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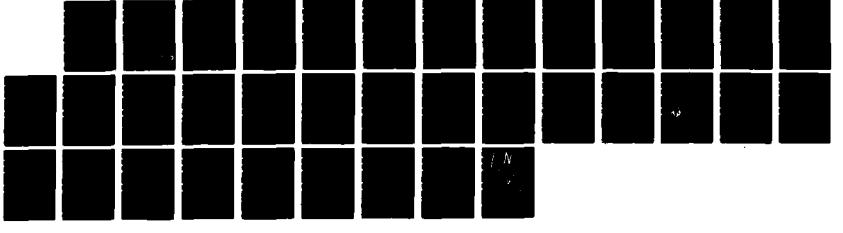

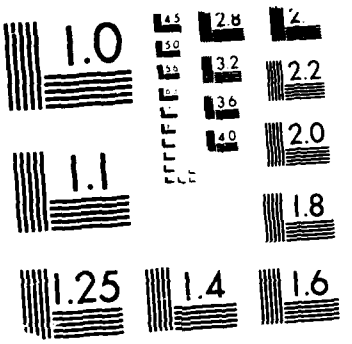

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NEW APPROACHES TO ATTENUATED HEPATITIS A VACCINE DEVELOPMENT: CLONING AND SEQUENCING OF CELL-CULTURE ADAPTED VIRAL cDNA

ANNUAL REPORT

Stanley M. Lemon, M.D.

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Research efforts under this contract have centered on the determination of molecular changes associated with adaptation of hepatitis A virus to growth in cell culture, and with attenuation of virus virulence. To determine the molecular changes associated with adaptation of hepatitis A virus (HAV) to growth in cell culture, the genome of a cell culture-adapted variant of HM175 strain HAV (p16 HM175, 16th *in vitro* passage level) was molecularly cloned and the complete nucleotide sequence of the virus determined. Compared with wild-type virus, p16 HM175 replicates efficiently in monkey kidney (BS-C-1) cells (approximately 58 RNA-containing particles per one infectious unit, compared with 2.4×10^5 for wild-type HM175). The nucleotide sequence of p16 HM175 revealed a total of 19 mutations from the wild-type genome, including five
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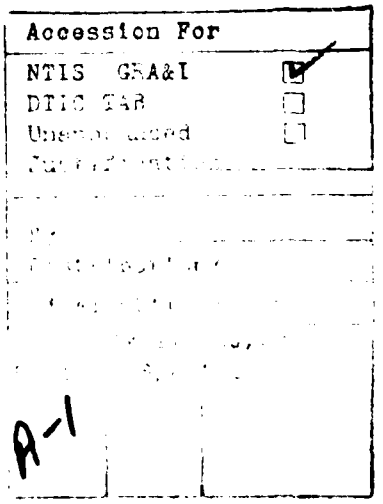
mutations in the 5' nontranslated region, one mutation in the 3' nontranslated region, and 13 mutations predicting 8 changes in the amino acid sequences of HAV proteins. Only one amino acid substitution occurred among the capsid proteins (VP2), while others involved proteins 2A, 2B, 2C, VPg and 3Dpol. When the sequence of p16 virus was compared with that reported previously for an independently isolated, cell culture-adapted variant of HM175 virus (Cohen *et al.*, 1987), there were three identical mutations in nontranslated regions of the RNA, and four mutations involving identical amino acids in proteins VP2, 2B and 3Dpol. The distribution of these mutations within the genome suggests that changes in RNA replication may be of primary importance in adaptation of the virus to growth *in vitro*. These data are thus helpful in understanding the molecular basis of adaptation of HAV to cell culture and, since attenuation frequently accompanies adaptation of virus to growth in cell culture, may be of benefit in planning for attenuated vaccine development.

In addition, a cell culture-adapted variant of hepatitis A virus (HM175/S18), selected for resistance to neutralizing monoclonal antibody, has been examined for virulence in the New World owl monkey model. Monkeys challenged with approximately 5×10^4 RFU of HM175/S18 developed liver enzyme elevations and virologic markers of infection resembling those previously noted following wild-type virus challenge. Fecal shedding of viral antigen may have been less following challenge with the S18 virus than with wild-type virus, however. Virus shed in the stools of monkeys challenged with HM175/S18 was found to have reverted to neutralization susceptibility. Thus, passage of HM175/S18 in the owl monkey was associated with selection of revertant virus.

Methods for direct sequencing of viral RNA by primer extension have been optimized, and it is now possible to obtain sequencing information from as little as 2 ng of RNA template. This method is currently being applied to determination of strain-specific differences among various HAV isolates, and HAV antigen-positive fecal samples.



SUMMARY

Research efforts under this contract have centered on the determination of molecular changes associated with adaptation of hepatitis A virus to growth in cell culture, and with attenuation of virus virulence. To determine the molecular changes associated with adaptation of hepatitis A virus (HAV) to growth in cell culture, the genome of a cell culture-adapted variant of HM175 strain HAV (p16 HM175, $16^{\underline{th}}$ *in vitro* passage level) was molecularly cloned and the complete nucleotide sequence of the virus determined. Compared with wild-type virus, p16 HM175 replicates efficiently in monkey kidney (BS-C-1) cells (approximately 58 RNA-containing particles per one infectious unit, compared with 2.4×10^5 for wild-type HM175). The nucleotide sequence of p16 HM175 revealed a total of 19 mutations from the wild-type genome, including five mutations in the 5′ nontranslated region, one mutation in the 3′ nontranslated region, and 13 mutations predicting 8 changes in the amino acid sequences of HAV proteins. Only one amino acid substitution occurred among the capsid proteins (VP2), while others involved proteins 2A, 2B, 2C, VPg and 3Dpol. When the sequence of p16 virus was compared with that reported previously for an independently isolated, cell culture-adapted variant of HM175 virus (Cohen *et al.*, 1987), there were three identical mutations in nontranslated regions of the RNA, and four mutations involving identical amino acids in proteins VP2, 2B and 3Dpol. The distribution of these mutations within the genome suggests that changes in RNA replication may be of primary importance in adaptation of the virus to growth *in vitro*. These data are thus helpful in understanding the molecular basis of adaptation of HAV to cell culture and, since attenuation frequently accompanies adaptation of virus to growth in cell culture, may be of benefit in planning for attenuated vaccine development.

In addition, a cell culture-adapted variant of hepatitis A virus (HM175/S18), selected for resistance to neutralizing monoclonal antibody, has been examined for virulence in the New World owl monkey model. Monkeys challenged with approximately 5×10^4 RFU of HM175/S18 developed liver enzyme elevations and virologic markers of infection resembling those previously noted following wild-type virus challenge. Fecal shedding of viral antigen may have been less following challenge with the S18 virus than with wild-type virus, however. Virus shed in the stools of monkeys challenged with HM175/S18 was found to have reverted to neutralization susceptibility. Thus, passage of HM175/S18 in the owl monkey was associated with selection of revertant virus.

Methods for direct sequencing of viral RNA by primer extension have been optimized, and it is now possible to obtain sequencing information from as little as 2 ng of RNA template. This method is currently being applied to determination of strain-specific differences among various HAV isolates, and HAV antigen-positive fecal samples.

