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Complete nucleotide sequence of an attenuated hepatitis A virus: Comparison with wild-type virus

(picornavirus/cell culture adaptation)

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ABSTRACT **The complete nucleotide sequence of an attenuated hepatitis A virus, HAV HM-175/7 MK-5, was determined from cloned cDNA. This virus was derived from wild-type HAV HM-175 after 32 passages in African green monkey kidney cells. The resultant cell culture-adapted virus is attenuated for chimpanzees. This virus was passaged an additional three times in monkey kidney cells to obtain sufficient virus for molecular cloning and was designated HM-175/7 MK-5. Three overlapping cDNA clones were obtained that together spanned the entire genome. Comparison of the nucleotide sequence of cDNA from wild-type virus (propagated in marmoset liver *in vivo*) with attenuated virus (grown in cell culture) showed 24 nucleotide changes distributed throughout the genome. Five base deletions occurred in the 5′ noncoding region, and 12 of the 16 base substitutions in the coding region resulted in amino acid changes. Amino acid changes occurred in viral capsid proteins VP1 and VP2 and several of the nonstructural proteins. Thus, a small number of nucleotide changes are responsible for adaptation to cell culture and attenuation of HAV strain HM-175.**

Hepatitis A virus (HAV) is responsible for more than 20,000 cases of hepatitis reported in the United States each year (1). Certain populations are at high risk for infection with HAV, including foreign travelers, children attending day-care centers and their close contacts, military personnel, and close contacts of patients with hepatitis A (2). Currently, passive immunization with immune serum globulin is the only effective measure for preventing hepatitis A infections in these individuals. Immune serum globulin, however, provides only low levels of neutralizing antibody (3) and must be administered repeatedly.

An effective vaccine would be useful for active immunization of populations at high risk. Inactivated HAV vaccines have been developed that have been shown to be immunogenic (4) and protective against challenge with live virus (5). These vaccines may prove effective; however, they may be costly, may fail to produce local (or secretory) immunity, and may require repeated administration. For these reasons, live HAV vaccines are under development in several laboratories. Provost *et al.* (6) have shown that a cell culture-adapted mutant of HAV strain CR-326 is attenuated in nonhuman primates and induces resistance to challenge with wild-type virus. Our laboratory has developed a cell culture-adapted mutant of HAV strain HM-175 that induces serum neutralizing antibody and is attenuated for chimpanzees. Chimpanzees inoculated with this virus developed little or no elevation in the level of liver enzymes in blood. They also shed less virus and exhibited a lower level of viral replication in the liver compared to animals infected with wild-type virus.†

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We have previously determined the complete nucleotide sequence of wild-type HAV HM-175, which was purified directly from infected marmoset liver (7). Here we report the nucleotide sequence of an attenuated mutant (derived from cell culture) and compare it to the sequence of wild-type virus. Although sequences of other cell culture-adapted strains of HAV have been reported (10, 16–19), it is not known whether these viruses were attenuated. Comparisons between attenuated polioviruses and their wild-type parents were reported previously (26–30); however, the latter viruses were already adapted to cell culture before molecular cloning was performed. In contrast, wild-type HAV HM-175 did not replicate efficiently in culture. Hence, comparison between wild-type and attenuated HAV HM-175 should reflect changes associated with both attenuation and cell culture adaptation.

MATERIALS AND METHODS

Virus, Virus Purification, and RNA Extraction. HAV strain HM-175 was recovered from the stool of a patient with hepatitis A and grown in primary African green monkey kidney (AGMK) cells (8). The virus was passaged 26 times in AGMK cells and then triply cloned by the terminal-dilution method. One resultant variant (clone 7) was then passaged three times in AGMK cells to prepare a stock of virus for studies in primates. This virus, like uncloned AGMK cell-adapted HAV (9), is attenuated for chimpanzees and partially attenuated for marmosets but has not yet been tested in man. It induces serum neutralizing antibodies in marmosets and chimpanzees (Karron *et al.*, see footnote†). The clone 7 variant was passaged three additional times in AGMK cells to produce sufficient HAV, designated HM-175/7 MK-5, for molecular cloning. HAV was purified by a modification of the procedure described by Linemeyer *et al.* (10). The final viral pellet was resuspended in TNE (10 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM EDTA) and HAV RNA was extracted as described (11).

