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Susceptibility of Nonprimate Cell Lines to Hepatitis A Virus Infection

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Hepatitis A virus (HAV) has been adapted to grow in primate cell cultures. We investigated replication of HAV in nonprimate cells by inoculating 20 cell lines from different species with the tissue culture-adapted HM175 strain. Slot blot hybridization and immunofluorescence analysis revealed that HAV replicated in GPE, SP 1K, and IB-RS-2 D10 cells of guinea pig, dolphin, and pig origin, respectively. Studies in IB-RS-2 D10 cells were discontinued because cultures were contaminated with classical swine fever virus. A growth curve showed that HAV grew poorly in GPE cells and intermediately in SP 1K cells compared with growth in FRhK-4 cells. Therefore, the cell surface receptor(s) and other host factor(s) required for HAV replication are present in nonprimate as well as primate cells.

Hepatitis A virus (HAV), a small nonenveloped positive-stranded RNA virus, is the prototype of the newly created *Hepatovirus* genus of the *Picornaviridae* family. This hepatotropic virus is a pathogen of medical importance that has been adapted to grow in cell cultures of primate origin (5, 20). Its genome of approximately 7,500 bases has been molecularly cloned, and full-length cDNA of tissue culture-adapted strain HM175 has been shown to be infectious in cell culture (4).

HAV replicates slowly in a number of primate cell types, often resulting in persistent infections and producing low yields of particles. The limited host range of the virus suggests that cellular determinants for HAV replication are also restricted to primates. To investigate this, we studied the susceptibility of nonprimate cell lines to infection with the tissue culture-adapted HM175 strain of HAV (tc-HAV) produced by transfecting infectious cDNA and its derived in vitro transcripts (4) into rhesus monkey kidney cells (FRhK-4 cells). The cDNA derived tc-HAV was inoculated into cell lines of mouse (Ltk^-, 3T6, YAC, and NCTC clone 1469), rat (RL-9), hamster (BHK-21 and CHO-K1), guinea pig (GPE), cat (CRFK), dog (MDCK), pig (IB-RS-2 D10 and ST), dolphin (SP 1K), turtle (TH-1), quail (QT6), sheep (SCP), bovine (MDBK), and horse (E. derm [ATCC]) origins. Confluent monolayers were infected with tc-HAV at a multiplicity of infection (MOI) of 1 to 10, adsorbed for 3 h at 34°C, washed three times with phosphate-buffered saline (PBS), and incubated with Dulbecco's modified Eagle's minimum essential medium (BioFluids) containing 10% fetal bovine serum (HyClone) at 34°C in a CO_2 incubator. Mock- and tc-HAV-infected monolayers were split weekly into thirds or sixths, depending on the cell line. Replication of HAV was studied by slot blot hybridization analysis of total cellular RNA obtained from cytoplasmic extracts at 0 and 6 weeks postinfection (p.i.). Slot blots were probed with full-length cDNA labeled with ^{32}P by random priming and hybridized at 50°C as described previously (14). At 6 weeks p.i., HAV-specific RNA was detected in nonprimate GPE, SP 1K, and IB-RS-2 D10 cells and in primate FRhK-4 cells, which were used as a positive control (Fig. 1). HAV-specific RNA was not detected in any of the remaining 17 cell lines (Fig. 1) or in any of the mock-infected cells. It should be pointed out that the low levels of HAV-specific RNA detected in GPE and SP 1K cells did not increase after longer incubation periods; therefore, replication of tc-HAV in these nonprimate cell lines was further confirmed by techniques of immunochemistry and classic virology (see below).

