Journal of General Virology (2001), **82**, 597–602. Printed in Great Britain

Adaptation of primate cell-adapted hepatitis A virus strain HM175 to growth in guinea pig cells is independent of mutations in the 5′ nontranslated region

Werner Frings† and Andreas Dotzauer

Department of Virology, University of Bremen, Leobener Straße/UFT, D-28359 Bremen, Germany

Previous studies of hepatitis A virus (HAV) genotypes after adaptation of wild-type virus to growth in cell cultures of primate origin identified determinants for growth in cell culture in the viral 2B and 2C protein-coding regions of the genome and demonstrated that an increased growth efficiency in a particular cell line was achieved by subsequent mutations in the 5′ nontranslated region (5′NTR). The results reported in this study demonstrate that the passage of HAV adapted to primate BS-C-1 cells in guinea pig cells resulted in increased growth efficiency in the rodent cells and decreased growth efficiency in BS-C-1 cells. This adaptation occurred without mutation in the 5′NTR, but the viral 2B and 2C proteins seem to play a role during adaptation to the new environment, as one mutation occurred in each protein. Although the data presented here do not clearly identify which region of the viral genome underwent mutations to improve the interaction of the viruses with guinea pig proteins, they do confirm that the 5′NTR is not the only region responsible for providing host cell-specific information.

Introduction

Hepatitis A virus (HAV), a hepatotropic human picornavirus, has been adapted to grow in a variety of primate cell lines (Siegl & Lemon, 1990). In contrast to that of other picornaviruses, the replication cycle of HAV in cell culture is slow and, with some exceptions (Brack *et al.*, 1998), a persistent infection with low virus yields is normally established. After infection with wild-type HAV isolated from infected human hosts, a minimum of 8 weeks elapses before isolation of HAV can be achieved in cell culture. Although replication is more rapid after serial virus passages in cultured cells, even the replication of these tissue culture-adapted HAV variants (tc-HAV) is not detectable within the first days after infection. The mechanisms of adaptation and the cause of inefficient growth in tissue culture are poorly understood. By sequence analysis of different tc-HAV variants (Paul *et al.*, 1987; Ross *et al.*, 1989; Emerson *et al.*, 1991, 1992; Day *et al.*, 1992; Chang *et al.*, 1993; Tedeschi *et al.*, 1993; Morace *et al.*, 1993; Graff *et al.*, 1994) and studies with chimeric HAV composed of sequences from wild-type and cell culture-adapted variants (Emerson *et al.*, 1991; Funkhouser *et al.*, 1994; Zhang *et al.*, 1995; Graff *et al.*, 1997), mutations that increased virus replication in cultured cells were identified. These mutations were found to occur within the 5′ nontranslated region (5′NTR), which contains the regulatory IRES element for virus translation (Brown *et al.*, 1991), and within the 2B and 2C protein-coding regions, which encode nonstructural proteins involved in RNA replication (Jansen *et al.*, 1988). While the mutations in the 2B and 2C protein-coding regions (mainly the mutation in protein 2B at nt 3889, aa 215) were determined to be necessary for the improvement of growth in cell culture of different tc-HAV variants, regardless of the cell line used, modifications in the 5′NTR appeared to be responsible for the specific host cell range of the virus, as differences in *in vitro* growth were documented to be related to the 5′NTR sequence (Emerson *et al.*, 1991, 1992; Funkhouser *et al.*, 1994; Zhang *et al.*, 1995; Graff *et al.*, 1997). The influence of the 5′NTR on differential growth in particular cell lines is attributed to its function in cap-independent initiation of translation through interaction with specific host cell proteins in addition to the canonical initiation factors (Pestova *et al.*, 1996). This, together with the cell type-specific action of the 5′NTR mutations, is supported by several studies showing that multiple proteins bind specifically to the 5′NTR (Chang *et al.*, 1993). Three of these proteins could be identified

Author for correspondence: Andreas Dotzauer.
Fax +49 421 218 4266. e-mail dotzauer@uni-bremen.de

†**Present address:** Institute of Experimental Dermatology, University of Muenster, von Esmarch-Str. 56, D-48149 Muenster, Germany.

0001-7391

as polypyrimidine tract-binding protein (Chang *et al.*, 1993; Yi *et al.*, 2000), glyceraldehyde-3-phosphate dehydrogenase (Schultz *et al.*, 1996; Yi *et al.*, 2000) and poly(rC)-binding protein (Graff *et al.*, 1998).