Molecular Cloning. HAV RNA (1.2 μg) served as a template for first-strand cDNA synthesis, using conditions described previously (11), except that the concentration of reverse transcriptase was increased to 1000 units/ml and the reaction mixture was incubated for 45 min. The reaction was terminated, RNA·cDNA hybrids were isolated, and second-strand cDNA was synthesized using RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase (12). The reaction was terminated and double-stranded cDNA was isolated (12). This cDNA was size-selected by use of a 1-ml

Abbreviation: HAV, hepatitis A virus.

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†Karron, R. A., Ticehurst, J., Daemer, R., Mihalik, K., Feinstone, S. & Purcell, R. H., Twenty-Sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, Sept. 28–Oct. 1, 1986, New Orleans, LA, p. 278 (abstr. 983).

Sepharose 4B column. The first four 65-μl fractions, containing double-stranded cDNA, were pooled and precipitated in ethanol after the addition of tRNA as carrier. Homopolymeric tails of dCMP were added to double-stranded cDNA by use of terminal deoxynucleotidyltransferase (11). The reaction was terminated, and oligo(dC)-tailed cDNA was phenol-extracted and ethanol-precipitated. Plasmid vector pBR322, cleaved at the *Pst* I site and tailed with dGMP, was hybridized to equimolar amounts of oligo(dC)-tailed double-stranded cDNA and used to transform *E. coli* HB101 according to standard procedures (13).

Analysis of cDNA Clones and Sequence Analysis. Bacterial colonies were transferred to nitrocellulose filters and lysed. The resulting DNA bound to the filters was analyzed with three HAV cDNA probes, which corresponded to the 5′, middle, and 3′ regions of wild-type HAV cDNA (11, 14). DNA fragments from plasmids $pHAV_{LB}113$ (*Nco* I/*Xba* I digest), $pHAV_{LB}228$ (*Pst* I digest), and $pHAV_{LB}93$ (*Pst* I digest) were isolated from agarose gels, nick-translated, and used as probes for these hybridizations. Approximately 5000 bacterial clones were screened by colony hybridization (14) with the radiolabeled wild-type HAV cDNA probes. Nucleotide sequence was determined directly from plasmid DNA, using reverse transcriptase, oligonucleotide primers, and dideoxynucleoside triphosphates (15).

RESULTS

A set of three cDNA clones was obtained that together spanned the entire HAV genome. There were 24 nucleotide changes distributed throughout the genome of attenuated HAV HM-175/7 MK-5 when compared to wild-type HM-175 (Table 1). Each of the nucleotide changes from the wild-type sequence was verified by replicate assays of at least two independent cDNA clones (except base 1742), and each of the nucleotide deletions was confirmed on three independent cDNA clones. More than 90% of the sequence was determined on both strands of cDNA.

Seven nucleotide changes occurred in the 5′ noncoding region, including five base deletions. At nucleotide positions 203–207 a single thymidine (out of five consecutive thymidines in wild-type HAV) was deleted in HM-175/7 MK-5. The genome of another cell culture-adapted mutant of HAV HM-175 has a deletion in the same region (17). This strain (HM-175/P59), isolated independently of HM-175/7 MK-5, was also derived from the same wild-type virus; supernatant virus after 6 passages *in vivo* and 59 passages *in vitro* was used for molecular cloning in this instance. HAV HM-175/P59 has seven other nucleotide differences from wild-type HAV HM-175 in the 5′ noncoding region; however, none of these differences correspond to those present in HM-175/7 MK-5. Another cell culture-adapted strain of HAV (LA), derived from a different wild-type HAV, has an adenosine at position 203 (16), instead of the thymidine present in wild-type HAV HM-175. Thus, the region of 203–207 differs from wild-type HAV HM-175 in each of the three independent cell culture-adapted HAV mutants that have been sequenced.

