JOURNAL OF VIROLOGY, Oct. 1994, p. 6147–6160
0022-538X/94/$04.00+0

Vol. 68, No. 10

Formation and Intracellular Localization of Hepatitis C Virus Envelope Glycoprotein Complexes Expressed by Recombinant Vaccinia and Sindbis Viruses

JEAN DUBUISSON,[1]† HENRY H. HSU,[2] RAMSEY C. CHEUNG,[2] HARRY B. GREENBERG,[2] DAVID G. RUSSELL,[1] AND CHARLES M. RICE[1]*

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093,[1] and Division of Gastroenterology, Stanford University School of Medicine, Palo Alto VA Medical Center, Palo Alto, California 94304[2]

Received 5 May 1994/Accepted 28 June 1994

Hepatitis C virus (HCV) encodes two putative virion glycoproteins (E1 and E2) which are released from the polyprotein by signal peptidase cleavage. In this report, we have characterized the complexes formed between E1 and E2 (called E1E2) for two different HCV strains (H and BK) and studied their intracellular localization. Vaccinia virus and Sindbis virus vectors were used to express the HCV structural proteins in three different cell lines (HepG2, BHK-21, and PK-15). The kinetics of association between E1 and E2, as studied by pulse-chase analysis and coprecipitation of E2 with an anti-E1 monoclonal antibody, indicated that formation of stable E1E2 complexes is slow. The times required for half-maximal association between E1 and E2 were 60 to 85 min for the H strain and more than 165 min for the BK strain. In the presence of nonionic detergents, two forms of E1E2 complexes were detected. The predominant form was a heterodimer of E1 and E2 stabilized by noncovalent interactions. A minor fraction consisted of heterogeneous disulfide-linked aggregates, which most likely represent misfolded complexes. Posttranslational processing and localization of the HCV glycoproteins were examined by acquisition of endoglycosidase H resistance, subcellular fractionation, immunofluorescence, cell surface immunostaining, and immunoelectron microscopy. HCV glycoproteins containing complex N-linked glycans were not observed, and the proteins were not detected at the cell surface. Rather, the proteins localized predominantly to the endoplasmic reticular network, suggesting that some mechanism exists for their retention in this compartment.

Hepatitis C virus (HCV) is a major causative agent of non-A, non-B hepatitis (15, 42). It is associated with the vast majority of cases of posttransfusion hepatitis and a significant proportion of community-acquired hepatitis worldwide (1, 2, 47). HCV infection usually results in a chronic infection, and a range of clinical conditions are observed (reviewed in reference 36), including a strong association with the development of hepatocellular carcinoma (reviewed in reference 68).

Since the initial molecular cloning of this agent, sequence data for a number of independent HCV isolates have been reported, and our knowledge of HCV molecular biology is advancing rapidly (reviewed in references 37 and 49). HCV is similar in genomic organization to the pestiviruses (16) and the flaviviruses (13), and these groups have recently been classified as three genera in the family *Flaviviridae* (24). HCV is an enveloped virus, and its genome is composed of a single-stranded, positive-sense RNA containing a long open reading frame of 9,030 to 9,099 nucleotides. This open reading frame is translated as a single polyprotein of 3,010 to 3,033 amino acids (37, 49) which is processed by a combination of host and viral proteinases (3, 27, 28, 32, 33, 46, 67, 74). Although it has not been possible to study authentic viral polypeptides produced in HCV-infected cells, a tentative map of the HCV cleavage products has been established by cell-free translation and cell culture transient expression assays (49). For the HCV strain H (HCV-H), the gene order is 5′-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ (27, 28, 29, 45) and additional processing may occur (34, 41). C, a basic protein, is believed to be the viral capsid protein; E1 and E2 are possible virion envelope glycoproteins; and NS2 through NS5B are putative nonstructural proteins. The predicted protein sequences of E1 and E2 contain 5 or 6 and 11 N-linked glycosylation sites, respectively (reviewed in reference 51). Previous studies indicate that cleavages generating the HCV structural proteins (C/E1, E1/E2, and E2/p7 and p7/NS2) occur after hydrophobic stretches which are catalyzed in the endoplasmic reticulum (ER) lumen by host signal peptidase (32, 45). Autoproteolytic cleavage at the 2/3 site is mediated by an HCV-encoded proteinase which encompasses the NS2 region and the N-terminal portion of NS3 (28, 33). A distinct serine proteinase activity, located in the N-terminal one-third of the NS3 protein, is responsible for the four downstream cleavages in the NS region (3/4A, 4A/4B, 4B/5A, and 5A/5B) (3, 19, 27, 33, 46, 74).

Although the physical properties of infectious HCV particles have been studied (6, 7, 50, 70, 78), the glycoprotein composition of the HCV virion is not known. This is due to the low levels of HCV particles in infected liver tissues or blood and the lack of an efficient system for cell culture replication. Previous reports suggest that E1 and E2 interact to form a complex (29, 44, 56). Purified HCV glycoprotein complexes expressed by using vaccinia virus are noncovalently associated (56). In contrast, a small fraction of E1 and E2 present in lysates of cells infected with vaccinia virus-HCV recombinants has been reported to be associated via disulfide linkages (29).

In this study, we further characterized the formation of HCV glycoprotein complexes in two different HCV strains, H (22)

* Corresponding author. Mailing address: Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., Box 8230, St. Louis, MO 63110-1093. Fax: (314) 362-1232.

† Present address: Unité d'Oncologie Moléculaire, Institut Pasteur, 59019 Lille cédex, France.

and BK (71), two transient expression vectors (vaccinia and Sindbis viruses), and three different cell lines (HepG2, BHK-21, and PK-15). The kinetics of association between E1 and E2, studied by pulse-chase analysis, indicated that the formation of stable E1E2 complexes is very slow. We also showed that the E1E2 complex formation follows two pathways. The dominant pathway involves noncovalent interactions to form nonionic detergent-stable E1E2 heterodimers, whereas a minor fraction of the complexes are stabilized by disulfide bonds, aggregated, and most likely misfolded. HCV glycoproteins were not detected at the cell surface, and the results of immunolocalization studies and glycan analysis suggest that the majority of them are localized in the ER and not translocated beyond the *cis* Golgi.

MATERIALS AND METHODS

Plasmid constructs. Sindbis virus-HCV recombinants were constructed by using pSINrep5 (8) and standard methodology (63). For pSINrep/HCV-H1-1207, a cDNA fragment from pBRTM/HCV1-1207 (28) encoding the first 1,207 amino acid residues of the HCV strain H (HCV-H) polyprotein, was subcloned into the polylinker of pSINrep5 (8). For pSINrep/HCV-BK1-1207, a cDNA fragment encoding amino acid residues 1 to 1207 from the BK-146 clone (71) generously provided by H. Okayama (Research Institute for Microbial Diseases, Osaka University, Japan) was amplified by PCR (62) using the following oligonucleotides: 5′-CCCTCTAGACAA TGAGCACGAATCCTAAA-3′ (positive sense) and 5′-CCC TCTAGATTAAGACCGCATAGTAGCTTC-3′ (antisense). These oligonucleotides introduced *Xba*I sites upstream of the initiation codon and downstream of the stop codon. The PCR fragment was then digested by *Xba*I and subcloned into *Xba*I-digested SINrep5. An *Aat*II (position 7713 in pSINrep/HCV-BK1-1207)-*Aat*II (position 11064 in pSINrep/HCV-BK1-1207) fragment was replaced with the equivalent fragment from BK-146, and the rest of the HCV-BK cDNA sequence in pSINrep/HCV-BK1-1207 was sequenced and compared with the HCV-BK-146 sequence (71). Two mutations in the NS3 region (Ser-1173 and Ala-1203) were inadvertently introduced during the PCR amplification. The Ser (instead of Phe) at position 1173 is commonly found in other HCV strains; the Ala (instead of Thr) at position 1203 lies at the C terminus of the NS3 serine protease domain, which has not been implicated in structural region processing (27). pSINrep/HCV-H171-1026 was generated by using plasmids pSINrep/HCV-H171-379 and pSINrep/HCV-H370-1026-myc (45a). pSINrep/HCV-H171-1026 has a c-*myc* epitope tag fused to the C terminus of the HCV sequence, which is recognized by monoclonal antibody (MAb) Myc1-9E10 (EQKLISEEDL) (20, 21).

Generation and growth of viruses. Stocks of vTF7-3, a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase (25), and vaccinia virus-HCV (vHCV1-1488), which contains sequences of HCV-H (29), were grown in BSC-40 monolayers and partially purified (38), and titers of infectious progeny were determined by plaque assay on BSC-40 cells (38).

Sindbis virus recombinants SINrep/HCV-H1-1207, SINrep/HCV-BK1-1207, and SINrep/HCV-H171-1026 were generated as described by Bredenbeek et al. (8), using DH-BB(5′SIN) helper RNA. To estimate the titers of the stocks of Sindbis virus-HCV recombinants, serial dilutions of each stock were adsorbed on chicken embryo fibroblasts (CEF) in 96-well plates. After a 10-h incubation at 37°C, plates were fixed for 15 min at 4°C with cold isopropanol, and the number of particles (infectious units) was determined by immunostaining using anti-HCV MAbs as described below or by immunoperoxidase (23) when human antiserum was used.

Cell cultures. The BHK-21 and PK-15 cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, Md. The BSC-40 cell line (10) was obtained from D. Hruby (Oregon State University), and the A16 subclone of the human hepatoma HepG2 cell line (ATCC) was generously provided by Alan Schwartz (Washington University, St. Louis, Mo.). Secondary CEF were also used for some experiments. Cell monolayers were grown in Eagle's minimal essential medium (MEM; BSC-40 and PK-15 cells and CEF), alpha-MEM (BHK-21 cells), or Dulbecco's modified MEM (DMEM; HepG2 cells) supplemented with 10% (BSC-40, BHK-21, PK-15, and HepG2 cells) or 4% (CEF) fetal bovine serum (FBS).

HCV-specific MAbs and human antisera. A baculovirus vector encoding the structural proteins (core, E1, and E2) derived from HCV-H (HCV-Bac 4) was used to express HCV-specific antigens (39). HCV E1 and E2 glycoproteins from lysates of SF-9 cells infected with recombinant baculovirus were partially purified by cutting the appropriate bands from a sodium dodecyl sulfate (SDS)-polyacrylamide gel followed by electroelution and concentration. BALB/c mice were immunized four times intraperitoneally with 10 to 30 μg of antigen per injection in Freund's adjuvant. Following a final intravenous boost, splenocytes from hyperimmune animals were fused with nonsecreting mouse myeloma cells (Fox-NY) and selected for growth in media containing hypoxanthine, aminopterin, and thymidine (31). HCV-specific, antibody-secreting hybridomas were identified by immunoblot screening using E1 and E2 glycoproteins expressed by the vHCV-H1-966 construct. A single hybridoma specifically recognizing E1 was found (A4), while two hybridomas directed against E2 (A11 and I19) were identified. All MAbs were immunoglobulin G subtype 1.