FOREWORD

The investigator has abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

TABLE OF CONTENTS

Report Documentation Page (DD Form 1473)

Summary----------2

Foreword----------3

Table of Contents----------4

Introduction----------5

Experimental Approach----------7

Research Progress----------7

Conclusions----------16

Tables: 1----------17

2----------18

3----------19

4----------20

5----------21

Figures: 1----------22

2----------23

3----------24

4----------25

5----------26

6----------27

Literature Cited----------28

Publications----------32

Personnel----------32

INTRODUCTION

Hepatitis A virus (HAV) is a human picornavirus with a worldwide distribution (Lemon, 1985). It poses a considerable threat to military forces of the United States due to its capacity for epidemic spread, and the fact that acute hepatitis A associated with primary infection with HAV is a protracted illness with a prolonged convalescence. At present, there is a low prevalence of antibody among active duty U.S. forces, indicating a high degree of susceptibility.

No vaccine is currently available for prevention of infection with this medically important virus. Three general approaches to HAV vaccines have been considered, including development of an inactivated, cell-culture derived vaccine (Binn *et al.*, 1985), an attenuated vaccine derived by extensive passage of virus *in vitro* (Provost *et al.*, 1982; Provost *et al.*, 1986), and more novel vaccines based on synthetic peptide or recombinant DNA technology. A detailed discussion of these approaches, all of which are beset with difficulties may be found in Report #1 of this contract, or in the review by Lemon (1985). This contract has focused on understanding the molecular basis of attenuation of HAV, as such an understanding might open new approaches to development of an economic and effective HAV vaccine.

Although attenuation of HAV has been shown to result from extended *in vitro* passage of virus and has been proposed as an approach to the development of attenuated vaccines (Provost *et al.*, 1982; Provost *et al.*, 1986). Wild-type HAV, recovered from feces or liver of infected primates, replicates slowly and to low titers in cell culture. Most strains of HAV do not induce cytopathic effects in cell cultures, and persistent infection is characteristic of this virus system. With successive passages, however, the virus becomes progressively adapted to growth *in vitro* (Binn *et al.*, 1984; Frösner *et al.*, 1979; Provost and Hilleman, 1979). Adaptation to growth in cell culture results in a shortening of the interval between inoculation of cultures and maximum virus yield as well as increases in the yield of virus, and appears to be a multi-step process. Passage of virus *in vitro* has also been associated with attenuation of virulence as evidenced by a reduction in the ability of the virus to induce liver injury in both experimentally challenged primates and man (Provost *et al.*, 1982; R.A. Karron, R. Daemer, J. Ticehurst, E. D'Hondt, H. Popper, K. Mihalik, J. Phillips, S. Feinstone, and R.H. Purcell, manuscript submitted). Extensively passaged virus may even be incapable of replication *in vivo*, as no clinical evidence of infection and no measurable antibody response has followed parenteral challenge of humans with very extensively passaged virus (Provost *et al.*, 1986). However, because some virus variants that are well adapted to growth in cell culture retain nearly unaltered virulence in some primate species (e.g., owl monkeys) (Lemon *et al.*, 1987a), attenuation and cell culture adaptation represent separate although closely related phenotypic characteristics of HAV.

The molecular mechanisms underlying either adaptation of HAV to growth in cell culture or attenuation of HAV remain unknown. Adaptation of HAV to growth in cell culture results in profound changes in the biologic characteristics of the virus and with continued passage of virus has been associated with a reduction in virulence in several different species of primates. A

primary effort under support of this contract has therefore been the cloning and sequencing of the genome of a cell culture-adapted variant of HM175 strain HAV, and the identification of mutations in this virus that were associated with adaptation of this virus to growth *in vitro*.

In addition, we have considered the possibility that selection of virus variants for resistance to neutralizing monoclonal antibody might be associated with attenuation of virus. Neutralization escape variants derived from a number of other viruses, including some picornaviruses, have been shown by other workers to be relatively attenuated. Along these lines, we recently isolated a number of variants of HM175 strain HAV that have been selected for resistance to murine monoclonal neutralizing antibodies (Stapleton, 1987). Neutralization-resistant variants of HAV were developed in an effort to further define the molecular structure of the major neutralization epitopes of the virus. Individual variants demonstrate resistance to each of a panel of four neutralizing monoclonal antibodies. Neutralization resistance is related to reduced binding of the monoclonal antibody to the mutant virus particles. Despite this, the variants grow well *in vitro* and are detectable in conventional immunoassays utilizing polyclonal antisera. Because the monoclonal antibodies against which these mutants were selected are directed against immunodominant domains on the virion surface, several variant viruses demonstrate a substantial level of resistance to polyclonal neutralizing antisera as well (Stapleton, 1987). This relative neutralization resistance extends even to pooled human immune serum globulin.

The fact that there is no epidemiologic or clinical evidence to suggest the evolution of antigenic variants of HAV *in vivo* suggests the existence of tight biologic constraints on the degree of variation permissible in the immunodominant epitopes of the virus. These constraints do not appear to be operative *in vitro*. One possible explanation is that the conserved antigen represents part of the hepatocyte receptor-binding domain on the virion surface. It was of considerable interest, therefore, to determine whether such variant viruses remain infectious or have altered pathogenicity in primate hosts. Accordingly, during the past contract year, we have collaborated with LTC J. LeDuc of USAMRIID and Dr. L. Binn of WRAIR in an effort to determine whether the S18 variant of HM175 virus, selected for high level resistance to monoclonal K2-4F2, is attenuated for New World owl monkeys, and whether infection of the owl monkey with this virus is associated with reversion of neutralization resistance.

As a third area of investigation, we have optimized methods permitting the direct sequencing of viral RNA by primer extension in the presence of dideoxynucleotides. We have thus far applied this approach only to the sequencing of RNA from cell culture-derived virus. However, the amounts of RNA required (2-10 ng) should permit sequencing of fecal virus after partial purification. Thus, in the next year of the contract, we intend to apply this approach to the determination of strain-specific differences in sequences of HAV strains.

EXPERIMENTAL APPROACH

1. We have determined the complete nucleotide sequence of HM175 virus at the 16th *in vitro* passage level, and have compared the sequence of this virus to that reported previously for its wild-type parent (Cohen *et al.*, 1987a). These studies have determined that adaptation of this virus to growth *in vitro* was associated with a limited number of mutations, and have suggested a possible molecular basis for attenuation of the virus.

2. We evaluated a cell culture-adapted, neutralization-resistant variant of HM175 strain HAV (HM175/S18) for hepatovirulence in seronegative owl monkeys. Approximately 5.3×10^4 radioimmunofocus-forming units of p1 HM175/S18 were given intravenously to 6 susceptible owl monkeys. Outcome was compared with that of 5 animals infected previously with wild-type (wt) HM175. Hepatovirulence of HM175/S18 was associated with reversion to the wild-type neutralization-susceptible phenotype.

3. We have optimized conditions for direct sequencing of viral RNA, and are now able to determine sequence by primer extension with as little as 2-10 ng viral RNA as template.

