JOURNAL OF VIROLOGY, May 1986, p. 522–525
0022-538X/86/050522-04$02.00/0

Vol. 58, No. 2

Pre-s_1 Antigens and Antibodies Early in the Course of Acute Hepatitis B Virus Infection

MO-QUEN KLINKERT,[1]* L. THEILMANN,[2] E. PFAFF,[1] AND H. SCHALLER[1]

Mikrobiologie and Zentrum für Molekulare Biologie,[1] and Medizinische Universitätsklinik,[2] University of Heidelberg, 69 Heidelberg, Federal Republic of Germany

Received 22 November 1985/Accepted 6 February 1986

The presence of the two "large" surface proteins of hepatitis B virus (HBV), P39 and GP42 of pre-s_1–hepatitis B surface antigen, was assayed in the serum of an experimentally infected chimpanzee by using antibodies to a pre-s_1-specific fusion protein synthesized in *Escherichia coli*. The immune response to pre-s_1–hepatitis B surface antigen was monitored by using the pre-s_1 fusion protein as an antigen. pre-s_1 proteins were detected in the serum early in the course of infection and prevailed as long as hepatitis B surface antigen did, together with hepatitis B e antigen and viral DNA. Thus, the pre-s_1 antigen can be considered a novel diagnostic marker for acute HBV infection. Antibodies to pre-s_1, both immunoglobulin M and G classes, were also detected early in infection, shortly after the appearance of the pre-s_1 antigen, suggesting its strong immunogenicity in vivo. The anti-pre-s_1 antibodies therefore also represent an early serological marker for acute HBV infection and, owing to their early appearance and persistence, may play a role in the neutralization of the virus.

The major surface antigen (hepatitis B surface antigen [HBsAg]) of hepatitis B virus (HBV) is composed of a polypeptide of 226 amino acids (P24) and its glycosylated form, GP27 (9). In addition, two other pairs of proteins have recently been detected in minor amounts and have been shown to consist of HBsAg with amino-terminal extensions of 55 amino acids in the pre-s_2 proteins (GP33 and GP36 [12]) (7) and a further 108 amino acids in the pre-s_1 proteins (P39 and GP42 [2]) (10, 17). In the HBV genome, these additional sequences are encoded in pre-s_1 and pre-s_2 regions, both preceding the *s* gene in the same reading frame (for a review, see reference 13).

HBsAg appears in the serum of HBV-infected individuals very early in the course of infection, acting as the first serological marker for the disease, and prevails in the serum of chronic carriers, often over a long period, in the absence of infectious viral particles (11). In contrast, pre-s_2 proteins are produced exclusively in the acute phase of HBV infection (3). A receptor of polymerized human serum albumin is believed to be located on the 55-amino-acid residues of pre-s_2, and its role may be to mediate virus uptake by the cell (5). The presence of the pre-s_1 proteins P39 and GP42 is closely correlated with hepatitis B e antigen (HBeAg) and HBV DNA, two serological markers for acute HBV infection [10; L. Theilmann, M.-Q. Klinkert, K. Gmelin, J. Salfeld, H. Schaller, and E. Pfaff, Hepatology (Baltimore), in press]. However, the time course of pre-s_1 expression after HBV infection has not been determined.

In an attempt to define the importance of pre-s_1–HBsAg and the corresponding antibodies in the diagnosis or prognosis of HBV or both, we investigated the appearance of both of these markers in the serum of a chimpanzee that had been experimentally infected with "clone-purified" HBV and had developed typical self-limited hepatitis (16). The acute phase of the disease (in chimpanzee 7 in reference 15) was observed at 5 weeks after infection, and all known serological markers were analyzed at weekly intervals for over 40 weeks (15). In this study, we used antibodies directed against pre-s_1-specific amino acid sequences synthesized in *Escherichia coli* to determine the appearance of the pre-s_1 proteins after infection and to correlate it with that of the other known HBV markers. In addition, we used the pre-s_1 fusion protein to detect the appearance of anti-pre-s_1 antibodies in the above-mentioned test animal. Our findings were confirmed with serum samples from another HBV-infected chimpanzee and from HBV-infected patients.

