

Rescue of *Hepatitis A virus* from cDNA-transfected but not virion RNA-transfected mouse Ltk- cells

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Summary. *Hepatitis A virus* (HAV) has stringent replication requirements and a restricted host-range. Mouse Ltk- cells do not support growth of HAV upon infection or transfection of virion RNA. However, low levels of HAV were rescued from Ltk- cells transiently transfected with its infectious cDNA. Ltk- stable transfectants that expressed HAV antigens and produced infectious HAV were selected and termed Ltk-pJH15 cells. After a few serial passages, HAV became undetectable in the Ltk-pJH15 cells. Multiple rounds of single cell cloning of HAV antigen positive Ltk-pJH15 cells resulted in the isolation of clone E8 that produced higher levels of HAV for at least 5 passages. HAV produced in E8 cells was similar to the parental virus as shown by infectivity assays. Luciferase assays using a bi-cistronic construct containing the HAV 5' noncoding region showed similar levels of HAV IRES-dependent translation in Ltk- and Ltk-pJH15 cells, which suggested that HAV IRES-dependent translation was not a limiting factor for HAV growth in these cells. The availability of the Ltk-pHJ15 cells will allow the identification of cellular factors required for HAV growth, which could lead to the development of a mouse model to study pathogenesis of HAV.

Introduction

Hepatitis A virus (HAV), an atypical picornavirus that causes acute hepatitis in humans [16], has a positive sense RNA genome of approximately 7,500 bases and a capsid formed by 60 copies of at least 3 viral proteins (VP1, VP2, and VP3). VP4, the fourth protein found in the capsid of picornaviruses, is very small (21–23 amino acids) in HAV and has not been detected in mature virions. After binding to a cellular receptor, identified in African green monkey (AGMK) cells as havcr-1

[18] and in human cells as huhavcr-1 [9], HAV enters the cell by an unknown mechanism. Although its replication is poorly understood, it is clear that HAV has stringent growth requirements. Cellular determinants for the efficient growth of HAV seem to be restricted to specific hosts and cell types, and HAV needs to accumulate cell-specific adapting mutations to grow in a new host. Most wild-type (wt) strains of HAV grow poorly in cell culture, however, passage of the virus in primate cell cultures results in the selection of attenuated variants that grow efficiently, produce higher viral yields, and in general do not cause cytopathic effect [4, 21]. The genomes of several cell culture-adapted strains of HAV have been sequenced and found to contain 19 to 55 mutations scattered all over the genome [3, 15, 17]. Mutations in the nonstructural P2 region of the genome are most critical for cell culture adaptation [7, 8]. Mutations in the 5' noncoding region also contribute to enhance growth *in vitro* [5, 7, 8] in a host cell dependent manner [5, 7, 8, 11, 12, 24]. In addition to the absolute presence or absence of cellular factors that may limit or enhance HAV replication, their relative abundance may also play a role in virus growth [14].

Pathogenesis of HAV is poorly understood, and primates are the only animal model for this virus. Therefore, development of a small animal model for pathogenesis of HAV is highly desirable. Preliminary work demonstrated that HAV can grow in guinea pig, pig, and dolphin cell lines [6] and indicated that cellular factors required for HAV replication are not restricted to primates. However, HAV does not grow efficiently in murine cells upon infection or transfection with virion RNA [6, 26]. In this paper we studied growth of HAV in mouse cells. We transfected the infectious cDNA of HAV into mouse Ltk- cells, rescued infectious virus, and showed that HAV IRES-dependent translation did not limit growth of HAV in the Ltk- cells. Therefore, it is likely that other steps of the life cycle of HAV, such as RNA replication and/or morphogenesis, are impaired in mouse Ltk- cells. Further studies of the Ltk- transfectants described in this paper could lead to the identification of cellular factors required for HAV replication in mouse cells and to the development of a murine model for HAV.

Materials and methods

Antisera

Rabbit anti-HAV antiserum was produced by immunizing animals with a commercially available HAV vaccine as described [25]. Human anti-HAV polyclonal antiserum was obtained from the HAVAB kit (Abbott Laboratories). Peroxidase-labeled goat anti-rabbit antibodies and FITC-labeled goat anti-human IgG antibody were used as suggested by the manufacturer (Kirkegaard and Perry Laboratories, Inc.). Murine anti-HAV neutralizing Mabs K2-4F2 and K3-4C8 [19] were purchased from Commonwealth Serum Laboratories, Melbourne, Australia.

Cells and viruses

Murine Ltk- cells, obtained from the American Type Tissue Collection (ATCC), and the continuous clone GL37 of African green monkey kidney (AGMK) cells [27], termed AGMK

GL37 cells, were grown in Eagle's minimal essential medium (EMEM) with 10% fetal bovine serum (FBS) at 37 °C in a CO₂ incubator.