Human sera containing antibodies against the E1 and E2 glycoproteins were found by screening chronically HCV-infected individuals for the presence of antibodies directed toward the baculovirus-expressed E1 and E2 antigens.

A MAb that recognizes the E1 glycoprotein of Sindbis virus (MAb 33) was kindly provided by A. L. Schmaljohn (65, 66).

Sedimentation analyses. HepG2, PK-15, or BHK-21 cells grown in 35-mm-diameter dishes were infected with vTF7-3 and vHCV1-1488, SINrep/HCV-H1-1207, or SINrep/HCV-BK1-1207 with a multiplicity of infection of 10 PFU per cell. At 10 to 12 h postinfection, monolayers were washed once with phosphate-buffered saline (PBS) and lysed with 0.5% Nonidet P-40 (NP-40)–50 mM Tris-Cl (pH 7.5)–150 mM NaCl–2 mM EDTA–20 μg of phenylmethylsulfonyl fluoride per ml. Cell lysates were clarified by centrifugation at 14,000 × *g* in an Eppendorf centrifuge for 5 min, and the supernatant was layered over a 10-ml gradient of 5 to 20% sucrose in PBS containing 0.1% Triton X-100. After centrifugation at 4°C for 24 h at 36,000 rpm in a Beckman SW41 rotor, 22 fractions were collected from the bottom of the gradient and analyzed by Western blotting (immunoblotting) or immunoprecipitation as described below. Molecular mass markers (Combithek, calibration protein I; Boehringer Mannheim) were sedimented in a parallel sucrose gradient. In some experiments, cells were disrupted with 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS) or 3% octylglucoside, and complexes were separated in 5 to 30% or 5 to 40% sucrose gradients containing the same concentrations of these detergents.

Metabolic labeling and immunoprecipitation. Subconfluent monolayers in 35- or 100-mm-diameter dishes were infected

with 5 to 10 PFU of the indicated recombinants per cell. After 1 h at room temperature, medium containing 2% FBS was added. At 4.5 h postinfection, monolayers were washed once with prewarmed medium lacking methionine and cysteine and incubated in the same medium for an additional half hour. Infected cells were then pulse-labeled for 5 min with 100 μCi of ^{35}S-Translabel (ICN) per ml. Cells were washed twice with prewarmed medium containing 100-fold excess methionine and cysteine and then chased for various times in medium with a 10-fold excess of these amino acids. In steady-state labeling, cells were labeled at 4 h postinfection with 25 μCi of ^{35}S-Translabel (2-h labeling period) per ml in medium containing 1/40 the normal concentration of methionine and 2% FBS or with 50 μCi of [^{3}H]glucosamine or [^{3}H]fucose (Amersham; 5-h labeling period) in regular medium containing 2% FBS.

Immunoprecipitations of nondenatured antigen were carried out as described previously (31), with some modifications (75). Infected cells were lysed with the NP-40-containing lysis buffer described above. Cell lysates were clarified by centrifugation in an Eppendorf centrifuge for 15 min and precleared by an overnight incubation at 4°C with fixed *Staphylococcus aureus* Cowan I (Calbiochem) followed by centrifugation. Four microliters of rabbit anti-mouse immunoglobulin G (Sigma) was incubated with protein A-agarose (Boehringer Mannheim) in 0.1 M phosphate buffer (pH 8.1) for 1 h at 4°C. This step was omitted when human serum was used. Beads were then incubated with 1 μl of MAb or 4 μl of an HCV-specific human antiserum, followed by the antigen (each step for 1 h at 4°C). Between each step, beads were washed twice with a solution containing 0.2% NP-40, 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 2 mM EDTA. After the last step, they were washed three times with this buffer, twice with a solution containing 0.2% NP-40, 0.2% SDS, 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 2 mM EDTA, twice with a solution containing 0.2% NP-40, 10 mM Tris-Cl (pH 7.5), 500 mM NaCl, and 2 mM EDTA, and finally once with distilled water. The precipitates were then solubilized by heating for 5 min at 96°C in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and run on a 10 or 13% polyacrylamide gel (43). After electrophoresis, gels were treated with sodium salicylate (12), dried, and exposed to Kodak XAR-5 film. ^{14}C-methylated protein molecular mass markers were purchased from Amersham. To estimate the half-maximal association time ($t_{1/2}$) for E1E2 complex formation, the intensity of E2 coprecipitated by anti-E1 MAb A4 was determined by densitometry of autoradiograms.

Immunoprecipitation of SDS-denatured antigens was done as described previously (29, 57).

Endoglycosidase digestions. Immunoprecipitated proteins were eluted from protein A-agarose in 30 μl of 0.5% SDS and 1% β-mercaptoethanol by heating for 10 min at 96°C. The protein samples were then divided into three equal portions for digestion with peptide *N*-glycosidase (PNGase) F, endo-β-*N*-acetylglucosaminidase H (endo H; New England Biolabs), or an undigested control. Digestions with PNGase F and endo H were carried out for 1 h at 37°C in the buffer provided by the manufacturer. Digested samples were mixed with equal volume of 2 × Laemmli sample buffer and analyzed by SDS-PAGE.

Western blotting. Analysis of proteins bound to nitrocellulose membranes (Hybond-ECL; Amersham) was performed by using enhanced chemiluminescence detection (ECL; Amersham) as recommended by the manufacturer. Briefly, after separation by SDS-PAGE (5 to 20% gradient gels), proteins were transferred to nitrocellulose membranes by using a Nova Blot apparatus (Pharmacia-LKB) and revealed with anti-E1 (dilution of 1/10,000) or anti-E2 (dilution of 1/5,000) HCV MAbs followed by goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (dilution of 1/1,000; Boehringer Mannheim). In some experiments, the relative protein levels were estimated by densitometric analysis.

Subcellular fractionation. HepG2 cells grown in a 150-mm-diameter dish were infected with vTF7-3 and vHCV1-1488 with a multiplicity of 5 PFU per cell and incubated for 10 to 12 h at 37°C in DMEM containing 4% FBS. Subcellular fractionation was conducted by using a modification of the technique described by Bole et al. (5). Prior to disruption, the cells were put on ice and then washed twice with 250 mM sucrose in water and once with 50 mM sucrose. Cells were then removed from the plates with a scraper and homogenized in 50 mM sucrose in a tight-fitting Dounce homogenizer (10 gentle strokes). Sucrose was then added to a final concentration of 250 mM in 5 mM HEPES (pH 6.8). Nuclei and cellular debris were removed by centrifugation at 800 × *g* for 10 min. The postnuclear supernatant was then layered over a discontinuous sucrose gradient consisting of 1 ml/2.0 M, 3.4 ml/1.3 M, 3.4 ml/1.0 M, and 2.75 ml/0.6 M sucrose in 5 mM HEPES (pH 6.8) in a Beckman tube (14 by 89 mm). After 3 h of centrifugation at 37,000 rpm in a Beckman SW41 rotor, 12 fractions containing 1 ml each were collected from the bottom of the tubes. Each fraction was assayed for enzymes characteristic of the ER and Golgi. NADPH cytochrome *c* reductase, a marker for ER membranes, was assayed as described by Omura and Takesue (53); galactosyltransferase, a marker for *trans* Golgi complex membranes, was assayed as described by Brew et al. (9), with some modifications (5).

Immunostaining. For immunostaining, monolayers of HepG2 or BHK-21 cells in 24-well plates were infected with vTF7-3 and vHCV1-1488 or SINrep/HCV-H1-1207 and incubated for 12 h at 37°C. Plates were then fixed for 15 min at 4°C with 4% paraformaldehyde for cell surface staining or cold isopropanol for internal staining. Some wells were left unfixed for cell surface staining. Immunostaining was then performed as described previously (18). Briefly, monolayers were incubated successively with the anti-E1 (dilution of 1/1,000) or anti-E2 (dilution of 1/400) MAb followed by biotinylated goat anti-mouse immunoglobulins (dilution of 1/200) each for 30 min at 37°C. Plates were then incubated with the Vectastain ABC alkaline phosphatase kit (Vector Laboratories) for 30 min at room temperature and revealed by using an Alkaline Phosphatase Substrate kit II (Vector Laboratories). Between steps, plates were rinsed three times with PBS, and the final color reaction was stopped by rinsing with distilled water. A vaccinia virus recombinant expressing Sindbis virus structural proteins (vTM3/SINS [55a]) and a Sindbis virus recombinant expressing the structural proteins of yellow fever virus (SINrep/YFS [18a]) were used for controls.

For immunofluorescence, subconfluent HepG2 cells on coverslips were infected with vTF7-3 and vHCV1-1488 or SINrep/HCV1-1207 and incubated in DMEM containing 4% FBS for 12 h at 37°C. Cells were washed three times with PBS and fixed with 3% paraformaldehyde in PBS for 20 to 30 min at room temperature. After being washed with PBS containing 10 mM glycine, cells were permeabilized with 0.1% Triton X-100 for 30 min. Cells were stained with the anti-E1 (dilution of 1/1,000) or anti-E2 (dilution of 1/400) MAbs followed by rhodamine-conjugated goat anti-mouse immunoglobulin G antibodies (dilution 1/100).

