Selective Synthesis and Secretion of Particles Composed of the Hepatitis B Virus Middle Surface Protein Directed by a Recombinant Vaccinia Virus: Induction of Antibodies to Pre-S and S Epitopes

KUO-CHI CHENG* and BERNARD MOSS*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

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Selective synthesis in mammalian cells of the hepatitis B virus middle surface (MS) protein, which is 55 amino acids longer than the major surface (S) protein, was achieved by using a recombinant vaccinia virus. The 33-kilodalton MS polypeptide was glycosylated and secreted as particles that resembled human hepatitis B surface antigen as well as particles composed solely of S protein with regard to antigenicity, buoyant density, size, and electron micrographic appearance. The MS particles differed from S particles, however, by binding to polymerized human albumin and inducing antibodies that reacted with a pre-S peptide and inhibited the binding of human plasma-derived hepatitis B surface antigen to polymerized human albumin.

Hepatitis B virus (HBV), an important cause of human liver disease and hepatocellular carcinoma, is composed of a DNA-containing core and a lipoprotein envelope (for a review, see reference 23). Both 44-nm infectious virions and 22-nm empty envelope particles (hepatitis B surface antigens [HBsAg]) may be present in the blood of infected individuals. The envelope contains a major surface (S) protein of 226 amino acids and two minor proteins of 281 amino acids (middle surface [MS]) and (dependent on the HBV serotype) 389 to 400 amino acids (large surface [LS]) that have the same carboxyl terminus (5, 8, 22). Disulphide-linked dimers of S polypeptides form the structural unit of the HBV envelope, whereas MS and LS polypeptides may be involved in virus attachment to hepatocytes (7, 15). The region of the HBV genome that encodes surface proteins contains one long open reading frame that may be divided into pre-S1, pre-S2, and S regions (23). All three domains compose the coding sequence for the LS polypeptide, whereas the MS polypeptide is encoded by pre-S2 plus S, and the S polypeptide is encoded by S alone. Transcripts with 5′ ends that map within or upstream of the pre-S regions have been described previously (3, 6, 10, 17–19, 21).

Although HBV cannot replicate in tissue culture cells, synthesis of HBsAg has been obtained by recombinant DNA methods (for a review, see reference 23). Selective expression of S, MS, and LS polypeptides would be desirable for studying the processing and transport as well as the physical and immunological properties of these proteins. However, in mammalian cells, only S or a mixture of S and MS polypeptides (1, 6, 10, 17) has been made. Although selective synthesis of MS has been achieved in yeast (24), HBsAg particles are not secreted by these lower eucaryotic cells but are obtained after cell disruption. Vaccinia virus vectors have been used to selectively express S (16, 20) and LS (4) polypeptides in mammalian cells. Both surface proteins were glycosylated, but only S was capable of forming secreted particles. We now describe a recombinant vaccinia virus that selectively synthesizes the MS polypeptide. Like S, this form of surface protein is secreted as particles of approximately 22 nm; however, additional binding sites are present on MS particles, and vaccinated animals produce antibodies to both S and pre-S epitopes. For vaccine purposes, recombinant vaccinia viruses that express MS or LS polypeptides may have advantages over ones that express only S.

Construction of MS recombinant vaccinia virus. We prepared a recombinant vaccinia virus (Wyeth strain) that expresses the HBV MS protein by using a protocol (9) that is similar to the one used for construction of recombinants that express S (20) and LS (4) polypeptides. A 1.2-kilobase-pair DNA segment starting 40 base pairs upstream of the pre-S2 translation initiation codon and terminating 250 base pairs downstream of the S coding region of HBV DNA (24) was inserted into a plasmid containing the vaccinia virus p7.5 promoter and RNA start site flanked by vaccinia virus TK sequences. The plasmid pKCl was shown by restriction endonuclease analysis to contain the pre-S2 region properly oriented downstream of the p7.5 promoter. When pKCl was transfected into vaccinia virus-infected cells, homologous recombination occurred, and a TK- recombinant virus designated vKCl containing HBV DNA was isolated and purified.