RESEARCH PROGRESS

Cloning and sequencing of p16 HM175 virus. For analysis of the genetic changes accompanying adaptation of HAV to growth *in vitro*, we determined the sequence of HM175 virus at the 16th *in vitro* passage level, and compared its sequence with that of wild-type virus. This virus was initially isolated in primary African green monkey kidney cells (AGMK) from an homogenate of liver tissue obtained from a marmoset inoculated with wild-type HM175 strain HAV (6th marmoset-passage), a gift of S.M. Feinstone and R.H. Purcell, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA. The origin of this strain of HAV has been described (Gust *et al.*, 1985). Virus was adapted to growth *in vitro* as described previously (Binn *et al.*, 1984), and after 10 AGMK passages was passed once in continuous green monkey kidney cells (BS-C-1). Virus was then twice plaque-purified (Lemon and Jansen, 1985), and amplified at the sixteenth *in vitro* passage level (p16 HM175 virus) for isolation of viral RNA. Wild-type HM175 virus, employed for studies determining the efficiency of wild-type virus replication *in vitro*, was recovered from the feces of a New World owl monkey (*Aotus trivirgatus*) inoculated intravenously with a human fecal suspension containing HM175 strain HAV (LeDuc *et al.*, 1983).

The efficiency of replication of p16 HM175 virus in BS-C-1 cells was compared with that of wild-type virus by determining the proportion of virus particles capable of inducing foci of infection detectable under agarose overlays 2 weeks after inoculation of cell cultures (Figure 1). The wild-type virus used for this experiment was a suspension of feces obtained from an owl monkey (*Aotus trivirgatus*) experimentally infected with HM175 virus from human stool (LeDuc *et al.*, 1983). The number of RNA-containing particles was estimated in both wild-type and p16 cell culture inocula by quantitative cDNA-RNA hybridization (Jansen *et al.*, 1985). Foci of viral replication, identified by staining infected cell sheets with radiolabelled polyclonal human antibody

(Lemon and Jansen, 1985), were considerably larger in cells inoculated with p16 virus than in cells inoculated with wild-type virus (Figure 1), indicating more efficient spread of the p16 virus to adjacent cells. Moreover, cDNA-RNA hybridization results (not shown) indicated that the p16 virus inoculum contained approximately 58 genome copies (i.e., RNA containing particles) per RFU, compared with 2.4×10^5 particles per RFU in the wild-type virus inoculum. Thus, there was an approximately 4000-fold difference in the ability of the cell culture-adapted (p16) virus and wild-type virus to initiate foci of replication in BS-C-1 cells.

Molecular cloning of p16 HM175 virus cDNA. cDNA clones were derived from p16 HM175 virus RNA by cloning cDNA-RNA hybrid molecules into the *PstI* site of plasmid pBR322. The position and orientation of insert fragments within the HAV genome was determined by slot-blot hybridization with wild-type cDNA probes and by selected mapping of restriction endonuclease sites (See Report #1 for this contract for details of this work). Precise mapping of cDNA inserts was accomplished by dideoxynucleotide sequencing of plasmid DNA using pBR322 *PstI* site-specific oligonucleotide primers (Pharmacia). Insert fragments from p16 cDNA clones were subcloned into the phage vector M13mp8 or M13mp19 and subjected to rapid sequencing using the dideoxynucleotide method of Sanger (Sanger *et al.*, 1977). As physical mapping indicated few differences between p16 virus cDNA and wild-type cDNA in terms of major restriction sites, a sequencing strategy was adopted which involved the subcloning and subsequent sequencing of overlapping restriction fragments. Where necessary, additional sequence information was obtained by direct sequence analysis of plasmid DNA using HAV-specific oligonucleotide primers prepared in the laboratory of C. Hutchison of the University of North Carolina or provided as a gift by J. Cohen of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. The derived sequence for p16 HAV cDNA was assembled with MicroGenie software (Beckman) and compared with that reported by Cohen *et al.* for wild type (3rd marmoset passage) HM175 virus (Cohen *et al.*, 1987a), and p35 chimpanzee-attenuated HM175 (Cohen *et al.*, 1987). Except where noted, mutations from the wild-type virus sequence were considered present only when found in at least two independent cDNA clones derived from p16 virus.

The cDNA-RNA cloning strategy adopted permitted clones bearing stable HAV cDNA inserts to be obtained from minimal quantities of viral RNA. This approach was taken in order to limit the number of virus passages between plaque purification and isolation of viral RNA for molecular cloning. Clones derived from cDNA synthesized with oligo-dT_{12-18} as primer were nearly always restricted to the 3' terminus of the genome, while clones of cDNA synthesized with random oligonucleotide primers were randomly distributed within the genome. Several clones containing 5' terminal sequences were identified, reflecting one advantage of the cloning of cDNA-RNA hybrid molecules (Cann *et al.*, 1983; Stanway *et al.*, 1983). Clones with overlapping inserts spanning the HAV genome were identified by hybridization, limited restriction mapping, and direct sequencing of plasmid DNA (Figure 2), and selected cDNA insert fragments were subcloned into M13 vectors for sequencing. The sequence of the complete genome was determined, with over 95% of the sequence confirmed in sequencing reactions involving both strands of cDNA.

Mutations in cell culture-adapted p16 HM175. A total of 19 mutations (20 base changes) was evident when the sequence of p16 HM175 was compared with that previously reported for wild-type HM175 virus (Cohen *et al.*, 1987a) (Table 1). Except where indicated in Table 1, each of these mutations was documented in at least two independent cDNA clones derived from p16 virus. Mutations clustered in the 5′ nontranslated region of the genome, and in the P2 and P3 regions (Figure 3). Altogether, there were 13 mutations in the large open reading frame, predicting 8 amino acid substitutions in the proteins of the p16 virus (Table 1).

(i) 5′ nontranslated RNA. There were five mutations (six nucleotide changes) in the 5′ nontranslated RNA of the p16 virus genome. At the 5′ terminus, there was the addition of a U, confirmed in multiple cDNA clones, and between base positions 203-7 a UU deletion (map positions refer to published wild-type HM175 sequence) (Cohen *et al.*, 1987a). Thus, the length of the 5′ nontranslated region was shortened by one base from the wild-type genome. The remaining changes included a G → A at position 8, an A → G at position 152 and a U → G at position 687.

(ii) P1 region. The capsid-encoding region was relatively free of mutations compared with other regions of the genome. One nucleotide substitution from the reported wild-type sequence was present in the putative VP3 coding region (position 1742), but did not predict a change in the amino acid sequence of the capsid protein. This silent "mutation" has been found in all cell-culture adapted HM175 cDNA clones reported to date (Cohen *et al.*, 1987; Ross *et al.*, 1986), and may in fact represent the true wild-type sequence, as the base in question has been determined in only a single wild-type cDNA clone (Cohen *et al.*, 1987). In addition, there was a mutation predicting a lysine to arginine substitution in amino acid residue 54 of VP2 (map position 964). This conservative substitution was the only predicted change in the capsid structure of the p16 virion.

(iii) P2 region. There were six mutations in the P2 region, predicting four amino acid substitutions. According to the HAV polyprotein cleavage sites proposed by Cohen *et al.* (1987a), these changes included one amino acid substitution in protein 2A, two in protein 2B and one in protein 2C (Table 1).

(iv) P3 region. In the P3 region, there were 5 nucleotide substitutions predicting three amino acid substitutions. There were no predicted amino acid substitutions in proteins 3A or 3Cpro, the putative HAV protease. In the VPg (3B) protein (Weitz *et al.*, 1986), however, there was a glutamine to histidine substitution at amino acid 11. Within the proposed polymerase coding region there were three mutations, two of which predicted amino acid substitutions in 3Dpol. These substitutions included a change from aspartic acid to glycine at residue 67 and from serine to threonine at residue 192.

(v) 3′ non-translated RNA. A single mutation was present in the 3′ nontranslated RNA, involving an A → G substitution at map position 7430.

