

Influence of the 5' noncoding region of hepatitis A virus strain GBM on its growth in different cell lines

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Previous sequence analysis of consecutive passages of the hepatitis A virus (HAV) strain GBM/WT in human embryonic kidney cells (HEK cells), human embryonic lung fibroblasts (HFS cells) and in FRhK-4 cells (foetal rhesus monkey kidney cells) pointed to a host cell dependent cell culture adaptation of GBM/WT in HFS cells involving mutations in the 5' noncoding region (5'NCR). Multiple nucleotide changes occurred in the 5'NCR of the GBM genome after the cell line used for virus passage was changed from HEK cells to HFS cells. In contrast, no mutations in the 5'NCR occurred during the first 20 passages of GBM/WT in FRhK-4 cells. In order to analyse the influence of the 5'NCR on host cell specific adaptation of HAV strain GBM in different

cell cultures, GBM/HM175 chimeras were constructed which contained 5'NCRs from different GBM variants by replacing the 5'NCR of the infectious clone pHAV/7. Parallel transfection assays in FRhK-4 and HFS cells, performed with transcripts from the chimeric GBM/HM175 constructs, showed that the 5'NCR of the GBM variant GBM/HFS is essential for virus growth in HFS cells. The GBM/HM175 chimeric RNA, which contained the 5'NCR of GBM/HFS, exclusively, was able to produce infectious virus after transfection of HFS cells. The growth of the different GBM/HM175 chimeras in FRhK-4 cells, in contrast, did not seem to be strongly influenced by a specific sequence of the 5'NCR.

Introduction

Hepatitis A virus (HAV), one of the viruses that cause acute hepatitis in humans, is classified as the sole member of the genus *Hepatovirus*, within the family *Picornaviridae* (Francki *et al.*, 1991). The genome of HAV is composed of single-stranded positive-sense RNA approximately 7500 nt in length with a structural organization and gene order characteristic of picornaviruses. HAV RNA has a single open reading frame divided into regions P1, P2 and P3 (Rueckert, 1990). The 3' noncoding region is 63 nt long, followed by a poly(A) tract. The 5' noncoding region (5'NCR) comprises about 10% of the genome. Like the other picornaviruses, the HAV 5'NCR contains several AUG codons and is predicted to form extensive secondary structures (Brown *et al.*, 1991). The 5'

terminus is covalently linked to the viral protein VPg and lacks a 5' m⁷GpppN cap structure (Weitz *et al.*, 1986). Initiation of translation is thought to occur by direct binding of the 40S ribosomal subunit within the internal ribosome entry segment (IRES) of the 5'NCR close to the start codon (Brown *et al.*, 1991, 1994; Glass & Summers, 1992; Glass *et al.*, 1993). HAV can be adapted to growth in a variety of primate (for review, see Siegl & Lemon, 1990) and nonprimate cell substrates (Dotzauer *et al.*, 1994) which allowed studies of the molecular biology of HAV. In contrast to other picornaviruses, the replication cycle of HAV in cell culture is relatively slow and the yield of virus is relatively low. Wild-type HAV isolated from infected human hosts grows poorly in cell cultures. A minimum of 8 weeks elapses before primary HAV isolation can be achieved. After adaptation of the virus growth through multiple virus passages in cultured cells, the growth cycle of HAV is typically 7–14 days. With some exceptions (Anderson *et al.*, 1987; Cromeans *et al.*, 1989; Lemon *et al.*, 1991; Nasser *et al.*, 1987; Venuti *et al.*, 1985), HAV does not produce cytopathic effects in cell cultures; rather HAV establishes a persistent, non-lytic infection after prolonged incubation times with no observable inhibition of host-cell macromolecular

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synthesis. Little is known about the mechanism of adaptation of HAV and the cause of delayed and inefficient growth.

Through sequence analysis of different cell culture adapted HAV variants (Cohen *et al.*, 1987*b*; Graff *et al.*, 1994*a, b*; Jansen *et al.*, 1988; Lemon *et al.*, 1991; Morace *et al.*, 1993; Paul *et al.*, 1987; Ross *et al.*, 1989; Tedeschi *et al.*, 1993) and experiments with chimeric HAV containing sequences for wild-type and cell culture adapted strain HM175 (Emerson *et al.*, 1991; Funkhouser *et al.*, 1994; Zhang *et al.*, 1995), mutations within the 5'NCR and within the 2B and 2C coding regions of HAV that enhanced virus replication *in vitro* were identified. Whereas one mutation in 2B at nt 3889 was determined to be required for promoting growth in cell culture of a variety of cell culture adapted HAV strains, regardless of the cell line used, the sequence of the 5'NCR appears to be determinant of the specific host range of the virus (Emerson *et al.*, 1991, 1992; Funkhouser *et al.*, 1994; Graff *et al.*, 1994*b*). Wild-type HAV 5'NCR sequences produced poor growth of HAV in CV-1 cells (Emerson *et al.*, 1991), and differences in the *in vitro* growth of cell culture adapted HAV HM175p16 in BS-C-1 cells and FRhK-4 (foetal rhesus monkey kidney) cells or of the HM175 variant MRC5/9 in MRC5 cells and FRhK-4 cells were documented to be related to the sequence of the 5'NCR (Day *et al.*, 1990, 1992; Funkhouser *et al.*, 1994). Specific mutations within the 5'NCR of HM175p16 were described to be relevant in enhanced translation of the virus in BT7-H cells which showed no effect on viral translation in FRhK-T7 cells (Schultz *et al.*, 1996).

