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Hepatitis B Virus Large Surface Protein Is Not Secreted but Is Immunogenic when Selectively Expressed by Recombinant Vaccinia Virus

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The envelope region of the hepatitis B virus (HBV) genome contains an open reading frame that begins upstream of the major surface protein gene. The two minor proteins that are initiated within this pre-s segment are immunogenic and may be involved in virus attachment to hepatocytes. We have constructed a recombinant vaccinia virus that contains the predicted coding segment for the large surface protein (LS) under control of a vaccinia virus promoter. Cells infected with the recombinant virus synthesized HBV polypeptides of 39 and 42 kilodaltons, corresponding to the unglycosylated and glycosylated forms of LS, respectively. The presence of pre-s epitopes in the 39- and 42-kilodalton polypeptides was demonstrated by binding of antibody prepared against a synthetic peptide. Synthesis of the 42-kilodalton species was specifically inhibited by tunicamycin, suggesting that it is N-glycosylated. Despite apparent glycosylation, LS was not secreted into the medium of infected cells. Nevertheless, rabbits vaccinated with the purified recombinant virus made antibodies that recognized s and pre-s epitopes. Antibody to the NH_2 terminus of LS appeared before or simultaneously with antibody that bound to the major surface protein. The additional immunogenicity provided by expression of LS may be advantageous for the development of an HBV vaccine.

Approximately 200 million people are chronically infected with hepatitis B virus (HBV), and numerous deaths result from fulminant hepatitis, cirrhosis, and primary hepatocellular carcinoma (1, 4). The 42-nm blood-borne infectious virus particle is composed of a nucleocapsid and a lipoprotein envelope. A 22-nm empty envelope particle is also found in the blood during acute infections as well as in chronic carrier states. These small noninfectious particles have been purified and used as a subunit vaccine (23). Despite the safety and efficacy of the blood-derived product, the limited quantities available and the high cost associated with purification and testing have so far prevented mass vaccination in areas where HBV is highly endemic. For these reasons, alternative methods of producing HBV surface antigens (HBsAg) are actively being sought.

The major envelope protein of HBV is 226 amino acids long. There are also two minor envelope proteins of 281 and (depending on the HBV serotype) 389 to 400 amino acids that have the same carboxyl terminus as the major polypeptide (3, 7, 22). We will refer to the major surface protein as S and the middle and large minor ones as MS and LS, respectively. S exists in both glycosylated ($gp27^s$) and unglycosylated ($p24^s$) forms. There are two glycosylated forms of MS ($gp33^s$ and $gp36^s$), and one glycosylated ($gp42^s$) and one nonglycosylated ($p39^s$) form of LS. Disulfide-bonded dimers of S form the structural unit of the envelope, whereas MS and LS may be involved in virus attachment to hepatocytes (7, 8, 15). Since the additional epitopes present in the NH_2-terminal regions of MS and LS are immunogenic, their inclusion in synthetic or recombinant vaccines has been suggested (12).

The region of the HBV genome that encodes surface proteins is divided into three continuous coding regions, pre-s1, pre-s2, and s, which form a long open reading frame (reviewed in reference 24). LS is encoded by all three segments, MS by pre-s2 plus s, and S by s alone. A 2.1-kilobase (kb) transcript with a 5′ end that maps about 20 bases upstream of the pre-s2 segment was isolated from the liver of HBV-infected chimpanzees (2). Similar transcripts were also found in transformed cell lines (2, 6, 11, 18, 21). Both MS and S could form from the 2.1-kb mRNA by translation initiation at the first and second AUG codons, respectively (17). Slightly smaller RNA transcripts that might express S exclusively have also been described (6, 21). A large RNA that has a 5′ end upstream of pre-s1 and which spans the entire s region was detected in transformed monkey cells (6) and in cell-free transcription systems (19).

Synthesis of S has been obtained in eucaryotic cells by recombinant DNA methods (reviewed in reference 24). In some cases (17, 18, 21) both MS and S were made, but in no case has LS been demonstrated. The object of the present study was to express LS by using a virus vector. Vaccinia virus has been used to express surface antigens from a variety of different viruses, including HBV (14, 16, 20). Previously, we constructed a recombinant vaccinia virus with the HBV S gene approximately 150 base pairs (bp) downstream of vaccinia virus transcriptional regulatory sequences (20). Both glycosylated and nonglycosylated forms of S were synthesized, and 22-nm HBsAg particles were secreted. In a similar fashion, we have now inserted the entire pre-s1–pre-s2–s region into the vaccinia virus genome and have obtained expression of LS. Although the predicted glycosylated and nonglycosylated forms were made, the LS remained intracellular. This may be an intrinsic property of LS which accounts for its presence in very low amounts in or absence from the 22-nm empty envelope particles of chronic HBV carriers (3). Secretion is evidently not required for immunogenicity, however, since rabbits that were inoculated with purified recombinant vaccinia virus made antibodies to epitopes located within the pre-s2 and s regions.