The secondary structures of the 5′ noncoding regions of wild-type HAV HM-175 and attenuated HM-175/7 MK-5 viral RNAs were markedly different when compared by

Table 1. Comparison of genome sequence of wild-type HAV HM-175, attenuated HAV HM-175/7 MK-5, and other cell culture-adapted strains of HAV

Nucleotide position	Location in HAV	HAV HM-175				Nucleotide of other cell culture-adapted HAV strains				
		Nucleotide		Amino acid						
		WT	Attenuated	WT	Attenuated	LA	CR-326	HM-175/P59	HAS-15	Italy
124	5′	T	C			T	—	T	—	T
131	5′	T	d			T	—	T	—	C
132	5′	T	d			T	—	T	—	T
133	5′	T	d			T	—	T	—	T
134	5′	G	d			G	—	G	—	G
152	5′	A	G			A	—	A	—	A
203–207	5′	T	d			A	—	d	—	—
964	VP2	A	G	Lys	Arg	A	A	A	A	—
1742	VP3	G	A			T	C	—	T	—
3025	VP1	A	T	Glu	Val	A	A	—	A	—
3196	2A	A	G	Asn	Ser	A	A	—	A	—
3889	2B	C	T	Ala	Val	C	—	—	C	—
3919	2B	G	C	Gly	Ala	G	—	—	G	—
4043	2C	T	C			T	—	—	T	—
4087	2C	A	T	Lys	Met	A	—	—	A	—
4185	2C	G	A	Glu	Lys	A (Asn)	—	—	A (Asn)	—
4222	2C	T	C	Phe	Ser	T	—	—	T	—
4563	2C	G	A	Val	Ile	G	—	—	—	—
5204	3A	G	A			A	—	—	—	—
5232	3B	C	T	His	Tyr	C	—	—	—	—
6147	3D	G	A	Asp	Asn	G	—	—	—	—
6522	3D	T	A	Ser	Thr	A (Thr)	—	—	—	—
7032	3D	T	C			T	—	—	—	—
7430	3′	A	G			A	—	—	—	—

The following HAV strains are compared: wild-type (WT) HAV HM-175 (7); attenuated HM-175/7 MK-5; LA (16); CR-326 (10); HM-175/P59 (17); HAS-15 (18); Italy (19). 5′ indicates 5′ noncoding region; 3′ indicates 3′ noncoding region; d indicates deletion; dash indicates nucleotide sequence not determined. Nucleotide positions correspond to numbering for wild-type HAV HM-175 (7), and amino acids in parentheses indicate those which differ from wild-type HAV HM-175. The sequence of wild-type HAV HM-175 was determined at each position on at least two independent cDNA clones (except base 1742). The nucleotide at position 4185 is G in wild-type HAV plasmid $pHAV_{LB}228$ and A in $pHAV_{LB}148$; the previously published wild-type HAV sequence (7) has a T at position 2864 ($pHAV_{LB}288$), but $pHAV_{LB}1688$ and $pHAV_{L}1307$ have an A at this position. The nucleotide sequences of the other cell culture-adapted strains (LA, CR-326, etc.) differ from wild-type HAV HM-175 in more positions than indicated in the table; nucleotides listed are those at which wild-type virus differs from HM-175/7 MK-5. It is unknown whether or not the other cell culture-adapted HAV strains are attenuated.

