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Enhancement of Hepatitis B Virus Infection by Noninfectious Subviral Particles

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The biological function of the huge excess of subviral particles over virions in hepatitis B virus infections is unknown. Using the duck hepatitis B virus as a model, we unexpectedly found that subviral particles strongly enhance intracellular viral replication and gene expression. This effect is dependent on the multiplicity of infection, the ratio of virions over subviral particles, and the time point of addition of subviral particles. Most importantly, we show that the pre-S protein of the subviral particles triggers enhancement and requires the presence of the binding regions for putative cell-encoded virus receptor proteins. These data suggest that enhancement is due either to the recently described transactivation function of the pre-S protein or to signaling pathways which become activated upon binding of subviral particles to cellular receptors. The findings are of clinical importance, since they imply that infectivity of sera containing hepadnaviruses depends not only on the amount of infectious virions but also decisively on the number of particles devoid of nucleic acids. A similarly dramatic enhancing effect of noninfectious particles in other virus infections is well conceivable.

Hepadnaviruses cause acute and chronic liver diseases in humans, rodents and birds. Infection always results in the synthesis of at least 1,000-fold more subviral particles (SVPs) than virions. SVPs consist of host cell-derived lipids and viral envelope proteins but, unlike virions, do not contain nucleocapsid protein and viral nucleic acids (13). The viral envelope proteins of hepatitis B viruses, designated small S and large S or pre-S proteins, have a variety of different functions in the viral life cycle. In the early steps of infection, they bind to cellular receptors and thus mediate the intracellular uptake of the virions. In the late stage of infection, they are essential for virion assembly and secretion (4, 5, 34). A block or deficiency in virion assembly and secretion due to inappropriate expression of pre-S proteins can lead to upregulation of the copy number of the intracellular viral DNA template of transcription (36). In addition, pre-S proteins can contribute to viral pathogenesis when expressed in mutated form or when accumulating intracellularly (4, 9, 10, 35, 47, 48). Recently, a transcriptional activating function was reported for the nonmutated full-length pre-S protein of human hepatitis B virus (HBV). This finding may implicate a role for this function in the normal viral life cycle and possibly also in hepatocarcinogenesis (18).

Putative receptor binding sites and neutralizing epitopes are known best for the pre-S envelope protein P36 of duck HBV (DHBV) (7, 20, 31, 32, 37, 49, 55) because primary hepatocytes permissive for infection are readily available from ducks but not from humans (50) and because ducks can be infected experimentally. Infection experiments with DHBV demonstrated that the pre-S proteins contain host range-determining sequences (19). It is not known why HBVs produce a huge excess of viral envelope proteins which end up mainly in the form of SVPs. It has been speculated that the large number of SVPs may act as a decoy for the immune system in vivo to hinder it from eliminating infectious virions. However, since virions and SVPs contain the same envelope proteins, they should bind to the same cellular receptors and thus interfere with efficient infection. Unexpectedly, this seems to be the case only when using a large excess of SVPs, as was examined at a high multiplicity of infection (MOI) (28). Therefore, we have investigated, whether SVPs influence infection differently at low MOI, a condition more closely reflecting natural infection. Surprisingly, we found that SVPs can strongly enhance infection under these experimental conditions and that this effect is mediated by a specific domain of the pre-S protein.

MATERIALS AND METHODS

Cells and virus. Primary hepatocytes from fetal ducks were prepared essentially as described by Köck and Schlicht (30). In brief, fertilized Peking duck eggs purchased from a commercial supplier were brooded in an automatic incubator at 37°C at 50% relative humidity. After incubation of the eggs for 21 days, livers were obtained from sacrificed embryos. The hepatocytes were isolated from the mechanically dissociated livers by digestion with 0.5% collagenase for 1 h at 37°C (Sigma, Deisenhofen, Germany). After the cells were washed twice with Williams' medium E (WME; GIBCO, Eggenstein, Germany), they were resuspended in WME and centrifuged through a cushion of 1% Percoll (Sigma) in the same medium. After 10^6 cells were plated into microplate wells 3.5 cm in diameter (Nunc, Wiesbaden, Germany), they were cultured for 24 h at 37°C and 5% CO_2 in modified WME containing 1.5% dimethyl sulfoxide (Sigma), 10^{-9} M insulin, 2 mM glutamine, 10 μM hydrocortisone, 15 mM HEPES (pH 7.2), and antibiotics (100 IU of penicillin per ml; 100 μg of streptomycin per ml).