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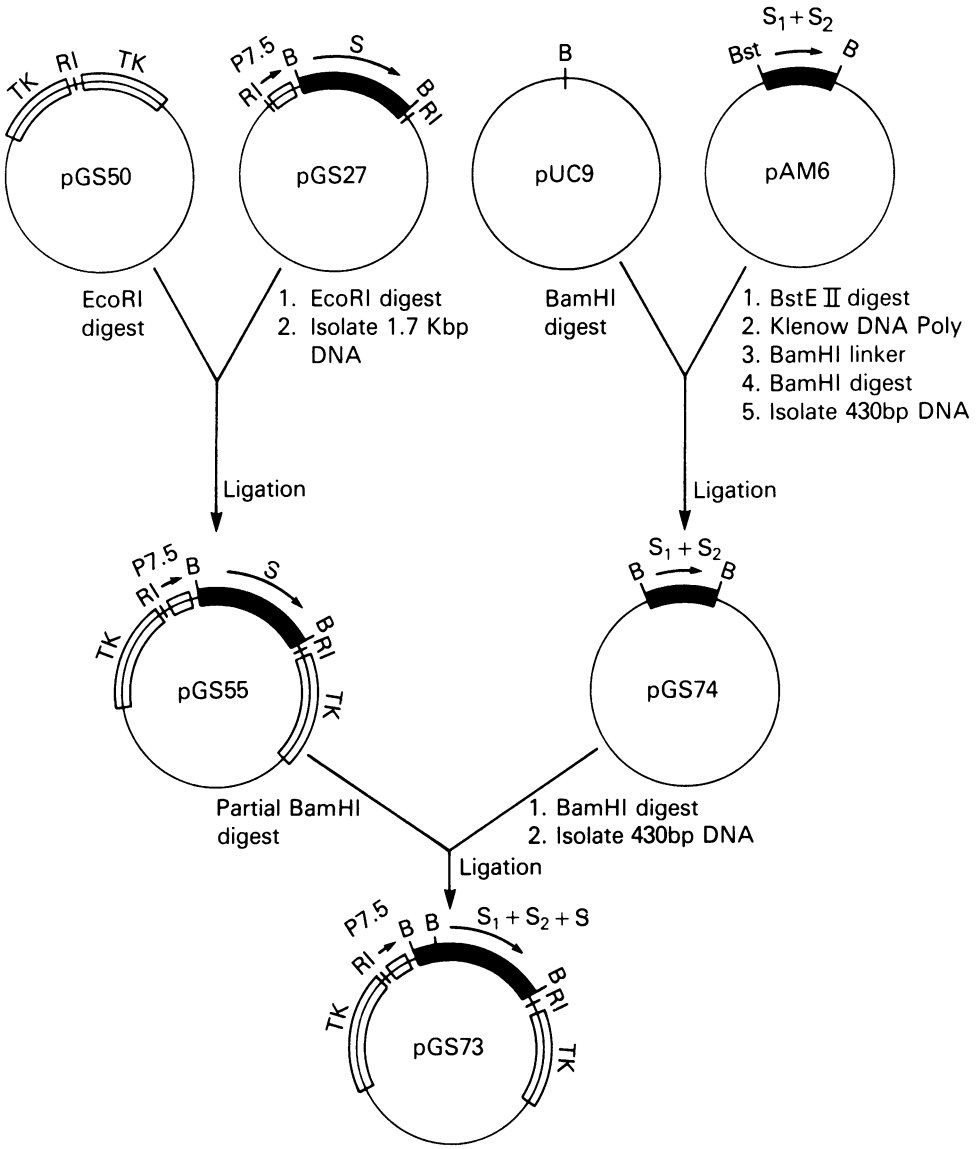

FIG. 1. Construction of recombinant plasmid vector. The entire HBV surface antigen coding region s1–s2–s was reconstructed from HBV sequences contained in pGS27 and pAM6 and placed downstream of the vaccinia virus promoter P7.5 and flanked by vaccinia virus TK gene sequences. Solid boxes represent HBV and open boxes represent vaccinia sequences used for the construction of pGS73. Abbreviations: B, *Bam*HI; Bst, *Bst*EII; RI, *Eco*RI; poly, polymerase.

MATERIALS AND METHODS

Construction of recombinant plasmids. A 1.7-kb *Eco*RI fragment containing the vaccinia virus P7.5 promoter ligated to the s gene of HBV type adw was excised from pGS27 (20) and inserted into the *Eco*RI site of the vaccinia virus thymidine kinase (TK) gene in plasmid pGS50 to form pGS55 (Fig. 1). HBV DNA in plasmid pAM6 (13) was cleaved with *Bst*EII, the termini were filled in with DNA polymerase, and *Bam*HI linkers were added. After cleavage with *Bam*HI, a 430-bp fragment containing the entire pre-s1 and part of pre-s2 was isolated and cloned into *Bam*HI-digested pUC9 to form pGS74 in order to amplify the HBV DNA fragment. Plasmid pGS55 was then linearized by partial digestion with *Bam*HI and ligated to the 430-bp *Bam*HI fragment from pGS74 to form pGS73. The latter contains the reconstructed pre-s1–pre-s2–s open reading frame downstream of the vaccinia virus P7.5 promoter as well as flanking vaccinia virus TK sequences. The correct orientation of the DNA segments was verified by restriction endonuclease analyses.

Construction of recombinant vaccinia virus. CV-1 cells were infected with vaccinia virus (WR strain) and transfected with pGS73 as previously described (9). TK^- recombinant vaccinia virus plaques were selected on TK^- 143 cells in the presence of 5-bromodeoxyuridine (BUdR) and checked for HBV DNA by dot-blot hybridization. After two plaque purifications, the virus stock, designated v73, was expanded in TK^- 143 cells and then HeLa cells.