In previous studies of cell culture adaptation of the HAV strain GBM/WT, we showed that different mutations in the 5'NCR appeared during consecutive passages of virus, dependent on the cell line used (Graff *et al.*, 1994*b*). Multiple nucleotide changes occurred in the 5'NCR of the GBM genome after the cell line for virus passaging was changed from HEK cells (human embryonic kidney cells) to HFS cells (human embryonic lung fibroblasts). In contrast, no mutations in the 5'NCR occurred during the first 20 passages of GBM/WT in FRhK-4 cells. However, GBM/WT yields a host range variant after multiple passages in FRhK-4 cells. No replication of this variant, GBM/FRhK, could be detected in HFS cells within 15 days post-infection (Graff *et al.*, 1994*a*).

To investigate the importance of a specific 5'NCR sequence for virus growth in a particular cell line, we constructed different GBM/HM175 chimeras, which contained various mutations in the 5'NCR of GBM/WT within the background of the infectious full-length clone pHAV/7 (Cohen *et al.*, 1987*c*). In this report we show that the analysis of the growth of these GBM/HM175 chimeras in FRhK-4 cells and HFS cells supports the role of the 5'NCR of GBM in selective cell growth of HAV. Whereas the growth of HAV in FRhK-4 cells seems not to be strongly influenced by an altered nucleotide sequence of the 5'NCR, mutations at nt 153, 178, 646 and 687 in the 5'NCR were of great importance for growth of the GBM/HM175 chimeric virus in HFS cells.

Methods

■ **Plasmids.** Plasmid pHAV/7 (Cohen *et al.*, 1987*c*), which was kindly provided by S. U. Emerson (NIAID, Bethesda, MD, USA) is a transcription vector harbouring the full-length infectious cell culture adapted HAV strain HM175 cDNA under the control of the SP6 promoter. HM175 was originally adapted to grow in African green monkey kidney (AGMK) cells (Daemer *et al.*, 1981) but grows with similar efficiency in BS-C-1 and FRhK-4 cells. The subclone pGBM<25> contains the 5'NCR and a portion of the capsid protein coding sequence from nt 25 to 788 of the HAV wild-type strain GBM/WT (Graff *et al.*, 1994*a*) or cell culture adapted variants of this strain, namely GBM/HFS, GBM/Fp27, GBM(46, 107) and GBM(107, 646) generated from viral RNA by RT-PCR and inserted into the vector pGEM-3Zf(+) (Promega). The nucleotide sequences of all PCR generated GBM subclones were verified by sequence analysis throughout the entire PCR fragment. Compared to pGBM<25>WT four nucleotide changes are present in pGBM<25>HFS (A153G, A178G, G646T and T687G) (Graff *et al.*, 1994*a*). The plasmid pGBM<25>Fp27 contains one substitution at nt 646, G646T (Graff *et al.*, 1994*b*), pGBM<25>(46, 107) and pGBM<25>(107, 646) contain mutations at the positions indicated (A46G, T107G, G646T). The mutations A46G and T107G are PCR generated.

The subclone pGBM<3'> (Graff *et al.*, 1994*a*), used as a negative control of the transfection assay, encompassed the 3' end region of the HAV strain GBM/WT from nt 6700 to the poly(A) tail under control of the T7 promoter.

All plasmids were propagated by standard procedures using *E. coli* DH5 α and ampicillin selection (Sambrook *et al.*, 1989).

■ **Construction of chimeric GBM/HM175 cDNA genomes.** Standard recombinant DNA techniques were used for construction of the chimeric GBM/HM175 cDNA genomes (Sambrook *et al.*, 1989).

The infectious clone of HAV strain HM175, pHAV/7, was used for the construction of recombinant viruses GBM/HM175, since it has been well characterized previously (Cohen *et al.*, 1987*c*). The cell culture adapted variants of both HAV strains, GBM and HM175, show the same growth characteristics in FRhK-4 cells (data not shown).

