral envelope proteins is responsible for hepatocyte-specific attachment, subviral particles containing only one of the two DHBV surface proteins were produced in *Saccharomyces cerevisiae*. In infectivity competition experiments, only particles containing the large pre-S/S protein were found to markedly reduce the efficiency of DHBV infection, while particles containing the small S protein had only a minor effect. Similarly, physical binding of radiolabelled serum-derived subviral particles to primary duck hepatocytes was inhibited well only by the yeast-derived pre-S/S particles. Together, these results strongly support the notion that hepadnaviral infection is initiated by specific attachment of the pre-S domain of the large DHBV envelope protein to a limited number of hepatocellular binding sites.

Hepadnaviruses are a family of small, enveloped animal viruses which cause acute and chronic liver infections in their respective hosts. While the human hepatitis B virus (HBV) is its prototype, this family also comprises several other members that have been well characterized, including HBVs that infect woodchucks, ground squirrels, and ducks. A narrow host range, a distinct organ tropism, and a replication strategy which involves a circular DNA genome and reverse transcription of a linear RNA intermediate are characteristics of these viruses. The hepadnavirus genome, one of the smallest viral genomes known at 3 kb, encompasses three extensively overlapping open reading frames common to all hepadnaviruses. These encode structural proteins with essential functions for the viral life cycle: the DNA polymerase/reverse transcriptase, the core protein that assembles into an icosahedral nucleocapsid, and three (only two in duck HBV [DHBV]) surface proteins present in the viral envelope. In the mammalian viruses, an additional open reading frame, the *X* gene, encodes a pleiotropic transcriptional activator of yet undefined function for the viral life cycle.

In recent years, analysis on the molecular level of the viral life cycle has relied extensively on functional analysis of manipulated viral DNA genomes transfected into cultured hepatoma cells to provide liver-specific transcription factors required to activate viral transcription. This approach helped to define, in increasing detail, the mechanisms controlling hepadnaviral transcription, gene expression, capsid assembly, and DNA synthesis (for recent reviews, see references 9 and 12). Little progress has been made in forwarding our knowledge of the early steps in the hepadnavirus life cycle because the cell lines used to support virus production from transfected viral DNA genomes apparently lack the cellular receptor(s) required for initiation of infection by virions. Consequently, little is known about the role played by hepadnaviral surface proteins in virus attachment to and uptake by hepatocytes. Infectivity studies to address these questions are presently only possible with primary hepatocytes.

For this type of experiment, DHBV provides the best model system, since primary duck hepatocytes are easily obtained and the conditions for their maintenance and DHBV infection have been investigated in considerable detail (3, 29). In DHBV, there are two nonglycosylated surface proteins embedded in the lipid envelope: the major S protein, a transmembrane protein of 167 amino acids, and the larger pre-S/S protein, containing an additional N-terminal extension of 161 amino acids (17, 21). From hydrophilicity and antigenicity profiles, the pre-S domain is predicted to be the major structural determinant on the virus surface (2, 33). Together with the high sequence variation of the pre-S domain between hepadnaviruses in different host species (25, 27), this suggests that the pre-S sequence probably also carries the site for host-specific attachment to hepatocytes.

The participation of pre-S sequences in the early steps in hepadnaviral infection is also suggested from the results of numerous studies analyzing the physical binding of particulate surface proteins of human HBV, obtained from patient sera or from recombinant vaccines, to plasma membrane fractions from human liver or to cultured cells of various origins. In these experiments, particle binding preferentially correlated with the presence of amino acid sequences unique to the N-terminal segments of the large surface polypeptide or located N terminally in the middle-sized one of the three HBV surface polypeptides (the pre-S1/pre-S2/S protein and the

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pre-S2/S protein, respectively), suggesting that two separate segments of the pre-S sequence participate in the initial virus-cell interaction (13–16). However, for technical reasons outlined above and below, binding experiments in the HBV system were carried out with nonpermissive cell lines and with noninfectious subviral particles (SVPs). Therefore, the significance of the results for our understanding of the hepadnaviral entry pathway remains unclear as long as they are not complemented by infectivity studies.

A feature characteristic of all hepadnaviral infections and complicating their study on the molecular level is the production of DNA-free SVPs, which consist of lipids and viral surface proteins only and are present in vast excess over virions in the blood of viremic hosts. This overproduction of surface proteins probably ensures the formation and budding of the enveloped virion into the endoplasmic reticulum lumen. It most likely also plays a role in inducing immunotolerance, leading to chronic viral infection. Whether these SVPs have any effect on the infection process is not known. Interestingly, the large pre-S protein, thought to have receptor functions, is present only in much reduced amounts in SVPs from human HBV (5). A similar reduction in the level of pre-S sequences is, however, not observed in SVPs from the avian virus DHBV; the SVPs show a protein composition very similar to that of the virion (28).

These observations from different experimental systems raise many interesting questions about the mechanisms and components involved in the initiation of hepadnaviral infection. Are all of the several viral surface proteins required for virus uptake? How does the virion avoid competition from the vast excess of SVPs? Are SVPs not recognized by the cellular receptor, or are they internalized and sorted out at a later stage of the infection process? To explore these and other questions, we have performed combined binding and infectivity studies with natural DHBV particles and primary duck hepatocytes. These studies were complemented by competition studies with virus-free DHBV SVPs which contain only a single DHBV surface protein produced in *Saccharomyces cerevisiae.*

MATERIALS AND METHODS

Cells. Primary duck hepatocytes were prepared and cultivated as described before (19). Briefly, hepatocytes, isolated by two-step collagenase perfusion, were seeded at a density of approximately 5×10^5 cells per well in 24-well plates (Costar) and incubated at 37°C. The medium was changed every 24 h for the first 2 days. Usually cells were used on the second day after plating for infection studies or binding assays. The medium for binding studies contained 1× Earl's saline (Serva) buffered with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and contained the same supplements as the medium used for maintenance of the cells.