Radioactive labeling and immunopurification of proteins. CV-1 cells were infected with 30 PFU of recombinant or wild-type vaccinia virus. After 2 h, 1.5 ml of RPMI medium (GIBCO Laboratories) containing 60 μCi each of [^{35}S]methionine and [^{35}S]cysteine was added. After 18 h, the medium was collected and unattached cells were removed by centrifugation. The cell monolayer was washed, harvested by centrifugation, and lysed with 0.5% Nonidet P-40. The

medium was also adjusted to contain the same detergent concentration. Insoluble material was removed by centrifugation for 10 min in a microcentrifuge, and 0.5 ml of supernatant was incubated for 4 h with 20 μl of normal guinea pig serum. After addition of 30 μl of protein A-Sepharose (1:1, vol/vol; Pharmacia), the mixture was incubated for a further 30 min, and any bound proteins were removed by centrifugation for 10 min. The supernatant was incubated for 15 h with guinea pig anti-HBsAg serum (Research Resources Section, National Institute of Allergy and Infectious Diseases) and then for 30 min with 30 μl of protein A-Sepharose. The protein A-Sepharose was collected by centrifugation and washed three times with 20 mM Tris hydrochloride (pH 7.5)–0.15 M NaCl–1% sodium deoxycholate–0.1% sodium dodecyl sulfate–1% Triton X-100 and twice with 20 mM Tris hydrochloride (pH 7.5)–2 M urea–0.4 M LiCl. The bound material was finally eluted from the Sepharose by heating at 100°C for 5 min in 30 mM Tris hydrochloride (pH 6.8)–3% sodium dodecyl sulfate–20% glycerol and applied to a 12.5% polyacrylamide gel (5). Afterward, the gel was soaked in En^{3}Hance (New England Nuclear Corp.), dried, and autoradiographed.

RIA. AUSRIA II and AUSAB radioimmunoassay (RIA) kits from Abbot Laboratories were used to measure HBsAg particles and antibody to HBsAg, respectively. Antibody directed to pre-s regions was measured in microtiter wells (Dynatech) that had been coated overnight with 0.1 ml of a solution containing 20 μg of synthetic peptide GTNLSVPNPLGFFPDHQLDPAGFANSNN or MQWNSTAFHQTLQDPRVRGLYLPAGGSS, prepared for us by L. Malloy (10). Antibody against vaccinia virus was measured in microtiter wells coated with 0.1 ml of a 1.2×10^9 PFU/ml suspension of wild-type virus. Remaining surface binding sites were blocked by incubation for 1 h with 3% gelatin in phosphate-buffered saline prior to addition of diluted serum samples containing 5% bovine serum albumin. After 2 h, the wells were washed and then incubated with ^{125}I-protein A (Amersham) for an additional 2 h. Radioactive material that remained bound to the wells after washing was measured with a gamma counter. All these incubations were carried out at room temperature.

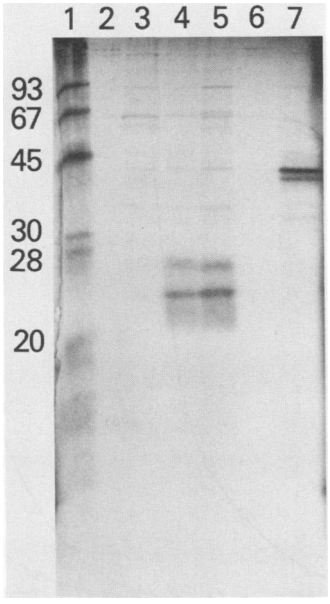

FIG. 2. Immunopurification of [^{35}S]methionine- and [^{35}S]cysteine-labeled HBV proteins. CV-1 cells were infected with wild-type vaccinia virus (lanes 2 and 3), S recombinant vaccinia virus v55 (lanes 4 and 5), or LS recombinant vaccinia virus v73 (lanes 6 and 7) and metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Proteins from the medium (lanes 2, 4, and 6) and cells (lanes 3, 5, and 7) were incubated with anti-HBV guinea pig serum, and proteins that bound to protein A-Sepharose were resolved by polyacrylamide gel electrophoresis and autoradiographed. Marker proteins are shown in lane 1 (in kilodaltons).

TABLE 1. RIA of s and pre-s antigen particles

Prepn	cpm
Negative control	120
Positive control[a]	8,600
VV-S-Ag	
Cell pellet[b]	38,220
Medium[c]	39,660
VV-LS-Ag	
Cell pellet[b]	760
Medium[c]	210

[a] Positive control contained HBsAg particle (Abbott) (20 ng/ml). VV-S-Ag and VV-LS-Ag, Materials obtained from cells infected with recombinant virus v55, which expresses s antigen, and v73, which expresses LS antigen, respectively.

[b] CV-1 cells (5×10^6 cells) were infected with recombinant virus. The cell pellets were suspended in 5 ml of medium and frozen and thawed three times, and then a 0.2-ml sample was used for RIA.

[c] CV-1 cells were infected as above, and 0.2 ml of medium (total, 5 ml) was used for RIA.

RESULTS

Construction of LS recombinant vaccinia virus. We have developed plasmid vectors to facilitate the transfer of foreign genes into vaccinia virus and their subsequent expression (9). These plasmids contain a vaccinia virus promoter, sites for insertion of DNA, and flanking segments of the vaccinia virus TK gene. Upon insertion of the desired foreign gene, the plasmid is transfected into cells that have been infected with vaccinia virus, and TK$^-$ virus recombinants are selected. In this manner, we previously inserted a 1.35-kbp cloned *Bam*HI fragment containing the s gene of HBV type adw into vaccinia virus (20). In that recombinant, the first ATG codon downstream of the vaccinia virus promoter and RNA start site initiates the S coding segment. As predicted, the 226-amino-acid S protein in both nonglycosylated (p24^s) and glycosylated (gp27^s) forms was made, assembled into 22-nm particles, and secreted. To synthesize the large 389-amino-acid LS protein, a 440-bp *Bst*EII-*Bam*HI DNA fragment containing the remainder of pre-s2 and the entire pre-s1 region was used to reconstruct the complete HBV envelope region downstream of the vaccinia virus promoter (Fig. 1). This plasmid (pGS73) was then used to form a new TK$^-$ recombinant vaccinia virus (v73) which was plaque-purified repeatedly on TK$^-$ cells in the presence of BUdR. The absence of contaminating TK$^+$ wild-type virus was suggested by equivalent plaque titers in the presence and absence of BUdR. Restriction endonuclease analysis and agarose gel electrophoresis confirmed that the 5.1-kbp *Hin*dIII fragment J of wild-type vaccinia virus had been replaced by one of 7.2 kbp that hybridized to HBV DNA (not shown).

