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Attenuation and Cell Culture Adaptation of Hepatitis A Virus (HAV): a Genetic Analysis with HAV cDNA

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RNA transcripts of hepatitis A virus (HAV) HM-175 cDNA from attenuated, cell culture-adapted HAV were infectious in cell culture. A full-length HAV cDNA from wild-type HAV (propagated in marmosets in vivo) was constructed. Chimeric cDNAs that contained portions of both wild-type and attenuated genomes were produced. Oligonucleotide-directed mutagenesis was used to engineer a point mutation into the VP1 gene of attenuated HAV cDNA, so that the sequence of this capsid protein would be identical to that of the wild-type virus. Transfection of monkey kidney cells with RNA transcripts from several of the chimeric cDNAs and from the mutagenized cDNA induced production of HAV. Comparison of the growth of attenuated, wild-type, chimeric, and mutant viruses in vitro indicated that the P2-P3 (nonstructural protein) region is important for cell culture adaptation of the virus; the 5′ noncoding region may also contribute to adaptation, but to a lesser extent. Inoculation of marmosets with transfection-derived virus also suggested that the P2-P3 region plays an important role in attenuation of HAV HM-175.

Hepatitis A virus (HAV) is responsible for about 40% of the cases of acute hepatitis in the United States each year (4). Live attenuated HAV vaccines are currently under development in several laboratories (3, 11, 12, 20). Many of the candidate vaccine viruses have been attenuated by passage in cell culture (11, 12, 20); however, one virus was attenuated for chimpanzees by rapid passage in marmosets (3). We have developed a live, attenuated candidate HAV vaccine by serial passage of HAV strain HM-175 in primary African green monkey kidney (AGMK) cells. After 32 passages in cell culture, the virus was adapted for growth in cell culture, attenuated for chimpanzees, and partially attenuated for marmosets (14). Since an attenuated HAV may be the basis for a future live-virus vaccine, it would be important to determine the molecular basis of attenuation.

Attenuation of HAV HM-175 must be due to changes in the genome which occur during adaptation to cell culture. Comparison of the nucleotide sequences of wild-type and attenuated HAV HM-175 shows 24 nucleotide changes in a total of 7,478 bases. Nucleotide substitutions are present in both the 5′ and 3′ noncoding regions, and five base deletions occur in the 5′ noncoding region of attenuated HAV. Twelve amino acid changes occur in the polyprotein, with amino acid substitutions in 7 of the 11 viral proteins (5). While attenuation is due to a small number of nucleotide changes, sequence comparison alone does not indicate which changes are responsible for attenuation.

Wild-type HAV HM-175 grows poorly in cell culture and requires weeks to achieve maximum virus titers. With increasing passage in cell culture, the virus adapts to growth in vitro with more rapid production of viral antigen and increasing virus titers. The molecular basis for cell culture adaptation is unknown. However, when developing live-HAV vaccines, it would be useful to determine whether the mutations responsible for attenuation are identical to those necessary for cell culture adaptation.

We have recently constructed a full-length copy of attenuated, cell culture-adapted HAV cDNA in an RNA transcription vector. RNA transcripts produced in vitro from this cDNA are infectious in cell culture; the resultant virus produced is partially attenuated for marmosets (6). Wild-type HAV HM-175 has been molecularly cloned (7, 26), and we produced a full-length cDNA corresponding to the genome of the wild-type virus. Chimeric cDNAs that contain portions of the wild-type and attenuated HAV genomes were constructed. In addition, oligonucleotide mutagenesis was used to engineer a specific mutation into the cDNA. RNA transcripts from several of these chimeric cDNAs and the mutagenized cDNA were infectious in cell culture. Analysis of the growth of these viruses in cell culture and inoculation of these viruses into primates provided insight into which portions of the HAV genome are responsible for cell culture adaptation and attenuation.

MATERIALS AND METHODS

Construction of full-length wild-type HAV cDNA. Molecular cloning of wild-type HAV HM-175 has been reported previously (7, 26). Six overlapping cDNA clones span the entire genome of wild-type HAV HM-175 (Fig. 1). Restriction endonuclease fragments from these clones were ligated together in a stepwise fashion to produce plasmid pHAV/WT-BR322, which contains a full-length copy of wild-type HAV HM-175 cDNA in plasmid pBR322 (Fig. 1). The HAV cDNA insert in this plasmid is bracketed by oligo(dG)-oligo(dC) tails that remain from the initial cloning procedure. The complete nucleotide sequence of pHAV/WT-BR322 was determined directly from plasmid DNA by the method of Zagursky et al. (28), and no changes were detected between this construct and its parent cDNA clones.