Virus and virus purification. DHBV particles were isolated from sera obtained from 3-week-old ducklings previously transfected with cloned DHBV-3 DNA (27). Portions (8 ml) of serum containing between 5×10^9 and 1×10^{10} genome equivalents were centrifuged at 190,000 × *g* for 2.5 h through 3 ml of 20% (wt/vol) sucrose onto a 2-ml 70% sucrose cushion. The fractions collected were analyzed for viral DNA by dot blot hybridization and for viral proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions positive for both markers were pooled, diluted, and centrifuged for 4 h at 190,000 × *g* through a sucrose step gradient consisting of layers of 20, 30, 40, 50, 60, and 70% sucrose. The fractions obtained were analyzed for viral DNA and proteins as described below. Fractions positive for both viral components were pooled and stored at −70°C. All centrifugation steps were carried out in an SW40 rotor (Beckman) at 20°C. Phosphate-buffered saline (PBS) with EDTA (PBSE; 140 mM NaCl, 10 mM phosphate [pH 7.4], 1 mM EDTA) was used as the buffer system for all steps.

Estimation of number of viral particles and SVPs. The number of viral particles contained in each preparation was calculated from the viral DNA content, determined by DNA dot blot hybridization (26), and quantitated by using standards of cloned DHBV DNA. The total amount of viral plus subviral particles was estimated from the pre-S/S protein content, as determined by Western blotting (immunoblotting) experiments with an anti-MS2/pre-S serum (21). Pre-S was quantitated by comparison with a dilution series of a DHBV particle preparation for which the pre-S/S protein content had been determined by SDS-PAGE relative to a carbonic anhydrase standard. Assuming that 25 of the ca. 100 surface proteins in the envelope of a DHBV particle are pre-S or S proteins (4), the estimated pre-S/S protein content was used to calculate the particle number (7×10^8 particles per ng of pre-S/S protein). When checked in a particular preparation by electron microscopy, the calculated value differed by a factor of only 1.3 from the number of particles determined relative to an internal standard of polystyrene-latex beads (0.091 μm diameter; 2.41×10^{12} beads per ml; Plano). For SVPs produced in yeast cells, particle numbers were determined similarly, again assuming that the envelope of each particle contains 100 surface protein molecules.

Radiolabelling of DHBV. DHBV particle preparations were dialyzed against PBSE with Centricon 30 centrifugal microconcentrators (Amicon) and used for the radiolabelling reaction with ^{125}I-Bolton-Hunter reagent (~2,000 Ci/mmol; Amersham) as recommended by the manufacturer. Unincorporated label was removed by gel filtration on a PD10 column (Pharmacia) equilibrated in $TNT_{0.05}$ (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% Tween 20). The specific activities of various preparations ranged from 2.5×10^{-4} to 6.2×10^{-4} cpm per particle.

Expression of DHBV pre-S and S protein in *S. cerevisiae.* DNA fragments encompassing the genes for both surface proteins were cloned into the unique *Bam*HI cloning site of plasmid pMB221-T (1) after *Bgl*II sites were introduced at their ends by polymerase chain reaction. pMB221-T contains, besides the inducible *GAL1* promoter and the alcohol dehydrogenase 1 gene terminator, a 2 μm replication origin, an Amp^r gene, and a *LEU2* selection marker. Both constructs, pMB-DPS and pMB-DS, were verified by sequencing. In all experiments, the yeast strain CY141 pmr1 (*MAT***a** *his3-11,15 leu2-3,112 trp1-1 ade2-1 ura3-1 can1-100 pmr1*::*URA3*) was used. *LEU2* transformants carrying the desired plasmid were selected, grown in SD medium supplemented with an amino acid mixture (20 to 375 μg/ml) lacking leucine for 2 days, and then transferred into YPD medium containing 2% galactose for a further 2 days. All media were prepared as described before (20). The cells were collected by centrifugation, resuspended in an equal volume of lysis buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM EDTA, 0.01% Triton X-100) containing a mixture of protease inhibitors (100 ng of chymostatin, 2 μg of aprotinin, 1 μg of pepstatin A, and 2.5 μg of antipain per ml plus 10 mM benzamidine and 10 mM sodium metabisulfite [Sigma]), and disrupted by rapid stirring with glass beads in a blender. The resulting extract was clarified by successive centrifugations for 5 min at 1,500 × *g*, 60 min at 12,000 × *g*, and 40 min at 100,000 × *g*. The final supernatant was layered onto a 2-ml 70% (wt/vol) sucrose cushion and centrifuged for 14 h at 20,000 × *g* in an SW28 rotor (Beckman). Fractions (2 ml) were collected from the bottom of the

centrifuge tube, diluted, and loaded onto a 20, 30, 40, and 50% (wt/vol) sucrose step gradient. Centrifugation was carried out in an SW40 rotor (Beckman) for 12 h at 190,000 × *g*. The gradient was fractionated from the bottom, and fractions of interest were pooled. The density of the pooled fractions was increased by the addition of an equal volume of 70% (wt/vol) sucrose, overlaid with 50, 40, 30, 20, and 10% (wt/vol) sucrose, and centrifuged for 8 h at 200,000 × *g*. Fractions containing the desired proteins were pooled, diluted, and pelleted through 20% sucrose onto 70% (wt/vol) sucrose by centrifugation for 2.5 h at 190,000 × *g*. All purification steps were monitored by SDS-PAGE. The pre-S/S protein was detected by Western blotting, and the S protein was visualized by Coomassie blue staining (see below). PBSE was used as a buffer system and included the protease inhibitors described for the lysis buffer. All operations were carried out at 4°C.

Detection of DHBV pre-S/S and S protein expressed in *S. cerevisiae*. For protein analysis, aliquots from the fractions obtained after the various purification steps were subjected to SDS-PAGE and either stained with Coomassie brilliant blue or detected by the more sensitive silver staining method (11). For Western blot analysis, samples separated by SDS-PAGE were transferred to nitrocellulose filters (Schleicher & Schüll) and detected with an anti-MS2/pre-S serum or anti-yeast-derived S serum followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The bands were visualized with 5-bromo-4-chloro-3-indolylphosphate-*p*-Nitro-Blue Tetrazolium chloride (Biomol) as the substrate. The immunoprecipitation analysis was performed as described before (21), and detection was done by Western blotting.

