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Induction of Intracellular Membrane Rearrangements by HAV Proteins 2C and 2BC

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Hepatitis A virus (HAV) is distinguished from other picornaviruses by its slow and relatively poor, noncytopathic growth in cultures of mammalian cells. The 2C and 2BC proteins of HAV have been implicated in the determination of virus growth in cultured cells. The homologous proteins from other picornaviruses, such as poliovirus, have been demonstrated to exhibit multiple activities, such as RNA binding, nucleotide binding and NTPase, and membrane binding and reorganization. At least some of these activities are required for viral RNA replication. We report here that HAV 2C and 2BC proteins, like their poliovirus counterparts, can induce rearrangement of intracellular membranes and directly or indirectly interact with membranes. Therefore, the inefficient replication properties of HAV are not consequences of the inherent ability of 2C (2BC) to interact with membranes. The effect of 2C (2BC) protein sequences derived from a cell culture-adapted (cc) strain of HAV was compared with that of corresponding protein sequences from either a wild-type (wt) strain of HAV or a faster replicating cytopathic (cp) strain. The analysis demonstrated that mutations acquired in wt virus during adaptation to cell culture do not change dramatically either the ability of these proteins to associate with membranes and induce membrane alterations or the specific architecture of the induced membrane structures. On the other hand, 2C, but not 2BC, protein from the cp strain of HAV induced different membrane structures.

INTRODUCTION

Hepatitis A virus (HAV) is the sole member of the hepatovirus genus of the *Picornaviridae* family (Murphy *et al.,* 1995). Despite the common pattern of gene organization shared with other picornaviruses, there is limited similarity of nucleotide or amino acid sequences, and HAV seems to be more distantly related than other members of the family (Ticehurst *et al.,* 1989). Sequence motifs that signify proteins required for specific functions are present in the HAV polyprotein in the same relative positions as identified for other picornaviral genomes (Cohen *et al.,* 1987c). This implies that a set of nonstructural proteins typical for picornaviruses must be produced during HAV infection. However, an attempt to identify nonstructural proteins in infected cells using specific antisera raised against different portions of the HAV polyprotein was not successful, despite demonstrated sensitivity of the antiserum (Updike *et al.,* 1991). Not surprisingly, little is known about the functions of these proteins.

A striking difference between HAV and other picornaviruses is its growth characteristics. Wild-type HAV isolated from infected individuals fails to replicate in cultured cells. Although HAV was adapted to grow in a variety of cell cultures by serial passage in the laboratory (Provost and Hilleman, 1979), adapted virus still grows relatively slowly and manifests an asynchronous replication cycle that results in persistent infection (Harmon *et al.,* 1989). Several cell culture-adapted (cc) viruses have been isolated and sequenced and shown to differ from the parent virus by about 22–30 nucleotides within the 7.5-kb genome (Cohen *et al.,* 1987c; Jansen *et al.,* 1988; Graff *et al.,* 1994). Studies of chimeric HAV viruses implicated the 2B/2C region and the 5′ NCR of the genome as major determinants responsible for the adaptation of wild-type virus to growth *in vitro* (Cohen *et al.,* 1989; Emerson *et al.,* 1991, 1992; Zhang *et al.,* 1995), although other mutations acquired during adaptation could also contribute to the efficiency of virus growth in cultured cells (Day *et al.,* 1992; Emerson *et al.,* 1993). Nothing is known about the mechanism of action of these mutations. One mutation at nucleotide 3889 (amino acid 216 of the 2B protein) appeared to be required for growth of a variety of cc-HAV strains, regardless of the cell line used, although this mutation by itself was not sufficient to induce growth in cell cultures (Emerson *et al.,* 1992).

Several HAV variants which emerged during continued passage of persistently infected cell cultures, have been reported to induce a cytopathic effect in specific cell lines (Venuti *et al.,* 1985; Anderson, 1987; Cromeans *et al.,*

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1987; Nasser and Metcalf, 1987; Lemon *et al.,* 1991). These cytopathic (cp) variants were distinguished from their noncytopathic precursors by a faster replication cycle, with maximum virus yields reported to be reached as early as 40 hr after inoculation of cells under one-step growth conditions (Cromeans *et al.,* 1989). Cell necrosis associated with these variants was demonstrated both by clearing of cells in radioimmunofoci assays and by formation of visible plaques when infected cells were maintained under agarose overlays. Complete or partial nucleotide sequences have been determined for several of these cytopathic virus strains (Lemon *et al.,* 1991; Morace *et al.,* 1993; Beneduce *et al.,* 1995) and 31–42 mutations from the wild-type strain of HAV were found distributed throughout the genome, some of which had been found previously in genomes of cc-viruses. Deletion of an amino acid residue in 3A was postulated to have a primary role in causing the cytopathic effects observed with some of these strains (Morace *et al.,* 1993). However, detailed studies of the phenotypes of different chimeric viruses indicated that mutations in the P2 region (namely in 2B and 2C coding sequences) were necessary for expression of the rapid replication properties of the virus, while mutations in the 5′ noncoding region (NCR) and P3 regions cooperate with those P2 mutations to facilitate replication (Zhang *et al.,* 1995).

Proteins from the P2 region of the picornaviral polyprotein are essential for replication of viral RNA, although their biochemical functions in this process have not been determined. In poliovirus-infected cells 2C and 2BC proteins are found associated with intracellular membrane vesicles that are induced after virus infection and serve as the site for viral RNA replication (Bienz *et al.,* 1987; Semler *et al.,* 1988). The vesicles have been proposed to be responsible for efficient synthesis of progeny plus strand RNA (Bienz *et al.,* 1990, 1992). Their continuous increase in number leads to a profound rearrangement of the intracellular membranes, which has been proposed to be an underlying mechanism of the "cytopathic effect" observed with enterovirus infection (Bienz *et al.,* 1983). Similar membrane rearrangements have not been observed in HAV-infected cells (Huang *et al.,* 1979; Asher *et al.,* 1988; Egger and Bienz, unpublished observations), although no morphologic studies of cells infected by the cp HAV strains have been reported. This failure to detect induced membrane alterations and formation of replication complexes in HAV-infected cells may result from inefficient production of viral gene products that induce membrane reorganization, or alternatively, from an inherent inability of HAV proteins to induce membrane alterations. The latter explanation might contribute to the cause of the inefficient replication phenotype.