Two plasmids, pHAV 5′ and pHAV 3′ (6), which con-

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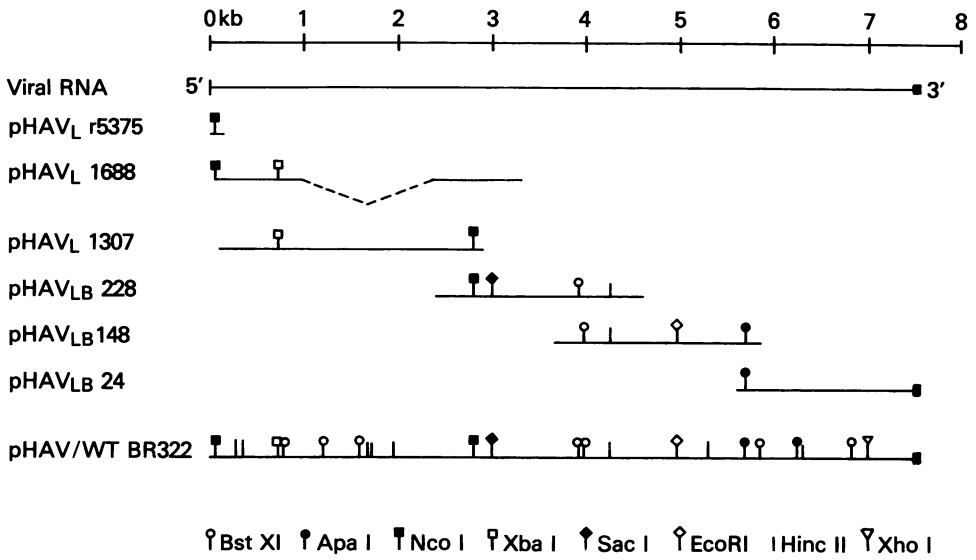

FIG. 1. Ligation of cDNA clones to produce full-length wild-type HAV HM-175 cDNA. Restriction endonuclease *Bst*XI was used to ligate pHAV$_{LB}$228 (at base 3931) to pHAV$_{LB}$148 (at base 3970). The resulting plasmid, pHAV-L1, has a 39-base deletion (bases 3931 to 3970). pHAV-L1 was ligated to pHAV$_{LB}$24 at their common *Apa*I site (base 5687). The resulting plasmid, pHAV-L2, was ligated to pHAV$_L$1307 at the *Nco*I site (base 2814) to produce pHAV L3. pHAV$_L$1688 was ligated to pHAV-L3 at the *Xba*I site (base 744). The resulting plasmid, pHAV-L4, was ligated to pHAV$_L$r5375 at the common *Nco*I site (base 45) to yield pHAV-L5. The deletion in pHAV-L5 (bases 3931 to 3970) was repaired by a three-fragment ligation of restriction endonuclease fragments from pHAV-L5 (*Sac*I [base 2989] to *Eco*RI [base 4977]), pHAV$_{LB}$228 (*Sac*I to *Hin*cII [base 4242]), and pHAV$_{LB}$148 (*Hin*cII to *Eco*RI). The resulting plasmid, pHAV/WT-BR322, contains full-length wild-type HAV HM-175 cDNA in plasmid pBR322. Partial restriction endonuclease digests were performed for plasmids pHAV$_{LB}$148 (*Bst*XI), pHAV$_{LB}$24 (*Apa*I), and pHAV-L4 (*Nco*I). The numbers at restriction sites refer to nucleotide positions in wild-type HAV HM-175 cDNA (7). kb, Kilobases.

tained the 5′ and 3′ termini of wild-type HAV cDNA inserted into RNA transcription vector pGEM1 were constructed. Restriction endonuclease fragments from pHAV-5′ (*Nco*I-*Bgl*I), pHAV/WT-BR322 (*Nco*I-*Xho*I), and pHAV-3′ (*Xho*I-*Bgl*I) were ligated together as described previously for attenuated HAV HM-175 cDNA (6). The resulting plasmid, pHAV/WT, contains a full-length cDNA copy of the wild-type HAV HM-175 genome in pGEM1. The nucleotide sequences of the ligation junctions were verified by sequencing the plasmid DNA (28).