In a previous study on the susceptibility of a variety of cell lines to HAV infection, it was demonstrated that HAV can infect not only cell cultures of primate origin but also guinea pig embryo cells (GPE), dolphin cells (SP 1K) and probably pig cells (Dotzauer *et al.*, 1994). As all data on HAV replication *in vitro* were obtained by using primate cell cultures, the nonprimate cell lines are of interest for performing investigations on HAV host range restriction, virulence and pathogenesis. To date, only nonhuman primates are available as animal models; thus guinea pigs would be a valuable model for experimental HAV infection and therefore the rodent GPE cell line is of particular interest. A previous study showed that after infection of GPE cells with HAV the virus grew poorly. Furthermore, blind rapid serial passaging of the virus did not result in adaptation of HAV for enhanced growth in GPE cells (Dotzauer *et al.*, 1994). However, as serial passages of tc-HAV in different primate cell lines resulted in an accumulation of mutations that enabled the virus to grow more efficiently, we applied a similar strategy to investigate the importance of a specific 5′NTR sequence for virus growth in a particular cell line and to determine whether similar mutations are responsible for the adaptation of HAV for growth in primate and nonprimate cells.

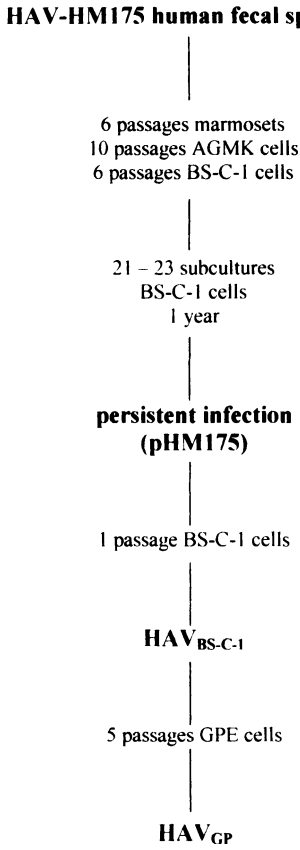

Fig. 1. Passage history of the HAV variants $HAV_{BS\text{-}C\text{-}1}$ and HAV_{GP}. An overview of the pedigree of HAV-HM175 variants was presented by Tedeschi *et al.* (1993).

Methods

■ **Growth and titration of HAV in GPE cells.** $HAV_{BS\text{-}C\text{-}1}$, obtained by infection of the African green monkey kidney cell line BS-C-1 with the pHM175 variant of HAV strain HM175 (Cromeans *et al.*, 1987) and therefore well adapted to growth in these primate cells, was used for primary infection of GPE cells (Fig. 1) at an m.o.i. of 1. In order to calculate the m.o.i., the 50% tissue culture infective dose ($TCID_{50}$) titre calculated by the Kärber method of the $HAV_{BS\text{-}C\text{-}1}$ inoculum, as determined in BS-C-1 cells, was used. Virus titre was assessed by inoculating cells grown in 96-well microtitre plates and virus infection was checked 2 weeks after inoculation by indirect immunofluorescence with the HAV-specific monoclonal antibody 7E7 (Mediagnost) and a fluorescein-labelled anti-mouse antibody (Kirkegaard & Perry) (Brack *et al.*, 1998).

HAV replication in GPE cells was continuously assayed by RT–PCR using primers corresponding to the 2C and VP3 protein-coding regions (Graff *et al.*, 1994) and HAV RNA obtained by phenol–chloroform extraction from cytoplasmic extracts (Dotzauer *et al.*, 1994).

Infected GPE cells were lysed by triple freeze–thaw cycles as soon as viral RNA was detectable. 10% of this material was used as the inoculum for the following passage. This procedure was repeated four times, resulting in the preparation of the passage five virus HAV_{GP} (Fig. 1).

■ **One-step growth curves.** Growth curves of HAV_{GP} and the parental virus, $HAV_{BS\text{-}C\text{-}1}$, were performed in both GPE and BS-C-1 cells under the same conditions. Cells were infected with an m.o.i. of 5; the inoculum titre of HAV_{GP} was determined in GPE cells (7×10^6 $TCID_{50}$/ml) and the titre of $HAV_{BS\text{-}C\text{-}1}$ was determined in BS-C-1 cells (4×10^6 $TCID_{50}$/ml). $TCID_{50}$ titres were assessed on days 2, 4, 8, 12, 16 and 20 post-infection (p.i.).