Immunoelectron microscopy. HepG2 cells infected with vTF7-3 and vHCV1-1488 or SINrep/HCV1-1207 recombinants were harvested at 12 h postinfection and processed for immunoelectron microscopy as described previously (60, 61). In

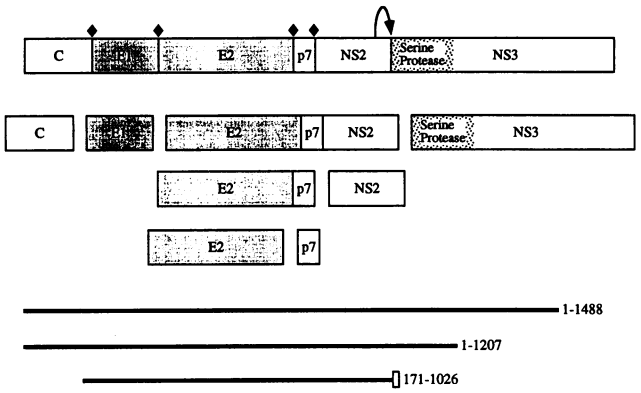

FIG. 1. HCV genome structure and expression constructs. A diagram of the HCV polyprotein (from C to NS3) and its cleavage products is shown on the top. The identities of the cleavage products are indicated (27, 28, 29, 45). Also shown are putative cleavage sites for host signalase (◆) (32, 45) and the NS2-3 proteinase (↓) (28, 33). E2-containing precursors are also shown. The HCV polyprotein expression constructs used in this study are shown below. HCV sequences present in each construct are indicated by black lines which are drawn to scale and oriented with respect to the diagram of the HCV polyprotein. The polyprotein encoded by vHCV1-1488 contains three extra amino acids present at the N terminus of the capsid protein (Met-Cys-Thr [29]). The C-terminal end of NS2 in construct 171-1026 is followed by the 10-residue c-*myc* epitope tag and is indicated by a box (EQKLISEEDL).

brief, cells were fixed in 4% formaldehyde–0.25% glutaraldehyde in HEPES saline (pH 7.0). The cells were scraped, pelleted, embedded in gelatin, and infiltrated with 2.3 M sucrose–20% polyvinylpyrolidone in PBS. The blocks were trimmed, frozen, sectioned with an RMC MT7/CR21 cryoultramicrotome, and probed with antibodies. The sections were then washed and embedded in 0.3% uranyl acetate in 2% polyvinyl alcohol.

HepG2 cells infected with vTF7-3 alone or SINrep/YFS were used as negative controls, and the number of gold particles per cell was between 2 and 9% of the labeling observed on the cells infected with the HCV glycoprotein expression constructs (data not shown).

RESULTS

HCV E1E2 complexes have been recently described (29, 44, 56), although differences regarding the presence of disulfide bonds stabilizing at least some of these complexes have been noted. To obtain a better picture of HCV glycoprotein oligomerization, we studied complex formation by using two different transient viral expression systems (vaccinia and Sindbis viruses), three different cell lines (HepG2, BHK-21, and PK-15), and two HCV isolates of different subtypes (HCV-H and HCV-BK [69]). In the hope of mimicking authentic processing, the HCV glycoproteins were produced via expression of polyproteins encompassing the entire HCV structural-NS2 region including an active NS2-3 proteinase to mediate autocatalytic cleavage at the 2/3 site (Fig. 1).

Kinetics of association of HCV E1 and E2. To study the kinetics of E1 and E2 association, we conducted pulse-chase experiments and monitored complex formation by coprecipitation of E2 with an anti-E1 MAb under nondenaturing conditions (Fig. 2). Previous experiments have shown that processing at the C/E1 and E1/E2 signalase cleavage sites and autocatalytic cleavage at the 2/3 site occur rapidly (45). For HCV-H, cleavages at the E2/p7 and the p7/NS2 signalase sites are delayed, leading to the production of discrete polyproteins (29, 45). The majority of E2-NS2 is a short-lived precursor (Fig. 3), whereas E2-p7 and E2, whose glycosylated forms are not readily resolved by SDS-PAGE, are stable species. For HCV-BK, signalase cleavage at the E2/p7 site is more efficient and E2-p7 is not readily observed (45) (see below). For simplicity, we will refer to the diffuse band at ~70 kDa as E2, although for HCV-H it consists of a mixture of E2 and E2-p7. As shown in Fig. 2, in HepG2 cells infected with vaccinia virus HCV-H recombinant vHCV1-1488, E1 was readily detected after a 5-min pulse; however, the intensity of E1 increased during the chase, suggesting that this MAb may not efficiently recognize newly synthesized E1 (Fig. 2A). Two major forms of E1 which differed in the extent of N-linked glycosylation were observed (see below), and the mobility of E1 increased during the chase, presumably as a result of trimming of mannose-rich core glycans. After a 15-min chase, a small fraction of the E2-NS2 present (data not shown) was coprecipitated by the anti-E1 MAb. Except for long chase periods, the level of E2-NS2 coprecipitated was unchanged despite the conversion of the majority of E2-NS2 to E2 during the first 2 h of chase (data not shown, Fig. 3, and reference 45). Beginning at 45 min of chase, increasing levels of coprecipitated E2 glycoprotein were detected. As assayed with this anti-E1 MAb, the $t_{1/2}$ for the HCV-H E1 and E2 glycoproteins in HepG2 cells was ≈85 min. Similar results were obtained during vHCV1-1488-mediated expression of HCV-H glycoproteins in PK-15 cells (Fig. 2B; $t_{1/2}$ of ≈75 min) and BHK-21 cells (Fig. 2C; $t_{1/2}$ of ≈60 min). Three smaller forms of E1 were reproducibly detected in PK-15 cells, suggesting that N-linked glycosylation of E1 was less efficient in this cell type, as observed for E1 expressed independently in insect or BHK-21 cells (44, 45, 48). When a Sindbis virus/HCV replicon (8) expressing residues 1 to 1207 of the HCV-H polyprotein (SINrep/HCV-H1-1207) was used to examine complex formation in BHK-21 cells, association of E1 and E2 was also found to be slow (Fig. 2D; $t_{1/2}$ of ≈75 min). The results of pulse-chase analyses using the Sindbis virus/HCV recombinant expressing the HCV-BK 1-1207 polyprotein were similar except that reactivity of the anti-E1 MAb with BK E1 was delayed and coprecipitated E2 appeared later and increased throughout the chase period (Fig. 2E; $t_{1/2}$ of >165 min). The higher molecular mass observed for HCV-BK E1 is believed to be due to the presence of an additional glycan compared with HCV-H (71).

Most E1E2 complexes are noncovalently associated. The fraction of E2-specific products associated with E1 via noncovalent interactions versus intermolecular disulfide bonds was examined by conducting immunoprecipitations under denaturing or nondenaturing conditions (Fig. 4). When the antigens were denatured with SDS prior to immunoprecipitation (Fig. 4B), only a small fraction of E2-NS2 and E2 were coprecipitated compared with the levels observed under nondenaturing conditions (Fig. 4A). To verify that the E2-specific products coprecipitated under denaturing conditions were not simply residual complexes resistant to SDS denaturation, the reducing agent was omitted from the SDS-PAGE sample buffer. In this case, E2 was not detected and instead heterogeneous bands of higher molecular mass were observed, suggesting the presence of intermolecular disulfide bonds (data not shown). Pretreatment of cells with *N*-ethylmaleimide and its inclusion in the lysis buffer yielded similar results, which indicates that these complexes were present in vivo and did not form during or after lysis (data not shown). These results provide evidence for at least two forms of HCV glycoprotein complexes: a predom-

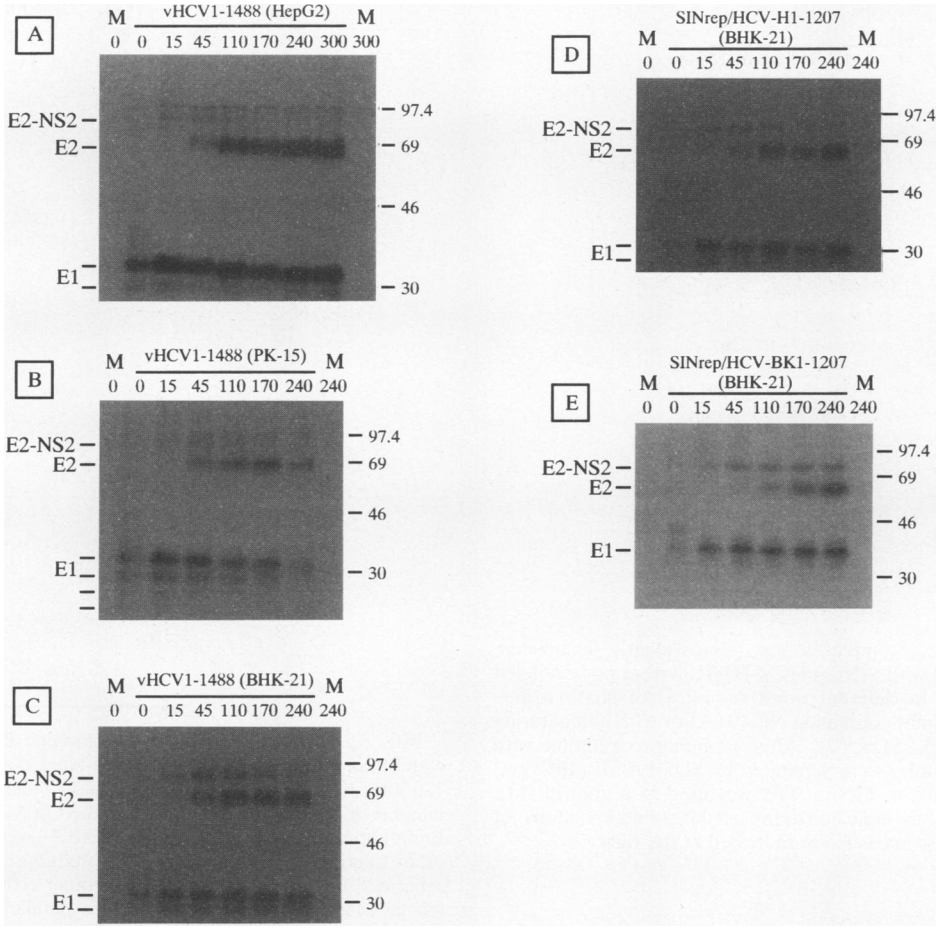

FIG. 2. Kinetics of association of E1 and E2. HepG2 (A), PK-15 (B), or BHK-21 (C to E) cells infected with vTF7-3 plus vHCV1-1488 (A to C), SINrep/HCV-H1-1207 (D), or SINrep/HCV-BK1-1207 (E) were pulse-labeled for 5 min and chased for the indicated times (in minutes). Cell monolayers were lysed in a nondenaturing lysis buffer containing NP-40 as described in Materials and Methods. After immunoprecipitation with anti-E1 MAb A4, samples were separated by SDS-PAGE (10% gel). vTF7-3 alone or a Sindbis virus replicon expressing the yellow fever virus structural proteins (SINrep/YFS [18a]) was used as a control (M). HCV-specific proteins are indicated at the left, sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

inant species stabilized by noncovalent interactions and a minor fraction stabilized by intermolecular disulfide bonds.