Human tissue culture adapted HM175 strain of HAV was derived from full-length *in vitro* synthesized RNA transcripts [2], and termed HAV/7. This virus was serially passaged approximately 100 times in BSC-1 cells and termed HAV PI (generous gift of S. Feinstone, FDA). Virus derived from Ltk- cells transfected with pJH15, which codes for the full-length tissue culture adapted HM175 strain of HAV [2], was termed HAV-L.

The Mahoney strain of poliovirus type 1 (PV1/M) was grown in HeLa cells, and the A59 strain of mouse hepatitis virus (MHV) was grown in Ltk- cells.

Titration of HAV

HAV titers were determined by an endpoint dilution assay in 96-well plates containing confluent monolayers of FRhK-4 cells, which were fixed with 90% methanol 2 weeks after infection and stained with ¹²⁵I-labeled human anti-HAV antibodies from the HAVAB kit [6]. Alternatively, HAV titers were determined by ELISA in an endpoint dilution assay [25] in 96-well plates containing confluent monolayers of AGMK GL37 cells, which were fixed 2 weeks after infection with 90% methanol, stained with rabbit anti-HAV antibodies and peroxidase-labeled goat anti-rabbit antibodies, and developed with TMB one component substrate as described by the manufacturer (Kirkegaard and Perry Laboratories, Inc.). Wells that developed at least 2.5 times the absorbance of the uninfected control wells were considered as positives and viral titers were determined using the Reed and Muench method [22].

Detection of HAV antigens by indirect immunofluorescence (IF) analysis

Cell monolayers grown on cover slips were fixed with cold acetone and stained with 1:250 dilution of human anti-HAV antibodies or negative control antibodies from the HAVAB kit (Abbott Labs). Cells were washed and then treated with 1:500 dilution of FITC-labeled goat anti-human antibodies. After washing extensively, cover slips containing the stained cells were mounted on microscope slides using Permafluor (Thomas Scientific, Inc.) and analyzed under a Zeiss Axioscope immunofluorescence microscope at 400× with an oil immersion objective.

Plasmid constructs

Recombinant DNA manipulations were done by standard methods [23]. Constructions were verified by automatic nucleotide sequencing. All plasmids were grown in *E. coli* DH5α and purified by chromatography as recommended by the manufacturer (Quiagen, Inc.). The following plasmids were constructed:

1. pJH15: The 7.5 kb full-length infectious cDNA of the HM175 strain of HAV contained in pHAV/7 [1] was subcloned into eukaryotic expression vector pR/cCMV under the control of a CMV promoter and termed pJH15. To do so, a 2 Kb HindIII fragment from pHAV/7 containing the 5' end of the HAV cDNA was subcloned into the HindIII site of pR/cCMV. The correct orientation, which contained the HAV 5' non-coding sequence adjacent to the CMV promoter, was selected. The resulting plasmid was termed pJH11 and contained XbaI restriction sites next to the initiation codon of the polyprotein of HAV and in the polylinker of the expression vector. A 6.8 Kb XbaI HAV cDNA fragment from pHAV/7, which contained the coding sequence and 3' end non-coding sequence of HAV, was cloned into XbaI cut pJH11. A construct containing the correct orientation coding for the full-length infectious cDNA of HAV under the control of the CMV promoter was selected and termed pJH15.

2. pJH16: Construction of control pJH16 and pJH15 were done similarly, but the 6.8 Kb XbaI HAV cDNA fragment was inserted in the inverse orientation in the XbaI site of pJH11. The resulting construct was termed pJH16 and coded for a noninfectious cDNA construct of HAV containing an inversion.
3. pRFLCMV-HAV IRES: A plasmid containing a bi-cistronic message coding for the *Renilla reniformis* (renilla) and *Photinus pyralis* (firefly) luciferases was constructed. To do so, the 0.75 kb cDNA fragment coding for the entire 5' non-coding region of HAV was cloned into the polilynker pRLCMV (Promega Corp.). The resulting construct, termed pRLCMV-HAV IRES, coded for the renilla luciferase followed by the 5' noncoding region of HAV. A PCR DNA fragment coding for the firefly luciferase amplified from pMMneoLuc (Clontech Labs) was cloned into pRLCMV-HAV IRES linearized with Xba I at the initiation codon of HAV. The resulting plasmid, termed pRFLCMV-HAV IRES, contains a CMV promoter that controls the expression of a bi-cistronic message coding for the renilla and firefly luciferases separated by the 5' non-coding region of HAV.

Transfections

Confluent monolayers of GL37 AGMK and Ltk- cells in 60 mm dishes were transiently transfected with 1 μ g of pJH15 or control pJH16 using 5–10 μ g of DOTAP (Roche, Inc.) as facilitator. Genomic RNA from purified HAV virions was extracted with phenol and transfected into cells using DEAE-Dextran as facilitator [6]. Cells were washed and harvested at different times post-transfection. Cell extracts were prepared by 3 freeze-and-thaw cycles. AGMK GL37 cells infected with the cell extracts were analyzed by IF. HAV present in the cell extracts was titrated in AGMK GL37 cells by the end-point assay in 96-well plates.