■ **Radioimmunofocus assay (RIFA).** RIFA (Lemon *et al.*, 1983) analyses were performed with HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$, 125 infectious units of each virus for each cell type, using parallel cultures of BS-C-1 and GPE cells. Cells were inoculated onto 60 mm cell culture dishes containing nearly confluent cell monolayers. After adsorption for 2 h, cells were overlaid with medium containing 0·5% agarose and incubated for 9 days, due to the limited viability of GPE cells under the solidified overlay. The overlay was removed and the cells were fixed with 80% acetone and stained with radioiodinated human anti-HAV IgG (Abbott). Foci were visualized by autoradiography and both the size and the intensity of the foci were used as parameters to assess virus replication.

■ **Dot blot hybridization.** Serial tenfold dilutions of virus inocula were added concurrently to parallel cultures of GPE and BS-C-1 cells grown in 24-well cell culture plates. After 10 days of incubation, which corresponds to the time at which the maximum $TCID_{50}$ titres were reached (see Fig. 2), viral RNA from each well was extracted with phenol–chloroform (Dotzauer *et al.*, 1994) and quantified by dot blot hybridization with full-length ^{32}P-HAV labelled cDNA (labelled by random priming) and the endpoint dilutions determined.

■ **Sequence analysis.** RT–PCR was used to isolate the genomic regions 5′NTR (nt 45–734), 2ABC (nt 3027–5000), 3AB (nt 5001–5291) and 3′NTR (nt 7416–7478) of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$. Sequencing of three independently isolated fragments for each region was performed

with the dideoxynucleotide method by using the sequencing kit from Pharmacia.

Results and Discussion

In order to study the adaptation of primate cell-adapted HAV to growth in a rodent cell line, $HAV_{BS\text{-}C\text{-}1}$ was passaged through GPE cells to produce HAV_{GP}. Virus replication was continuously assessed by RT-PCR and viral RNA was found to be detectable 20 days p.i. in the first passage. However, in the course of the passages, the incubation time decreased from 13 days p.i. during passage two to 5 days p.i. during passage five until HAV RNA was detectable for the first time by RT–PCR. This indicated that HAV grew increasingly better in the nonprimate cell line; however, as the virus was passaged without knowing the titre, the earlier detection of HAV in the course of the passages could reflect a higher m.o.i. Therefore, in order to obtain significant information about changes in the pattern of virus growth, we recorded one-step growth curves for both HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$. The growth curves revealed that the potential of HAV to replicate in nonprimate GPE cells was increased significantly after five passages in these cells (Fig. 2*a*) and showed the same growth characteristics as the parent $HAV_{BS\text{-}C\text{-}1}$ virus in BS-C-1 cells (Fig. 2*b*), whereas the ability of HAV_{GP} to multiply in primate BS-C-1 cells was reduced dramatically (Fig. 2*b*). The inversion of the growth pattern of HAV_{GP} and the parent virus $HAV_{BS\text{-}C\text{-}1}$ in GPE cells as compared with growth in BS-C-1 cells showed that adaptation to the nonprimate cell line occurred after just five passages.

In order to confirm the data generated from the one-step growth curves, RIFA analyses were also carried out. Again, HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$ displayed a notable difference in their abilities to grow in BS-C-1 and GPE cells (Fig. 3), reflecting the differences in the growth kinetics of the two HAV variants as determined from one-step growth curves. $HAV_{BS\text{-}C\text{-}1}$ ex-

