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Nucleotide Sequence of Wild-Type Hepatitis A Virus GBM in Comparison with Two Cell Culture-Adapted Variants

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In order to study cell tropism and attenuation of hepatitis A virus (HAV), the genome of HAV wild-type GBM and two cell culture-adapted variants, GBM/FRhK and GBM/HFS, were cloned and sequenced after amplification by reverse transcriptase-PCR. During virus cultivation, the HAV variant GBM/FRhK had a strict host range for FRhK-4 cells, in contrast to GBM/HFS, which can be grown in HFS and FRhK-4 cells. The HAV variant GBM/HFS was shown to be attenuated when inoculated into chimpanzees (B. Flehmig, R. F. Mauler, G. Noll, E. Weinmann, and J. P. Gregerson, p. 87–90, *in* A. Zuckerman, ed., *Viral Hepatitis and Liver Disease*, 1988). On the basis of this biological background, the comparison of the nucleotide sequences of these three HAV GBM variants should elucidate differences which may be of importance for cell tropism and attenuation. The comparison of the genome between the GBM wild type and HAV wild types HM175 (J. I. Cohen, J. R. Ticehurst, R. H. Purcell, A. Buckler-White, and B. M. Baroudy, J. Virol. 61:50–59, 1987) and HAV-LA (R. Najarian, O. Caput, W. Gee, S. J. Potter, A. Renard, J. Merryweather, G. Van Nest, and D. Dina, Proc. Natl. Acad. Sci. USA 82:2627–2631, 1985) showed a 92 to 96.3% identity, whereas the identity was 99.3 to 99.6% between the GBM variants. Nucleotide differences between the wild-type and the cell culture-adapted variants, which were identical in both cell culture-adapted GBM variants, were localized in the 5′ noncoding region; in 2B, 3B, and 3D; and in the 3′ noncoding region. Our result concerning the 2B/2C region confirms a mutation at position 3889 (C→T, alanine to valine), which had been shown to be of importance for cell culture adaptation (S. U. Emerson, C. McRill, B. Rosenblum, S. M. Feinstone, and R. H. Purcell, J. Virol. 65:4882–4886, 1991; S. U. Emerson, Y. K. Huang, C. McRill, M. Lewis, and R. H. Purcell, J. Virol. 66:650–654, 1992), whereas other mutations differ from published HAV sequence data and may be cell specific. Further comparison of the two cell culture-adapted GBM variants showed cell-specific mutations resulting in deletions of six amino acids in the VP1 region and three amino acids in the 3A region of the GBM variant GBM/FRhK.

Human isolates of hepatitis A virus (HAV) possess a single serotype, but more than one genotype of HAV is responsible for HAV infection in different parts of the world (31). These genotypic differences may result in different biologic properties of the virus, some of which can be measured, such as in vitro growth characteristics or virulence differences.

HAV is classified as a member of the family *Picornaviridae* under the new genus *Hepatovirus* (24). Like other picornaviruses, HAV is a nonenveloped particle and contains a single-stranded plus-sense RNA genome of 7,478 nucleotides with a long 5′ noncoding region (5′NCR) comprising approximately 10% of the genome and terminating at the 3′ end in a poly(A) tail. The genome contains a single long open reading frame that codes for a polyprotein of 2,227 amino acids (6). Although the genome organization is similar to those of other picornaviruses, there is little identity to other picornaviruses at the nucleotide or amino acid sequence level. The growth characteristics of HAV are drastically different from those of other picornaviruses. Wild-type HAV grows poorly in cell culture, does not cause cytopathic effects, and tends to establish persistent infection only after prolonged incubation times in which the cells shed low amounts of virus (9, 13, 22, 29, 35, 37). Although HAV can be adapted to a variety of cell culture substrates such as human kidney, fetal rhesus monkey kidney, African green monkey kidney, and human fibroblasts, adapted HAV still grows relatively slowly in tissue culture, requiring 1 to 2 weeks to reach maximum titers. Only a few cell culture-adapted HAV variants which replicate rapidly and cause cytopathic effects in vitro have been isolated (1, 7, 36).