Construction of chimeric HAV cDNAs. Construction of full-length attenuated HAV cDNA (pHAV/7) has been described previously (6). Chimeric HAV cDNAs were produced by using restriction endonuclease sites shared by wild-type and attenuated HAV cDNAs (Fig. 2). Three restriction fragments (*Bgl*I-*Hpa*I, *Hpa*I-*Avr*II, and *Avr*II-*Bgl*I) from both wild-type and attenuated HAV cDNAs were isolated from low-melting-point agarose and used for ligations. Six chimeric cDNAs were constructed: pHAV-AAW, pHAV-AWA, pHAV-AWW, pHAV-WAA, pHAV-WAW, and pHAV-WWA. A indicates attenuated HAV cDNA, and W indicates wild-type cDNA. The first position after pHAV corresponds to restriction fragment *Bgl*I-*Hpa*I, the second position is *Hpa*I-*Avr*II, and third position is *Avr*II-*Bgl*I (Fig. 3). The nucleotide sequences of the chimeric cDNAs were determined at selected sites where wild-type and attenuated cDNAs have different sequences. Each of the chimeras had the appropriate nucleotide sequence corresponding to the donor cDNA for each of the three segments used to construct the chimera.

Mutagenesis. A *Sac*I restriction fragment from pHAV/7 (base 2984 of HAV to 27 bases after the 3′ end of the HAV insert) was isolated from low-melting-point agarose and cloned into the *Sac*I site of plasmid pTZ18R (Phar-

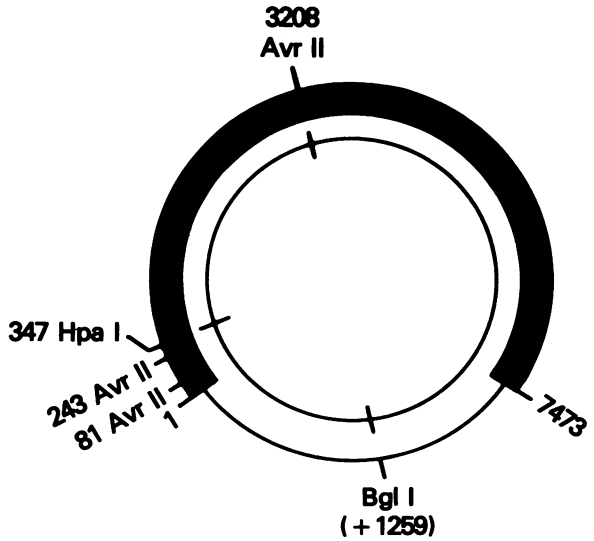

FIG. 2. Restriction endonuclease sites common to wild-type and attenuated HAV cDNAs used to produce chimeric cDNAs. In the outer circle, the solid bar indicates HAV cDNA, and the thin line indicates pGEM1 DNA. The inner circle indicates restriction fragments used for ligations. The numbers refer to nucleotide positions in attenuated HAV HM-175 cDNA (6), and the number in parentheses refers to a nucleotide position in pGEM1. *Avr*II cuts HAV cDNA at three positions; however, only the *Avr*II site at base 3208 was used to produce restriction fragments.

