JOURNAL OF VIROLOGY, Nov. 1985, p. 579–588
0022-538X/85/110579-10$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 56, No. 2

Isolation and Molecular Cloning of a Fast-Growing Strain of Human Hepatitis A Virus from Its Double-Stranded Replicative Form

A. VENUTI,[1] C. DI RUSSO,[2] N. DEL GROSSO,[3] A.-M. PATTI,[2] F. RUGGERI,[4] P. R. DE STASIO,[1] M. G. MARTINIELLO,[1] P. PAGNOTTI,[1] A. M. DEGENER,[1] M. MIDULLA,[3] A. PANÀ,[2] AND R. PEREZ-BERCOFF[1]*

The Institute for Virology, University of Rome,[1] Department of Public Health, 2nd University of Rome,[2] Institute of Experimental Medicine, Consiglio Nazionale delle Richerche,[3] and Laboratory of Ultrastructure, Istituto Superiore di Sanità,[4] 00185 Rome, Italy

Received 3 April 1985/Accepted 2 July 1985

A fast-growing strain of human hepatitis A virus was selected and characterized. The virus has the unusual property of developing a strong cytopathic effect in tissue culture in 7 to 10 days. Sequences of the viral genome were cloned into recombinant plasmids with the double-stranded replicative form as a template for the reverse transcription of cDNA. Restriction analysis and direct sequencing indicate that this strain is different from that described by Ticehurst et al. (Proc. Natl. Acad. Sci. USA 80:5885–5889, 1983) in the region that presumptively codes for the major capsid protein VP1, but both isolates have conserved large areas of homology in the untranslated 5′-terminal sequences of the genome.

Described in the Babylonian Talmud after its most conspicuous clinical symptom ("yarkon" = jaundice), related by ancient Romans to the living conditions of the armies ("ictero castrensis"), and suspected by Pope Zacharias (7th century A.D.) to be a transmissible condition ("pestilentia"), human hepatitis A (HHA) is still in our day a widely spread infectious disease which is endemic in large areas of the world. In Southern Europe and the Mediterranean area, more than 90% of the young adult population bear serological evidence of past infection (3% in Scandinavia and 40% in the United States).

The etiological agent of HHA is a picornavirus of the enterovirus group (3, 6, 13, 26). Electronmicroscopic examination of virions isolated from fecal specimens revealed naked, icosahedral particles, about 27 to 32 nm in diameter (4, 25). The viral genome is a single-stranded, infectious RNA molecule, about 7,500 to 8,000 bases long, of "positive" polarity (3, 4, 26). Four polypeptides of molecular sizes 30, 24, 21, and 7 to 10 kilodaltons form the capsid (2, 3, 26, 29). By analogy with other picornaviruses (polio, foot-and-mouth disease, rhinoviruses, etc.), it is reasonable to assume that the largest structural protein (VP1) contains the antigenic determinants responsible for viral neutralization.

Transmission is supposed to occur through the oral-fecal route, in areas where poor hygienic conditions favor the pollution of water. There is, so far, no specific prophylaxis against HHA.

Several groups have succeeded in growing the virus in either primary or established cell lines (5, 8, 9, 13, 25), but apparently all of these isolates were only able to undergo a limited ("restricted") replication. Viral growth (as revealed by immunofluorescence or enzyme-linked immunoassays) was very slow (6 to 10 weeks), without any detectable sign of cytopathic effect (CPE) (3, 13, 26), suggesting that optimal conditions for stable, high-level replication were still to be determined (6).

The question to be addressed, therefore, was whether such unusual behavior constituted an intrinsic characteristic of HHA virus or whether it merely reflected the lack of a suitable, fully permissive cell line. We report here the adaptation of HHA virus to a simian cell line, resulting in the production of infectious virions and typical CPE in 7 to 10 days.

These properties made the fast-growing strain a suitable isolate to study the biochemistry of HHA virus replication. Accordingly, as a first step toward the analysis of the genome structure and organization, we cloned the viral genome. To overcome the difficulties involved in the reverse transcription of a 7.5- to 8-kilobase-long RNA template, we developed a procedure that allowed us to insert into recombinant plasmids cDNA copies of the hepatitis genome obtained by reverse transcription of both strands of the virus-induced, double-stranded (ds) replicative form (RF).

(Part of this research was presented at the Sixth International Conference of Virology, Sendai, Japan, 1 to 7 September 1984.)

MATERIALS AND METHODS

Cells. Monolayers of human diploid, Vero, and RC-37 (cercopithecus) cells were routinely maintained in Eagle minimum essential medium (Hanks salts) containing 10% calf serum and antibiotics. Frp/3 is a subline derived in our laboratory from Frhk/4 (rhesus). After 6 months in spinner culture, Frhk/4 cells were plated, and single-cell clones were selected by standard procedures after the culture underwent its third crisis. Frp/3 cells have been maintained since 1980 in Stocker-McPherson medium supplemented with nonessential amino acids, antibiotics, and either 10% calf serum and 0.11% bicarbonate (growing medium) or 2% serum and twice the normal amount of bicarbonate (maintenance medium).

Immune sera. Three sets of human negative and convalescent sera from certified patients with acute hepatitis A were used throughout these studies. They were generously provided by B. Flehmig (Tübingen, Federal Republic of Germany) and M. Jung (Virion AG, Zurich, Switzerland).

HHA viral antigens. HHA viral antigens were detected either by indirect immunofluorescence (IIF) as previously described (19) or by means of a standard enzyme-linked immunosorbent assay (ELISA) (Hepanostika; Organon, B. V. Boxten, Holland), according to the instructions of the manufacturer.