The 2C protein is the most highly conserved protein among all picornaviruses. It likely has multiple functions. Recently it was shown (Cho *et al.,* 1994; Aldabe and Carrasco, 1995) that poliovirus 2C or 2BC protein expressed in mammalian cells in the absence of other polioviral proteins associates with intracellular membranes and induces striking reorganization of these membranes into structures that may sometimes resemble the vesicles formed in infected cells. Previous analysis of multiple sequence alignments of 2C proteins from representatives of all genera of picornaviruses has revealed a highly conserved central region, encompassing amino acid residues 117–268 (poliovirus numbering), flanked by two nonconserved regions at the N- and C-terminal ends (Gorbalenya *et al.,* 1990; Teterina *et al.,* 1997). Sequences in both termini have been implicated in the membrane association properties of the poliovirus 2C protein (Echeverri and Dasgupta, 1995; Teterina *et al.,* 1997). The HAV 2C protein shares with other picornavirus relatives from 23 to 29% identical residues. This represents significantly less similarity than is shared among most other picornaviral 2C proteins, but represents one of the highest similarities between any HAV protein and its relatives (Ticehurst *et al.,* 1989; Gorbalenya, unpublished observations). In the N-terminal and C-terminal domains of 2C molecules, however, no residues have been identified that are absolutely conserved among all picornaviruses. In this report, we demonstrate that, like its poliovirus counterparts, HAV 2C and 2BC proteins have the intrinsic property to similarly associate with and induce structural rearrangements of intracellular membranes in HeLa as well as FRhK-4 cells. Thus, the absence of membranous replication complexes in HAV-infected cells is not due to a fundamental difference in membrane association activity of the HAV 2C protein sequences.

MATERIALS AND METHODS

Construction of plasmids

pTM-HAV-2C.* HAV cDNAs corresponding to nt 3996–5000 (the nucleotide numbering used below corresponds to the sequence of the plasmid pHAV/wt which contains cDNA of the wild-type strain HM175 (Cohen *et al.,* 1989) were amplified by polymerase chain reaction (PCR) from pHAV/wt (Cohen *et al.,* 1989), pHAV/7 (Cohen *et al.,* 1987a) (plasmid containing cDNA of cell culture adapted virus HAV175, passage 35), or PCR amplified P2 region from cytopathogenic mutant strain HM175/24a of HAV (Lemon *et al.,* 1991), kindly provided by Dr. S. Emerson. The sense primer #1, 5′-GAGTTAAGGATCCACATATG-AGTTTTTCCAACTGGTTAA-3′ was designed to create an *Nde* I site (underlined) immediately upstream of the 2C coding sequence (shown in bold). The antisense primer #3, 5′-ATTATCAAGATCTGCAGTCACTGAGA-CCACAACTCC-3′ was designed to introduce a stop codon followed by a *Pst*I site (underlined) at the end of the 2C coding sequence (shown in bold). The amplification was done for 20 cycles of denaturation at 94° for 1 min,

annealing at 53° for 1 min, and elongation at 72° for 2 min in the presence of Vent DNA polymerase (New England Biolabs). The resulting DNA fragments were cut with restriction enzymes *Nde*I and *Pst*I, gel-purified, and inserted between the corresponding sites in plasmid pTM-*Nde*I (Cho *et al.,* 1994) to produce plasmids pTM-HAV2C(wt), pTM-HAV2C(cc), and pTM-HAV2C(cp) bearing sequences from pHAV/wt, pHAV/7, and HAV175/24a, correspondingly.

pTM-HAV-2BC. HAV cDNAs corresponding to nt 3243–5000 were amplified as described above using a sense primer #2, 5′-GGGACTGTTTTCCATGGCCAAAATTTCTC-3′ that was designed to create an *Nco*I site (underlined) at the 5′ end of the 2B coding sequence (shown in bold) and negative sense primer #3. The resulting DNA fragments were hydrolyzed with restriction endonucleases *Nco*I and *Pst*I, gel-purified, and inserted between the corresponding sites in plasmid pTM-1 (Moss *et al.,* 1990), resulting in plasmids pTM-HAV2BC(wt), pTM-HAV2BC(cc), and pTM-HAV2BC(cp). Sequence analyses of the entire coding regions of 2C and 2BC demonstrated that no mutations were introduced during PCR amplification.

Generation of recombinant vaccinia virus vHAV2C(cc)

Recombinant vaccinia virus vHAV2C(cc) was generated by homologous recombination between pTM-HAV2C(cc) and TK^+ wild-type vaccinia virus as described previously (Cho *et al.,* 1994). Putative recombinant vaccinia viruses selected in TK^- 143 cells in the presence of 5-bromo-2′-deoxyuridine (BUdR, 25 μg/ml, US Biochemicals, Cleveland, OH) were isolated by plaque purification and identified by their ability to express HAV 2C protein, analyzed by Western immunoblot.

Expression of HAV 2C or 2BC proteins in HeLa cells

The transient gene expression system described by Fuerst *et al.* (1986) was used with slight modifications. HeLa cells in 35-mm plates were transfected with various plasmids using Lipofectin (Gibco/BRL) and simultaneously infected with recombinant vaccinia virus vTF7-3. Briefly, 7–8 μg of supercoiled plasmid DNA in 50 μl of water were mixed with 20 μl of Lipofectin in 100 μl of water and mixtures incubated at room temperature for 20 min. Mixtures were diluted with 0.5 ml of MEM, and vTF7-3 was added at 5 PFU per cell before applying to cell monolayers for 1 hr at 37° with constant rocking. Two milliliters of MEM were added and incubation was continued for 2–4 hr. The medium was removed and 2 ml of MEM with 3% fetal bovine serum was added. Cells were incubated at 37° for 12–16 hr and then harvested for protein analysis or fixed for immunofluorescence or EM analysis. In some experiments, cells were coinfected with vHAV2C(cc) and vTF7-3. Expressed proteins were analyzed by Western immunoblot analysis using anti-HAV2C antiserum (Updike *et al.,* 1991) as previously described (Tesar *et al.,* 1994).

Indirect immunofluorescence microscopy

For immunofluorescence (IF) microscopy, cells were grown and transfected in 35-mm dishes on 22-mm^2 coverglasses. HeLa cells, transfected with HAV 2C or 2BC expression plasmids and infected with the recombinant vTF7-3 were washed once with PBS buffer and fixed in acetone-methanol (1:1) for 10 min at room temperature. IF was performed as previously described (Cho *et al.,* 1994) using rabbit anti-HAV2C serum as primary antibodies and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL) as secondary antibodies.