MATERIALS AND METHODS

Serum samples. Sera used for the characterization of pre-s_1 proteins and for the detection of anti-pre-s_1 antibodies were obtained from patients or from chimpanzees with acute hepatitis B. A chimpanzee from a previous study (chimpanzee 7) was inoculated intravenously with serum containing HBV produced in another chimpanzee (chimpanzee 1) which had been inoculated by intrahepatic injection of cloned HBV DNA and of liver cells transfected with cloned HBV DNA (15, 16).

Purification of the pre-s_1 fusion protein and preparation of the anti-pre-s_1 antiserum. Part of the pre-*s* region spanning amino acids 20 to 120 was cloned in an *E. coli* expression plasmid, pPlc24, as previously described (10). Cells were grown overnight at 28°C; the expression of the MS2 polymerase–pre-s_1 fusion protein was induced by incubating the overnight culture (diluted 1:5 in fresh medium) for 2 h at 42°C. The cells were lysed with lysozyme and by sonication, and the fusion protein was extracted with 5% Triton X-100, 1 M urea, and 7 M urea. After purification by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the 7 M urea fraction was used to immunize rabbits, and the antiserum obtained was designated antiserum 80.

Detection of pre-s_1 proteins in serum. pre-s_1 proteins were detected by Western blotting as described previously (10). Proteins from 10 μl of serum were separated by SDS-PAGE (4) and transferred to nitrocellulose filters (14). After each filter was blocked with 30% fetal calf serum for 1 h, it was

* Corresponding author.

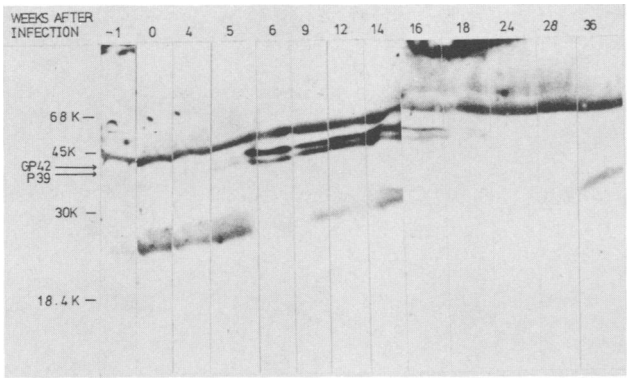

FIG. 1. Western blot analysis showing the reactivity of anti-fusion protein antiserum 80 with serum proteins from a chimpanzee experimentally infected with cloned HBV DNA. Serum samples taken every week for over 40 weeks after infection were analyzed up to week 36 in a Western blot, but only 13 samples are shown; therefore, lanes −1 to 14 and lanes 16 to 36 are from separate gels and do not exactly align. The numbers on top refer to the weeks after infection, −1 being the week prior to infection. The positions of the two large pre-s_1 proteins are marked P39 and GP42. A band around 45K reacting nonspecifically with the antiserum is present throughout the blot. ^{14}C-labeled molecular weight standards (Amersham Corp.) are designated on the left.

probed overnight with antiserum 80, which contained antibodies against the MS2 polymerase–pre-s_1 fusion protein and was diluted 1:5,000 in 30% fetal calf serum–0.05% Nonidet P-40–0.1% sodium azide in phosphate-buffered saline; the filter was then incubated with iodinated anti-rabbit immunoglobulin for 3 h. After being washed thoroughly, the filter was subjected to autoradiography.

Detection of pre-s_1 proteins in the liver. A surgical biopsy specimen was taken from the chimpanzee at the peak of infection. A 1-g liver sample was homogenized mechanically in 10 ml of phosphate-buffered saline containing 1% SDS as described previously (Theilmann et al., in press). The homogenate (20 μl) was processed for gel electrophoresis and Western blotting as described above.