Isolation of human hepatitis virus. Feces samples from a

* Corresponding author.

young relative of a patient with acute hepatitis A were collected during what was suspected to be the incubation period and stored at −70°C. The stool samples from days 6 and 4 preceding the onset of clinical symptoms were used to prepare a 10% (wt/vol) dispersion in phosphate-buffered saline containing penicillin (200 μg/ml), streptomycin (200 μg/ml), kanamycin (100 μg/ml), and amphotericin B (Fungizone) (100 μg/ml). The suspension was clarified by low-speed centrifugation (900 × *g*, 10 min), and then the supernatant was subjected to a second centrifugation (5,000 × *g*, 30 min).

Barely confluent monolayers of the simian cell line Frp/3 were infected with 0.2 ml of the above-mentioned fecal suspension. The virus was allowed to adsorb at 4°C for 1 h before adding 1.5 ml of prewarmed Stocker-McPherson-modified minimum essential medium containing 2% fetal calf serum, nonessential amino acids, and 0.22% bicarbonate.

At regular intervals, the growth of the virus was monitored by IIF. Four to eight weeks after the infection, when the cells showed typical images of specific cytoplasmic fluorescence, the cultures were frozen and thawed thrice, and the clarified supernatants were used to infect a fresh set of cultures.

Addition of poly(A) sequences to the ds HHA RF. The reaction mixture (final volume, 100 μl) contained the following: 100 mM Tris hydrochloride (pH 7.5), 0.5 mM EDTA, 5 mM $MgCl_2$, 5.5 mM ATP, 10 μCi of [^{3}H]ATP (specific activity, 50 mCi/mmol), 8 mM AMP, 40 U of RNasin, 5 U of myokinase (EC 2.7.4.3), 0.5 U of polynucleotide phosphorylase (EC 2.7.7.8), and 5 to 20 μg of HHA ds RF. The reaction was incubated 15 min at 32°C. The kinetics of incorporation was monitored by spotting at intervals onto DE-81 paper disks duplicate 1-μl samples. The filters were washed by suction with two 15-ml portions of 6% Na_2HPO_4, rinsed with 20 ml of water, dried, and counted by liquid scintillation. To the bulk of the reaction (about 90 μl), EDTA and sodium sarcosinate were added to 5 mM and 0.5%, respectively. The polyadenylate [poly(A)]-tailed HHA RF was then extracted three times with an equal volume of phenol-chloroform. The organic phases and the thin interphase were sequentially washed with 50 μl of 10 mM Tris hydrochloride (pH 7.5), the wash and the final aqueous phase were combined, potassium acetate was made 500 mM, and the poly(A)-tailed HHA RF was precipitated with 2.5 volumes of ethanol (−20°C overnight or −70°C for 2 to 3 h), collected by centrifugation (10,000 × *g*, 4°C, 1 h), dried under vacuum, and dissolved in 10 μl of 10 mM Tris hydrochloride (pH 7.5)–20% dimethyl sulfoxide.

Synthesis of DNA complementary to both strands of HHA virus RF. The poly(A)-tailed HHA virus RF was first heat denatured (96°C for 90 s, 0°C for 2 to 3 min); 5 μl (9 μg) of oligo(dT)$_{12-18}$ and 1.6 μl of 2.5 M KCl were then added, and the reaction was incubated for 2 min at 65°C. The hybridization of the oligo(dT) primer to the poly(A)-tailed template was allowed to proceed for 5 to 10 min at 60°C, 20 min at 55°C, and 45 min at 42°C. One microliter (40 U) of RNasin was added, and a reaction mixture (final volume, 50 μl) was constructed that contained the following: 40 U of RNasin, 50 mM Tris hydrochloride (pH 8.2), 10 mM $MgCl_2$, 80 mM KCl, 2 mM dithiothreitol, 0.8 to 1 mM each dCTP, dGTP, and dTTP, 100 μCi of [^{32}P]dATP (specific activity, 1,200 Cerenkov cpm/pmol), and 20 U of reverse transcriptase (Seikagaku, St. Petersburg, Fla.). Early experiments had shown that the presence of a residual 4% dimethyl sulfoxide in the reaction does not affect adversely the process of reverse transcription. To monitor the kinetics of the reaction, duplicate 1-μl samples were spotted at intervals onto DE-81 paper disks, counted as Cerenkov units to check the uniformity of the sample size, and washed with 6% Na_2HPO_4 as above; the radioactivity bound to the filters was counted as Cerenkov radiation. To the bulk of the reaction, 32 μg of phenol-purified commercial carrier RNA was added, EDTA and sodium sarcosinate were brought to 5 mM and 0.5%, respectively, and the nucleic acids were extracted three times with 100 μl of phenol-chloroform as in the previous step. After ethanol precipitation, the template RF was eliminated by alkaline hydrolysis (300 mM NaOH and 2 mM EDTA at 100°C for 45 s, immediately followed by neutralization with HCl and Tris hydrochloride [pH 8.2] brought to 400 and 200 mM, respectively), and the cDNA was resolved from unincorporated deoxynucleoside triphosphates and degraded RNA by gel filtration (Sephadex G-50, fine). The peak fractions of the void volume were combined, 30 μg of carrier RNA was added, and the nucleic acids were ethanol precipitated.

RESULTS

Selection of the fast-growing strain of HHA virus. Isolated HHA virus (Fig. 1) was serially passaged in Frp/3 cells, a subline of Frhk/4 cells. During the first six to eight passages, a typical immunofluorescent granulation appeared in the cytoplasm of infected cells after 3 to 4 weeks at 34°C and was maximal within 8 to 10 weeks postinfection (Fig. 1). As the percentage of immunofluorescent cells increased, so also did the amounts of cell-bound HHA viral antigens quantitated by a specific ELISA (Fig. 1C). Beginning from serial passage 12, as soon as 25 to 30% of the cells were positive in the IIF test, HHA virus-infected cultures were frozen and thawed, and the clarified supernatants were used to infect a fresh culture. This procedure resulted in the progressive shortening of the time between the infection of the culture and the production of HHA antigens as revealed by either IIF or ELISA. Eventually, it was possible to reduce the time between serial passages of the virus to roughly 3 weeks.