Expression of the HBV coding sequence. Synthesis of LS was demonstrated by metabolically labeling CV-1 cells with [^{35}S]methionine and [^{35}S]cysteine after they had been infected with the LS recombinant vaccinia virus. Two labeled polypeptides of 39 kilodaltons (kDa) and 42 kDa were

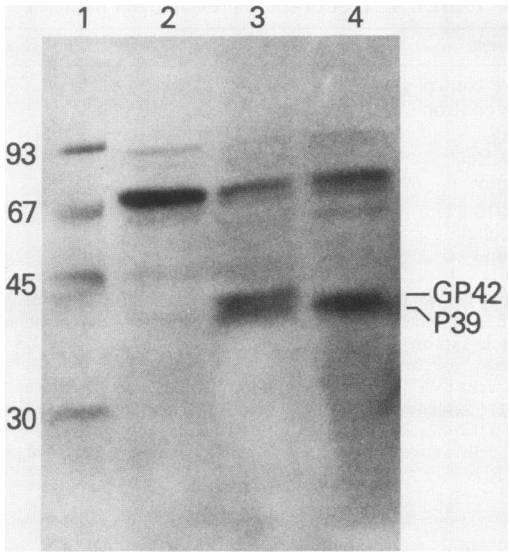

FIG. 3. Immunoblot of LS and S incubated with antibody raised against synthetic peptide corresponding to amino acids 13–40 from the NH_2 terminus of LS. Lane 1, ^{14}C-labeled molecular weight markers of 93, 67, 45, and 30 kDa. Lane 2, Solubilized CV-1 cells infected with recombinant virus v55 expressing only S; lane 3, solubilized CV-1 cells infected with recombinant virus v73 expressing LS; lane 4, solubilized CV-1 cells infected with recombinant virus v73 expressing LS in the presence of tunicamycin (1 μg/ml). The positions of $gp42^s$ and $p39^s$ are indicated.

immunopurified from cell lysates with anti-HBV guinea pig serum and resolved by polyacrylamide gel electrophoresis (Fig. 2, lane 7). These sizes corresponded to the unglycosylated ($p39^s$) and glycosylated ($gp42^s$) forms of LS (3). Despite the apparent glycosylation, the LS polypeptides were not secreted into the medium of infected cells (Fig. 2, lane 6). By comparison, the $p24^s$ and $gp27^s$ synthesized by our previous S recombinant vaccinia virus were present intracellularly and extracellularly (Fig. 2, lanes 4 and 5). The absence of $p24^s$ and $gp27^s$ from lysates of LS recombinant vaccinia virus was noteworthy and indicated exclusive synthesis of the LS polypeptides.

A sandwich-type RIA designed to measure HBsAg particles detected very low levels of reactive materials in lysates and none at all in the medium of cells infected with the LS recombinant virus (Table 1). Since $p39^s$ and $gp42^s$ were readily detectable by immunoprecipitation of infected-cell lysates (Fig. 2), we attribute the negative RIA results to the nonparticulate form of the LS proteins.

Further characterization of LS. We sought direct evidence that the 39- and 42-kDa proteins synthesized by the recombinant vaccinia virus contained the NH_2-terminal sequence of LS. Proteins from LS recombinant virus-infected cells were resolved by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Antiserum obtained by immunizing a rabbit with a synthetic peptide corresponding to amino acids 13 to 40 from the NH_2 terminus of LS was incubated with the blotted proteins. Specific binding of immunoglobulin G (IgG) to the 39- and 42-kDa polypeptides from LS recombinant virus was then detected by incubation with ^{125}I-protein A (Fig. 3). (A 70-kDa band was nonspecifically labeled in all samples.)

Both metabolic labeling (Fig. 2) and immunoblotting (Fig. 3) suggested that the 42-kDa polypeptide was present in equal or greater amounts than the 39-kDa polypeptide. Because of its size, we suspected that the larger polypeptide was glycosylated. Evidence for N-glycosylation was obtained by infecting cells with the LS recombinant vaccinia virus in the presence of tunicamycin. Under these conditions, only the unglycosylated 39-kDa polypeptide was formed (Fig. 3, lane 4). Based on electrophoretic mobilities, binding to antipeptide antibody, and the effect of tunicamycin, we concluded that the LS proteins $p39^s$ and $gp42^s$ were made by the recombinant vaccinia virus.

Induction of antibody reactive with HBsAg particles. Animals infected with the recombinant vaccinia virus would be expected to mount an immune response to both vaccinia virus and HBV proteins. RIAs (AUSAB, Abbott Laboratories) were performed on serial dilutions of serum from rabbits 10 weeks after intradermal inoculation with LS or S recombinant vaccinia viruses or with unmodified vaccinia virus as a control. Based on a Bureau of Biologics human antibody standard (Fig. 4), the anti-HBsAg levels in sera from rabbits immunized with LS and S recombinant virus were calculated to be in the same general range, e.g., approximately 720 and 2,000 mIU/ml, respectively. For comparison, the anti-HBsAg titer in the human plasma supplied in the RIA kit was about 44 mIU/ml, and levels above 10 mIU/ml have been shown to confer passive resistance to HBV.