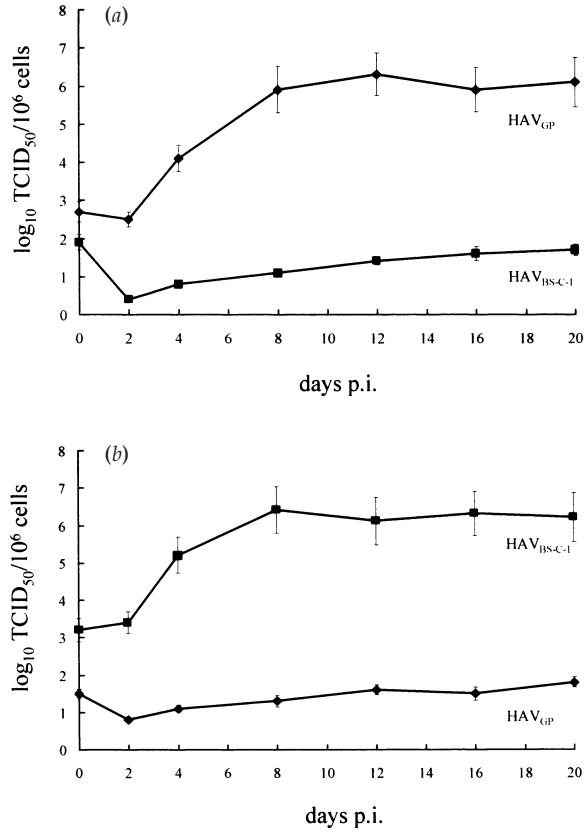

Fig. 2. Replication kinetics under one-step growth curve conditions for HAV_{GP} in GPE (*a*) and BS-C-1 (*b*) cells in comparison with the parent virus $HAV_{BS\text{-}C\text{-}1}$. The kinetics show the $TCID_{50}$/ml titres related to 10^6 HAV-producing cells over the course of 20 days. Cells were infected with an m.o.i. of 5 and the $TCID_{50}$ titre was determined by indirect immunofluorescence at the times indicated in the corresponding cell line 2 weeks after inoculation. Each data point is an average obtained from two separate experiments. Error bars indicate SD of the means.

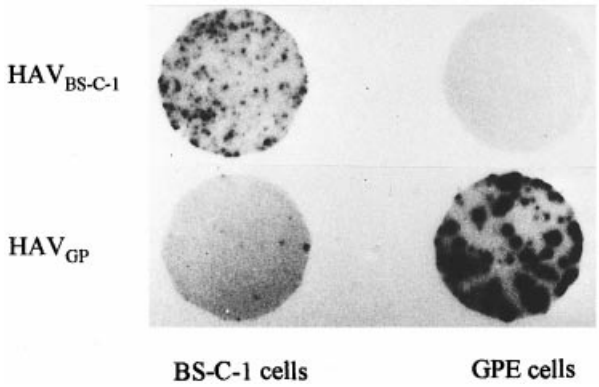

Fig. 3. Replication of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$ analysed by RIFA. Both GPE and BS-C-1 cells were infected with identical amounts of the virus variants in parallel. Replication was analysed at day 9 p.i. using ^{125}I-labelled anti-HAV antibodies.

Table 1. Comparison of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$ growth in GPE and BS-C-1 cells by dot blot hybridization of virus titrations

Parallel cultures of GPE and BS-C-1 cells in 24-well culture plates were inoculated with serial tenfold dilutions of virus inocula and incubated at 34 °C. Cytoplasmic RNA was extracted 10 days p.i., blotted onto nitrocellulose membranes, probed with full-length HAV cDNA labelled with ^{32}P by random priming and assayed by auto-radiography. The results shown were identical in two separate experiments.

	Titre (endpoint dilution)		
Virus	GPE cells	BS-C-1 cells	GPE/BS-C-1*
HAV_{GP}	10^3	10^2	10^1
$HAV_{BS\text{-}C\text{-}1}$	10^2	10^4	10^{-2}

* Ratio of endpoint dilutions in GPE cells to endpoint dilutions in BS-C-1 cells.

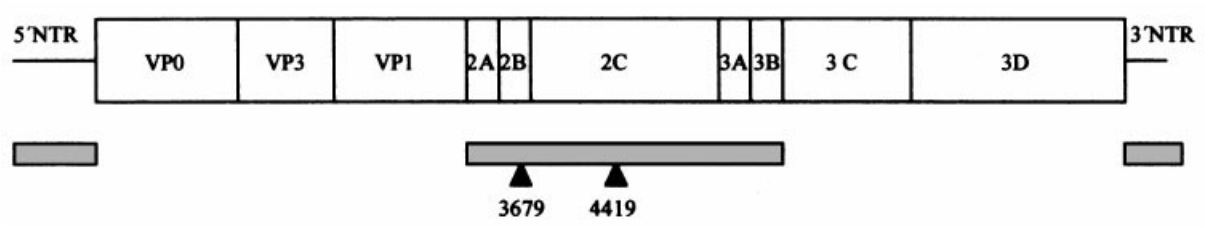

Fig. 4. Schematic representation indicating the sequenced genomic regions of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$ (grey bars) and the locations of mutations (black triangles) found in the HAV_{GP} genome as compared with the parent $HAV_{BS\text{-}C\text{-}1}$ genome.