After 20 more passages of HHA virus in Frp/3 cells, a clear CPE developed in these cultures in response to the infection. Typically, nuclei became pycnotic (6 to 9 days), the cells rounded up, and by 12 to 15 days most of them had either detached or undergone total degeneration (Fig. 2). To check whether the observed CPE was induced specifically by HHA virus, three human, high-titer anti-HHA sera were used in classical neutralization tests. Figure 3 depicts a representative experiment in which the hyperimmune serum prevented simultaneously the development of CPE in Frp/3 cells and the synthesis of HHA antigens as revealed by either the ELISA or the IIF test. None of the negative sera had the slightest effect on the outcome of the infection, even at the lowest dilution (Fig. 3). The role of the host cell was then investigated. Cultures of human diploid fibroblasts, Vero, or RC-37 cells were infected with the fast-growing strain of HHA after passage 31 on Frp/3 cells. Three weeks later, more than 50% of the cells showed specific cytoplasmic IIF. None of the above cell lines, however, developed any CPE.

Purification of HHA virus. HHA virus was extracted from Frp/3 cells which had been infected for 9 to 12 days by two cycles of freezing and thawing, followed by mild detergent treatment (Dounce homogenization in 10 mM Tris hydrochloride plus 0.2% Triton X-100 [pH 7.5]). After pelleting the particulate material and eliminating the light contaminants by centrifugation through a 20% sucrose cushion, the viral

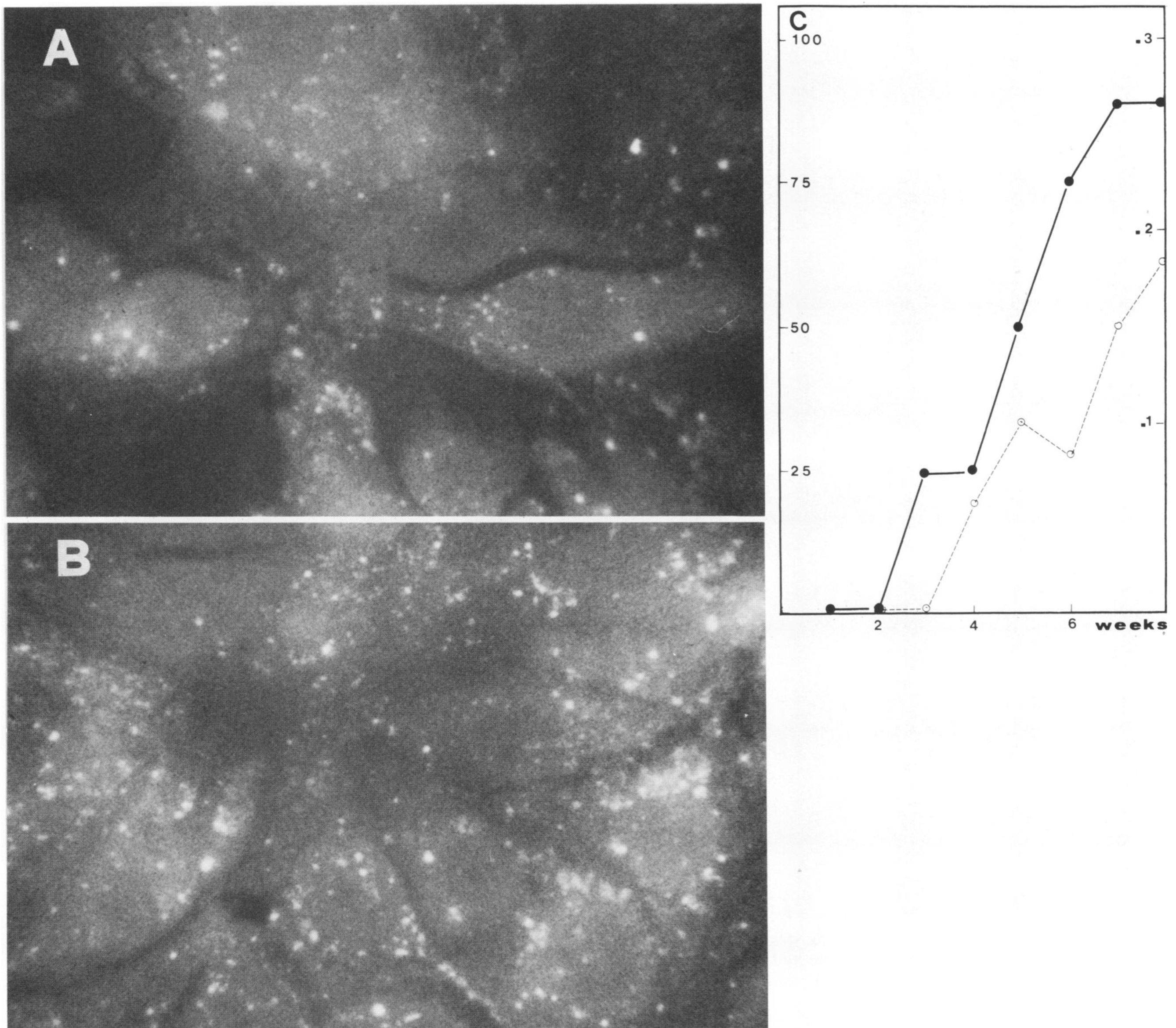


FIG. 1. Isolation of HHA virus. Barely confluent monolayers of Frp/3 cells were infected as described in Materials and Methods. Four (A) and eight (B) weeks after infection, the presence of HHA-related antigens was revealed by IIF or by means of a standard ELISA. (C) Percentage of cells found positive in the IIF test (●) and antigens detected by ELISA (○) (optical density at 494 nm).

