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The Hepatitis A Virus Polyprotein Expressed by a Recombinant Vaccinia Virus Undergoes Proteolytic Processing and Assembly into Viruslike Particles

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Hepatitis A virus (HAV) contains a single-stranded, plus-sense RNA genome with a single long open reading frame encoding a polyprotein of approximately 250 kDa. Viral structural proteins are generated by posttranslational proteolytic processing of this polyprotein. We constructed recombinant vaccinia viruses which expressed the HAV polyprotein (rV-ORF) and the P1 structural region (rV-P1). rV-ORF-infected cell lysates demonstrated that the polyprotein was cleaved into immunoreactive 29- and 33-kDa proteins which comigrated with HAV capsid proteins VP0 and VP1. The rV-P1 construct produced a 90-kDa protein which showed no evidence of posttranslational processing. Solid-phase radioimmunoassays with human polyclonal anti-HAV sera and with murine or human neutralizing monoclonal anti-HAV antibodies recognized the rV-ORF-infected cell lysates. Sucrose density gradients of rV-ORF-infected cell lysates contained peaks of HAV antigen with sedimentation coefficients of approximately 70S and 15S, similar to those of HAV empty capsids and pentamers. Immune electron microscopy also demonstrated the presence of viruslike particles in rV-ORF-infected cell lysates. Thus, the HAV polyprotein expressed by a recombinant vaccinia virus demonstrated posttranslational processing into mature capsid proteins which assembled into antigenic viruslike particles.

Hepatitis A virus (HAV) is a human pathogen which causes disease throughout the world. Currently, prevention of hepatitis A requires the timely administration of immune globulin, which, though inexpensive and efficacious, must be administered soon after exposure and has a limited duration of protection (30, 39). Both live-attenuated and inactivated vaccines have been developed (10, 26, 38, 48), but production of HAV is hindered by its slow growth in tissue culture. Consequently, a recombinant subunit or particle vaccine would be desirable.

HAV is classified as a member of the picornavirus family. The 7,478-nucleotide (nt)-long, single-stranded, plus-sense RNA genome encodes a single long open reading frame (ORF) (6). In other picornaviruses, translation of the ORF produces a polyprotein of approximately 220 kDa which is posttranslationally processed into a variety of precursor polyproteins and finally into mature viral proteins (18). Capsid proteins are assembled into subviral particles and mature virions (for a review, see reference 33). Proteolytic processing of HAV has been difficult to study since HAV replicates slowly in tissue culture, requiring several weeks of growth to produce relatively small yields of HAV (maximum of 10^8 to 10^9 50% tissue culture infective doses per ml) (37). Additionally, in vitro translation of HAV RNA demonstrated incomplete precursor processing or internal initiation of protein synthesis (11, 38a). The 3C coding region of HAV contains His and Cys amino acids which are homologous to the catalytic sites of other picornavirus 3C proteases (6); thus, it is thought that 3C-mediated HAV processing is similar to that of other picornaviruses (12, 18, 28, 45).

HAV infection elicits neutralizing antibodies directed toward an immunodominant neutralization antigenic site (13, 41). Analysis of neutralization escape mutants suggests that two regions of the HAV capsid contribute to this neutralization site; one epitope includes amino acid 70 of capsid protein VP3 and the second includes amino acids 102 and 114 of VP1 (7, 31). These epitopes are thought to be conformationally dependent (13, 41), and immunization with individual capsid proteins or synthetic peptides elicits only low levels of neutralizing antibodies or primes for an enhanced immune response to subsequent challenge with subimmunogenic doses of HAV (7, 9, 17, 32). Thus, assembly of capsid proteins into subviral or virion structures may be necessary for the generation of HAV epitopes which efficiently elicit neutralizing antibodies.

We recently reported that recombinant baculoviruses which expressed the entire ORF of HAV produced antigenic proteins of the same molecular mass as HAV proteins (42). Several larger HAV proteins were detected in cells infected with the recombinant baculovirus, suggesting that posttranslational processing was inefficient or retarded in *Spodoptera frugiperda* cells. Similar processing of influenza virus hemagglutinin in insect cells is also inefficient (21). We wished to examine if the HAV polyprotein expressed in mammalian cells would undergo more complete proteolytic processing and to determine if assembly into viruslike particles occurred. Recombinant vaccinia viruses expressing the HAV ORF and P1 structural protein precursor were constructed. The expressed polyprotein was processed into capsid proteins which assembled into antigenic material which migrated in sucrose gradients with HAV empty capsids and pentameric precursors. Immune electron microscopic analysis confirmed the generation of viruslike particles.