For electron microscopy, preparations of yeast-derived pre-S/S protein were further purified by immunoaffinity chromatography by a modification of the method described previously (31). Briefly, anti-pre-S antiserum was covalently coupled to protein A-Sepharose beads (22), the yeast pre-S protein preparation was adsorbed to the column, and the column was washed with 2 column volumes of $TNT_{0.05}$ and eluted with 3 M ammonium thiocyanate. Fractions containing pre-S/S protein were sedimented on a sucrose cushion. (As the yield of recovery of the pre-S/S protein by this method was only approximately 10%, this procedure was not feasible for large-scale preparations.) For electron microscopy, fractions diluted 1:10 with PBS were loaded onto Cu-carbon grids and negatively stained with 2% uranyl acetate. Electron microscopic recording, carried out by H. Zentgraf, was done at a magnification of ×20,100.

Binding assay. Two days after being plated, the primary duck hepatocytes (5 × 10^5 cells per well) were washed two times with PBS and incubated at 20°C for 12 h with 250 μl of a mixture containing radiolabelled DHBV particles, unlabelled particles, and medium per well. The cells were washed three times with PBS and lysed by the addition of TN-N40 (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40), and the cell-associated radioactivity was counted in a multicrystal gamma counter. Each value was determined in triplicate, and the results obtained were reproduced at least twice.

Infection assay. On the second day after plating, primary duck hepatocytes (5 × 10^5 cells per 25-mm well) were inoculated with 250 μl of a mixture containing 5 μl of the purified virus stock, various amounts of competitor particles, and supplemented William's medium E. After 12 h of incubation at 37°C, the inoculum was removed and the cells were washed two times with PBS. The cells were kept in maintenance medium for a further 7 days, and then half of the supernatant from each well was filtered with a dot blot manifold (Schleicher & Schüll) onto nitrocellulose filters. Filters were treated as described before (19). Briefly, after blocking, the filters were incubated with rabbit anti-DHBeAg-DHBV core antigen antiserum and then with ^{125}I-labelled protein A. The quantity of radioactivity associated with each dot was determined. The values shown represent averages for three independent determinations.

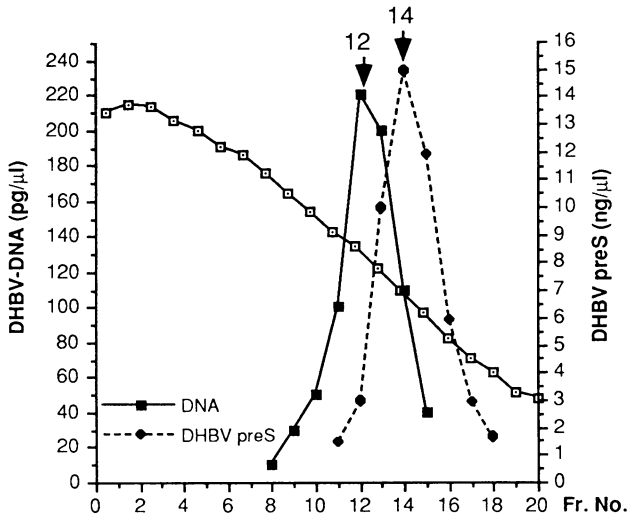

FIG. 1. Partial separation of DHBV virus and SVPs from duck serum by sucrose density gradient sedimentation. For each fraction, DHBV DNA and pre-S levels and the sucrose density (□) were determined as described in Materials and Methods. The fractions used for the infection experiment (Table 1) are marked by arrows.

RESULTS

Influence of natural DHBV SVPs on infection. In DHBV, SVPs and virions are very similar in size and density, and it is difficult to separate them completely (8, 23). Therefore, for initial competition experiments, the relative infectivity of virus preparations containing distinctly different ratios of SVPs was compared. Two such DHBV preparations, which differed 10-fold in the ratio of pre-S antigen to DHBV DNA and thus in the ratio of SVPs to virions, were obtained from DHBV-positive duck serum by sucrose density gradient centrifugation (Fig. 1A, fractions 12 and 14). From these, equivalent amounts of DNA-containing particles were inoculated into cultures of primary duck hepatocytes, and 7 days later, the level of DHBV e antigen (DHBeAg), a DHBV core gene product secreted into the culture medium, was quantitated by immunoblotting. As shown before, this assay provides a reliable indicator for ongoing virus replication in that DHBeAg secretion parallels the amount of intracellular viral DNA (19). This assay, or the similarly indirect in situ hybridization assay (17, 18), is required to monitor the establishment of hepadnaviral infections in vitro because infection does not cause cytopathic effects in the absence of the cellular immune system.

As shown in Table 1, the two virus preparations displayed comparable infectivities when assayed at a multiplicity of infection (MOI) of 60, indicating that DHBV infection was only slightly affected by increasing the excess of SVPs over virions from 65- to 650-fold. However, at a 10-fold-higher particle input (MOI, 600), relative infectivity was reduced more than twofold at the high SVP/virion ratio. These results suggest that SVPs indeed interfere with productive DHBV infection, provided that their concentration is high enough to

TABLE 1. Influence of DHBV SVPs on DHBV infectivity[a]

DHBV inoculum[a]			Relative infectivity (DHBeAg production)[b]	
Fraction no.	MOI[c]	No. of SVPs/cell[d]	cpm	%
12	60	4×10^3	663	100
14	60	4×10^4	620	94
12	600	4×10^4	1,813	273
14	600	4×10^5	830	125

[a] Fractions from the sucrose gradient shown in Fig. 1 were used to infect primary duck hepatocytes. Fraction 12 (1 or 10 μl) and fraction 14 (2 or 20 μl) were used to inoculate 5×10^5 cells per well.

[b] DHBeAg production values represent averages for two independent determinations. The value elicited by fraction 12 at an MOI of 60 was taken as 100%.

[c] The MOI was based on DHBV DNA, as determined by DNA dot blot hybridization.

[d] The number of SVPs per cell was based on the pre-S content, determined by Western blotting.

saturate a cellular component required to initiate productive DHBV infection. The data in Table 1 indicate that this critical concentration is reached in our experimental system at about 10^8 particles per ml, which corresponds to approximately 10^4 particles per hepatocyte.