particles were sedimented to equilibrium in a CsCl gradient at pH 7.5. HHA antigens were assayed in each fraction with a standard ELISA kit. The following three unequal components were clearly resolved (Fig. 4): a major peak (1.31 to 1.32 g/ml), a smaller one of denser particles (1.39 to 1.41 g/ml), and a broad irregular band (distributed between 1.28 through 1.24 g/ml) that tended to vary from one preparation to another. A similar distribution has been reported for HHA particles directly isolated from fecal specimens or purified from tissue culture (7, 26; A. G. Coulepis, Ph.D. thesis, Monash University, Melbourne, Australia, 1981). The viral particles present in each peak were pelleted through a 40% sucrose cushion. Solid-phase immune electron microscopy was used to characterize the virions. Naked particles, similar in size and shape to poliovirions and of typical icosahedral symmetry and no discernible structure, were readily observed (Fig. 4B). Like other picornaviruses, the bulk of the infectivity was confined to the standard-density particles, the denser and light components being barely infectious (data not shown).

Upon phenol extraction, the virions of either density yielded intact RNA that migrated in neutral agarose gels under nondenaturing conditions as a single band slightly behind the 9.2-kilobase fragment of *Hin*dIII-digested lambda DNA (Fig. 4C).

HHA viral RNA was digested to completion with RNase T_1, the resulting fragments were labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP, and the large T_1-resistant oligonucleotides were resolved by two-dimensional polyacrylamide gel electrophoresis as previously described (22). The complexity of the fingerprint depicted in Fig. 4D is similar to that of the T_1 fingerprints of the genome of other picornaviruses (22). Moreover, the large T_1 oligonucleotides appear to be present in substantially equimolar amounts.

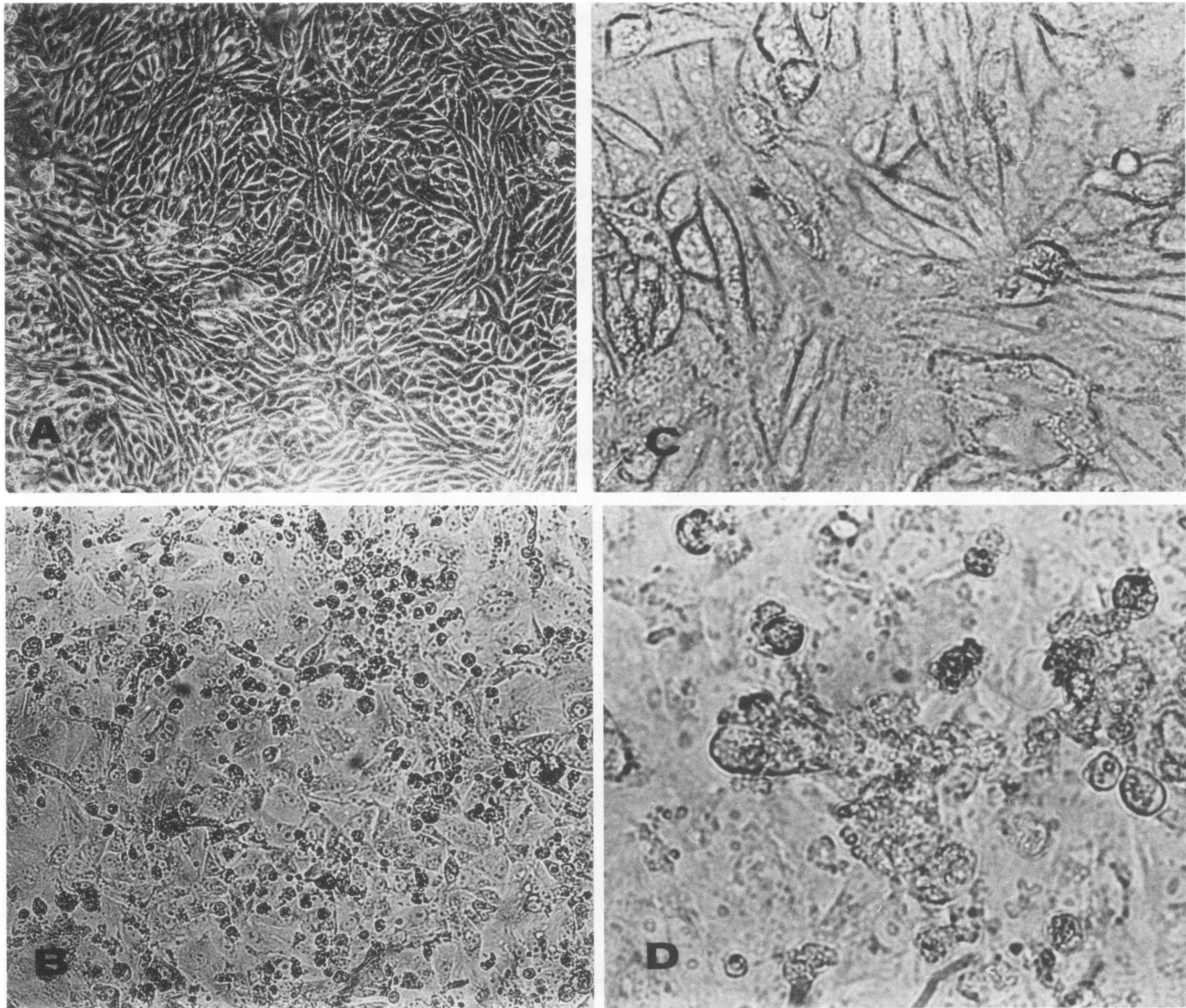


FIG. 2. CPE induced by the fast-growing strain of HHA virus. Monolayers of Frp/3 cells were infected (B and D) with a 1:50 dilution of passage 34 of HHA virus or were mock-infected (A and C) with spent medium. The cultures were incubated 15 days at 34°C. Panels C and D show selected fields from panels A and B, respectively, at larger magnification.