Expression of MS. Since the first translation initiation codon downstream of the vaccinia p7.5 RNA start site begins the pre-S2 open reading frame, expression of authentic MS was predicted. To demonstrate this, CV-1 cells were infected with the MS recombinant vaccinia virus and metabolically labeled with [35S]methionine and [35S]cysteine. Parallel cultures were infected with vaccinia recombinants that were previously shown to express S or LS polypeptides. In each case, proteins present in the medium and in cell lysates at 24 h after infection were incubated with guinea pig antiserum to human HBsAg, and the antigen-antibody complexes were then bound to staphylococcal protein A-Sepharose. The bound proteins were eluted with sodium
dodecyl sulfate and resolved by polyacrylamide gel electrophoresis. The recombinant virus that was engineered to express MS synthesized an appropriate size protein of 33 kilodaltons (kDa) (Fig. 1A, lanes 5 and 6). At this time, 24 h after infection, MS was detected in the medium but was still predominantly intracellular. Little or no p24 and gp27 were made by the recombinant, indicating selective expression of the MS polypeptide. Glycosylation of the 33-kDa protein was deduced by using tunicamycin to inhibit N-glycosylation. A polypeptide of 30 kDa replaced the one of 33 kDa (Fig. 1A, lanes 7 and 8). These data indicate that the 33-kDa polypeptide corresponds to gp33. Human HBsAg particles contain MS proteins of 33 and 36 kDa, the latter being more heavily glycosylated (5, 22). A minor diffuse band above gp33 was detected in cells infected with the MS recombinant (Fig. 1A, lane 5); however, the presence of background bands at this position in other lanes made interpretation difficult. The autoradiograph confirmed previous results and demonstrated the presence of p24 and gp27 (Fig. 1A, lanes 1 and 2) in the lysates and medium of cells infected with the S recombinant (20). Although both glycosylated (gp42) and nonglycosylated (p39) forms of MS were made, their distribution was entirely intracellular (Fig. 1A, lanes 3 and 4) as previously shown (4).

Kinetics of synthesis and secretion of MS. The time course of synthesis and secretion of S particles in CV-1 cells infected by a recombinant vaccinia virus is shown in Fig. 1B. As previously reported (20), S is rapidly detected intracellularly and within a few hours appears in the medium. Between 8 and 16 h, the level of S in the medium exceeded that in the cells. Secretion of MS particles appeared to occur more slowly so that the amount in the medium did not exceed the amount in the cells until 48 h (Fig. 1C). The total amount of MS, determined by the AUSRIA II test (Abbott

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amt of bound HBsAg (cpm)</th>
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<tr>
<td>Negative control</td>
<td>106</td>
</tr>
<tr>
<td>S particlea</td>
<td>1,546</td>
</tr>
<tr>
<td>MS particlec</td>
<td>12,627</td>
</tr>
<tr>
<td>HBsAg particlec</td>
<td>5,053</td>
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a Microtiter wells were coated with 0.1 ml of human albumin (10 mg/ml) that had been polymerized with glutaraldehyde and then incubated with test particles for 2 h at room temperature. After the wells were washed with phosphate-buffered saline, 0.1 ml of 125I-labeled antibody to HBsAg from an AUSRIA II kit was added. The wells were washed again and counted.

b No antigen particle was used.

c Purified from medium of cells infected with recombinant vaccinia virus that expressed S or MS.

d Purified from human plasma.

FIG. 1. Expression of HBV polypeptides by recombinant vaccinia viruses. (A) Polyacrylamide gel electrophoresis. CV-1 cells were infected with recombinant vaccinia viruses that express S (lanes 1 and 2), LS (lanes 3 and 4), or MS (lanes 5 to 8) and that were metabolically labeled with [35S]methionine and [35S]cysteine. In lanes 7 and 8, the cells were infected in the presence of 1 mg of tunicamycin per ml. After 24 h, the media were removed, and the cells were washed and lysed. Cell-associated (odd-numbered lanes) and secreted (even-numbered lanes) proteins were incubated with anti-HBsAg guinea pig serum, and then immune complexes were bound to protein A-Sepharose. After the beads were washed, the bound proteins were eluted with sodium dodecyl sulfate, analyzed by polyacrylamide gel electrophoresis, and autoradiographed. M, Marker proteins with molecular sizes in kilodaltons. The positions of HBV polypeptides are indicated by arrowheads. In lane 8, the faint designated band was not reproduced photographically. (B and C) CV-1 cells were infected with S recombinant vaccinia virus (panel B) or MS recombinant vaccinia virus (panel C). Intracellular and extracellular antigen was measured by RIA with an AUSRIA II kit. Inset is an electron micrograph (magnification, ×133,500) of MS particles, from recombinant vaccinia virus-infected CV-1 cells, that were purified by CsCl-gradient centrifugation, deposited on collagen-coated grids, and stained with uranyl acetate.
phosphate-buffered saline containing was used in panel A because of the large difference in antibody to vaccinia virus. All serum samples were diluted to HBsAg. (B) RIA for antibody to human plasma-derived HBsAg particles (data not shown). We previously demonstrated that the commercial antibody to S and MS particles was not determined. Whether "sandwich" RIA does not detect nonparticulate LS protein. Physical properties of MS particles. The sedimentation of MS particles, the presence of nonparticulate MS that is poorly detected by the radioimmunoassay (RIA), or differences in the binding of antibody to S and MS particles was not determined. In this regard, we previously demonstrated that the commercial "sandwich" RIA does not detect nonparticulate LS protein.