For infection, virions and/or SVPs in WME were incubated with the cells for 4 h at room temperature with slight agitation. Nonadsorbed viral particles were removed by washing the cells three times with WME. The cells were then cultured for 7 days in modified WME. The medium was removed for further analysis, and the cells were washed once with phosphate-buffered saline (PBS) (pH 7.5), scraped off, and washed again three times with PBS. The hepatocytes were infected with DHBV present in duck serum at MOIs of 0.01, 0.1, and 1 (for definitions, see below). UV-irradiated viremic sera containing different amounts of noninfectious viral particles (see below) served as a source of SVPs. UV inactivation was achieved by exposure of sera, diluted in 1 ml of WME in a petri dish (diameter, 5 cm), to UV light (Mineralight; Ultraviolet Products, San Gabriel, Calif.) for 30 min at a distance of 10 cm. This procedure has previously been used successfully for inactivation of lymphocytic choriomeningitis virus (LCMV) (46).

Purification of virus and subviral particles. Viral particles were isolated from positive duck sera obtained 2 or 3 weeks after infection with DHBV. First the serum was clarified by low-speed centrifugation in a tabletop centrifuge (5,000 rpm for 20 min). Then the viral particles of the supernatant were centrifuged through a cushion of 20% sucrose in GNTE buffer (pH 7.5), consisting of 0.2 M

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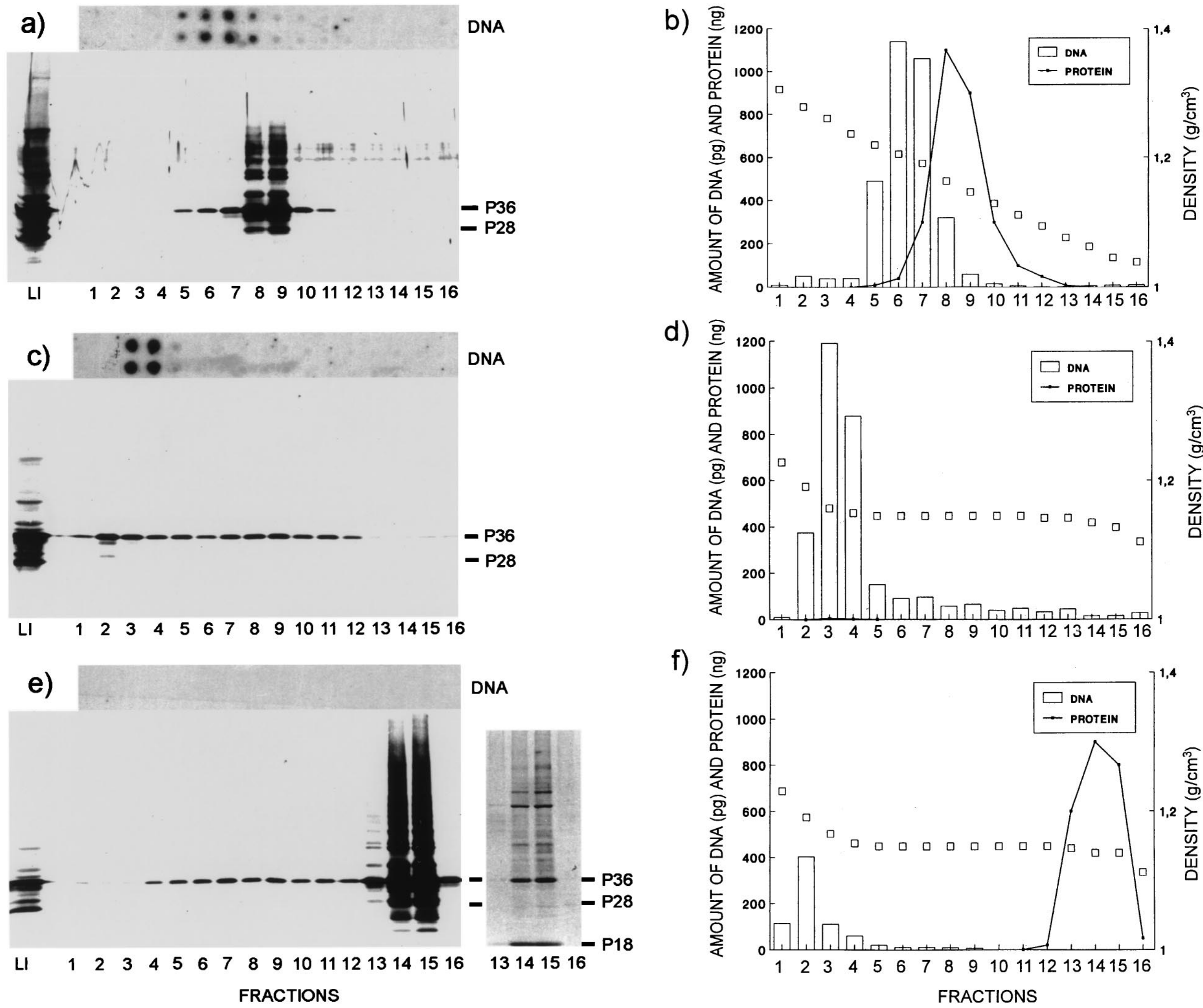