Replication of tc-HAV in nonprimate cell lines was further studied by indirect immunofluorescence. At 6 weeks p.i., eight-well slide chambers (Costar) containing mock- and HAV-infected confluent monolayers were acetone fixed, treated with a mixture of HAV-neutralizing murine monoclonal antibodies K2-4F2, K3-4C8, and K3-2F2 (17) at a concentration of 1 μg/ml, and stained with a 1:1,000 dilution of fluorescein-labeled affinity-purified goat anti-mouse antibody mixed with a 1:500 dilution of Evans blue dye (Sigma). Staining with contrast Evans blue dye, which fluoresced as a diffuse red background, allowed visualization of both uninfected and infected cells bodies (Fig. 2). At 6 weeks p.i., 80 to 100% of HAV-infected FRhK-4, GPE, SP 1K, and IB-RS-2 D10 cells showed the characteristic green granular cytoplasmic fluorescence of HAV-infected cells. HAV-inoculated Ltk^- cells and mock-infected cells (Fig. 2) as well as the remaining 16 cell lines studied in this work did not show any HAV-specific immunofluorescence. The immunofluorescence result of susceptibility to HAV infection of GPE, SP 1K, and IB-RS-2 D10 cells correlated with the data obtained by slot blot analysis (Fig. 1). Although differences in the amount of HAV-specific RNA (Fig. 1) were not reflected by the immunofluorescence assay as variations in the intensity of HAV-specific fluorescence (Fig. 2), such differences were detected by a radioimmunoassay of HAV-specific antigens and by the infectivity titers of HAV at 42 days p.i. (Fig. 3).

Because of contamination with classical swine fever virus at the source, all stocks of IB-RS-2 D10 cells in the United States were recalled and destroyed by the U.S. Department of Agriculture, and we were therefore unable to complete our studies of this cell line. However, we continued the characterization of HAV infection in GPE and SP 1K cell lines.

Replication of tc-HAV in GPE and SP 1K cells was analyzed by a growth curve and compared with replication in FRhK-4 (positive control) and Ltk^- (negative control) cells. Monolayers were infected with tc-HAV at an MOI of 10 as above

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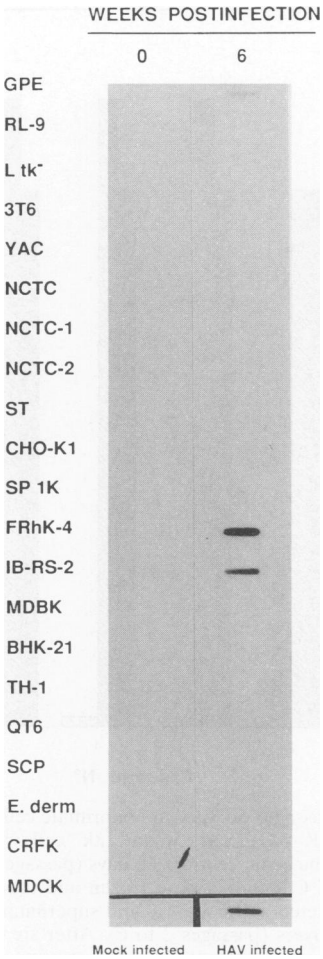

FIG. 1. Slot blot analysis of total cell RNA from infected cell lines. Cell lines were obtained from the American Type Culture Collection, except YAC, 3T6, CHO, MDCK, MDBK, and GPE cells, which were obtained from the FDA Center for Biologics Evaluation and Research repository. NCTC-1 and NCTC-2 cell lines are single cell clones obtained from NCTC clone 1469. Confluent monolayers were inoculated with the tissue culture-adapted HM175 strain of HAV. Cytoplasmic extracts from equal amounts of HAV-inoculated cells were prepared at 0 and 6 weeks p.i., and total cell RNA was extracted with phenol-chloroform–1% SDS. Cell equivalents of total RNA were blotted onto nitrocellulose by using a Schleicher & Schuell slot blotter, baked at 80°C for 2 h, and hybridized with full-length ^{32}P-labeled cDNA as described previously (14). Primate FRhK-4 cells were used as a positive control of infection at 0 and 6 weeks p.i. Cytoplasmic extracts of mock and HAV-infected FRhK-4 cells obtained at 6 weeks p.i. were used as negative and positive hybridization controls, respectively.

