Molecular Cloning of cDNA from Hepatitis A Virus Strain HM-175 after Multiple Passages in vivo and in vitro

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SUMMARY

Hepatitis A virus (HAV) strain HM-175 was passaged six times in marmosets, 59 times in cell culture and purified from infected cell culture supernatant fluid. The viral RNA was extracted, copied into cDNA and the cDNA:RNA hybrids were cloned into the PstI site of plasmid pBR322. The cDNA clones were authenticated by hybridization to RNA extracted from HAV-infected cells and clones representing the 3' end of the genome were identified using a previously authenticated cDNA clone. The clones represented all but 29 bases of the HAV genome. They were compared to HAV strain HM-175 cDNA cloned from viral RNA after three passages in marmosets on the basis of restriction endonuclease mapping and DNA sequencing. No differences were found in either the presence or absence of restriction endonuclease sites using 33 different restriction enzymes. Sequencing of cDNA representing bases 29 to 1002 of the HAV genome revealed eight base changes all of which were within the 5' non-coding region.

The replication of hepatitis A virus (HAV) in cell culture is characterized by a relatively poor yield of virus which has hampered attempts to study its biology (Provost, 1984). The first report of successful propagation of HAV in cell culture was by Provost & Hilleman (1979). Since that time, HAV has been serially passaged in cell culture by many groups of investigators of which some have reported changes in the viral phenotype such as adaption (Frosner et al., 1979; Daemer et al., 1981), attenuation (Feinstone et al., 1983) and cytopathology (Venuti et al., 1985).

In order to study the genetic changes that occur upon serial passage in cell culture we cloned the genome of HAV strain HM-175 after six passages in marmosets and 59 passages in cell culture. This enabled us to compare the genome of highly cell culture-adapted HM-175 to that of HM-175 which has been previously cloned (Ticehurst et al., 1983) and partially sequenced (Baroudy et al., 1985) using virus purified after only three passages in marmosets.

The HM-175 strain of HAV originated from a patient admitted to Fairfield Hospital, Melbourne, Australia, in 1979 (Gust et al., 1985) and a 20% (w/v) faecal suspension was sent to the National Institutes of Health (NIH), Bethesda, Md., U.S.A., where it was passaged six times in marmosets, 17 times in primary African green monkey kidney and 40 times in continuous African green monkey kidney (BSC-1) cells. Infected cells at this passage level were obtained from NIH and passaged a further two times in BSC-1 cells. HAV-infected cells were then split into two Nunc cell factories with the culture medium decanted at weekly intervals for 6 weeks. A total of 22 litres of infected cell culture medium was concentrated by ultrafiltration to 200 ml and subjected to ultracentrifugation. The resultant virus pellet was resuspended and further purified by chloroform extraction, differential centrifugation, agarose gel filtration and isopycnic ultracentrifugation as previously described (Coulepis et al., 1978; Locarnini et al., 1978). A 1 ml suspension of purified virions was treated with micrococcal nuclease (P-L Biochemicals) in 5 mM-CaCl₂ and 20 mM-Tris-HCl pH 9-0 for 30 min at 37 °C. The RNA was extracted from the virions by adding 3 ml of a 4 M-guanidine thiocyanate buffer (Maniatis et al., 1982) followed by hot phenol extraction. After ethanol precipitation, the RNA served as a template for cDNA
synthesis using 100 units/ml reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) and oligo(dT)$_{15}$ as primer. Homopolymeric dCMP tails were then added to the 3' ends of the cDNA:RNA hybrids and were annealed to dGMP-tailed, PstI-cut plasmid pBR322. The resultant recombinant molecules were used to transform *Escherichia coli* strain MC1061.