FIG. 1. Separation of DHBV DNA-containing particles and SVPs by two Urografin-gradient ultracentrifugations. (a) DHBV DNA dot blot (top) and pre-S immunoblot (bottom) performed with fractions of DHBV virions and SVPs separated by centrifugation through a Urografin-gradient (0 to 40%) at 38,000 rpm for 18 h. The viral DNA-containing particles are present mainly in fractions 5 to 7, whereas SVPs are concentrated in fractions 8 and 9, as evident from the locations of the viral DNA and pre-S proteins. (c) DHBV DNA dot blot (top) and pre-S immunoblot (bottom) performed with fractions obtained by mixing partially purified virions from fractions 5 to 7 of the first gradient with 26.5% Urografin and ultracentrifugation. Viral DNA-containing particles are present mainly in fractions 3 and 4, as evident from the DNA dot blot. Pre-S protein of particles without or with only very little DNA is present in fractions 1 to 12. The amount of proteins in the fractions of this gradient was too small for visualization by silver staining when 50% of each fraction was analyzed on a SDS-polyacrylamide gel (data not shown). A nonlinear shallow gradient is formed during centrifugation. (e) DHBV DNA dot blot (top) and pre-S immunoblot (bottom) performed with fractions obtained by mixing partially purified SVPs from fractions 8 and 9 of the first gradient with 26.5% Urografin and ultracentrifugation. Silver staining of the proteins separated on an SDS-polyacrylamide gel run in parallel (50% of each fraction was loaded) revealed proteins only in fractions 13 to 16 (small panel). In these fractions, the small envelope and pre-S protein are major bands. Another major band migrating at 70 kDa reacted with antibodies to heat shock protein 70 (HSC70) on an immunoblot (data not shown). Consistent with and extending recently reported data for HBV and DHBV (38), this observation indicates that HSC70 is a major component of DHBV SVPs. (b, d, and f) Quantification of the amount of DHBV DNA and total protein in the fractions of all three gradients. LI, protein extract from the liver of an infected duck used as a positive control in the immunoblot.

glycine, 0.2 M NaCl, 0.02 M Tris, and 0.002 M EDTA, for 3 h at 200,000 $\times$ *g*. The pelleted material was then soaked in GNTE buffer overnight, and viral aggregates were disrupted with an ultrasonic drill applied three times for 10 s each. The suspended particles were then placed on top of a 0 to 40% linear Urografin (Schering AG, Berlin, Germany) gradient and centrifuged to equilibrium at 38,000 rpm for 18 h similar to the method described for LCMV (3). Thus, an incomplete separation of virions from SVPs was achieved as demonstrated by DNA dot blotting and a pre-S immunoblot (Fig. 1a and b). To separate the two types of particles more effectively, fractions containing viral particles with or without detectable viral DNA were collected separately, mixed with 26.5% Urografin, and centrifuged at 38,000 rpm for 18 h. Thus, as reported for the isolation of the infectious LCMV and its interfering particles (46), a much improved separation of viral particles with or without viral DNA was achieved (Fig. 1c to f), as shown by the analysis of the fractions for DHBV DNA by dot blotting and for pre-S proteins by immunoblotting. The purified virions in fractions 3 and 4 of the gradient in Fig. 1c and d and the SVPs in fractions 14 and 15 of the gradient in Fig. 1e and f were used for the infection experiments. To get rid of the Urografin, the fractions were diluted fivefold in GNTE buffer, pelleted by ultracentrifugation at 200,000 $\times$ *g*, and stored at −70°C until use. All ultracentrifugation steps were carried out in an SW50 rotor (Beckman, Munich, Germany) at 4°C.

Measurement of the number of SVPs and complete viral particles. The number of viral genome molecules as determined by dot blot hybridization was considered equivalent to the number of virions since viremic sera seem to contain very few defective genomes (21, 51). The number of SVPs was experimentally determined in a pre-S protein-specific immunodot blot with, for standardization, a defined number of virions purified by Urografin centrifugation as described above. The same signal intensity for virion and SVP preparations in this assay was taken as evidence for identical particle numbers because the two types of viral particles are similar in size and seem to contain a similar ratio of pre-S to S proteins. According to this assay, the viremic serum used for the infection experiments contained approximately 1,000-fold more SVPs than virions, which

is consistent with previously published data for other DHBV-containing sera (21, 39).

