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Morphogenesis of Hepatitis A Virus: Isolation and Characterization of Subviral Particles

DAVID A. ANDERSON* AND BRUCE C. ROSS

Macfarlane Burnet Centre for Medical Research, Fairfield Hospital, Yarra Bend Road, Fairfield, Victoria 3078, Australia

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The morphogenesis of hepatitis A virus (HAV) in BS-C-1 cells was examined by immunoblotting with antisera to capsid proteins and labeling of virus-specific proteins with L-[^{35}S]methionine. Antiserum to VP2 detected two virus-specific proteins with apparent molecular masses of 30.6 and 30 kDa, representing VP0 and VP2, while antiserum to VP1 detected proteins with molecular masses of 33 and 40 kDa, representing VP1 and a virus-specific protein which we designated PX, respectively. Sedimentation of cell lysates revealed the presence of virions, procapsids, and pentamers, but particles analogous to the protomers of other picornaviruses were not detected. Although provirions and virions were not found as discrete species in our gradient system, it was evident that the rate of sedimentation was proportional to the relative amounts of VP0 and VP2 in particles, with slower-sedimenting particles (provirions) containing predominately VP0 rather than VP2. Procapsids contained VP0 in addition to VP1 and VP3. Pentamers also contained VP0, but PX was present rather than VP1. These results suggest that PX is a precursor to VP1 and is most likely 1D2A. Primary cleavage of the viral polyprotein also occurs at the 2A-2B junction in cardioviruses and aphthoviruses, but assembly of pentamers containing 1D2A has not been reported for those viruses. The absence of detectable levels of protomers suggests a high efficiency of pentamer formation, which may be related to the high efficiency of viral RNA encapsidation for HAV (D. A. Anderson, B. C. Ross, and S. A. Locarnini, J. Virol. 62:4201–4206, 1988). The results of this study reveal further unusual aspects of the HAV replicative cycle which distinguish it from other picornaviruses and may contribute to its restricted replication in cell culture.

Picornaviruses are among the most extensively studied animal viruses. They are often characterized by rapid and efficient replicative cycles in permissive cells, with production of 10^5 particles per cell within 8 h of infection being common (15). Hepatitis A virus (HAV) has a number of characteristics which distinguish it from other picornaviruses, such as extreme thermal stability (1, 18), a tendency to establish persistent infections of cell cultures (6, 8, 9, 13, 17, 20, 21), and a protracted replicative cycle which produces relatively little virus. In addition, while its physical characteristics are most like those of enteroviruses, its greatest nucleotide sequence homology appears to be shared with the cardioviruses (12). Studies of the replicative cycle of HAV (2, 3, 6, 9, 21) have provided further evidence for the divergence of HAV from other picornaviruses.

We have previously used nucleic acid probes to detect HAV-specific RNA in cells (2, 3), and those studied showed that most of the viral RNA is present within mature virions throughout the replicative cycle. On the basis of this finding, we have proposed a model for the restriction of HAV replication whereby progeny positive-strand RNA molecules are preferentially encapsidated rather than being used as templates for further RNA synthesis throughout the replicative cycle. We believe that this may be related to the thermal stability of the virus, reflecting a high affinity of the viral capsid proteins for one another and/or viral RNA. This hypothesis also predicts an excess of capsid protein precursors in the cell to allow sequestration of the RNA into virions and that capsid precursors have a high affinity for one another and/or viral RNA.

To test these predictions, we used immunoblot analysis and metabolic labeling to examine the morphogenesis of HAV in infected cells. The results are consistent with our hypothesis but also suggest that the proteolytic processing pathway of HAV is similar to that of the cardioviruses (10) and aphthoviruses (19), in which primary cleavage occurs at the junction of 2A and 2B (by the L434 nomenclature of Rueckert and Wimmer [16]).