Physical properties of MS particles. The sedimentation of MS particles was visualized by electron microscopy and appeared identical to human HBsAg (Fig. 1C, inset).

Previous studies have shown that HBsAg particles specifically bind to polymerized human albumin and that binding sites are present in the pre-S2-encoded peptide (7, 8, 10, 15, 17). If the pre-S2 epitopes were located on the surface of MS particles, binding to polymerized human albumin should occur. The binding, to microtiter wells coated with polymerized human albumin, of human plasma-derived HBsAg particles (containing approximately 15% MS) and of particles (made by recombinant vaccinia viruses) that are entirely S or MS was compared. The data in Table 1 demonstrate minimal binding of S particles, significant binding of human HBsAg, and highest binding of MS particles.

Induction of antibody reactive with HBsAg and pre-S epitopes. Recombinant vaccinia viruses, although somewhat attenuated (2), retain infectivity for animals as well as cultured cells. To determine the immunogenicity of the MS particles, rabbits were inoculated intradermally with 10⁷ PFU of purified virus at four (rabbit A) or one (rabbit B) site. Typical pox lesions developed in the skin during week 1. Sera were tested for antibodies reactive with human HBsAg by using the commercial AUSAB (Abbott) RIA. A second RIA, with a synthetic peptide corresponding to amino acids 1 to 27 of MS, was performed to detect antibody to epitopes in the pre-S2 region. Elevated titers to both HBsAg and pre-S2 peptides were detected in 1 week and plateaued in 2 to 3 weeks (Fig. 2A and B). Rabbit A had higher antibody titers to HBsAg and peptide than did rabbit B at all times. To determine whether this difference also was reflected in antibody titers to vaccinia virus, an RIA was developed by immobilizing purified wild-type vaccinia virions to microtiter wells. Compared with rabbit A, rabbit B had a considerably delayed onset of production of antibody, although the final values measured by this RIA were similar to each other (Fig. 2C).

Antibody blockade of HBsAg binding to polymerized human albumin. We wished to determine whether antibodies made by rabbits inoculated with the MS recombinant vaccinia virus could block the binding of HBsAg particles to polymerized human albumin. This experiment was carried out by preincubating HBsAg with diluted serum samples and then adding the mixture to microtiter wells coated with polymerized human albumin. A block in binding was observed (Fig. 3). This was evidently due to antibody to pre-S2 epitopes since antisera from rabbits inoculated with S recombinant vaccinia virus (Fig. 3) or from unimmunized rabbits (data not shown) did not prevent binding in this assay. Antiserum from a rabbit immunized with an LS recombinant vaccinia virus, however, also blocked binding of HBsAg to polymerized human albumin (Fig. 3).

Conclusion. Selective synthesis of MS was achieved by placing a vaccinia virus promoter just upstream of the pre-S2 open reading frame. Evidently, downstream initiation at the start of the S open reading frame was infrequent or did not occur. This permitted, for the first time, analysis of MS particle formation and secretion in mammalian cells. The MS polypeptide was glycosylated, and the particles were similar to those made up predominately or solely of S. The binding of MS particles to polymerized human albumin, however, indicated the presence of the pre-S2 portion on the surface. In addition, rabbits vaccinated with the recombinant virus made antibodies to both S- and pre-S2-encoded epitopes. Because pre-S-derived peptides may contain sites for binding of HBV to hepatocytes (15, 17) and are highly immuno-

FIG. 2. Time course of antibody induction in rabbits vaccinated with MS recombinant vaccinia virus. (A) AUSAB test for antibody to HBsAg. (B) RIA for antibody to pre-S2 peptide. (C) RIA for antibody to vaccinia virus. All serum samples were diluted 1:32 with phosphate-buffered saline containing 3% bovine serum albumin. Rabbit vaccinated at four sites (C) and at one site (A). A log scale was used in panel A because of the large difference in antibody responses to HBsAg in the two rabbits.
The inclusion of the MS and LS proteins or peptides derived from them may be desirable in vaccines. Evidence was previously obtained that chimpanzees inoculated with a recombinant vaccinia virus that selectively expressed S were protected from clinical signs of hepatitis after an intravenous challenge with HBV (13). Rather than stimulating the production of neutralizing antibodies, however, the vaccination primed the animals so that they exhibited a rapid and protective anamnestic response to HBsAg upon HBV challenge. Whether MS or LS recombinant vaccinia viruses provide more complete immunizing effects in primates is currently under investigation.

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LITERATURE CITED