Isolation and analysis of DHBV DNA. Replicative intermediates of DHBV were purified from infected cells as described previously (12). In brief, cells were lysed in 50 mM Tris-HCl (pH 8.0)–1 mM EDTA–1% Nonidet P-40, and centrifuged for 1 min at 10,000 × *g* in an Eppendorf centrifuge. The proteins of the supernatants were digested with 500 μg of proteinase K (Sigma) per ml overnight at 37°C, and the viral DNA was extracted. The nucleic acids were separated on 1% agarose gels, transferred to Hybond N (Amersham) membranes, and cross-linked by UV light. For detection of DHBV DNA on Southern and dot blots, a gel-purified full-length *Eco*RI fragment of cloned DHBV-26 DNA was labeled with [α-^{32}P]dCTP (Megaprime; Amersham) and used for hybridization. Defined amounts of DHBV-26-containing plasmid DNA were used for quantitation.

Detection of DHBV pre-S protein. For detection of intracellular pre-S protein, 2.5×10^5 cells were lysed in 20 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (4% SDS, 10% β-mercaptoethanol, 0.05 M Tris-HCl [pH 7.0]), boiled for 3 min, and separated on SDS–5 to 20% polyacrylamide gradient gels (33). For immunoblotting, the proteins were transferred to nitrocellulose filters (Schleicher & Schüll, Darmstadt, Germany) and the filters were incubated first with a rabbit antibody against recombinant pre-S protein (12) and then with a peroxidase-labeled anti-rabbit immunoglobulin G (Medac, Hamburg, Germany). Bound antibodies were visualized by enhanced chemiluminescence (with a kit available from Amersham). The pre-S proteins of purified DHBV and SVPs were analyzed after SDS-PAGE either by staining with silver nitrate (40) or by immunoblotting as described above.

Generation of DHBV pre-S deletion mutants and transfection experiments. The DHBV pre-S deletion mutants were created by oligonucleotide-directed mutagenesis of a DHBV-26 genome (45), cloned as an *Eco*RI fragment in the same orientation into the polylinker of the two phasmid vectors pMa5-8 and pMc5-8, respectively. Deletions were introduced into the DNA of the resulting two phasmids, pMaV26-1 and pMcV26-1, by an oligonucleotide-directed mutagenesis procedure used previously for the construction of other DHBV pre-S mutants (12). Correct introduction of the deletions was ascertained by DNA sequencing of the plasmids. For production of viral particles, 10 μg of the plasmids previously linearized by *Eco*RI digestion were transfected into LMH cells by calcium phosphate precipitation (11, 15). The cell culture media of the transfected LMH cells were harvested 4 days after transfection and used directly for the infection experiments without further purification of the viral particles. The number of viral particles in the culture media was similar for all DHBV pre-S mutant genomes as determined by immunoblotting of pelleted particles with anti-pre-S and by a DHBV DNA determination in a dot blot.

RESULTS

SVPs enhance infection at low MOI. Primary duck hepatocytes were infected with diluted duck serum containing 0.01, 0.1, or 1 virion/cell as defined by determination of DHBV DNA genome equivalents in a dot blot. Dilutions of UV-irradiated viremic serum containing different numbers of noninfectious viral particles served as a source of SVPs, which were coincubated with the dilutions of the infectious serum. As control, dilutions of UV-irradiated DHBV-positive serum with 10^3 and 10^4 SVPs/cell or DHBV-negative duck serum were added. The presence of intracellular pre-S proteins detected by immunoblotting was taken as indirect evidence for productive infection. No pre-S protein was observed in control cells (Fig. 2a, lane 10), whereas increasing levels of the full-length pre-S protein P36 and its degradation product P28 (12) were observed in cells infected with 0.01, 0.1, and 1 virion/cell (lanes 1 to 9). Incubation of cells with a small amount of DHBV (0.01 and 0.1 virion/cell) and simultaneously with 10^3 or 10^4 SVPs/cell resulted in much higher levels of intracellular pre-S proteins (lanes 2 and 3 and lanes 5 and 6) than did incubation without additional SVPs (lanes 1 and 4). In contrast, the pre-S protein levels remained unaltered when a larger amount of DHBV (1 virion/cell) was coincubated with SVPs (lanes 7 to 9). These experiments indicate that SVPs enhance virus infection or intracellular pre-S protein synthesis at low MOI when present in appropriate excess over virions.

Enhancement of infection by SVPs is dose dependent. To investigate whether there is an SVP dose-dependent enhancement of DHBV infection, different amounts of SVPs, concentrated by being pelleted through a 20% sucrose cushion, and serum with DHBV were used for coinfection of hepatocytes.