The RNAs extracted from the standard or denser virions yielded undistinguishable T_1 fingerprints.

Molecular cloning of HHA virus sequences from its ds RF. The RF of picornaviruses is a very stable ds RNA molecule continuously produced during the process of replication that tends to accumulate in membrane-bound structures. It contains an intact copy of the genomic RNA, hydrogen bound to a full-length complementary strand (21). The RF of HHA virus was extracted as previously described (17) from the cellular debris of Frp/3 cultures which had been infected for 12 days. After LiCl precipitation, cellulose chromatography, and glycerol gradient centrifugation, it was tailed by the addition of ^{3}H-labeled poly(A) residues at the 3′ end of both strands in a reaction that used the enzymes polynucleotide phosphorylase and myokinase. As we found it very difficult to remove the traces of nuclease activity that contaminated commercial preparations of myokinase, the elongation of HHA RF was carried out at 32°C for only 15 min. The poly(A)-tailed HHA RF migrated in neutral agarose gels under nondenaturing conditions as a broader band than native RF and slightly behind it (Fig. 5). HHA RF was heat denatured and hybridized to oligo(dT), and both strands were reverse transcribed into cDNA. In this reaction, the 7.5- to 8-kilobase-long RF was transcribed to an extent that appeared to depend upon the degree and stability of the intramolecular folding of the template. In early experiments it was noticed that the conditions used to hybridize the oligo(dT) primer to the RNA template (ionic strength, temperature, reaction volume) played a major role in this respect, being critical for both the efficiency of the process of transcription and the length of the product obtained. Accordingly, conditions were routinely used that yielded the longest transcripts (see Materials and Methods).

The cDNA so constructed contained sequences complementary to both strands of HHA RF that, under proper conditions, would hybridize to form a partial ds structure, provided the 3′-proximal sequences of the cDNA copies overlapped at least in part. The absolute prerequisite for this to occur was that the reverse transcription of each strand be completed for at least a minimum of half its length. Apparently, however, this was not the case, and the longest cDNA molecules obtained after the first run of transcription were