MATERIALS AND METHODS

Cells and viruses. Cytopathic HAV strain HM175A.2 and poliovirus type 1 (PV; Mahoney) were propagated in BS-C-1 cells as described previously (1), except that the incubation temperature was 37°C. Stocks of infectious virus were prepared from cells as follows. Infected cell monolayers were dispersed with a mixture of 0.125% trypsin–0.02% EDTA, washed with Eagle minimum essential medium (MEM) containing 5% fetal bovine serum, and pelleted by centrifugation for 1 min at 6,500 rpm in an MSE microcentrifuge. The cell pellet was suspended in 5 volumes of NT (100 mM NaCl, 10 mM Tris [pH 7.4]), and 10% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 1% to lyse the cell membranes. After brief mixing at 24°C, the nuclei were removed by centrifugation at 13,000 rpm in the MSE microcentrifuge for 2 min and 10% *N*-lauroylsarcosine, sodium salt (Sarkosyl; Sigma), was added to the cytoplasmic extract to a final concentration of 1%. Six-milliliter cell lysate samples were layered on top of discontinuous sucrose-glycerol gradients in Beckman SW41 tubes prepared by layering 0.5 ml of 80% (vol/vol) glycerol in 100 mM Tris (pH 7.4), 2 ml each of 30 and 20% (wt/vol) sucrose in NT, and 2 ml of 10% sucrose in NT containing 1% sodium dodecyl sulfate (SDS). After centrifugation at 37,000 rpm (170,000 × *g*) for 5 h at 18°C, virus in the glycerol cushion (total volume, 1 ml) was collected by puncturing the bottom of the tube and this purified virus preparation was stored in aliquots at −70°C.

* Corresponding author.

Infectious virus was quantitated by plaque assay as described previously (3). Virus inocula were prepared by extraction of purified virus with an equal volume of chloroform, followed by dilution in MEM containing 1% fetal bovine serum. Pools of HAV prepared as described typically contained 6×10^9 PFU/ml.

Single-cycle infection of cells. Monolayers of BS-C-1 cells were inoculated with 10 PFU of HM175A.2 or PV per cell for 1 h at 37°C, washed with 0.85% NaCl, and maintained in MEM at 37°C. The cells were harvested by addition of 0.5 ml of NPT (100 mM NaCl, 0.5% Nonidet P-40, 10 mM Tris [pH 7.4]) per 75 cm^2 of cell monolayer (approximately 2×10^7 cells). Nuclei were removed by centrifugation as described above, and SDS was added to the cytoplasmic extract to a final concentration of 1%.

Labeling of proteins with [^{35}S]methionine. Cells were fed methionine-free MEM (Flow Laboratories, Inc., McLean, Va.) for 3 h and then similar medium containing 150 μCi of L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham International plc, Amersham, England) per ml for a further 3 h. Incorporation was stopped by addition of MEM containing 2 mM methionine. All cultures were harvested and processed as described above.

Sucrose density gradient ultracentrifugation. Ultracentrifugation was performed in a Beckman SW41 rotor at 4°C with 0.5-ml samples. Gradients were either 5 to 30% (wt/vol) sucrose in NT, which were centrifuged for 3 h at 35,000 rpm (150,000 × *g*) for isolation of virions and procapsids, or 5 to 20% (wt/vol) sucrose in NT, which were centrifuged for 18 h at 37,000 rpm (170,000 × *g*) for isolation of smaller subviral particles. Fractions of 0.5 ml were collected from the bottom of each tube and analyzed for viral RNA and proteins.

Hybridization of viral RNA. Positive-strand HAV RNA was detected in gradient fraction samples by hybridization with ^{32}P-labeled RNA probes as described previously (3). Probes were transcribed by using the T7 promoter of plasmid pGEMHAV digested with *Hin*dIII, and they represented sequences from 2.0 to 5.0 kb of the HAV genome.

Electrophoresis. Gradient fraction samples (typically, 0.4 ml) were mixed with 20 μl of 0.05% bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) and 9 volumes of methanol at −20°C. After incubation overnight at −20°C, the protein precipitate was collected by centrifugation at 2,500 rpm in an MSE Mistral 4L centrifuge at −10°C and dried under vacuum. The pellet was dissolved in 30 μl of LB (2% SDS, 8% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol, 50 mM Tris [pH 6.8]). Crude cell lysates were mixed with 0.25 volume of 5× LB, and samples were boiled for 5 min before electrophoresis in SDS-polyacrylamide gels containing 12% acrylamide with the Laemmli buffer system (11). Resolution of HAV-specific proteins was facilitated by inclusion of urea in the gels at a final concentration of 3.5 M (submitted for publication), but urea was omitted from gels of PV proteins, as it caused compression of some bands. Electrophoresis and electrophoretic transfer of proteins were performed with the Mini Protean system (Bio-Rad Laboratories, Richmond, Calif.). After electrophoresis, gels containing radiolabeled proteins were fixed in 10% acetic acid–20% methanol and dried for autoradiography.