Comparison of p16 virus sequence with that of other cell-culture adapted HM175 variants. The complete sequence of an HM175 variant that had been adapted to growth in African green monkey kidney cells was recently reported

by Cohen *et al.* (1987). This p35 HM175 variant was isolated directly from human feces and adapted to growth *in vitro* without prior marmoset passage. It has been shown to be attenuated in chimpanzees, and to a lesser degree in marmosets (R.A. Karron, R. Daemer, J. Ticehurst, E. D'Hondt, H. Popper, K. Mihalik, J. Phillips, S. Feinstone, and R.H. Purcell, manuscript submitted). It is likely that this virus is significantly more attenuated than the p16 variant described in this paper (see Discussion). In addition, the partial sequence of a third variant (p59) of HM175 virus has been reported by Ross *et al.* (1986). The p59 virus had been adapted to growth in monkey kidney cells following six marmoset passages, *in vivo* passages shared in common with the wild-type virus (first 3 marmoset passages) cloned by Ticehurst et al. (Cohen *et al.*, 1987a) and the p16 virus (all 6 marmoset passages). The passage history of these three cell culture-adapted HM175 variants, each of which has been independently adapted to growth in primary or continuous monkey kidney cells, is depicted in Figure 4. Mutations common to these indeperdently isolated viruses can be expected to have special relevance to the ability of virus to replicate in cell culture.

A comparison of the available sequence data shows that HM175 variants sequenced at the 16th, 35th and 59th passage levels have 6, 7, and 8 base substitutions, deletions or additions respectively, within the 5' nontranslated region of the viral genome. (The 5' terminal 29 bases have not been reported for the p59 virus.) The p16 and p35 sequences share a common mutation at base position 152 (A → G) and a common U deletion between 203-7. This latter change is also found in p59 virus, and is the only 5' mutation common to all three variants. The U → G substitution found at map position 687 in p16 virus is also present in the p59 variant, but is not found in p35 virus. Therefore, this mutation may have occurred during the last three marmoset passages which the p16 and p59 viruses shared in common (Figure 4). The region representing map positions 124 through 207 appears especially prone to develop mutations during *in vitro* passage of the virus (Jansen *et al.*, 1987). Within this region, 3.6% of base residues were altered in p16 virus, as were 8.4% in the p35 sequence, and 2.4% in the p59 sequence. This mutational "hot spot" also contains two of the three 5' region mutations (map positions 152 and 203-7) common to two or more variants.

The full-length sequences of the p16 and p35 HM175 variants are remarkably concordant in terms of the distribution of mutations within the genome and share a striking number of identical or very similar mutations. In addition to the two identical mutations in the 5' nontranslated region, there are four mutations involving identical amino acids in the proteins of p16 and p35 viruses (Table 2). This represents 50% of the predicted amino acid substitutions present in p16 virus, and 33% of those present in p35 virus. In the P1 region, the single mutation (lysine → arginine) in VP2 of p16 virus is also present in p35 virus; a second mutation found in VP1 of p35 virus is not present in p16 HM175. In the P2 region, each variant has one mutation in protein 2A, and two in protein 2B (including a common alanine → valine substitution at residue 72). The greatest difference between the two viruses is in the 2C protein, in which there is one predicted amino acid substitution in p16 virus and four substitutions in the p35 variant. Neither virus has amino acid substitutions in proteins 3A or 3Cpro, but each virus has one substitution (at different sites) among the 23 amino acids of VPg (protein 3B). Of particular

interest, there are two predicted amino acid substitutions in 3Dpol of both p16 and p35 viruses. These substitutions involve identical amino acid residues, including an identical substitution (serine → threonine) at residue 192, and different mutations involving aspartic acid 67. In p16, a mutation at map position 6148 predicts a substitution of this residue with glycine, while in p35 virus a mutation in the adjacent nucleotide (6147) predicts that this amino acid is replaced with asparagine. Lastly, a single, common mutation (A → G at position 7430) is present in the 3' non-translated region of both variants. With the possible exception of the apparent change at map position 1742, these changes do not reflect errors in the reported sequence of wild-type HM175, as the wild-type sequence at the seven other sites of common mutations indicated in Table 2 was confirmed by Cohen *et al.* in at least two independent wild-type cDNA clones (Cohen *et al.*, 1987).

Mechanisms of cell culture-adaptation and attenuation of HAV. The mutations identified in the p16 variant of HM175 virus provide information that is helpful in understanding the molecular basis of adaptation of HAV to cell culture. Because adaptation of HAV to growth in cell culture has been the first step in the development of all attenuated variants examined to date (Provost *et al.*, 1982; Provost *et al.*, 1986; R.A. Karron, R. Daemer, J. Ticehurst, E. D'Hondt, H. Popper, K. Mihalik, J. Phillips, S. Feinstone, and R.H. Purcell, manuscript submitted), an understanding of the molecular basis of attenuation of HAV is dependent on understanding the events accompanying adaptation of the virus to cell culture.

The p16 variant is highly adapted to growth *in vitro*. However, experimental infection of New World owl monkeys with a neutralization-resistant variant of HM175 virus that is closely related by passage history to p16 virus has recently demonstrated nearly unaltered virulence at the 22nd *in vitro* passage level (Lemon *et al.*, 1987a) (see below). This suggests that the mutations present in p16 virus have not resulted in significant attenuation for owl monkeys. Thus, comparison of the p16 virus (cell culture adapted, but most likely not attenuated) sequence with that of the p33 virus (cell culture adapted, attenuated) (Cohen et al., 1987) provides useful insights into the possible molecular basis of cell culture adaptation and attenuation of HAV.

The lack of mutations predicting changes in the surface structures of the p16 virus argues against alterations in virus receptor-binding activity as a mechanism of adaptation to growth *in vitro*. The mutation in VP2 that is common to both p16 and p35 variants appears unlikely to have induced changes in the receptor-binding activity of the virus, given that the position of this residue is probably deep within the capsid structure according to computer-generated alignments derived by A. Palmenberg (personal communication) and the known crystal structure of mengovirus (Luo *et al.*, 1987; M. Rossmann, personal communication). However, the precise location of this VP2 residue in the HAV capsid and the possible functional relevance of this mutation must await crystallographic determination of the HAV capsid structure. This change in VP2 does not appear to be essential for adaptation of virus to growth in cell culture, however, as it is not present in the cell culture-adapted p59 variant of HM175 (Ross *et al.*, 1986). Similarly, the absence of mutations in the putative protease, 3Cpro, or at proposed cleavage sites for polyprotein processing

in HAV (Cohen *et al.*, 1987a), suggests that changes in posttranslational processing do not play major roles in adaptation of virus to cell culture.