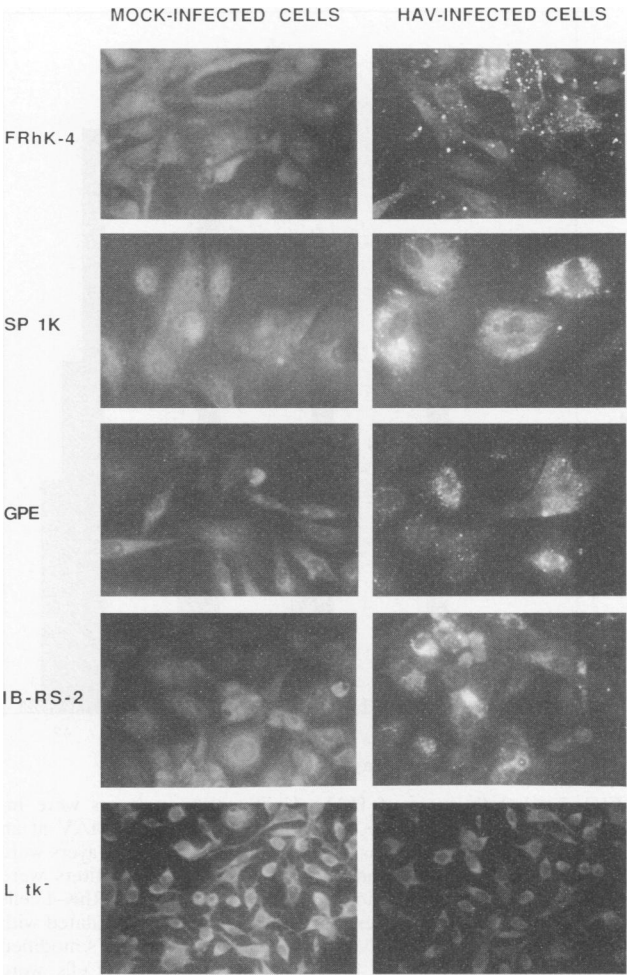

FIG. 2. Immunofluorescence analysis of HAV-inoculated cells. Mock- and HAV-infected FRhK-4, GPE, SP 1K, IB-RS-2 D10, and Ltk$^-$ cell lines were grown in eight-well slides and acetone fixed at 6 weeks p.i. HAV-specific antigens were detected by indirect immunofluorescence in fixed cells treated with a mixture of HAV-neutralizing murine monoclonal antibodies K2-4F2, K3-4C8, and K3-2F2 (17) at a concentration of 1 μg/ml and stained with a 1:1,000 dilution of fluorescein-labeled affinity-purified goat anti-mouse antibody mixed with a 1:500 dilution of contrast Evans blue dye.

mentioned, and at 0, 2, 4, 8, and 42 days p.i., cell extracts were prepared as follows: culture media was removed, monolayers were scraped into 6 ml of PBS, cells were frozen and thawed three times, and cellular debris was pelleted at 1,000 × *g* for 30 min at 4°C. Titers of HAV were determined by an endpoint assay in 96-well plates containing FRhK-4 confluent monolayers. After 2 weeks p.i., monolayers were fixed with 80% methanol, and HAV-specific antigens were detected by treatment with ^{125}I-labeled human anti-HAV antibodies taken from the HAVAB kit (Abbott). At 42 days p.i., HAV grew in GPE, SP 1K, and FRhK-4 cells to titers of 10^2, 10^3, and 10^5 50% tissue culture infective doses per ml, respectively, whereas no growth was detected in Ltk$^-$ cells (Fig. 3). HAV grew poorly and slowly in GPE cells and was detected only after 42 days p.i. In SP 1K cells, HAV grew intermediately, but in spite of HAV being detected early, at 4 days p.i., growth never reached a plateau. HAV always grew the best in FRhK-4 cells and was detected in the first point of the growth curve, reaching a maximum titer at 8 days p.i. Although tc-HAV did infect GPE and SP 1K cells, we did not detect replication of the wild-type HM175 strain of HAV in these cell lines under similar experimental conditions.

Low rates of growth and lower titers of HAV in GPE and SP 1K cells suggested that the virus could be adapted to grow more efficiently by serial passaging in the same cell lines. Passage 1 was obtained by infecting monolayers with viral stocks prepared in the same cell line at 42 days p.i.; weekly passages were done by infecting fresh monolayers with one-third of the virus stock from the preceding passage, washing off