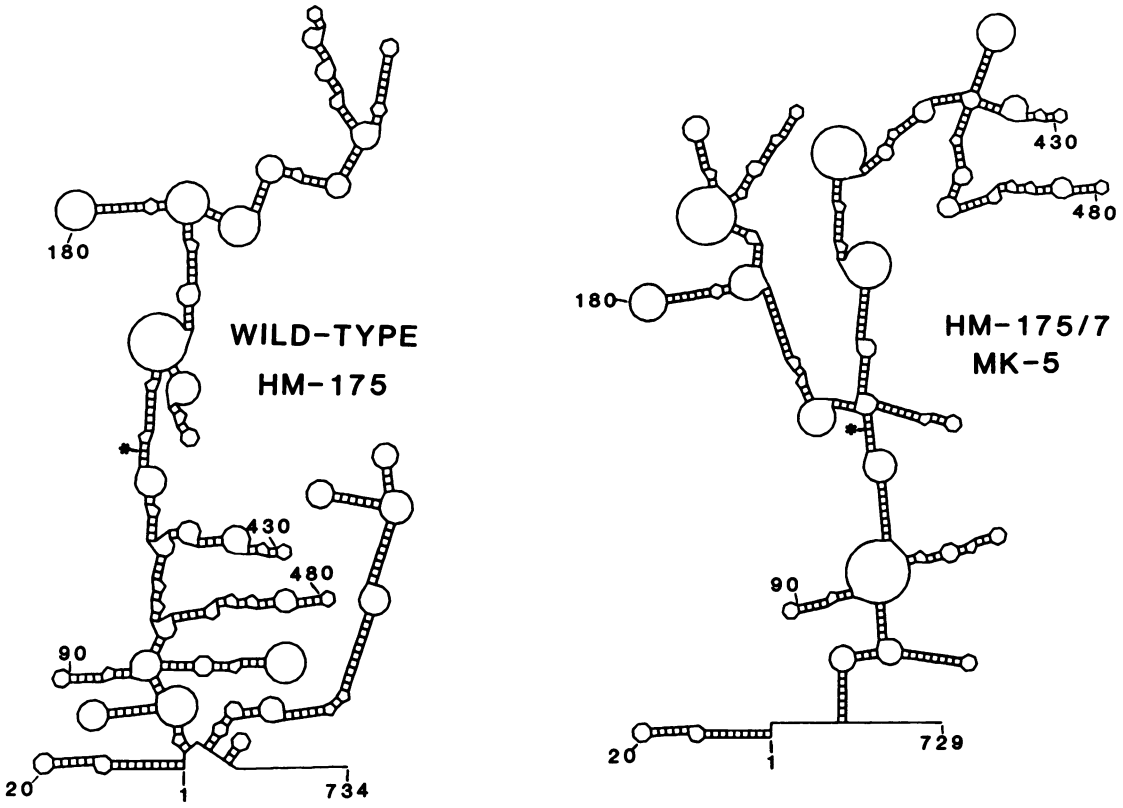

FIG. 1. Predicted secondary structure for RNA from 5′ end of wild-type HAV HM-175 and attenuated HM-175/7 MK-5, based on folding and display algorithms (20, 21). Conserved structures are indicated with numbered bases; asterisk indicates base 124.

computer analysis (Fig. 1). Most of the difference in predicted secondary structure of HM-175/7 MK-5 results from a single nucleotide substitution at base 124 (data not shown).

Comparison of wild-type HAV HM-175 and attenuated HM-175/7 MK-5 revealed 16 nucleotide changes in the coding region. Twelve of these base changes resulted in a change in amino acid sequence. Seven of these changes yielded an amino acid with a difference in charge.

There were three nucleotide differences between wild-type HAV HM-175 and HM-175/7 MK-5 in the capsid region, and one of these changes, at position 1742, did not alter the amino acid sequence. All of the other cell culture-adapted HAV strains that have been sequenced in the capsid region (10, 16, 18) also exhibit a nucleotide difference from wild-type HAV HM-175 at position 1742 that does not affect the amino acid sequence. The nucleotide substitution at position 3025 in the region encoding VP1 changed glutamic acid in wild-type HM-175 to valine in HM-175/7 MK-5. This is the only amino acid change in the capsid region that substituted an amino acid of different charge.