The 5'NCR from nt 25 to 739 of pHAV/7 (*MroI*-*AflIII* fragment) was replaced with the homologous sequences derived from different subclones pGBM<25> of the HAV strain GBM, namely pGBM<25>HFS, pGBM<25>Fp27, pGBM<25>(46, 107) and pGBM<25>(107, 646). Each GBM *MroI*-*AflIII* fragment was used for ligation with the HM175/*AflIII*-*AccI* fragment (nt 740–2024) and the HM175/*AccI*-*MroI* fragment (nt 2025–24 including the vector pGEM-1) to generate the full-length GBM/HM175 chimeric plasmids – pCHMG_{WT}, pCHMG₆₄₆, pCHMG_{46,107}, pCHMG_{107,646} and pCHMG_{153,178,646,687}. The indices of the chimeric plasmids indicate the nucleotide positions of the mutations compared to the 5'NCR of GBM/WT. Competent *E. coli* DH5 α cells (Gibco BRL) were used for propagation of each plasmid. The chimeric plasmids were purified by Qiagen plasmid kit according to the manufacturer's directions (Qiagen).

Further chimeric GBM/HM175 cDNAs – pCHMG_{153,178}, pCHMG_{646,687}, pCHMG₁₀₇ and pCHMG_{107,646,687} – with single base changes from the 5'NCR of GBM/WT at nt 153 and 178, nt 646 and 687, nt 107 alone as well as nt 107, 646 and 687 were generated by substitution of the *MroI*-*HpaI* fragment (nt 25–354) of the plasmid pCHMG_{WT} or pCHMG_{153,178,646,687} with the equivalent fragment of the subclone pGBM<25>HFS, pGBM<25>WT or pGBM<25>(107, 646).

The appropriate nucleotide sequences of all generated chimeric plasmids were verified throughout the entire 5'NCR and the junctions of the religated fragments using Sequenase version 2.0 (US Biochemical) and [α -³⁵S]dATP according to the manufacturer's instructions. The

Table 1. 5'NCR mutations of the constructed GBM/HM175 chimeric plasmids

Nucleotide positions of the mutations in the chimeric plasmids in comparison to the wild-type sequence of GBM/WT are shown. Nucleotide numbering is based on the genome map of HM175 (Cohen *et al.*, 1987*a*). (1) The sequence from nt 25–739 is identical to the 5'NCR of GBM/WT (EMBL accession number X75215), which shows 96.4% identity to the 5'NCR of HM175. (2) The sequence from nt 25–739 is identical to the 5'NCR of GBM/HFS (EMBL accession number X75216).

Chimeric plasmid pcHMG _x	nt:	5'NCR mutations					
		46	107	153	178	646	687
1	pcHMG _{WT}	A	T	A	A	G	T
	pcHMG ₆₄₆	*	*	*	*	T	*
	pcHMG _{16,107}	G	G	*	*	*	*
	pcHMG _{107,646}	*	G	*	*	T	*
2	pcHMG _{153,178,646,687}	*	*	G	G	T	G
	pcHMG _{153,178}	*	*	G	G	*	*
	pcHMG _{646,687}	*	*	*	*	T	G
	pcHMG ₁₀₇	*	G	*	*	*	*
	pcHMG _{107,647,687}	*	G	*	*	T	G

* The nucleotide is identical to pcHMG_{WT}.

different single base mutations within the 5'NCR of GBM/WT of the constructed chimeras compared to GBM/WT are listed in Table 1.

In vitro transcriptions and RNA transfection assays. Purified plasmid DNAs of the GBM/HM175 chimeras were linearized using the restriction endonuclease *HaeII*, precipitated with ethanol and transcribed *in vitro* using a commercially available kit (Riboprobe System II, Promega). Run-off transcripts from 2 µg linearized plasmid were synthesized using 30 units of SP6 RNA polymerase in a 100 µl reaction volume for 2 h at 37 °C following the supplier's instructions. DNase digestion and RNA purification were omitted. Transcripts were analysed by agarose gel electrophoresis to confirm their integrity and size. The plasmid DNA of the clone pGBM<3'>, which is under the control of the T7 promoter, was linearized with *SalI* and transcribed *in vitro* using T7 RNA polymerase under the conditions described.

One half of the resulting transcription mixture was used for transfection of 25 cm² confluent monolayers of HFS or FRhK-4 cells in the presence of DEAE-dextran as described (Emerson *et al.*, 1991). Briefly, the cell monolayer was washed twice with PBS and incubated at 37 °C with 568 µl of transfection mixture, containing 50 µl of the transcription mixture, 490 µl PBS and 28 µl DEAE-dextran (10 µg/µl). After 30 min, 2 ml of MEM/Hanks (Gibco BRL) was added, and incubation was continued for 2 h at 37 °C. The DEAE-dextran mixture was removed, cell monolayers were washed twice with MEM/Hanks and 5 ml of fresh MEM/Hanks supplemented with 10% foetal calf serum (FCS, Gibco BRL) was added. The incubation was continued at 37 °C. Medium was changed to MEM/Earles with 3% FCS for long-term cell cultures after the cell monolayers became confluent. Transfection assay of HFS and FRhK-4 cells with each chimeric plasmid were performed at least in triplicate.