Expression of DHBV surface proteins in yeast cells. To ascertain whether SVPs compete with virions during DHBV infection, SVPs were produced independently from virus replication by heterologous gene expression in yeast cells, as done previously to produce a subviral HBV vaccine (10, 30, 31). This approach promised to allow not only testing of virion-free DHBV SVPs but also dissection of the contributions of each of the two viral surface proteins contained in the viral envelope. For this purpose, the genes encoding the DHBV pre-S/S and the DHBV S protein were cloned into an inducible yeast expression vector (Fig. 2A) and transfected into yeast cells. Transformants showing high yields in small-scale experiments were grown preparatively and then induced for several days to express the viral proteins. Assuming that the membrane proteins produced were retained intracellularly as membranous particles (as known from the expression of the corresponding HBV pre-S/S genes [30]), yeast cell extracts were prepared and subjected to several centrifugation steps established previouslyfor the purification of natural SVPs from DHBV-positive duck serum (for details, see Materials and Methods). Samples from crude cell extracts and from purified fractions were then analyzed and compared with serum-derived DHBV particles by SDS-PAGE, Western blotting, and immunoprecipitation.

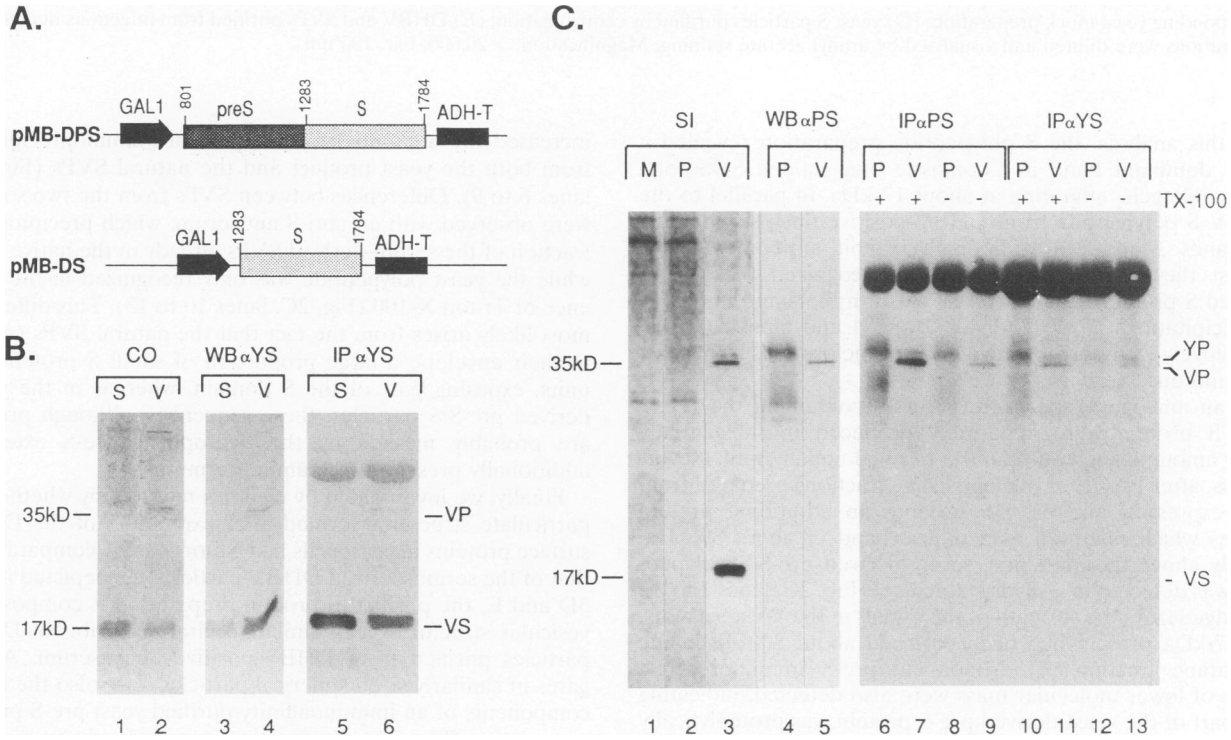

FIG. 2. Analysis of DHBV surface proteins expressed in yeast cells. (A) Relevant segments of the inducible yeast expression constructs pMB-DPS and pMB-DS. DHBV open reading frames are indicated by dotted boxes and nucleotide positions in the DHBV genome. An arrow symbolizes the inducible *GAL1* promoter, and a black box indicates the alcohol dehydrogenase 1 gene terminator (ADH-T). (B) Analysis of S protein produced in yeast cells. Gradient-purified yeast S polypeptide (lanes S) and serum-derived DHBV SVPs (lanes V) were subjected to SDS-PAGE, and proteins were either stained directly with Coomassie blue (CO) or analyzed by Western blotting with anti-yeast-derived S antiserum (αYS); the latter was also used for the detection of proteins immunoprecipitated by anti-yeast-derived S antiserum (IPαYS). The positions of pre-S (VP) and S (VS) are marked. (C) Analysis of DHBV pre-S protein produced in yeast cells. A yeast pre-S protein preparation (lanes P), a yeast mock preparation (lanes M), and purified DHBV particles (lanes V) were resolved by SDS-PAGE, and proteins were visualized either by silver staining (SI) or by Western blotting with an anti-MS2/pre-S antiserum (WBαPS). Immunoprecipitations were carried out with anti-MS2/pre-S antiserum (IPαPS) or anti-yeast-derived S antiserum (IPαYS) in the presence (+) or absence (−) of 0.1% Triton X-100 (TX-100). The positions of serum-derived viral pre-S (VP) and S (VS) polypeptides are marked, and their molecular masses are indicated (in kilodaltons).