Binding of rabbit anti-HBsAg in the RIA was inhibited by addition of HBsAg particles containing only S protein, indicating that pre-s epitopes did not play a quantitatively significant role in this assay (data not shown). The HBsAg

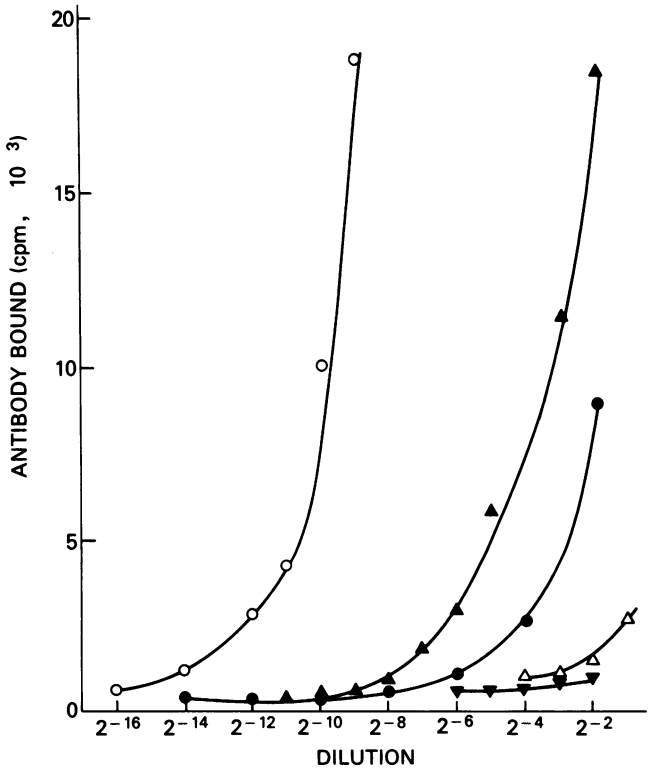

FIG. 4. Induction of antibody reactive with HBsAg particles in rabbits vaccinated with LS recombinant vaccinia virus. RIAs were performed with serum from a rabbit (rabbit B, see Fig. 6) vaccinated with LS recombinant vaccinia virus v73 (●), S recombinant vaccinia virus v55 (▲), or wild-type vaccinia virus (▼), with human HBV-positive plasma from the RIA kit (△), or with human immunoglobulin standard reference 2 from the Bureau of Biologics (○).

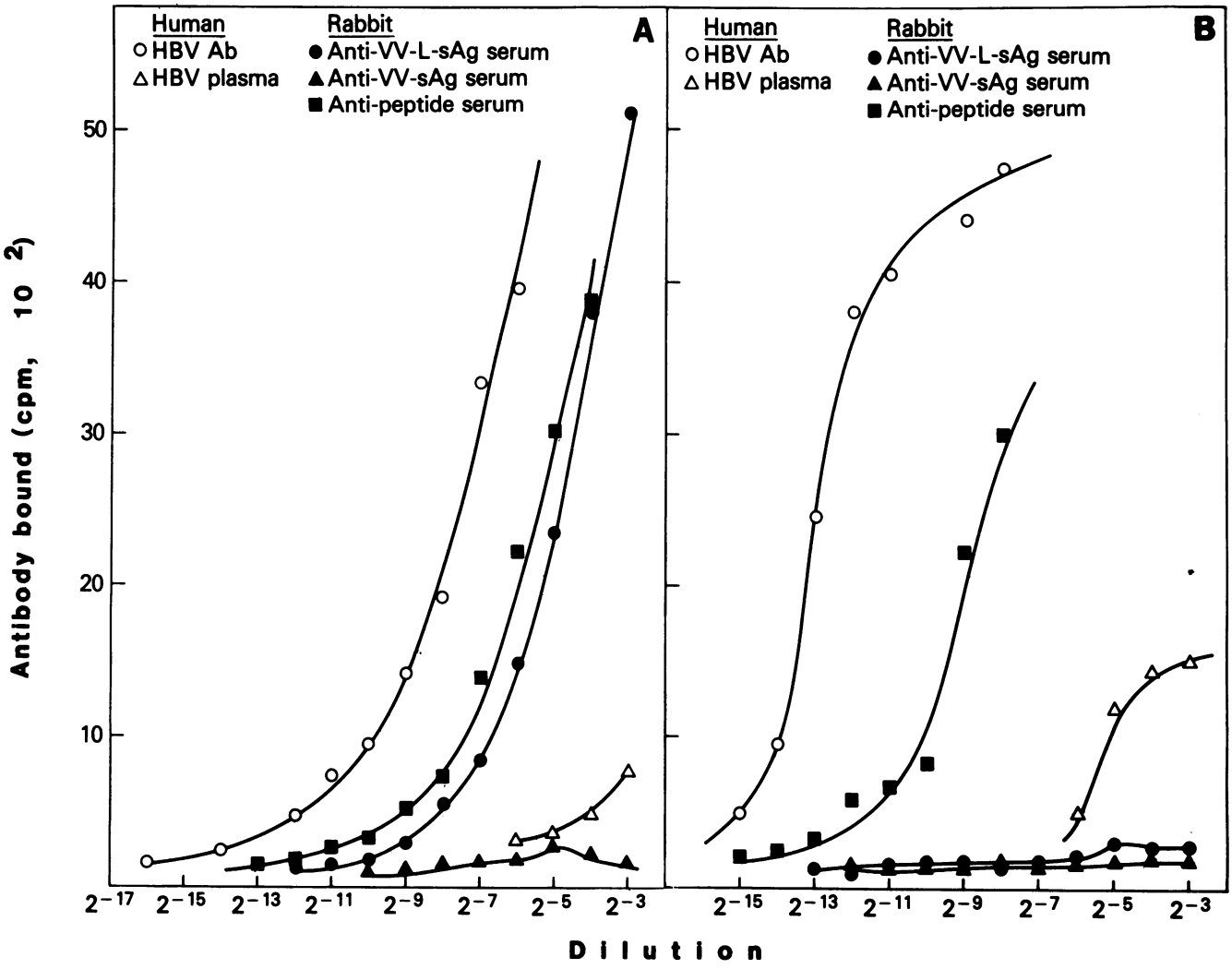

FIG. 5. Induction of antibody to pre-s epitopes. RIAs were performed in microtiter wells coated with peptides from the pre-s1 (panel A) or pre-s2 (panel B) regions, as described in the text.