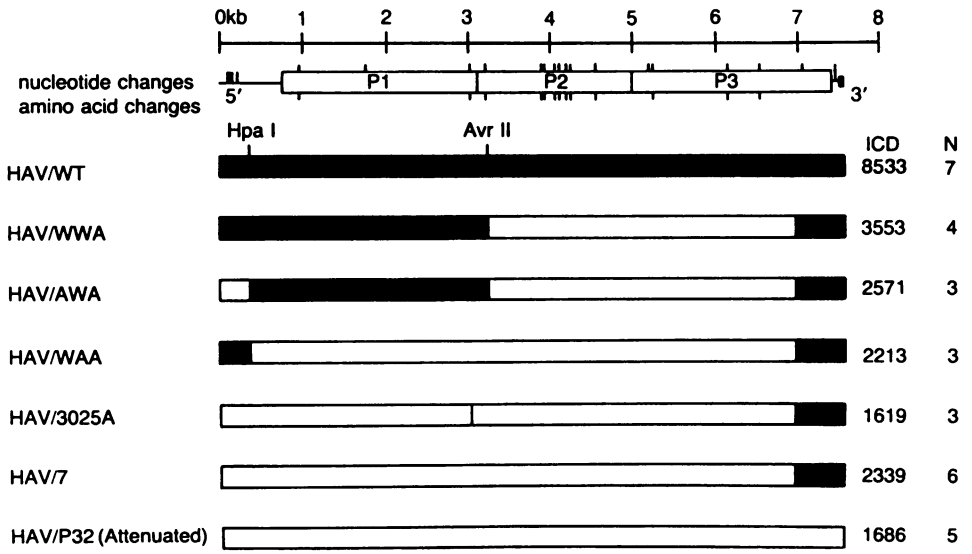

FIG. 3. Genome structures of chimeric HAV cDNAs. The top line shows the length of the HAV genome in kilobases (kb) from the 5′ terminus. The second line depicts the genome organization of HAV and the putative gene assignments (7) with nucleotide (and amino acid) differences between wild-type and attenuated HAV HM-175 above (and below) the genome. The genome organization of infectious chimeric viruses is shown with attenuated HAV sequences (open bars) and wild-type sequences (closed bars), along with the restriction endonuclease sites used to divide the cDNAs. *Hpa*I cuts attenuated HAV cDNA at base 347; all of the nucleotide changes between wild-type and attenuated HAV in the 5′ noncoding region are 5′ to this site. *Avr*II cuts attenuated HAV cDNA at base 3208; all of the nucleotide changes in the capsid region and one of the nucleotide changes in the gene that encodes protein 2A are between the *Hpa*I and *Avr*II sites. The region of the HAV genome 3′ to the *Avr*II site contains the nucleotide changes in the P2 and P3 regions. Two of the nucleotide changes from wild-type HAV, bases 7027 and 7425, are not present in any of the chimeric cDNAs. The full-length attenuated HAV genome used to construct the chimeras does not contain these latter two base changes (6); hence, all of the constructs have wild-type bases at these two positions. ICD, Geometric mean ICD peak; N, number of marmosets inoculated.

macia Inc., Piscataway, N.J.). The resulting plasmid, pTZHAV*Sac*Ids, was used to transform *Escherichia coli* JM109. After superinfection with helper bacteriophage, single-stranded plasmid pTZHAV*Sac*Iss was isolated. An oligonucleotide (3025A, CATTGATTCAGTGGAT) was synthesized (380A DNA synthesizer; Applied Biosystems, Foster City, Calif.) which corresponds to the complementary strand of attenuated HAV HM-175 cDNA at bases 3012 to 3027, except for the thymidine (underlined) at base 3025 seen in wild-type HAV cDNA. A 5′ phosphate residue was added to the oligonucleotide by using polynucleotide kinase.

Site-directed mutagenesis was performed by the method of Zoller and Smith (29). Briefly, an oligonucleotide (15-mer) was synthesized whose sequence corresponded to attenuated HAV at about 500 bases 5′ to the sequence of the kinase-treated oligonucleotide. The kinase-treated oligonucleotide and its corresponding 15-mer were annealed to plasmid pTZHAV*Sac*Iss. The large (Klenow) fragment of *E. coli* DNA polymerase I and T4 DNA ligase were added, and the mixture was incubated at 15°C overnight. About 10 ng of the reaction was used to transform *E. coli* HB101, and the resulting bacterial colonies were screened for plasmids containing HAV inserts with the mutation. Screening was performed with the oligonucleotide (3025A) that had been kinase treated with [γ-^{32}P]ATP.

The mutation was verified by sequencing by the method of Zagursky et al. (28). A plasmid containing the mutation was digested with *Sac*I, and the HAV cDNA insert was purified from low-melting-point agarose and ligated to the appropriate *Sac*I restriction fragment from pHAV/7 to produce full-length HAV with the desired mutation. The full-length plasmid was screened with additional restriction enzymes to ensure that the *Sac*I insert was in the correct orientation, and the mutation was again confirmed by sequencing.