Immunoblot analysis of HAV proteins. Proteins for immunoblot analysis were transferred to nitrocellulose (HyBond C-extra; Amersham) filters for 2 h at 60 V in a buffer of 20% methanol–0.15 M glycine–25 mM Tris (pH 7.5). The filters were dried at room temperature and incubated for 1 h at 37°C with shaking in 0.1 M Tris (pH 7.5) containing 3% (wt/vol) casein. Rabbit antisera raised to recombinant proteins containing sequences of VP1 and VP2 of HAV (14) were diluted in TBT (0.05% Tween 20, 0.1 M Tris [pH 7.5]) containing 1% casein and incubated with the filters for 1 h at 37°C. The filters were then washed six times for 5 min each time in TBT with gentle agitation. Antiserum to rabbit immunoglobulin G (Bio-Rad) was iodinated and diluted to 10^6 cpm/ml in TBT containing 1% casein and incubated with the filters for 1 h. After being washed six times with TBT, the filters were dried and exposed to preflashed X-Omat RP film (Eastman Kodak Co., Rochester, N.Y.) at −70°C between Cronex Lightning-Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) or at room temperature without screens.

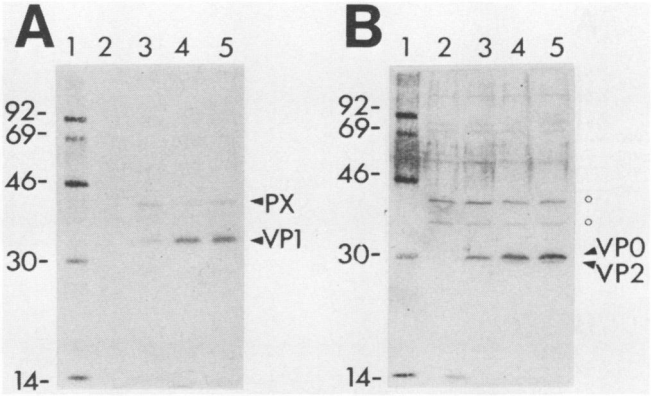

FIG. 1. Accumulation of HAV-specific proteins in BS-C-1 cells. Cells were infected with 10 PFU of HM175A.2 and harvested after 24 (lane 3), 48 (lane 4), or 72 (lane 5) h or mock infected and harvested after 72 h (lane 2). After electrophoresis in the presence of 3.5 M urea, proteins were transferred to nitrocellulose and immunoblotted with antiserum specific to VP1 (A) or VP2 (B) as described in Materials and Methods. Two cellular proteins which were detected by anti-VP2 serum in infected and uninfected cells are marked with circles. The molecular masses of marker proteins (lane 1) are indicated in kilodaltons.

RESULTS

Detection of viral proteins in infected cells. Accumulation of HAV-specific proteins in infected cells during a single growth cycle was examined by immunoblot analysis using antisera raised to recombinant HAV proteins (14) to detect VP1 and VP2 (Fig. 1). Antisera to VP2 (Fig. 1B) detected two virus-specific proteins (lanes 3 to 5) migrating as a doublet with apparent molecular masses of 30.6 and 30 kDa. The slower-migrating protein, which we believe to be VP0, was present in considerable excess throughout the 3-day study, while VP2 (30 kDa) was first detected at 48 h postinfection and was the minor band at all times. Two additional proteins were detected by this antiserum in both uninfected (lane 2) and infected cells, and they presumably represent cross-reactivity with the large excess of cellular protein present on the filter. This reactivity was not removed by absorption of antisera with acetone-fixed BS-C-1 cells (data not shown).