Monitoring of progeny HAV. Production of progeny HAV after transfection of HFS and FRhK-4 cells with *in vitro* transcribed GBM/HM175 chimeric RNA was monitored by HAV antigen-capture

PCR (AC-PCR) of the cell culture supernatant every third day and by indirect immunofluorescence microscopy of the transfected cells after 2–4 weeks.

An 80 µl aliquot of the supernatant of the transfected cells was subjected to AC-PCR as described previously (Graff *et al.*, 1993) using HAV specific oligonucleotide primers corresponding to nt 6700–6721 within the 3D coding region of the HAV genome (sense primer) and the poly(A) tail of the genome (antisense primer). A 10 µl aliquot of the amplified DNA product, yielding a 793 bp fragment, was analysed by 1% agarose gel electrophoresis.

In order to detect cell associated progeny HAV by indirect immunofluorescence microscopy, the transfected HFS and FRhK-4 cells were split 1:2 2 or 4 weeks post-transfection, seeded on microscope slides and allowed to grow overnight or until confluent. The cell monolayer was fixed with cold acetone and HAV was detected using human anti-HAV IgG positive serum and FITC-conjugated rabbit anti-human IgG as secondary antibody (Dako) as described previously (Flehmg, 1980).

Infection of HFS and FRhK-4 cells. In an attempt to ascertain the infectivity of the GBM/HM175 chimeric viruses (vpHMG_x) and vpHAV/7, produced by transfection of HFS and FRhK-4 cells with the different run-off transcripts of the pcHMG_x plasmids and of pHAV/7, AC-PCR positive cell culture supernatants were used to infect FRhK-4 and HFS cells. Briefly, confluent FRhK-4 and HFS cell monolayers (T25 flasks) were overlaid with a 1 ml aliquot of the AC-PCR positive cell culture supernatant derived from transfected cell monolayers and incubated for 2 h at 37 °C. After the incubation time the inoculum was removed and the cells were fed with MEM/Earles supplemented with 3% FCS. Production of progeny virus was again monitored by AC-PCR from the cell culture supernatant every second or third day as described above.

Using the conditions described, AC-PCR positive supernatant containing HAV GBM/Fp3, Fp5, Fp9, Fp20, Fp30 or Fp37, representing

GBM/WT passaged 3, 5, 9, 20, 30 or 37 times in FRhK-4 cells, were tested for infectivity in HFS cells.

Results

Mutations in the 5'NCR of GBM/WT continuously passaged in HFS cells

In previous experiments, wild-type virus GBM/WT was grown for eight passages in HEK cells, before changing the host cell to HFS cells. After a further three passages in HFS cells, mutations A153G, A178G, G646T and T687G were identified in the resulting virus, adapted to grow in HFS cells (Graff *et al.*, 1994b). To confirm that growth in HFS cells was the basis for these particular mutations wild-type virus GBM/WT was also passaged directly in HFS cells. GBM/WT propagated in HFS cells showed the same growth characteristics as virus passaged in HEK cells or FRhK-4 cells. Sequence analysis of the 5'NCR of the HFS-passaged virus demonstrated the identical mutations, A153G, A178G, G646T, T687G, after eight passages of GBM/WT in HFS cells as were detected after the cell change from HEK cells to HFS cells.

Analysis of transfection of FRhK-4 cells with GBM/HM175 chimeric plasmids

RNA transcribed *in vitro* from each of the nine constructed

GBM/HM175 chimeric plasmids as well as from pHAV/7 and the subclone pGBM<3'> was used to transfect FRhK-4 cell monolayers. The supernatants of the transfected cells were tested for progeny HAV every third day by AC-PCR over a time period of 28 days in FRhK-4 cells. The *in vitro* transcribed proviral RNA of pHAV/7 served as a positive control for the transfection assay and RNA transcribed from the subclone pGBM<3'>, comprising the same genome region amplified by AC-PCR in the detection assay, was used as negative control to exclude false positive results due to the detection of transfected RNA. The day of the first detectable progeny HAV in the supernatant of the transfected FRhK-4 cells investigated by AC-PCR with HAV specific oligonucleotide primers is indicated in Table 2. The first detection of HAV in FRhK-4 cells was obtained 10 days after transfection with RNA transcribed from pCHMG_{WT}, pCHMG_{646,687}, pCHMG_{107,646} as well as from pHAV/7. The amount of detectable HAV increased from 10 to 17 days post-transfection (Fig. 1). RNA from the chimeric plasmid pCHMG_{153,178,646,687} induced low amounts of detectable HAV in FRhK-4 cells after 14 days. RNA from the remaining chimeric plasmids – pCHMG₆₄₆, pCHMG_{153,178}, pCHMG_{46,107}, pCHMG₁₀₇ and pCHMG_{107,646,687} – did not generate detectable HAV within 28 days, even when AC-PCR was performed on total infected cell material, disrupted by three cycles of freeze-thawing, instead of supernatant (data

