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Antigenic and Genetic Variation in Cytopathic Hepatitis A Virus Variants Arising during Persistent Infection: Evidence for Genetic Recombination

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Variants of hepatitis A virus (pHM175 virus) recovered from persistently infected green monkey kidney (BS-C-1) cells induced a cytopathic effect during serial passage in BS-C-1 or fetal rhesus kidney (FRhK-4) cells. Epitope-specific radioimmunofocus assays showed that this virus comprised two virion populations, one with altered antigenicity including neutralization resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics. Replication of the antigenic variant was favored over that of virus with the normal antigenic phenotype during persistent infection, while virus with the normal antigenic phenotype was selected during serial passage. Viruses of each type were clonally isolated; both were cytopathic in cell cultures and displayed a rapid replication phenotype when compared with the noncytopathic passage 16 (p16) HM175 virus which was used to establish the original persistent infection. The two cytopathic virus clones contained 31 and 34 nucleotide changes from the sequence of p16 HM175. Both shared a common 5′ sequence (bases 30 to 1677), as well as sequence identity in the P2-P3 region (bases 3249 to 5303 and 6462 to 6781) and 3′ terminus (bases 7272 to 7478). VP3, VP1, and $3C^{pro}$ contained different mutations in the two virus clones, with amino acid substitutions at residues 70 of VP3 and 197 and 276 of VP1 of the antigenic variant. These capsid mutations did not affect virion thermal stability. A comparison of the nearly complete genomic sequences of three clonally isolated cytopathic variants was suggestive of genetic recombination between these viruses during persistent infection and indicated that mutations in both 5′ and 3′ nontranslated regions and in the nonstructural proteins 2A, 2B, 2C, 3A, and $3D^{pol}$ may be related to the cytopathic phenotype.

Hepatitis A virus (HAV) is unique among human picornaviruses with respect to its tropism for liver cells and its capacity to induce acute hepatocellular injury (25). A variety of primate cell types have been shown to be permissive for HAV, but most wild-type virus isolates replicate very slowly in cell culture (4, 11, 17, 35). Although more rapid replication and higher final yields are achieved with virus that has been adapted to growth in cell culture, even highly cell culture-adapted HAV variants replicate considerably more slowly and less efficiently than poliovirus. In almost all cases, wild-type or low-passage virus does not induce visible cytopathic effects, and there is no evidence that HAV interferes with host cell macromolecular synthesis (19). In vitro infections generally result in the establishment of persistent infection (41), a curious finding since viral persistence is not known to occur in vivo (25).

Several HAV variants have been reported to induce a cytopathic effect in monkey kidney cell and diploid human lung fibroblast cultures (1, 10, 21, 32, 42). In at least three instances, these cytopathic variants have emerged during continued passage of persistently infected cell cultures (1, 10, 32). An important attribute of these cytopathic variants, each derived from the HM175 strain of HAV, is that they appear to be uniquely well adapted to growth in cell culture. One such variant (10) replicates significantly more rapidly than its noncytopathic precursor, with maximum virus yields reached as early as 40 h after inoculation of cells under one-step growth conditions (9). In further characterizing this cytopathic variant, we noted that the virus had spontaneously acquired a mutation within an immunodominant neutralization site (34). In this report, we describe the emergence of this spontaneous neutralization escape mutant during persistent infection in cell culture and report the nearly complete nucleotide sequence of three clonally isolated cytopathic HM175 strain variants. We show that these cytopathic variants of HAV arise by a combination of point mutations and genetic recombination.

MATERIALS AND METHODS

Cells. Continuous green monkey kidney (BS-C-1) cells were obtained from the American Type Culture Collection; high-passage-level (passage 50 [p50] to p70) fetal rhesus kidney (FRhK-4) cells were the gift of B. Flehmig. Both cell lines were grown in Eagle minimal essential medium (Earle's salts) supplemented with 10% fetal calf serum.

Antibodies. Monoclonal antibodies to HAV (K24F2 and K34C8) (31) were purchased from the Commonwealth Serum Institute, Melbourne, Australia. Other monoclonal antibodies were obtained from sources described previously (34) and from R. H. Decker of Abbott Laboratories, North Chicago, Ill. (12).

Virus. HM175 strain HAV was adapted to growth in primary African green monkey kidney (AGMK) cells as described previously (4). p16 HM175 virus is a clonally isolated variant that has undergone a total of 16 cell culture

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passages: 6 passages in BS-C-1 cells following 10 passages in AGMK cells (22). Persistent infection of BS-C-1 cells was established with uncloned p16 HM175 virus as described previously (9, 10). The cytopathic pHM175 virus (10) was recovered by disruption of these persistently infected cells after 21 to 23 subculture passages made at 2- to 4-week intervals. Unless otherwise noted, pHM175 virus stock had been serially passaged four times at low multiplicity of infection in FRhK-4 cells. By serial passage, we mean the passage in uninfected cells of virus recovered by disruption of infected cells. Two cytopathic clonal variants were selected from pHM175 harvests as described in Results. Clone A (HM175/43c virus) was twice plaque purified from neutral red-stained overlays of FRhK-4 cells inoculated with pHM175 virus and was selected for its ability to induce clearly visible plaques in this cell line. Clone A stock virus was propagated in FRhK-4 cells. Clone B (HM175/18f virus) was twice clonally isolated from agarose overlying foci of viral replication in radioimmunofocus assays by using previously described techniques (29); it was selected for continued reactivity with monoclonal antibody K24F2 (see Results). Clone B stock virus was propagated in BS-C-1 cells. In addition, a third clonal isolate, clone C (HM175/24a) was isolated from pHM175 virus stock by classical plaque purification techniques and propagated in FRhK-4 cells (9).

For comparison of the growth properties of pHM175 antigenic variants during serial and persistent passage, 25-cm^2 flasks containing nearly confluent cultures of FRhK-4 or BS-C-1 cells were inoculated with virus at an estimated multiplicity of infection of 0.01 to 0.1. Virus was harvested after 3 to 4 days (serial passage) or at 7- to 14-day intervals (persistent infection, when cells were subcultured) by freeze-thawing cells in 2 to 5 ml of Hanks balanced salt solution. Virus harvests were clarified by centrifugation in a microcentrifuge for 15 min and then extracted with an equal volume of chloroform before assay.