The molecular basis of adaptation resulting in an improvement in the rate of viral growth from more than 2 months to less than 2 weeks as well as attenuation of HAV virulence is still not completely understood. In this study we analyzed and compared the nucleotide sequence of wild-type HAV strain GBM with the sequences obtained after adaptation of this strain to FRhK-4 cells (fetal rhesus monkey kidney-derived cells) or HFS cells (human lung fibroblast cells). The two GBM cell culture-adapted variants, GBM/FRhK and GBM/HFS, differ in their biologic characteristics. While GBM/FRhK showed a strict host range for FRhK-4 cells, GBM/HFS replicated in both cell types. Furthermore, GBM/HFS has been shown to be attenuated after inoculation into chimpanzees (16). GBM/FRhK has not been investigated for attenuation. Comparison of the biological characteristics and the nucleotide sequences of the GBM variants may reveal genome regions important for cell tropism and attenuation.

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Virus samples. HAV strain GBM was recovered from a human fecal specimen, collected in the preicteric phase of the infection (14), and is named GBM wild type (GBM/WT) in this paper. The strain has been adapted for growth in HFS and/or FRhK-4 cells with a selection procedure for rapid growth and release of virus into cell culture supernatant. Two GBM variants, GBM/HFS and GBM/FRhK, were attained after cell

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culture propagation. GBM/HFS was propagated eight times in human embryonic kidney cells and then passaged 23 times in HFS cells and is used as an inactivated vaccine (15). GBM/FRhK was isolated and propagated in FRhK-4 cells (13) and after 63 passages exhibited a strict host range behavior. No replication was observed in HFS cells. The isolation and passage history of HAV strain GBM have been previously described (13–15, 19).

Growth of GBM/FRhK and GBM/HFS in different cell cultures. The growth of the two cell culture-adapted GBM variants was tested on FRhK-4 and HFS cells for the presence of HAV released into the supernatant by antigen radioimmunoassay (Ag-RIA) by the method of Flehmig et al. (17), and the infectivity was measured by the method of Vallbracht et al. (35). Fourteen cell culture flasks (25 cm^2 each) of confluent monolayer cultures of both cell types were each infected with 1 ml of cell culture supernatant containing $10^{4.15}$ 100% tissue culture infective doses per ml of either GBM/FRhK or GBM/HFS, which corresponds approximately to a multiplicity of infection of 0.05 to 0.1. The cell culture supernatant was tested for released HAV each day over a period of 14 days after inoculation by Ag-RIA (Fig. 1A). Figure 1B shows the growth characteristics of the cell culture-adapted variants GBM/HFS and GBM/FRhK in HFS and FRhK-4 cells, expressed as 100% tissue culture infective doses per 100 μl of cell-associated virus over the same period. Figure 1 illustrates that the variant GBM/HFS replicated well in HFS cells, as detected by Ag release in the supernatant and high infectivity titer of cell-associated virus. Replication of GBM/HFS was not as efficient in FRhK-4 cells as in HFS cells, as shown in Fig. 1A. The presence of HAV released into the supernatant of FRhK-4 cells showed only a borderline positive result. Cell-associated virus, however, was detectable in both cell types with only minor differences (Fig. 1B). The variant GBM/FRhK, in contrast, showed optimal growth only during cultivation in FRhK-4 cells, whereas no detectable replication of this variant could be obtained in HFS cells. Thus, the variant GBM/FRhK has a known host range limited to FRhK-4 cells.