Expression of HAV 2C or 2BC proteins in FRhK-4 cells

Avian host-restricted vaccinia virus recombinant MVA/T7 (Wyatt *et al.,* 1995) was used instead of vTF7-3 for expression of proteins in FRhK-4 cells. Transfections were done as described above for HeLa cells.

Electron microscopy (EM) and EM immunocytochemistry (IEM)

HeLa cells were fixed for EM or IEM 16 hr after transfection with plasmids or infection with recombinant vaccinia virus as described (Cho *et al.,* 1994; for review see Bienz and Egger, 1995)). Briefly, for conventional EM, cells were fixed in glutaraldehyde/osmium tetroxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). For IEM, cells were fixed in 2% paraformaldehyde followed by fixation in 0.2% uranyl carbonate and embedded in LRGold (London Resin Company) at −20°.

For immunocytochemical labeling, polyclonal rabbit antibody against HAV 2C was used (Updike *et al.,* 1991). Labeling of the sections was done by the indirect method with GAR-G10 gold label (Amersham, UK).

Comparative sequence analysis

The nucleotide and amino acid sequences of picornavirus 2C proteins were extracted from the GenBank and Swissprot databases, respectively. Global multiple sequence alignments were produced with the CLUSTALV program (Higgins *et al.,* 1992). The program was run in conjunction with the different amino acid scoring tables of the PAM (Dayhoff *et al.,* 1978) and Blossum (Henikoff and Henikoff, 1992) families. Analyses of multiple sequence alignments were performed within the Wisconsin GCG package version 8, 1994 (Genetics Computer Group, 575 Science Drive, Madison, WI), using the EGCG package interface. Secondary structure predictions were produced with the help of the PHD program (Rost and Sander, 1993) through the EMBL-network server. Calcu-

TABLE 1

Variant HAV 2C and 2BC Expression Plasmids

Designation	Plasmids	Source of coding sequence
cc	pTM-HAV2C(cc)	pHAV/7
	pTM-HAV2BC(cc)	
wt	pTM-HAV2C(wt)	pHM175/wt
	pTM-HAV2BC(wt)	
cp	pTM-HAV2C(cp)	HM175/24a
	pTM-HAV2BC(cp)	

lation of the hydrophobic/hydrophilic profile of proteins was made using a scoring table of Eisenberg *et al.* (1984).

RESULTS

Expression of HAV 2C and 2BC proteins in HeLa cells

The aim of this study was to analyze the ability of HAV 2C and 2BC proteins to associate with intracellular membranes and to test whether the proteins were able to induce morphological rearrangements. We selected the transient expression system based on recombinant vaccinia virus containing the T7 RNA polymerase gene (Fuerst *et al.,* 1986), which has been used extensively for expression of foreign proteins in mammalian cells and which was used previously for studies of poliovirus (PV) 2C protein (Cho *et al.,* 1994; Aldabe and Carrasco, 1995). Initially, we constructed plasmids containing the sequence coding for 2C protein from the attenuated cc-HM175, passage 35, strain of HAV (from pHAV/7 (Cohen *et al.,* 1987b) cloned in the expression vector, pTM-1. Since 2C sequences may also function during virus infection as a component of the 2BC protein, we also engineered plasmids that contained sequences coding for HAV 2BC protein. The cleavage site between HAV 2A and 2B proteins has been determined recently (Martin *et al.,* 1995; Gosert *et al.,* 1996) to be different from that proposed earlier (Cohen *et al.,* 1987c). For subsequent studies to compare 2C sequences from the wild-type strain of HM175 HAV, and from a cytopathic variant of the virus, strain HM175/24a (Lemon *et al.,* 1991), similar constructs containing 2C and 2BC sequences from these variants were also prepared (Table 1).

Plasmid pTM-HAV2C(cc) was used to generate recombinant vaccinia virus vTM-HAV2C(cc). HAV proteins were expressed in HeLa cells either by transfection of pTM-HAV2C or pTM-HAV2BC with infection by vTF7-3 or by coinfection of vTF7-3 and vTM-HAV2C(cc). Immunofluorescence of cells probed with anti-HAV2C serum confirmed expression of 2C protein sequences. Coinfection with two recombinant vaccinia viruses yielded more than 80% of cells expressing HAV 2C antigen, compared to 25–30% of cells expressing antigen after simultaneous plasmid transfection and infection. The latter level of expression proved sufficient for further analyses, however, and obviated the need to isolate multiple recombinant vaccinia viruses, so the transfection/infection protocol was used for most subsequent studies.

Expression of HAV proteins was generally detectable by 8 hr posttransfection, and increased during the first 24–36 hr. Figure 1 shows an analysis of protein expression using the transfection/infection protocol. Cell lysates from mock-infected cells (lane 1) or vTF7-3-infected cells (lane 8) contain a background of four cellular proteins that immunoreact with the anti-HAV 2C rabbit serum. 2C protein from cc-virus (lane 2), wild-type (wt) virus (lane 4), and the cytopathic (cp) variant (lane 6) and 2BC proteins from the same sources (lanes 3, 5, 7, respectively) were readily detected. Expression levels were comparable for all 2C and slightly lower for all three 2BC proteins (compare lanes 2, 4, 6 and lanes 3, 5, 7, respectively). Synthesis of 2C protein from cc-virus also generated a smaller immunoreactive protein, which is likely due to initiation of translation from the second AUG codon, located at codon 31 in the 2C sequence of cc-virus. Neither the wt nor cp strains of HAV contain this AUG codon (Table 2), and no smaller immunoreactive protein was detected in the cell extracts (Fig. 1, lanes 2, 4, and 6). A similar utilization of the second AUG for initiation of translation has been described previously for the poliovirus 2C protein (Cho *et al.,* 1994).