hibited small, clearly visible foci in BS-C-1 cells and barely detectable foci in GPE cells. After five passages of adaptation (HAV_{GP}), virus replication was dramatically enhanced in GPE cells, inducing large and intense foci, whereas HAV_{GP} in BS-C-1 cells induced barely visible foci, demonstrating that a change in cell tropism had occurred.

In addition, the growth of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$ in GPE and BS-C-1 cells was compared by dilution dot blot hybridization (Table 1). The important measure was the ratio of the endpoint dilutions of each virus determined in GPE cells to that determined in BS-C-1 cells. We obtained identical results in two separate experiments. Again, the growth ratio of 10^{1} obtained with HAV_{GP} compared with the growth ratio of 10^{-2} obtained with $HAV_{BS\text{-}C\text{-}1}$ reflected the adaptation of HAV_{GP} to grow in GPE cells and supports both the one-step growth kinetics data and the RIFA data. Therefore, after five passages of $HAV_{BS\text{-}C\text{-}1}$ in GPE cells, the host cell-specific growth pattern changed dramatically in favour of the rodent cell line and was accompanied by restricted growth in the former primate BS-C-1 cell line. This shows adaptation of the parent virus to the new host cells.

In order to investigate whether the altered tissue culture host range of HAV is reflected by mutational changes in the 5′NTR, which are thought to be responsible for adaptation of tc-HAV to distinct cell lines, and whether related mutations are responsible for the adjustment of HAV for growth in cultured cells of primate and nonprimate origin, we isolated and sequenced selected genomic regions (5′NTR, nt 45–734; 2ABC, nt 3027–5000; 3AB, nt 5001–5291 and 3′NTR, nt 7416–7478) of HAV_{GP} and $HAV_{BS\text{-}C\text{-}1}$. The results, obtained with three independently isolated fragments for each region, revealed that HAV_{GP} has only two mutational changes as compared with $HAV_{BS\text{-}C\text{-}1}$ (Fig. 4). The sites mutated are at nucleotide position 3679 with an exchange from T to C, resulting in an exchange from Val to Ala at amino acid position 145 in protein 2B, and at nucleotide position 4419 with an exchange from T to C, resulting in an exchange from Tyr to His at amino acid position 141 in protein 2C. These data show that the 2B and 2C proteins, which are involved in HAV genome replication (Porter, 1993), are important not only for adaptation to growth in primate cells but also for adaptation to growth in the rodent GPE cells. Interestingly, the Val at amino acid position 215 in protein 2B (Ala in wild-type HAV), which results from a mutation at nucleotide 3889 and which is considered to be the main determinant for virus growth in cell culture (Emerson *et al.*, 1992; Tedeschi *et al.*, 1993), is not present in either HAV_{GP} or $HAV_{BS\text{-}C\text{-}1}$; $HAV_{BS\text{-}C\text{-}1}$ contains Glu at this position and this is preserved in HAV_{GP}. In the 5′NTR, which is demonstrated to be dominantly involved in the adaptation to growth of tc-HAV in distinct primate cell lines, no mutation occurred, although the host cell-specific growth pattern changed significantly.

The regions that were sequenced were chosen because they contain the determinants ascertained to be important for growth in primate cell cultures. We found that mutations in the viral proteins 2B and 2C proteins occurred during adjustment of HAV to a nonprimate cell line, as is also the case for adaptation to growth in cultured primate cells. We did not find an involvement of the 5′NTR sequence in the adaptation of HAV to growth in certain cell lines, which shows that this is not the only region responsible for providing host cell-specific information. A similar finding that changes in the sequence of the 5′NTR of HAV did not correlate with changes in the growth characteristics of HAV in certain cell lines is reported not for HAV strain HM175 but for strain GBM (Graff *et al.*, 1997). This finding supports our interpretation that there are different ways to improve HAV fit with a specific complement of host cell factors and that growth restrictions in defined host cells are not determined solely by regulatory interactions of cellular factors with 5′NTR sequences, which concerns mainly cap-independent translation initiation. As the viral proteins 2B and 2C, in which different combinations of mutations accumulate during adaptation to cultured cells, are considered to play a role during interaction with cellular membranes (Jecht *et al.*, 1998), HAV replication may be influenced by interactions with cell type-specific membrane components.