Purification of virus particles and RNA extraction. The cell culture-adapted HAV variants GBM/HFS and GBM/FRhK were propagated in HFS or FRhK-4 cells at 37°C in cell factories (Nunc, Copenhagen, Denmark) for mass production. The infected cells were lysed, and the virus particles were separated over cesium chloride gradient centrifugation as described by Heinricy et al. (19). Fractions were collected from the top of the tube and dialyzed against phosphate-buffered saline (PBS). The protein-containing fractions with a buoyant density of around 1.32 g/cm^3 were assayed for HAV antigen by Ag-RIA. Peak fractions of the antigen were pooled and treated with 100 μg of proteinase K per ml in a buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM NaCl, and 1% sodium dodecyl sulfate for 3 h at 37°C. Viral RNA was extracted twice with phenol-chloroform and then ethanol precipitated. After centrifugation at 12,000 × *g* for 30 min, the pellet was resuspended in TE (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) and used directly for assays or stored at −70°C. An aliquot of the isolated RNA was identified by slot blot hybridization with a radiolabelled HAV cDNA probe, which corresponded to the VP1 region. The HAV fragment was isolated from the plasmid $pHAV_{1.B}1307$ (34), which was kindly provided by J. Ticehurst (Walter Reed Army Institute of Research, Washington, D.C.).

Wild-type RNA was extracted from a 50-μl aliquot of the stool suspension (10% in PBS) containing HAV strain GBM/WT with guanidinium isothiocyanate by the method by Chirgwin et al. (4). After phenol-chloroform extraction and ethanol precipitation, the RNA was resuspended in TE and used directly for reverse transcriptase (RT)-PCR or stored at −70°C.

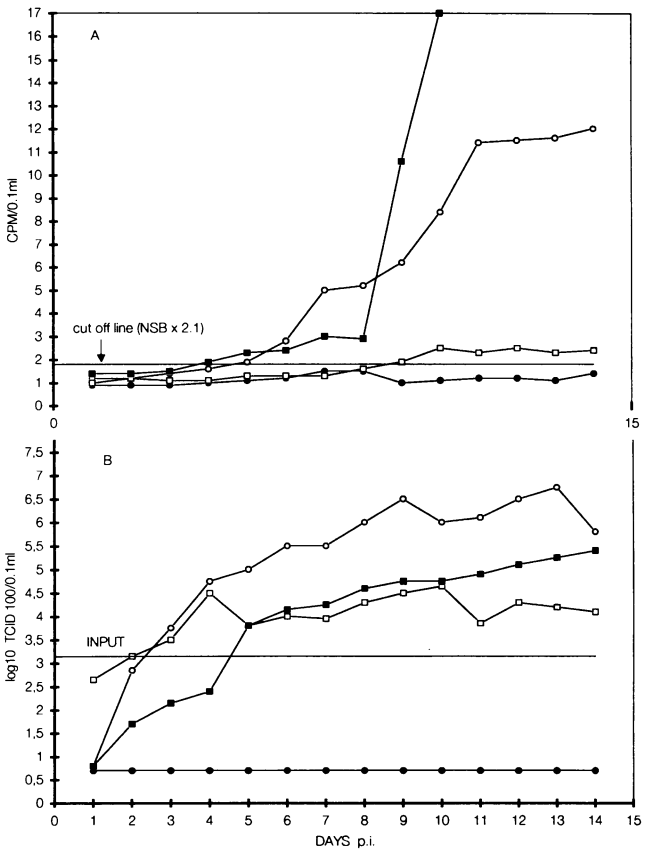

FIG. 1. Growth behavior of the two cell culture-adapted GBM variants in different cell lines over a period of 14 days. Detection of HAV is shown by GBM/FRhK propagated in FRhK-4 (○) and HFS cells (●) and by GBM/HFS propagated in FRhK-4 (□) and HFS cells (■). (A) HAV released in cell culture supernatant was detected by Ag-RIA. Samples were considered positive if the counts per minute measured were greater than the counts per minute of anti-HAV-negative serum (NSB) × 2.1 (Cutoff line = counts per minute of anti-HAV negative serum × 2.1). (B) Viral replication was determined by detection of infectivity measured in 100% tissue culture infective doses (TCID 100)/0.1 ml of cell-associated viruses. GBM/HFS shows a normal growth in both cell types, whereas GBM/FRhK replicates only in FRhK-4 cells (input = amount of infectious virus used as an inoculum). p.i., postinfection.