Plaque assay for cytopathic HAV. Conventional plaque assays were done with either FRhK-4 or BS-C-1 cells. Following inoculation with pHM175 virus, cell sheets were overlaid with 0.5% agarose (Seakem ME, Rockland, Maine) containing 2.0% heat-inactivated fetal calf serum and placed at 35°C in a humidified environment with 5% CO_2. Six days later, a second overlay containing 0.5% agarose, 25 mM $MgCl_2$, and neutral red (final concentration, 0.02%) was added. Visible plaques were counted 6 to 24 h later. In some instances, the agarose overlays were subsequently removed from the cell sheets and the cells were processed for immunologic detection of viral replication foci as described below.

Radioimmunoassays for HAV antigen. Suspensions of gradient-purified virus (clones A and B and p16) were standardized for viral RNA content by cDNA-RNA hybridization. Serial dilutions of standardized virus suspensions were then tested for antigen content by a solid-phase radioimmunoassay employing human polyclonal antibody for both virus capture and detection (30) and for ability to bind HAV-specific murine monoclonal antibodies by an indirect immunoassay method (34).

Radioimmunofocus assays for HAV. Quantitation of infectious virus was based on the autoradiographic detection of foci of HAV replication developing in cell sheets (BS-C-1 or FRhK-4) maintained beneath 0.5% agarose overlays, following fixation of the cell sheet with acetone and staining with ^{125}I-labeled antibody to HAV (JC, polyclonal postconvalescent human immunoglobulin G) (27, 29). Cell sheets were assayed for replication foci 5 to 7 days after inoculation with rapidly replicating cytopathic pHM175 virus (or clonal variants A and B) or 14 days after inoculation with the more slowly replicating p16 HM175 virus. Results are presented in terms of radioimmunofocus-forming units (RFU) of virus. A modification of the radioimmunofocus procedure resulted in an epitope-specific assay (28). After a conventional radioimmunofocus assay with ^{125}I-labeled monoclonal anti-HAV (K24F2), cell sheets were counterstained with ^{125}I-labeled polyclonal anti-HAV and exposed once more to film. Foci derived from virus having a normal antigenic phenotype were visualized equally on both exposures, whereas foci of virus lacking the K24F2 epitope were only visualized after staining with the polyclonal antibody.

Growth and purification of HAV. Virus was propagated in 850-cm^2 roller bottle cultures of BS-C-1 cells. The medium was removed 2 to 3 weeks after inoculation, and cells were washed twice with phosphate-buffered saline (PBS) and subjected to three freeze-thaw cycles in a total volume of 35 ml of PBS. The lysate was centrifuged at 500 × *g* for 20 min. The membrane (pellet) fraction was resuspended in 10 ml of TN buffer (50 mM Tris, 50 mM NaCl) with 0.1% sodium lauroylsarcosine and subsequently processed in parallel with the supernatant fraction. DNase (50 μg/ml) in 10 mM Tris with 10 mM $MgCl_2$ was added to both fractions, followed by incubation at 37°C for 60 min. Virus was pelleted by centrifugation at 148,000 × *g* for 14 h, resuspended in 3.5 ml of TN buffer with 0.1% sodium lauroylsarcosine, sonicated for 60 s, extracted with an equal volume of chloroform, and layered onto a 31-ml combination sucrose-cesium chloride gradient in an SW28 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) (14). Following centrifugation at 65,000 × *g* for 24 h, fractions were collected from the bottom of the gradient and assessed for HAV antigen content by a solid-phase radioimmunoassay (30). Two peaks of antigen activity were present in each gradient, representing full and empty HAV particles as determined by cDNA-RNA hybridization of gradient fractions. Fractions composing the first peak (complete HAV particles) were pooled for extraction of RNA.

Nucleotide sequence analysis of cytopathic variants. The 5′ nontranslated and P1 genomic regions of clone A and B viruses were sequenced by primer extension of virion RNA. Gradient-purified virus was treated with sodium dodecyl sulfate-proteinase K, followed by extraction of RNA with phenol-chloroform (40). HAV RNA was twice ethanol precipitated and sequenced by primer extension (approximately 10 ng of RNA per reaction), using high concentrations of primers relative to template and all four deoxynucleoside triphosphates labeled to high specific activities with ^{35}S. The P2, P3, and 3′ nontranslated regions of these virus genomes were sequenced from cDNA amplified by an antigen-capture polymerase chain reaction (PCR) method (23). Sequence was determined by direct primer extension of amplified cDNA transcripts, using a modified Sequenase protocol (U.S. Biochemicals). The nearly complete genomic sequence of clone C virus was also obtained by a PCR-based method, but in a separate laboratory employing different PCR primer sets which contained *Eco*RI sites. For clone C virus, amplified transcripts were digested with *Eco*RI and cloned into pGEM3Zf+. Individual cDNA clones were sequenced by primer extension. To eliminate the possibility of errors in the sequence due to PCR, we sequenced at least three individual clones for each region of the genome containing mutations from the parent virus.

Thermal stability of HAV. Small-volume aliquots of crude infected cell lysates, clarified as described above, were subjected to temperatures ranging from 40 to 90°C for 10 min in a programmable thermal cycler. Thermal stability of virus

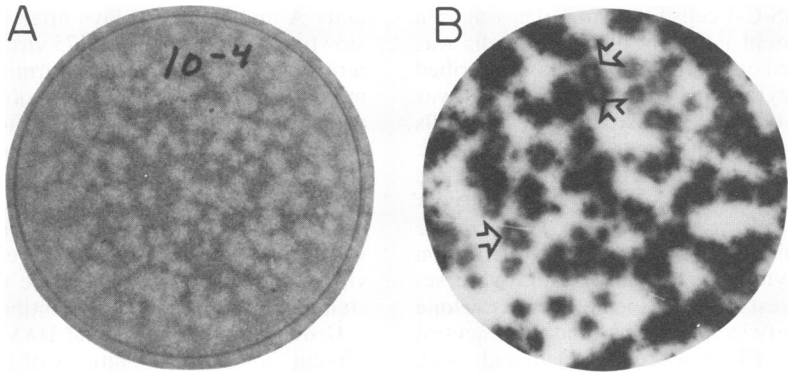

FIG. 1. (A) Neutral red-stained plaque assay of cytolytic pHM175 virus (virus clone A, see text) in FRhK-4 cells (6 days postinoculation). (B) Radioimmunofocus assay of cytolytic pHM175 virus (virus clone B) in BS-C-1 cells, demonstrating central clearing of cells in radioimmunofoci (7 days postinoculation).

was tested in the presence or absence of added 1 M $MgCl_2$ (38). Samples were kept at 0°C before and after heating and were assayed immediately for infectious HAV by radioimmunofocus assay and for HAV antigen by solid-phase radioimmunoassay (30).