Immunofluorescent microscopic analysis of cells expressing 2C and 2BC proteins

To investigate the subcellular localization of HAV 2C and 2BC proteins we examined HeLa cells expressing

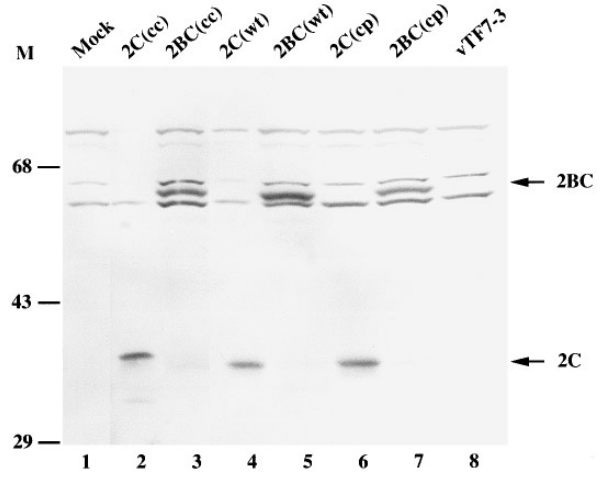

FIG. 1. Immunoblot analysis of proteins in HeLa cells transfected with 2C or 2BC expression plasmids. HeLa cell monolayers were transfected with the plasmids described in Table 1, in the presence of recombinant vaccinia virus encoding T7 RNA polymerase. After 10 hr, cells were harvested and extracts were subjected to SDS–PAGE and immunoblot analysis with anti-HAV 2C serum.

TABLE 2

Amino Acid Differences in 2B and 2C Proteins from wt, cc, and cp Variants of HAV HM175

Amino acid residue	HM 175 wild-type	HM 175 HAV/7	HM175/24a cytopathic
2B			
2	Lys	Lys	*Asn*
13	Ile	Ile	*Met*
105	Asp	Asp	*Glu*
157	Asp	Asp	*His*
216	Ala	*Val*	*Val*
226	Gly	*Ala*	Gly
2C			
22	Ala	Ala	*Gly*
24	Tyr	Tyr	*Cys*
31	Lys	*Met*	Lys
64	Glu	*Lys*	*Lys*
76	Phe	*Ser*	*Ser*
93	His	His	*Tyr*
125	Ser	Ser	*Phe*
142	Tyr	Tyr	*His*
190	Val	*Ile*	Val
320	Glu	Glu	*Asp*

Note. The spaces separate the predicted N-terminal, central, and C-terminal domains. Amino acid residues different from the wild-type sequence are indicated in bold and italic.

these proteins by IF microscopy using anti-HAV 2C serum (Updike *et al.,* 1991). Figure 2 shows the IF pattern manifested by the 2C(cc) and 2BC(cc) proteins. Both proteins were localized throughout the cytoplasm. Both exhibited a highly punctate pattern, with numerous predominantly spherical spots, which were more densely localized to the perinuclear region. Expression of 2C protein from wt virus generated a similar IF pattern, whereas the IF pattern of 2C from the cp variant was more fibrillar, often appearing as long "thread-like" formations, although in some cells a pattern resembling that of 2C cc was also observed (data not shown; see below). Thus IF analysis of cells expressing HAV 2C and 2BC proteins suggested an uneven distribution of these proteins in subcellular compartments. To determine if these proteins induced ultrastructural changes in cells, we performed EM studies.

Membrane rearrangements in HAV 2C- and 2BC-expressing cells

Cells infected with recombinant vaccinia virus containing HAV2C(cc) cDNA or transfected with plasmid pTM-HAV2BC(cc) were harvested after 16 hr and were examined by electron microscopy. Figures 3A and 3B show the formation of extensive networks of membrane structures by HAV 2C and 2BC proteins, respectively. Both proteins induce a highly regular coiling of membranes, devoid of ribosomes, into large aggregates, averaging 1–2 μm in diameter. The curvature of the 2BC-induced coils is tighter than the 2C-induced coils. In addition, the 2C-induced coiled membranes often seem to extend into the lumen of the endoplasmic reticulum (ER), whereas the 2BC-induced coils arise from a juxtaposition of undulating smooth ER tubules. The appearance of the 2C-induced coils seems identical to the structures desig-

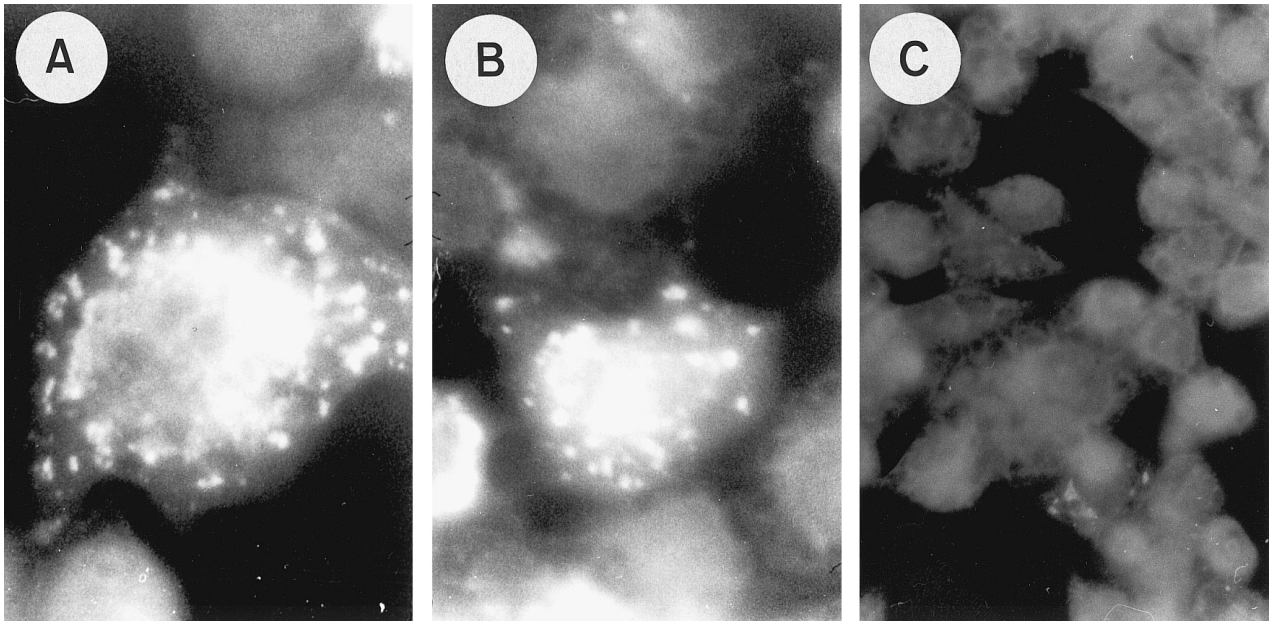

FIG. 2. HAV 2C and 2BC protein expression in HeLa cells shown by indirect immunofluorescence. HeLa cells were grown on chamber slides, infected with vTF7-3, and transfected with (A) pTM-HAV2C(cc), (B) pTM-HAV2BC(cc), or (C) mock transfected. The cells were fixed with acetone/methanol (50/50) and stained using anti-HAV2C serum, followed by FITC goat anti-rabbit antibodies.