Table 2. Transfection of FRhK-4 and HFS cells with GBM/HM175 chimeric plasmids

AC-PCR and indirect immunofluorescence microscopy (IF) were used to determine HAV growth in FRhK-4 and HFS cells transfected with RNA transcribed *in vitro* from the GBM/HM175 chimeric plasmids, pHAV/7 and <pGBM3'>. Indirect IF was performed 14 days post-transfection for FRhK-4 cells and 28 days post-transfection for HFS cells.

GBM/HM175 chimera	Detection of HAV from transfected cells by:			
	AC-PCR*		Indirect IF	
	FRhK-4 cells	HFS cells	FRhK-4 cells	HFS cells
pCHMG _{WT}	10	-†	10%	10% -
pCHMG _{153,178,646,687}	14	22	2%	-
pCHMG _{646,687}	10	-	ND	5%
pCHMG ₆₄₆	-‡	-	-	-
pCHMG _{153,178}	-	-	-	-
pCHMG _{46,107}	-	-	-	-
pCHMG ₁₀₇	-	-	-	-
pCHMG _{107,646}	10	-	10%	-
pCHMG _{107,646,687}	-	-	-	-
pHAV/7	10	-	5%	-
Neg. ctr.: pGBM3'	-	-	ND	ND
Mock	-	-	-	-

* Days post-transfection of first detectable HAV.

† No detectable virus within 5 days post-transfection in HFS cells.

‡ No detectable virus within 28 days post-transfection in FRhK-4 cells.

ND, Not determined.