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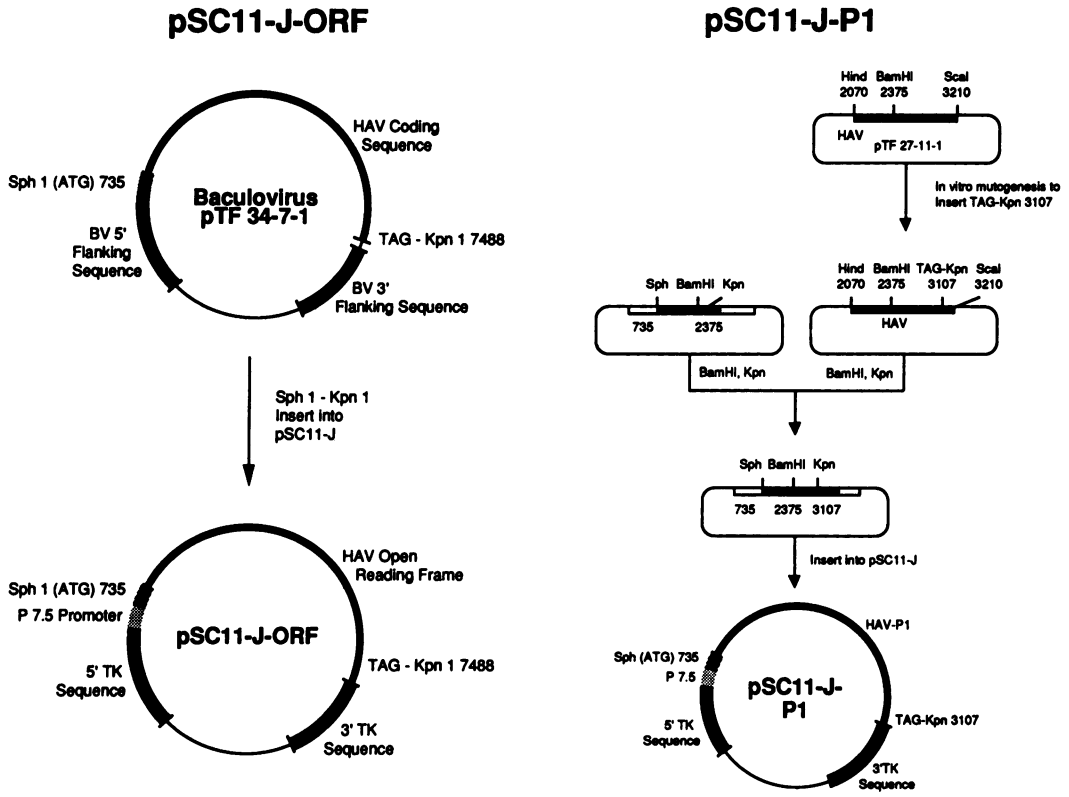

FIG. 1. Construction of pSC11-J-ORF and pSC11-J-P1 vaccinia virus shuttle vectors. The entire ORF, nt 735 to 7488, was subcloned from baculovirus vector pTF 34-7-1 (42). The coding sequence for the capsid protein precursor P1, nt 735 to 3107, was constructed as diagrammed.

MATERIALS AND METHODS

Modification of the pSC11 vaccinia virus shuttle vector. The construction of pSC11-J and the pSC11-J-HAV plasmids was performed by using standard cloning methods (34). pSC11 was generously provided by B. Moss, National Institutes of Health, Bethesda, Md. This plasmid contains a unique *Sma*I site which is immediately downstream from the vaccinia virus early-late p7.5 promoter (3). It is flanked by sequences from the vaccinia virus thymidine kinase gene which direct the insertion of the foreign gene into the nonessential thymidine kinase gene of vaccinia virus. It also expresses β-galactosidase, which can be used to select recombinant viruses. The pSC11 vector was cleaved at nt 6500 with *Sma*I, and the following oligonucleotide was inserted:

5′ GGGCATGCAATCGCGAGATCTAGGTACCTAATTGA 3′

3′ CCCGTACGTTAGCGCTCTAGATCCATGGATTAACT 5′

The resulting pSC11-J vector maintains the *Sma*I site at nt 6500 followed by unique *Sph*I, *Nru*I, *Bgl*II, and *Kpn*I restriction sites. Additionally, the inserted oligonucleotide contains termination codons for reading frame 1 (TAA) and 3 (TGA), and the downstream pSC11 DNA contains a termination codon for reading frame 2. pSC11-J was sequenced through the new polylinker site by using standard methods (Sequenase; United States Biochemical, Cleveland, Ohio).