Nucleotide sequence accession numbers. Nucleotide sequences of three cytopathic HM175 virus variants have been submitted to GenBank under the following accession numbers: M59809, HAV HM175/43c (clone A); M59808, HAV HM175/18f (clone B); M59810, HAV HM175/24a (clone C).

RESULTS

Altered antigenicity of cytopathic HAV variant. pHM175 virus was recovered from BS-C-1 cells that had been persistently infected with the HM175 strain of HAV for approximately 1 year (10). We confirmed that pHM175 virus was cytopathic by establishing a conventional plaque assay in FRhK-4 cells with virus recovered from BS-C-1 cells at cell passage 21 to 23 following persistent infection (Fig. 1A). A clonal isolate (HM175/43c virus, virus clone A) was twice plaque purified from the pHM175 virus harvest. When cell monolayers inoculated with clone A virus and overlaid with agarose were sequentially stained with neutral red and then processed for immunologic detection of radioimmunofoci, there was excellent concordance between visible plaques and radioimmunofoci (data not shown). In plaque reduction neutralization tests, clone A virus was susceptible to neutralization with the HAV-specific monoclonal antibody K34C8 (83% plaque reduction at 0.1 mg of antibody per ml). However, this virus clone was found to be resistant to similar concentrations of a second monoclonal antibody, K24F2 (no significant plaque reduction). These results were confirmed in radioimmunofocus inhibition assays, which also demonstrated that K24F2 was capable of neutralizing standard virus under identical conditions (data not shown). Thus, passage of HM175 virus in persistently infected BS-C-1 cells, in the absence of antibody pressure, resulted in the selection of a spontaneous neutralization escape variant resistant to monoclonal antibody K24F2.

Replication advantage of antigenic variant during persistent infection. We utilized an epitope-specific radioimmunofocus assay (28) to determine the titer and proportion of antigenically variant virus present at various passage levels in persistently infected BS-C-1 cells and in harvests made from subsequent serial passages of pHM175 virus in FRhK-4 cells (see Materials and Methods). These results are shown in Fig. 2 and 3. Approximately 99% of infectious virus particles found in disrupted, persistently infected BS-C-1 cells (23 cell passages after establishment of persistent infection) were of the escape mutant (clone A) phenotype (Fig. 3A). However,

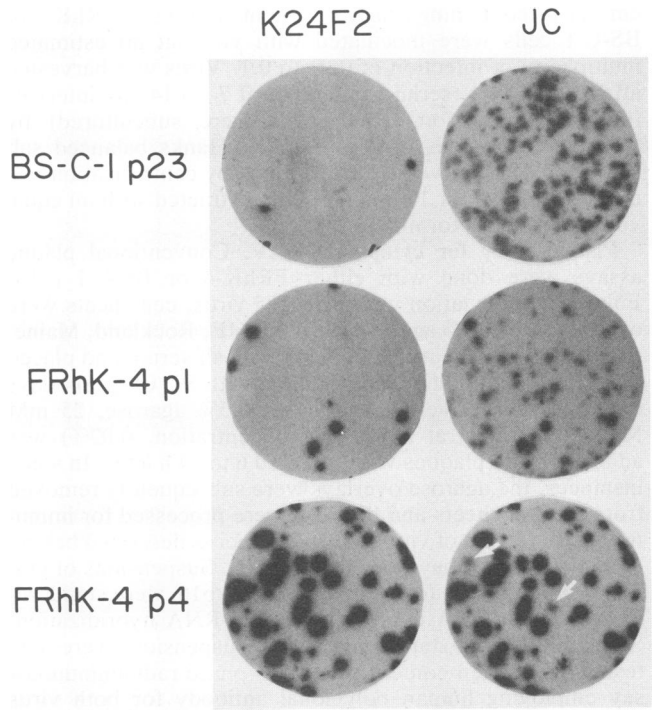

FIG. 2. Epitope-specific radioimmunofocus assays demonstrating growth advantage of antigenically normal virus during serial passage of pHM175 virus in FRhK-4 cells. Autoradiograms are of identical acetone-fixed cell sheets inoculated with the indicated virus harvests and stained, after 7 days of incubation under agarose, with ^{125}I-labeled monoclonal antibody (K24F2) and subsequently with ^{125}I-labeled polyclonal anti-HAV (JC). The mutant, clone A-like virus is not detected by the monoclonal antibody K24F2 and is only visualized with polyclonal antibody (arrows in lower panel). BS-C-1 p23, pHM175 virus recovered from persistently infected BS-C-1 cells after 23 subculture passages; FRhK-4 p1 and FRhK-4 p4, virus harvests from FRhK-4 cells after 1 and 4 serial passages of pHM175 virus, respectively. Assays were done in BS-C-1 cells.