Mutations within the 5′ and 3′ nontranslated regions of the genomes of p16 and p35 viruses could affect priming or initiation of both positive and negative strand RNA replication. In addition, since guanidine resistance of poliovirus maps to the 2C protein of that virus (Pincus *et al.*, 1986), mutations present in the analogous HAV protein may also affect RNA replication. Lastly, the coevolution of related mutations in the putative polymerase (3Dpol) proteins of both p16 and p35 viruses (Table 2) argues that changes in polymerase function and hence RNA replication may be central to efficient growth *in vitro*. Such mutations could result in more efficient interactions between viral proteins, viral RNA and cell-specific proteins suspected to be involved in replication of picornaviral RNA (Andrews *et al.*, 1986; Morrow *et al.*, 1985). This interpretation is consistent with evidence suggesting that the restrictive event in HAV replication *in vitro* may be replication of the viral RNA (Anderson *et al.*, 1987). Host cell factors necessary for replication of viral RNA in cultured monkey kidney cells may be different from those available to the virus in the human hepatocyte, the cell type within which virus replication normally occurs *in vivo*. If so, mutations occurring during adaptation of virus to growth in cell culture and permitting more efficient utilization of host cell factors *in vitro* might be expected to lead to a reduction in the ability of virus to replicate within the hepatocyte *in vivo*. This hypothesis provides a possible explanation for the attenuation of HAV that has been associated with passage of the virus in cell cultures. It is consistent with the observation that attenuated HAV (specifically, the F′ variant of CR326 strain HAV) replicates only to low levels in nonimmune humans, as evidenced by delayed and relatively low level antibody responses (Provost *et al.*, 1986; S.M. Lemon and P. Provost, unpublished data). Attenuation of HAV, like attenuation of poliovirus (Sabin, 1985), may thus be related to mutations in several regions of the genome (Minor *et al.*, 1986; Omata *et al.*, 1986).

An effect on ribosome recognition or initiation of translation cannot be ruled out for the nucleotide changes found in the 5′ nontranslated region of the genomes of cell culture-adapted HAV variants, however, as the functions of the nontranslated RNA of picornaviruses remain poorly defined. Nor can the influence of mutations on viral assembly, or on the efficiency of virus uncoating following penetration be ascertained. The recent availability of an infectious molecular construct derived from the p35 variant of HM175 (Cohen *et al.*, 1987b) should, however, allow more precise mapping of viral functions involved in adaptation of virus to *in vitro* growth and may possibly shed light on their relevance to attenuation. These studies will be pursued in the next year of this contract.

Experimental infection of New World owl monkeys with neutralization resistant HM175/S18 virus. HM175/S18, a neutralization resistant variant of HM175 virus was isolated by repetitive cycles of neutralization with monoclonal antibody K2-4F2, followed by amplification and plaque-purification as described previously (Stapleton, 1987). Monoclonal K2-4F2 ascitic fluid (MacGregor, 1983) was purchased from the Commonwealth Serum Institute, Melbourne, Australia. HM175/S18 (total of 21 *in vitro* passages) was passaged

once at low multiplicity in the absence of antibody to produce a virus inoculum for primate infection. Virus (p1 HM175/S18) was harvested as cell culture supernatant fluid, extracted with an equal volume of chloroform, and stored frozen at −70 °C. Neutralization resistance was assessed by determining the reduction in titer of virus following a 2 hr incubation with a standardized concentration of antibody (Stapleton, 1987).

Experimental infection of healthy, seronegative New World owl monkeys (*Aotus trivirgatus*) was carried out as described previously (LeDuc, 1983). Baseline serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured weekly over a 6 week period preceding challenge. Approximately 5.3×10^4 RFU of p1 HM175/S18 virus was inoculated intravenously into each of six owl monkeys. Sera were collected twice weekly postinoculation and tested for liver enzyme (AST and ALT) activities and anti-HAV detectable by solid-phase radioimmunoassay or radioimmunofocus inhibition test. Feces were collected approximately 5 days each week and assayed for HAV antigen and infectious virus after preparation of 10% (w/w) fecal suspensions. Selected serum specimens were similarly assayed for infectious virus.

Induction of hepatitis in experimentally infected owl monkeys. These studies were done in collaboration with LTC J. LeDuc of USAMRIID and Dr. L. Binn of WRAIR. Six seronegative owl monkeys were inoculated intravenously with 5.3×10^4 RFU of p1 HM175/S18 virus. All six monkeys seroconverted to anti-HAV positivity in the HAVAB radioimmunoassay by 32 days postinoculation. Elevations of serum ALT activities were noted in all six monkeys, as was fecal shedding of HAV antigen detectable by solid-phase radioimmunoassay (Table 3). The response to intravenous inoculation of HM175/S18 virus was compared to that following intravenous inoculation of wild-type HM175 virus (human fecal suspension), given to five owl monkeys in a previous study (LeDuc, 1983). The mean maximum fold-increase in serum ALT in monkeys infected with HM175/S18 was 6.5 (range 3.8-11.3), compared with 8.5 (range 2.6-16.5) in monkeys infected with wild-type virus. The period from inoculation of HM175/S18 to maximal ALT elevation was 28.2 days (range 22-33), compared with 34.8 days (range 25-43) for wild-type virus. Antigen shedding in feces appeared to be reduced in HM175/S18 virus-infected monkeys (mean maximum S/N 16.2, range 3.4-36.4) compared with monkeys infected with wild-type virus (mean maximum S/N 73.2, range 24-112). Because HM175/S18 virus is highly adapted to growth in cell culture, it was possible to efficiently recover virus from fecal suspensions and serum specimens collected from infected owl monkeys (Table 4). In three monkeys, virus was first noted at low titers in fecal suspensions 4 days after intravenous inoculation and was present in the feces of all monkeys by day 6. Fecal virus shedding peaked in all monkeys prior to maximum elevations of serum aminotransferase activities, but sporadic shedding of small quantities of virus occurred as late as 35-42 days after inoculation (Figure 5). Fecal virus shedding was not noted in subsequent specimens collected between 75 and 102 days. Viremia was documented in all six monkeys inoculated with HM175/S18 virus. The course of the viremia generally paralleled fecal shedding of virus, but the quantity of virus present in serum was always several orders of magnitude less than that in feces. Neutralizing antibody was present in each of the owl monkeys by 35 days after inoculation, and by 21 days in animals I2, A5, and PN2. The appearance of neutralizing antibody preceded, but was correlated closely with, antibody detectable by radioimmunoassay as described pre-

viously (Lemon, 1983b). The development of antibody was associated with a sharp reduction in the magnitude of the viremia. In five monkeys, however, one or more serum specimens contained both infectious virus and neutralizing antibody directed against HAV.

Neutralization phenotype of HM175/S18 virus after monkey passage. To ascertain whether the hepatovirulence, viremia and fecal shedding of HAV in HM175/S18-infected monkeys was associated with reversion of HM175/S18 to neutralization-sensitivity, virus present in feces collected from owl monkeys J1 and PN2 were tested for resistance against neutralization mediated by the cognate monoclonal antibody, K2-4F2. Virus present in fecal specimens collected 21 days postinoculation from both monkeys was tested in a log-reduction neutralization assay, and compared with p1 HM175/S18 virus and its p15 HM175 parent. The dilution of ascitic fluid was 10^{-2} in these experiments (Table 5). Whereas the mutant p1 HM175/S18 virus demonstrated resistance to K2-4F2 mediated neutralization (-0.10 to 0.28 $\log_{10}$ titer reduction in replicate assays), fecal virus from PN2 and J1 demonstrated a neutralization susceptibility (1.16 and 1.12 $\log_{10}$ reduction) approaching that of the p15 HM175 parent virus (1.57 and 1.36 $\log_{10}$ reduction). Thus, passage of HM175/S18 in the owl monkey was associated with selection of revertant virus.