Construction of pSC11-J-ORF and pSC11-J-P1. The vaccinia virus shuttle vector containing the HAV ORF was created by cleaving bp 735 to 7488 from pTF 34-7-1 (a baculovirus vector previously described) (42). Briefly, pTF 34-7-1 was constructed by cleaving the infectious HAV clone pHAV7 (6) with *Bam*HI. The 5.2-kb fragment from nt 2375 to 7488 was isolated and inserted into a *Bam*HI-*Bgl*II-restricted baculovirus transfer vector, which resulted in the loss of the 3′ *Bam*HI site (pTF 32-19-1). The 1.7-kb *Bam*HI fragment from nt 633 to 2375 was cloned into M13mp19. Oligonucleotide-directed mutagenesis (20) was used to insert a new *Sph*I site which maintained the ATG initiation codon of the HAV ORF. The only codon of the pHAV7 plasmid DNA (6) used for these studies which was changed by our cloning strategy resulted in the substitution of a histidine for an arginine at the predicted second amino acid of the HAV ORF. The resulting vector (pTF 33-6-1) was restricted with *Sph*I and *Bam*HI. The 1.6-kb HAV fragment was ligated to an *Sph*I-*Bam*HI-restricted pTF 32-19-1 containing HAV nt 2375 to 7488. This baculovirus vector (pTF 34-7-1) contained the HAV sequence from nt 735 to 7488. The HAV ORF coding sequence was removed from pTF 34-7-1 with *Sph*I and *Kpn*I and ligated into *Sph*I-*Kpn*I-digested pSC11-J (Fig. 1). Sequencing of all cloning sites was performed (Sequenase; United States Biochemical) to verify that no artifacts were generated during cloning.

A vector which contained the predicted coding region for the polyprotein precursor of the viral structural proteins (P1) was also constructed. Oligonucleotide-directed mutagenesis (20) was used to insert a translational termination codon (TAG) followed by a unique *Kpn*I site at the predicted 3′ end of P1, nt 3107 (6). The *Bam*HI-*Kpn*I fragment (nt 2375 to 3107) was ligated with a previously constructed vector which contained the HAV sequence from nt 735 to 2375. The resulting HAV sequence from nt 735 to 3107 was subcloned into pSC11-J, and the new vector pSC11-J-P1 was sequenced across the *Sph*I, *Bam*HI, and *Kpn*I sites (Fig. 1).

Selection of recombinant vaccinia viruses. Vaccinia virus recombinants were produced by using standard techniques (25). Recombinant viruses were selected for thymidine kinase-negative phenotype by using media containing 5'-bromodeoxyuridine and for expression of β-galactosidase activity. HeLa cells were infected with wild-type vaccinia virus (WR strain; American Type Culture Collection [ATCC], Rockville, Md.) by using a multiplicity of infection of 0.05. The infected cells were transfected with 8 or 16 μg of recombinant pSC11-J-ORF or pSC11-J-P1 DNA by using Lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.). The cells were incubated for 3 days, and cell lysates were obtained. Selective amplification in human TK-143 cells grown in media containing 5'-bromodeoxyuridine (50 μg/ml; Sigma, St. Louis, Mo.) was carried out, and recombinant viruses were plaque purified twice in TK-143 cells. Blue plaques were identified by overlaying the top agar with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (0.2 mg/ml in sterile phosphate-buffered saline [PBS]; Sigma). Viruses were selectively amplified twice in TK-143 cells and nonselectively amplified once in HeLa cells. Cells were lysed by freeze-thaw three times and sonicated for 20 s (60 Hz).

Cells. HeLa and MRC5 cells (ATCC) were grown in Eagle minimal essential medium (MEM) (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma) and with penicillin-streptomycin (100 U/ml–100 μg/ml). Human TK-143 cells (ATCC) were grown and maintained in Eagle MEM supplemented with 8% FCS and 50 μg of 5'-bromodeoxyuridine per ml. Vero cells (ATCC) were grown in Eagle MEM supplemented with 5% FCS and penicillin-streptomycin. BS-C-1 cells were grown as previously described (39). All cells were grown at 37°C with 5% CO_2 and humidity.

Virus. HM-175 HAV (23) and WR strain vaccinia virus (ATCC) were used for these studies.

Antibodies. Antisera raised against recombinant HAV fusion proteins were generously provided by Jeffrey Johnston and Thomas Powdrill, Burroughs Wellcome, Research Triangle Park, N.C. (17, 32). One serum sample recognized VP1 very well but VP3 only weakly (rabbit anti-trpE/HAV VP3,1), and another recognized VP0 (rabbit anti-trpE/HAV VP0). Murine anti-HAV monoclonal antibodies (K3-4C8, K2-4F2, and K3-2F2) were purchased from Commonwealth Serum Laboratories, Melbourne, Australia; B5-B3 antibody was a gift from Richard Tedder and Bridget Ferns; and MPO monoclonal antibody, which recognizes myeloperoxidase, was a gift from William Nauseef and served as a negative control monoclonal antibody. Human anti-HAV monoclonal antibody 3.2.4 was generously provided by Steven Day and Stanley Lemon, University of North Carolina, Chapel Hill, N.C. (8). Polyclonal human sera obtained from patients convalescing from hepatitis A (JC-pcAb and Mexico 6-pcAb) (40, 41) and nonimmune serum obtained from a healthy volunteer who was vaccinated in childhood with vaccinia virus (BV-niAb) were used in these studies.