particles used for competition were purified from cells infected with our previously described recombinant vaccinia virus (20).

Induction of antibody to pre-s epitopes. A synthetic 28-mer peptide corresponding to amino acids 13 to 40 from the NH_2 terminus of LS was used in an RIA to measure the induction of antibody to pre-s1 epitopes (Fig. 5A). Antibody to the peptide was detected in a 1:1,024 dilution of a serum obtained 10 weeks after inoculation of a rabbit with LS recombinant vaccinia virus. This level of antibody was only slightly less than that obtained after repeated immunization with peptide-keyhole limpet hemocyanin conjugates in Freund adjuvant. As expected, no antibody to this peptide was detected in serum from the rabbit vaccinated with the S recombinant vaccinia virus. The presence of antibody to this peptide in HBV-positive human plasma confirmed a recent report (15).

We were also interested in determining whether antibody to pre-s2 epitopes was induced by the LS recombinant. For this purpose another peptide, corresponding to amino acids 1 to 27 from the NH_2 terminus of MS, was synthesized. The presence of antibodies to this peptide in HBV-positive human plasma (Fig. 5B) also confirmed a previous report (8). Surprisingly, however, we could not detect a significant amount of antibody to the pre-s2 peptide in the sera from two rabbits vaccinated with the LS recombinant. Control experiments indicated that human HBV plasma contained antibodies that reacted with the peptide and that the peptide itself was immunogenic in rabbits.

Kinetics of antibody induction. Following intradermal inoculation of two rabbits with purified infectious LS recombinant vaccinia virus, the induction of antibodies that bind to vaccinia virus, HBsAg particles, and pre-s peptides was determined at weekly intervals (Fig. 6). Rabbits vaccinated with LS recombinant vaccinia virus had a delayed onset of production of the antibody that binds to S antigen particles (Fig. 6A) compared with those infected with an S recombinant vaccinia virus (20). A low level of such antibody was detected in the serum of rabbit A by week 3 after vaccination and was threefold higher after 6 weeks. Antibody was first detected in the serum of rabbit B at 8 weeks after vaccination, but the level at 14 weeks was much higher than that of rabbit A. The delay in production of the antibody that binds to the S antigen particle may result from the failure of LS to be secreted and a consequent difference in the presentation of the antigen. The kinetics of production of antibody