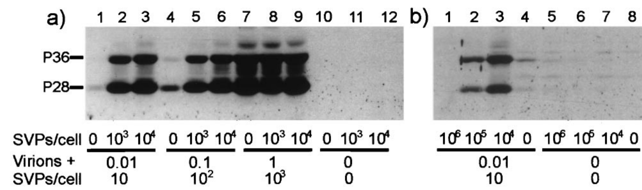


FIG. 2. Dose-dependent enhancement or inhibition of DHBV infection by SVPs. (a) Detection of pre-S proteins (P36 and P28) by immunoblotting with extracts of cells incubated with duck serum containing either no DHBV particles (lane 10), various amounts of live virus including SVPs (lanes 1 to 9), or UV-inactivated virus particles, designated SVPs (lanes 2, 3, 5, 6, 8, and 9). Enhancement of infection by SVPs is seen at low but not high MOI. (b) Pre-S immunoblot with extracts from cells infected with a DHBV-positive or -negative serum (lanes 1 to 4 and 5 to 8, respectively) and various amounts of UV-irradiated, partially purified SVPs, both added simultaneously. Inhibition of DHBV infection by SVPs is observed when 10^6 SVPs/cell are added, whereas gradual enhancement is seen with 10- and 100-fold fewer SVPs. SVPs alone do not lead to detectable levels of intracellular pre-S proteins (lanes 5 to 8).

As expected, no pre-S proteins were observed in any of the extracts from cells incubated with different amounts of SVPs alone (Fig. 2b, lanes 5 to 7). In cells infected with 0.01 DHBV virion plus 10 SVPs per cell alone, only a small amount of pre-S protein was seen (lane 4). Addition of 10^5 SVPs/cell strongly increased pre-S protein levels, and this effect was even more pronounced when 10^4 SVPs/cell were added (lanes 2 and 3, respectively). In contrast, when 10^6 SVPs/cell were added, intracellular pre-S proteins were no longer seen (Fig. 2b, lane 1). These data indicate that enhancement of infection by SVPs at an MOI of 0.01 is seen only when they are present at roughly up to 10^5 SVPs/cell whereas 10-fold-higher levels are strongly inhibitory. The latter is probably due to saturation of all specific and nonspecific virion- and SVP-binding sites on the surface of cells by SVPs.

Enhancement of infection by SVPs is time dependent. Next we asked whether the SVPs exert their effect before, during, or after infection. In addition, we wanted to know whether nonspecific serum proteins play a role in infection enhancement. Therefore, highly purified virus particles (see Materials and Methods) were used. Purified SVPs (10^2/cell) were incubated with the hepatocytes before, simultaneously with, or after infection with purified virions (0.1 virion/cell). The number of intracellular DHBV DNA replicative intermediates as a measure of replication was then analyzed by Southern blotting with total-cell DNA hybridized with a ^{32}P-labeled DHBV DNA probe. The very small amount of cell-associated viral DNA observed when virions alone were added was no longer detected when a 1,000-fold excess of SVPs was added 24 h before infection (Fig. 3, lanes 2 and 4). This demonstrates that SVPs administered long before infection do not enhance virus production. In contrast, increasing concentrations of replicative intermediates were found in cells incubated with the same number of SVPs shortly before (−4 h), simultaneously with (0 h), and after (+4 and +24 h) infection (lanes 6, 9, 11, and 13). The intracellular pre-S protein levels tested in parallel by immunoblotting showed similar kinetics (data not shown). These findings indicate that SVPs can enhance productive DHBV infection very weakly when added shortly before infection, strongly when added at the time of infection, and most strongly when given a short time after virus entry. Addition of SVPs to cells 48 and 72 h after infection had a similar effect to that when they were given 24 h postinfection and simultaneously, respectively, as revealed on the pre-S protein level by immunoblotting (data not shown). The decrease in enhancement after 48 h indicates a time-limited duration of the effect of

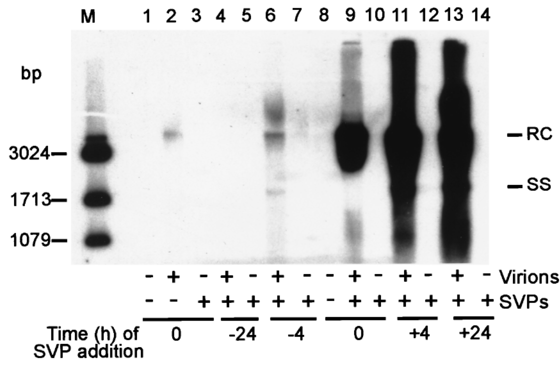


FIG. 3. Time dependence of infection enhancement by SVPs. Southern blot analysis of replicative intermediates reveals strong enhancement of infection by highly purified SVPs only when coincubated with purified virions or when added after infection. Cells were either mock infected (lanes 1 and 8) or infected with 0.1 DHBV virion/cell and either with (lanes +) or without (lanes −) 10^2 SVPs/cell. SVPs were either added before (lanes 4 to 7), simultaneously with (lanes 9 and 10), or after (lanes 11 to 14) infection. The positions of relaxed-circular (RC) and single-stranded (SS) DHBV DNA are indicated.

SVPs after infection. What is more, the lack of a significant effect when cells were preincubated with the SVPs 4 or 24 h before infection strongly suggests that the SVP-enhanced infection is not due to long-term upregulation of cellular virus receptors or of receptor-associated proteins or to long-lived activation of signalling pathways. Moreover, the data imply that enhancement is not mediated by nonspecific serum proteins.