Amplification of HAV RNA. Enzymatic amplification by RT-PCR of the isolated HAV RNA was performed to obtain the desired fragments in amounts adequate for cloning and sequencing. The oligonucleotide primers necessary for these amplifications were designed to produce subgenomic overlapping HAV fragments, with an average size of 1,200 bp, which cover the entire HAV genome. The primers were corresponding to the nucleotide sequence of HAV strain HM175 (6) or to the successive available sequence of the GBM strains (Table 1). The isolated RNA was reverse transcribed into cDNA and then amplified in a single-step assay mixture (100 μl) including 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 0.25 mM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia); 300 nM positive-sense primer; 300 nM negative-sense primer; 40 U of RNase inhibitor (RNasin;

TABLE 1. Genome positions of the oligonucleotides primers and reaction conditions for amplification of HAV cDNA of the GBM variants

Clone[a]	Viral primers[b]	Position (nucleotides)[c]	Predicted size (bp) of PCR product	Annealing temp (°C)	Extention time (min)
<25>	Pos	25–46	763	55	1
	Neg	747–788			
<22>	Pos	719–735	772	55	1
	Neg	1470–1491			
<23>	Pos	1431–1451	1,313	55	2
	Neg	2724–2744			
<4>	Pos	2500–2521	1,200	50	2
	Neg	3679–3700			
<24>	Pos	3540–3570	1,500	50	2
	Neg	5020–5040			
<6>	Pos	4600–4621	1,200	55	2
	Neg	5779–5800			
<21>	Pos	5270–5292	2,150	50	3
	Neg	7399–7400			
<3′>	Pos	6700–6721	792	55	1
	Neg	Poly(A)			

[a] Eight overlapping subclones of the entire genomes of GBM/WT and of the cell culture-adapted variants, GBM/FRhK and GBM/HFS, could be obtained by RT-PCR following molecular cloning, except for the first 45 nucleotides of the HAV genome.

[b] The viral primer set for clone <25> was kindly provided by S. U. Emerson, National Institute of Allergy and Infectious Diseases, Bethesda, Md. The sequence of the viral primer set for clone <24> is complementary to GBM/HAV. All other primer sequences are complementary to HM175 (6). Pos, positive sense; Neg, negative sense.

[c] Nucleotide numbering is based on the genome map of HM175 (6).

TABLE 2. Percent nucleotide and amino acid identity of GBM/WT to wild-type HAV strain HM175 and HAV-LA

Genomic region	% Nucleotide identity		% Amino acid identity	
	HM175	HAV-LA	HM175	HAV-LA
5′NCR (bases 46–734)	96.4	99.0		
VP4	92.6	100.0	95.7	100.0
VP2	95.8	96.4	99.1	99.5
VP3	88.1	97.2	99.6	100.0
VP1	91.0	94.4	99.3	95.0
Total P1	91.5	96.0	99.2	98.0
2A	90.1	95.9	98.4	98.5
2B	92.5	95.9	100.0	100.0
2C	89.3	96.2	97.9	99.7
Total P2	90.1	96.1	98.4	99.4
3A	93.7	96.4	97.3	97.3
3B	89.9	97.1	100.0	100.0
3C	92.1	96.5	100.0	100.0
3D	92.8	96.3	98.6	99.4
Total P3	92.6	96.4	98.9	99.4
3′NCR	96.9	87.3		
Total genome (bases 46–7478)	92.0	96.3	97.8	98.9

Promega Corp.); 5 U of RT from avian myoblastosis virus (Promega Corp.); and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was incubated at 43°C for 30 min for reverse transcription and then subjected to 30 automated cycles of denaturation at 94°C for 1 min, annealing at 50 or 55°C for 1 min, and extention at 72°C for 1 to 3 min, depending on the melting temperature of the primer and the length of the target region (Table 1). To amplify the 5′NCR of HAV, 5 μl of dimethyl sulfoxide was added to the reaction mixture to reduce the secondary structure of this genome region. A 10-μl aliquot of each PCR product was evaluated by gel electrophoresis through 1% agarose (SeaKem GTG; FMC Bioproducts) in Tris acetate-EDTA (TAE) buffer and visualized under UV light after being stained with ethidium bromide to analyze the PCR product.