Antiserum to VP1 (Fig. 1A) detected two virus-specific proteins (lanes 3 to 5). The faster-migrating protein with a molecular mass of 33 kDa probably represents VP1, but the slower-migrating protein with a molecular mass of 40 kDa was not identified in a previous study using these antisera to characterize mature virions (submitted for publication). This

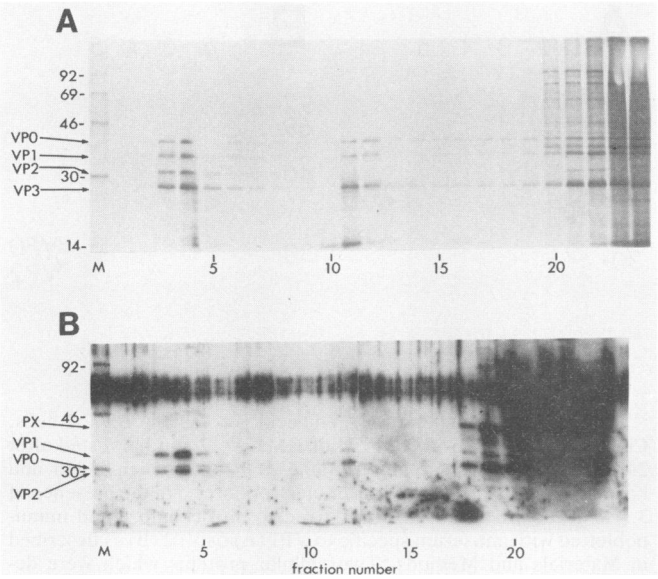

FIG. 2. Sucrose density gradient ultracentrifugation of intracellular viral proteins. Cell lysates were centrifuged at 150,000 × *g* for 3 h at 4°C on linear 5 to 30% sucrose gradients in Beckman SW41 tubes. Fractions of 0.5 ml were collected from the bottom of each tube, and proteins were precipitated with methanol and separated by SDS-polyacrylamide gel electrophoresis. (A) PV-infected cells labeled with [^{35}S]methionine from 3 to 7 h postinfection. (B) HAV-infected cells harvested at 48 h postinfection and virus-specific proteins detected with a mixture of antisera to VP1 and VP2 as described in Materials and Methods. M, Molecular mass markers (sizes are indicated at the left in kilodaltons). Sedimentation was from right to left.

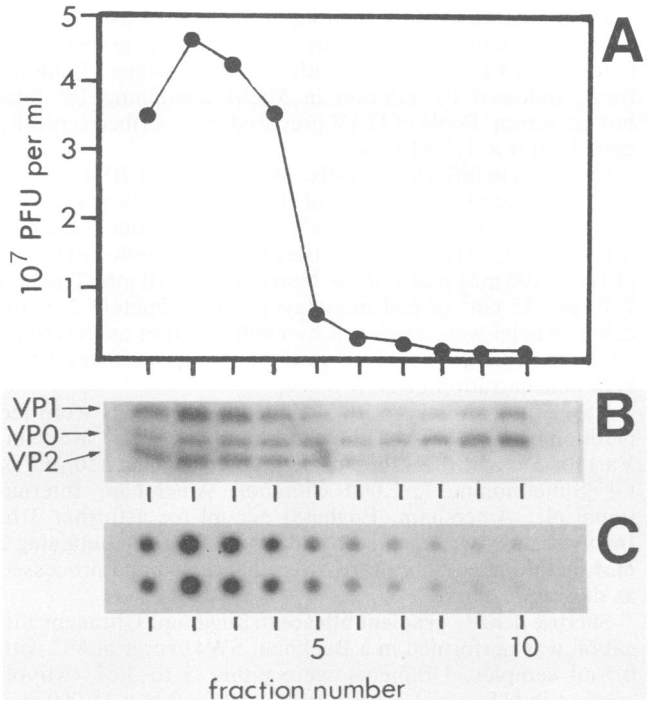

FIG. 3. Sucrose density gradient ultracentrifugation of intracellular infectious virus, viral proteins, and RNA. HAV-infected cells were harvested at 48 h postinfection, cell lysates were centrifuged, and fractions were collected as described in the legend to Fig. 2. Panels: A, infectious virus; B, viral capsid proteins; C, positive-strand RNA detected as described in Materials and Methods. Only the first 10 fractions from the bottom of the tube are shown.

suggests that this protein, which we have designated PX, is a precursor of VP1. The kinetics of accumulation of PX and VP1 are also consistent with a precursor-product relationship, as the level of PX did not increase beyond 24 h postinfection, while the level of VP1 increased approximately fivefold from 24 to 72 h postinfection, consistent with the processing of PX to VP1.