Neutralization resistance of HAV and hepatovirulence. Previous studies with neutralization-escape mutants of other RNA viruses (including some picornaviruses) have demonstrated reduced virulence in comparison with normal virus (Prabhakar, 1987). Our results, however, demonstrate unequivocably that p1 HM175/S18 remains hepatovirulent. Although fecal shedding was apparently reduced in comparison with studies conducted previously with a wild-type HM175 inoculum, the incubation period and magnitude of liver enzyme elevations were similar in monkeys infected with wild-type and neutralization-resistant HM175 virus. Virus shed in the feces of HM175/S18 infected monkeys had reverted to a neutralization-susceptible phenotype. It is unclear, therefore, whether hepatovirulence should be attributed to the HM175/S18 neutralization-resistant virus *per se* or to the revertant virus that was apparently selected for in the inoculated monkeys. It seems likely, that hepatitis in these monkeys occurred as a result of infection with revertant virus inasmuch as virus, tested for neutralization resistance and found to be susceptible to K2-4F2 neutralization, was collected from both animals J1 and PN2 prior to the height of liver enzyme elevations. The reversion of virus to a neutralization-susceptible phenotype in each of these monkeys supports the hypothesis that the major antigenic domains of HAV are highly conserved because they are essential for replication *in vivo*. Such a restriction to replication apparently occurs at the hepatocyte level, as in all six monkeys HM175/S18 virus was inoculated intravenously, effectively bypassing any putative gastrointestinal replication site.

Because HM175/S18 virus is highly adapted to cell culture, yet retains much of the pathogenic potential of wild-type virus, infection of owl monkeys with this variant presented a unique opportunity to monitor virological events during acute hepatitis A. This generally has not been possible previously, because replication of wild-type virus in cell culture is extremely inefficient and viral isolation has very poor sensitivity as a diagnostic procedure. Isolation of virus from HM175/S18 infected owl monkeys, however, demonstrated

the presence of virus in feces within four days of intravenous inoculation, and the appearance of detectable viremia (>20 RFU/ml) three to seven days later. Viremia generally paralleled the shedding of virus in feces, but was quantitatively less by several orders of magnitude. It is likely, therefore, that both viremia and fecal shedding of virus represent intra-hepatic replication of virus. The timing of viremia observed in inoculated monkeys is consistent with clinical observations made many years ago (Krugman, 1967). Viremia and fecal shedding of virus began to abate with the appearance of serum neutralizing antibody, but both persisted (albeit at reduced levels) for a number of days after the development of substantial titers of neutralizing antibody in serum. As previously suggested, the simultaneous presence of neutralizing antibody and infectious virus in some serum samples may be related to protective membrane fragments associated with virus in blood (Lemon, 1985), although in these experimentally infected owl monkeys the possibility that this viremia represents persistence of neutralization-resistant virus has yet to be excluded.

Direct sequencing of HAV RNA. Direct sequencing of viral RNA by extension of specific synthetic oligodeoxynucleotide primers in the presence of dideoxynucleotide triphosphates has proven to be a useful procedure for analysis of genomic RNA from a variety of viruses. This approach has been employed to characterize the degree of relatedness among different virus types or strains, and to identify mutations responsible for the neutralization-resistance of spontaneous virus escape mutants selected for resistance to monoclonal antibodies. We have modified this technique to allow the reliable sequencing of very small quantities of viral RNA. By increasing the concentration of primer relative to RNA template, and by including all four deoxynucleotides as high-specific activity, [^{35}S]-labelled triphosphates in the reaction mixture, we were able to obtain readable sequence from as little as 2 ng of RNA. Figure 6 depicts an autoradiogram of a sequencing gel loaded with products of primer-extension reactions containing 2 ng hepatitis A virus RNA (500 pg/lane). Seven different complementary synthetic oligonucleotides (− sense) and two genomic sense (+ sense) oligonucleotides were used as primers. The RNA was extracted from purified virions, and was therefore exclusively single-stranded (+ sense). Extension leading to an identifiable array of oligodeoxynucleotide products occurred only with the complementary primers, and resulted in sequence identical to that determined from cloned cDNA. Under the conditions employed, the reaction was highly dependent upon both the concentration of RNA template and, to a lesser extent, the concentration of the priming oligodeoxynucleotide.

We have recently used this method to identify mutations present in the capsid-encoding region of RNA from several variants of hepatitis A virus (HAV), demonstrating the applicability of this approach to the sequence analysis of RNAs available only in limited quantities. In the next contract year, we will apply this method to identification of sequences differences between HAV strains.

CONCLUSIONS

1. Adaptation of hepatitis A virus to growth in cell culture is associated with a relatively small number of mutations in the viral genome. These mutations are scattered across the 5′ nontranslated region, the 3′ nontranslated region, and P2 and P3 genomic regions. The location of mutations present in cell culture-adapted virus suggests that adaptation to growth in cell culture may be associated with changes in virus components involved in the RNA replication complex. These data are helpful in understanding the possible molecular basis of attenuation of HAV, since attenuation frequently accompanies adaptation and extensive passage of virus in cell culture.

2. Infection of susceptible primates with a variant of hepatitis A virus selected for resistance to monoclonal neutralizing antibody resulted in typical primate hepatitis A and shedding of neutralization-susceptible revertant virus. The antigenic sites of HAV appear to be under tight biologic constraints *in vivo* (explaining the high degree of conservation in the HAV antigen) and spontaneous, neutralization-resistant variants of HAV do not appear to be viable candidates for attenuated vaccine development.

3. A method for direct sequencing of viral RNA has been developed which should be capable of specific identification of various HAV strains in future studies. This method will be a useful tool in field trials of new vaccines.

Table 1. Comparison of wild-type and cell culture-adapted p16 HM175 virus

Region		Nucleotide			Amino acid		
		wt pos	wt	p16	pos	wt	p16
5′		0	–	U			
		8	G	A			
		152	A	G			
		203-7	UU	dd			
		687	U	G			
P1	VP2	964	A	G	54	lys	arg
	VP3	1742	G	A*			
P2	2A	3281	A	G	58	ile	met
	2B	3711	G	A	13	asp	asn
	2B	3867	U	C*			
	2B	3889	C	U*	72	ala	val
	2C	4049	C	U*			
	2C	4426	A	C	144	lys	thr
P3	3A	5204	G	A			
	VPg	5255	A	U	11	gln	his
	3Dpol	6148	A	G	67	asp	gly
	3Dpol	6216	U	C			
	3Dpol	6522	U	A	192	ser	thr
3′		7430	A	G			

* Identified in only one clone from p16 virus; all other mutations confirmed in at least two clones. The mutation at 1742 has been confirmed by direct sequencing of viral RNA (unpublished data).

d = Deletion

Table 2. Mutations in independently isolated cell culture-adapted HM175 variants

Region		nucleotide or amino acid position*	wt	p16	p35	
5′		0	–	U		
		8	G	A		
		124	U		C	
		131-134	UUUG		dddd	
		152	A	G	G	†
		203-207	UU	dd	dU	†
		687	U	G		
P1	VP2	54	lys	arg	arg	†
	VP1	273	glu		val	
P2	2A	30	asn		ser	
	2A	58	ile	met		
	2B	13	asp	asn		
	2B	72	ala	val	val	†
	2B	82	gly		ala	
	2C	29	lys		met	
	2C	64	glu		lys	
	2C	76	phe		ser	
	2C	144	lys	thr		
	2C	190	val		ile	
P3	VPg	4	his		tyr	
	VPg	11	gln	his		
	3Dpol	67	asp	gly	asn	†
	3Dpol	192	ser	thr	thr	†
3′		7430	A	G	G	†

* Based on polyprotein cleavage sites proposed by Cohen *et al.* (1987a).