There were 10 amino acid differences between wild-type HAV HM-175 and HM-175/7 MK-5 in the P2 and P3 regions. Amino acid changes occurred in proteins 2A, 2B, 2C, 3B, and 3D. Four of the 12 amino acid changes occurred in protein 2C. By analogy with poliovirus (22), HAV protein 2C may be involved in transcription. Amino acid changes did not occur in proteins 3A and 3C; wild-type and vaccine strains of poliovirus types 1 and 3 also did not sustain changes in these proteins. None of the amino acid changes occurred at putative dipeptide cleavage sites or at proposed enzymatic sites on the polyprotein (7). The nucleotide difference at 6522 changed a serine in protein 3D of wild-type HAV HM-175 to a threonine in HM-175/7 MK-5. A different cell culture-adapted HAV strain (16) exhibits the same difference from wild-type HAV HM-175. This is the only amino acid difference of HM-175/7 MK-5 from wild-type HM-175 that has been identified in another cell culture-adapted HAV strain.

When the 12 amino acids that differed in wild-type HAV HM-175 and HAV HM-175/7 MK-5 were analyzed using the mutation data matrix (23), only two amino acid changes (glutamic acid to valine in VP1; phenylalanine to serine in 2C) had a negative score. This matrix compares amino acid sequences from pairs of proteins on phylogenetic trees; a negative score means that the pair would be expected to occur less frequently in related sequences than random chance would predict.

One nucleotide change in the 3′ noncoding region was identified in HM-175/7 MK-5. One of the three cloned cDNAs from HAV HM-175/7 MK-5 had an additional thymidine at the 3′ end of the genome, immediately preceding the poly(A) tail. An additional nucleotide was also seen in the only other cell culture-adapted HAV strain (16) that has been sequenced in this region.

DISCUSSION

Wild-type HAV strain HM-175 was difficult to isolate in cell culture; however, after serial passage the virus grew with increased efficiency *in vitro* and at the same time became attenuated for chimpanzees (24). Comparison of the nucleotide sequence of wild-type HAV HM-175 and its AGMK cell-adapted, attenuated mutant indicated that 24 nucleotide changes (resulting in 12 amino acid changes) had occurred during the serial passages that yielded the mutant.

The number of changes that were identified when wild-type and attenuated (vaccine) strains of type 1 and type 3 polioviruses were compared bracket those identified in HAV 175/7 MK-5. The vaccine (Sabin) strain of poliovirus type 1 was derived from its wild-type (Mahoney) parent after 14 *in vivo* and 54 *in vitro* passages (25). Comparison of these two strains showed 57 nucleotide differences resulting in 21 amino

acid changes (26–28). The vaccine strain of poliovirus type 3 was derived from its wild-type (Leon) parent after 21 *in vivo* and 53 *in vitro* passages (25). Comparison of the two strains showed 10 nucleotide changes resulting in three amino acid changes (29, 30). Thus, although wild-type HAV HM-175 had never been in cell culture before molecular cloning, the number of nucleotide differences between wild-type and attenuated HAV HM-175 was in the same range as that observed for wild-type and attenuated poliovirus strains.

The exact nucleotide changes responsible for attenuation of polioviruses have been difficult to map. Using neurovirulent revertants of the vaccine strain of poliovirus type 3, Almond *et al.* (31) have suggested that nucleotide substitutions in the 5′ noncoding region and in the regions encoding VP1 and VP3 may be responsible for attenuation of the Sabin vaccine virus. Omata and colleagues (32), using recombinant viruses derived from infectious cDNA clones of wild-type virus and attenuated virus, have shown that attenuation of poliovirus type 1 is associated with multiple nucleotide changes scattered over most of the genome.