Isolation of virions and procapsids. To examine the distribution of viral proteins in more detail, lysates of cells infected with HAV or PV were sedimented over sucrose density gradients to separate viral and subviral particles. When the cell lysates were sedimented over 5 to 30% sucrose gradients for 3 h, four pools of virus-specific particles were detected in PV-infected (Fig. 2A) and HAV-infected (Fig. 2B) cells. As expected on the basis of published studies (reviewed by Rueckert [15]), PV-infected cells contained virions (VP1, VP2, VP3, and traces of VP0; fractions 2 to 5), provirions (VP1, VP0, VP3, and traces of VP2; fractions 6 to 9), procapsids (VP0, VP1, and VP3; fractions 11 to 13), and slower-sedimenting particulate material (containing VP0, VP1, and VP3; fractions 18 to 20) which was poorly resolved from nonparticulate proteins under these conditions. Similarly, HAV-infected cells (Fig. 2B) contained virions sedimenting at 160S containing VP1 and a mixture of VP0 and VP2 (fractions 2 to 4), together with slower-sedimenting species containing VP1, VP0, and traces of VP2 (fraction 5) or PX and VP0 (fractions 6 and 7). These particles will be referred to as provirions (7) (containing VP1 and VP0) and preprovirions (containing PX and VP0). It should be noted that the proportion of VP2 to VP0 appeared to be greatest in the fastest-sedimenting particles. A discrete peak of empty capsids (procapsids; fractions 11 to 13) sedimenting at 70S and containing VP1 and VP0 was detected. A fourth species, sedimenting slightly faster than the bulk of cellular protein and containing PX, VP0, and traces of VP1 (fractions 18 to 20), was detected in these gradients. Note that the antiserum used in these immunoblots does not detect VP3 but VP3 was detected in virions and procapsids by labeling in cell culture (see below).

Association of RNA and infectivity with particles. The distribution of viral RNA and infectivity was examined in gradients of cell lysates similar to those shown in Fig. 2, with samples of each gradient fraction being assayed for viral positive-strand RNA by dot blot hybridization and for infectious virus by plaque assay, in addition to immunoblotting of viral proteins. The particles sedimenting at 70S and containing VP0 and VP1 contained no RNA and were not infectious, confirming their identity as procapsids or empty capsids, while both virions (fractions 1, 2, and 3, containing VP1 and predominately VP2) and provirions (fractions 4 to 7, containing VP1 and predominately VP0) contained RNA and were infectious (Fig. 3). Preprovirions (containing PX and VP0) were not detected in this experiment, and their infectivity has yet to be established.

Isolation of pentamers. The material near the tops of gradients (e.g., Fig. 2) was resolved more effectively when centrifugation was extended to 18 h over 5 to 20% sucrose gradients (Fig. 4). Under these conditions, virions and procapsids were recovered as a pellet (P) which contained VP1, VP0, and VP2 (not visible in this autoradiograph). The presence of an excess of VP0 over VP2 in the pellet was consistent with the sum of these proteins from virions,