The majority of E1E2 complexes are heterodimers. The formation and distribution of oligomers was then examined by conducting pulse-chase experiments followed by separation of nonionic detergent-solubilized complexes by sedimentation through sucrose gradients. Association of E2 with E1 was again monitored by coprecipitation of E2-specific products with the E1 MAb (Fig. 5). After a 5-min pulse, mainly glycosylated E1, which sedimented between molecular mass standards 68K (68 kDa) and 158K, was detected. When the reducing agent was omitted from the SDS-PAGE sample buffer, a smear was observed in the upper part of the gel for the same fractions, and the intensity of E1 migrating as a monomer was much lower (data not shown). This finding suggests that immediately following the pulse, E1 is present in heterogeneous complexes stabilized by disulfide bonds, which may explain its detection between molecular mass standards 68K and 158K in the sucrose gradient. These complexes could consist of E1 linked to itself, to E2-NS2 (which also forms a smear after a short pulse; Fig. 3), or to unlabeled host polypeptides. After a 45-min chase, E2-NS2 and E2 were coprecipitated with E1, and these complexes also sedimented between molecular mass standards 68K and 158K. At the same time, E1 was also detected in fractions that sedimented more slowly than 68K, perhaps representing monomeric E1. After a 120-min chase,

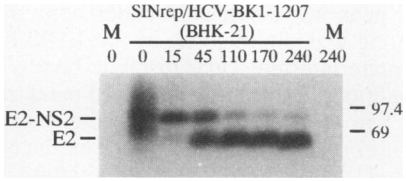

FIG. 3. Kinetics of E2-NS2 cleavage for the BK strain. BHK-21 cells infected with SINrep/HCV-BK1-1207 were pulse-labeled for 5 min, chased for the indicated times (in minutes), and lysed with a denaturing buffer containing 0.5% SDS. After immunoprecipitation with anti-E2 MAb I19 as described in Materials and Methods, samples were separated by SDS-PAGE (10% gel). SINrep/YFS was used as a control (M). HCV-specific proteins are indicated at the left; sizes (in kilodaltons) of protein molecular mass markers are indicated at the right. Similar results were obtained with SINrep/HCV-H1-1207 (data not shown).

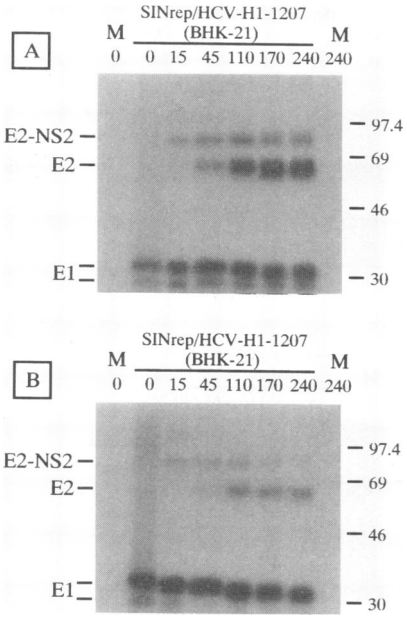

FIG. 4. Most E1E2 complexes are noncovalently associated. BHK-21 cells infected with SINrep/HCV-H1-1207 were pulse-labeled for 5 min and lysed at different times (minutes) of chase with a nondenaturing lysis buffer containing NP-40 (A) or with a denaturing buffer containing 0.5% SDS (B). After immunoprecipitation with anti-E1 MAb A4, samples were separated by SDS-PAGE (10% gel) under reducing conditions. SINrep/YFS was used as a control (M). HCV-specific proteins are indicated at the left; sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

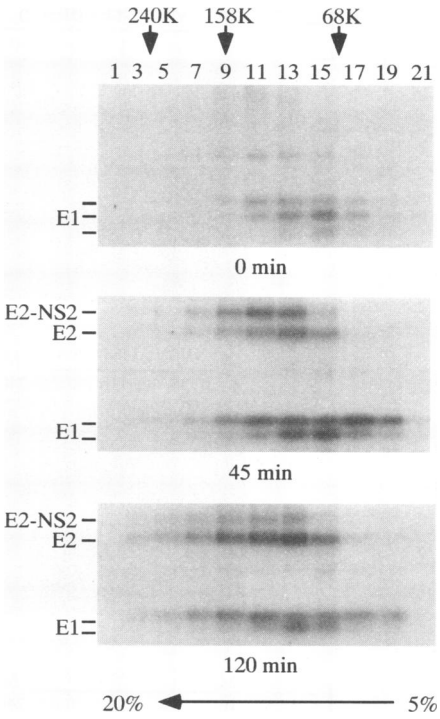

FIG. 5. Formation of discrete oligomers. BHK-21 cells infected with SINrep/HCV-H1-1207 were pulse-labeled and chased for 0, 45, or 120 min. Cells were lysed with a buffer containing NP-40 and sedimented in sucrose gradients as described in Materials and Methods. Immunoprecipitations with anti-E1 MAb A4 were performed on every other fraction under nondenaturing conditions, and the samples were separated by SDS-PAGE (10% gel) under reducing conditions. Molecular mass markers sedimented in a parallel sucrose gradient are indicated at the top; HCV-specific proteins are indicated at the left.

the intensity of the coprecipitated E2 band sedimenting between molecular mass standards 68K and 158K increased, suggesting the formation of E1E2 heterodimers (predicted molecular mass, 100K).

Since our anti-E2 MAbs did not react with E2 under nondenaturing conditions, we could not perform the complementary coprecipitation analyses to examine the distribution of E2 complexes and monomers (data not shown). However, Western blot analyses using both E1- and E2-specific MAbs were used to compare the distribution of the glycoproteins and glycoprotein complexes, using the different expression vectors and cell types (Fig. 6). Similar distributions were observed in HepG2, BHK-21, and PK-15 cells when either the Sindbis virus or vaccinia virus expression construct was used. By densitometric analysis of the blots, the majority of E2 was found to sediment between molecular mass standards 68K and 158K, at the expected position of E1E2 heterodimers. A lagging shoulder (particularly apparent in SINrep/HCV-H1-1207-infected BHK-21 cells) which may represent monomeric E2 was observed. For E1, two unresolved peaks were observed: one which comigrated with the major E2 peak and another which was closer to the top of the gradient, which probably represents E1 monomers. Additional minor peaks were also apparent, especially for vHCV1-1488-infected PK-15 cells. Together with the results of the pulse-chase analyses (Figs. 2, 4, and 5), the comigrating E1 and E2 peaks most likely represent E1E2 heterodimers. For HCV-BK (SINrep/HCV-BK1-1207-infected BHK-21 cells), the anti-E1 MAb A4 did not recognize HCV-BK E1 (on Western blots), but the blot revealed with anti-E2 MAb I19 showed the same result as that obtained for SINrep/HCV-H1-1207 (data not shown).

To see if higher-order oligomers which were disrupted by treatment with Triton-X 100 or NP-40 might exist, we tested other mild detergents (3% octylglucoside or 2% CHAPS) commonly used to study membrane protein complexes. Similar results were obtained with these detergents (data not shown).

The majority of the E1E2 heterodimer is noncovalently linked. To compare the size distribution of covalent versus noncovalently linked complexes, duplicate samples from sucrose gradient fractions were separated by SDS-PAGE with or without reducing agent in the sample buffer and then analyzed by Western blotting with a mixture of E1- and E2-specific MAbs. As shown in Fig. 7, the majority of E1 and E2 in the heterodimer peak was dissociated under nonreducing conditions, indicating that the majority of the E1E2 heterodimers does not contain intermolecular disulfide bonds. Under nonreducing conditions, the intensity of the E1 band was very weak in fractions 1 to 13 and bands of high molecular mass were observed (Fig. 7). For fractions 1 to 5, unreduced complexes barely entered the separating gel and diffuse bands were observed in fractions 6 to 13. These diffuse species may indicate heterogeneity in disulfide bond formation, which would suggest formation of misfolded complexes. Some blots were revealed with an anti-E1 MAb, and then the membrane was stripped of bound antibody and reprobed with an anti-E2 MAb. Under these conditions, the bands of high molecular mass detected under nonreducing conditions by the anti-E1 or anti-E2 MAb were the same (data not shown), suggesting that aggregates involved intermolecular disulfide bonds between E1

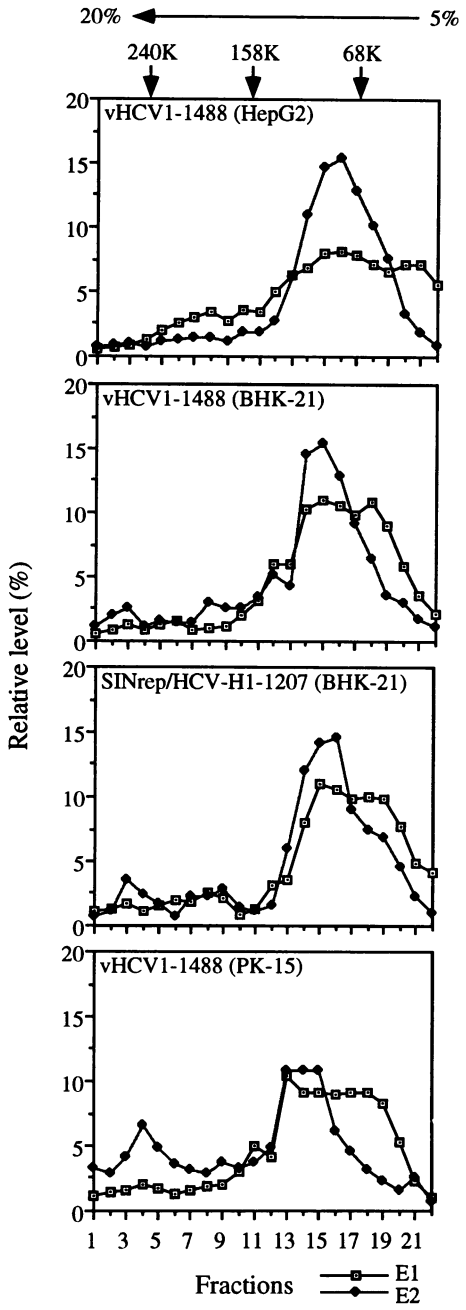

FIG. 6. Similar complexes are formed in several different mammalian cell lines. HCV glycoproteins were expressed by infecting HepG2, BHK-21, or PK-15 cells with vTF7-3 plus vHCV1-1488 or SINrep/HCV-H1-1207. Infected cells were lysed with NP-40 and sedimented through 5 to 20% sucrose gradients as described in Materials and Methods. Samples of gradient fractions were separated on SDS–5 to 20% gradient polyacrylamide gels and analyzed by Western blotting with anti-E1 (A4) and -E2 (A11) MAbs. Relative amounts of the E1- or E2-specific products were estimated by densitometry of the autoradiograms. Molecular mass markers sedimented in a parallel sucrose gradient are indicated at the top.

and E2. Similar aggregated species were observed in HepG2 (vHCV1-1488), BHK-21 (SINrep/HCV-H1-1207, SINrep/HCV-BK1-1207, vHCV1-1488), and PK-15 (vHCV1-1488) cells (Fig. 7 and data not shown). Together, these results suggest that the formation of heterogeneous disulfide-linked HCV glycoprotein complexes is a minor but general phenomenon when these proteins are expressed at high levels in mammalian cells.

