Sequence analysis of a new hepatitis A virus naturally infecting cynomolgus macaques (*Macaca fascicularis*)

Omana V. Nainan,¹,²* Harold S. Margolis,¹ Betty H. Robertson,¹ Mikhail Balayan² and Margo A. Brinton³

¹Hepatitis Branch, Division of Viral and Rickettsial Diseases, Center for Infectious Diseases, Centers for Disease Control (World Health Organization Collaborating Centre for Research and Reference in Viral Hepatitis), Atlanta, Georgia 30333, U.S.A., ²Institute of Poliomyelitis and Viral Encephalitides, Moscow 142782, U.S.S.R. and ³Department of Biology, Georgia State University, Atlanta, Georgia 30303, U.S.A.

A new isolate of hepatitis A virus (HAV), CY-145, was isolated from stool specimens obtained from cynomolgus macaques naturally infected with this agent. Sequence analysis of the capsid region of the genome indicated that this virus differed from other sequenced HAV strains by about 20% at the nucleotide level and 7% at the amino acid level. Two amino acid residues (residues 70 of VP3 and 102 of VP1), previously identified as constituting an immunodominant site and conserved in all sequenced HAVs, were changed in the CY-145 virus. Sequence analysis of a second cynomolgus HAV isolate (CY-55), which came from a different geographical location, showed the same amino acid replacement at these two sites. In addition both isolates had an amino acid substitution at the VP3–VP1 cleavage site. These data suggest that the cynomolgus HAV differs genetically and antigenically from all other sequenced HAVs.

Humans have been considered the natural host for hepatitis A virus (HAV), although non-human primates can be infected experimentally (Dienstag et al., 1975). Human HAV is a single serotype and different isolates have shown a high degree of nucleotide and amino acid conservation. HAV infections in wild-caught Panamanian owl monkeys (*Aotus trivirgatus*) have been thought to be caused by a host-specific HAV, designated PA 21 (Brown et al., 1989). Although the nucleic acid sequence of the capsid region of PA 21 differs by more than 17% from most of the sequenced human HAV isolates, this genotype has now been identified in patients with hepatitis A (Jansen et al., 1990; Robertson et al., 1991).

Antibody to HAV has been detected in newly captured cynomolgus macaques (*Macaca fascicularis*) and was thought to be the result of infection with human HAV (Burke & Heisey, 1984). We have sequenced the capsid region of human HAV isolates, this genotype has now been identified in patients with hepatitis A (Jansen et al., 1990; Robertson et al., 1991).

Cyno-HAV was immunocaptured in microfuge tubes coated with polyclonal rabbit anti-human-HAV, as previously described (Robertson et al., 1991). Viral RNA for amplification by polymerase chain reaction (PCR) was isolated by digestion with proteinase K (2 mg/ml; Boehringer Mannheim) in 20 mM-Tris–HCl pH 7.5, containing 10 mM-EDTA, 0.1% SDS and 1% vanadyl-ribonucleoside complexes at 42 °C for 1 h, using β-globin mRNA (BRL) as a carrier, followed by serial extraction with phenol–chloroform and chloroform, and ethanol precipitation. The RNA was resuspended in 50% DMSO, incubated at 60 °C for 25 min before annealing with an appropriate oligonucleotide primer at 65 °C for 15 min, and reverse-transcribed, as described previously (Margolis & Nainan, 1990; Robertson et al., 1991). The resulting cDNA was ethanol-precipitated, resuspended in PCR buffer containing the amplification primers, dNTPs and Taq polymerase, and amplified for 30 cycles using optimal conditions calculated according to the primer's Tₘ and the expected length of the amplified fragment (Fig. 2).
PCR products were purified on a 4.5% acrylamide gel. DNA from ethidium bromide-stained bands of the appropriate size was eluted as described (Sambrook et al., 1989). The eluted samples were passed through Spinnewx columns (Costar) to remove residual polyacrylamide. DNA was purified by serial phenol–chloroform and chloroform extraction, ethanol-precipitated, and resuspended in TE buffer (10 mM-Tris–HCl, 1 mM-EDTA, pH 8) and quantified by the DNA dipstick method (Invitrogen).