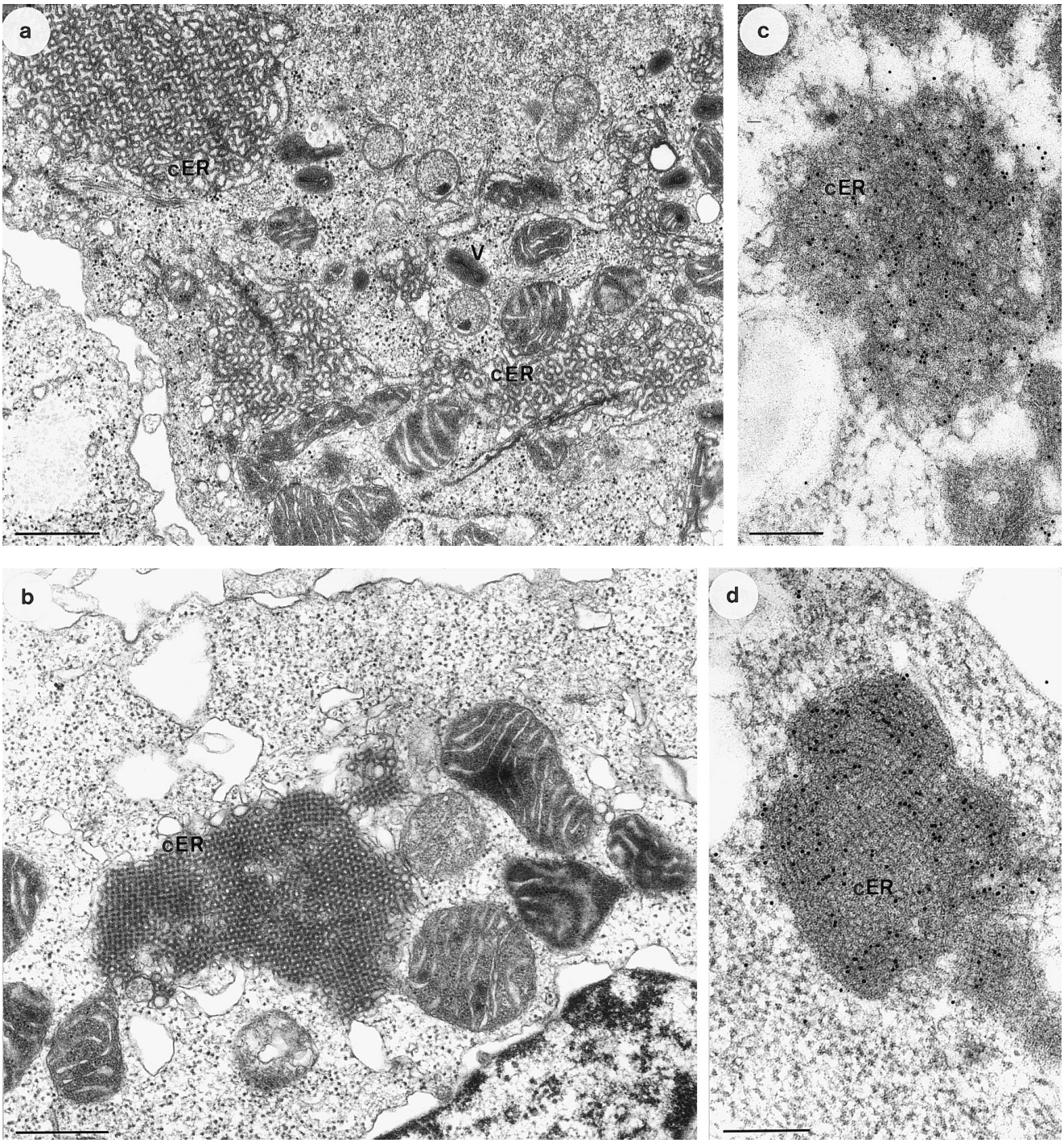

FIG. 3. Expression of HAV 2C or 2BC(cc) in Hela cells, 16 hr posttransfection. V, vaccinia virus; cER, crystalloid ER. (a) Conventional EM of a HAV 2C(cc) expressing cell, showing crystalloid ER scattered throughout the cytoplasm. Bar, 500 nm. (b) Same as (a), expressing HAV 2BC(cc). Note that crystalloid ER is more dense and shows more narrow curvature of the membranes as compared to (a). Bar, 500 nm. (c) Immunoelectron micrograph of a cell as in (a). HAV 2C(cc) is labeled using rabbit anti-HAV 2C serum, visualized with goat anti-rabbit antibodies coupled to 10 nm gold. The label is found over crystalloid ER. Bar, 200 nm. (d) Immunoelectron micrograph as in (c), but of cell expressing HAV 2BC(cc). Again, immunocytochemical label is over crystalloid ER. Bar, 200 nm.

nated as crystalloid ER (Chin *et al.,* 1982, see Discussion), which are formed by ER invaginations similar to the formation of the tubular structures found in cells expressing poliovirus 2C (Cho *et al.,* 1994). Although quite similar in aspect, the 2BC-induced membrane coils seem to represent a different type of crystalloid ER-like struc-