† Similar or identical mutations (only mutations in 5′ and 3′ nontranslated RNA, and nonsilent mutations in the translated regions of the genome, are listed). The p35 sequence was determined by Cohen *et al.* (1987).

d = Deletion

Table 3. Maximal serum ALT elevations and fecal HAV antigen shedding in six owl monkeys inoculated with HM175/S18 virus.

	serum ALT (U/liter)			fecal HAV antigen	
Owl monkey	mean baseline preinoculation	maximum postinoculation	days to maximum	maximum S/N*	days to maximum
I2	41	463	22	3.4	14
I3	49	291	33	4.4	25
A5	55	350	22	6.1	21
A112	68	259	33	12.7	21
J1	54	286	26	34.6	20
PN2	46	295	33	36.4	21

*S/N = sample/negative control ratio

Table 4. Maximal fecal virus shedding and viremia in six owl monkeys inoculated with HM175/S18 virus

	fecal virus		viremia	
owl monkey	maximum RFU/gm*	day	maximum RFU/ml†	day
I2	6.90	13	2.98	14
I3	5.90	19	3.06	21
A5	6.64	20	4.00	14
A112	6.75	21	3.90	21
J1	6.71	18	4.08	18
PN2	7.53	18	4.15	18

*$\log_{10}$ radioimmunofocus-forming units/gm stool (tested as 10% suspension)

†$\log_{10}$ radioimmunofocus-forming units/ml serum

Table 5. Neutralization of HM175 virus variants with monoclonal antibody K2-4F2

Virus	Experiment	$\log_{10}$ reduction*
p15 HM175	1	1.57
	2	1.36
p1 HM175/S18	1	0.28
	2	-0.10
J1 feces d21	2	1.16
PN2 feces d21	2	1.12

*$\log_{10}$ reduction in virus titer (RFU/ml) following 2 hr incubation with 0.01% K2-4F2 ascitic fluid

Figure 1. HAV radioimmunofocus assay (Lemon and Jansen, 1985) of BS-C-1 cells inoculated 14 days previously with (A) p16 HM175 virus, (B) wild-type HM175 virus (1p owl monkey fecal virus) and (C) control, uninoculated cells. Quantitative cDNA-RNA hybridization indicated the inoculum for A was approximately 1.2×10^3 RNA-containing particles, and for B approximately 3.3×10^7 RNA-containing particles.

Figure 2. Physical map of $pHAV_{CH}$ clones containing inserts of p16 HM175 cDNA.

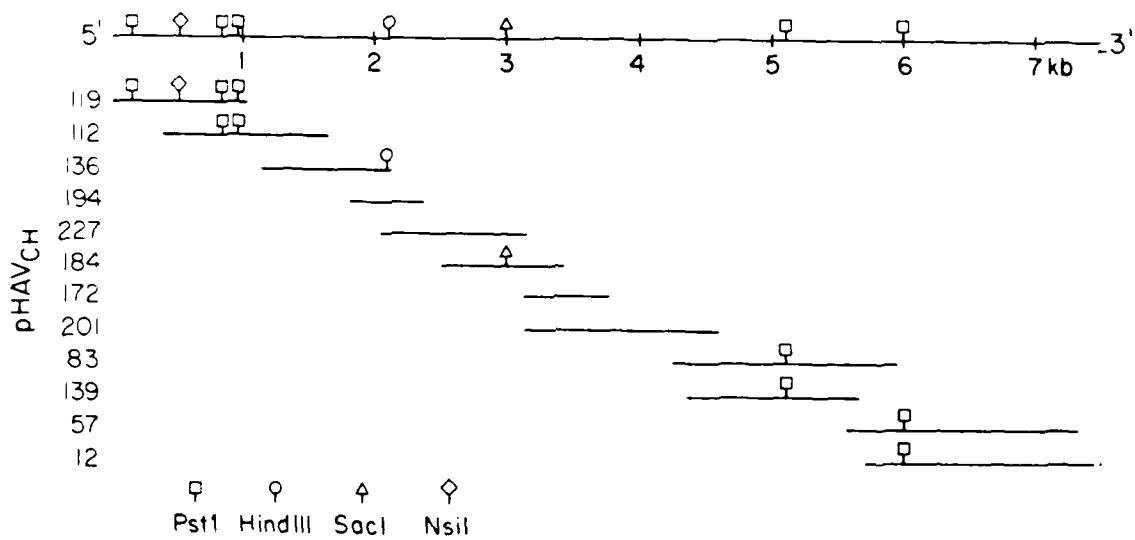


Figure 3. Genomic location of mutations present in p16 HM175 virus. Putative protein assignments are as described by Cohen *et al.* (1987a). The sequence of p16 HM175 virus was compared with that reported for wild-type HM175 (3 marmoset passages).

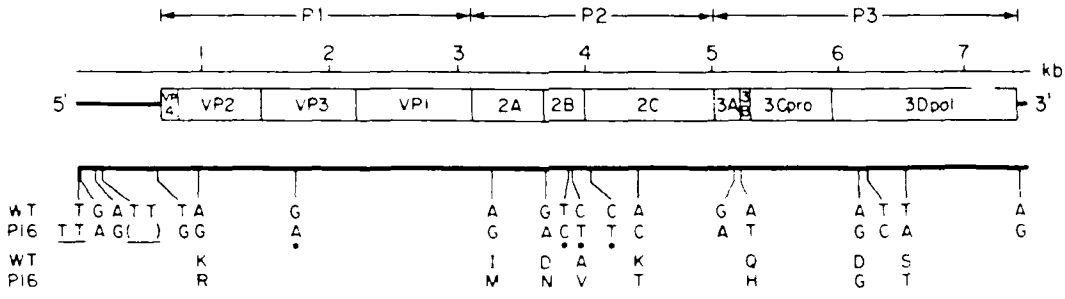


Figure 4. Passage history and relation between variants of HM175 virus for which partial or complete nucleotide sequences have been determined. Variants for which sequence is known are enclosed in boxes. Literature citations for these HM175 variants are as follows: wild-type HM175 (human feces) Gust *et al.* (1985); wild-type HM175 (3p marmoset) Ticehurst *et al.* (1983), Cohen *et al.* (1987a); p16 HM175, this paper; p35 HM175, Cohen *et al.* (1987); p59 HM175, Ross *et al.* (1986).