By analogy with poliovirus, three areas of the HAV genome may contain determinants of attenuation. The RNAs of the 5′ noncoding regions of wild-type HAV and HM-175/7 MK-5 differ in predicted secondary structure. However, it is not known whether such structures have significance or even exist in the native molecule. Changes in predicted secondary structures were also detected when RNAs of the wild-type and vaccine strains of poliovirus types 1 and 3 were compared. A single nucleotide substitution (base 472) was associated with a decrease in neurovirulence and a change in predicted secondary structure for the 5′ noncoding region of the poliovirus type 3 genome (33). The 5′ noncoding region of HAV HM-175/7 MK-5 has five nucleotide deletions (one four-base and one single-base deletion) when compared to wild-type HM-175. The 5′ noncoding region has the highest degree of nucleotide conservation among different strains of HAV (7); however, when wild-type and attenuated HM-175 are compared, 29% of the nucleotide differences (7 out of 24 bases) are located in the 5′ noncoding region, which contains less than 10% of the viral genome. Thus, this relatively small area of the genome may be important both for cell culture adaptation and for attenuation.

The capsid region of HAV may also be an important target for attenuation. HAV HM-175/7 MK-5 exhibits two amino acid differences from wild-type HM-175 in the capsid region. The nucleotide substitution at position 3025 changes amino acid 273 of VP1 from glutamic acid (negatively charged) in wild-type HM-175 to valine (nonpolar) in HM-175/7 MK-5. A. Palmenberg (personal communication) has aligned the amino acid sequence of HAV capsid proteins with two other picornaviruses (poliovirus type 1 Mahoney and rhinovirus type 14) for which the three-dimensional structures have been determined (34, 35). HAV VP1 amino acid 273 aligns with an antigenic site on poliovirus type 1 (amino acids 271–295) and with a portion of the "canyon wall," thought to be the receptor binding site, on rhinovirus type 14 (amino acids 254–272). In addition, when wild-type and vaccine strains of poliovirus type 3 are compared, one of the three amino acid differences (VP1 amino acid 287) aligns with an antigenic site. Wild-type and vaccine strains of poliovirus type 1 exhibit a difference in amino acids in three distinct antigenic sites. Thus, as for the live poliovirus vaccine strains, one of the amino acid differences between wild-type and attenuated HAV HM-175 may be located at a site important for binding of antibody.

One of three cloned cDNAs of HM-175/7 MK-5 has an additional thymidine at the 3′ end of the genome, when compared with wild-type HM-175. When wild-type and vaccine strains of poliovirus types 1 and 3 were compared, the vaccine strains also had an additional nucleotide at the 3′ terminus (26, 27, 29, 30). However, a second sequence reported for wild-type poliovirus type 1 (28) has the additional nucleotide at the 3′ end of the genome. Thus, the terminal 3′ nucleotide may be related to attenuation or may reflect sequence variation in a heterogeneous population of viruses.

The exact nucleotide changes responsible for cell culture adaptation and attenuation of HAV may be difficult to determine. Identification of the precise changes for HAV must await recombination experiments between infectious cDNAs from wild-type and attenuated (cell culture-adapted) viruses. The observation that HAV HM-175/7 MK-5 has five nucleotide deletions would be of particular importance if it can be shown that these mutations contribute to attenuation, because neither the type 1 nor the type 3 attenuated poliovirus vaccine strains contain internal deletion mutations. Deletion mutations that specify attenuation should confer a more stable phenotype than base substitution mutations, because reversion to a wild-type genotype is unlikely. However, the attenuated phenotype could be reversed by second-site mutation(s). Construction of infectious HAV cDNAs would allow determination of the changes in HAV HM-175 that are responsible for attenuation. Such cDNAs might also be useful for generation of additional candidate live vaccine viruses by *in vitro* mutagenesis.

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