Molecular cloning of subgenomic HAV fragments and sequence analysis. The PCR products were separated by electrophoresis through 1 to 2% SeaPlaque low-melting-point agarose (FMC Bioproducts) in TAE buffer. The DNA, representing the exact molecular weight, was recovered and purified from the gel by Spin Bind DNA extraction units (BIOzym Diagnostic) by the protocol of the manufacturer. The purified DNA was treated with the Klenow fragment of DNA polymerase (Boehringer Mannheim) for 1 h at 16°C to create uniformly blunt ends and then subjected to phosphorylation of the 5′ end with T4 polynucleotide kinase (Boehringer Mannheim) for 1 h at 37°C. This fragment was ligated into the *Sma*I site of the vector pGEM3Zf+ (Promega Corp.) with T4 DNA ligase (Boehringer Mannheim) overnight at 4°C (23). The Klenow and kinase reactions were each terminated by chloroform extraction, precipitated with ethanol, and redissolved in double-distilled water. The resulting recombinant plasmids were used to transform competent DH5α cells (GIBCO/BRL). Plasmid DNA (1 μg) with an insert of the predicted size obtained from the PCR-generated clones was sequenced after minipreparation (2) and alkali denaturation by the dideoxynucleotide chain termination method (33) with the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio) in the presence of [^{35}S]dATP (Amersham). Oligonucleotide primers specific for HAV and primers corresponding to the T7 and Sp6 promoter region of the plasmid were used to sequence the inserted HAV fragment. To eliminate the possibility of errors in the sequence due to *Taq* polymerase used for PCR, at least three individual clones of each amplified fragment, derived from two individual PCR products, were sequenced. Nucleotide separation was performed on a 6% acrylamide gel containing 8 M urea in the presence of 1× TBE (90 mM Tris-borate, 90 mM boric acid, 2 mM EDTA) at 1,500 V. After exposure overnight, the nucleotide sequences were visualized by autoradiography.

Analysis, alignment and translation in the amino acids of the obtained nucleic acid sequences were done by using the sequence analysis computer program Gene Jockey (Biosoft).

Sequence comparison between GBM/WT and other wild-type strains of HAV. The nearly complete nucleotide and predicted amino acid sequences of GBM/WT, except for the first 45 nucleotides of the HAV genome, have been compared with those of two wild-type strains, HM175 and HAV-LA (Table 2). The predicted junctions of the genome encoding each structural and nonstructural protein correspond to the prediction described by Cohen et al. (6). Nucleotide identity between the three strains is 92% for GBM/WT versus HM175 and 96.3% for GBM/WT versus HAV-LA, whereas the predicted amino acid identity was 97.8% for GBM/WT versus

HM175 and 98.9% for GBM/WT versus HAV-LA. Among the noncoding regions, GBM/WT shows a nucleotide diversity in the 5′NCR compared with that of HM175 of 96.4%, whereas the sequences of HAV-LA and GBM/WT are very similar, with 99% identity. The nucleotide sequence of the 3′NCR, in contrast, shows a higher diversity between GBM/WT and HAV-LA (88.9%) than between GBM/WT and HM175 (96.9%). A 100% identity of the predicted amino acid sequences was found in the region coding for the capsid proteins VP4 and VP3 between GBM/WT and HAV-LA. Among the nonstructural proteins, GBM/WT has a 100% identity of the predicted amino acids in 2B, 3B, and 3C compared with those of HM175 and HAV-LA. The comparisons have shown that the only region with identical nucleotide sequence between GBM/WT and HAV-LA was the region coding for VP4.