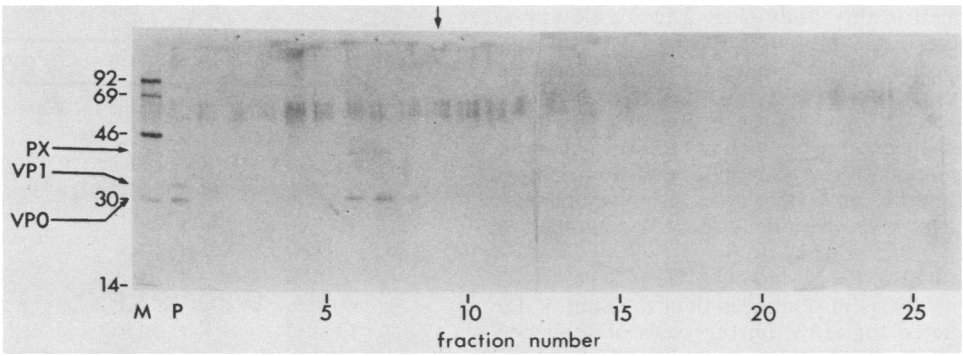

FIG. 4. Sucrose density gradient ultracentrifugation of subviral HAV-specific structural proteins. Cell lysate was centrifuged at 170,000 × *g* for 18 h at 4°C on linear 5 to 20% sucrose gradients in Beckman SW41 tubes. Fractions were collected and analyzed as described in the legend to Fig. 2. M, Molecular mass markers (sizes are given at the left in kilodaltons); P, suspended pellet. The vertical arrow marks the position of catalase (10S) in a parallel gradient.

provirions, and procapsids of HAV but varied between experiments. Only one other virus-specific species, containing VP0 and PX, was detected. By comparison with catalase (10S) in a parallel gradient, this material was estimated to have a sedimentation coefficient of approximately 14S to 17S, which suggests that the particles are analagous to the pentamers described for other picornaviruses (15). Unexpectedly, no protomers (5S) or free capsid proteins were detected in any of our experiments. Although not visible in this exposure, traces of VP1 were sometimes detected in the pentameric HAV material (for example, fractions 18 to 20 of Fig. 2B).

Labeling of HAV-specific proteins in cell culture. As the immunoblot analyses do not allow a study of the kinetics of viral assembly, we investigated the labeling of HAV-specific proteins with L-[^{35}S]methionine in infected cells to establish conditions for such a study. Monolayers of BS-C-1 cells in 3-cm-diameter petri dishes were infected with 10 PFU of HAV per cell and labeled with 150 μCi of [^{35}S]methionine per ml from 15 to 18 h postinfection. Cells were harvested after 4 or 24 h of a cold chase, and lysates were analyzed by ultracentrifugation and SDS-polyacrylamide gel electrophoresis (Fig. 5). When cells were harvested after a 4-h chase, very little labeled virus-specific protein was detected (Fig. 5, lanes 2 to 7), although most of the intracellular positive-strand RNA was present within virions at that time (data not shown), as has been reported previously (3). In contrast, after a 24-h chase (lanes 8 to 13), significant amounts of virions and provirions (lanes 8 to 10) and procapsids (lanes 11 to 13) were detected. HAV virions contained VP1, VP0, and VP2, as shown earlier, as well as VP3 (27 kDa); which was not detected in our immunoblot system. Faster-sedimenting particles also contained higher proportions of VP2 to VP0 (Fig. 5, compare lanes 8 and 10). The relative intensities of the bands are consistent with the number of methionine residues predicted from the nucleotide sequence of the parental HM175 strain reported by Cohen et al. (5; 8 in VP1, 3 in VP0, 2 in VP2, and 7 in VP3). VP1 migrated as a doublet in some experiments, but the reason for this is not known. It is not clear whether the labeled protein migrating at approximately 38 kDa is virus specific.

Samples of labeled cell lysates were also centrifuged for 18 h to allow isolation of pentamers, but under these conditions, it was not possible to detect labeled HAV-specific proteins against the background of labeled host cell proteins (data not shown).

DISCUSSION

The detection of viral macromolecules within cells is a prerequisite for a detailed understanding of viral replication, and detection of HAV RNA by hybridization with radiolabeled probes has been used extensively for this purpose (2, 3, 6, 9). Studies of HAV-specific proteins have been limited to detection of viral antigens rather than individual proteins (2, 6, 17, 21), but with the development of immunoblot assays for individual HAV proteins (9, 14; submitted), it is now possible to detect the capsid proteins irrespective of their conformation within the cell. This report provides the first description of the synthesis and assembly of viral capsid proteins in cells infected with HAV.