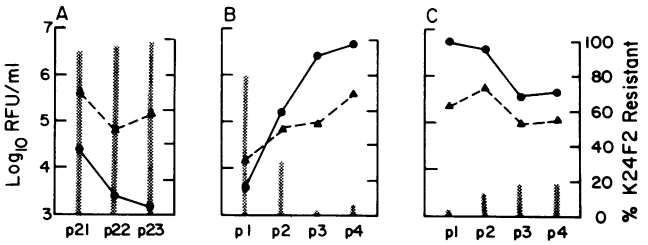

FIG. 3. Clone A-like virus (▲----▲) and antigenically normal virus (●——●) populations in pHM175 virus harvests after persistent infection in BS-C-1 cells (A and C) and serial passage in FRhK-4 cells (B). Bars (▩) represent the percentage of each virus harvest that had the clone A phenotype. Panel A represents the last three passages (p21 to p23) of pHM175 in the persistently infected BS-C-1 cell line (10). In panel B, virus derived from the BS-C-1 cell p23 persistent infection harvest (A) was passed in serial fashion with disruption of cells. In panel C, virus recovered from the serial p4 FRhK-4 harvest (B) was used to reestablish persistent infection in BS-C-1 cells.

subsequent serial passage of this virus in FRhK-4 cells at 3-day intervals resulted in selection of virus with a normal antigenic phenotype (Fig. 3B). Virus with the escape mutant phenotype represented only 3 to 8% of all infectious virus after three to four serial passages in FRhK-4 cells, although both viral phenotypes replicated to higher titers upon recovery from persistently infected BS-C-1 cells and subsequent serial passage in FRhK-4 cells.

To determine whether virus with the escape phenotype could be reselected if persistent infection was reestablished with virus recovered from FRhK-4 cells after serial passage, we inoculated BS-C-1 cells with virus harvested after serial passage 4 in FRhK-4 cells (92% wild-type antigenic phenotype, Fig. 3B). These cells were subcultured at weekly intervals for 4 weeks. The yield of each virus type dropped significantly in harvests made after the third subculture of these infected cells (Fig. 3C). The proportion of virus with the escape phenotype increased with time, however. One month (four cell culture passages) after infection, such virus represented 19% of the total virus yield. Further subculture of these persistently infected cells was not done. Thus, the clone A escape variant appeared to have a slight replication advantage over nonmutant virus during persistent infection in BS-C-1 cells.

We did further experiments to determine whether the selection of viruses with different antigenic phenotypes shown in Fig. 3B and 2C reflected a change in host cell type (BS-C-1 cells versus FRhK-4 cells) or a change in passage conditions (persistently infected cell passage versus serial virus passage). First, pHM175 virus harvested from p23 of the persistently infected BS-C-1 cells (Fig. 3A, 99% escape mutant phenotype) was serially passaged in BS-C-1 or FRhK-4 cells at 4-day intervals (Table 1). In both cell types, the wild-type antigenic phenotype demonstrated a selective advantage over virus with the escape mutant phenotype under these growth conditions. The proportion of mutant virus dropped from 99% in the inoculum to 22% (p5) in BS-C-1 cells and 75 to 85% (p4 to p5) in FRhK-4 cells. The lower rate of selection of the nonmutant phenotype in FRhK-4 cells in the experiment shown in Table 1 compared with that in Fig. 3B may reflect different lengths of culture for each passage (4 days in Table 1 versus 3 days in Fig. 3B) or subtle differences in the cells used in these experiments. In a separate experiment, pHM175 virus recovered from

TABLE 1. Selection of pHM175 virus variants with wild-type and escape mutant antigenic phenotypes under different cell culture conditions

Passage	BS-C-1 cells		FRhK-4 cells	
	Yield[a]	% Mutant	Yield[a]	% Mutant
Serial passage of pHM175 from p23 persistently infected BS-C-1 cells[b]				
1	5.33	91	6.33	96
2	6.24	88	6.54	94
3	6.15	66	6.50	89
4	6.90	55	7.34	75
5	6.56	22	6.32	85
Persistent passage of pHM175 from p3 serial passage in FRhK-4 cells[c]				
1	5.11	50	5.81	81
2	5.16	58	ND[d]	ND
3	6.17	76	5.15	63
4	5.15	71	4.96	65
5	6.13	68	5.26	91

[a] Log_{10} RFU/ml.
[b] Inoculum was 99% mutant phenotype.
[c] Inoculum was 3% mutant phenotype.
[d] ND, Not determined.

serial passage 3 in FRhK-4 cells (Fig. 3B, 3% mutant phenotype) was used to establish persistent infection in BS-C-1 cells, which were then subcultured at 14-day intervals (Table 1). Under these conditions of persistent infection, there was a striking increase in the proportion of virus with the escape mutant phenotype, which reached 68 to 78% in BS-C-1 cells and 63 to 91% in FRhK-4 cells. These results indicate that the escape mutant has a replication advantage during persistent infection in either cell type, whereas replication of virus with the normal antigenic phenotype is favored during serial passage of pHM175.

Cytopathogenicity of pHM175 virus is not restricted to the escape mutant phenotype. Two additional virus clones picked from plaques forming in FRhK-4 cells were shown to be antigenic variants similar to clone A (data not shown). However, the data shown in Fig. 3B suggested that virus in pHM175 harvests which retained normal antigenicity shared a capacity for relatively rapid replication with the escape mutant. Thus, we clonally isolated antigenically normal virus (HM175/18f virus, or clone B) from radioimmunofocus assay overlays after infection of BS-C-1 cells with pHM175 virus. Unlike clone A virus, which was selected for its ability to induce distinct plaques, clone B virus was selected for its continuing ability to bind K24F2 antibody. However, clone B virus generated distinct plaques in BS-C-1 cells maintained under agarose and stained with neutral red (data not shown). Like clone A, clone B virus had a rapidly replicating phenotype, forming large radioimmunofoci within 7 days of inoculation of cells. Furthermore, autoradiograms of the ^{125}I-anti-HAV-stained cell sheets often showed replication foci with central areas of cell necrosis (Fig. 1B). This observation confirmed the HAV-specific nature of the cytopathic effect and indicated that extensive accumulation of intracellular viral antigen preceded cell lysis.

To compare the cytopathic effects induced by clones A and B, we inoculated parallel cultures of FRhK-4 cells with either virus at a low multiplicity of infection (approximately 0.01). Additional FRhK-4 cells were inoculated with p16

TABLE 2. Cytopathic effects and intracellular and extracellular HAV antigen accumulation after infection of FRhK-4 cells at low multiplicities[a]

Virus	Cytopathic effects (day p.i.)[b]						HAV antigen (cpm)[c] (day p.i.)	
	0	2	4	6	7	9	Cells	Media
p16 HM175	0	0	0	0	0	0	454 (9)	76 (9)
Clone A	0	0	0	+(+)	++	ND	2,145 (7)	72 (7)
Clone B	0	0	0	(+)	+	ND	3,623 (7)	820 (7)

[a] Parallel cultures of FRhK-4 cells were established in 25-cm^2 flasks and inoculated with 580 RFU of clone A virus, 460 RFU of clone B virus, or 1,500 RFU of p16 HM175 virus.