Sequence comparison between GBM/WT and GBM/FRhK. The genome sequence of the host range variant GBM/FRhK showed a total of 56 nucleotide mutations compared with that of GBM/WT (Table 3) which correspond to an identity of 99.3% among these strains. Eight of the mutations occur in the 5′NCR and one occurs in the 3′NCR. Of 48 nucleotide mutations in the coding region, 15 have been determined to be nonsilent. The resulting 13 amino acid changes are localized in VP1 (Met→Ile and Asp→His), 2B (Ile→Val and Ala→Val), 2C (Lys→Gln, Lys→Thr, and Asn→Ser), 3A (Gly→Ala), 3B (His→Tyr), 3C (Gly→Asp and Thr→Ser), and 3D (Thr→Ser and Cys→Tyr). In addition, two in-frame deletions occur in GBM/FRhK in comparison to the sequence of GBM/WT. One of the deletions is localized in VP1 at nucleotide positions 2283 to 2301, resulting in the loss of six amino acids. The other deletion is a three-amino-acid deletion near the amino terminus of 3A. Only 5 nucleotide mutations of 56 in total are silent mutations.

Sequence comparison between GBM/WT and GBM/HFS. The cell culture-adapted and attenuated variant GBM/HFS shows fewer nucleotide mutations from GBM/WT than the host range variant GBM/FRhK. A total of 27 nucleotide mutations (99.6% identity to GBM/WT) have been determined (Table 3). Four of them occur in the 5′NCR, 2 occur in the 3′NCR, and 10 are silent mutations in the coding region. The 11 nonsilent mutations were found in VP1 (Thr→Ile), 2B (Ala→Val), 2C (Phe→Leu), 3A (Asp→Tyr, Gly→Ala, and Ala→Thr), 3B (His→Tyr) and 3D (Thr→Ser, Met→Leu, Cys→Tyr, and Ser→Gly). In contrast to the host range variant GBM/FRhK, no deletions appeared within the sequence of GBM/HFS compared with that of GBM/WT. However, there is a mutation located at nucleotide position 5013, the beginning of the deletion in 3A from GBM/FRhK, which resulted in an amino acid change from aspartic acid to tyrosine.

Sequence comparison between the GBM variants. Nucleotide sequence analysis of the three GBM variants reveals an identity between GBM/WT and GBM/FRhK of 99.3%, between GBM/WT and GBM/HFS of 99.6%, and between the two cell culture-adapted variants of 99.2%. The 5′NCR shows a high percentage of nucleotide differences. Five identical nucleotide changes in the noncoding region, and two silent and five nonsilent mutations in the protein coding region could be found common in both cell culture-adapted variants, GBM/FRhK and GBM/HFS, compared with those of the wild-type (Table 3). The identical amino acid changes are located in the 2B, 3A, 3B, and 3D regions. The genome region P1, coding for the structural proteins of HAV, shows a high similarity between the GBM variants. Amino acid mutations were found only in VP1. In contrast, in the genome regions P2 and P3, only protein 2A is free of any mutation. There is only one nucleotide position (5013) which leads to an amino acid deletion in GBM/FRhK and also to an amino acid change from aspartic acid to tyrosine in the other cell culture-adapted variant, GBM/HFS.

Conclusions. The HAV wild-type strain GBM and the cell culture-adapted GBM variants, the host range variant GBM/FRhK and the attenuated variant GBM/HFS, were used for defining cell tropism and attenuation on the molecular basis of HAV.

The alignment of the genome sequence of the GBM wild type with other HAV wild-type sequences has shown a 92% (HM175) to 96.3% (HAV-LA) identity and classifies the GBM strain as subgenotype IA within the seven genotypes of human HAV isolated from different geographical regions (31). Subgenotype IA contains most of the HAV isolates of human origin. The comparison of the consensus sequence, a 168-nucleotide region encoding the putative VP1-2A junction (31), shows that wild-type HAV-LA (26) is also a member of subgenotype IA but differs in comparison with the sequence of GBM/WT. HM175 (6), another wild-type HAV strain with a completely known nucleotide sequence, is grouped into subgenotype IB.