A specific region of the pre-S protein is essential for SVP-mediated infection enhancement. Binding of the SVPs to cellular virus receptors and intracellular uptake of SVPs may be a prerequisite for their enhancing effect on virus production. To test this possibility, we next performed infection experiments to investigate whether putative receptor binding sites on the pre-S proteins which are present on SVPs (20, 31, 32, 37, 49) are essential. For these experiments, we used UV-irradiated virus particles of various DHBV pre-S mutants which were produced by transfection of the chicken hepatoma cell line LMH (23) with *Eco*RI-linearized DHBV DNA genomes containing various deletions within the pre-S region (for details of construction, see Materials and Methods; the location of the deletions is indicated in Fig. 4a). In some of the mutants, part of the putative receptor binding sites and almost all the known neutralizing epitopes are deleted (Fig. 4a). In addition to the DHBV mutants, two nonmutated infectious viral genomes, DHBV-3 and DHBV-1, isolated from a duck (WT3T) (44) and a goose (WT1T) (45), respectively, were tested as controls. For infection, virions (0.01/cell) purified by the procedure described above from a naturally infected duck and 10^2 UV-inactivated viral particles per cell of the various DHBV mutants in WME-diluted LMH cell culture media were used.

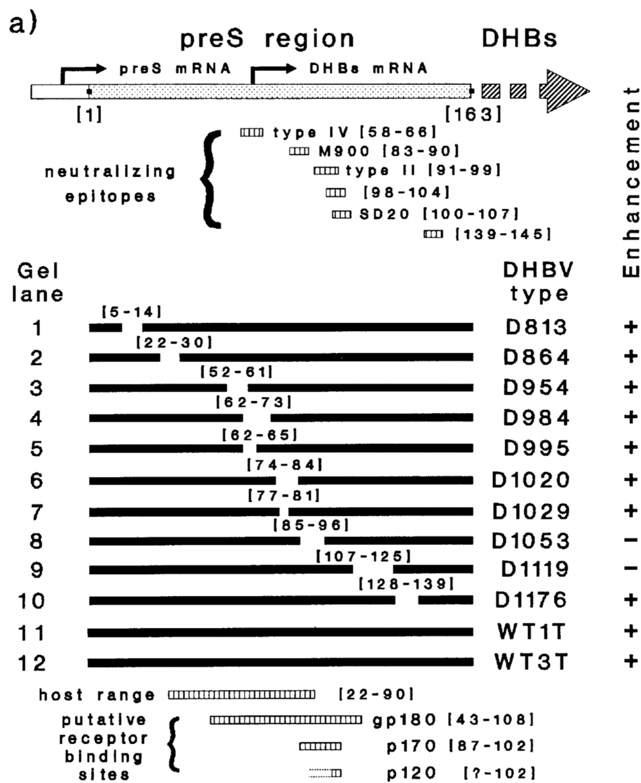
Inactivated viral particles from all except two of the pre-S deletion mutants and from the reference DHBV genomes strongly enhanced DHBV infection, as shown at the pre-S protein expression level (Fig. 4b). This was confirmed for some mutants by analysis of replicative intermediates (Fig. 4c). In mutants D1053 and D1119, which did not enhance infection, pre-S amino acids 85 to 96 and 107 to 125, respectively, are deleted (Fig. 4). Interestingly, the corresponding region represents at least part of the binding domain for two putative cell receptors, the P120 and P170/GP180 proteins (20, 31, 32, 37, 49) (Fig. 4a). In addition, most of the known neutralization sites (7, 37, 55) are deleted in the pre-S proteins of these mutants (Fig. 4a). Taken together, these data indicate that the SVP helper effect is dependent on a specific pre-S protein region, which is believed to bind to cellular virus receptors and which is a target for neutralizing antibodies.

DISCUSSION

In this study, we have investigated whether noninfectious SVPs can affect hepadnavirus infection. It was previously speculated that they may serve as a decoys to adsorb neutralizing antibodies. Moreover, competition of SVPs with infectious virions for viral receptors was previously demonstrated experimentally (28, 40). The data obtained in this study indicate that DHBV SVPs can both stimulate and inhibit DHBV infection in vitro depending on the MOI and the ratio of SVPs to virions. SVP-mediated enhancement of infection was found to require pre-S protein, was abrogated when a specific region of the pre-S protein was deleted, and was observed only when SVPs were added at the beginning of or shortly after infection but not when they were added long before infection. Taken together, these data strongly suggest that SVPs can enhance infection under specific conditions by short-lived, pre-S protein-dependent signalling or transactivation mechanisms which become active either immediately after binding of SVPs to cellular receptors or following SVP uptake.