Adaptation of HAV to growth in certain host cells seems to be achieved by different sets of multiple interacting mutations. With regard to this, it might be difficult to identify marker mutations for growth in cell culture and therefore determinants for attenuation of HAV, which is connected with the adaptation of HAV to replicate in cultured cells (Funkhouser *et al.*, 1994, 1996). With regard to marker mutations, similar results were obtained when analysing the genotype of cytopathogenic tc-HAV variants, which represent highly cell culture adapted, rapidly replicating viruses (Brack *et al.*, 1998).

Therefore, we did not construct recombinants incorporating the observed changes to investigate whether the mutations seen in proteins 2B and 2C have any relation to the altered tropism because this would not lead to the identification of specific determinants for growth in tissue culture and attenuation of HAV. However, further studies addressing the involvement of the polymerase and the structural proteins in the adaptation of HAV to nonprimate cells will be required.

Finding that HAV can be adapted to growth in guinea pig tissue culture cells demonstrates that HAV replication is not restricted to primates. In the further course of our experiments we succeeded in infecting guinea pigs with HAV (Hornei *et al.*, in press). After intraperitoneal and oral infection of the animals, virus replication occurred, with shedding of the virus in stools and viraemia between weeks 3 and 7 p.i. The animals showed an active, clinically inapparent infection with specific histopathological changes of the liver. HAV RNA and histopathological alterations were detected not only in the liver, but also in extrahepatic tissues such as spleen, regional lymph nodes and intestinal tract. The guinea pig model (cell culture and animal) therefore seems of value for the investigation of mechanisms and determinants of HAV cell and tissue tropism and the examination of still unanswered questions of pathogenicity concerning the HAV hepatotropism and the primary extrahepatic site of HAV replication. The guinea pig model may also be valuable for the study of HAV virulence.

This work was supported by the Tönjes-Vagt-Stiftung, Bremen, Germany. We thank Mediagnost, Tübingen, Germany for providing the monoclonal anti-HAV antibody 7E7.

References

Brack, K., Frings, W., Dotzauer, A. & Vallbracht, A. (1998). A cytopathogenic, apoptosis-inducing variant of hepatitis A virus. *Journal of Virology* **72**, 3370–3376.

Brown, E. A., Day, S. P., Jansen, R. W. & Lemon, S. M. (1991). The 5′ nontranslated region of hepatitis A virus RNA: secondary structure and elements required for translation *in vitro*. *Journal of Virology* **65**, 5828–5838.

Chang, K. H., Brown, E. A. & Lemon, S. M. (1993). Cell type-specific proteins which interact with the 5′ nontranslated region of hepatitis A virus RNA. *Journal of Virology* **67**, 6716–6725.

Cromeans, T., Sobsey, M. D. & Fields, H. A. (1987). Development of a plaque assay for a cytopathic, rapidly replicating isolate of hepatitis A virus. *Journal of Medical Virology* **22**, 45–56.

Day, S. P., Murphy, P., Brown, E. A. & Lemon, S. M. (1992). Mutations within the 5′ nontranslated region of hepatitis A virus RNA which enhance replication in BS-C-1 cells. *Journal of Virology* **66**, 6533–6540.

Dotzauer, A., Feinstone, S. M. & Kaplan, G. (1994). Susceptibility of nonprimate cell lines to hepatitis A virus infection. *Journal of Virology* **68**, 6064–6068.

Emerson, S. U., McRill, C., Rosenblum, B., Feinstone, S. M. & Purcell, R. H. (1991). Mutations responsible for adaptation of hepatitis A virus to efficient growth in cell culture. *Journal of Virology* **65**, 4882–4886.

Emerson, S. U., Huang, Y. K., McRill, C., Lewis, M. & Purcell, R. H. (1992). Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. *Journal of Virology* **66**, 650–654.

Funkhouser, A. W., Purcell, R. H., D'Hondt, E. & Emerson, S. U. (1994). Attenuated hepatitis A virus: genetic determinants of adaptation to growth in MRC-5 cells. *Journal of Virology* **68**, 148–157.