In vitro transcription and transfection. Full-length HAV cDNAs were digested with *Hae*II [cuts immediately after the poly(A) tail of HAV], and transcriptions were performed as described previously (6). The resulting RNA was transfected into AGMK cells (second passage) (6). When maximal levels of HAV antigen were detected in cells by fluorescein-labeled antibody (16), the cells were harvested and suspended in 1 ml of Dulbecco modified Eagle medium with 10% (vol/vol) fetal bovine serum. Cells were lysed by three cycles of freezing, thawing, and sonication.

Titration of HAV. After transfection of AGMK cells with RNA transcripts from full-length attenuated HAV cDNA (pHAV/7), the virus titer in the cell lysate was determined in BSC-1 cells. This known-titer, transfection-derived virus was used as a reference virus for additional titrations.

AGMK cell lysates were prepared from cells that had been transfected with RNA transcripts from (i) attenuated HAV cDNA (reference virus), (ii) chimeric HAV cDNAs, (iii) mutagenized cDNA, and (iv) noninfectious HAV cDNA (control). In addition, a homogenate containing wild-type virus was prepared from marmoset liver. This wild-type virus, not derived from transfection, had a titer of 10^3 chimpanzee 50% infectious doses (14). The AGMK cell lysates and liver homogenate were incubated with proteinase K and sodium dodecyl sulfate, and the nucleic acid was extracted with phenol and chloroform (25). Half-log dilutions of the mixtures were applied to nitrocellulose paper in a manifold well apparatus, and the paper was baked under vacuum. Nucleic acid on the nitrocellulose paper was hybridized with a radiolabeled HAV cDNA probe (prepared from a restriction fragment of pHAV/J [25]), and autoradiography was performed. The intensities of the signals in cell lysates containing chimeric and mutagenized HAVs and in homogenates containing wild-type HAV were compared

with those of the reference virus lysate and the control lysate.

Titration of HAV based on quantitation of viral RNA by hybridization shows a linear correlation with virus infectivity in cell culture (25). Although titrations of HAV were performed in an attempt to prepare inocula with identical titers for animal studies, this may not have been necessary; increasing the inoculum of HAV may shorten the incubation period for hepatitis but generally does not result in an increase in the incidence or severity of hepatitis (14).

Cell culture studies. Secondary AGMK cells were infected with virus dilutions from attenuated, chimeric, mutagenized, and wild-type viruses that contained the same amount of HAV RNA as in 5×10^5 50% tissue culture infectious doses ($TCID_{50}$) of the reference virus lysate. Cells were incubated with the virus for 45 min, medium was added, and the cells were plated onto glass cover slips. Cells infected with each virus were assayed each week with fluorescein-labeled antibody (16) for production of HAV antigen.

Animal studies. Cell lysates from AGMK cells infected with attenuated (HAV/7), mutated (HAV/3025A), or chimeric viruses were diluted with fetal bovine serum to produce a 0.5-ml inoculum containing the same amount of HAV RNA as in 10^5 $TCID_{50}$ of the reference virus lysate. The diluted lysates were inoculated intravenously into marmosets (*Saguinus mystax*).

Fifteen micrograms of pHAV/WT RNA or 45 μg of pHAV/7 RNA was dissolved in 0.5 ml of HBSS buffer (21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose [pH 7.05]) with DEAE-dextran (0.5 mg/ml) and inoculated into marmosets by direct intrahepatic injection. In addition, 45 μg of a pool of pHAV-AWW, pHAV-AAW, and pHAV-WAW with DEAE-dextran was inoculated into marmosets by intrahepatic injection. Forty micrograms of pHAV/WT DNA or 45 μg of pHAV/7 DNA was dissolved in 0.5 ml of HBSS buffer, and 31.2 μl of 2 M $CaCl_2$ was added to form a calcium phosphate precipitate (21). The DNA precipitate was inoculated into marmosets by intrahepatic injection. The animals were bled weekly for determination of isocitrate dehydrogenase (ICD) and anti-HAV antibody (HAVAB; Abbott Laboratories, North Chicago, Ill.) in serum. The data are expressed as geometric means $\times/\div$ the relative standard errors.