Our investigations of the nearly entire sequence of the three GBM variants have shown that the 5′NCR; 2B, 3A, 3B, and 3D regions; and the 3′NCR contain nucleotide or amino acid mutations common in both cell culture-adapted variants. One of these common mutations is identical to the one found by Emerson et al. (12) at position 3889 in the 2B region, which results in an amino acid change from alanine to valine. This mutation seems to be essential for cell culture adaptation, whereas other mutations in the 2C region at nucleotide position 4087 and 4222, which are described as relevant for cell culture adaptation (10), do not appear in the GBM variants. Only the host range variant GBM/FRhK has a mutation at position 4086 which results in the substitution of the same amino acid, lysine, described by Emerson et al., but the exchanged amino acid is different (glutamine instead of methionine). We therefore think that different mutations present in the cell culture-adapted variants in combination with the mutation at nucleotide position 3889 in 2B may influence viral growth in cell culture but may be cell specific and/or related to alteration during the virus passages. Recently, this was shown with chimeric viruses constructed from HAV wild-type HM175 and cell culture-adapted mutants of the HM175 strain (11).

The different biologic behavior of the two cell culture-adapted GBM variants correlates with a diverse genome sequence. The amount of mutations found in GBM/HFS is comparable to those in other cell culture-adapted human HAV strains described so far (5, 20, 32). In contrast, the host range variant GBM/FRhK has more mutations compared with the wild-type than does GBM/HFS. In addition, there are in-frame deletions in the sequence of GBM/FRhK which do not occur in GBM/HFS. The six-amino-acid deletion at the amino terminus of VP1 is also described for the HAV strain HAS-15 (26), a strain which is adapted to growth on FRhK-6 and FRhK-4 cells (30, 37). The Asp-Asp-Asn deletion at the amino terminus of the 3A region has not been described for other HAV strains. However, there is a deletion of an aspartic acid described in two cytopathogenic strains, HM175m6 p18f (21) and an Italian variant, FG (25), one position before the described deletion in GBM/FRhK. The combination with the other deletion and/or other mutations may be important for the host range behavior of GBM/FRhK. Interestingly, GBM/HFS contains a nucleotide mutation resulting in an amino acid change in 3A which is located at nucleotide position 5013, the beginning of the deletion in the variant GBM/FRhK. This different exchange in the GBM variants may indicate an important role for cell

TABLE 3. Differences in the genome sequence and predicted amino acids of the host range variant GBM/FRhK and the attenuated variant GBM/HFS from wild-type HAV GBM/WT[a]

Polyprotein, region, and function	Residue (amino acid)	Position (nucleotide)	Nucleotide			Amino acid		
			GBM/WT	GBM/FRhK	GBM/HFS	GBM/WT	GBM/FRhK	GBM/HFS
5′NCR		46	A	G				
		153	A	G	G			
		178	A	G	G			
		245	T	A				
		327	G	A				
		377	T	C				
		646	G	T	T			
		687	T	G	G			
P1								
VP2, capsid	6	821	A		G			
	18	857	T		C			
	168	1307	T		A			
VP1, capsid	26	2283	A	DEL[b]		Thr	DEL	
		2284	C	DEL				
		2285	A	DEL				
	27	2286	A	DEL		Thr	DEL	
		2287	C	DEL				
		2288	C	DEL				
	28	2289	A	DEL		Met	DEL	
		2290	T	DEL				
		2291	G	DEL				
	29	2292	A	DEL		Arg	DEL	
		2293	G	DEL				
		2294	G	DEL				
	30	2295	G	DEL		Asp	DEL	
		2296	A	DEL				
		2297	C	DEL				
	31	2298	C	DEL		Leu	DEL	
		2299	T	DEL				
		2300	A	DEL				
	98	2503	C		T	Thr		Ile
	155	2675	G	C		Met	Ile	
	214	2853	G	C		Asp	His	
	223	2879	C	T				
P2								
2B, transcription	21	3737	T		C			
	34	3774	A	G		Ile	Val	
	72	3889	C	T	T	Ala	Val	Val
2C, viral transcription	8	4019	C	T				
	22	4061	C		A			
	30	4086	A	C		Lys	Gln	
	64	4188	T		C	Phe		Leu
	143	4426	A	C		Lys	Thr	
	198	4592	C	T	T			
	265	4793	T	C				
	287	4858	A	G		Asn	Ser	
P3								
3A, pre-VPg	5	5013	G	DEL	T	Asp	DEL	Tyr
		5014	A	DEL				
		5015	T	DEL				
	6	5016	G	DEL		Asp	DEL	
		5017	A	DEL				
		5018	C	DEL				
	7	5019	A	DEL		Asn	DEL	
		5020	A	DEL				
		5021	T	DEL				
	55	5164	G	C		Gly	Ala	
	73	5217	G		A	Ala		Thr
3B, VPg	4	5232	C	T	T	His	Tyr	Tyr