Selection of Ltk- cell transfectants expressing full-length HAV mRNA

Ltk- cells were transfected with pJH15 or control pJH16 using DOTAP as indicated above. After overnight incubation at 37 °C, cells were washed, and fresh growth medium was added to the cells. Neomycin resistant cells were selected in the presence of 800 μ g/ml G-418 (Gibco-BRL). Cell extracts of G-418 resistant Ltk- transfectants prepared by 3 freeze-and-thaw cycles were titrated on AGMK GL37 cells by the end-point assay in 96-well plates. Cell clones that produced infectious HAV were termed Ltk-pJH15 cells. Production of HAV was monitored by IF, and Ltk-pJH15 cells that expressed HAV antigens were frequently single-cell cloned in 96-well plates by end-point dilution. Subcloning of the 1C3 clone of Ltk-pJH15 cells resulted in the isolation of the single cell clone E8 that expressed higher levels of HAV.

Dual-luciferase assay

AGMK, Ltk-, 1C3 and E8 cells grown in 6-well plates were transfected with pRFLCMV-HAV IRES as described above. Two days after transfection, cells were washed with PBS, cell extracts were prepared, and the renilla and firefly luciferase activities were measured sequentially from a single sample using the Dual-luciferase assay as recommended by the manufacturer (Promega Biotech). The luciferase activities were determined using a Turner TD20e Luminometer. Cap-dependent translation of the renilla luciferase was compared to translation of the firefly luciferase directed by the HAV internal ribosomal entry site (IRES). The relative activity of the HAV IRES in the different cell lines was expressed as the ratio of firefly luciferase activity to renilla luciferase activity times 1000. The mean luciferase activities of triplicate determinations were calculated. The luciferase experiment was repeated twice with similar results.

Results

Rescue of virus from Ltk- cells transiently transfected with the infectious cDNA of HAV

HAV does not grow in mouse Ltk- cells after infection or transfection of virion RNA [6]. To determine whether transfection of the infectious cDNA of HAV could bypass the block(s) to viral growth in Ltk- cells, we subcloned the full-length cDNA of cell culture adapted HAV [2] into the eukaryotic expression vector pR/cCMV (Invitrogen, Inc.) under the control of a CMV promoter. The resulting plasmid, termed pJH15, was transfected into Ltk- cells using the cationic lipid reagent facilitator DOTAP. As a positive control, pJH15 was transfected into AGMK GL37 cells, which are susceptible cells to HAV infection. Ltk- and AGMK GL37 cells were also transfected with virion RNA using DEAE-dextran as facilitator to compare rescue of HAV from cDNA- and RNA-transfected cells. At 0 and 2 weeks posttransfection, cells extracts were prepared by 3 freeze-and-thaw cycles, cell debris were pelleted and discarded, and HAV was titrated on 96-well plates containing confluent monolayers of AGMK GL73 cells [25]. As expected, HAV was rescued from AGMK GL37 cells transfected with pJH15 and virion RNA but not from Ltk- cells transfected with virion RNA (Fig. 1). Interestingly, we rescued HAV from Ltk- cells transfected with pJH15, which indicated that the cDNA transfection was able to trigger growth of HAV in these cells. Neutralization of virus produced in pJH15-transfected Ltk- cells with

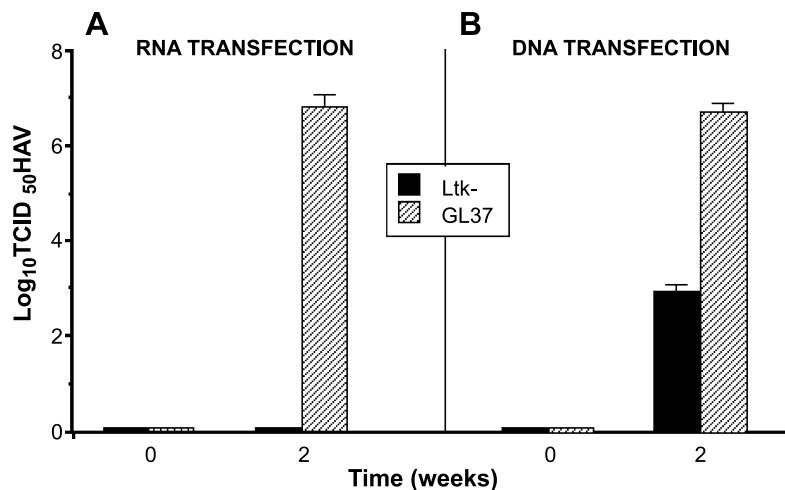


Fig. 1. Rescue of HAV from Ltk- cells transfected with its infectious cDNA. Ltk- and control AGMK GL37 cells were transfected with RNA extracted from HAV virions (A) or pJH15 (B), a plasmid coding for the infectious cDNA of cell culture adapted strain HM175 of HAV under the control of a CMV promoter. At 0 and 2 weeks after transfection, cells were washed and cell extracts prepared by 3 freeze-and-thaw cycles. HAV present in the cell extracts was titrated by ELISA using an endpoint dilution assay in 96-well plates containing AGMK GL37 cell monolayers. Values are the log₁₀ of the HAV titers determined by the Reed and Muench method [22], and the standard deviations are shown as error bars