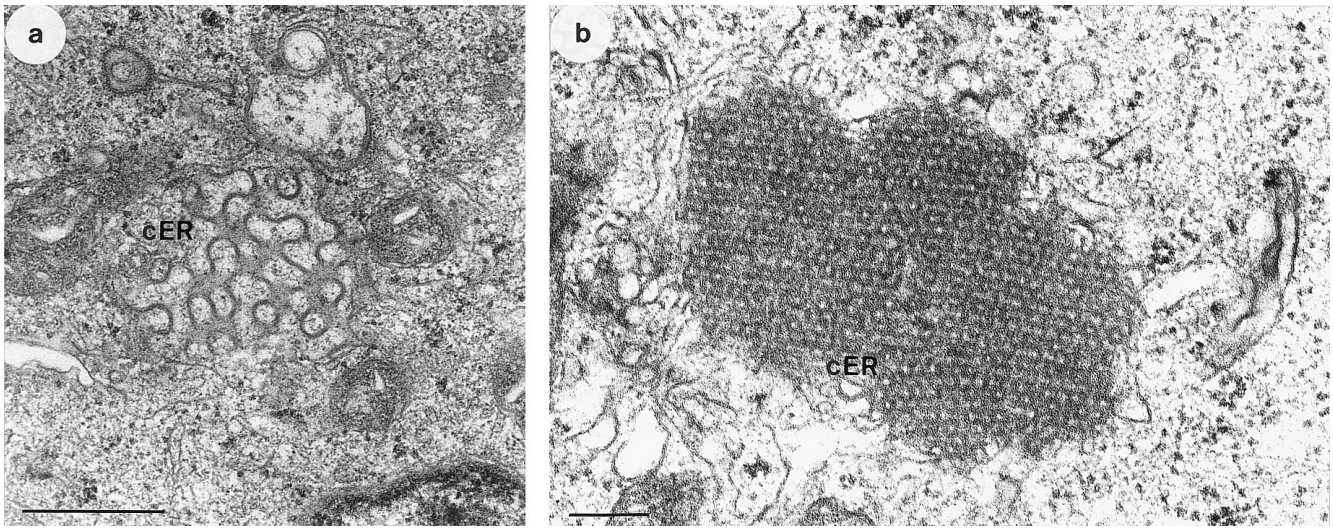

FIG. 4. Expression of HAV 2C or 2BC(cc) in FRhK-4 cells, 16 hr posttransfection; cER, crystalloid ER. (a) Electron micrograph of a HAV 2C(cc) expressing cell. Note a piece of the loose crystalloid ER similar to that found in Hela cells transfected with the same plasmid. Bar, 500 nm. (b) Electron micrograph of a HAV 2BC(cc) expressing FRhK-4 cell showing tight crystalloid ER. Bar, 200 nm.

ture. Golgi stacks are noticeably absent in these cells, suggesting that multiple components of the cell's organellar network contribute to the 2C-induced rearrangements, as were shown to occur in PV-infected cells (Schlegel *et al.,* 1996).

Immunoelectron microscopy performed with antibody against HAV 2C showed a dense concentration of viral protein associated with the crystalloid structures (Figs. 3C and 3D). Presumably, interaction of the 2C-containing sequences with cellular membranes is required for the structural rearrangements to occur.

HeLa cells have been used previously to visualize membrane changes induced by expression of poliovirus 2C protein (Cho *et al.,* 1994; Aldabe and Carrasco, 1995; Teterina *et al.,* manuscript in preparation). These cells are not permissive hosts for growth of HAV, however, and no data are available regarding possible host cell membrane specificity for 2C protein interaction. We attempted to repeat the experimental protocol described in Fig. 3 using FRhK-4 and BS-C-1 cells, both of which are routinely used to support growth of cc-HAV HM175. Both of these cell lines manifested a significantly greater sensitivity to vaccinia virus infection compared to HeLa cells, and failed to sustain morphological integrity long enough to produce 2C protein and permit preparation for electron microscopic examination. We therefore used a recombinant avian vaccinia virus engineered to produce T7 RNA polymerase, which was reported to be less cytopathic in mammalian cells (Wyatt *et al.,* 1995). BS-C-1 cells still tended to disintegrate after infection, but FRhK-4 cells withstood avian vaccinia virus infection to permit fixation and EM analysis of cells transiently expressing HAV 2C and 2BC protein. Induction of coiled membrane structures occurred in FRhK-4 cells (Figs. 4A and 4B; 2C and 2BC), as well as in HeLa cells. The expression of protein and appearance of crystalloid structures were similar in both cell types.

2C protein sequences from wild-type, cell culture-adapted, and cytopathic strains of HAV

Important determinants of the growth phenotype of HAV in cultured cells have been mapped to the 2B and 2C coding regions. Most of the mutations that were acquired during cell culture adaptation are localized in the N-terminal portion of 2C, which, for the poliovirus 2C protein, has been shown to contain sequences conferring membrane binding properties (Echeverri and Dasgupta, 1995; Teterina *et al.,* manuscript in preparation). For example, three of four mutations in cell culture-adapted HM175 p35 (in pHAV/7) and six of eight mutations in the cytopathic variant HAV175/24a are located in the N-terminal third of the protein sequence (Table 2). Cell-culture adapted and cytopathic viruses also harbor one mutation in the central region of 2C, and HAV175/24a bears an additional mutation in the C-terminal region. Table 2 lists the mutations and amino acid residue changes in the three variant 2C protein sequences.

Effects of different HAV 2C sequences on membrane rearrangements

To explore the possibility that the ability of the virus's 2BC and 2C sequences to induce membrane alterations might correlate with its ability to grow, we examined cells transfected with plasmids encoding the wt 2C and 2BC sequences (pTM-HAV2C(wt) and pTM-HAV2BC(wt). Expression of the various forms of the proteins was similar (compare Fig. 1, lanes 4–7). Figures 5A and 5B show