Protein analysis. Cell lysates were electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Proteins were electrophoretically transferred onto nitrocellulose (Schleicher & Schuell, Keene, N.H.) in Tris-glycine-methanol buffer (44). Nitrocellulose was probed with antisera, and bound antibodies were detected with iodinated staphylococcal protein A followed by autoradiography (15).

Solid-phase RIAs. The ability of human polyclonal and murine monoclonal antibodies to bind HAV and rV-ORF lysates was determined by solid-phase radioimmunoassays (RIAs) as previously described (22).

Sucrose gradient analysis. HAV, rV-ORF, rV-P1, and wild-type vaccinia virus-infected cell lysates and uninfected HeLa cell lysates were layered onto a 7.5 to 45% (wt/vol) sucrose gradient in NT buffer (10 mM Tris, 100 mM NaCl [pH 7.2]). Gradients were centrifuged for 165 min at 215,000 × *g* and at 10°C (SW41 rotor; Beckman Instruments, Palo Alto, Calif.), and fractions (250 μl) were collected from the bottom and analyzed by RIA.

Electron microscope imaging. Fifty microliters of rV-ORF and wild-type vaccinia virus-infected cell lysates was incubated with 7.5 μl of human polyclonal anti-HAV serum (JC-pcAb) or nonimmune serum (BV-niAb) in a total volume of 75 μl for 1 h at 22°C followed by 14 h at 4°C. The samples were diluted in PBS (Sigma) and centrifuged at 105,000 × *g* for 30 min (SW41 rotor; Beckman Instruments). The pellets were resuspended in 50 μl of 0.1 M phosphate buffer, pH 7.4. Solid-phase immune electron microscopy was performed as described by Humphrey et al. (14). Briefly, carbon-coated Formvar grids (400 mesh) were applied to 25 μl of protein A (10 μg/ml in 0.1 M phosphate buffer, pH 7.4) for 10 min, washed in five changes of 0.1 M phosphate buffer (pH 7.4), and applied for 20 min to the antigen-antibody mixture described above. These grids were washed five times and stained with 2% phosphotungstic acid in water, pH 6.5, prior to examination with a Hitachi H7000 (Tokyo, Japan) electron microscope (Electron Microscope Facility, University of Iowa College of Medicine).

RESULTS

Immunoblot analysis. Expression of the HAV polyprotein and the P1 capsid precursor protein was assessed by immunoblot analysis. rV-P1-infected HeLa cell lysates demonstrated an immunoreactive 90-kDa protein which was recognized by both the anti-VP3,1 serum and the anti-VP0 serum (17, 32) (Fig. 2). This is consistent with the predicted molecular mass of the P1 polyprotein, 88,171 Da (IBI Pustell Sequence Analysis Program, version 2.02). rV-ORF-infected HeLa cell lysates demonstrated two proteins of 33 and 42 kDa which were recognized by the anti-VP3,1 sera (Fig. 2). The 33-kDa protein was of the same molecular mass as the capsid protein VP1 from HAV-infected BS-C-1 cell lysates (predicted molecular mass, 33,134 Da). Also present in the rV-ORF-infected HeLa cell lysates was a 42-kDa protein which comigrated with an antigenic protein in the HAV-infected BS-C-1 cell lysates. When probed with anti-VP0 serum (32), the rV-ORF lysate showed a 29-kDa protein which comigrated with VP0 of HAV. The anti-VP0 serum did not bind to the 42-kDa protein. VP0 and VP2 frequently comigrate in immunoblot studies. As with poliovirus, Anderson et al. demonstrated that subviral particles contain a predominance of VP0, whereas only mature virions contain VP2, suggesting that cleavage of VP0 to VP2 and VP4 requires RNA encapsidation (2). As described below, the rV-ORF virus contains empty capsids and pentamers, suggesting that the 29-kDa protein represents VP0. None of these proteins were recognized by antibodies from three different HAV antibody-negative human sera (data not shown).

Solid-phase RIA evaluation of rV-ORF. rV-ORF-infected cell lysates were evaluated for their antigenicity in solid-phase RIAs. Flasks (75 cm^2) of HeLa, MRC5, and Vero cells were infected with equal volumes of rV-ORF-infected TK-143 cell lysates and were maintained under identical condi-