Consistent with previous reports which demonstrated that both virions and SVPs bind specifically to susceptible hepatocytes and compete for the same hepatocyte receptor molecules (28, 41), we observed inhibition of DHBV infection when we used a great excess of SVPs over virions (10^6 SVPs compared to only 0.01 virion per cell [Fig. 2b]). If the reported rough estimate of the number of viral receptors of 10^4 per hepatocyte is correct (28), efficient inhibition of infection was achieved in our experiment only when the ratios of SVPs to receptor and virions to receptor were 100 and 10^{-6}, respectively (Fig. 2b). Interestingly, when 10 times fewer SVPs were used in the same type of experiment, infection was enhanced (Fig. 2b), although in this case most of the receptors should be blocked by SVPs. This observation could be explained by assuming the presence of approximately 10^6 virus receptor molecules on our hepatocytes. In this case, no competition for viral receptor molecules would take place because SVPs and virions would all find an unblocked receptor. Since we established primary hepatocyte cultures from embryonal livers, whereas the cells in the previous studies were prepared from the livers of young ducklings, the number of viral receptors in our cells may be greater. However, a large number of nonspecific binding sites with affinities for pre-S protein which are similar to that of the receptor(s) and other differences in experimental conditions could also account for the divergent observations.

We have tried to exclude the possibility that serum components are responsible for the SVP-mediated enhancement of infection. A two-step ultracentrifugation procedure, previously used for separation of infectious RNA viruses from interfering particles (3, 46), was applied to separate DHBV virions from SVPs. Thus, a dramatic improvement in the separation of these particles was achieved. The SVPs purified by this method were free of detectable viral DNA (no signals were observed even after a long exposure of the dot blot [data not shown]) and were not infectious, whereas the virion fraction appeared to contain few, if any, SVPs (compare Fig. 1c and d with Fig. 1e and f). The enhancement achieved with these highly purified SVPs and virion preparations strongly argue against the possibility that a serum component is responsible for or contributes to the SVP-mediated enhancement of infection. How-



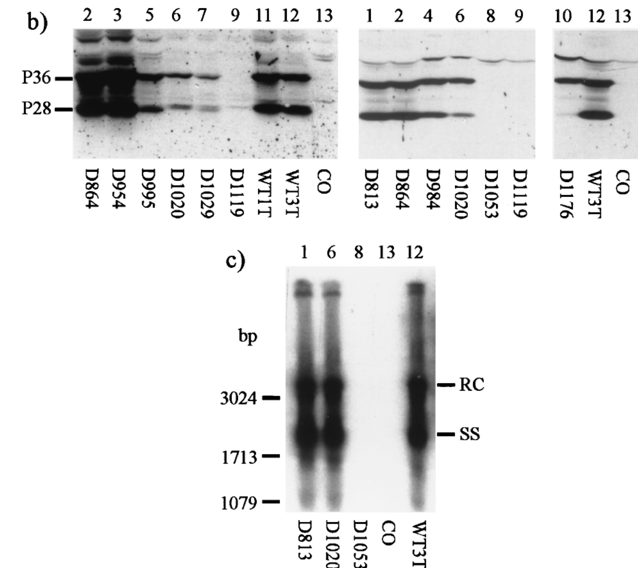


FIG. 4. Effect of UV-irradiated virus particles of natural DHBV isolates and of pre-S mutants on DHBV infection. (a) Virus particles were first produced by transfection of LMH chicken hepatoma cells with DHBV DNA cloned from an infected duck (WT3T) or goose (WT1T) and mutants of DHBV-26 with various deletions (amino acids deleted are in parentheses) introduced into the pre-S region. (b and c) The virus particles from the culture medium, harvested 3 days after transfection, were concentrated by being pelleted through a sucrose cushion and were UV irradiated before use. Similar numbers of viral particles were applied in each experiment (0.1 virion and 100 SVPs/cell), as deduced from the presence of similar amounts of DHBV DNA and pre-S proteins detected by DNA hybridization and immunoblotting, respectively (data not shown). The LMH cell-derived SVPs were mixed with highly purified DHBV virions (0.01 genome equivalent/cell) and used for infection as described in the legend Fig. 2. Intracellular pre-S in the infected hepatocytes was detected by immunoblotting (b), and the amount of replicative intermediates was detected by Southern blotting (c).

ever, a role of serum proteins strongly adhering to the surface of SVPs is not fully excluded by our experiments.