We have previously reported that restriction of HAV replication is due to inefficient RNA replication (2, 3) and suggested that this was a result of efficient encapsidation of progeny positive-strand RNA at the expense of the replicative pool (3). One prediction of this hypothesis, that there must be a pool of excess capsid proteins within infected

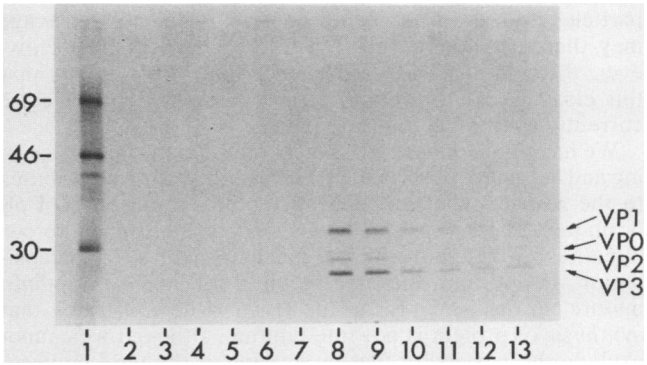

FIG. 5. Sucrose density gradient ultracentrifugation of HAV labeled with [^{35}S]methionine. Cells were infected with 10 PFU of HM175A.2 per cell and labeled with [^{35}S]methionine from 15 to 18 h postinfection. Incorporation of the label was stopped by addition of excess cold methionine, and cell lysates were centrifuged as described in the legend to Fig. 2. Adjacent fractions were pooled for analysis, and only the first 12 fractions are shown. Lanes: 1, molecular weight markers (sizes are indicated to the left in kilodaltons); 2 to 7, fractions 1 plus 2 to 11 plus 12, respectively, of cells harvested after a 4-h chase; 8 to 13, fractions 1 plus 2 to 11 plus 12, respectively, of cells harvested after a 24-h chase.

cells, was confirmed in this study (Fig. 2 to 4). However, detection of virus-specific protein PX (Fig. 1) was unexpected. We suggest that this protein is a precursor of VP1 on the basis of (i) reactivity with antiserum raised to recombinant VP1 protein (14) (Fig. 1); (ii) the presence of PX in pentamers and some provirions in which VP1 was absent or present in trace amounts (Fig. 2 and 4); (iii) the kinetics of its accumulation within the cell, where it ceased to accumulate by 24 h postinfection while the amount of VP1 continued to increase (Fig. 1).

With a mass of 40 kDa, PX is unlikely to be 1CD (16) (VP1 plus VP3, 60 kDa). PX is also smaller than the sum of 1D plus the 2A predicted for HAV on the basis of sequence alignments with PV (5; total mass, approximately 53 kDa), but the limited homology between HAV and other picornaviruses leaves this prediction open to question. We suggest instead that HAV 2A has a mass of approximately 7 kDa, with primary cleavage of the polyprotein occurring at the 2A-2B junction, as has been demonstrated for cardioviruses (10) and aphthoviruses (19).

Although such a 2A would be much smaller than the corresponding protease in enteroviruses and cardioviruses, it has been reported that the 16-amino-acid 2A of aphthoviruses retains activity (4). However, whereas aphthovirus 2A is highly homologous to a region of cardioviral 2A which has therefore been proposed as the active motif (4), this motif is not present in the sequence of HAV. As a result, we cannot exclude the possibility that cellular proteases or sequences distal to the putative 2A in PX are involved in the processing of the polyprotein and/or PX. Definitive identification of PX may depend on demonstration of self-cleavage activity or modification of the 1D2A junction to abolish its processing to VP1. It would be interesting to see whether such a mutant assembled large amounts of preprovirions containing PX rather than VP1, like those detected in small amounts in this study (Fig. 2B, fractions 6 and 7).

The relative proportions of VP0 and VP2 in HAV virions and provirions (Fig. 2, 3, and 5) may be relevant to the protracted uncoating which has been observed for HAV (2, 21). It appears on the basis of the increased sedimentation of virions containing higher proportions of VP2 that cleavage of VP0 results in some conformational change, yet all of these particles appear to be equally infectious and this cleavage may therefore not be required for receptor binding. However, the role of VP4 in virion uncoating (15) suggests that this cleavage is a prerequisite for disassembly, and we are currently examining that possibility.