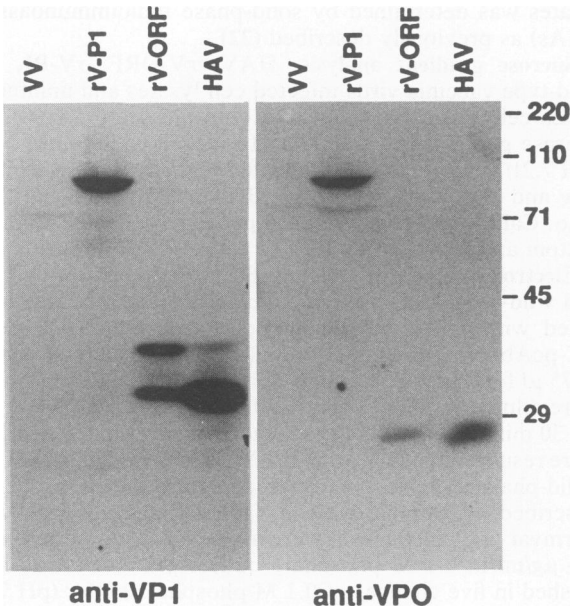

FIG. 2. Immunoblot analysis of HeLa cell lysates infected with wild-type vaccinia virus (VV), vaccinia virus containing the HAV P1 structural protein precursor (rV-P1), vaccinia virus containing the HAV ORF (rV-ORF), and BS-C-1 cell lysates infected with HM-175 strain HAV (HAV). Cell lysates were electrophoresed on SDS–10% polyacrylamide gels and electrophoretically transferred onto nitrocellulose. The left panel was probed with rabbit anti-trpE/HAV VP3,1 serum, which predominantly recognizes VP1 (17). The right panel was probed with rabbit anti-trpE/HAV VP0 serum (32). Molecular mass markers are indicated on the right in kilodaltons.

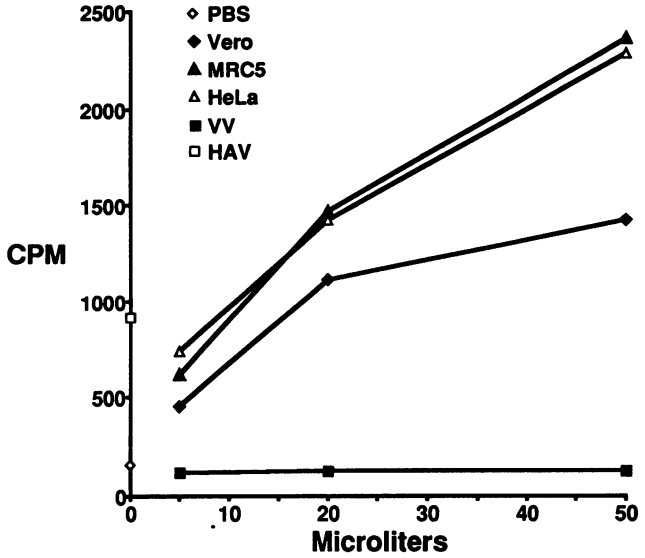

FIG. 3. Solid-phase RIA of rV-ORF-infected Vero, MRC5, and HeLa cell lysates. HeLa and Vero cell lysates which were infected with wild-type vaccinia virus were indistinguishable from the wild-type vaccinia virus-infected MRC5 infected-cell lysate (VV) shown. Human anti-HAV (JC-pcAb) serum was used as the capture antibody. Aliquots of 5, 20, or 50 μl of cell lysates were applied, and specific binding was recognized with ^{125}I-labelled human anti-HAV (Mexico 6-pcAb) serum. Residual bound counts are portrayed graphically. Controls included PBS and 10^5 $TCID_{50}$ of HAV. When serum from an HAV nonimmune individual was used as the capture antibody, none of these samples produced more than 200 cpm of bound ^{125}I.

tions. The infected cells were harvested at 72 h, and each cell pellet was resuspended in 1 ml of RPMI medium without FCS. Cells were lysed by freeze-thaw and sonication. Human convalescent serum (JC-pcAB) was applied to 96-well plates and, after extensive washing, 5-, 20-, and 50-μl samples of each cell lysate were added as previously described (40). Specific binding was detected by ^{125}I-labelled immunoglobulin G (IgG) from another human convalescent serum (Mexico 6-IgG). Previous studies have shown this serum to bind to cell culture-derived HAV in solid-phase RIA (40). PBS, wild-type vaccinia virus-infected cell lysates, and uninfected HeLa cell lysates served as the negative controls, and BS-C-1 cell culture-derived HAV served as the positive control antigen. Figure 3 demonstrates that HAV-specific antigen was detected in the rV-ORF-infected cells. Uninfected HeLa cell lysates yielded counts per minute of ^{125}I bound comparable to those of the wild-type vaccinia virus-infected HeLa cell lysates.