[b] Cytopathic effects were scored as: 0, none; +, 10% cells detached; ++, 25% cells detached; p.i., postinoculation; ND, not done.

[c] Antigen was determined by radioimmunoassay.

HM175 which, although well adapted to growth in cell culture (22), had never been passaged in persistently infected cells. This p16 HM175 virus is closely related to the virus used to initiate the pHM175 persistent infection. Cultures were monitored for cytopathic effect, release of viral antigen into supernatant fluids, and accumulation of intracellular antigen (Table 2). Cytopathic effects similar to those described previously (1, 10) were seen with both clone A and B viruses and included the rounding up of cells followed by their release from the cell sheet. Such cytopathology was not seen with p16 HM175 virus, however, even when infected cells were held for 3 weeks or longer. Release of viral antigen into cell culture supernatant fluids was noted with clone B but not clone A virus, and intracellular antigen accumulation was greater with clone B than with clone A (Table 2). However, the detection of antigen from clone A was likely to have been biased by its altered antigenicity, which was evident even in polyclonal radioimmunoassays (see below).

Taken together, these results indicate that the cytopathic effect of pHM175 virus variants occurs in both cell types and is not restricted to FRhK-4 cells as first suggested (10). Furthermore, the neutralization escape phenotype of clone A was neither necessary for nor specifically associated with the ability of virus to replicate rapidly and generate a cytopathic effect in cell culture.

Comparative antigenicity and thermal stabilities of cytopathic virus clones A and B. We did a detailed analysis of the antigenicity of virus clones A and B in comparison with that of the noncytopathic p16 HM175 virus, using a panel of neutralizing murine monoclonal antibodies assembled from several laboratories. Standardized quantities of each virus (determined by cDNA-RNA hybridization) were tested for ability to bind monoclonal antibodies in an indirect radioimmunoassay (34). While these assays demonstrated that clone A virus was poorly recognized by many of the monoclonal antibodies, the clone B variant was indistinguishable from the noncytopathic p16 HM175 virus (Fig. 4). These findings were confirmed in subsequent monoclonal antibody neutralization assays (data not shown). When standardized quantities of each virus were tested in a polyclonal radioimmunoassay, there was a marked reduction in the amount of antigenic activity associated with the clone A virus (Fig. 5). This finding supports the concept that the HAV capsid displays an immunodominant antigenic site on its surface (34, 39).

Since it has been suggested that cytopathic HAV variants have reduced virion stability at elevated temperatures (1), we examined the thermal stability of virus clones A and B.

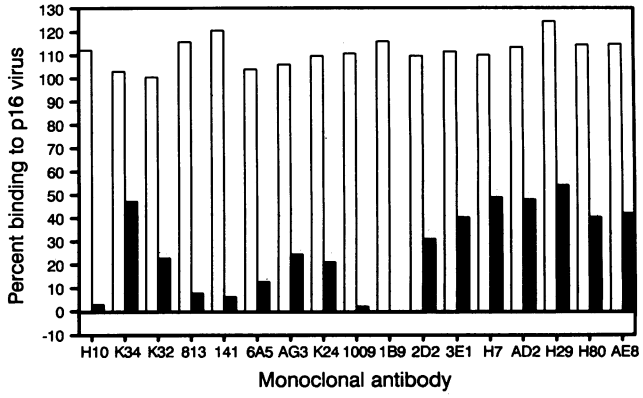

FIG. 4. Binding of murine monoclonal antibodies to clone B (□) and clone A (■) viruses, determined by an indirect radioimmunoassay. Antibody binding is expressed as the percentage of binding to equivalent amounts of p16 HM175 virus in companion assays. Gradient-purified virus suspensions tested in the assays were standardized by cDNA-RNA hybridization.

Crude cell lysates containing p16, clone A, and clone B viruses were heated for 10 min at temperatures ranging from 40 to 90°C and then assayed for infectious virus (Fig. 6A and B) and for HAV antigen detectable in the polyclonal solid-phase radioimmunoassay (Fig. 6C and D). These studies failed to demonstrate reproducible differences between the thermal stability of the p16 virus and that of the cytopathic clone A and clone B variants, in terms of either loss of infectivity or loss of antigenicity. The addition of 1 M Mg^{2+} (Fig. 6B and D) resulted in an increase of approximately 20°C in the 50% survival point of all three viruses, as might be expected from previous studies (38).

Mutations present in cytopathic variants. We determined the nearly complete genomic sequences (nucleotides 30 to 7478) of virus clones A and B, as well as HM175/24a, a third virus clone (clone C) that was conventionally plaque purified from pHM175 virus stock after serial passage in FRhK-4 cells (9). These sequences were compared with those previously determined for wild-type HM175 virus (three passages in marmosets) (8) and cell culture-adapted p16 HM175 virus (22), which is closely related to the virus used to initiate the

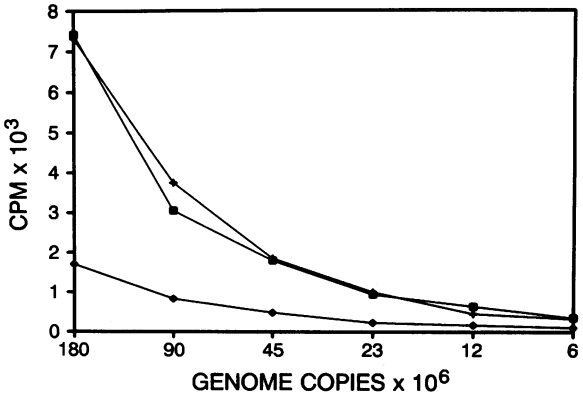

FIG. 5. Standardized quantities of isopycnic gradient-purified p16 HM175 (■), clone A (◆), and clone B (+) viruses detected by a solid-phase radioimmunoassay employing human convalescent antibody for capture and detection of virus.