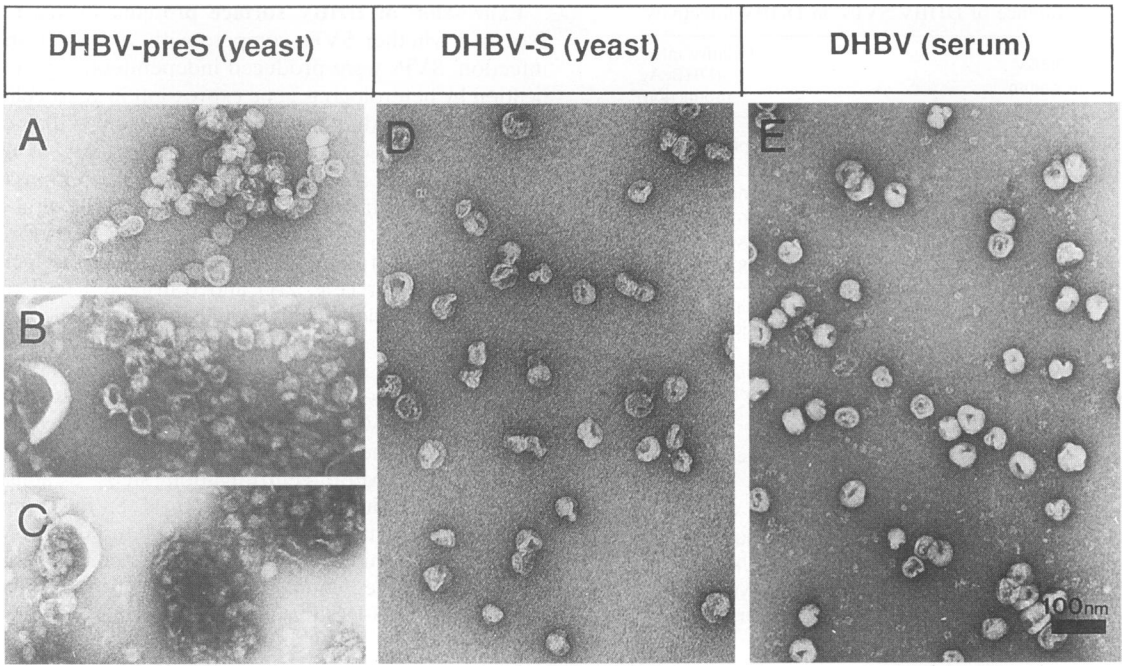

FIG. 3. Electron microscopy of DHBV pre-S and S protein preparations from yeast cells in comparison with serum-derived DHBV particles. (A) Yeast pre-S preparation purified by immunoaffinity chromatography; (B) yeast pre-S preparation enriched by centrifugation; (C) corresponding yeast mock preparation; (D) yeast S particles purified by centrifugation; (E) DHBV and SVPs purified from infectious duck serum. Preparations were diluted and visualized by uranyl acetate staining. Magnification, ×20,000; bar, 100 nm.

In this analysis, the S polypeptide preparation revealed a single dominant band in Coomassie blue-stained SDS-polyacrylamide gels, migrating at about 17 kDa, in parallel to the DHBV S polypeptide from DHBV-positive duck serum (Fig. 2B, lanes 1 and 2). Additionally, rabbit antibodies raised against the yeast-derived S protein recognized the serum-derived S protein equally well in Western blots and in immunoprecipitations (Fig. 2B, lanes 3 and 4 and lanes 5 and 6, respectively), supporting the identity of the proteins from yeast cells and duck serum.

In an analogous approach, particles containing the larger pre-S/S protein were apparently produced in substantially lower amounts and could not be purified equally well. Nevertheless, after PAGE of the membrane fractions prepared from cells expressing the pre-S/S protein, an additional protein species which migrated as a diffuse band of about 37 kDa, slightly above the authentic, serum-derived pre-S/S polypeptide, was detected in a silver-stained gel (Fig. 2C, lanes 1 to 3). As judged from the strength of the signals in the Western blots, the 37-kDa protein had been enriched about 80-fold in the membrane fraction (6). Minor anti-pre-S immunoreactive bands of lower molecular mass were also detected, indicating that part of the yeast-derived pre-S protein was proteolytically degraded during synthesis and/or purification. The 37-kDa protein was also immunoprecipitated by antibodies directed against different parts of the pre-S sequence (6). It therefore most likely represents a full-length pre-S/S protein whose electrophoretic migration is slightly affected by a posttranslational modification of an unknown nature. The yeast pre-S/S product was also similar to the natural SVPs in that it was immunoprecipitated with anti-pre-S antibodies in the native state, indicating that at least part of the pre-S domain was localized at the outside of the membranous particles (Fig. 2C, lane 8). Addition of the nonionic detergent Triton X-100 increased the amounts of pre-S protein immunoprecipitated from both the yeast product and the natural SVPs (Fig. 2C, lanes 6 to 9). Differences between SVPs from the two sources were observed with an anti-S antiserum, which precipitated a fraction of the serum-derived SVPs already in the native state, while the yeast polypeptide was only recognized in the presence of Triton X-100 (Fig. 2C, lanes 10 to 13). This difference most likely arises from the fact that the natural SVPs contain in their envelope a large proportion of small S protein subunits, exposing part of the S domain, whereas in the yeast-derived pre-S/S particles, these sequences, although present, are probably masked by the hydrophylic pre-S extension additionally present at the amino terminus.

Finally, we investigated by electron microscopy whether the particulate structures formed after expression of the DHBV surface proteins in yeast cells had a morphology comparable to that of the serum-derived DHBV particles. As depicted in Fig. 3D and E, the purified S protein preparation is composed of vesicular structures very similar to viral and subviral DHBV particles purified from DHBV-positive duck serum. Aggregates of similarly sized spherical particles were also the major components of an immunoaffinity-purified yeast pre-S protein preparation (Fig. 3A). Preparations purified only by centrifugation also contained other vesicular structures that were present in the mock preparation included as a control (Fig. 3B and C). Together with the centrifugation steps used for preparative purification, this analysis confirmed that both DHBV surface proteins were produced in yeast cells as part of lipoprotein particles with properties similar to those of serum-derived SVPs. For the design and evaluation of the competition experiments described below, yeast protein concentrations were therefore converted into particle numbers, as was done for the natural particles, again using the assumption that 100