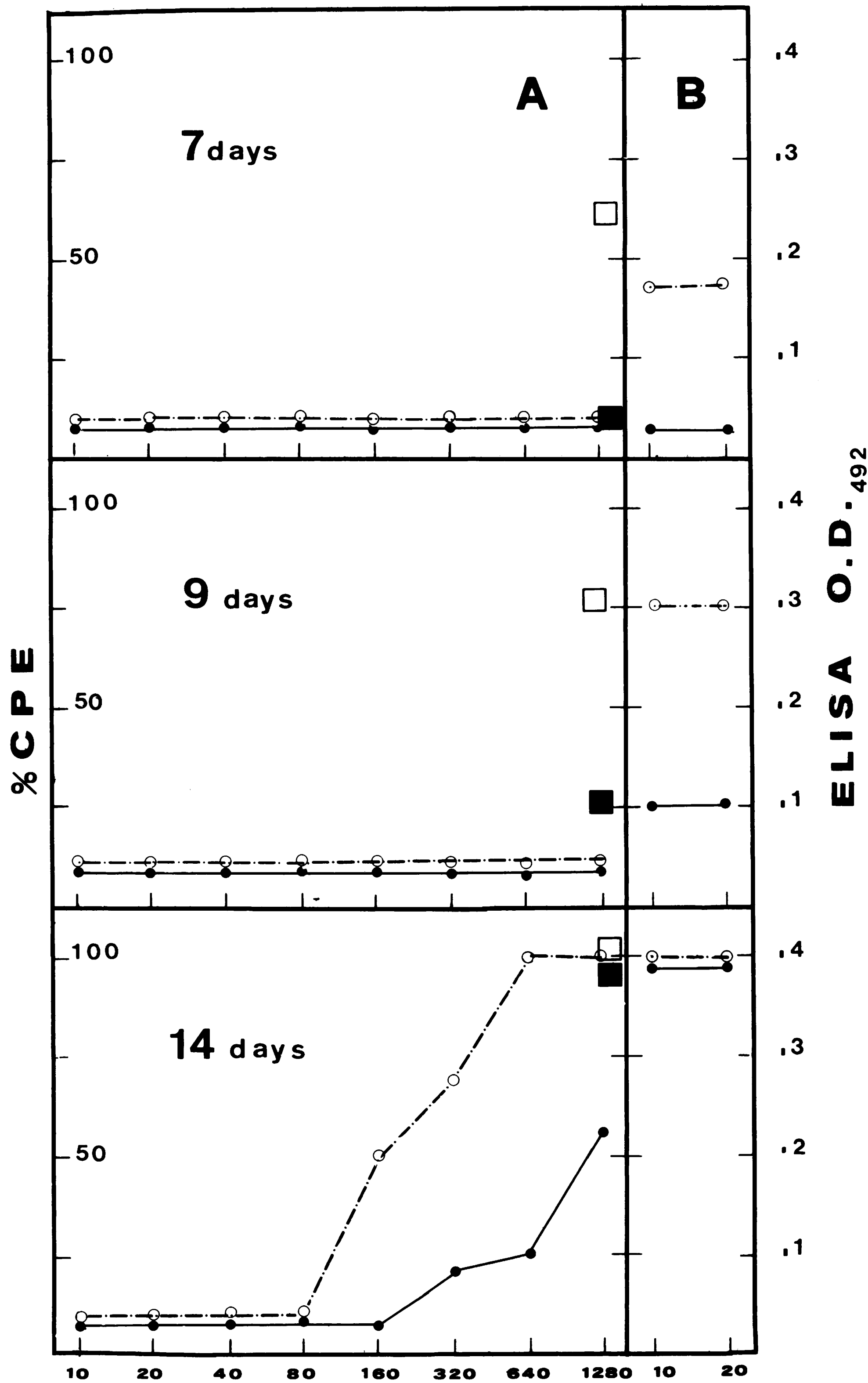
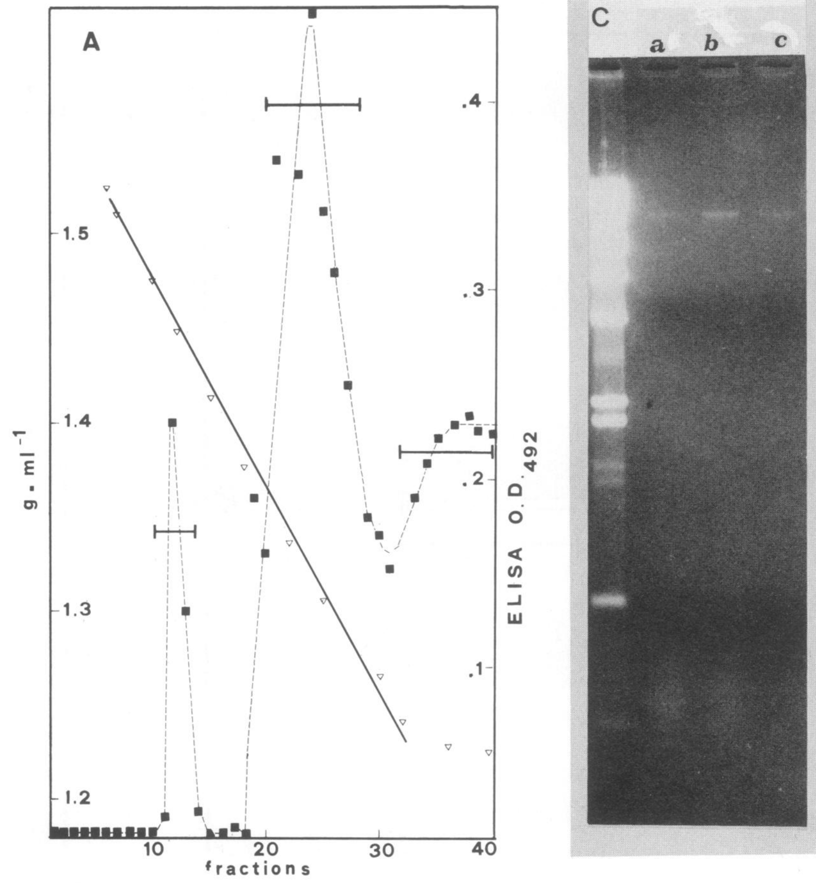
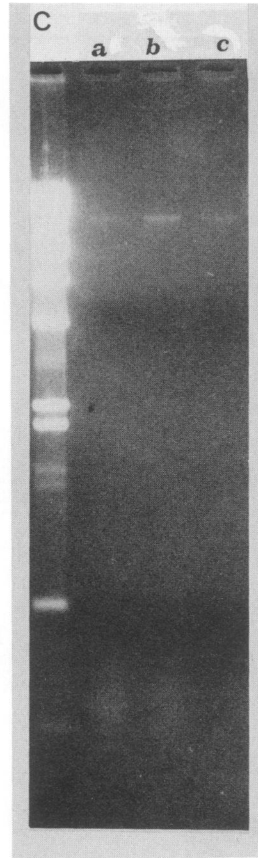
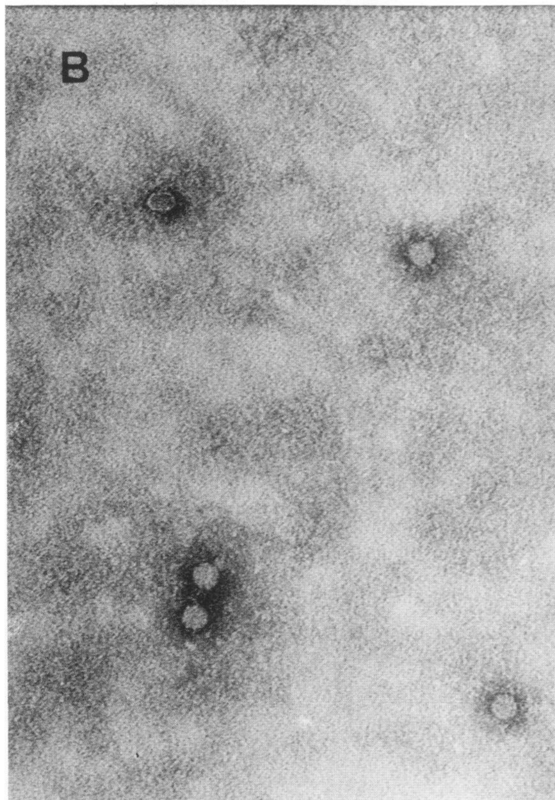
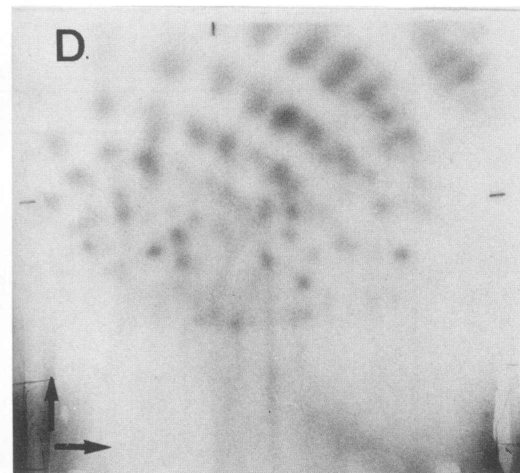


FIG. 3. Neutralization of HHA virus-induced CPE. A 1:25 dilution of HHA virus was incubated 1 h at 37°C with an equal volume of serial dilutions of a set of positive and preimmune anti-HHA sera. Triplicate cultures of Frp/3 cells were then infected as described in the legend to Fig. 1 and incubated at 34°C. The synthesis of HHA-specific antigens (○) was monitored by ELISA at 7, 9, and 14 days thereafter. Also shown is the percentage of cells that developed CPE (●). The values of CPE (□) and ELISA (■) reached by cultures infected with untreated virus are included. The reciprocal of the dilution of the hyperimmune (column A) and preimmune (column B) sera are reported in the abscissa.