The bound antigen (in counts per minute) at various concentrations of each rV-ORF-infected cell lysate was determined and compared with that of BS-C-1 cell culture-derived HAV. rV-ORF (1.27×10^4 PFU) grown in Vero cells produced the same counts per minute of ^{125}I bound by HAV-specific antigen as did 1×10^5 $TCID_{50}$ of HAV by RIA. Likewise, 9.92×10^4 PFU of rV-ORF-infected MRC5 cells and 1.04×10^5 PFU of rV-ORF-infected HeLa cells contained a similar amount of antigen. Thus, by immunoassay the amount of HAV antigen produced by 1 infectious vaccinia virus unit was approximately equal to 1 infectious HAV unit. Denaturation of HAV- or rV-ORF-infected cell lysates by heating to 85°C for 1 or 5 min followed by immediate cooling to 4°C reduced the counts per minute (antigen) bound in the RIA by 31 and 97%, respectively (data not shown). Thus, the polyclonal anti-HAV sera appeared to recognize HAV antigen only in its native state. Nonimmune human serum (BV-niAb) did not capture antigen from the rV-ORF-infected cell lysates or the HAV-infected cell lysates (data not shown).

rV-ORF-infected cell lysates were also recognized by neutralizing murine and human monoclonal anti-HAV antibodies (Table 1). Monoclonal antibodies were able to capture rV-ORF and compete with polyclonal human IgG for binding to the rV-ORF antigen. These antibodies recognize functionally overlapping epitopes which contribute to the HAV immunodominant neutralization antigenic site (8, 41). Of note is that individual HAV capsid proteins and denatured HAV lysates have not been recognized by these monoclonal antibodies (13, 24, 41), suggesting that the rV-ORF-infected cell lysate contained assembled (conformational) HAV epitopes.

rV-P1-infected cell lysates were also evaluated by RIA, and conflicting results were obtained. Two of seven rV-P1 infections resulted in counts per minute of antigen bound greater than two times background counts, whereas five infections did not. Upon repeat testing (following additional cycles of freeze-thaw) and by using HAV monoclonal antibody to capture HAV antigen, these two infections were below the positive cutoff (defined as counts per minute for sample/counts per minute for negative control ≥ 2). However, the counts per minute of ^{125}I-labeled anti-HAV IgG bound to rV-P1 cell lysates relative to that of the negative

TABLE 1. Anti-HAV antibody recognition of HAV and recombinant vaccinia virus antigens

Capture antibody	cpm of antigen/cpm of control[a]				
	HAV	rV-ORF	rV-P1	VV	UI
Polyclonal[b]					
JC-pcAb	6.3	3.7	1.4	0.7	1.0
BV-niAb	0.9	1.0	1.4	0.7	0.8
Monoclonal[c]					
K3-4C8	3.6	2.2	1.1	0.9	1.0
K3-2F2	3.9	7.9	0.7	0.7	0.9
3.2.4	7.2	21.0	2.0	0.9	1.1
K2-4F2	4.7	7.7	0.7	1.0	0.8
B5-B3	4.4	3.1	0.7	0.7	0.7
MPO	0.8	1.2	0.5	0.7	0.9

[a] HAV antigen derived from infected BS-C-1 cell lysates which were partially purified by isopycnic banding in cesium chloride (41). HeLa cells (5×10^5) infected with rV-ORF, rV-P1, or wild-type vaccinia virus (VV) or uninfected (UI) cells served as the antigen source. Values represent counts per minute of sample divided by the counts per minute of the negative (PBS) control. A value of ≥2 was considered positive. Samples were run in duplicate, and have been confirmed on several occasions.

[b] HAV human convalescent serum (JC-pcAb) or HAV nonimmune human serum (BV-niAb) served as the capture antibody.

[c] Anti-HAV monoclonal antibodies from cell culture supernatant fluid (3.2.4, concentration 1 μg/ml) or ascitic fluid (K3-4C8, K3-2F2, K2-4F2, and B5-B3) were used as the capture antibody. HAV antibodies have been described previously (8, 24, 41). Anti-myeloperoxidase monoclonal antibody (MPO) served as a negative control capture antibody. Antibody concentrations were diluted to yield optimal capture of HAV.