N-linked glycosylation and subcellular localization of HCV glycoproteins. As an indicator of intracellular trafficking of the HCV E1 and E2 glycoproteins, we began by examining the acquisition of endo H resistance. Endo H removes the chitobiose core of high-mannose and some hybrid forms of N-linked sugars but not the complex forms (58). In contrast, PNGase F removes both high-mannose and complex forms of N-linked sugars (72). Hence, resistance to digestion with endo H but not PNGase F is indicative that glycoproteins have moved from the ER to at least the medial and *trans* Golgi where complex sugars are added. In steady-state labeling experiments, no endo H-resistant forms were detected (Fig. 8A). In pulse-chase studies (Fig. 8B and C), no endo H-resistant forms were detected even after 4 h of chase, suggesting that E1E2 complexes are not transported through the medial Golgi. Similar results were obtained in HepG2 (vHCV1-1488), BHK-21 (vHCV1-1488, SINrep/HCV-H1-1207 and SINrep/HCV-BK1-1207), and PK-15 (vHCV1-1488) cells. For BHK-21 cells infected with SINrep/HCV-BK1-1207, a more slowly migrating form of E1 was observed after PNGase F treatment, suggesting that one of the glycans was resistant to PNGase F treatment under our experimental conditions or that completely deglycosylated HCV-BK E1 migrates anomalously. As mentioned earlier, E2-p7 was barely detected for HCV-BK, whereas it remained present until the end of the chase for HCV-H, suggesting that the E2/p7 cleavage is more efficient for HCV-BK (see also reference 45).

Since endo H treatment was done after immunoprecipitation with the anti-E1 MAb A4, it is possible that the epitope recognized by this MAb is blocked by processing of high-mannose glycans to complex or hybrid forms. To address this concern, the experiments were repeated with serum from an HCV-positive patient. Since this serum recognizes additional HCV-specific cleavage products which would complicate the analysis, we used another Sindbis virus-HCV recombinant, SINrep/HCV-H171-1026, which expresses a truncated polyprotein beginning with the E1 signal sequence and extending through the C-terminal residue of NS2 (Fig. 1). Similar to the previous results, no endo H-resistant forms could be detected with the human antiserum (data not shown).

Since glycoproteins migrating through the Golgi apparatus are usually fucosylated in the *trans* Golgi, we attempted to label HCV glycoproteins with [^{3}H]fucose. As shown in Fig. 9, although the HCV glycoproteins were readily labeled with [^{3}H]glucosamine, E1 and E2 did not incorporate [^{3}H]fucose, again suggesting that these glycoproteins are arrested in a pre-*trans* Golgi compartment. Although these results are consistent with the endoglycosidase and subcellular fractionation data (see below), it is possible that E1 and E2 glycans are not substrates for fucosyltransferase.

Intracellular localization of E1 and E2 was also examined directly by subcellular fractionation. For these analyses, vHCV1-1488-infected HepG2 cells were used, and as shown in Fig. 10, the bulk of E1 and E2 was found in the rough and smooth ER fractions. Some E1 and E2 could also be detected in Golgi fractions, but this may be due to slight contamination of these fractions with smooth ER membranes (Fig. 10; note the profile for the ER marker enzyme, NADPH cytochrome *c* reductase). Fractions from this subcellular fractionation experiment were treated with endo H and analyzed by Western blotting (data not shown). No endo H-resistant forms were detected in the Golgi fraction, suggesting that if small amounts

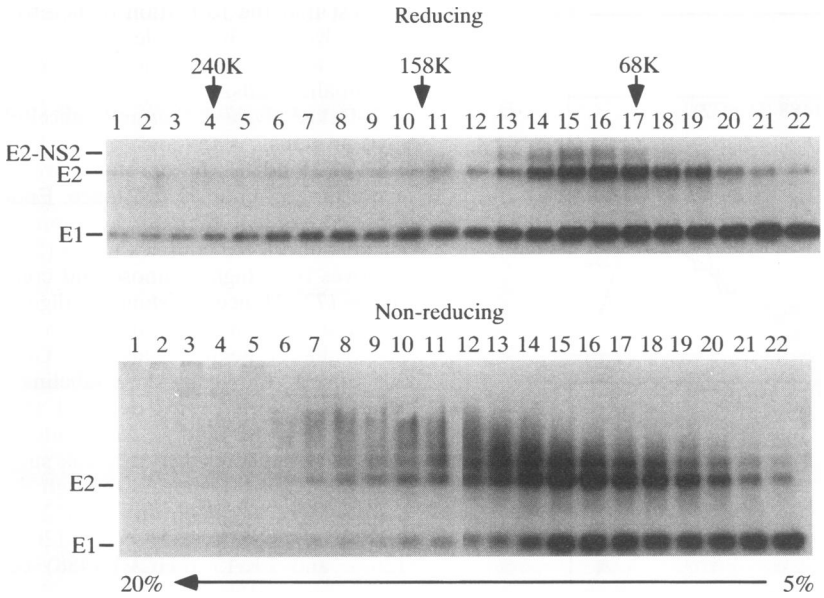

FIG. 7. Size distribution of noncovalently associated versus disulfide-linked complexes. HCV glycoproteins were expressed by coinfecting HepG2 cells with vTF7-3 plus vHCV1-1488. Infected cells were lysed with NP-40 and sedimented through sucrose gradients as described in Materials and Methods. Samples of gradient fractions were separated on SDS–5 to 20% polyacrylamide gels, using a reducing or nonreducing Laemmli sample buffer, and analyzed by Western blotting with anti-E1 (A4) and anti-E2 (A11) MAbs. Molecular mass markers sedimented in a parallel sucrose gradient are indicated at the top; HCV-specific proteins are indicated at the left.

of HCV E1 and E2 are exported to the Golgi complex, they are most likely localized in the *cis* Golgi.

Glycoprotein antigens localized by immunofluorescence of paraformaldehyde-fixed cells permeabilized with Triton X-100 were concentrated mainly in the perinuclear space and exhibited a granular pattern indicative of a vesicular localization (Fig. 11A). Immunoelectron microscopy confirmed this distribution (Fig. 12). Both E1 and E2 glycoproteins were detected in vesicles in close proximity to the ER near the nucleus. We did not observe labeling in the Golgi apparatus, consistent with the biochemical and cell fractionation data described above.

HCV glycoproteins are not detected on the cell surface. Neither E1 nor E2 was detected when MAbs A4, A11, and I19 were used for cell surface immunostaining of HepG2 or BHK-21 cells infected with vHCV1-1488 (Fig. 11B, lower right) or SINrep/HCV-H1-1207, whereas the glycoproteins of Sindbis virus, which buds from the plasma membrane, were readily detected when expressed in the vaccinia virus/T7 expression system (Fig. 11B, lower left). Since epitopes could have been altered by paraformaldehyde fixation, unfixed infected cells were also immunostained, and the same result was obtained (data not shown). We also failed to detect either glycoprotein on the cell plasmalemma by immunoelectron microscopy (data not shown). These results suggest that at least under the conditions of these expression studies, neither E1 nor E2 is exported to the cell surface.

DISCUSSION

Results obtained from experiments using viral transient expression systems to study heterologous viral glycoprotein processing and oligomerization generally mimic the situation in authentic virus-infected cells. For HCV, such a comparison is not yet possible since an efficient cell culture replication system is lacking. To obtain a more realistic picture of HCV glycoprotein biogenesis, we have compared glycoprotein biosynthesis and oligomerization for two HCV strains, using vaccinia virus and Sindbis virus expression systems and several different cell lines (HepG2, BHK-21, and PK-15 cells). The HepG2 human hepatoma line was chosen since HCV replicates in human and chimpanzee hepatocytes (44, 52). Similarly, porcine kidney PK-15 cells have been reported to support HCV replication in vitro (4). Our results of assays using the different expression systems and cell types were remarkably consistent and indicate that the majority of HCV E1 and E2 associate slowly to form noncovalent nonionic detergent-stable heterodimers. Besides these noncovalent heterodimers, intermolecular disulfide bonds are also formed to generate heterogeneous complexes. Analysis of N-linked glycans and localization of E1 and E2 indicated that the HCV-H glycoproteins were not present at the cell surface and did not migrate further than the *cis* Golgi, which is consistent with a previous report for HCV-1 glycoproteins expressed by vaccinia virus (67).