The pre-S protein was identified unequivocally to be the component of the SVPs which is essential for the enhancement effect. Moreover, deletion of specific pre-S sequences (amino acids 85 to 96 and 107 to 125) from viral particles was shown to abrogate the enhancing effect of SVPs. The overlap of these sequences with well-characterized neutralizing epitopes (7, 37, 55), with binding sites of two putative receptor proteins (20, 31, 32, 37, 49), and with part of the host range-determining sequences (19) suggests that SVPs bind to these bona fide viral receptors via these pre-S sequences. However, potential proteolytic cleavage sites (17), a phosphorylation site in the pre-S region (16), and pre-S sequences located further up- and downstream may also play a role in SVP-mediated infection enhancement. Whatever the precise function of the pre-S sequences may be in infection, pre-S protein-mediated binding of SVPs to cellular receptors with or without subsequent SVP uptake is a likely prerequisite for the potential to enhance infection. Consistent with this assumption is the observation that enhancement of infection was strongest when SVPs were added postinfection. Due to the unusual cellular entry mechanism of hepadnavirus particles, which appears to be a pH-independent, ATP-dependent endocytosis process (29), it is not clear in which cellular compartment the pre-S proteins of the SVPs will end up and exert their enhancing effect. In any case, the failure to enhance infection by SVPs when added long before incubation of the cells with an infectious inoculum suggests that the signals transmitted by SVPs are short-lived.

The transmission of signals by binding of viral particles to receptors independent of viral gene expression and replication, as proposed here for DHBV, is known for a number of different viruses, and a few examples are given below. Epstein-Barr virus modulates various isoforms of protein kinase C and tyrosine kinases by binding to its receptor (42) and leads to phosphorylation of an intracellular protein, pp105 (2). The envelope protein of human cytomegalovirus activates tyrosine kinase(s) and protein kinase C, which phosphorylate a 92.5-kDa putative cell membrane protein receptor (24). Binding of the envelope protein gp52 of mouse mammary tumor virus to its receptor is known to stimulate several cellular biological activites, including amino acid accumulation (1). The soluble glycoprotein sgp41 of human immundeficiency virus type 1 was reported to enhance major histocompatibility complex class I and intercellular cell adhesion molecule type 1 expression in some cells (8). Furthermore, binding and aggregation of receptors by replication-defective reovirus is sufficient to transmit the signal for inhibition of cellular DNA synthesis (14). Herpes simplex virus can induce the expression of cellular genes without prior viral protein synthesis (26). Taken together, the transmission of biologically significant signals mediated by interaction of envelope proteins with virus receptors is fairly well established for a number of viruses. Therefore, the assumption that the induction of intracellular signaling events occurs after binding of the pre-S protein of DHBV SVPs to virus receptors is not far-fetched. This could imply that the DHBV receptor has itself signal-transmitting potential or is associated with signal-transmitting components.

We can only speculate on the type of mechanisms causing SVP-mediated enhancement of infection. After cell entry of the SVPs, the complete or truncated fragments of pre-S proteins could be released into the cytoplasm and/or nucleus,

where they might directly or indirectly transactivate viral transcription. The transactivation activity of the intracellularly produced pre-S protein of DHBV (43) and pre-S1 protein of HBV (27), as well as of the truncated HBV pre-S/S protein (6, 25), is consistent with this speculation. The cellular transcription factors most recently reported to be activated by HBV pre-S1 protein-induced stress in the endoplasmic reticulum (52) could also enhance infection. However, other cellular signaling events, which might be induced after the binding of pre-S to cellular receptors, such as activation of proteolytic enzymes, kinases, and phosphatases or changes in the concentration of small molecules, are also conceivable and are consistent with the apparently short-lived nature of the signal induced by SVPs. If this is true, it is possible that very early steps in viral infection such as uncoating of the viral genome, transport into the nucleus, and covalently closed circular DNA (cccDNA) formation are enhanced. In this case, enhanced phosphorylation of core particles could facilitate release of the genome from cores and accelerate nuclear transport. Indirect evidence for the regulation of genome uncoating and nuclear transport by phosphorylation and dephosphorylation (22, 53, 54) makes this an attractive hypothesis. Thus, the copy number of the cccDNA form of the viral DNA, which is of central importance for the efficiency of virus production, would be upregulated. Interestingly, truncated pre-S proteins expressed from some mutant DHBV genomes were shown to increase cccDNA formation because pre-S protein appears to have a matrix protein-like function required for cccDNA copy number control (48). Consistent with our speculation, the same authors speculated in a follow-up study that the DHBV pre-S protein may have an additional activity which can lead to upregulation of the cccDNA copy number (36).

Provided that HBV and DHBV SVPs have a similar effect on infection, our data predict that the levels of SVPs in viremic sera from chronic HBV carriers codetermine the degree of infectivity. Moreover, enhancement of infection could be even more important for successful HBV transmission because many sera of chronic carriers contain a billion-fold excess of SVPs and very small amounts of viral genomes which often includes a large variety of defective genomes. In contrast, most DHBV sera are of very high titer with, usually, only a 1,000-fold excess of SVPs and contain very few defective genomes (21). Infection experiments with mammalian hepadnaviruses including HBV, as well as with viral particles from other viruses, are required to elucidate the extent to which the enhancement of infection by DHBV SVPs can be extrapolated to other viral systems.

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