anti-HAV monoclonal antibodies K2-4F2 and K3-4C8 confirmed that these cells produced infectious HAV (data not shown). In addition, rescue of HAV from Ltk- cells transfected with pJH15 but not pJH16, a construct similar to pJH15 but containing an inversion of a large portion of the HAV cDNA, showed that rescue of virus from Ltk- cells depended on transfection with an infectious cDNA of HAV. It should also be pointed out that transfection of Ltk- cells with control poliovirus cDNA or RNA resulted in the rescue of poliovirus infectious particles, which indicated that neither viral cDNA transcription nor RNA stability was impaired in the Ltk- cells.

Ltk- cell transfectants produce low levels of HAV

After 3–5 passages, HAV became undetectable in the Ltk- cells transfected with pJH15, which suggested that HAV could not spread to other cells in the culture. It is also possible that the HAV-infection triggered antiviral mechanism(s) that limited the viral infection in the Ltk- cells. To further study the HAV infection in Ltk-cells, we decided to establish stable Ltk- cell transfectants expressing the HAV cDNA. To do so, Ltk- cells were transfected with pJH15 and neomycin-resistant cells were selected using 800 µg/ml of the antibiotic G418 (Gibco-BRL). Sixty independent G418-resistant colonies were isolated and subjected to Southern and Northern blot analysis using ³²P-labeled HAV cDNA probes (data not shown). Five of the G418 resistant Ltk- clones, 1A2, 1C3, 1C4, 2C3, and 5C2 that contained the full-length cDNA of HAV and produced full length HAV RNA were termed Ltk-pJH15 cells. Titration of cell extracts showed that the Ltk-pJH15 cells produced approximately 2.5–3.5 log of HAV (Fig. 2A), which was similar to the amount of produced in the Ltk- cells transiently transfected with pJH15 (Fig. 1) and significantly lower than the 7 log of HAV produced in susceptible GL37 AGMK cells. Few Ltk-pJH15 cells (less than 1%) contained HAV-specific antigens as assessed by indirect immunofluorescence (IF) analysis using human anti-HAV antibodies and FITC-labeled goat anti-human antibodies (data not shown). To rule out the possibility that the limited HAV infection of the Ltk-pJH15 cells was due to a contamination with cells of primate origin, we first infected the cells with *Poliovirus* for 3 days and then performed the IF analysis. *Poliovirus* infection did not reduce the HAV-specific IF of the Ltk-pJH15 cells, which indicated that these cells were not of primate origin (data not shown). Infection of Ltk-pJH15 with the A59 strain of *Murine hepatitis virus* (MHV), a coronavirus that only infects murine cells, induced 100% cytopathic effect and further confirmed the murine origin of the Ltk-pJH15 cells.

Interestingly, HAV titers and the number of cells expressing HAV antigens decreased upon serial passaging of the Ltk-pJH15 cells. Periodic single cell cloning of cells expressing HAV antigens was required to maintain the HAV infection in the Ltk-pJH15 cells. The instability of the HAV infection in the Ltk-pJH15 cell clones is puzzling since these cells contain the infectious cDNA of HAV and express full-length HAV RNA.