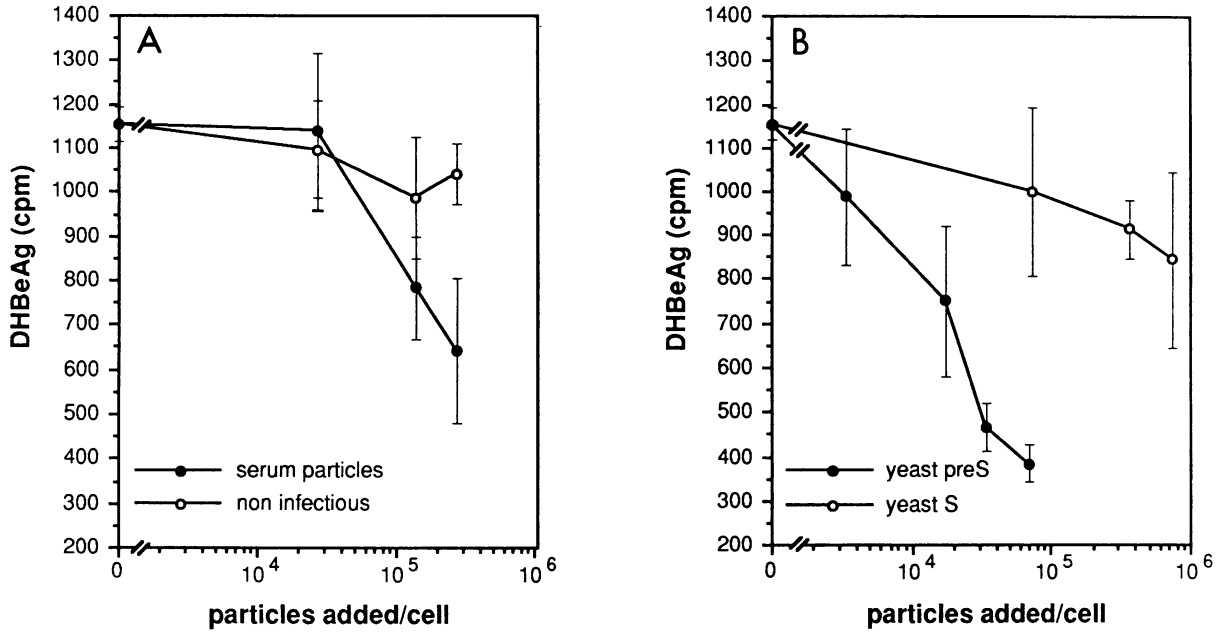

FIG. 4. Competition with DHBV infection of primary duck hepatocytes. Hepatocytes (5×10^5 cells per well) were incubated with 2×10^{10} DHBV particles (1×10^8 DNA-containing particles) in the presence of increasing amounts of competitor: (A) a preparation enriched for natural DHBV SVPs (10^{10}/μl; 7×10^6 DNA-containing particles per μl) or a negative control preparation obtained from noninfectious serum and (B) yeast pre-S particles (1.7×10^9/μl) or yeast S particles (3.5×10^{10}/μl). The relative infectivity was determined in the DHBeAg assay as described in Materials and Methods. Values are not corrected for the background obtained with noninfected cells (200 cpm) (nor, for serum-derived competing particles, for the effects caused by the additional virus contained in this preparation). Values plotted represent the mean ± standard deviation for three independent determinations. The calculated number of particles added to the inoculum is depicted as number of particles added per hepatocyte.

surface protein molecules are contained in these virus-like particles.

Inhibition of infection by virus-like particles from yeast cells. To determine which of the DHBV surface proteins contributes to virus uptake, virus-like particles from yeast cells were analyzed for their capacity to compete with DHBV during infection. Primary duck hepatocytes were inoculated with a DHBV preparation at an MOI of 200, a ratio at which (as shown in Table 1) the accompanying SVPs did not significantly affect viral infectivity (2×10^4 particles per cell). In addition, increasing amounts of the yeast DHBV surface proteins were added to concentrations of 7×10^5 S particles or 7×10^4 pre-S/S particles per cell (Fig. 4A and B, respectively). In parallel positive control experiments, natural SVPs were added to concentrations as high as 4×10^5 particles per cell (Fig. 4). At 7 days postinfection, the cultures were analyzed for ongoing virus replication by the DHBeAg assay.

In these experiments, yeast pre-S/S particles were found to be most effective in interfering with infecting DHBV virions, since they reduced production of secreted DHBeAg even at relatively low concentrations (20 and 40% reduction at 3×10^3 and 1.5×10^4 particles per cell, respectively). Thus, they were significantly more effective than the natural SVPs, for which about 10^5 particles per cell were required to achieve an approximately 50% reduction in DHBV infection. This difference can be explained by the fact that the natural SVP preparation used for competition still contained infectious virions, which also contributed to the viral infectivity determined (6). Furthermore, the yeast particles contain about fourfold more pre-S protein than the natural SVPs, and therefore, a single pre-S/S particle may block more binding sites on the hepatocyte surface than natural particles.

In contrast to the efficient competition by the pre-S/S protein, only a minor reduction in infectivity was observed after addition of the yeast S protein preparation (20% reduction at 10^5 particles per cell; control, Fig. 4B). This effect can probably be regarded as nonspecific, since similar minor reductions were also observed in the negative controls with fractions from sucrose gradients not containing any DHBV proteins. Together, these results indicate that the pre-S polypeptide is the part of the viral envelope that is essential for interacting with the hepatocyte early during infection.

Physical binding of radiolabelled DHBV particles to primary duck hepatocytes. The results from the infectivity studies suggest that DHBV virions and natural SVPs bind comparably well via their pre-S domains to a limited number of cellular receptor sites. Thus, SVPs should provide a readily available substitute for the virus in monitoring virus-hepatocyte attachment. To explore this possibility, serum-derived DHBV SVPs were radiolabelled with ^{125}I-labelled Bolton-Hunter reagent (6). Analysis of the viral proteins by SDS-PAGE (Fig. 5A) revealed as major labelled components a 17-kDa band (the S protein) and a weaker one at 35 kDa (the pre-S/S protein). Several other polypeptides, in particular one migrating at about 10 kDa, remained tightly associated with the DHBV particles during all purification steps after the labelling reaction. Of these, the 10-kDa polypeptide, which was present in various amounts in different preparations, is probably a cleavage product of the DHBV S protein, since it was not immunoprecipitated with anti-pre-S antiserum (Fig. 5A and B) and was also produced from yeast S protein (Fig. 5B). At any rate, these copurifying proteins could be considered integral parts of the SVPs and expected not to obscure the binding studies to follow.