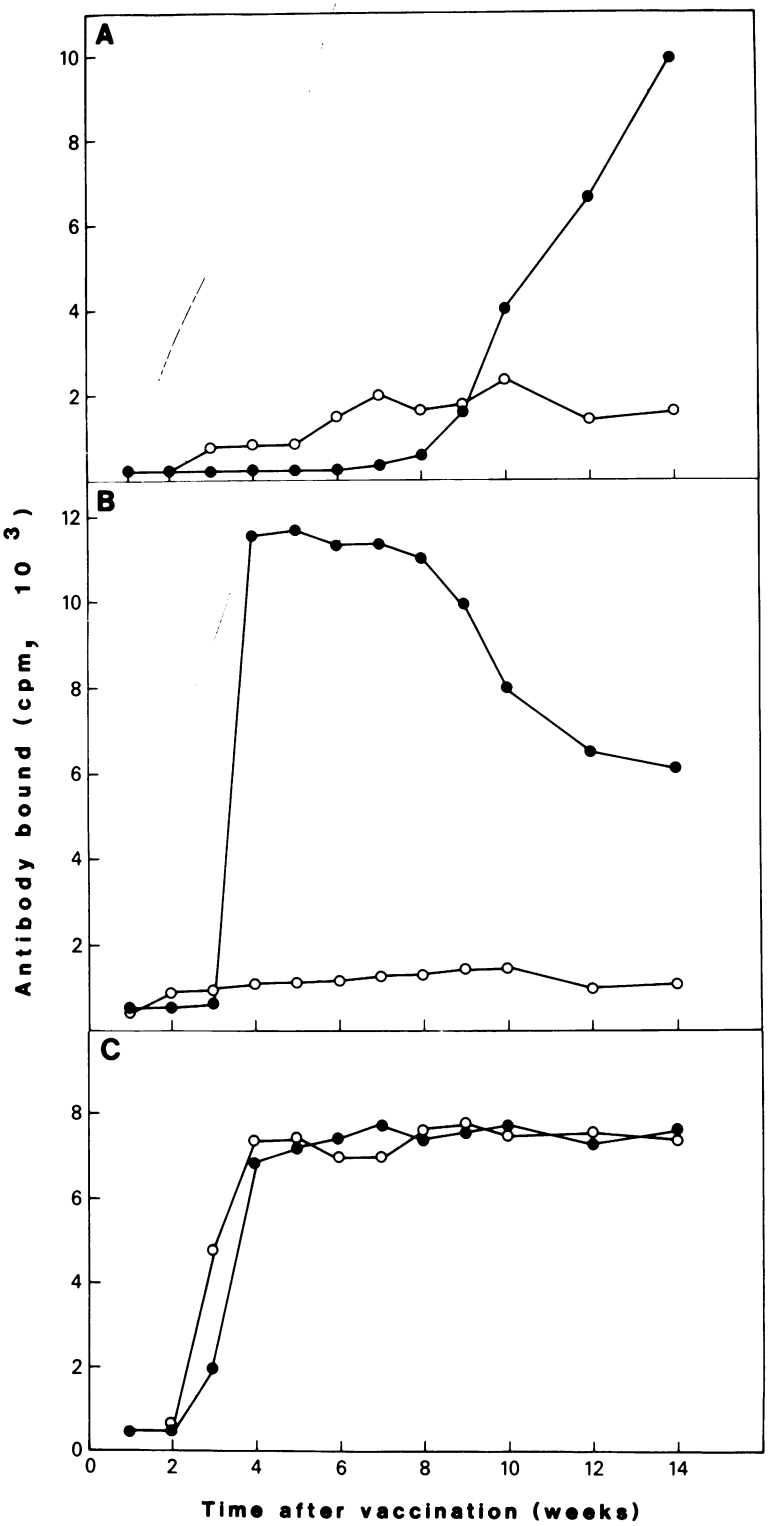

FIG. 6. Time course of antibody response in rabbits vaccinated with LS recombinant vaccinia virus. Rabbits A (○) and B (●) were injected intradermally at four sites with 10^8 PFU of the LS recombinant vaccinia virus per site. Serum samples were collected at weekly intervals. RIAs were performed for (A) antibody to HBsAg particles (AUSAB, Abbott Laboratories), (B) pre-S1 peptide, and (C) vaccinia virus particles. Sera were diluted 1:8 in phosphate-buffered saline containing 3% bovine serum albumin prior to the RIA.

reactive with the pre-s1 peptide are shown in Fig. 6B. Rabbit A started to produce some antibody against the peptide at week 2 after vaccination. The level increased slightly and then leveled off at week 10. Rabbit B, on the other hand, was a much better producer of anti-pre-s1 antibody. The level of antibody increased dramatically at week 3 after vaccination. In this case, production of anti-pre-s antibody occurred faster than that of anti-s antibody. Anti-vaccinia virus anti-

body was detected at week 3 after inoculation of both rabbits and soon saturated our assay (Fig. 6C), indicating good vaccinations. We do not understand the basis of the variable antibody response of outbred rabbits to LS, but have also noted variable responses to S (20) and MS (Cheng and Moss, unpublished).

DISCUSSION

Under conditions of active HBV infection, the major surface protein (S) as well as the minor ones (MS and LS) are synthesized in the liver. Until now, however, only MS and S have been expressed by recombinant DNA techniques. Since HBV does not replicate in cultured cells, there has been little opportunity to study the biosynthesis and properties of LS. By constructing a recombinant vaccinia virus that contains the entire HBV envelope region and has a vaccinia virus promoter just upstream of pre-s1, we have obtained synthesis of LS in infected cells. Neither MS nor S was detected, consistent with little or no use of internal mRNA translation initiation sites. Presumably, shorter mRNAs needed for the synthesis of MS and S were not made because HBV RNA start sites located within pre-s2 or pre-s1 cannot be utilized by the vaccinia virus transcriptional apparatus. The evident failure of the cellular RNA polymerase to recognize the HBV RNA start sites incorporated within vaccinia virus DNA is probably due to the cytoplasmic location of the latter, although inhibition of host transcription may also be relevant.

Two LS polypeptides corresponding to the natural unglycosylated ($p39^s$) and glycosylated ($gp42^s$) forms were made in recombinant vaccinia virus-infected cells. This result was analogous to the synthesis of glycosylated and unglycosylated S polypeptides by a previous recombinant vaccinia virus that contained the S gene (20). N-Glycosylation of LS indicates that the protein did undergo membrane translocation, although it was not secreted. The absence of LS from the medium is unlikely to be due to inhibitory effects of vaccinia virus infection, since HBsAg particles containing S were assembled and secreted by a previous recombinant vaccinia virus. We suspect that the transport block is an intrinsic property of the NH_2-terminal portion of the LS protein. To determine whether physical association with S might facilitate secretion, we coinfected cells with recombinant vaccinia viruses that expressed S and LS. Under these conditions, the amount of HBsAg particles in the medium was significantly reduced from the amount obtained by infection with the s recombinant alone, and analysis of the small amount of particle-associated polypeptides in the medium revealed that they were predominantly S (K.-C. Cheng and B. Moss, manuscript in preparation). It may be pertinent that LS is found in higher amounts in infectious HBV than in HBsAg empty envelope particles from chronic carriers (3). The presence of core proteins or other factors associated with a productive virus infection may facilitate the incorporation of LS into the virus particle. As a practical point, we noted that the nonparticulate form of LS was readily detected by radioimmunoprecipitation and immunoblotting but not by RIA. We believe that this discrepancy results from the sandwich-type design of the RIA.

Intradermal inoculation of rabbits with the infectious recombinant vaccinia virus induced antibodies to pre-s1 and s epitopes. Assuming that the behavior of LS in tissue culture cells and animals is similar, active secretion of the LS protein is apparently not required for immunogenicity. Unexpectedly, we did not detect antibody that recognized a synthetic peptide corresponding to the NH_2 terminus of MS. Since human sera contain antibodies to this peptide (15), our result may signify that folding of LS interferes with the immunogenicity of this pre-s2 epitope and that such antibody is formed only in response to MS. Significantly, antibody to pre-s2 epitopes is made when rabbits are inoculated with a recombinant vaccinia virus that expresses MS (K.-C. Cheng and B. Moss, manuscript in preparation).