Detection of anti-pre-s_1 antibodies. A crude extract from *E. coli* cells (2×10^8 cells per slot) induced to express the 26K MS2 polymerase–pre-s_1 fusion protein as described above was subjected to SDS-PAGE and analyzed by Western blotting. The filter was incubated for 1 h in 30% fetal calf serum and then probed overnight with serial serum samples from the chimpanzee or from HBV-infected individuals (diluted 1:1,000 in 30% fetal calf serum–0.05% Nonidet P-40–0.1% sodium azide in phosphate-buffered saline) for the presence of antibodies to the fusion protein. After excess antibodies were washed off, the filter was incubated with a second antibody, either iodinated anti-human immunoglobulin G (IgG) or anti-human IgM coupled to peroxidase. The antiserum against the 26K fusion protein raised in a rabbit showing an immune reaction with the fusion protein band acted as a control for the presence of antibodies specifically recognizing pre-s_1-encoded amino acids.

RESULTS

Presence of pre-s_1 proteins in chimpanzee serum and liver. Chimpanzee serum proteins were separated by SDS-PAGE, transferred to nitrocellulose paper, and probed with antiserum 80, an anti-pre-s_1 fusion protein antiserum (see Materials and Methods). Two bands corresponding to the pre-s_1 proteins P39 and GP42 were detected at week 5 after infection and remained in the sera until week 18 (Fig. 1, lanes 5 through 18). The pre-s_1 proteins were not detected in serum samples taken early, i.e., in the first 4 weeks, after infection (Fig. 1, lanes −1, 0, and 4) or after week 18 (Fig. 1, lanes 24, 28, and 36). The two bands reacted specifically with antiserum 80 (Fig. 1) but not with the corresponding preimmune serum (data not shown).

The time of appearance of the pre-s_1 proteins in the chimpanzee during the HBV infection coincided with the production of HBsAg, HBeAg, and HBV DNA, markers characteristic of an acute hepatitis infection and ongoing HBV replication (see Discussion). A similar correlation of the pre-s_1 proteins with the above-mentioned markers was also observed in serum samples selected from a second chimpanzee (chimpanzee 1 from a previous study [15]) at various times during the infection. The peak of infection extended over a period from weeks 10 to 24, during which time this chimpanzee was found to be HBsAg positive. Furthermore, the sera were HBeAg positive from weeks 11 to 18 and positive for HBV DNA from weeks 12 to 17. A serum sample taken at week 13 was found to contain pre-s_1, as expected. In two more samples taken at week 22 (at which time the chimpanzee was still HBsAg positive but was HBV DNA negative) and week 36 (9 months postinfection, only anti-HBs positive), pre-s_1 proteins were no longer detected.

A liver tissue specimen taken from chimpanzee 7 at the peak of infection and analyzed by Western blotting with antiserum 80 was also found to contain two bands corresponding to the pre-s_1 proteins P39 and GP42 (Fig. 2, lane 1). Previous incubation of the antiserum with an excess of homologous pre-s_1 fusion protein decreased the signal (Fig. 2, lane 2), whereas a heterologous fusion protein had no effect (Fig. 2, lane 3), thus verifying the specificity of the reaction.

Anti-pre-s_1 antibody response in HBV-infected subjects. To reveal the presence of antibodies to the pre-s_1 proteins, we

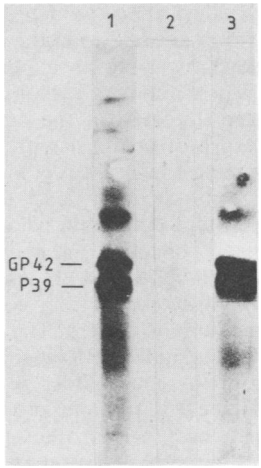

FIG. 2. Specificity of anti-fusion protein antiserum 80 for a liver tissue sample from chimpanzee 7. Liver proteins were separated by SDS-PAGE and transferred to a nitrocellulose filter for Western blot analysis. The nitrocellulose strips were incubated with antiserum against the pre-s_1 fusion protein (antiserum 80) (lane 1), antiserum 80 blocked with the pre-s_1 fusion protein (lane 2), and antiserum 80 preincubated with a nonrelated MS2 polymerase fusion protein (lane 3). The positions of the two large pre-s_1 proteins P39 and GP42 are indicated on the left.