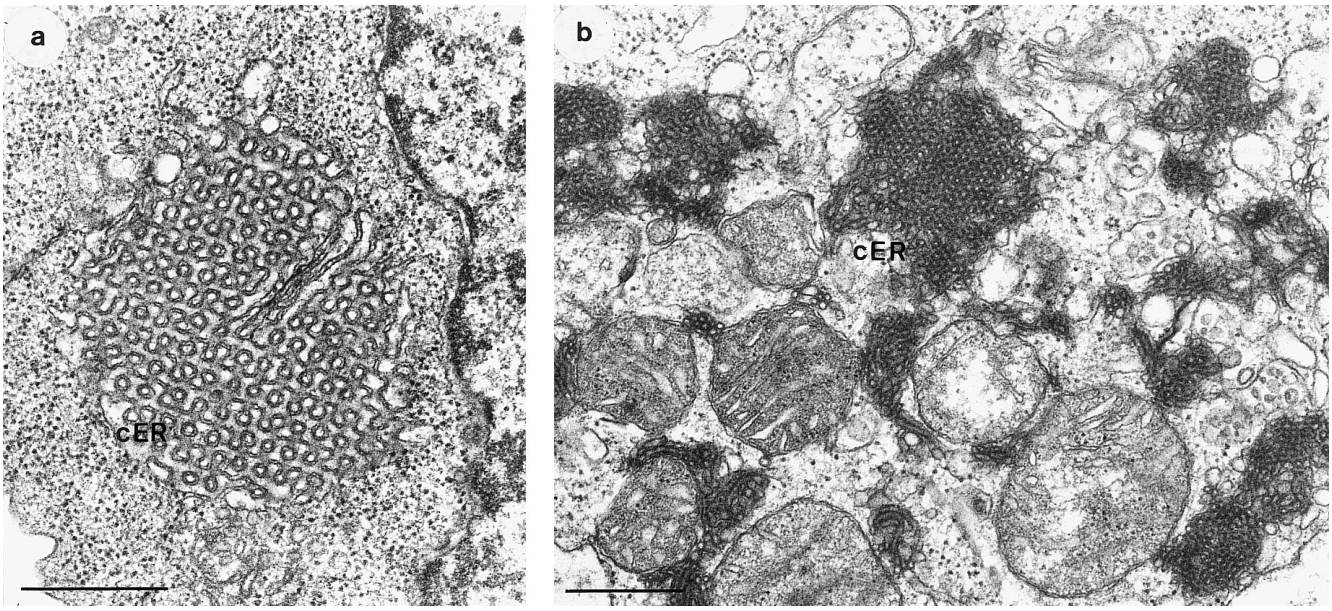

FIG. 5. Expression of (a) HAV 2C(wt) or (b) 2BC(wt) in Hela cells, 16 hr posttransfection. The crystalloid ER patterns found are comparable to those observed in cells transfected with the corresponding plasmids derived from HAVcc strains. Bars, 500 nm.

that the wt HAV 2C and 2BC proteins induced the same membrane rearrangements in transfected cells as occurred in cells transfected with the plasmids encoding 2C proteins from the cell culture-adapted strain.

In contrast, 2C sequences derived from a rapidly replicating, cytopathic strain of HAV, encoded in pTM-HAV2C(cp), did not induce crystalloid ER but generated membrane structures with an extended, thread-like appearance (Fig. 6A). This distinction was consistent with the more fibrillar pattern observed for these cells by IF microscopy. pTM-HAV2BC(cp), however, produced the same regular crystalloid networks observed with wt or cc sequences of 2BC (compare Figs. 3B, 5B, and 6B). The lack of crystalloid ER formation with the 2C(cp) sequences does not seem to be due to a low expression of the corresponding protein since IEM pictures show a heavy labeling of the thread-like inclusions (data not shown).

Additionally, 2BC-containing plasmids induced varying amounts of heterogeneously sized vesicles. They comprise larger vacuoles of dilated rER and smaller smooth-surfaced vesicles, also presumably of ER origin. The number and location of all vesicular structures differed with the three HAV strains compared. 2BC of cc-HM175 and of wt-HM175 tend to induce fewer of the larger vacuoles than the cp variant. The latter induced comparatively more smooth vesicles, which often appeared to be associated in a central region of the cell. In all cases, however, these vesicles constituted a minor fraction of the induced membrane changes in the transfected cells, with crystalloid ER networks representing the major structures formed.

DISCUSSION

Expression of 2C and 2BC proteins from either poliovirus or hepatitis A virus in mammalian cells induces striking alterations in the organization and architecture of intracellular membranes. The specific morphology of the structures formed varies with the protein sequence. Highly ordered crystalloid, myelin-like membrane swirls, clusters of vesicles, and random membrane fragments have all been observed to accumulate following expression of different 2C sequences, mutated 2C sequences, or partial 2C sequences. The ability to induce these membrane rearrangements requires the presence in the protein of domains or regions that associate tightly with the lipid bilayer. In the case of the picornaviral 2C protein, the N-terminal region has been predicted to form an amphipathic helix (Paul *et al.,* 1994; Teterina *et al.,* 1997), believed to account for the association with membranes. *In vitro,* deletion of the N-terminal 54 amino acids from poliovirus 2C protein synthesized in rabbit reticulocyte lysates eliminated its capacity to bind to exogenous membrane preparations (Echeverri and Dasgupta, 1995). In transfected cells, the N-terminal domain alone (amino acids 1–122) or any N-terminal domain-containing portion of poliovirus 2C became associated with membrane structures (Teterina *et al.,* 1997). Although no biochemical evidence has been reported for other picornaviral 2C protein membrane interactions, comparative amino acid sequence analysis predicted that the N-terminal domains of all 2C proteins can accommodate an α/α-fold organization (Orengo, 1994) with a potential for membrane association (Paul *et al.,* 1994; data not shown).