Double-stranded PCR products were sequenced using a modification of the dideoxynucleotide chain termination method (Sanger et al., 1977; Winship, 1989). Purified PCR-DNA and primer (10 to 20 pmol) were dried with 10 μCi [35S]dATP (Amersham), and the sequencing reaction was done using a Sequenase kit (USB). Single-strand binding protein (0.5 μg, USB) was added to the annealed template–primer mixture, and concentrations of nucleotides were reduced sixfold from those described in the original procedure (Winship, 1989). After the reaction had been stopped, the samples were treated with proteinase K as described previously (Robertson et al., 1991). Samples were denatured at 78 °C for 2 min and snap-cooled, and electrophoresis was performed at 1500 V on a 6% polyacrylamide gel containing 8 M-urea.

Initial sequencing of each fragment was done with the primers used for PCR amplification; the sequencing of the fragment was then extended using primers derived from the sequence obtained. Fragments were amplified at least twice from viral RNA and both strands of amplified DNA were sequenced. The complete sequence of the capsid region of CY-145 and a partial sequence of JM-55/CY-55 are shown in Fig. 3. The nucleic acid sequence was analysed using computer algorithms provided by the Genetics Computer Group, University of Wisconsin (Devereux et al., 1983). When compared with human HAV isolates, no insertions or deletions were present in the capsid region of the cynomolgus isolates. CY-145 differs in 446 (18.8%) of the 2373 capsid region nucleotides from the human HAV isolate HM175 (Cohen et al., 1987). However, 370 of these changes are at the third position of a codon and do not alter the amino acid composition of the translated protein. The majority of the amino acid changes observed were located within the VP3 and VP1 proteins.

Analysis of the proposed cleavage sites of the capsid proteins (Linemeyer et al., 1985; Cohen et al., 1987) showed an amino acid substitution at the VP3–VP1 cleavage site. In all human HAV isolates sequenced to date, a Gln–Val pair is cleaved at this site, whereas in the cynomolgus isolates, the valine is replaced by threonine. The amino acid composition of this cleavage site is analogous to that of foot-and-mouth disease virus (Strohmaier et al., 1978). The other two cleavage sites of the capsid protein of CY-145 are identical to those in human HAV. A three-nucleotide deletion was observed near the 5' end of the CY-145 non-structural region (Fig. 3).

A comparison of amino acid and nucleotide identity between the capsid region of CY-145 and other HAV isolates is shown in Table 1. All human HAV isolates diverge from each other by 5 to 12% in the nucleotide sequence of the capsid region and have only one to three amino acid changes (Robertson et al., 1988). The putative New World monkey isolate PA 21 diverges from human isolates by about 17% at the nucleotide level and by 2.9 to 3.8% at the amino acid level (Brown et al., 1977).
Fig. 3. Nucleotide and predicted amino acid sequence of the capsid region of CY-145. The right-angled arrows indicate cleavage sites of capsid polyproteins. The vertical arrow indicates the position of a three-nucleotide deletion, relative to human HAV, at the 5' end of sequence. Asterisks indicate amino acids involved in the proposed immunodominant site. Dots indicate identical sequence and changes region P2. A partial sequence for the CY-55/JM-55 capsid region is shown above (nucleotides) or below (amino acids) the CY-145 sequence.
Table 1. Percentage identity in amino acid and nucleotide composition of the capsid region of human HAV isolates compared with simian HAV isolates CY-145 and PA 21