To summarize our current view of E1E2 oligomer formation, E1 and E2-NS2 are rapidly released from the polyprotein by host signal peptidase and autocatalytic cleavage at the 2/3 site. These primary cleavage products are core glycosylated and appear heterogeneous, perhaps because of formation of aberrant intermolecular and/or intramolecular disulfide bonds. The production of properly folded E1 and E2 glycoproteins may involve rearrangement of initially formed disulfide bonds by ER-localized protein disulfide-isomerase. A small fraction of these complexes is resistant to disulfide bond rearrangement and persists in the form of heterogeneous disulfide-linked aggregates. The majority of the E1 and E2 slowly form a nonionic detergent-stable heterodimeric complex. Although further processing of E2-NS2 at the E2/p7 and p7/NS2 signalase sites occurs more rapidly than formation of these E1E2 complexes, it is not known if such cleavages are required for proper glycoprotein folding and complex formation. In the case of HCV-H, in which processing at the E2/p7 site is incomplete (this work and reference 45), we observed E1E2-p7

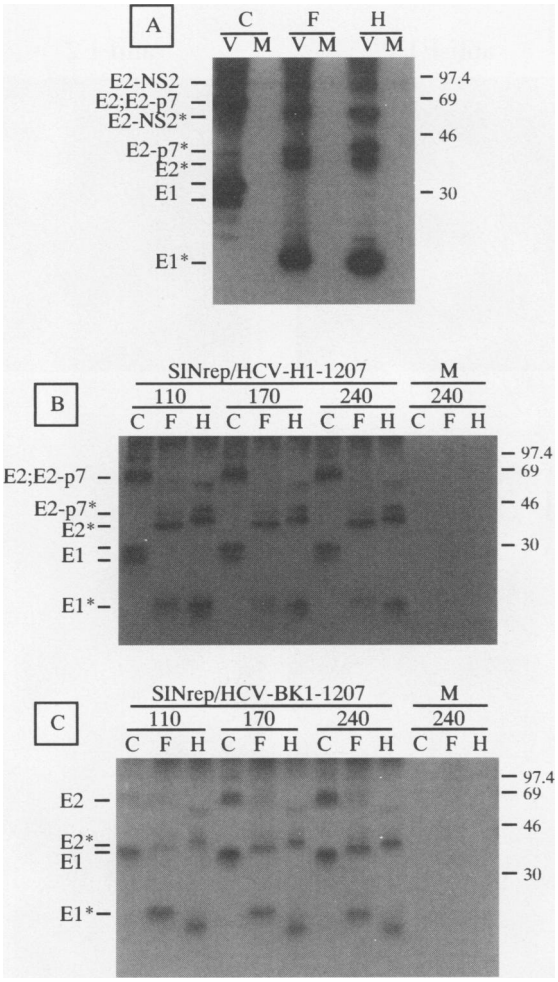

FIG. 8. Complex N-linked glycans are not detected on HCV glycoproteins. Metabolically labeled lysates of infected cells were immunoprecipitated with anti-E1 MAb (A4) and then treated with endo H (H), or PNGase F (F) or incubated without enzyme in endo H digestion buffer (C) as described in Materials and Methods. (A) HepG2 cells were coinfected with vTF7-3 plus vHCV1-1488 (V) or vTF7-3 alone (M) and labeled from 4 to 6 h postinfection before immunoprecipitation. (B and C) BHK-21 cells were infected with SINrep/HCV-H1-1207 (panel B), SINrep/HCV-BK1-1207 (panel C), or SINrep/YFS (M), pulse-labeled for 5 min, and chased for the indicated times (in minutes). Samples were separated by SDS–13% PAGE under reducing conditions. HCV-specific proteins are indicated at the left (deglycosylated proteins are indicated by asterisks); sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

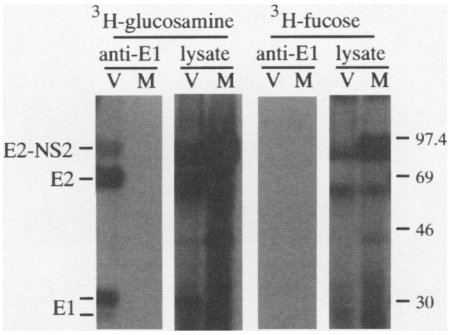

FIG. 9. Metabolic labeling of HCV glycoproteins with [^{3}H]glucosamine or [^{3}H]fucose. HepG2 cells coinfected with vTF7-3 plus vHCV1-1488 (V) or vTF7-3 alone (M) were labeled with [^{3}H]glucosamine or [^{3}H]fucose. Cell lysates were separated by SDS-PAGE (10% gel) directly (lysate) or after immunoprecipitation with the anti-E1 MAb (A4) (anti-E1). HCV-specific proteins are indicated at the left; sizes (in kilodaltons) of protein molecular mass markers are indicated at the right.

complexes, suggesting that this cleavage is probably not required.

The formation of E1E2 heterodimers for both HCV-H and HCV-BK (this study) and HCV-1 (56), as well as the ability of such complexes to elicit protective immunity (14), suggests that this complex may represent a functional subunit of the HCV virion envelope. It seems likely, by analogy to other enveloped viruses, that higher-order oligomers may exist for E1E2 in mature HCV virions. Such structures may have been missed in our studies if they are stabilized by interactions which are disrupted by nonionic detergents. Consistent with this idea, higher-molecular-mass complexes have been reported after sedimentation of HCV glycoproteins through glycerol gradients lacking detergent (56). Such complexes may reflect higher-order structures which mimic those present on HCV virions. Alternatively, they may have been formed by nonspecific hydrophobic interactions due to removal of the detergent. Further experiments examining the glycoprotein components of authentic HCV virions are clearly needed but have not been possible because of the low levels of HCV particles which can be purified from infected liver or blood.

At least in heterologous expression systems, the ER localization and lack of complex glycans suggests that the HCV glycoproteins were not translocated beyond the *cis* Golgi. Although many enveloped viruses bud from the plasma membrane, there are a number of viral families whose members bud into intracellular lumenal compartments (30, 35, 55). In such cases, the viral membrane proteins which participate in these budding events contain signals which allow their retention in the appropriate subcellular compartment. The fact that the HCV glycoproteins do not migrate further than the *cis* Golgi suggests that HCV budding, as suggested for other flaviviruses, may occur in the ER or a subcompartment located between the ER and the *cis* Golgi (reviewed in reference 55). For the flaviviruses, virions formed in an intracellular compartment appear to be released from cells via the exocytosis pathway. Complex glycans are acquired in the Golgi (for some flaviviruses), and the virion glycoprotein precursor prM is cleaved in a late compartment just prior to release of mature virus. In the case of HCV, a recent study indicates that mature virions contain complex N-linked glycans, although these carbohydrate moieties were not shown to be specifically associated with E1 and/or E2 (64). If correct, this observation indicates that the later stages in HCV glycoprotein maturation, which are perhaps linked to normal virion assembly and release, did not occur in the heterologous expression studies reported thus far. In lieu of an efficient cell culture replication assay, it may be useful to try alternative strategies for production of HCV-like virion particles, including the expression of additional HCV RNA sequences.

A minor fraction of the complexes formed between E1 and E2 were found to contain intermolecular disulfide bonds. This was observed during expression of both HCV-H and HCV-BK polyproteins and did not appear to be an artifact of the expression vector or host cell used for expression. Although such disulfide-linked complexes might be incorporated into

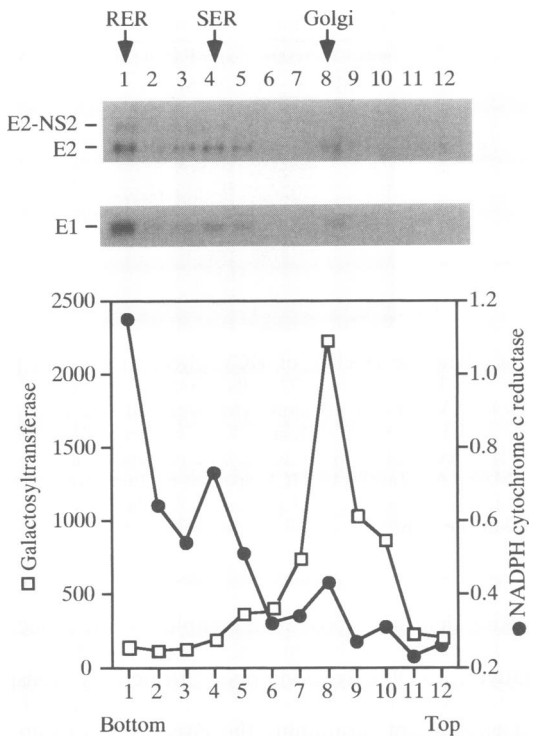

FIG. 10. Distribution of HCV glycoproteins by subcellular fractionation. HepG2 cells were coinfected with vTF7-3 plus vHCV1-1488. At 12 h postinfection, cells were disrupted and the postnuclear supernatant was layered over a discontinuous sucrose gradient as described in Materials and Methods. After centrifugation, membrane-containing fractions were collected from the bottom of the tube. Marker enzymes for the ER (NADPH cytochrome *c* reductase) and the Golgi apparatus (galactosyltransferase) were assayed as described in Materials and Methods. Samples of gradient fractions were also separated by SDS-PAGE (5 to 20% gradient gel) and analyzed by Western blotting with anti-E1 (A4) or anti-E2 (A11) MAbs. HCV-specific proteins are indicated at the left. RER, rough ER; SER, smooth ER.

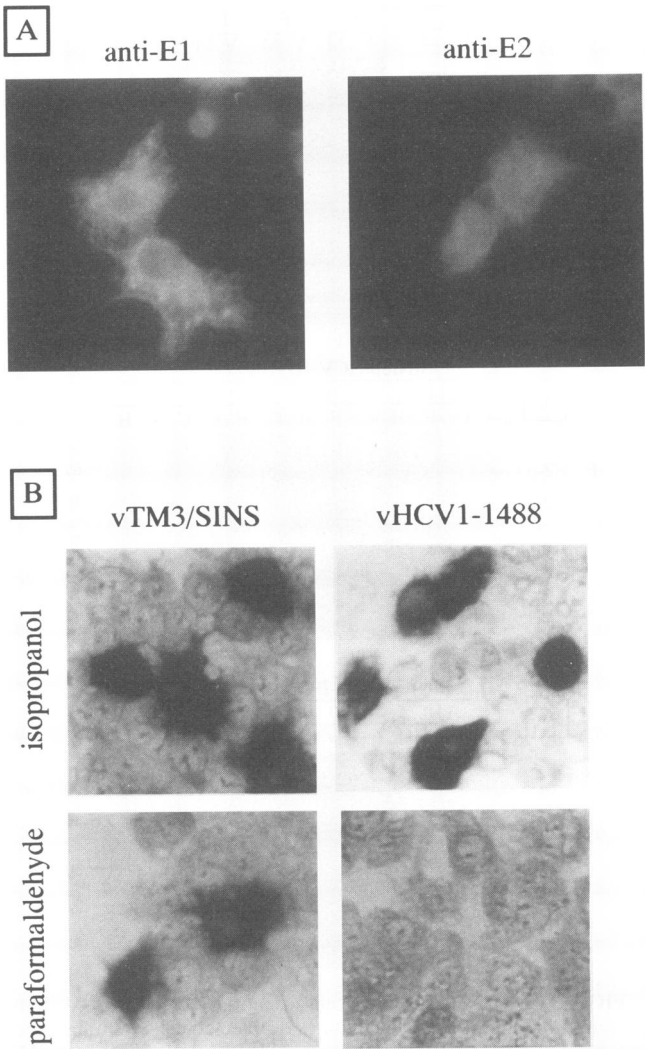

FIG. 11. Localization of HCV E1 and E2 by immunofluorescence and immunostaining. (A) HepG2 cells coinfected with vTF7-3 plus vHCV1-1488 were fixed with paraformaldehyde at 12 h and permeabilized with Triton X-100, and distribution of E1 or E2 was examined by immunofluorescence as described in Materials and Methods. (B) HepG2 cells coinfected with vTF7-3 plus vHCV1-1488 or with vTF7-3 plus vTM3/SINS (a vaccinia virus recombinant expressing Sindbis virus structural proteins) were fixed with isopropanol (internal staining) or paraformaldehyde (cell surface staining) at 12 h postinfection. The presence of HCV glycoprotein E1 (vHCV1-1488) or Sindbis virus glycoprotein E1 (vTM3/SINS) was revealed by immunostaining with anti-HCV E1 MAb A4 or anti-Sindbis E1 MAb 33, respectively. Control cells infected with vTF7-3 alone did not show any staining (data not shown).