A calculated 31 ng of cDNA synthesized yielded 2344 tetracycline-resistant colonies of which 90% were ampicillin-sensitive. Initially the cDNA clones were screened by colony hybridization (Grunstein & Hogness, 1975) for the presence of 3' end sequences using a previously authenticated cDNA clone, pHAV(LB)207 (Ticehurst *et al.*, 1983), which was kindly provided by Dr J. R. Ticehurst. A series of overlapping clones representing the HAV genome was obtained by re-probing the cDNA library with the most 5' proximal fragment determined by restriction enzyme mapping. Sizeable clones were further authenticated by hybridization to an RNA species present in HAV-infected BSC-1 cells with an intact mol. wt. of approx. 7.5 kb (data not shown).

Five overlapping clones representing the HAV genome were selected to construct a detailed cDNA restriction map from single and double restriction enzyme digests (Fig. 1). The cDNA clones were digested with each enzyme used to generate the map of HM-175 cDNA published previously by Ticehurst *et al.* (1983). No differences were found between the two maps after digestion with 33 different enzymes.

The restriction mapping data suggested that there was little difference in sequence between low and high passage HM-175 and this was confirmed by partial sequencing of the cDNA. Cloned HAV cDNA was subcloned into mp8 and mp9 derivatives of phage M13 (Messing *et al.*, 1981) and sequenced by the dideoxy nucleotide method (Sanger *et al.*, 1977). The subcloning strategy and comparison of the sequence with the previously published HM-175 sequence is depicted in Fig. 2. Sequence analysis of the 5' end of clone pHM-14 indicated that this clone was 29 bases shorter than the corresponding portion of the low passage HM-175 genome but was probably not the 5' terminus of the genome since the UU dinucleotide required for picornavirus replication was not present. The HAV cDNA sequenced represents bases 29 to 1002 of the previously published sequences and includes the 5' non-coding region, with the first 270 bases of the putative coding region. We found seven point mutations and a single base deletion when compared to low passage HM-175. All of these changes were in the 5' non-coding region and were sequenced in both directions for confirmation.
Fig. 2. The sequencing strategy and result of sequence comparison with the previously published HM-175 sequence (Baroudy et al., 1985) at the 5' end of the HAV genome. The start of the coding region shown is based upon the start of an open reading frame predicted from sequence determinations in three independent studies (Baroudy et al., 1985; Linemeyer et al., 1985; Najarian et al., 1985). The clone used for sequence analysis (pHM-14) is 29 bases short of the 5' terminus of the HAV genome. The sequencing strategy is illustrated by bars and arrows representing restriction sites used in subcloning and the extent of sequence obtained. The abbreviations used for restriction enzymes are: H, HaeIII; N, NcoI; P, PstI; Pv, PvulI.

An analogy may be drawn between our observations and those of Schild et al. (1984) who compared the sequence of a poliovirus type 3 vaccine strain and its neurovirulent progenitor. They found only 10 base changes along the entire genome and more recently have reported that one of these changes is responsible for a near complete reversion to virulence (Evans et al., 1985). This crucial base change in poliovirus occurs in the 5' non-coding region of the genome at position 472 which is 271 bases from the start of the open reading frame. It is interesting to note that the eight base changes we detected with HAV occur in the 5' non-coding region and none has been found in the coding region sequenced thus far.

The base changes we report for HAV are not consistently found in the sequences of other cell culture-adapted strains of HAV (Najarian et al., 1985; Linemeyer et al., 1985). This may imply that changes elsewhere in the genome are responsible for changes in viral phenotype during serial passage or that it is a species-specific phenomenon. It should be noted that although these strains were cloned from virus purified after cell culture passaging, no details were given as to the passage history or biological characteristics of the viral phenotype. Currently we are completing the comparative sequence analysis of HM-175 in the coding region and cloning cDNA from further strains of HAV after multiple passages in cell culture to determine whether base changes are consistently clustered in the 5' non-coding region or occur randomly throughout the genome. We are also attempting to clone cDNA from strains of HAV before and after defined phenotypic changes occur in order to precisely correlate genetic variations with changes in biological characteristics.

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REFERENCES


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