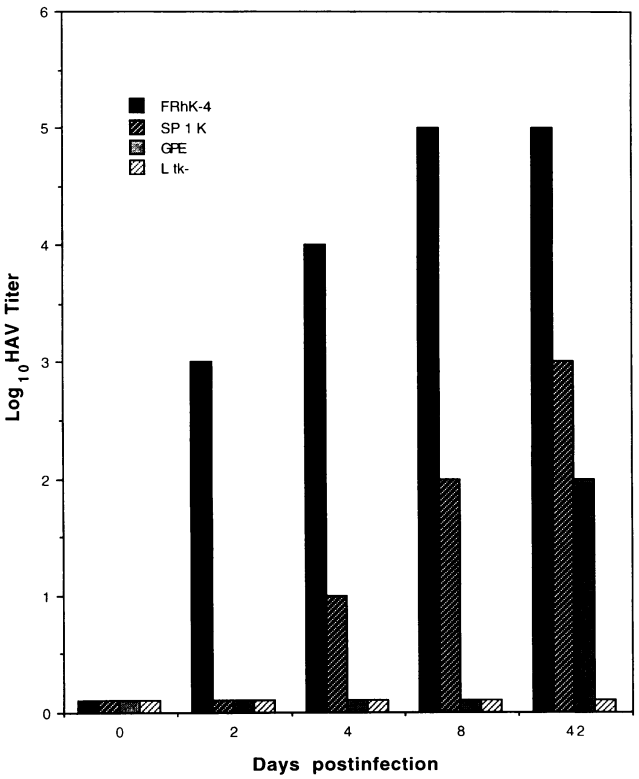

FIG. 3. Growth curve of HAV. Confluent monolayers were infected with the tissue culture-adapted HM175 strain of HAV at an MOI of 10. The inoculum was washed out, and the monolayers were incubated for 0, 2, 4, 8, and 42 days at 34°C. HAV titers were determined by endpoint dilution assay in monolayers of FRhK-4 cells grown in 96-well culture plates. Duplicate wells were inoculated with 0.2 ml of 10-fold dilutions of viral suspension in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were incubated at 34°C for 14 days, the medium was removed, cells were fixed with 200 μl of 80% methanol in PBS for 1 h at 4°C, the monolayers were washed three times with PBS, and the wells were blocked with 5% bovine serum albumin–0.1% NaN_3 in PBS. Monolayers were treated with 50 μl of a 1:5 dilution of human anti-HAV ^{125}I-labeled antibody (0.038 μCi per well) from HAVAB radioimmunoassay test (Abbott) for 2 h at 37°C. After three washes with PBS, skirts of 96-well plates were torn out, and the plates were exposed to X-Omat AR film (Eastman Kodak Co.) with intensifying screens for 12 to 24 h. Endpoint titers were established by densitometric analysis of autoradiography as the inverse of the highest dilution at which HAV antigen could be detected compared with a mock-infected control. Endpoint dilution assays were repeated at least two times. Each datum point represents the mean titer for two replicate assay determinations. Results were reproducible.

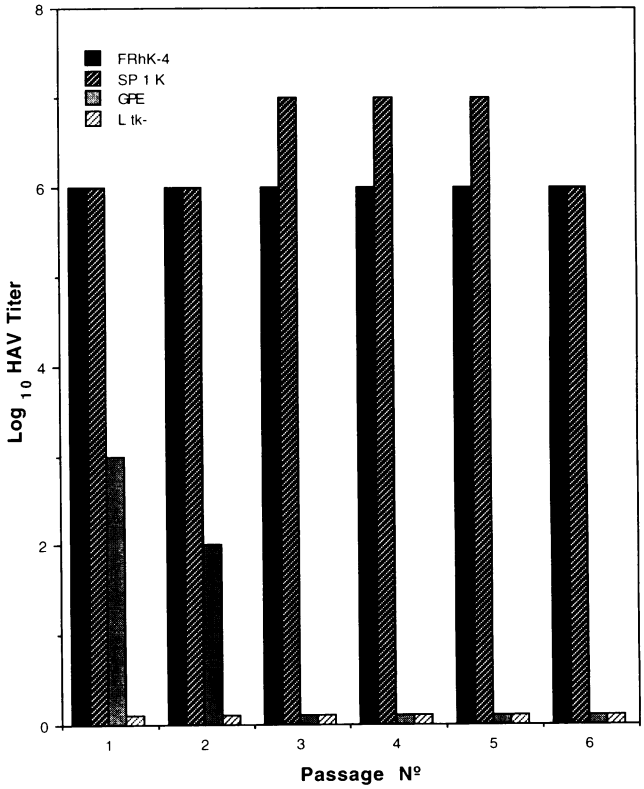

FIG. 4. Serial passages of HAV in nonprimate cell lines. Confluent monolayers of FRhK-4, GPE, SP 1K, and Ltk$^-$ cells were infected with tc-HAV grown in the same cells for 42 days (passage 1). After 7 days of incubation at 34°C, cultures were frozen and thawed three times, cell debris was pelleted at 1,000 × *g*, and supernatants were used to infect fresh monolayers (passages 2 to 6). After six weekly passages, HAV was titrated by using the same endpoint assay as that described for Fig. 3.