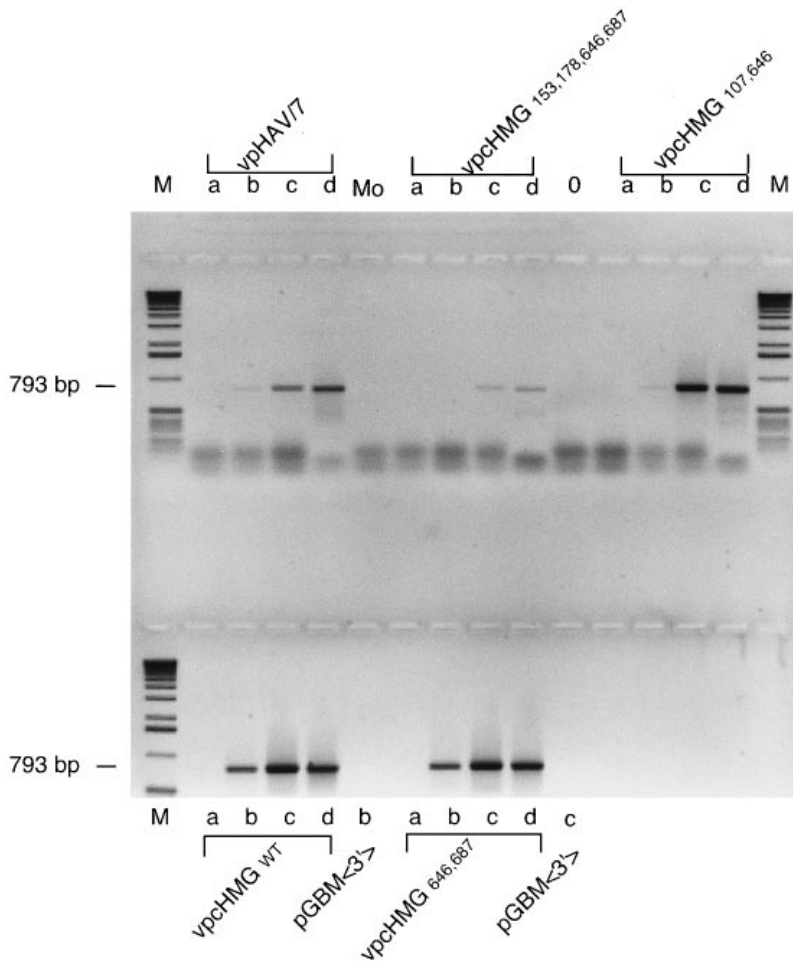


Fig. 1. Negative image of electrophoretic separation of AC-PCR amplified segments on a 1% agarose gel obtained from cell culture supernatant of transfected FRhK-4 cell monolayers. FRhK-4 cells were transfected with RNA transcribed *in vitro* from GBM/HM175 chimeric clones – pcHMG_x, pHAV/7 or the negative control pGBM<3'> as indicated in the figure. Cell culture supernatant was analysed by AC-PCR with HAV specific oligonucleotides 7 (a), 10 (b), 14 (c) and 17 (d) days post-transfection. The predicted size of the HAV specific AC-PCR amplified segments is 793 bp. Lane M was loaded with 1 kbp ladder DNA-marker (Gibco), lane Mo was loaded with the AC-PCR amplified segments of mock transfected FRhK-4 cell supernatant and lane O represents the water control of the AC-PCR.

not shown). In order to identify progeny HAV in FRhK-4 cells transfected with the GBM/HM175 chimera *in situ*, indirect immunofluorescence microscopy was performed 14 days after transfection. The percentage of HAV producing cells ranged from 2% to 10%. Consistent with the results obtained by AC-PCR, the chimera pcHMG_{153,178,646,687} showed the least efficient virus growth. None of the negative transfections, repeatedly determined by AC-PCR, showed detectable virus by indirect immunofluorescence microscopy.

Analysis of transfection of HFS cells with GBM/HM175 chimeric plasmids

RNA transcribed from each of the nine constructed GBM/HM175 chimeric plasmids as well as from pHAV/7 and the subclone pGBM<3'> were also used to transfect HFS cell monolayers. The supernatants of the transfected HFS cells were tested for progeny HAV every third to seventh day by AC-PCR over a time period of 5 days. The detection of progeny virus by AC-PCR of the supernatant and the indirect immunofluorescence microscopy results of the transfected HFS cells are also shown in Table 2. The first detection of HAV in

HFS cells was obtained 22 days after transfection with RNA transcribed from pcHMG_{153,178,646,687}. The indirect immunofluorescence microscopy of the HFS cells transfected with this chimera showed 5% of the cells containing HAV after 28 days. The plasmid pcHMG_{153,178,646,687} which contains the 5'NCR identical to the sequence of the cell culture adapted variant GBM/HFS (Graff *et al.*, 1994b), was the only construct which induced progeny virus after transfection of RNA into HFS cells. All other attempts to transfect HFS cells with RNA transcribed from the remaining eight GBM/HM175 chimeric plasmids or pHAV/7 did not yield detectable HAV over a time period of 5 days after transfection.

Infectivity of GBM/HM175 chimeric virus rescued after transfection of HFS and FRhK-4 cells

In order to verify the production of infectious progeny HAV, chimeric virus (vpCHMG_x), rescued from cell culture supernatant 17 days after transfection of FRhK-4 cell monolayers or 50 days after transfection of HFS cell monolayers with infectious RNAs derived from the GBM/HM175 chimeric plasmids and from pHAV/7 was used to infect parallel cultures

Table 3. Infectivity of GBM/HM175 chimeric viruses

Cell culture supernatant of transfected FRhK-4 (17 days post-transfection) or HFS cells (50 days post-transfection), determined HAV positive by AC-PCR, was used to infect FRhK-4 and HFS cells. Determination of successfully infected cells was by AC-PCR.

Rescued virus used for infection	Infection of:	
	FRhK-4 cells	HFS cells
FRhK-4- vpcHMG _{WT}	Positive	Negative*
FRhK-4- vpcHMG _{153,178,646,687}	Positive	Positive
HFS- vpcHMG _{153,178,646,687}	Positive	Positive
FRhK-4- vpcHMG _{646,687}	Positive	Negative
FRhK-4- vpcHMG _{107,646}	Positive	Negative
FRhK-4- vpHAV/7	Positive	Negative
Mock	Negative	Negative

* No detectable virus within 40 days post-infection.

of HFS and FRhK-4 cell monolayers. Table 3 shows the results of the infections of the FRhK-4 and HFS cells detected by AC-PCR with HAV specific oligonucleotides. The supernatant of the infected cells was investigated every fourth day until 40 days post-infection. HAV was detectable in FRhK-4 cells after 8–20 days post-infection with all of the investigated GBM/HM175 chimeras and vpHAV/7. Since no positive result was obtained by AC-PCR by the fourth day post-infection, detection of inoculated HAV could be excluded. Only progeny virus was detectable by this method. The infection of HFS cell monolayers with the GBM/HM175 chimeras, in contrast, was only successful with vpcHMG_{153,178,646,687}, the chimeric virus possessing the 5'NCR derived from the HFS cell culture adapted variant GBM/HFS, confirming that only transfection of HFS cells with RNA transcribed from pchMG_{153,178,646,687} permitted HAV growth. Also the cell culture adapted virus of HM175, which was successfully obtained from transfection of FRhK-4 cells with pHAV/7 derived RNA (vpHAV/7), was only able to infect FRhK-4 cells. No virus could be detected in HFS cell culture supernatant or in cell associated preparations within 40 days post-infection by AC-PCR or indirect immunofluorescence microscopy.