subjected an *E. coli* extract containing the fusion protein (see Materials and Methods) to Western blotting (Fig. 3, WB). The nitrocellulose paper was blotted with the different chimpanzee sera taken throughout the course of infection. Antibodies recognizing the fusion protein carrying pre-s_1-specific amino acids were detected with iodinated anti-human IgG. Such antibodies appeared at week 8, as indicated by the vertical arrow in Fig. 3, and persisted throughout the study (Fig. 3, lanes 8 through 36). For the detection of antibodies of the IgM class, the immune reaction was carried out as described above but with anti-human IgM coupled to peroxidase as the second antibody. The specific color staining of the 26K fusion protein was observed with sera starting from week 8 after the start of infection (data not shown). Both immune reactions with the 26K fusion protein could be blocked by previous incubation of the chimpanzee sera with the homologous pre-s_1 fusion protein but not with the heterologous MS2 polymerase fusion protein (10), thus providing evidence for its specificity.

These results indicate the presence of IgM and IgG antibodies reacting specifically with the pre-*s* sequence. Antibodies to pre-s_1 were detected 2 weeks after the appearance of the pre-s_1 proteins in the samples. Furthermore, anti-pre-s_1 antibodies prevailed in the serum long after recovery, like the other antibody markers, antibody to hepatitis B core antigen (anti-HBc), antibody to hepatitis B e antigen (anti-HBe), and antibody to hepatitis B surface antigen (anti-HBs) (Fig. 4).

As confirmation of the above results, serum samples taken from a second chimpanzee (chimpanzee 1) at weeks 13, 22, and 36 were all positive for anti-pre-s_1 antibodies. In addition, the sera of four patients with acute hepatitis B assayed for the presence of anti-pre-s_1 antibodies, both IgM and IgG, were also found to be specifically reactive with the 26K fusion protein (data not shown). This finding strongly suggests that the anti-pre-s_1 antibody response is related to the recovery from an HBV infection, the possible role of the antibody being in the neutralization of the virus.

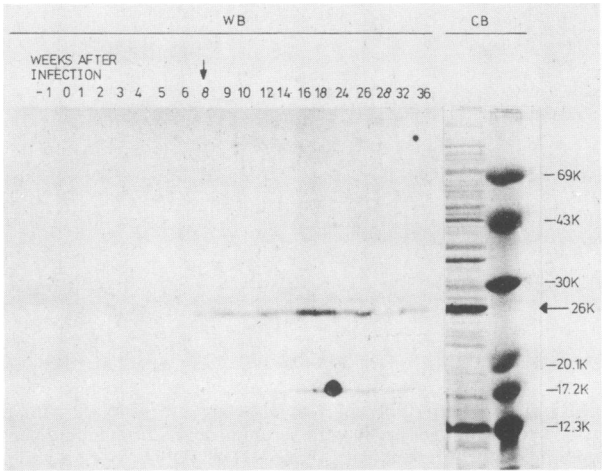

FIG. 3. Development of antibodies to the pres_1 region during acute HBV infection in a chimpanzee. Total *E. coli* cell extracts were separated by SDS-PAGE and either stained with Coomassie blue (CB) or transferred to nitrocellulose filters for Western blot analysis (WB) with serial serum samples from chimpanzee 7. The numbers on top refer to the weeks in the course of the disease. The vertical arrow (at week 8) indicates the time when antibodies which reacted with the pre-s_1 fusion protein (26K) were first detected. Molecular weight standards (Sigma Chemical Co.) are designated on the right.

DISCUSSION

In this report, we described the detection of both pre-s_1 proteins and anti-pre-s_1 antibodies in the context of all other known HBV markers by using an experimentally infected chimpanzee from a previous study as a model system. The pre-s_1 proteins were detected in the serum as circulating antigens by Western blot analysis with an antiserum to a fusion protein covering the pre-*s* region. The reliability of pre-s_1 as a serum marker may lead to the use of this anti-pre-s_1 fusion protein antiserum as a specific probe for the detection of pre-s_1-encoded antigens in HBV-infected persons. Anti-pre-s_1 antibodies were assayed by Western blotting by probing an *E. coli* extract containing the pre-s_1 fusion protein with chimpanzee sera. The complete course of infection, including the new HBV-specific markers pre-s_1 antigens and anti-pre-s_1 antibodies, in the serial serum samples of chimpanzee 7 is summarized in Fig. 4.