Previous studies indicated that a single inoculation with a live recombinant vaccinia virus containing the s gene significantly protected two chimpanzees against hepatitis B (14); nevertheless, the immunogenicity was low, as measured by antibody production, and the vaccination may not have prevented some initial HBV replication. Recent studies suggest that the pre-s region is highly immunogenic; in one patient antibodies to pre-s epitopes were detected before those directed to s (15). A similar phenomenon occurred in one of the two rabbits inoculated with the LS recombinant vaccinia virus. Since antibodies to pre-s epitopes have been shown to prevent the attachment of HBV to hepatoma cells in vitro (15), protection studies with the LS recombinant vaccinia virus in chimpanzees should be of considerable interest.

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LITERATURE CITED

1. **Beasley, R. P., and L.-Y. Huang.** 1984. Epidemiology of hepatocellular carcinoma, p. 275–305. *In* G. N. Vyas, J. L. Dienstag, and J. H. Hoofnagle (ed.), Viral hepatitis and liver disease. Grune and Stratton, New York.
2. **Cattaneo, R. O., H. Will, N. Hernandez, and H. Schaller.** 1983. Signals regulating hepatitis B surface antigen transcription. Nature (London) **305**:336–338.
3. **Heermann, K. H., U. Goldman, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-s sequance. J. Virol. **50**:396–402.
4. **Hoofnagle, J. H., and H. J. Alter.** 1984. Chronic viral hepatitis p. 97–114. *In* G. N. Vyas, J. L. Dienstag, and J. H. Hoofnagle (ed.), Viral hepatitis and liver disease. Grune and Stratton, New York.
5. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
6. **Laub, O., L. B. Rall, M. Truett, Y. Shaul, D. N. Standring, P. Valenzuela, and W. J. Rutter.** 1983. Synthesis of hepatitis B surface antigen in mammalian cells: expression of the entire gene and the coding region. J. Virol. **48**:271–280.
7. **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.
8. **Machida, A., S. Kishimoto, H. Ohnuma, K. Baba, Y. Ito, H. Miyamoto, G. Funatsu, K. Oda, S. Usuda, S. Togami, T. Nakamura, Y. Miyakawa, and M. Muyumi.** 1984. A polypeptide containing 55 amino acid residues coded by the pre-s region of hepatitis B virus deoxyribonucleic acid bears the receptor for

polymerized human as well as chimpanzee albumins. Gastroenterology **86**:910–918.
9. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for the production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
10. **Merrifield, R. B., and A. Marglin.** 1970. Chemical synthesis of peptides and proteins. Annu. Rev. Biochem. **39**:841–866.
11. **Michel, M-L., P. Pontisso, E. Sobczak, Y. Malpièce, R. E. Streek, and P. Tiollais.** 1984. Synthesis in animal cells of hepatitis B surface antigen particles carrying a receptor for polymerized human serum albumin. Proc. Natl. Acad. Sci. USA **81**:7708–7712.
12. **Milich, D. R., G. B. Thornton, A. R. Neurath, S. B. Kent, M.-L. Michel, P. Tiollais, and F. V. Chisari.** 1985. Enhanced immunogenicity of the pre-s region of hepatitis B surface antigen. Science **228**:1195–1199.
13. **Moriarty, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer.** 1981. Expression of hepatitis B virus surface antigen in cell culture using a simian virus 40 vector. Proc. Natl. Acad. Sci. USA **78**:3403–3407.
14. **Moss, B., G. L. Smith, J. L. Gerin, and R. H. Purcell.** 1984. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. Nature (London) **311**:67–69.
15. **Neurath, A. R., S. B. H. Kent, N. Strick, P. Taylor, and C. E. Stevens.** 1984. Hepatitis B virus contains pre-s gene-encoded domains. Nature (London) **315**:154–156.
16. **Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali.** 1984. Construction of live vaccines using genetically engineered poxvirus: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **81**:193–197.
17. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1984. A frameshift mutation in the pre-s region of the human hepatitis B virus genome allows production of surface antigen particles but eliminates binding to polymerized albumin. Proc. Natl. Acad. Sci. USA **82**:3440–3444.
18. **Pourcel, C., A. Louise, M. Gervais, N. Chenciner, M.-F. Dubois, and P. Tiollais.** 1982. Transcription of the hepatitis B surface antigen gene in mouse cells transformed with cloned viral DNA. J. Virol. **42**:100–105.
19. **Rall, L. B., D. N. Standring, O. Laub, and W. J. Rutter.** 1983. Transcription of hepatitis B virus by RNA polymerase II. Mol. Cell. Biol. **3**:1766–1773.
20. **Smith, G. L., M. Mackett, and B. Moss.** 1983. Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature (London) **302**:490–495.
21. **Standring, D. N., W. J. Rutter, H. E. Varmus, and D. Ganem.** 1984. Transcription of the hepatitis B surface antigen gene in cultured murine cells initiates within the presurface region. J. Virol. **50**:563–571.
22. **Stebbe, W., and W. H. Gerlich.** 1983. Structural relationships between minor and major proteins of hepatitis B surface antigen. J. Virol. **46**:626–628.
23. **Stevens, C. E., P. E. Taylor, M. J. Tong, P. T. Toy, and G. N. Vyas.** 1984. Hepatitis B vaccine: an overview, p. 275–305. *In* G. N. Vyas, J. L. Dienstag, and J. H. Hoofnagle (ed.), Viral hepatitis and liver disease. Grune and Stratton, New York.
24. **Tiollais, P., C. Pourcell, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.