the inoculum, and incubating cells for 1 week at 34°C. HAV grew in SP 1K cells to titers similar to those in FRhK-4, suggesting that tc-HAV was fully adapted to grow in SP 1K cells after only few passages (Fig. 4). This rapid serial passaging did not succeed in adapting tc-HAV to grow better in GPE cells. On the contrary, HAV titers declined and became undetectable at passage 3 (Fig. 4). It is possible that HAV requires more than 1 week to produce infectious particles in GPE cells; therefore, the low rate of growth of tc-HAV in GPE cells (Fig. 3) could explain the disappearance of HAV upon serial passaging. HAV did not grow in Ltk$^-$ cells even after six weekly passages, indicating once more that this cell line is not permissive to HAV infection (Fig. 4).

We used three criteria to rule out the possibility that replication of HAV detected in GPE and SP 1K cell was due to contamination with cells of primate origin. First, we tested for susceptibility to poliovirus infection by inoculating GPE, SP 1K, and FRhK-4 cells with P1/Mahoney at a MOI of 10. Poliovirus replication was studied at different times p.i. by both observation for cytopathic effect and slot blot analysis of total cellular RNA by using a ^{32}P-labeled cDNA probe (14). Our analysis showed that poliovirus replicated in FRhK-4 cells, as expected, but it did not grow in GPE and SP 1K cells (data not shown).

Isoenzyme analysis (18, 19) was the second criterion. Electrophoretic mobility in starch gels of the polymorphic enzymes nucleoside phosphorylase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, mannose phosphate isomerase, peptidase B, glutamate oxaloacetate transaminase, and lactate dehydrogenase was determined by using the AuthentiKit System (Innovative Chemistry, Inc., Marshfield, Mass.) as suggested by the manufacturer. Isoenzyme analysis of GPE, SP 1K, and IB-RS-2 D10 cells and cell line controls from ATCC revealed that our cell lines were not contaminated with cells of other origins. For instance, the lactate dehydrogenase zymogram (Fig. 5) shows that GPE and the guinea pig control have the same pattern of bands (compare lanes 8 and 9), which differs from the pattern shared by SP 1K cells and the dolphin

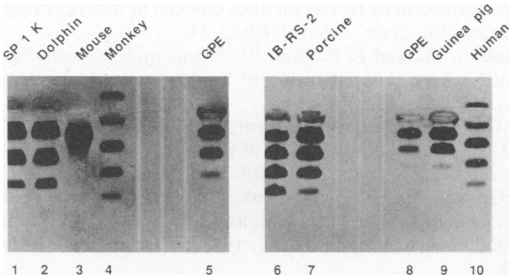

FIG. 5. Lactate dehydrogenase isoenzymes analysis. Isoenzyme analysis was done at the American Type Culture Collection (Rockville, Md.) using the AuthentiKit System (Innovative Chemistry). The following cell lines were used as controls: murine NCTC clone 929 cells (CCL1), human HeLa cells (CCL2), porcine PK 15 cells (CCL3), monkey COS-7 cells (CRL 1651), guinea pig GCP-16 cells (CCL 242), and dolphin SP 1K cells (CCL 78).

control (compare lanes 1 and 2) and the one shared by IB-RS-2 D10 cells and the porcine control (compare lanes 6 and 7). The distinctive band patterns for dolphin, mouse, monkey, guinea pig, porcine, and human cells and the lack of mixed patterns in all zymograms confirmed the origin and purity of cells lines used in this work.

The third criterion was the sequence analysis of the cytochrome *b* gene, a mitochondrial gene that has been extensively studied and used for phylogenic analysis. The entire mitochondrial cytochrome *b* gene was amplified by PCR (13), and 300 bases from the 3′ end of the gene were sequenced by the dideoxynucleotide sequencing method (21). Our results showed that the nucleotide sequence of the GPE and SP 1K genes were similar to the published sequences for the guinea pig and dolphin cytochrome *b* genes, respectively (13, 16).