Funkhouser, A. W., Raychaudhuri, G., Purcell, R. H., Govindarajan, S., Elkins, R. & Emerson, S. U. (1996). Progress toward the development of a genetically engineered attenuated hepatitis A virus vaccine. *Journal of Virology* **70**, 7948–7957.

Graff, J., Normann, A., Feinstone, S. M. & Flehmig, B. (1994). Nucleotide sequence of wild-type hepatitis A virus GBM in comparison to two cell culture-adapted variants. *Journal of Virology* **68**, 548–554.

Graff, J., Normann, A. & Flehmig, B. (1997). Influence of the 5′ noncoding region of hepatitis A virus strain GBM on its growth in different cell lines. *Journal of General Virology* **78**, 1841–1849.

Graff, J., Cha, J., Blyn, L. B. & Ehrenfeld, E. (1998). Interaction of poly(rC)-binding protein 2 with the 5′ noncoding region of hepatitis A virus RNA and its effects on translation. *Journal of Virology* **72**, 9668–9675.

Hornei, B., Kämmerer, R., Moubayed, P., Frings, W., Gauss-Müller, V. & Dotzauer, A. (2001). Experimental hepatitis A virus infection in guinea pigs. *Journal of Medical Virology* (in press).

Jansen, R. W., Newbold, J. E. & Lemon, S. M. (1988). Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for *in vitro* replication. *Virology* **163**, 299–307.

Jecht, M., Probst, C. & Gauss-Müller, V. (1998). Membrane permeability induced by hepatitis A virus proteins 2B and 2BC and proteolytic processing of HAV 2BC. *Virology* **252**, 218–227.

Lemon, S. M., Binn, L. N. & Marchwicki, R. H. (1983). Radioimmunofocus assay for quantitation of hepatitis A virus in cell cultures. *Journal of Clinical Microbiology* **17**, 834–839.

Morace, G., Pisani, G., Beneduce, F., Divizia, M. & Panà, A. (1993). Mutations in the 3A genomic region of two cytopathic strains of hepatitis A virus isolated in Italy. *Virus Research* **28**, 187–194.

Paul, A. V., Tada, H., von der Helm, K., Wissel, T., Kiehn, R., Wimmer, E. & Deinhardt, F. (1987). The entire nucleotide sequence of the genome of hepatitis A virus (isolate MBB). *Virus Research* **8**, 153–171.

Pestova, T. V., Hellen, C. U. T. & Shatsky, I. N. (1996). Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Molecular and Cellular Biology* **16**, 6859–6869.

Porter, A. G. (1993). Picornavirus nonstructural proteins: emerging roles in virus replication and inhibition of host cell functions. *Journal of Virology* **67**, 6917–6921.

Ross, B. C., Anderson, B. N., Edwards, P. C. & Gust, I. D. (1989). Nucleotide sequence of high-passaged hepatitis A virus strain HM175: comparison with wild-type and cell culture-adapted strains. *Journal of General Virology* **70**, 2805–2810.

Schultz, D. E., Hardin, C. C. & Lemon, S. M. (1996). Specific interaction of glyceraldehyde 3-phosphate dehydrogenase with the 5′-nontranslated RNA of hepatitis A virus. *Journal of Biological Chemistry* **271**, 14134–14142.

Siegl, G. & Lemon, S. M. (1990). Recent advancement in hepatitis A vaccine development. *Virus Research* **17**, 75–92.

Tedeschi, V., Purcell, R. H. & Emerson, S. U. (1993). Partial characterisation of hepatitis A viruses from three intermediate passage levels of a series resulting in adaptation to growth in cell culture and attenuation of virulence. *Journal of Medical Virology* **39**, 16–22.

Yi, M., Schultz, D. E. & Lemon, S. M. (2000). Functional significance of the interaction of hepatitis A virus RNA with glyceraldehyde 3-phosphate dehydrogenase (GAPDH): opposing effects of GAPDH and polypyrimidine tract binding protein on internal ribosome entry site function. *Journal of Virology* **74**, 6459–6468.

Zhang, H., Chao, S.-F., Ping, L.-H., Grace, K., Clarke, B. & Lemon, S. M. (1995). An infectious cDNA clone of a cytopathic hepatitis A virus: genomic regions associated with rapid replication and cytopathic effect. *Virology* **212**, 686–697.

Received 5 September 2000; Accepted 8 November 2000