We observed that the pre-s_1 proteins in the chimpanzee sera appeared concomitantly with other early HBV markers, such as HBsAg and HBeAg (as determined by a radioimmunoassay) and the virus (measured as viral DNA by dot blot hybridization with nick-translated cloned HBV DNA as a probe) (Fig. 4). pre-s_1–HBsAg was detected early in infection (week 5), 2 weeks after HBsAg was detected. This apparent 2-week delay in the appearance of pre-s_1 proteins may only be a question of sensitivity, with the radioimmunoassay method of detection of HBsAg being far more sensitive than Western blotting. Pre-s_1 proteins persisted to week 18 of infection, parallel to HBsAg.

In addition, anti-pre-s_1 IgM and IgG antibodies were detected early, when HBsAg production was reaching its peak, in contrast to the late appearance of anti-HBs directed against the major P24-GP27 HBsAg component of the viral envelope (Fig. 4). Like anti-HBs, both anti-pre-s_1 IgM and IgG prevailed in the serum long after recovery. This finding is consistent with the analysis of sera of patients with acute resolving hepatitis B, in whom anti-pre-s_1 antibodies have also been detected (data not shown).

Until now, the role of the pre-s_1 proteins in HBV-related disease has not been defined. The participation of the pre-s_1 region in the binding to hepatocytes via polyalbumin to mediate entry of the virus into the cell has only been speculation, as has its involvement in any other step in virus-host interactions. An indication for a possible role of the pre-s_1 proteins came from a recent investigation of serum samples from HBV-infected patients which suggested that the presence of pre-s_1 proteins correlates with active HBV replication (10). This notion is confirmed by the results from the present study with chimpanzees; the detection of pre-s_1 in a liver tissue specimen taken from a chimpanzee at the peak of infection is further evidence for the expression of pre-s_1 proteins during viral replication. Thus, the expression of pre-s_1 early in infection could indicate the acute stage of an HBV infection. The presence or absence of pre-s_1 has not yet been correlated with HBV replication during chronic HBV infection, and the possibility of its usefulness as a serum marker for the discrimination of the different types of carriers remains open.

So far, an anti-pre-s_1 response has not been detected in HBV-infected individuals, and no information is available on the time of appearance of anti-pre-s_1 antibodies during the course of the disease. Alberti and co-workers previously reported on antibodies, distinct from anti-HBs, which were reactive with Dane particles and suggested their similarity to

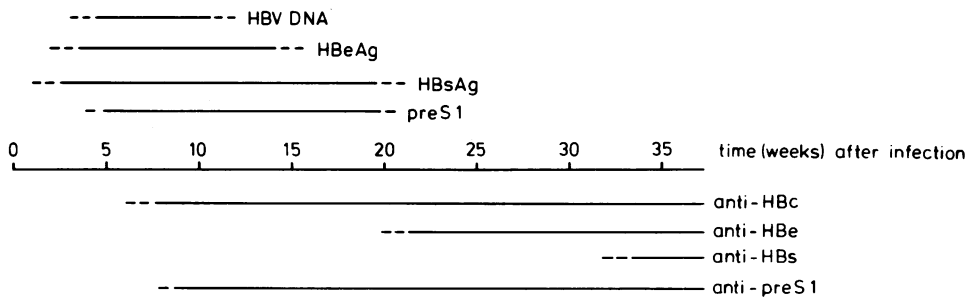

FIG. 4. Time course of HBV markers in the sera of a chimpanzee with an experimentally induced HBV infection. The times during which the pre-s_1 proteins and the anti-pre-s_1 antibodies are present are indicated by the horizontal lines, along with the other known serological markers, as determined in a previous study (15).

anti-pHSA receptor antibodies (1) such as pre-s_2-specific antibodies (6). However, in view of their early appearance, the possibility of the antibodies being indistinguishable from anti-pre-s_1 antibodies cannot be ruled out.