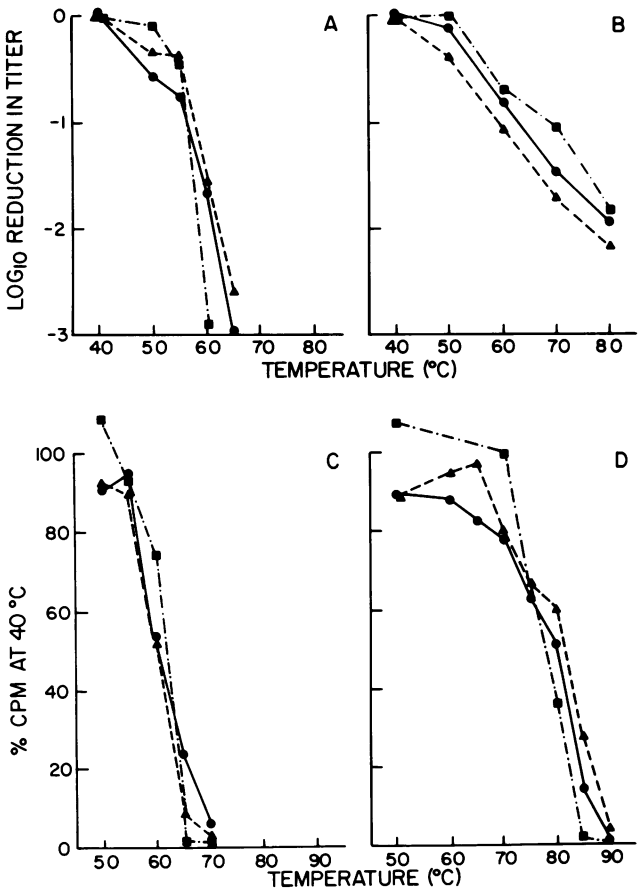

FIG. 6. Thermal stabilities of noncytopathic p16 HM175 virus (●) and the rapidly replicating cytopathic clone A (■) and clone B (▲) viruses. Crude cell lysates were heated at the indicated temperature for 10 min in the absence (A and C) or presence (B and D) of excess 1 M Mg^{2+} and subsequently assayed by radioimmunofocus assay for infectious virus (A and B) or by radioimmunoassay for HAV antigen (C and D). Virus incubated at 40°C was unchanged in infectious titer and antigen content from control virus held at 0°C.

persistent pHM175 virus infection. Results are shown in Tables 3 (nucleotide changes) and 4 (amino acid changes). Mutations present in virus clones A and B will be discussed first.

Mutations present in p16 HM175 virus (from wild-type sequence) and associated with initial adaptation of the virus to growth in cell culture (22) were found to be generally present in both virus clones A and B, as might be expected. However, both cytopathic virus clones were found to have a number of additional mutations in all regions of the genomes. Thus, the nucleotide sequence of clone A contains a total of 34 mutations from that of p16 virus, predicting 19 amino acid changes. Clone B has 31 mutations, predicting 17 amino acid changes. Virus clones A and B were found to share an identical 5′ sequence (bases 30 to 1677). Within the 5′ nontranslated region, both viruses had four mutations in addition to those already present in p16 HM175. The most remarkable of these mutations is a 14-base insertion between bases 154 and 155, representing a direct repeat of bases 141 to 154. The sequences of the two viruses differed within the P1 region, with the escape mutant, clone A, having nonsilent mutations resulting in amino acid substitutions at residues 3-070 (Asp to Ala) (by convention, residue 3-070 is residue 70 of VP3), 1-197 (Asn to Ser), and 1-276 (Met to Val), as previously reported (34). Within the P2 region, Lys-47 of protein 2A was changed to Asn in both viruses, although the nucleotide substitution responsible for this change was different in the two virus clones (A to C at base 3248 of clone A, and A to U in clone B). 3′ of this map position, the sequences of the two viruses again became identical (bases 3249 through 5303). 2C was the most altered protein in either virus clone, with 8 amino acid substitutions of a total of 335 residues (including reversion of the Thr-144 in p16 HM175 to the wild-type Lys-144.) From bases 5304 to 7271, the two virus clones contained distinctly different mutations, except for two conserved changes at 6619 ($3D^{pol}$) and 6633 (silent). Beyond 7272, the sequences of the two virus clones again became identical and included two mutations within the 3′ nontranslated region at 7429 and 7433, near the mutation already present in p16 HM175 virus at 7430.

With regard to clone C, a total of 42 nucleotide mutations (from the p16 HM175 sequence) were found, resulting in a predicted change in 21 amino acid residues (Tables 3 and 4). In general, mutations in clone C were more closely related to those present in clone A than to those in clone B. Two exceptions to this statement included the base 3248 change responsible for the conserved Lys-to-Asn substitution at residue 47 of 2A, which is A to U in clone C (see above), and a $3C^{pro}$ coding sequence which was identical to that of clone B. Within the P1 region, mutations present in clone A were also found in clone C, although there were a number of additional mutations present in clone C. To ensure that these differences were not sequencing errors related to PCR amplification of clone C cDNA (see Materials and Methods), we also sequenced the region between bases 2646 and 3033 of the clone C genome by primer extension of virion RNA. These results confirmed the presence of the additional mutations at bases 2646, 2780, and 2930 in clone C and thus validated the results of PCR-based sequencing.

DISCUSSION

The spontaneous emergence of an antigenic variant during persistent HAV infection, in the absence of selective antibody pressure, contrasts with the absence of significant antigenic variation evident among different wild-type human HAV strains (26). This finding suggests that HAV strains that have undergone considerable passage in cell culture (particularly as persistent infections) should be carefully examined for altered antigenicity before they are considered for use in diagnostic assays or as vaccines. Based on mutations we have identified previously in purposefully selected HAV neutralization escape variants (34), the mutation at residue 3-070 is most likely responsible for the antigenic variation displayed by clone A virus. However, a role for the other capsid mutations (especially that at 1-276) has not yet been excluded.