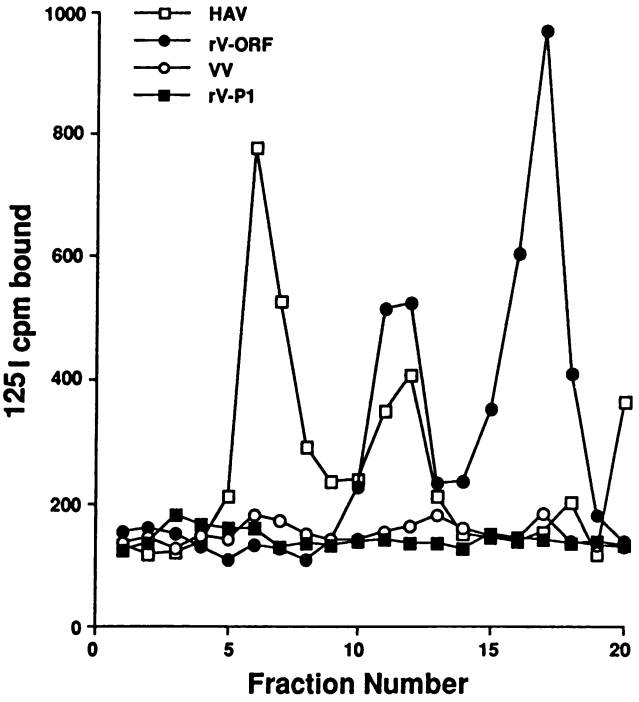

FIG. 4. Sucrose density gradient ultracentrifugation of HAV, rV-ORF, rV-P1, and wild-type vaccinia virus. Infected-cell lysates were prepared as described in Materials and Methods, layered onto 7.5 to 45% sucrose gradients, and centrifuged for 165 min. Fractions were collected from the bottom of the tube and assayed by RIA for HAV antigen. Equivalent numbers of vaccinia virus-infected cells were used in these studies.

(PBS) control was ≥1.4 in all seven infections. This level of binding has never been observed with wild-type vaccinia virus-infected or uninfected cell lysates, suggesting that there may be epitopes present on the P1 protein which may be weakly recognized by human HAV convalescent sera.

Sucrose density gradient analysis. To determine if the antigenic cell lysates contained viruslike structures, the sedimentation characteristics of the antigenic material were evaluated by sucrose gradient centrifugation. Cell lysates were sedimented over a 7.5 to 45% sucrose gradient for 165 min. RIA with human convalescent serum detected two major antigenic peaks in HAV-infected cell lysates with sedimentation coefficients of approximately 150S and 70S, consistent with those of virions and empty capsids (Fig. 4) (36, 37). Simultaneous gradients loaded with rV-ORF-infected cell lysates demonstrated two antigenic peaks with estimated sedimentation coefficients of approximately 70S and 15S. The latter antigenic peak sedimented between IgG (7S) and IgM (19S) markers run in simultaneous gradients. No antigenic material was detectable in the sucrose gradients of wild-type vaccinia virus-infected cell lysates or rV-P1-infected cell lysates. These data demonstrated that the rV-ORF-expressed polyprotein was processed and assembled into antigenic subviral particles. An alternative explanation is that the processed capsid proteins formed aggregates which sedimented at the same rate as HAV empty capsids.

Electron microscopy. To further characterize the products expressed in the rV-ORF-infected cells, immune electron microscopy was performed. Carbon-Formvar-coated copper grids (400 mesh) precoated with protein A were applied to rV-ORF- or vaccinia virus-infected cell lysates which had been precipitated with immune or nonimmune human sera (JC-pcAb or BV-niAb). Viruslike particles of approximately 25 nm were precipitated from the rV-ORF-infected cell lysates by immune sera; however, the rV-ORF-infected cell lysate incubated with nonimmune serum showed no evidence of viruslike particles (Fig. 5). Likewise, control grids of wild-type vaccinia virus-infected cells showed no evidence of virus particles (data not shown).

DISCUSSION

Several model expression systems have been used to generate picornavirus particles. Palmenberg successfully produced assembled encephalomyocarditis virus subviral particles in a cell-free translation of viral RNA (29). Similar experiments have been performed with foot-and-mouth disease virus (5). Attempts to generate HAV particles by using in vitro translation systems have not proven successful (11, 38a). A recombinant baculovirus system has recently been used to generate poliovirus empty capsids (47), and we have also used the baculovirus system to express the HAV polyprotein (42).

In this study we explored vaccinia virus expression of the HAV ORF. Immunoblot analysis of rV-ORF-infected cell lysates demonstrated that the major antigenic proteins were the same size as HAV capsid proteins. Only small quantities of high-molecular-mass precursors were seen, suggesting that processing of the HAV polyprotein was nearly complete in this system. It also appeared that processing intermediates were less common in mammalian cells than in the baculovirus expression system, although direct comparisons have not been undertaken. Cells infected with rV-ORF produced HAV antigen detected by solid-phase RIA. This antigen was recognized by human polyclonal anti-HAV sera and by