HCV virus particles, their heterogeneous size distribution on sucrose gradients and the diffuse bands observed by SDS-PAGE are more suggestive of misfolded complexes. This could be caused by overexpression of the glycoproteins and titration of a limiting host component, perhaps an ER molecular chaperone, which is required for proper folding of E1 and E2 and complex formation.

Although the E1-specific MAb and the conditions used for nondenaturing coprecipitation may have precluded detection of intermediate complexes, the assembly of detergent-stable HCV E1E2 heterodimers appears to be rather slow when compared with that in other viruses (17). Slow association of the pestivirus E1 and E2 glycoproteins, which are present as disulfide-linked heterodimers in infected cells and virions (73, 76), has also been reported (59). The rate of HCV E1 and/or E2 folding, which probably depends on interactions with molecular chaperones in the ER, may limit the rate of complex formation since properly folded subunits are usually required for specific recognition and stable association (17).

The inefficient assembly of HCV E1E2 complexes and the presence of misfolded disulfide-linked aggregates are not uncommon for viral glycoproteins (17). The measles virus HN glycoprotein folds slowly with low efficiency; less than 50% of the molecules have acquired processed glycans by 5 h after synthesis. The remaining molecules appear to be retained in the ER in a misfolded state (11, 40). Some retroviruses have also been shown to have inefficient processing of their glycoproteins. Only a small percentage (5 to 15%) of human immunodeficiency virus type 1 gp160 glycoprotein is cleaved to produce the mature gp120 component (77), and aberrant intermolecular disulfide bonds, similar to what we obtained for the HCV glycoproteins, have been observed for gp160 (54). Less than 5% of the Env protein of Friend spleen focus-forming virus leaves the ER and folds correctly (26). In

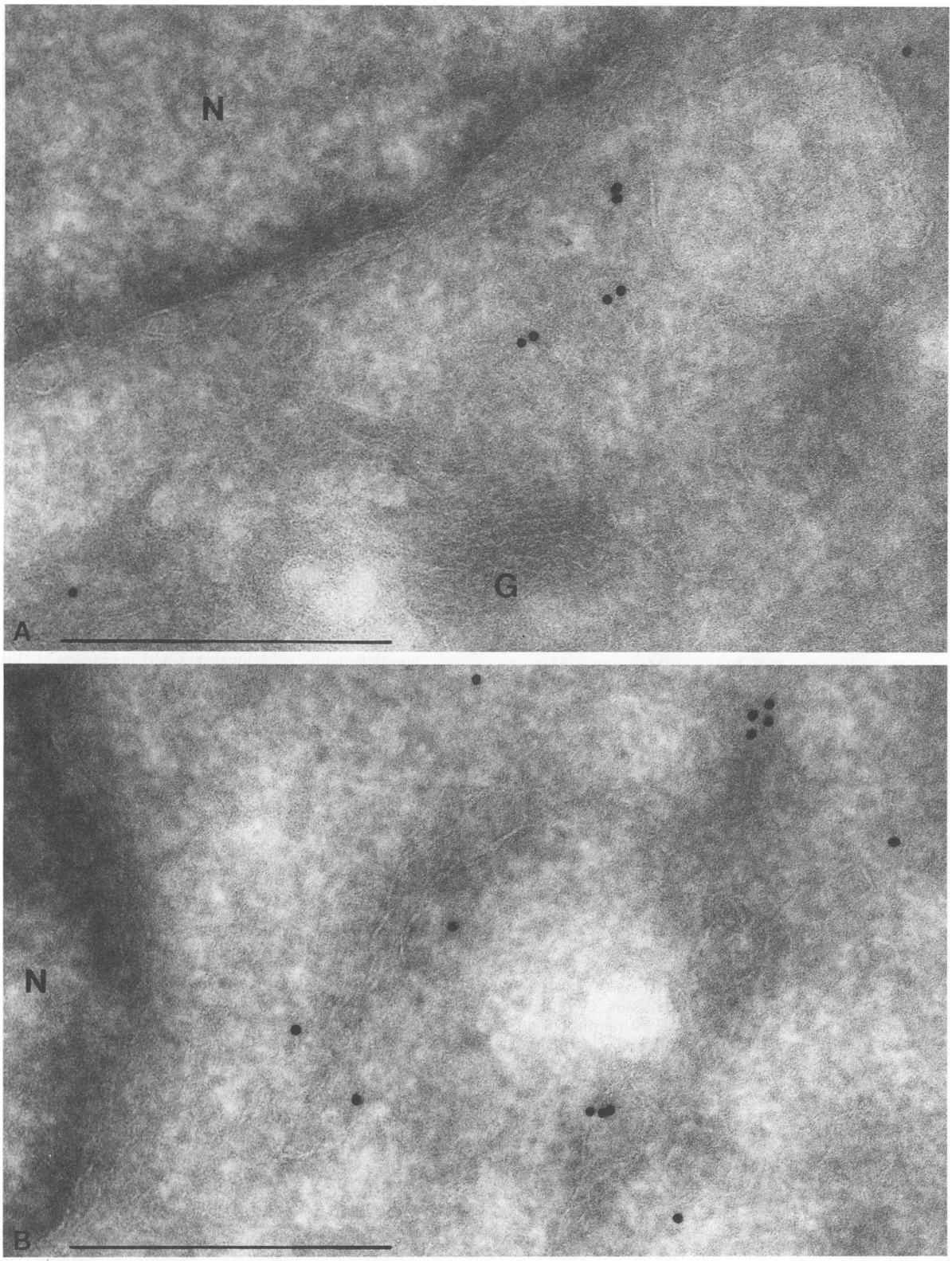

FIG. 12. Localization of HCV glycoproteins by immunoelectron microscopy. Shown are immunoelectron micrographs from frozen thin sections from HepG2 cells infected with SINrep/HCV1-1207. The sections were probed with MAbs against both E1 and E2, followed by 18-nm gold conjugated to rabbit anti-mouse immunoglobulin G. (A) The MAb (A4) against the E1 glycoprotein labeled vesicles in close proximity to the nucleus (N) and endoplasmic reticulum. No labeling was detected on the Golgi apparatus (G). (B) Similar labeling of perinuclear vesicles was also observed with the antibody against E2 (MAb A11). Bars = 0.5 μm.

potential conflict with our results, Ralston et al. (56) did not observe intermolecular disulfide bonds in purified HCV-1 E1E2 complexes. This could reflect strain- or cDNA clone-specific differences or might result from different expression levels of the HCV glycoproteins. Alternatively, aggregates and misfolded forms might have been lost during purification of the HCV-1 glycoprotein complexes.

Besides providing possible insight into the site of HCV virion assembly, the intracellular sequestration of HCV glycoproteins could also play a role in avoiding immune surveillance. The absence of HCV glycoproteins at the plasma membrane would be expected to reduce the chance of complement-mediated lysis of HCV-infected cells, thereby facilitating the establishment and maintenance of chronic infections in immunocompetent hosts.

ACKNOWLEDGMENTS

We thank Olga Strots and Songmei Xu for excellent technical assistance, our colleagues for critical reading of the manuscript, and H. Okayama for providing the BK-146 clone.

This work was supported by Public Health Service grant CA57973. J.D. was supported by the Fonds National Belge de la Recherche Scientifique as a senior research assistant, by the Keck Foundation, by NATO, and by a Fogarty International Research Fellowship (F05TW04765) from the Public Health Service.

REFERENCES

1. **Alter, H. J., R. H. Purcell, J. W. Shih, J. C. Melpolder, M. Houghton, Q.-L. Choo, and G. Kuo.** 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A non-B hepatitis. N. Engl. J. Med. **321:**1494–1500.
2. **Alter, M. J., S. C. Hadler, F. N. Judson, A. Mares, W. J. Alexander, P. Y. Hu, J. K. Miller, L. A. Moyer, H. A. Fields, D. W. Bradley, and H. S. Margolis.** 1990. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C virus infection. JAMA **264:**2231–2235.
3. **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J. Virol. **67:**3835–3844.
4. **Beach, M. J., and D. W. Bradley.** Personal communication.
5. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102:**1558–1566.
6. **Bradley, D., K. McCaustland, K. Krawczynski, J. Spelbring, C. Humphrey, and E. H. Cook.** 1991. Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. J. Med. Virol. **34:**206–208.
7. **Bradley, D. W., K. A. McCaustland, E. H. Cook, C. A. Schable, J. W. Ebert, and J. E. Maynard.** 1985. Posttransfusion non-A, non-B hepatitis in chimpanzees: physicochemical evidence that the tubule-forming agent is a small, enveloped virus. Gastroenterology **88:**773–779.
8. **Bredenbeek, P. J., I. Frolov, C. M. Rice, and S. Schlesinger.** 1993. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. J. Virol. **67:**6439–6446.
9. **Brew, K., J. Shaper, K. W. Q. Olsen, I. O. Trayer, and R. L. Hill.** 1975. Cross-linking of the components of lactose synthetase with dimethylpimelimidate. J. Biol. Chem. **250:**1434–1444.
10. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. USA **71:**942–946.
11. **Cattaneo, R., and J. K. Rose.** 1993. Cell fusion by the envelope glycoproteins of persistent measles viruses which caused lethal human brain disease. J. Virol. **67:**1493–1502.
12. **Chamberlain, J. P.** 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. Anal. Biochem. **98:**132–135.
13. **Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice.** 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44:**649–688.
14. **Choo, Q.-L., G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, C. Kuo, J. Kansopon, J. McFarland, A. Tabrizi, K. Ching, B. Moss, L. B. Cummins, M. Houghton, and E. Muchmore.** 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc. Natl. Acad. Sci. USA **91:**1294–1298.
15. **Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.
16. **Collett, M. S.** 1992. Molecular genetics of pestiviruses. Comp. Immun. Microbiol. Infect. Dis. **15:**145–154.
17. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
18. **Dubuisson, J., and C. M. Rice.** 1993. Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. J. Virol. **67:**3363–3374.