All three virus clones that we studied were cytopathic and had a rapid replication phenotype (replication foci developing in less than 7 days). A significant contribution of the P1 region mutations to this replication phenotype seems unlikely, given the fact that these virus clones have different mutations in the capsid proteins. However, the pattern of mutations within the P1 region was similar in the virus clones (Table 4). Despite a relative paucity of changes in the capsid proteins of virus clones A and B, both viruses have undergone mutation within or near the putative B-C loop of VP3 (residues 3-070 and 3-091), as suggested by the alignments of

TABLE 3. Nucleotide changes in cytopathic HM175 virus variants[a]

Base position	Wild type	p16	Clone A (HM175/43c)	Clone B (HM175/18f)	Clone C (HM175/24a)
5′ NTR[b]					
117/118	—[c]	—	—	—	U
152	A	G	G	G	G
154/155	—	—	UUGUAAAUAUUGAU	UUGUAAAUAUGAU	UUGUAAAUAUUGAU
203–204	UU	dd[c]	dd	dd	dd
551	A	—	G	G	G
591	A	—	G	G	G
647	A	—	C	C	C
687	U	G	G	G	G
P1					
964	A	G	G	G	G
987	U	—	—	—	C[d]
1064	A	—	—	—	G[d]
1678	A	—	C	—	C
1741	C	—	—	A	—
1742	G	A[d]	A[d]	A[d]	A[d]
2036	A	—	—	—	G[d]
2646	A	—	—	—	G
2684	C	—	—	U[d]	—
2780	C	—	—	—	U[d]
2797	A	—	G	—	G
2864	U	—	—	—	A[d]
2930	A	—	—	—	G[d]
3018	U	—	—	C	—
3033	A	—	G	—	G
P2					
3248	A	—	C	U	U
3281	A	G	G	G	G
3557	U	—	—	—	G
3711	G	A	C	C	C
3867	U	C[d]	C[d]	C[d]	C[d]
3889	C	U	U	U	U
4049	C	U[d]	—	—	—
4060	C	—	G	G	G
4066	A	—	G	G	G
4185	G	A	A	A	A
4222	U	—	C	C	C
4272	C	—	U	U	U
4369	C	—	U	U	U
4419	U	—	C	C	C
4426	A	C	—	—	—
4607	U	—	C[d]	C[d]	C[d]
4955	A	—	U	U	U
P3					
5010	GAU	—	ddd	ddd	ddd
5172	U	—	G	G	G
5194	G	—	A	A	A
5204	G	A[d]	A[d]	A[d]	A[d]
5255	A	U	U	U	U
5304	A	—	G	—	—
5592	C	—	—	G	G
5757	A	—	G	—	—
6074	U	—	—	—	C[d]
6148	A	G	G	G	G
6216	U	C[d]	C[d]	C[d]	C[d]
6461	A	—	—	G[d]	—
6522	U	A	A	A	A
6619	G	—	A	A	A
6633	U	—	C[d]	C[d]	C[d]
6782	U	—	C[d]	—	—
6813	C	—	—	—	U[d]
6827	U	—	C[d]	—	C[d]
6920	C	—	—	U[d]	—
7226	U	—	C[d]	—	C[d]
7247	U	—	—	C[d]	—
7253	A	—	G[d]	—	—

Continued on following page

TABLE 3—*Continued.*

Base position	Wild type	p16	Clone A (HM175/43c)	Clone B (HM175/18f)	Clone C (HM175/24a)
7271	U	—	C[d]	—	C[d]
7304	U	—	C[d]	C[d]	C[d]
3′ NTR					
7429	C	—	U	U	U
7430	A	G	G	G	G
7433	U	—	C	C	C

[a] Boxed areas represent regions in cytopathic virus clones with mutations (from p16 HM175) identical to those in clone B. Mutations which occurred during the primary adaptation of HM175 to growth in cell culture (i.e., wild type to p16) are included in the table.
[b] NTR, Nontranslated region.
[c] —, No change from wild type; d, deletion.
[d] Silent mutation within polyprotein-encoding region of genome.

Palmenberg (33), and near the carboxy terminus of VP1 (residues 1-271 and 1-276). Mutations predicting amino acid substitutions at similar sites in HAV capsid proteins (residue 3-070, 1-273, or 1-276) have been reported in cDNA derived from other independently isolated and cell culture-adapted virus variants (7, 36). Thus, these regions of the capsid proteins appear to be particularly prone to mutation during adaptation and passage of virus in cell culture.

TABLE 4. Amino acid alterations in cytopathic HM175 virus variants[a]

Residue	Wild type	p16	Clone A (HM175/43c)	Clone B (HM175/18f)	Clone C (HM175/24a)
VP2 (54)	Lys	Arg	Arg	Arg	Arg
VP3					
70	Asp	—	Ala	—	Ala
91	Thr	—	—	Lys	—
VP1					
147	Ile	—	—	—	Val
197	Asn	—	Ser	—	Ser
271	Ser	—	—	Pro	—
276	Met	—	Val	—	Val
2A					
47	Lys	—	Asn	Asn	Asn
58	Ile	Met	Met	Met	Met
150	Asp	—	—	—	Glu
2B					
13	Asp	Asn	His	His	His
72	Ala	Val	Val	Val	Val
2C					
22	Ala	—	Gly	Gly	Gly
24	Tyr	—	Cys	Cys	Cys
64	Glu	Lys	Lys	Lys	Lys
76	Phe	—	Ser	Ser	Ser
93	His	—	Tyr	Tyr	Tyr
125	Ser	—	Phe	Phe	Phe
142	Tyr	—	His	His	His
144	Lys	Thr	—	—	—
320	Glu	—	Asp	Asp	Asp
3A					
4	Asp	—	d	d	d
58	Phe	—	Val	Val	Val
65	Arg	—	His	His	His
3B (11)	Gln	His	His	His	His
$3C^{pro}$					
5	Ile	—	Val	—	—
101	Gln	—	—	Glu	Glu
156	Thr	—	Ala	—	—
$3D^{pol}$					
67	Asp	Gly	Gly	Gly	Gly
192	Ser	Thr	Thr	Thr	Thr
224	Arg	—	Lys	Lys	Lys

[a] —, No change from wild type; d, deleted.