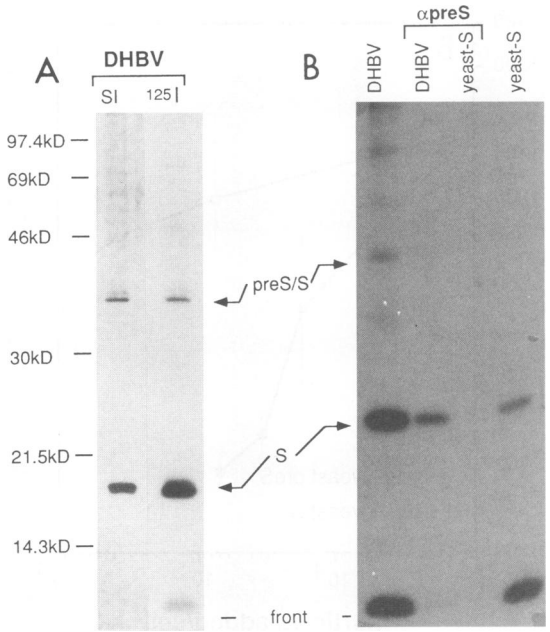

FIG. 5. Analysis of ^{125}I-labelled serum-derived DHBV particles. (A) Proteins were separated by SDS–12.5% PAGE and analyzed by autoradiography (^{125}I) or (B) visualized by silver staining (SI). (B) ^{125}I-labelled DHBV particles and yeast S particles were radiolabelled and analyzed either directly by SDS-PAGE or after immunoprecipitation with an anti-MS2/pre-S serum (αpreS). The DHBV surface proteins pre-S/S and S are marked by arrows, and the front of the gel is indicated. The positions of molecular mass markers are given in kilodaltons.

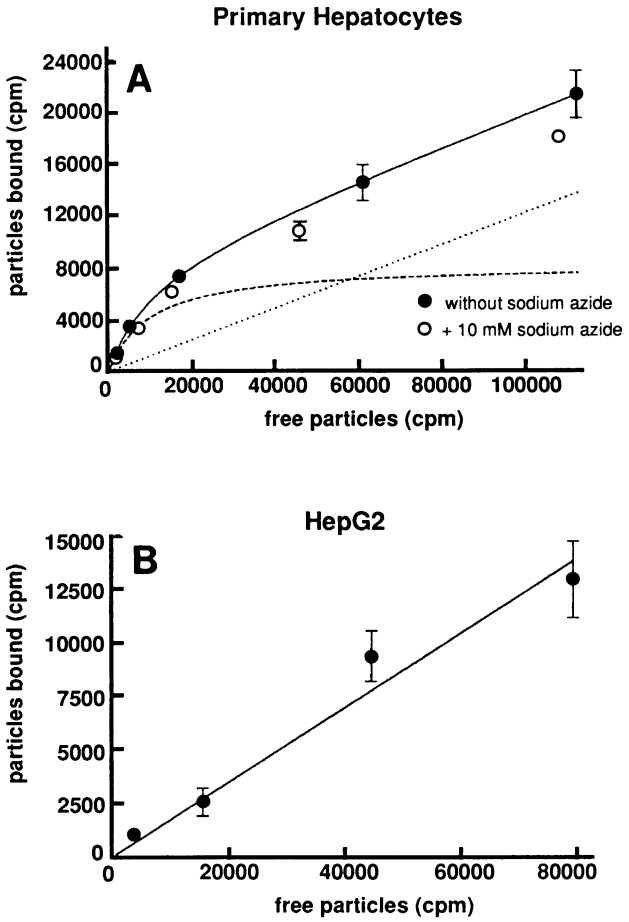

FIG. 6. Saturability of binding of ^{125}I-DHBV particles to primary duck hepatocytes (A) and HepG2 cells (B). Confluent monolayers in 24-well plates (5×10^5 cells per well) were incubated with radiolabelled DHBV particles for 12 h at 20°C. The unbound particles were washed off, and free and cell-associated radioactivity were determined. The specific activity was used to calculate the number of particles contained in each fraction, and the values were normalized to particles per cell by the number of cells per well. Each data point represents the mean ± standard deviation of triplicate values. The data were analyzed by nonlinear regression with the aid of the computer program Graph Pad (9) to yield curves representing saturating (- - -) and nonsaturating (· · ·) components of particles binding (see text).

In direct binding experiments, radiolabelled DHBV particles were incubated with primary duck hepatocyte cultures. To optimize binding, incubation was carried out for 12 h and the temperature was lowered to 20°C to reduce particle uptake. After binding, the radioactivity bound to cells and remaining in the supernatant was determined, and the data were converted into free and bound particles, respectively (for details, see Materials and Methods). When plotted against each other, as shown in Fig. 6, this analysis revealed that the fraction of particles bound increased in a biphasic manner with decreasing particle input, indicating a saturating and a linear component, reflecting specific and nonspecific binding, respectively. Nonspecific binding was relatively low and accounted for less than 13% of total binding. With a nonlinear regression program (7), curves representing these components were calculated, as indicated in Fig. 6A; specific binding was related to 8×10^3 sites per cell and to a K_d of 3.5×10^{-11} M. Qualitatively similar results (6×10^3 binding sites per cell and a K_d of 3.8×10^{-11}) were obtained in binding studies performed in the presence of 10 mM azide (Fig. 6B), indicating that energy-dependent uptake steps play no major role in particle binding under the conditions used.

In a negative control, a quite different profile of DHBV particle binding was observed with HepG2 cells, a human hepatoma cell line that cannot be infected by DHBV, although it supports DHBV replication after transfection with viral genomic DNA (3). In such experiments (Fig. 6B), about 15% of input ^{125}I-DHBV particles attached to the cells over a wide concentration range, indicating exclusively nonsaturable, and therefore nonspecific, binding.