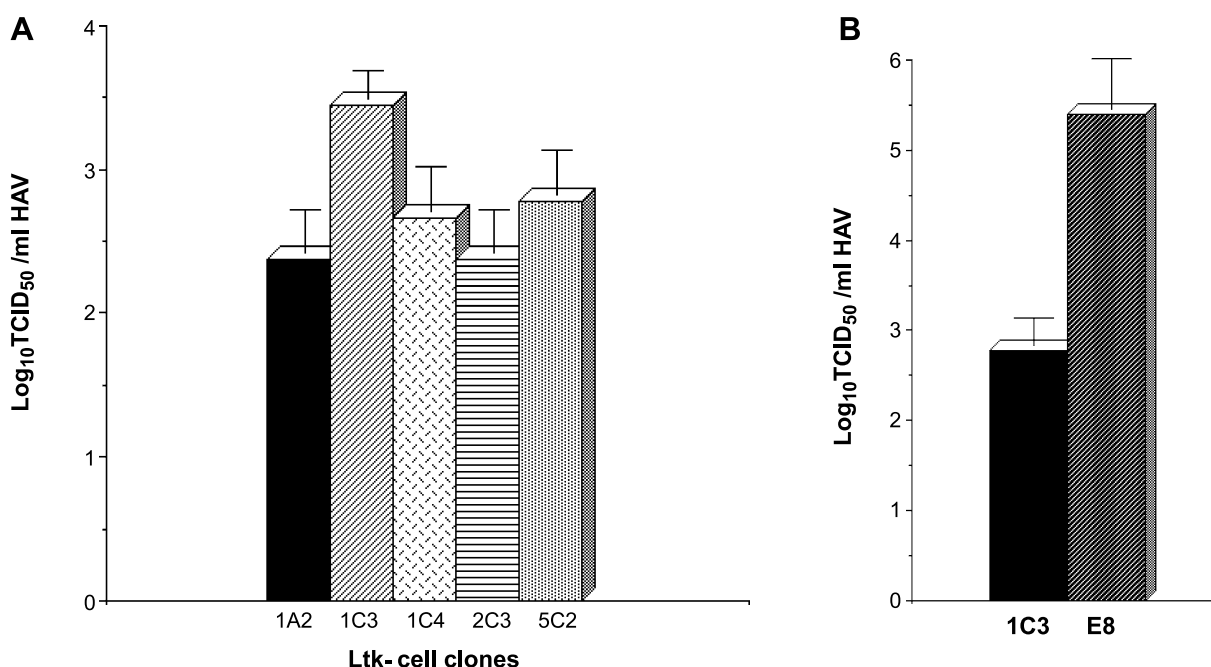


Fig. 2. Virus production in Ltk- cell clones stably transfected with the infectious cDNA of HAV. **A** Analysis of Ltk- cell transfectants. Ltk- cells transfected with pJH15, which contains the infectious cDNA of HAV, were selected with the antibiotic G-418 and termed Ltk-pJH15 cells. Cell extracts of clones 1A2, 1C3, 1C4, 2C3, and 5C2 of Ltk-pJH15 cells were produced by 3 freeze-and-thaw cycles and titrated by ELISA using an endpoint dilution assay in 96-well plates containing AGMK GL37 cell monolayers. Values are the log₁₀ of the HAV titers determined by the Reed and Muench method [22], and the standard deviations are shown as error bars. **B** Selection of Ltk-pJH15 cells that produce higher levels of HAV. Clone 1C3 of Ltk-pJH15 cells was subcloned multiple times by endpoint dilution in 96-well plates. Single cell clones were analyzed by IF for the expression of HAV antigens. Single cell clones that expressed HAV antigens were grown and further subcloned. After 8 cycles of subcloning, we isolated clone E8 that produced approximately 3 log more HAV than its parental clone 1C3. Clones 1C3 and E8 were grown in 6 cm plates, cell extracts were prepared by 3 freeze-and-thaw cycles, and HAV present in the extracts was titrated by ELISA using an endpoint dilution assay in 96-well plates containing AGMK GL37 cell monolayers. Values are the log₁₀ of the HAV titers determined by the Reed and Muench method [22], and the standard deviations are shown as error bars

Isolation of Ltk- transfectants that support the efficient growth of HAV

In an attempt to isolate Ltk-pJH15 cells capable of supporting the HAV persistent infection for several passages, clone 1C3 was further subcloned by end-point dilution. We selected single-cell clones derived from 1C3 cells that produced the highest HAV titers. After 7 rounds of subcloning, we isolated clone E8 that produced approximately 5.5 log of HAV (Fig. 2B), which yielded 2.5 log more HAV than the parental 1C3 cells. IF analysis staining with human anti-HAV antibodies and FITC-labeled goat anti-human antibodies (Fig. 4) showed that

about 5–10% of the E8 cells expressed HAV antigens (A) whereas no HAV-specific immunofluorescence was detected in the parental Ltk- cells (B). Control AGMK GL37 cells infected with HAV (C) showed the characteristic cytoplasmic immunofluorescence of HAV-infected cells whereas mock-infected AGMK GL37 cells (D) did not show HAV-specific immunofluorescence. Interestingly, expression of HAV antigens and virus production remained constant for 5 serial passages of the E8 cells, which indicated that the HAV persistent infection in these cells was stable.

Characterization of HAV rescued from Ltk- cell transfectants

It was of interest to characterize the HAV produced in the E8 cells, which we termed HAV-L. To do so, we compared growth of HAV-L and the parental HAV in one-step growth curves (Fig. 4) by infecting confluent monolayers of AGMK GL37