RESULTS

Transfections. Chimeric cDNAs were constructed by dividing the HAV genome into three portions: the 5' noncoding region, the P1 region (which encodes viral capsid proteins), and the P2-P3 region (which encodes nonstructural proteins). By using wild-type (W) and attenuated (A) HAV cDNAs, the six possible combinations of chimeric full-length HAV cDNA were prepared. RNA transcripts from three of the chimeric cDNAs induced production of HAV antigen in cell culture (Table 1). Virus subsequently obtained from these cells was infectious in marmosets (see below).

HAV antigen was detected in AGMK cells transfected with RNA transcripts from pHAV-WWA, pHAV-AWA, and pHAV-WAA by 3 weeks posttransfection. The three different transfected cell cultures were all harvested at 7 weeks posttransfection. AGMK cells transfected with cDNA from HAV/WT or RNA transcripts from pHAV/WT, pHAV-AAW, pHAV-AWW, or pHAV-WAW all failed to produce HAV antigen (Table 1). Cells that received the latter three RNA transcripts were monitored for 11 weeks posttransfection.

TABLE 1. In vitro transfections with HAV cDNA and its RNA transcripts

Transfection of secondary AGMK cells	HAV production
pHAV/7 RNA	+
pHAV/7 DNA	+
pHAV/3025A RNA	+
pHAV/WAA RNA	+
pHAV/WWA RNA	+
pHAV/AWA RNA	+
pHAV/AAW RNA	−
pHAV/AWW RNA	−
pHAV/WAW RNA	−
pHAV/WT RNA	−
pHAV/WT DNA	−

A point mutation at base 3025 was engineered into the attenuated cDNA so that the sequence of capsid protein VP1 would be identical to that of the wild-type virus. Transfection of RNA transcripts from this mutagenized cDNA into AGMK cells induced HAV production.

Titration of HAV. Titrations were performed by quantitation of HAV RNA, since quantitation by cell culture titer could have resulted in a higher apparent titer for cell culture-adaptated virus (attenuated HAV) than for viruses less well adaptated to growth in vitro (chimeric HAVs). The signal intensities observed on autoradiograms of extracts of cell lysates from chimeric viruses were compared with the signal of the reference virus (attenuated HAV lysate). On the basis of this comparison, 1-ml lysates obtained from 25-cm^2 flasks (containing about 10^6 AGMK cells) transfected with RNA transcripts from pHAV-AWA, pHAV-WAA, and pHAV-WWA each contained the same amount of HAV RNA as in 1 ml of the reference virus lysate. Since the reference virus lysate had $10^{7.8}$ $TCID_{50}$ of HAV per ml, cell lysates from each chimeric virus contained HAV at a titer of about $10^{7.8}$/ml. By the same comparison, AGMK cells transfected with RNA transcripts from noninfectious HAV cDNA (prepared from an earlier HAV cDNA construct) contained less than 10^3 $TCID_{50}$ of HAV per ml (25).

Cell culture studies. At 1 week postinoculation with transfection-derived viruses, about 20% of cells infected with HAV/7, HAV-AWA, or HAV/3025A produced HAV antigen. In contrast, after 1 week, less than 5% of cells infected with the wild-type virus (not derived from transfection) or with HAV-WAA or HAV-WWA produced viral antigen. With increasing time, cells infected with transfection-derived viruses produced increasing amounts of HAV antigen; by 3 weeks, at least 70% of cells produced viral antigen, and by 4 weeks, nearly 100% of cells produced viral antigen. In contrast, HAV antigen was undetectable in cells infected with the wild-type virus from weeks 2 to 4 postinoculation, and by week 6, only 10% of the cells produced viral antigen.

Animal studies. Two marmosets inoculated with transfection-derived HAV/7 (containing HAV RNA equivalent to 10^5 $TCID_{50}$ of the reference virus) developed anti-HAV antibodies within 10 weeks of inoculation (Table 2). When combined with results obtained with four marmosets inoculated with $10^{6.9}$ $TCID_{50}$ of HAV/7 from a previous study (6), the geometric mean ICD peak for the six animals was 2,339 $\times/\div$ 1.22 sigma U/ml. The geometric mean ICD peak was 1,619 $\times/\div$ 1.19 sigma U/ml for animals that received HAV/3025A, 2,213 $\times/\div$ 1.21 sigma U/ml for animals that received HAV-WAA, 2,571 $\times/\div$ 1.79 sigma U/ml for animals that received HAV-AWA, and 3,553 $\times/\div$ 1.15 sigma U/ml for animals that received