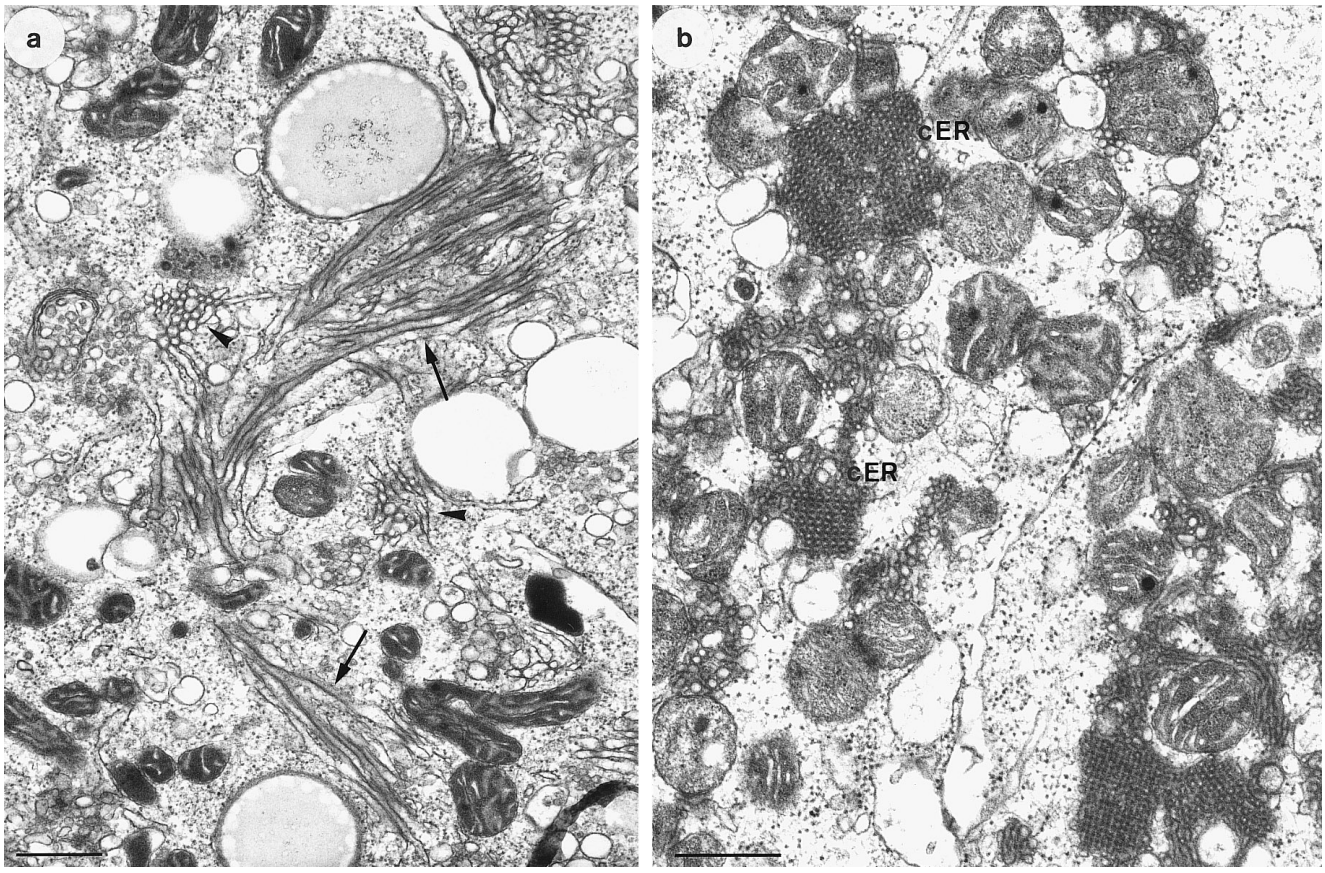

FIG. 6. Expression of HAV 2C(cp) or 2BC(cp) in Hela cells, 16 hr posttransfection. (a) HAV 2C(cp) does not induce crystalloid ER but rather extended unfolded membraneous threads, derived from the rER (arrows). In cross-sections, the tubular structure of the ER-derived threads is still recognizable (arrowheads). Bar, 500 nm. (b) HAV 2BC(cp) induces crystalloid ER very similar to HAV 2BC(cc) and HAV 2BC(wt). Bar, 500 nm.

The relative abundance of mutations scattered over the N-terminal domain of 2C in cell culture-adapted strains of HAV may indicate a role of this domain in the determination of growth efficiency of HAV in cultured cells. This possibility prompted us to perform an analysis of the effect of these mutations on the arrangements of hydrophobic/hydrophilic residues of the N-terminal domain. The analysis of the hydrophilic/hydrophobic profiles (Eisenberg *et al.,* 1984) of the different 2C protein sequences present in wild-type, cell culture-adapted, and cytopathic virus variants indicated that the local secondary structure organization of 2C of cell culture-adapted strains of HAV may be affected by the mutations and that the propensity to form amphipathic helices appears to decline in an inverse correlation with the ability of the virus to replicate in cell culture (data not shown). The relationships between the predicted structural features of the 2C protein, the morphologic arrangements of the induced membrane structures, and the growth properties of the viruses carrying specific 2C sequences remain to be determined.

The striking coiled formation of the HAV 2C-induced membrane structure is reminiscent of the formation of crystalloid ER by 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, a resident protein of the ER with seven transmembrane domains, which has been investigated in some detail (Anderson *et al.,* 1983; Pathak *et al.,* 1986). In the presence of an inhibitor of reductase activity, the enzyme is overproduced, albeit inactive, leading to the formation of crystalloid ER harboring large amounts of HMG CoA. This is supported by the observation that elimination of membrane-spanning domains 4 and 5 abolishes the ability of the protein to induce crystalloid ER, and cells that express high levels of a soluble form of the reductase show no hypertrophied or crystalloid ER (Jingami *et al.,* 1987), indicating that the membrane domain of the reductase is required to form this structure. In yeast, overproduction of HMG CoA reductase induces so called karmellae (Writght *et al.,* 1988; Lum and Write, 1995; Hampton *et al.,* 1996). Karmellae arrise in an identical way as crystalloid ER, but they are in essence only flat ER sheets not being folded into the regular undulating tubules of the crystalloid ER. There are only few reports of other than HMG CoA reductase induced crystalloid ER or karmellae. Restrepo-Hartwig and Carrington (1994) observed nucleus-associated

membrane proliferation with a striking resemblance to karmellae in transgenic tobacco plants, overexpressing the replication-associated 6-kDa protein of tobacco etch potyvirus. Wanker *et al.* (1995) found karmaellae induced in yeast by the 180-kDa ribosome receptor. In *E. coli,* overproduction of two different membrane protein complexes, ATP synthase and fumarate reductase, leads to formation of intracellular membrane tubules (Von Meyenberg *et al.,* 1984; Weiner *et al.,* 1984). Naturally occurring crystalloid ER is found in the spermatogonia of the firebug *Pyrrhocoris apterus* (Wolf and Motzko, 1995). The formation of the crystalloid ER induced by an overproduction of HMG CoA reductase is reversible in the process of vesiculation and tubulation. This indicates the membranes of the crystalloid ER are not permanently damaged but retain their ability to assume multiple configurations.

It is possible that production of relatively large amounts of any protein that interacts with the cell's internal membrane compartments will induce rearrangements or alterations of the membrane structure. We have observed that expression of recombinant cytochrome b5 protein in HeLa cells generates a juxtaposition of endoplasmic reticulum leading to swirls of membrane material (unpublished observations). Presumably, insertion or association of new proteins with preexisting membrane structures causes, by virtue of new protein–protein or protein–lipid interactions, adjustments in curvature and flexibility of the entire bilayer. It is surprising that even relatively small changes in protein sequence may lead to significant variations in membrane organization; for example, the replacement of eight amino acids in 2C from the cytopathic strain of HAV175/24a eliminated the organization into crystalloid structures and generated instead obviously ER-derived and straight tubules lacking the narrow curvature of crystalloid ER. Such profound modulation of membrane organization induced by relatively small changes in the protein sequence supports a genuine membrane-associates role for HAV 2C.

The HAV 2C and 2BC proteins demonstrate efficient membrane association properties and causes major rearrangement of the endoplasmic reticulum and probably Golgi and perhaps other intracellular membrane compartments. The slow growth phenotype of this virus is therefore not likely due to the inability of this protein to induce membrane changes per se; although it remains possible that the specific nature of the membrane interactions and the different membrane formations induced by variants of the 2C protein may favor or constrain fast and efficient RNA replication. However, the absence of any detectable membrane alterations and structural rearrangement into viral replication complexes in HAV-infected cells is likely due to production of very low amounts of these and other viral proteins in the infected cells.

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