The absence of significant antigenic variation among different wild-type human HAV strains suggests that the survival of HAV variants with mutations involving capsid antigenic sites is restricted under natural conditions. Primate challenge experiments with a plaque-purified neutralization escape variant confirm this view (28). As capsid mutations which confer antigenic variance do not affect the thermal stability of the viral capsid (Fig. 6) nor apparently interfere with viral replication in cell culture (uncoating, or viral assembly), it is reasonable to speculate that the natural restriction to such mutations may be at the level of viral attachment and entry. Thus, the conserved nature of the HAV capsid may reflect stringent requirements for recognition of cellular virus receptors. This restriction would not exist during passage of virus within persistently infected cells but could explain the reemergence of the wild-type antigenic phenotype during serial passage (Fig. 3B and Table 1). Significantly, there is strong evidence for involvement of antigenic sites in the receptor binding domains of other picornaviruses (18, 20). There are few other mutations that could be responsible for the differential growth properties of clones A and B: the nontranslated regions of the genomes of these viruses are identical (Table 3), and the only amino acid differences in proteins other than the capsid proteins are in $3C^{pro}$ (Table 4). Less efficient processing of the P1 polyprotein by the $3C^{pro}$ of clone A could explain the reduced efficiency of replication of this virus clone during serial passage. Furthermore, mutations in $3C^{pro}$ might also affect viral RNA synthesis, as Andino et al. (2) have demonstrated a role for $3C^{pro}$ of poliovirus in RNA replication. Further studies will be required to determine whether it is the P1 or $3C^{pro}$ mutations that are responsible for the different growth properties of these viruses.

A comparison of the nearly complete genomic sequences of all three cytopathic virus clones revealed discontinuous regions of identity and nonidentity with respect to mutations from the p16 genomic sequence (Table 3). These findings provide strong, albeit indirect, evidence for genetic recombination among these HAV variants (Fig. 7). Although the temporal order in which these mutations accumulated during persistent infection is not known, it is much less likely that the observed sequence microheterogeneity occurred simply as the result of the selection of favorable random mutations followed by additional mutations in each variant. Such a hypothesis fails to explain the striking pattern of identity and nonidentity with respect to both silent and nonsilent nucleotide base changes. Recombination is well recognized among certain other picornaviruses (24), and it is not sur-

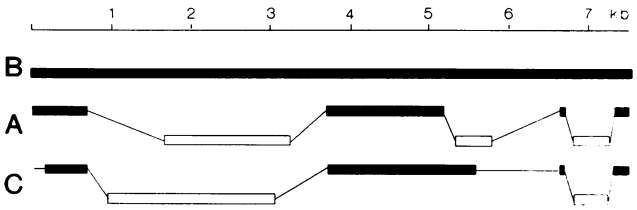

FIG. 7. Proposed recombination map for virus clones A, B, and C. Clone B virus (HM175/18f) has been arbitrarily selected as the reference sequence (shaded). Genomic regions of clones A and C which are identical in sequence with clone B, and which terminate at the site of common nucleotide base changes from the parental p16 HM175 sequence, are similarly shaded. Genomic regions of clones A and C which terminate at unique mutations from p16 virus (and contain no mutations in common with clone B) are shown as open boxes. Intervening sequences are of ambiguous origin, containing either no mutations or a mixture of shared and unique mutations, and are probable sites of crossover events.

prising to find it among HAV variants replicating over a prolonged time in persistently infected cells. The recombination partners would have been RNA molecules which had acquired new point mutations during persistent infection. The conditions of persistent infection would have selected those recombinants with superior replication properties, provided such recombinants did not induce rapid cell death. Multiple crossover events, as suggested in Fig. 7, would be expected given the lengthy nature of the persistent infection (approximately 1 year). If this hypothesis is correct, the conserved regions of the genomes of these three cytopathic virus clones should contain the mutations responsible for their rapid replication and cytopathic phenotype. Virus clones A and C are similar but, as indicated in Table 3 and Fig. 7, probably derived from distinct recombination events (different crossover points) within the P3 region.

Analysis of the nucleotide sequences of these three cytopathic virus clones thus suggests that mutations in the 5′ and 3′ nontranslated and P2/P3 regions are primarily responsible for their growth characteristics. Of all the viral proteins, the changes in 2C are most striking in both number and type (Table 4). Of the eight amino acid substitutions (from p16 HM175) present in 2C of all three viral clones, five are nonconservative in nature. Two sites within the protein appear particularly affected, with mutations at residues 22 and 24 and at 142 and 144. Almost all the mutations are in the amino half of the protein and none are near Gly-197 or Pro-245, which align with guanidine resistance and guanidine dependence sites, respectively, that have been identified in 2C of type 1 poliovirus (15). A role for 2C in the structural organization of picornaviral replication complexes has recently been proposed, with 2C putatively interacting jointly with viral RNA and membrane protrusions on the rough endoplasmic reticulum (3). Such a role would make 2C a likely determinant of host range. Indeed, host range mutants of human rhinovirus have been shown to have mutations within protein 2C (43).

Our findings are consistent with the idea that evolution of the rapid replication and cytopathic phenotype of HAV represents a further adaptation of the virus to cell culture. Mutations within 2B and 2C and in the 5′ nontranslated region of HAV have been shown to be associated with enhanced growth in cell culture (6, 7, 13, 16, 22). The involvement of the 5′ nontranslated region and 2B and 2C suggests that adaptation primarily affects viral RNA replication (22). The 3′ nontranslated region may also be important in this process, as mutations in this region are conserved in all three cytopathic clonal isolates. Moreover, changes in the 3′ nontranslated region occur within a short segment that has been identified as a binding site for an HAV-specific RNA binding protein that is expressed by infected cells (37). However, at least one mutation that has been shown to positively influence growth of the virus in cell culture (13) lies within the probable translational control element of the 5′ nontranslated region (5). Although the findings we report here are helpful in indicating which mutations may be responsible for the cytopathic and rapid replication phenotype, the construction and analysis of infectious cDNA recombinants, probably within a p16 HM175 genetic background, will be required to firmly establish the contribution of individual mutations to the cytopathic phenotype.

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