The finding that pre-s_1 antigen and the corresponding antibody are concomitantly present in the serum for several weeks may be explained by the existence of an immune complex between pre-s_1 antigen and antibody. While anti-pre-s_1 antibody is in excess in the immune complex in relation to pre-s_1 antigen, the detection of the latter in a Western blot may be facilitated by its release from the complex under denaturing conditions. HBsAg complexed with anti-HBs has also been reported (8). Moreover, Dane particles and antibodies reacting with Dane particles have been found to coexist over a period of time in patients with acute resolving hepatitis B (1).

Further studies will have to be conducted to determine the importance of these antibodies in the neutralization of HBV and to determine whether their appearance in the early stages of infection could have diagnostic and prognostic implications, as suggested for the pre-s_1 proteins. Finally, it will be of particular interest to determine whether the absence of anti-pre-s_1 antibodies can act as a prognostic marker in predicting the development of a chronic course of infection.

ACKNOWLEDGMENTS

We thank H. Will for discussion and critical reading of the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (Forschergruppe Genexpression).

LITERATURE CITED

1. **Alberti, A., P. Pontisso, E. Schiavon, and G. Realdi.** 1984. An antibody which precipitates Dane particles in acute hepatitis type B: relation to receptor sites which bind polymerized human serum albumin on virus particles. Hepatology (Baltimore) **4**:220–226.
2. **Heermann, K. H., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-*s* sequence. J. Virol. **52**:396–402.
3. **Imai, M., Y. Yanase, T. Nojiri, Y. Miyakawa, and M. Mayumi.** 1979. A receptor for polymerized human and chimpanzee albumins on hepatitis B virus particles co-occurring with HBeAg. Gastroenterology **76**:242–247.
4. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
5. **Machida, A., S. Kishimoto, H. Ohnuma, H. Miyamoto, K. Baba, K. Oda, T. Nakamura, Y. Miyakawa, and M. Mayumi.** 1983. A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology **85**:268–274.
6. **Milich, D. R., G. B. Thornton, A. R. Neurath, S. B. Kent, M. L. Michel, P. Tiollais, and F. W. Chisari.** 1985. Enhanced immunogenicity of the preS region of hepatitis B surface antigen. Science **228**:1195–1199.
7. **Neurath, A. R., S. B. H. Kent, N. Strick, P. Taylor, and C. E. Stevens.** 1985. Hepatitis B virus contains preS gene-encoded domains. Nature (London) **315**:154–156.
8. **Palla, M., R. Rizzi, M. Toti, P. Almi, M. Rizetto, F. Bonino, and R. Purcell.** 1983. Complexes of hepatitis B surface antigen and immunoglobulin M in the sera of patients with hepatitis B virus infection. Infect. Immun. **41**:950–958.
9. **Peterson, D. L.** 1981. Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. J. Biol. Chem. **256**:6975–6983.
10. **Pfaff, E., M.-Q. Klinkert, L. Theilmann, and H. Schaller.** 1986. Characterization of large surface proteins of hepatitis B virus by antibodies to preS-S encoded amino acids. Virology **148**:15–22.
11. **Robinson, W. S.** 1983. Hepatitis B virus, p. 57–116. *In* F. Deinhardt and J. Deinhardt (ed.), Viral hepatitis: laboratory and clinical science. Marcel Dekker, Inc., New York.
12. **Stibbe, W., and W. H. Gerlich.** 1982. Variable protein composition of hepatitis B surface antigen from different donors. Virology **123**:436–442.
13. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.
14. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
15. **Will, H., R. Cattaneo, G. Darai, F. Deinhardt, H. Schellekens, and H. Schaller.** 1985. Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. Proc. Natl. Acad. Sci. USA **82**:891–895.
16. **Will, H., R. Cattaneo, H.-G. Koch, G. Darai, H. Schaller, H. Schellekens, P. M. C. A. van Eerd, and F. Deinhardt.** 1982. Cloned HBV DNA causes hepatitis in chimpanzees. Nature (London) **299**:740–742.
17. **Wong, D. T., N. Nath, and J. J. Sninsky.** 1985. Identification of hepatitis B virus polypeptides encoded by the entire pre-*s* open reading frame. J. Virol. **55**:223–231.