Infection of HFS cells with FRhK-4 cell culture adapted virus

The biological characterization of the cell culture adapted HAV GBM/FRhK, which was attained after propagation in FRhK-4 cells for 63 passages, had exhibited a restriction in its growth in HFS cells (Graff *et al.*, 1994a). No replication was observed in HFS cells during 15 days, whereas virus is detectable after 7–10 days if GBM/FRhK is propagated in

FRhK-4 cells or if the HFS cell culture adapted virus GBM/HFS is propagated in either HFS or FRhK-4 cells. In order to evaluate whether this host-range behaviour is related to mutations in the entire genome which appeared during high passage cell culture adaptation of the HAV strain GBM/WT or to a sequence of the 5'NCR specifically compatible with HFS cells different early passages of GBM/WT propagated in FRhK-4 cells were examined for their infectivity of HFS cells. The infections were performed in five parallel cultures for each virus. Virus passage GBM/Fp3, Fp5, Fp9, Fp20, Fp30 and Fp37 were selected since they had developed new nucleotide mutations in different regions of the genome (Graff *et al.*, 1994b). None of the virus passages has a 5'NCR sequence identical to GBM/HFS. The infectivity of each virus was determined by AC-PCR of the HFS cell culture supernatant every week until week 22. Cell associated virus was also examined every second or third week, when the cells were subcultured. Detectable virus from infected HFS cells could only be determined after an incubation time longer than 100 days. This long incubation time is comparable to an infection of FRhK-4, HEK and HFS cell cultures with wild-type GBM/WT and not with cell culture adapted virus, which replicated within 7–14 days. Thus, pointing to the importance of the appropriate sequence of the 5'NCR for a HFS cell specific growth of HAV strain GBM.

Discussion

Investigations of the 5'NCR of HAV strain HM175 showed that this genome region is involved in regulatory mechanisms of the cell culture adaptation of HAV and its translation (Brown *et al.*, 1991; Chang *et al.*, 1993; Day *et al.*, 1990, 1992; Emerson *et al.*, 1991; Funkhouser *et al.*, 1994; Glass & Summers, 1992; Glass *et al.*, 1993; Schultz *et al.*, 1996; Shaffer *et al.*, 1994, 1995; Whetter *et al.*, 1994). The initiation of viral translation of HAV is by a cap independent mechanism, which is controlled by an internal ribosome entry segment (IRES) within the 5'NCR as has been shown for other picornaviruses. The IRES of HAV is localized in the 5'NCR between nt 152 and 734 (Brown *et al.*, 1994; Glass *et al.*, 1993).

Previous sequence analysis of the HAV strain GBM/WT and consecutive virus passages in HFS and FRhK-4 cells compared with the growth characteristics of the wild-type virus and the cell culture adapted variants showed that the 5'NCR of this HAV strain is influenced by the cell line used (Graff *et al.*, 1994b). After the switch from HEK cells as the host cell to HFS cells during consecutive passaging of GBM/WT, several mutations in the 5'NCR occurred which could be correlated with a change in the growth behaviour of the virus. These mutations were also obtained if GBM/WT was passaged only in HFS cells. In the case of GBM/WT passaged exclusively in FRhK-4 cells, however, no changes in the sequence of the 5'NCR correlated with changes in growth characteristics. Based on these observations we constructed

GBM/HM175 chimeric plasmids which contained various mutations in the 5'NCR of GBM/WT in the background of the infectious full-length clone pHAV/7 (Cohen *et al.*, 1987c).

The transfection studies with the chimeric viral RNA showed that changes in the sequence of the 5'NCR of HAV strain GBM concerning the growth of HAV in FRhK-4 cells did not play an important role. Transfection of FRhK-4 cells with the GBM/HM175 chimeric RNA possessing the wild-type 5'NCR sequence of GBM/WT or the 5'NCR sequence of the cell culture adapted variant GBM/HFS (with mutations at nt 153, 178, 646 and 687) as well as from the chimeras pCHMG_{646,687} and pCHMG_{107,646} resulted in viable, infectious virus as did the transcripts derived from pHAV/7 in FRhK-4 cells. These results indicate that the infection was not inhibited by a variety of alterations within the 5'NCR sequence. However, some of the generated mutations had an effect on HAV growth in FRhK-4 cells. Surprisingly, the single mutation at position 646 did not yield viable virus in FRhK-4 cells, indicating that this mutation alone interfered with detectable production of progeny virus in the background of the pHAV/7 sequence in contrast to the homologous virus GBM/Fp27. The viability of a chimeric virus with a mutation at nt 646 (G to U) could be rescued with one additional mutation at nt 107, but not with a total of three mutations at nt 107, 646 and 687. When the mutations in the 5'NCR were only located upstream or at the 5' end of the IRES as in the GBM/HM175 chimeras pCHMG_{46,107}, pCHMG₁₀₇ and pCHMG_{153,178}, no viable virus was produced in FRhK-4 cells.