Continued on following page

TABLE 3—*Continued.*

Polyprotein, region, and function	Residue (amino acid)	Position (nucleotide)	Nucleotide			Amino acid		
			GBM/WT	GBM/FRhK	GBM/HFS	GBM/WT	GBM/FRhK	GBM/HFS
3C, viral protease	133	5689	G	A		Gly	Asp	
		5690	A	C				
	141	5715	A	T		Thr	Ser	
	165	5789	A		G			
3D, RNA-dependent RNA polymerase	7	5970	A		T	Thr	Ser	Ser
		5971	C	G				
	39	6066	A		T	Met		Leu
	296	6838	G	A		Cys	Tyr	
	335	6956	G	A				
	351	7004	C		T			
	400	7151	T		C			
	425	7224	A		G	Ser		Gly
3′NCR		7468	C	T	T			
		7469	G		A			

[a] The predicted junctions of the genome encoding each structural and nonstructural protein correspond to the prediction by Cohen et al. (6).
[b] DEL, deleted.

culture adaptation within the HAV genome. Functional analysis will show the significance of these mutations for cell culture adaptation of HAV strain GBM. The mutation at nucleotide position 5970 or 5971 (3D) is particularly interesting. The exchanged A for T at position 5970 leads to a threonine-to-serine change in GBM/HFS compared with that in GBM/WT. In the variant GBM/FRhK, however, the serine is achieved by exchange from C to G at position 5971, suggesting a possible role for this serine residue in cell culture adaptation. The 3D region, coding for the RNA-dependent RNA polymerase, is also the only genome region in GBM/HFS which contains more nucleotide mutations with resulting amino acid changes than GBM/FRhK. Even if the percentage of the amino acid exchanges is low (0.3%), the resulting amino acid sequence of the polymerase could lead to a different replication behavior of the virus.

The 5′NCR of the GBM variants contains a high variability of the nucleotide sequence compared with that of GBM/WT. Investigations by different groups (3, 8, 12, 18) have shown that this region is involved in an important regulatory mechanism for cell culture adaptation, attenuation, and translation. Like other picornaviruses such as poliovirus, foot-and-mouth disease virus, or encephalomyocarditis virus, it is suggested that HAV is translated by a cap-independent mechanism. The 5′NCR is proposed to contain an internal ribosome entry site, the so-called IRES element (3, 18). The secondary structure in the 5′NCR is assumed to be responsible for the regulation of translation. Only a few nucleotide mutations could lead to significant changes in the secondary structure resulting in a different growth behavior of the cell culture-adapted variants compared with that of the wild-type. There are only two of the eight mutations in the 5′NCR of the GBM variants which are described for another HAV system. The cell culture-adapted variant HM175m6p59 (32) showed a nucleotide exchange at position 646 from G to A, which is a G-to-T exchange in the GBM variants and an exchange from T to G at position 687 which is identical in the GBM variants. This T-to-G exchange is also found in the variant HM175m6 p16 (20) and the cytopathic variant HM175m6 p18f (21). The other mutations found in the 5′NCR of HAV GBM differ from described data.

Compared with published data on adaptation and attenuation, we found in our system significant differences from other HAV strains. Further analysis is now necessary to demonstrate the individual importance of each of the described mutations for the growth behavior in cell culture, the host range restriction, and the attenuation.

Nucleotide sequence accession number. Nucleotide sequences of the HAV wild-type GBM/WT and the two cell culture-adapted GBM variants will appear in the EMBL nucleotide sequence data base under the following accession numbers: GBM/WT, X75215; GBM/FRhK, X75214; GBM/HFS, X75216.

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