<table>
<thead>
<tr>
<th>Capsid protein</th>
<th>HM-175</th>
<th>CR326</th>
<th>HAS-15</th>
<th>MMB</th>
<th>LA</th>
<th>PA 21</th>
<th>CY-145</th>
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</thead>
<tbody>
<tr>
<td>VP0 CY-145</td>
<td>96.0</td>
<td>93.9</td>
<td>95.9</td>
<td>95.9</td>
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<td>–</td>
<td>95.9</td>
</tr>
<tr>
<td>VP3 CY-145</td>
<td>92.7</td>
<td>92.7</td>
<td>93.1</td>
<td>92.7</td>
<td>93.1</td>
<td>92.3</td>
<td>–</td>
</tr>
<tr>
<td>VP3 PA 21</td>
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<td>98.0</td>
<td>98.0</td>
<td>98.4</td>
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<td>–</td>
<td>92.3</td>
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<tr>
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<td>92.4</td>
<td>91.4</td>
<td>92.9</td>
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<td>92.3</td>
<td>–</td>
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<tr>
<td>VP1 PA 21</td>
<td>95.3</td>
<td>95.0</td>
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<td>92.3</td>
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Nucleotides

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<th>CR326</th>
<th>HAS-15</th>
<th>MMB</th>
<th>LA</th>
<th>PA 21</th>
<th>CY-145</th>
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<td>82.4</td>
<td>81.7</td>
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<tr>
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<tr>
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<td>86.0</td>
<td>85.5</td>
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1989). However, PA 21 is serotypically identical to human HAV, since all monoclonal antibodies to human HAV tested have been shown to bind to this isolate (Brown et al., 1989). The nucleotide sequence to the VP3 and VP1 region of CY-145 differs from all sequenced HAV strains by 18 to 20%, and by more than 7% at the amino acid level. The partial sequence of the Indonesian isolate (CY-55/JM-55) showed an 18% nucleotide variation and a 3 to 4% amino acid variation when compared with other HAV strains, including CY-145.

Some of the amino acid changes common to CY-145 and CY-55/JM-55 appear important to the final antigenic structure as defined by binding to neutralizing monoclonal antibodies raised against human HAV. Antibody binding was assessed by enzyme immunoassay using microtitre wells (Immulon II; Dynatech) coated with monoclonal antibody; biotinylated anti-HAV IgG (chimpanzee) and streptavidin–horseradish peroxidase were used as the detector. Polyclonal rabbit anti-HAV showed 100% binding to human HAV (cell culture-adapted strain HAS-15), CY-145 and CY-55/JM-55. HAS-15 had 95% and 72% binding, respectively, to monoclonal antibodies H7C27 (Dawson et al., 1984) and K24F2 (MacGregor et al., 1983). However, both cynomolgus viruses showed only 1 to 4% binding to these monoclonal antibodies; findings similar to these were described by Karetnyi et al. (1989). Two amino acid residues have been identified as part of the immunodominant region in human HAV using escape mutants to monoclonal antibody K24F2 (Ping et al., 1988). Both of these amino acids were substituted in the cyo-HAV isolates (Fig. 3). The presence of amino acid changes in the immunodominant region of cyo-HAV that are similar to those found in antibody-resistant mutants suggest these changes may be responsible for the lack of monoclonal antibody binding. Antigenic escape mutants are currently being generated for neutralizing monoclonal antibodies to delineate these epitopes further.

Pairwise comparisons (Rico Hesse et al., 1987) of the nucleotide sequence of the capsid regions of the cyo-HAV isolates, human isolates, and PA 21 isolate are shown in Fig. 4. This analysis indicates that cyo-HAV isolates vary from each other by approximately 20%, that they vary from the majority of human isolates by about 20%, and that they vary from the PA 21 isolate by 20%. The diversity observed between the two cynomolgus monkey viruses is probably due to their geographical isolation.

In general, nucleotide variation among human HAV isolates in the capsid region ranged from 5 to 12% except for the PA 21 isolates which have been found in owl monkeys and humans. However none of the human HAV isolates, including PA 21, appears to have the amino acid changes at the identified immunodominant region, and none has shown decreased binding to monoclonal antibodies. No other HAV isolates have the unique amino acid substitution at the VP3–VP1 cleavage site, as do the cynomolgus isolates. The genetic and antigenic divergence of the cyo-HAV from human isolates and the genetic diversity among geographically isolated cyo-HAV isolates strongly suggest that distinct simian hepatitis A viruses exist.
Fig. 4. Relationship among the nucleotide sequences of the capsid regions of HAV isolates obtained by pairwise analysis. The percentage variation is the horizontal distance connecting any two isolates.

**References**


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