Investigations by Carneiro *et al.* (1995) with bicistronic constructs harbouring different mutations in the first pyrimidine-rich tract of the HAV 5'NCR showed that this tract is involved in internal initiation of translation in COS-7 cells. Shaffer *et al.* (1994, 1995) showed that deletion mutations in the first pyrimidine-rich tract affect translation efficiency and that sequences downstream of the pyrimidine-rich tract between nt 140–144 are critical for viral RNA replication in BS-C-1 and FRhK-4 cells.

In contrast to the results obtained by the transfections of FRhK-4 cells, transfection of HFS cells with the GBM/HM175 chimeras were strongly influenced by the 5'NCR sequence. Only chimeric viruses possessing the 5'NCR derived from the cell culture adapted variant GBM/HFS were consistently infectious in HFS cells. Transfection of HFS cells with transcripts derived from any of the other constructed GBM/HM175 chimeric plasmids or from pHAV/7 did not permit productive HAV infection. In addition, any infection of HFS cells with rescued chimeric virus different from vpCHMG_{153,178,646,687} derived successfully from FRhK-4 cells, failed.

Chimeric studies with the HAV strain HM175 passaged in AGMK cells and MRC-5 cells, a human lung fibroblast strain like HFS cells, revealed similar results. Funkhouser *et al.* (1994) showed that mutations in the 5'NCR which occur during virus passage in MRC-5 cells are required for virus growth in these

cells, whereas the 5'NCR sequence for growth in FRhK-4 cells is not as important. A mutation in 2C of the cell culture adapted virus MRC-5/9 in addition to the MRC-5 specific 5'NCR sequence improved the growth of the chimeric constructs. Two of the 5'NCR mutations of MRC-5/9 are at identical nucleotide positions, 646 and 687, as identified in the HFS cell culture adapted variant of the GBM virus.

Schultz *et al.* (1996) described a cell-type specific effect for translation of HAV. Translation of HM175p16 was enhanced in BT7-H cells by an UU deletion at nt 203 and 204 and a U to G substitution at nt 687, whereas these mutations did not affect translation in FRhK-T7 cells. The U687G substitution is one of the mutations in the 5'NCR of HAV strain GBM/WT which is important for virus growth in HFS cells.

Consistent with the results of the GBM/HM175 chimeras are the data obtained from infection of HFS cells with FRhK-4 cell culture adapted homologous HAV – GBM/Fp3, Fp5, Fp9, Fp20, Fp30 and Fp37. These FRhK-4 cell culture adapted viruses, which possess 5'NCR sequences different from GBM/HFS, are also restricted in their growth in HFS cells. None of these FRhK-4 cell derived GBM variants replicates in HFS cells better than wild-type HAV.

The different cell tropism of HAV for FRhK-4 and HFS cells indicates that specific host factors might control the outcome of infection. An unknown host factor which is essential for correct translation of the viral genome could be missing in the case of the HFS cells. Another hypothesis is that an additional host factor in HFS cells blocks a binding site necessary for translation initiation. Different cell lines seem to vary with respect to their complement of ribosome-associated proteins that bind to the 5'NCR of HAV. Chang *et al.* (1993) identified three proteins, p30, p39 and p110, from ribosomal salt wash prepared from BS-C-1 and FRhK-4 cells, which bound to multiple sites within the 5'NCR of the HAV strain HM175 by UV cross-linking. On the other hand p110 and p57 (PTB) were cross-linked to HAV 5'NCR in HeLa cell extracts or rabbit reticulocyte lysates. The proteins p30 and p39 were specifically associated with BS-C-1 and FRhK-4 cells.

The inability of HAV to adapt to growth in HFS cells without the appropriate sequence of the 5'NCR could also be caused by inefficient viral RNA replication. In the case of poliovirus, it was shown that the first 88 nt of the 5'NCR are necessary for RNA replication (Andino *et al.*, 1993). Even certain regions of the IRES of poliovirus seem to be involved in viral RNA synthesis as shown by Borman *et al.* (1994). Further studies addressing the involvement of the 5'NCR in cell tropism of HAV GBM on the level of translation or replication and of HAV specific host cell factors for translation initiation will be required.

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