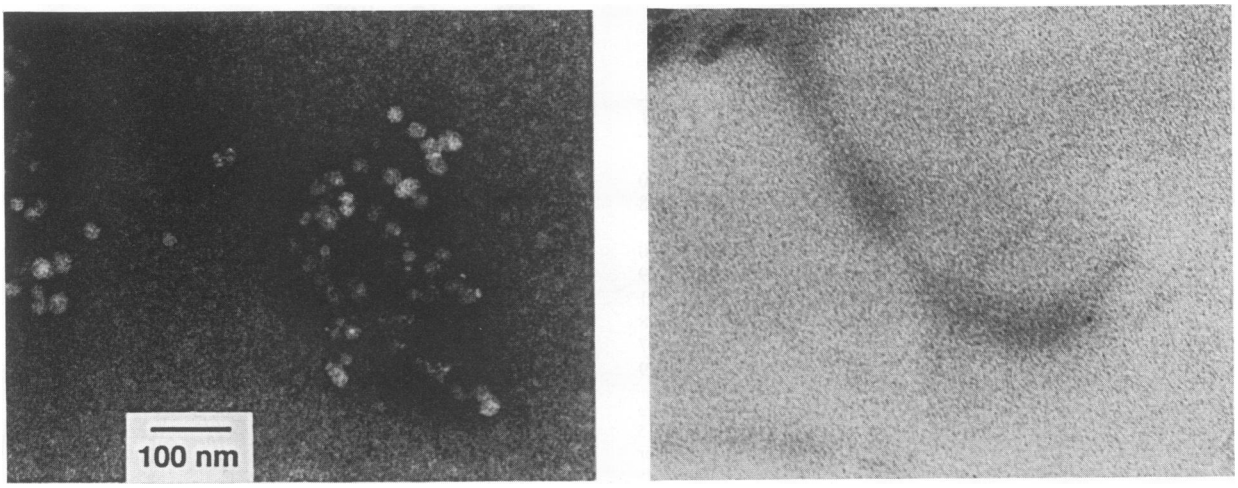

FIG. 5. rV-ORF-infected cell lysates were incubated with human polyclonal anti-HAV serum (JC-pcAb, left panel) or human nonimmune serum (BV-niAb, right panel). Carbon-Formvar-coated grids were pretreated with protein A and applied to precipitated rV-ORF-infected cell lysates. Grids were negatively stained with phosphotungstic acid. Magnification, ×60,000.

neutralizing murine and human monoclonal antibodies. Thus, the epitopes involved in HAV neutralization which form a major immunodominant HAV neutralization site (13, 41) were generated in the rV-ORF-infected cells. Additionally, sucrose gradient analysis demonstrated that the processed proteins assembled into particles with sedimentation coefficients consistent with those of empty capsids and pentamers (27). Vero and MRC5 cells have been used to produce clinically important vaccines (1, 35). Generation of HAV antigen in these cell lines by recombinant vaccinia virus may allow the development of a feasible recombinant-based HAV vaccine.

A 42-kDa protein which reacted with anti-VP3,1 sera was detected in the rV-ORF-infected cell lysates. This protein was not recognized by the anti-VP0 serum. Anderson and Ross described a 40-kDa protein, titled PX, which was present in HAV subviral particles (2). They postulated that this protein is a precursor to VP1 which most likely represents VP1 plus a truncated 2A (1D2A) and speculated that primary processing of the HAV polyprotein occurs at 2A-2B, similar to that seen with encephalomyocarditis virus and foot-and-mouth disease virus. Thus, HAV 2A protein may be smaller than predicted (6). Cho and Ehrenfeld also detected a 38-kDa protein in HAV-infected cells which reacted with anti-VP1 sera (4). We believe that the 42-kDa protein demonstrated in our rV-ORF-infected cell lysates corresponds to the PX or 38-kDa protein, and further characterization of this protein is under way.

rV-ORF contains the entire HAV genome except for the 5′ nontranslated region. Sequence analysis of different HAV strains has demonstrated that the 5′ noncoding region is the most highly conserved region in the HAV genome (16). The 5′ noncoding region of poliovirus is thought to be important for efficient translation and virulence (43, 46), and deletion mutations in the 5′ noncoding region of poliovirus demonstrated that the 5′ noncoding region is essential for production of infectious virions (19). Additionally, Urakawa et al. reported that a recombinant baculovirus which contained poliovirus cDNA lacking the 5′ noncoding region expressed polioviruslike empty capsids. These particles did not contain RNA and were noninfectious (47). By deleting nt 1 to 734 in our vaccinia virus construct, infectivity should be abolished. This was difficult to prove in the recombinant vaccinia virus system, as vaccinia virus-induced cytopathic effect prevented HAV infectivity measurements. However, HAV RNA (assessed by cDNA-RNA dot hybridization analysis) (41) was not detected in rV-ORF gradient fractions exhibiting peak levels of HAV antigen (data not shown).

Comparison of HAV-specific antigen from rV-ORF-infected cell lysates with HAV suggested that the antigen yield from 1 infectious vaccinia virus unit was approximately equal to 1 infectious HAV unit. The vaccinia virus early-late p7.5 promoter directs the synthesis of foreign proteins for approximately 24 to 48 h, and production of recombinant vaccinia virus peaks at approximately 72 h. Vaccinia virus titers of 10^8 PFU/ml are easily achieved in 72 h. HAV, on the other hand, requires 2 to 3 weeks of cell culture, and titers of 10^6 to 10^7 are common. Therefore, recombinant vaccinia viruses are potentially capable of producing greater antigen yields than HAV in cell culture in a significantly shorter period of time. The subviral particles produced may be useful as an antigen source for diagnostic assays or potentially as an immunogen for a recombinant-based HAV vaccine.

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