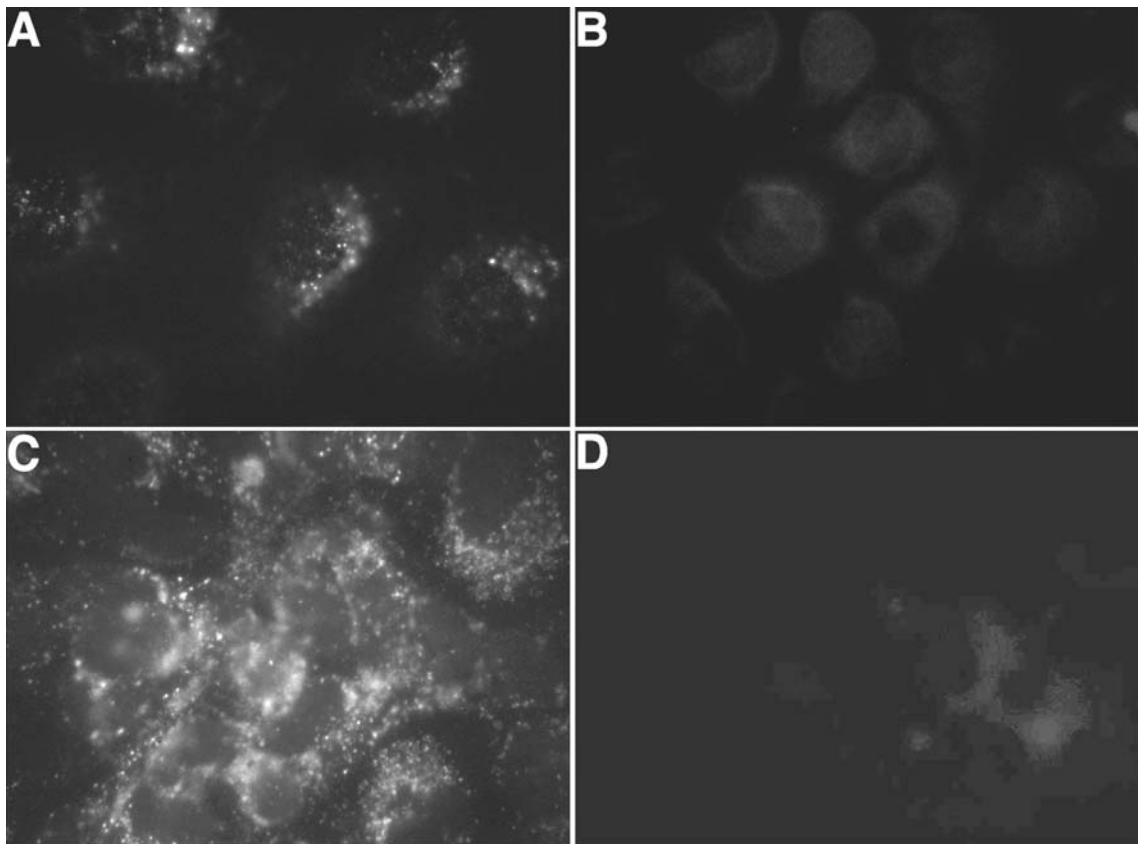


Fig. 3. Immunofluorescence analysis of Ltk- cell transfectants producing HAV. Clone E8 of Ltk-pJH15 cells (A), Ltk- cells (B), and HAV- (C) or mock-infected (D) AGMK GL37 cells grown on cover slips were fixed with acetone. Cells were stained with human anti-HAV antibodies and FITC-labeled goat anti-human Ig antibodies. Immunofluorescent micrographs were taken with a Zeiss Axioscope microscope at 400 \times magnification using an oil immersion objective

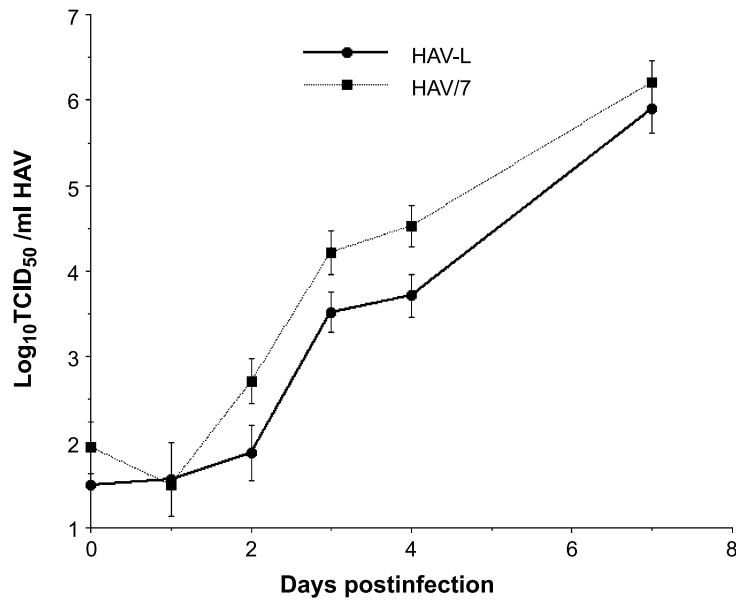


Fig. 4. Single-step growth curve of HAV-L. Growth of HAV-L produced in clone E8 of Ltk-pJH15 cells was compared with growth of its parental virus in AGMK GL37 cells. Monolayers of AGMK GL37 cells in 12-well plates were infected with an m.o.i of 5 of HAV-L or HAV. At different times postinfection, cell extracts were prepared by 3 freeze-and-thaw cycles and HAV was titrated by ELISA using an endpoint dilution assay in 96-well plates containing AGMK GL37 cell monolayers. Values are the log₁₀ of the HAV titers determined by the Reed and Muench method [22], and the standard deviations are shown as error bars

cells in 12-well plates with a m.o.i of 5 of HAV or HAV-L. After 4 h adsorption, monolayers were washed extensively, and cell extracts were prepared at different times post infection by 3 freeze-and-thaw cycles. The virus present in the cell extracts was titrated by end-point dilution in AGMK GL37 cells. Similar growth curves of HAV and HAV-L were observed in AGMK GL37 cells (Fig. 3), which suggested that these two viruses were entirely similar. Transfection of virion RNA extracted from HAV-L and HAV into naïve Ltk- cells did not result in the production of HAV as assessed by titration in AGMK GL37 cells, which indicated that HAV-L did not accumulate host range mutations that allowed it to grow more efficiently in Ltk- cells and suggested that HAV-L is phenotypically indistinguishable from the parental HAV.