18a. **Dubuisson, J., and C. M. Rice.** Unpublished data.

19. **Eckart, M. R., M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, G. Kuo, M. Houghton, and Q.-L. Choo.** 1993. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. Biochem. Biophys. Res. Commun. **192:**399–406.
20. **Ellison, M. J., and M. Hochstrasser.** 1991. Epitope-tagged ubiquitin. J. Biol. Chem. **266:**21150–21157.
21. **Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop.** 1985. Isolation of monoclonal antibodies specific for human c-*myc* proto-oncogene product. Mol. Cell. Biol. **5:**3610–3616.
22. **Feinstone, S., H. J. Alter, H. P. Dienes, Y. Shimizu, H. Popper, D. Blackmore, D. Sly, W. T. London, and R. H. Purcell.** 1981. Non-A, non-B hepatitis in chimpanzees and marmosets. J. Infect. Dis. **144:**588–598.
23. **Fitzpatrick, D. F., T. Zamb, M. D. Parker, S. van Drunen Little-van den Hurk, L. A. Babiuk, and M. J. P. Lawman.** 1988. Expression of bovine herpesvirus 1 glycoproteins gI and gIII in transfected murine cells. J. Virol. **62:**4239–4248.
24. **Francki, R. I. B., C. M. Fauquet, D. L. Knudson, and F. Brown.** 1991. Classification and nomenclature of viruses: fifth report of the international committee on taxonomy of viruses. Arch. Virol. **2:**(Suppl.)223.
25. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8126.
26. **Gliniak, B. C., S. L. Kozak, R. T. Jones, and D. Kabat.** 1991. Disulfide bonding controls the processing of retroviral envelope glycoproteins. J. Biol. Chem. **266:**22991–22997.
27. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. **67:**2832–2843.
28. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. A second hepatitis C virus-encoded proteinase. Proc. Natl. Acad. Sci. USA **90:**10583–10587.
29. **Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice.** 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. J. Virol. **67:**1385–1395.
30. **Griffiths, G., and P. Rottier.** 1992. Cell biology of viruses that assemble along the biosynthetic pathway. Semin. Cell Biol. **3:**367–381.
31. **Harlow, E., and D. P. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. Proc. Natl. Acad. Sci. USA **88:**5547–5551.
33. **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two

distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. **67**:4665–4675.

34. **Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. USA **90**:10773–10777.
35. **Hobman, T. C.** 1993. Targeting of viral glycoproteins to the Golgi complex. Trends Microbiol. **1**:124–130.
36. **Hollinger, F. B.** 1990. Non-A, non-B hepatitis viruses, p. 2239–2273. *In* B. N. Fields (ed.), Virology, Raven Press, Ltd., New York.
37. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q.-L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology **14**:381–388.
38. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. J. Virol. **29**:705–715.
39. **Hsu, H. H., M. Donets, H. B. Greenberg, and S. M. Feinstone.** 1993. Characterization of hepatitis C virus structural proteins with a recombinant baculovirus expression system. Hepatology **17**:763–771.
40. **Kohama, T., T. A. Sato, R. Kobune, and A. Sugiura.** 1985. Maturation of measles virus hemagglutinin glycoprotein. Arch. Virol. **85**:257–268.
41. **Kolykhalov, A. A., E. V. Agapov, and C. M. Rice.** 1994. Specificity of the hepatitis C virus NS3 serine proteinase: effects of substitutions at the 3/4A, 4A/4B, 4B/5A, and 5A/5B cleavage sizes on polyprotein processing. Submitted for publication.
42. **Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W.-S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244**:362–364.
43. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
44. **Lanford, R. E., L. Notvall, D. Chavez, R. White, G. Frenzel, C. Simonsen, and J. Kim.** 1993. Analysis of hepatitis C virus capsid, E1, and E2/NS1 proteins expressed in insect cells. Virology **197**: 225–235.
45. **Lin, C., B. D. Lindenbach, B. Pragai, D. W. McCourt, and C. M. Rice.** 1994. Processing of the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. J. Virol. **68**:5063–5073.

45a. **Lindenbach, B. D., S. M. Feinstone, and C. M. Rice.** Unpublished data.

46. **Manabe, S., I. Fuke, O. Tanishita, C. Kaji, Y. Gomi, S. Yoshida, C. Mori, A. Takamizawa, I. Yoshida, and H. Okayama.** 1994. Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. Virology **198**:636–644.
47. **Mast, E. E., and M. J. Alter.** 1993. Epidemiology of viral hepatitis: an overview. Semin. Virol. **4**:273–283.
48. **Matsuura, Y., S. Harada, R. Suzuki, Y. Watanabe, Y. Inoue, I. Saito, and T. Miyamura.** 1992. Expression of processed envelope protein of hepatitis C virus in mammalian and insect cells. J. Virol. **66**:1425–1431.
49. **Matsuura, Y., and T. Miyamura.** 1993. The molecular biology of hepatitis C virus. Semin. Virol. **4**:297–304.
50. **Miyamoto, H., H. Okamoto, K. Sato, T. Tanaka, and S. Mishiro.** 1992. Extraordinarily low density of hepatitis C virus estimated by sucrose density centrifugation and polymerase chain reaction. J. Gen. Virol. **73**:715–718.
51. **Miyamura, T., and Y. Matsuura.** 1993. Structural proteins of hepatitis C virus. Trends Microbiol. **1**:229–231.
52. **Negro, F., D. Pacchioni, Y. Shimizu, R. H. Miller, G. Bussolati, R. H. Purcell, and F. Bonino.** 1992. Detection of intrahepatic replication of hepatitis C virus RNA by *in situ* hybridization and comparison with histopathology. Proc. Natl. Acad. Sci. USA **89**:2247–2251.
53. **Omura, T., and S. Takesue.** 1970. A new method for simultaneous purification of cytochrome b5 and NADPH-cytochrome c reductase from rat liver microsomes. J. Biochem. **67**:249–257.
54. **Owens, R. J., and R. W. Compans.** 1990. The human immunodeficiency virus type 1 envelope glycoprotein precursor acquires aberrant intermolecular disulfide bonds that may prevent normal proteolytic processing. Virology **179**:827–833.
55. **Pettersson, R. F.** 1991. Protein localization and virus assembly at intracellular membranes. Curr. Top. Microbiol. Immunol. **170**:67–104.

55a. **Pragai, B., and C. M. Rice.** Unpublished data.

56. **Ralston, R., K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q.-L. Choo.** 1993. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J. Virol. **67**:6753–6761.
57. **Rice, C. M., and J. H. Strauss.** 1982. Association of Sindbis virion glycoproteins and their precursors. J. Mol. Biol. **154**:325–348.
58. **Robbins, P. W., R. B. Timble, D. F. Wirth, C. Hering, F. Maley, G. F. Maley, R. Das, B. W. Gibson, N. Royal, and K. Biemann.** 1984. Primary structure of the *Streptomyces* enzyme endo-β-N-acetylglucosaminidase H. J. Biol. Chem. **259**:7577–7583.
59. **Rümenapf, T., G. Unger, J. H. Strauss, and H.-J. Thiel.** 1993. Processing of the envelope glycoproteins of pestiviruses. J. Virol. **67**:3288–3294.
60. **Russell, D. G.** Immunoelectron microscopy of endosomal trafficking in macrophages infected with microbial pathogens. Methods Cell Biol., in press.
61. **Russell, D. G., S. Xu, and P. Chakraborty.** 1992. Intracellular trafficking and the parasitophorous vacuole of *Leishmania mexicana*-infected macrophages. J. Cell Sci. **103**:1193–1210.
62. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
63. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
64. **Sato, K., H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, and S. Mishiro.** 1993. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. Virology **196**:354–357.
65. **Schmaljohn, A. L., E. D. Johnson, J. M. Dalrymple, and G. A. Cole.** 1982. Nonneutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis. Nature (London) **297**:70–72.
66. **Schmaljohn, A. L., K. M. Kokubun, and G. A. Cole.** 1983. Protective monoclonal antibodies define maturational and pH-dependent antigenic changes in Sindbis virus E1 glycoprotein. Virology **130**:110–119.
67. **Selby, M. J., Q.-L. Choo, K. Berger, G. Kuo, E. Glazer, M. Eckart, C. Lee, D. Chien, C. Kuo, and M. Houghton.** 1993. Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J. Gen. Virol. **74**:1103–1113.
68. **Shimotohno, K.** 1993. Hepatocellular carcinoma in Japan and its linkage to infection with hepatitis C virus. Semin. Virol. **4**:305–312.
69. **Simmonds, P., E. C. Holmes, T.-A. Cha, S.-W. Chan, F. McOmish, B. Irvine, E. Beall, P. L. Yap, J. Kolberg, and M. S. Urdea.** 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS5 region. J. Gen. Virol. **74**:2391–2399.
70. **Takahashi, K., S. Kishimoto, H. Yoshizawa, H. Okamoto, A. Yoshikawa, and S. Mishiro.** 1992. p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts. Virology **191**:431–434.
71. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65**:1105–1113.
72. **Tarentino, A. L., G. Quinones, A. Trumble, L.-M. Changchien, B. Duceman, F. Maley, and T. H. Plummer.** 1990. Molecular cloning and amino acid sequence of peptide-N-(N-acetyl-β-D-glucosaminyl) asparagine amidase from *Flavobacterium meningosepticum*. J. Biol. Chem. **265**:6961–6966.
73. **Thiel, H.-J., R. Stark, E. Weiland, T. Rümenapf, and G. Meyers.**

1991. Hog cholera virus: molecular composition of virions from a pestivirus. J. Virol. **65:**4705–4712.

74. **Tomei, L., C. Failla, E. Santolini, R. deFrancesco, and N. LaMonica.** 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. J. Virol. **67:**4017–4026.
75. **Wahlberg, J. M., W. A. M. Boere, and H. Garoff.** 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to low pH during virus maturation. J. Virol. **63:**4991–4997.
76. **Weiland, E., R. Stark, B. Haas, T. Rümenapf, G. Meyers, and H.-J. Thiel.** 1990. Pestivirus glycoprotein which induces neutralizing antibodies forms part of a disulfide-linked heterodimer. J. Virol. **64:**3563–3569.
77. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
78. **Yuasa, T., G. Ishikawa, S. Manabe, S. Sekiguchi, K. Takeuchi, and T. Miyamura.** 1991. The particle size of hepatitis C virus estimated by filtration through microporous regenerated cellulose fibre. J. Gen. Virol. **72:**2021–2024.