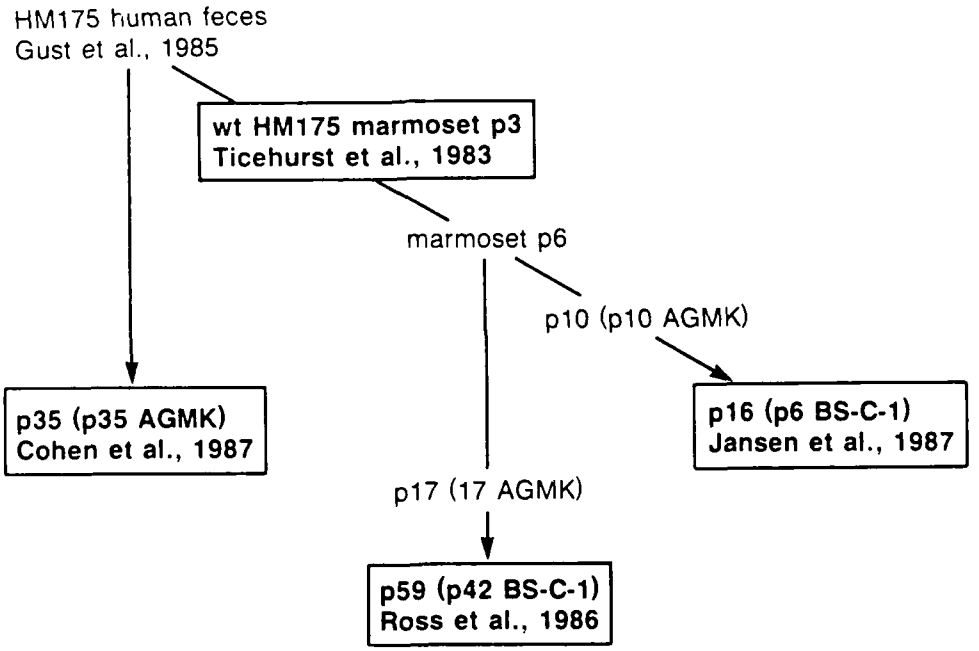


Figure 5. Infection of a seronegative owl monkey (*Aotus trivirgatus*) with pl HM175/S18 virus. Serum ALT activity and anti-HAV detected by radioimmunoassay (HAVAB) are shown: greater than 50% inhibition in the radioimmunoassay indicates the presence of antibody. The circles depict semiquantitative virus isolations attempted from serum specimens, while the squares represent attempted isolations from fecal suspensions: clear = no growth of virus (<20 RFU/ml serum or gm feces), striped = isolated foci on radioimmunofocus assay culture dishes (>20 but ≤2000 RFU/ml or RFU/gm), cross-hatched = semiconfluent HAV foci (>2000 RFU/ml or RFU/gm), solid = confluent radioimmunofoci.

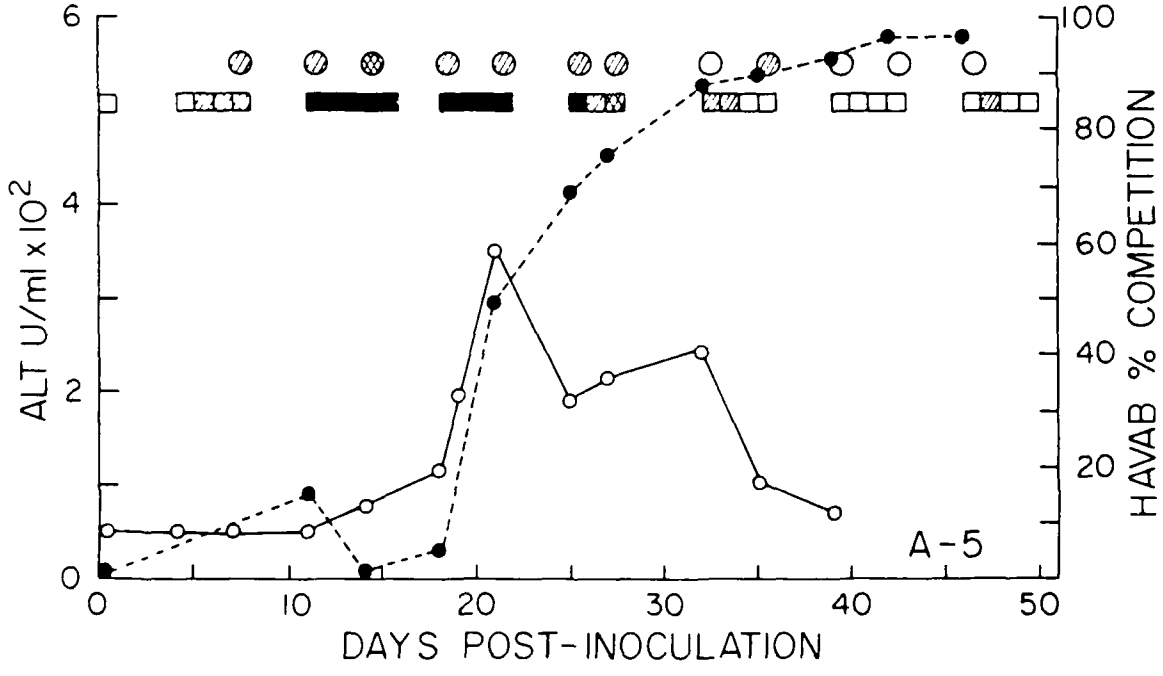
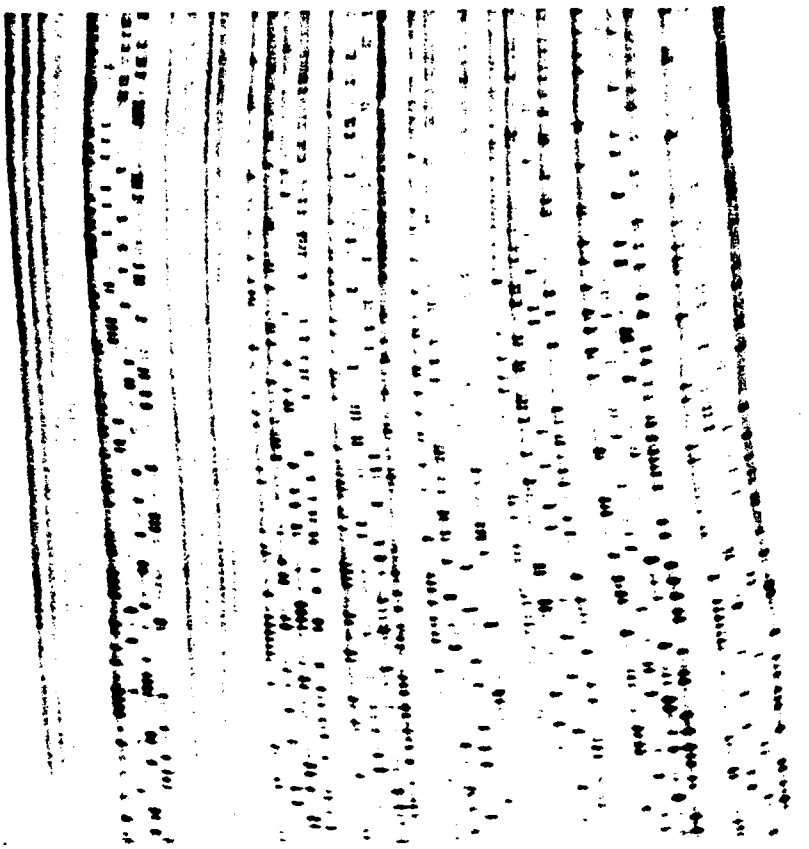


Figure 6. Products of nine different sequencing reactions, each of which contained 2 ng HM175 strain hepatitis A virus RNA (500 pg/lane). Virus was produced in cell culture and purified by centrifugation through rate-zonal sucrose gradients prior to extraction of RNA. Oligonucleotide primers used in reactions A and C (from left to right) were genomic-sense (+2189 and +2392 respectively), while primers in the other reactions were complementary to genomic RNA (A,-2366; D,-2436; E,-2566; F,-2797; G,-2949; H,-3174; and I,-2436) (oligonucleotides are designated (- or +) by sense relative to virion RNA and by map position relative to the 5' end of the genomic sequence). Exposure was for 60 hrs.



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S.M. Lemon, S-F. Chao, R.W. Jansen, L.N. Binn and J.W. LeDuc. Genomic heterogeneity among human and nonhuman strains of hepatitis A virus. *J Virol* **61**:735-742, 1987.

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