Analysis of HAV IRES-dependent translation in Ltk- cells

To study whether translation of HAV was more efficient in Ltk-pJH15 cells than in naïve Ltk- cells, we constructed a plasmid coding for a bi-cistronic message containing two reporter genes separated by the 5' noncoding region of HAV. The resulting construct, termed pRFLCMV-HAV IRES, coded for the renilla and firefly luciferases separated by the 5' noncoding region of HAV (Fig. 5A). Transfection of pRFLCMV-HAV IRES into cells allowed the quantitation of the

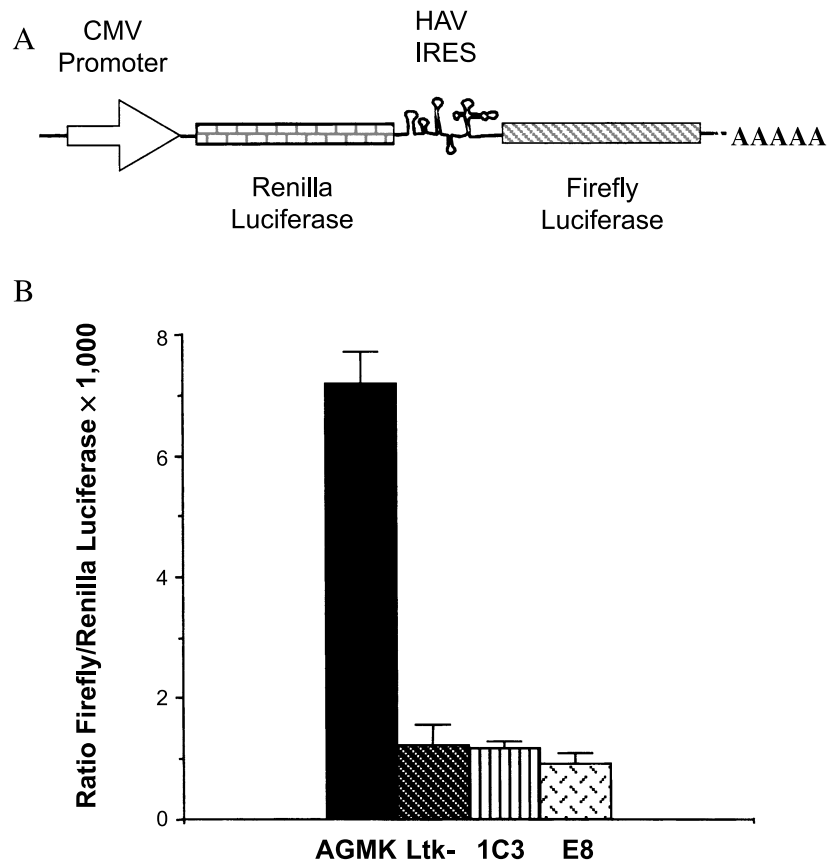


Fig. 5. Analysis of HAV IRES-dependent translation in Ltk- cell transfectants. Clones 1C3 and E8 of Ltk-pJH15 cells, parental Ltk- cells, and control AGMK GL37 cells were transfected with pRFLucHAV-IRES, a plasmid coding for a bi-cistronic message containing the HAV IRES under the control of a CMV promoter (A). Two days after transfection, cells were harvested and renilla and firefly luciferase activity were assayed using a dual-luciferase assay. The relative activity of the HAV IRES in the different cell lines was expressed as the ratio of firefly luciferase activity to renilla luciferase activity times 1,000 (B). The luciferase activity was determined in a Turner TD20e luminometer. The luciferase assay was determined by triplicates and the mean activity of each cell line was calculated. The assay was repeated twice, and the standard deviations of the ratios are shown as error bars

HAV IRES-dependent translation of the firefly luciferase compared to the cap-dependent translation of the renilla luciferase. HAV IRES-dependent translation in AGMK, Ltk-, 1C3, and E8 cells, was analyzed by transfecting cell monolayers in 6-well plates with 1 μ g of pRFLCMV-HAV IRES using 6 μ l of the Fugene-6 as facilitator. Two days after transfection, cells were washed with PBS and the renilla and firefly luciferase activities were assessed sequentially in triplicate samples using the respective substrates from the Dual-luciferase assay kit (Promega). The luciferase activities were determined using a Turner TD20e Luminometer. The HAV IRES-dependent translation in the different cell lines was expressed as the ratio of firefly luciferase activity, which translation is driven by the HAV

IRES, to renilla luciferase activity times 1000 (Fig. 5B). The HAV IRES-dependent translation was 4 to 7 times higher in AGMK cells than in any of the mouse cell lines tested, which correlated with the efficient replication of HAV in this primate cell line. Surprisingly, similar low levels of HAV IRES-dependent translation were observed in the naive Ltk- cells and the 1C3 and E8 clones of Ltk-pJH15 cells, which indicated that there was not a correlation between translation and viral replication. Consequently, it is most likely that block(s) in steps of the viral life cycle unrelated to translation are responsible for limiting HAV growth in Ltk- cells.