TABLE 2. Liver enzymes of marmosets that received transfection-derived viruses

Inoculum and marmoset no.	Peak ICD level (sigma U/ml)	No. of weeks postinoculation until:	
		Peak ICD level	Positive anti-HAV antibody titer
HAV/7			
460	3,230	9	8
517	2,023	10	10
HAV/3025A			
501	1,419	8	6
506	1,315	2	5
534	2,276	12	8
HAV/WAA			
532[a]	1,523	11	11
552	2,430	5	3
485	2,930	8	6
HAV/AWA			
479	8,147	7	7
489	1,676	7	7
522	1,245	9	7
HAV/WWA			
525	3,265	5	5
526	3,173	8	5
528	5,298	5	4
529	2,904	7	5

[a] Serum for ICD determination was not obtained 4 weeks before seroconversion for this animal.

HAV-WWA. In contrast, animals that received wild-type HAV HM-175 (not derived from transfection) had a geometric mean ICD peak of 8,533 $\times / \div$ 1.34 sigma U/ml (14; unpublished data). Wild-type virus obtained from marmoset liver was used to inoculate animals, because virus could not be obtained from transfection of cells with cDNA or RNA transcripts from pHAV/WT. Inoculation of animals with wild-type virus was performed before and shortly after, but not concurrently with, inoculation with transfection-derived viruses. Attempts to infect marmosets by intrahepatic inoculation of HAV cDNA or RNA transcripts derived from HAV cDNA were unsuccessful.

DISCUSSION

Comparison of the nucleotide sequences of wild-type and attenuated HAV HM-175 indicates that at most, 24 nucleotide changes are responsible for cell culture adaptation and attenuation of the virus in marmosets (5). Construction of chimeric HAVs containing portions of wild-type and attenuated genomes and oligonucleotide-directed mutagenesis of HAV cDNA allow assessment of which mutations contribute to cell culture adaptation and attenuation. Comparison of the growth in cell culture and infection of marmosets with these viruses suggests that the P2-P3 region of the genome plays a major role in cell culture adaptation and attenuation.

Similar studies have been performed with infectious cDNA derived from wild-type and attenuated (Sabin) strains of type 1 and type 3 poliovirus. There are 10 nucleotide differences between wild-type and attenuated type 3 poliovirus (23); however, only two of these changes are responsible for attenuation. Nucleotide changes at bases 472 (in the 5′ noncoding region) and 2034 (which results in an amino acid change in capsid protein VP3) account for the attenuation phenotype (1). There are 57 nucleotide differences between wild-type and attenuated poliovirus type 1 (18). In contrast to poliovirus type 3, attenuation of the vaccine (Sabin) strain of type 1 poliovirus is due to several nucleotide changes distributed over wide areas of the genome. The 5′ noncoding region has the strongest influence on attenuation; however, nucleotide changes in the P1, P2-P3, and 3′ noncoding regions all contribute to attenuation (17, 19).

On the basis of the findings that the 5′ noncoding region and capsid region are primarily responsible for attenuation of poliovirus types 1 and 3, we constructed chimeric HAVs in which the 5′ noncoding, P1 (capsid), and P2-P3 (nonstructural protein) regions were derived from either attenuated (A) or wild-type (W) virus.

The P2-P3 region may be responsible for cell culture adaptation of HAV HM-175 to AGMK cells; the 5′ noncoding region may also contribute to adaptation, but to a lesser degree. All three constructs in which the P2-P3 region was derived from wild-type HAV cDNA (pHAV-AAW, pHAV-AWW, and pHAV-WAW) failed to produce HAV antigen in cell culture, while the three constructs in which this region was derived from attenuated (cell culture-adapted) cDNA were infectious. Thus, the three viruses with wild-type sequences in this region may have grown so poorly in cell culture that they could not be detected. The infectivity of picornavirus RNA is several orders of magnitude lower than the infectivity of the virus (15). Since wild-type HAV HM-175 requires up to 11 weeks to reach maximum titers in cell culture (8), the transfection assay used may not have been sensitive enough for constructs with wild-type sequences in the P2-P3 region to yield detectable HAV. Alternatively, an unidentified mutation in the parent wild-type cDNA used to construct these clones may have resulted in production of a nonviable virus. This mutation, if present, occurred during cDNA synthesis from HAV RNA, since the sequence of the P2-P3 region of pHAV/WT was identical to that of the parent clones used to construct full-length cDNA. It is uncertain why intrahepatic inoculation of HAV cDNA and RNA (including RNA that was infectious in cell culture) failed to produce infection in the animals.