Competition with physical binding of labelled DHBV particles by yeast-derived SVPs. From the infectivity competition studies described above, we inferred that there were on the average about 10^4 binding sites per hepatocyte. To identify these sites also by binding competition studies, primary duck hepatocytes were inoculated with amounts of labelled SVPs well below saturation (calculated as 120 particles per cell) together with increasing amounts of the various unlabelled DHBV-like particles that had already been analyzed before for their potential to act as competitors of DHBV infection (Fig. 4). As expected, with the serum-derived DHBV particles (Fig. 7A), competition became apparent only at concentrations significantly exceeding 10^4 particles per cell and reached 60% inhibition of binding at 9×10^5 particles per cell (Fig. 7A). This agrees quite well with the result from infectivity experiments, as shown in Fig. 4, in that we observed no inhibition at 2×10^4 and about 60% inhibition at 4×10^5 competing particles per cell. Together, these results demonstrate that the

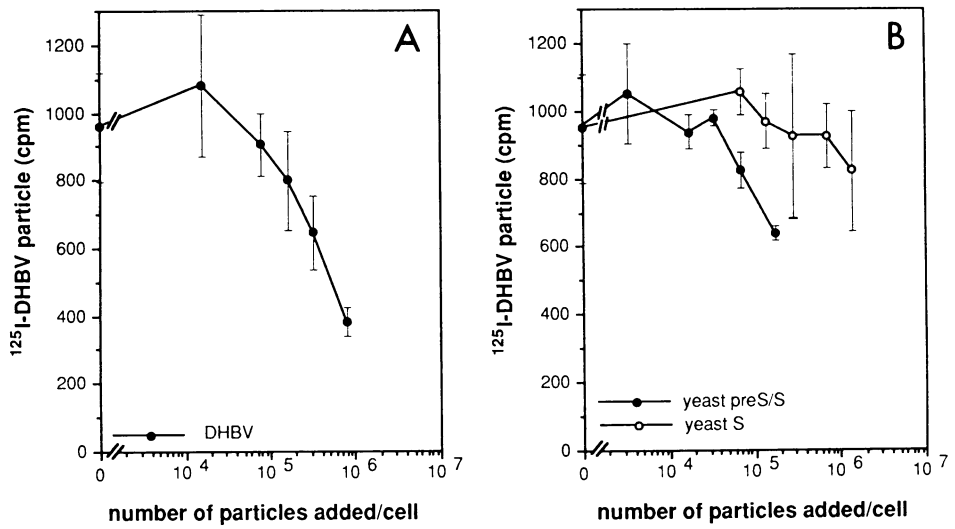

FIG. 7. Physical binding of hepatocytes by SVPs. Samples containing 6×10^7 ^{125}I-DHBV particles (specific radioactivity, 2×10^{-4} cpm per particle) were incubated with 5×10^5 primary duck hepatocytes for 12 h at 20°C in the presence of increasing amounts of unlabelled (A) serum-derived DHBV particles (1×10^{10}/μl) or (B) yeast pre-S particles (1.7×10^9/μl) or yeast S particles (3.5×10^{10}/μl). After washing, the cell-associated radioactivity was measured. The calculated number of unlabeled particles added per cell is indicated. Each point represents the mean ± standard deviation for triplicate determinations.

binding properties of labelled and unlabelled SVPs were comparable and thus not significantly altered by the radiolabelling procedure. Furthermore, they indicate that the physical binding of DHBV particles monitored in our binding assay parallels productive DHBV infection, as determined by DHBeAg secretion.

The latter conclusion was further substantiated by the results from competition experiments with yeast-derived SVPs. As shown in Fig. 7B, yeast pre-S particles inhibited the binding of ^{125}I-DHBV particles with initial dose-response characteristics similar to those of natural SVPs. Inhibition reached about 40% at 1.5×10^5 particles per cell (Fig. 7B); no further increase in competitor could be tested, as the particle concentration in the pre-S preparation was fourfold lower than for the serum-derived particles. In contrast, only an insignificant reduction in binding (less than 20% inhibition at 10^6 particles per cell) was observed after the addition of yeast S particles (Fig. 7B). Finally, addition of equivalent volumes of a yeast-derived mock preparation had no influence on the binding ability of ^{125}I-DHBV particles. These data provide further evidence for the notion that DHBV binding and cellular uptake are correlated and that both require the pre-S domain on the particle surface.

DISCUSSION

One of the essential functions of a viral surface protein is the recognition of specific receptors on the target cell. The specificity of this interaction is a major determinant of the host range, tissue specificity, and pathogenicity of viral infections. Receptor-virus interaction is therefore the subject of intensive research. For HBV, a major human pathogen, these interactions are still only poorly understood because there are no permanent cell lines allowing in vitro infection studies. We have therefore chosen to examine receptor attachment of DHBV, an avian hepadnavirus for which infectable cultured hepatocytes are readily available. Our results indicate that this virus attaches to its cellular receptor through the pre-S domain of the large envelope protein. Furthermore, we have also positively answered the long-standing question of whether the SVPs that accompany hepadnaviruses in vast excess are equivalent to the infectious virion in receptor binding and therefore potentially competitive inhibitors of infection. These observations are in keeping with earlier studies monitoring the physical binding of HBV SVPs to a variety of noninfectable mammalian cell lines. They go beyond, in linking particle binding and viral infection by using infectable target cells, and they also demonstrate for the first time that the number of sites available for productive virus attachment on the hepatocyte is limited.

From both the physical binding and infectivity competition studies, we estimate that there are about 10^4 productive binding sites per hepatocyte. This number appears to be sufficiently high to minimize interference during viral infection in vivo by SVPs, which, although cotransferred in large excess relative to the infecting virions, are expected to be present in amounts well below those required to saturate 10^4 sites per infectable hepatocyte. It is conceivable, however, that the much higher concentrations of SVPs circulating in the infected animal during acute infection (up to 10^{12} particles per ml) may help to reduce abortive infection of nonhepatic tissues with low receptor density. Assuming that there is a similar density of receptors for the HBV pre-S polypeptide on the human hepatocyte, competition with infecting virus by SVPs appears to be even less likely, since the predominant SVP species, the 22-nm S particle, carries only a few pre-S polypeptide chains (5).

In addition to productive virus binding to a relatively low number of cellular attachment sites, we have also observed physical binding of DHBV particles to a second class of sites which are characterized as nonspecific for infection by their large, nonsaturable number ($>10^5$ sites per cell) and also by being present at a similar density on a noninfectable human hepatoma cell line. Reversible binding to these nonspecific, primary sites may nevertheless contribute to the final, essentially irreversible, specific attachment leading to virus entry and productive infection in that it may initially provide a high local virus concentration at the cell surface. In keeping with such a two-step mechanism for virus attachment, DHBV infection in

vitro has been found to require prolonged incubation (~10 h) and high virus concentrations to be maximally productive (6, 17–19). Similar primary virus binding to nonproductive, high-density attachment sites has also recently been observed and characterized in detail in two other viral systems (24, 32). Thus, studies characterizing hepadnavirus receptors by physical binding should also take into consideration virus attachment to such primary binding sites.

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