The three tests used to determine the identity and purity of GPE and SP 1K cells clearly showed that these cell lines are of guinea pig and dolphin origin, respectively, and that they are not detectably contaminated with primate cells. Since the immunofluorescence studies showed that 80 to 100% of the GPE and SP 1K cells were infected with tc-HAV, growth of HAV in these cell cultures is clearly not due to undetected low levels of contamination with primate cells.

To analyze whether cell-derived lipids and proteins associated with HAV particles (15, 22, 24) played a role in the susceptibility of GPE and SP 1K cells to tc-HAV infection, we investigated tc-HAV replication upon infection with purified virions and transfection with genomic RNA phenol-extracted from virions. Viral stocks were incubated for 2 h at room temperature with a mixture of detergents (0.5% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Nonidet P-40), extracted with chloroform until there was no interface, pelleted through a 40% sucrose–0.5% sarkosyl cushion by centrifugation at 40,000 rpm for 5 h at 4°C in an SW40 rotor, and sedimented through a 5 to 30% sucrose gradient (1). Since tc-HAV replicated in GPE and SP 1K cells after infection with purified virions and transfection with virion RNA, we concluded that neither proteins or lipids associated with the virion nor interaction with cellular receptors is a requirement for the initial round of replication in GPE and SP 1K cells.

We did not detect replication of HAV upon transfection of virion RNA into Ltk^-, CHO-K1, 3T6, MDBK, BHK-21, SCP, *E. derm*, QT6, and PK 15 cell lines by slot blot and immunofluorescence analyses (data not shown). Using similar experimental conditions, we detected poliovirus replication in these cell lines after transfection of poliovirion RNA or cotransfection of poliovirion and hepatitis A virion RNA; these results clearly showed that the cells were not refractory to uptake of RNA and the preparation of HAV RNA did not contain inhibitory factors. The lack of detectable levels of replication of HAV upon transfection of virion RNA does not allow us to definitely conclude that there are intracellular blocks to HAV replication in these cell lines, as positive results might be attainable under other experimental conditions. It is possible that host-specific mutations necessary for replication of tc-HAV in primate cells (6–11, 23) are also required for growth in nonprimate cells; therefore, it is conceivable that such host-specific mutations were not generated or selected during the limited replication of HAV triggered by transfection of virion RNA. The introduction of genetic material coding for the cellular receptor(s) for HAV into nonpermissive cells might help to elucidate the mechanism(s) involved in the restricted growth of HAV; these experiments will have to await the characterization and molecular cloning of such a receptor.

It is believed that under natural conditions, HAV can infect only humans and certain species of nonhuman primates. There is no substantial evidence that would suggest infection of species other than primates with HAV (for a review, see reference 2). This restricted host range has led to adaptation of HAV to cell cultures of primate origin (for a review, see reference 12) but not to cell cultures of other origins. Recently, it has been shown that HAV can efficiently bind to murine and canine cell lines, but neither attachment to specific cellular receptor(s) nor susceptibility of these cell lines to HAV infection was established (24). In this work, we studied the susceptibility of a wide variety of cell lines of different origins to HAV infection. The results presented here show that tc-HAV can grow in guinea pig (GPE), dolphin (SP 1K), and probably pig (IB-RS-2 D10) cells. It is possible that serial passages could adapt tc-HAV to grow more efficiently in GPE cells; a similar strategy of serial passages of tc-HAV in different primate cell lines resulted in the accumulation of mutations that enabled the virus to grow more efficiently (3, 6–11, 23). It will be interesting to determine whether the same mutations are responsible for adaptation of HAV for growth in primate and nonprimate cells.

Our results clearly show that cellular factors such as cell surface receptors and intracellular host-factors required for HAV replication are not restricted to primate cells. Replication of HAV in cultures of guinea pig, pig, and dolphin cells does not imply that this virus could infect animals of the same species, although this remains an open question. Finally, the cell lines studied in this work will be of interest for elucidating the mechanism of the life cycle of HAV responsible for host range restriction, virulence, and pathogenesis; moreover, it is possible that adaptation of HAV for growth in nonprimate cell lines may result in the selection of mutants that could be evaluated for their potential as live attenuated vaccines.

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