A
g · ml⁻¹
ELISA O.D.492
fractions
1.5
1.4
1.3
1.2
.4
.3
.2
.1
10
20
30
40


C
a
b
c


B


D

still considerably shorter (1.9 to 2.4 kilobases) (Fig. 5). Accordingly, the cDNA was reannealed to denatured HHA RF and further extended with reverse transcriptase. The cDNA obtained by this procedure consisted of a collection of transcripts of various sizes (Fig. 5B, lane b), representing sequences derived from both strands of HHA RF. Some of the transcripts were long enough to contain 3'-proximal sequences complementary to each other. Accordingly, after phenol extraction, alkaline hydrolysis of the template RF, and gel filtration, the cDNA was hybridized and elongated in both directions by using the large (Klenow) fragment of the enzyme DNA polymerase. The untranscribed portions of HHA cDNA were trimmed with nuclease S1, homopolymeric sequences of deoxycytidine were added with terminal deoxynucleotidyl transferase, the "tailed" cDNA was inserted into the polydeoxyguanosine-tailed *Pst*I site of the plasmid pBR322, and *Escherichia coli* HB101 cells were transformed with the recombinant plasmids by published procedures (15).

Identification of HHA-related sequences. Plasmid DNA extracted from minipreparations of about 800 Tet^r Amp^s colonies was analyzed. To ascertain that the recombinants contained HHA virus-specific sequences, the DNA was immobilized onto Biodyne membranes (Pal Biodyne, Glen Cove, N.Y.) and probed by dot hybridization with 5'-labeled fragments of HHA virion RNA (Fig. 6). One hundred seventy-five recombinants were used to construct a restriction map of the genome of the fast-growing strain of HHA virus. Overlapping fragments were oriented and lined up as described previously (28). The restriction map of the fast-growing strain of HHA virus is similar to that published by Ticehurst et al. (28). Around the *Nco*I site (map position, about 2.9 kilobases), however, we noticed that all of the recombinants derived from the fast-growing strain of HHA virus lacked the site for *Pvu*II (map position, 3.3 kilobases) (Fig. 6). This was confirmed by DNA sequencing as described by Maxam and Gilbert (16).

The untranslated 5'-terminal sequences of the fast-growing strain of HHA virus RNA. Reverse transcription of the ds RF (instead of virion RNA) was devised as a method to facilitate the molecular cloning of the 5'-terminal sequences of the genome, which in principle should be represented even in the shortest cDNA transcripts of the negative strand. As expected, a series of recombinant plasmids were identified that carried inserts derived from the 5' end of HHA virus RNA. These sequences, determined as described by Maxam and Gilbert (16), are presented in Fig. 7.

A comparison of these sequences with those of three other isolates of HHA virus that became available after submission of this manuscript (1, 12, 18) revealed that the fast-growing strain of HHA virus and two of the other isolates (1, 18) have conserved extended areas of homology in the noncoding 5'-terminal region of the genome.

DISCUSSION

Infection of susceptible cells with a picornavirus usually results in a strong inhibition of the host cell protein and nucleic acid synthesis ("shutoff"), which eventually leads to cell death (14). Intriguingly, HHA virus has not been so far reported to induce CPE in vitro. In contrast to all known picornaviruses, HHA appears to be the only representative of this group that undergoes an exceedingly slow replication cycle (10).

In principle, the absence of CPE may be ascribed to some intrinsic deficiency of the viral strain (e.g., overproduction of defective interfering particles), to the lack of a suitable fully permissive host cell line (27), or to the conditions of the infection (temperature too high or low, multiplicity of infection, physiological conditions of the cell, etc.).

Therefore, the fast-growing strain described in this report appears as the first cytopathogenic isolate of HHA, and several lines of evidence indicate that it is indeed the agent responsible for the observed phenomenon. In fact, the synthesis of HHA-specific antigens (detected by either IIF or ELISA) parallels the development of CPE. Moreover,

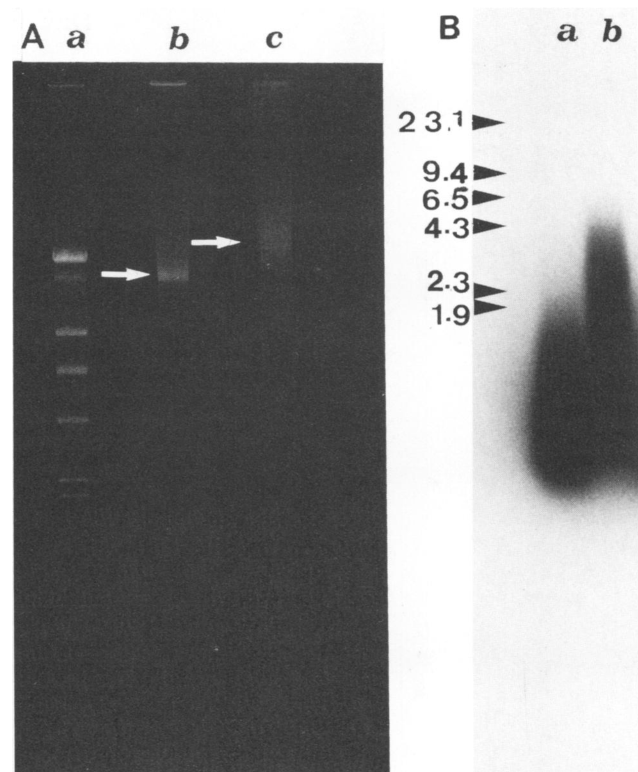


FIG. 5. Panel A, Neutral agarose gel electrophoresis of the HHA virus ds RF before (lane b) and after (lane c) the addition of poly(A) sequences at the 3' end of both strands. Also shown is *Hin*dIII-digested lambda DNA (lane a). Panel B, Alkaline agarose gel electrophoresis of ^{32}P-labeled cDNA transcripts of the poly(A)-tailed HHA ds RF before (lane a) and after being reannealed and extended (lane b). Arrowheads indicate the position of the *Hin*dIII-digested lambda DNA fragments in kilobases.