Discussion

The life cycle and pathogenesis of HAV is poorly understood, and major elements of the HAV replication strategy are still enigmatic. For instance, growth of cell culture adapted HAV requires cell-type specific mutations, and HAV adapted to grow in a primate cell system needs to be readapted before it can grow efficiently in a similar cell line. Much less is known about the growth requirements in non-primate cells [6, 10]. Adaptation of HAV to murine cell lines is highly desirable because it could help develop a mouse model to study pathogenesis of HAV to complement the currently available monkey models, which have obvious limitations. Few groups have attempted to study growth of HAV in murine cells. It has been shown that HAV does not grow in mouse Ltk- cells upon infection or transfection of virion RNA [6], and that mouse L929 and NIH/3T3 cells are almost completely refractory to HAV infection [26]. The nature of the strong block to HAV growth in murine cells is unknown but most likely is at the cell entry and/or intracellular levels. In an attempt to define these block(s), we decided to further study replication of HAV in Ltk- cells. We found that low levels of virus could be rescued from Ltk- cells transfected with the HAV infectious cDNA but not with virion RNA. This finding was intriguing because rescue of HAV from susceptible AGMK GL37 cells is more efficient after transfection of virion RNA than transfection of HAV cDNA contained in pJH15 (J. Lu and G. Kaplan, unpublished results). This is also the case for *in vitro* RNA transcripts compared to their cDNA templates [2]. It is not clear why the cDNA transfection was able to overcome the block(s) to HAV growth in Ltk- cells. It is possible that the continuous delivery of HAV RNA transcripts from the cellular nucleus to the cytoplasm was instrumental in jump-starting the HAV infection. This contrasts with the delivery of viral RNA to the cytoplasm of cells in culture after infection or transfection with RNA, which most likely occurs within a brief period not enough to trigger the HAV infection of the Ltk- cells. It should be pointed out that we could not rule out the possibility that HAV cDNA nuclear transcripts were translated via a cap-dependent mechanism and resulted in the production of infectious HAV. Consequently, the Ltk- cell transfectants that supported the efficient growth of HAV may produce higher levels of HAV transcripts and/or support higher levels of cap-dependent translation of the nuclear HAV transcripts. However, there is no current evidence to support the notion that packaging of picornaviral genomes could occur in the absence of active viral replication [20].

Our study did not provide conclusive evidence on the lack of HAV receptors in Ltk- cells. Since HAV did not grow in Ltk- cells transfected with virion RNA, it is likely that the infection of Ltk- cells via a putative active cellular receptor would have encountered a similar intracellular block(s) to HAV replication. Consequently, further studies will be required to determine whether Ltk- cells code for functional HAV receptors. These intracellular block(s) to HAV replication are most likely responsible for the instability of the HAV infection in the Ltk-pJH15 cell clones. The increased and stable production of HAV in E8 cells, a subclone of the Ltk-pJH15 cells, suggested that E8 cells may lack or contain weaker intracellular block(s) to HAV replication than the parental clones. Although it is conceivable that spontaneous mutations could have adapted HAV to grow more efficiently in E8 cells, this possibility was ruled out after we found that HAV did not replicate in Ltk- cells transfected with RNA extracted from virions grown in E8 cells.

The nature of the block(s) to HAV replication in Ltk- cells was also analyzed. Previous work showing that HAV translation was stimulated in a rabbit reticulocyte lysate by extracts prepared from mouse liver [13] suggested that the level of HAV IRES-dependent translation in Ltk- cells could be lower than that in the Ltk-pJH15 cell clones. Our results using bi-cistronic messages coding for dual luciferases showed that HAV IRES-dependent translation was similar in the E8, 1C3, and naive Ltk- cells. Therefore, we concluded that translation of HAV is not the limiting factor restricting growth of HAV in Ltk- cells. The similar levels of HAV IRES-dependent translation in the Ltk- and Ltk-pKJH15 cells suggested that block(s) in other steps of the growth cycle of HAV, such as in RNA transcription, are limiting the HAV growth in Ltk- cells. The availability of the E8 cells, which contain the intracellular factors required for HAV growth, and the parental Ltk-cells, which contain block(s) to HAV growth, represent a unique experimental system to study factors required for HAV growth using differential cloning and/or microarray technology.

Further characterization of the Ltk-pJH15 cells described in this work, especially the E8 cells that supported the highest level of HAV growth, will help identify cellular factors involved in host-cell interactions and develop a mouse model to study pathogenesis of HAV.

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