The complete nucleotide sequences of two cell culture-adapted HAV HM-175 viruses have been determined. When the predicted amino acid sequence of wild-type HAV HM-175 was compared with that of the cell culture-adapted viruses, the P2-P3 region contained 87.5% (7 of 8) of the amino acid changes for virus passaged 16 times in cell culture (13) and 83.3% (10 of 12) of the changes for virus passaged 35 times in cell culture (5). In contrast, the P2-P3 region contained only 64.5% of the amino acids of the HAV polyprotein. Thus, the P2-P3 region, which may be responsible for cell culture adaptation, sustained a disproportionately large number of amino acid changes in this region during passage in cell culture.

The 5′ noncoding region may also play a role in cell culture adaptation. Attenuated (HAV/7), mutagenized (HAV/3025A), and HAV-AWA viruses (all of whose 5′ noncoding regions were derived from attenuated cDNA) grew more efficiently than HAV-WWA or HAV-WAA during the first 2 weeks in cell culture. Thereafter, cells infected with HAV-WWA or HAV-WAA produced levels of viral antigen similar to those of cells infected with HAV/7 or HAV-AWA. The sequence of the 5′ noncoding region of HAV HM-175 has been determined for three cell culture-adapted viruses all derived from the same parent isolate. When the sequence of the 5′ noncoding region of wild-type HAV HM-175 was compared with those of the cell culture-adapted viruses, there were 6, 7, and 8 nucleotide changes for viruses passaged 16 (13), 35 (5), and 59 (22) times in cell culture, respectively. Many of these changes associated with cell

culture adaptation occur in a small area (bases 124 to 207) of the 5′ noncoding region (13).

The 5′ noncoding region and the P1 (or capsid) region of HAV HM-175 do not seem to play a major role in attenuation of HAV HM-175. Marmosets inoculated with HAV-WAA and HAV-AWA had liver enzyme levels that were similar to those seen in animals inoculated with attenuated virus. The change at base 3025 (glutamic acid to valine in VP1), which causes the only change in the charge of an amino acid in the capsid region, did not affect attenuation of the virus. Marmosets that received HAV-WWA had liver enzyme levels that were slightly greater than those seen in animals inoculated with attenuated virus. Since the P2-P3 region of HAV-WWA contains attenuated sequences, the P2-P3 region must play an important role in attenuation of the virus.

The importance of the P2-P3 region for cell culture adaptation and attenuation of HAV emphasizes that intracellular events during viral replication play a role in attenuation. This region is thought to encode a viral protease, a polymerase, a genome-linked protein (VPg), and other proteins important for replication (7, 24, 27). The P2-P3 region plays a minor role in attenuation of poliovirus type 1 (17, 19) and has no contribution to attenuation of poliovirus type 3 (1).

The exact nucleotide changes in the P2-P3 region responsible for attenuation of HAV HM-175 are unknown. Identification of these changes will have important implications for development of live-HAV vaccines. Location of these changes would provide genetic markers which could be useful for testing vaccine lots and for monitoring virus excreted from vaccinees. Passage of attenuated HAV HM-175 in a chimpanzee did not result in loss of attenuation (10). Although passage of poliovirus type 3 in humans resulted in a more neurovirulent virus (9), neurovirulent mutants of poliovirus type 1 are very rare (2). These findings suggest that attenuation of HAV HM-175 is more likely to resemble attenuation of poliovirus type 1 in which multiple nucleotide changes contribute to attenuation. If the results of further studies of attenuation of HAV support this prediction, these findings could be relevant for development of future HAV vaccines. Thus, if several nucleotide changes are responsible for attenuation of HAV HM-175, a live-virus vaccine derived from this virus may have a low risk for reversion to virulence in humans.

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