We have no evidence to suggest that the unusual processing and assembly observed for HAV in this study contributes to the restriction of its replication, but the absence of 5S promoters in HAV-infected cells lends some support to our hypothesis of a highly efficient mechanism of RNA encapsidation. In addition, the time required for label to flow into mature virions and procapsids (Fig. 4) demonstrates that synthesis of protein is not rate limiting, although we cannot exclude the possibility that processing of the viral polyprotein is a limiting factor.

The morphogenic pathway of HAV outlined in this report may prove to be a useful model for studying picornavirus assembly despite the small yield of virus-specific products in cells. In particular, the disparity between the protein contents of procapsids (VP0, VP1, and VP3) and pentamers (VP0, PX, and presumably VP3) should allow the true precursor of virions to be determined, as procapsids and pentamers cannot be in equilibrium in this system. The presence of a small quantity of preprovirions containing PX rather than VP1 (Fig. 2B, fractions 6 and 7) suggests that pentamers are the precursor for virion assembly in HAV-infected cells.

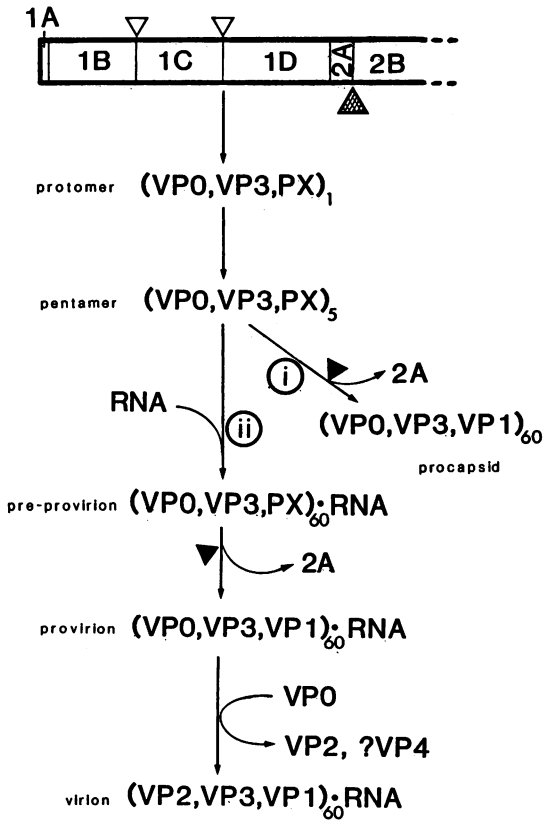

FIG. 6. Model of processing and assembly of HAV capsid proteins. The polyprotein is initially cleaved at the 2A-2B junction by an unknown protease (▲), yielding P12A. Cleavage between 1AB-1C (VP0-VP3) and 1C-1D (VP3-VP1) is probably catalyzed by $3C^{pro}$ (△), yielding protomers containing VP0, VP3, and PX (1D2A). These are rapidly assembled into pentamers, which may be assembled into procapsids with the loss of 2A (i) or with RNA into preprovirions (ii) containing PX, VP0, and VP3. Cleavage of PX by an unknown protease (▲) yields provirions, and autocatalytic cleavage of VP0 to VP2 and VP4 generates the mature virion containing VP1, VP2, and VP3; VP4 has not been detected in the virion and may be lost from the particle.

On the basis of these observations, we propose a model for the morphogenesis of HAV (Fig. 6). Primary cleavage of the polyprotein occurs at the junction of 2A and 2B, yielding 1ABCD2A, and 3C then cleaves between 1B-1C and 1C-1D, yielding 1AB (VP0), 1C (VP3), and 1D2A (PX), which remain associated as a protomer. These protomers are rapidly assembled into pentamers, which may then be assembled in either of two mutually exclusive pathways, i.e., (i) into empty capsids with loss of 2A yielding 1D (VP1) or (ii) with viral RNA into preprovirions containing RNA and 60 copies each of PX, VP0, and VP3, followed by loss of 2A to yield provirions and cleavage of VP0 to VP2 and VP4 to form mature virions containing RNA and VP1, VP2, and VP3. Further studies are required to determine the fate and function of the small VP4 peptide liberated from VP0, the protease(s) responsible for the cleavages of the polyprotein, and the identity and function of the proposed 2A residues in PX.

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