FIG. 4. Purification of the fast-growing strain of HHA virus. (A) HHA virus, extracted and purified as described in the text, was sedimented to equilibrium in a CsCl gradient (pH 7.5). Shown are the distribution of HHA antigens (■) and the density of each fraction (△). Denser (11–14), standard (21–26), and light (32–38) particles were pelleted from the fractions indicated by horizontal bars. (B) Standard-density HHA virions were searched for by solid-phase immune electron microscopy. (C) The RNAs extracted from the denser particles (lane a), standard virions (lane b), and the light component (lane c) were analyzed by electrophoresis through a neutral 0.8% agarose gel. (D) RNase T_1 fingerprint of HHA virus RNA.

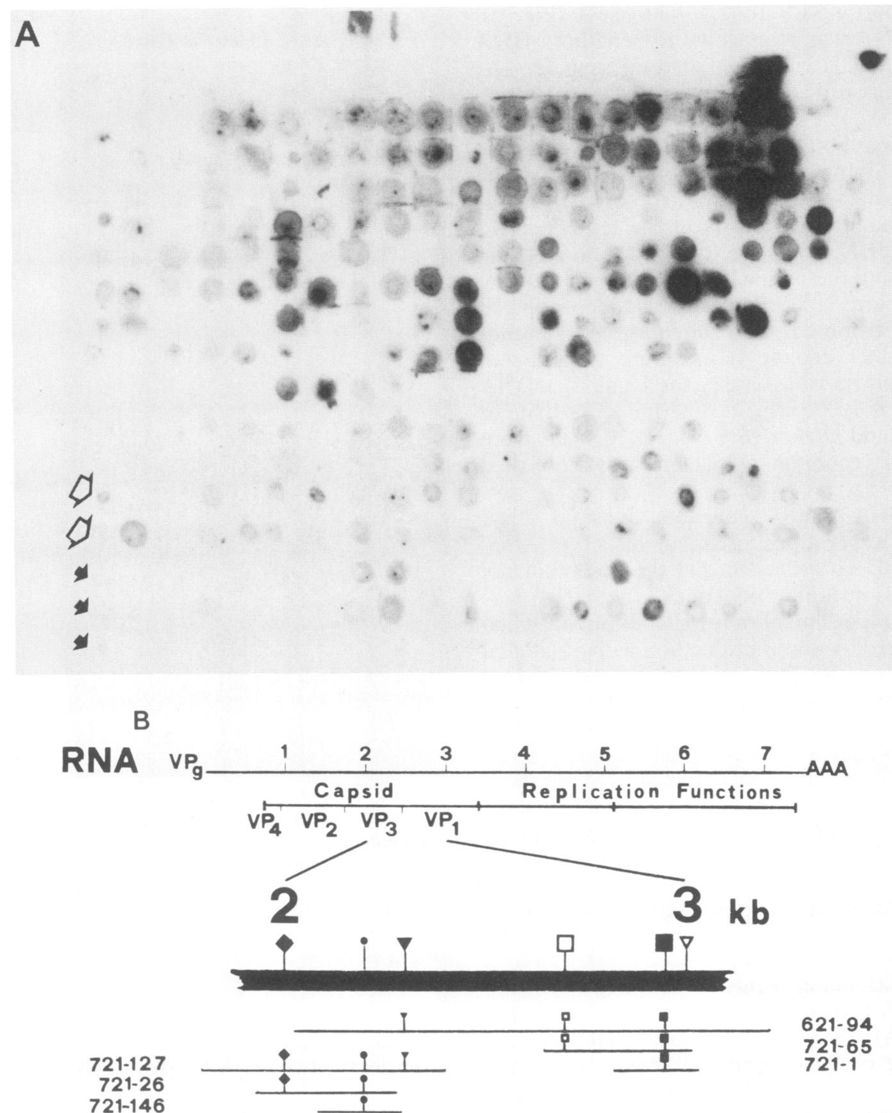


FIG. 6. Panel A, Identification of recombinant plasmids containing HHA virus-specific sequences. Plasmid DNA extracted from Tet[r] Amp[s] cells was immobilized onto membranes and probed by dot hybridization with 5′-labeled fragments of HHA virion RNA. Arrows indicate dots of DNA from colonies of *E. coli* HB101 (solid arrows) and pBR322-transformed bacteria (open arrows). Panel B, General organization of the picornavirus genome. In the expanded view, a map (uppermost) constructed from published data (28) is compared with the restriction map of plasmids 621-94, 721-65, 721-127, 721-26, 721-146, and 721-1 derived from the fast-growing strain of HHA virus. Symbols: ▽, *Pvu*II; ■, *Nco*I; □, *Bgl*I; ▼, *Bam*HI; ↑, *Hin*dIII; ◆, *Hin*cII.

three sets of human anti-HHA sera (but none of the preimmune ones) collected in totally different geographical areas (Switzerland and Germany) neutralized the infectivity of the virus (i.e., its ability to replicate and to induce the synthesis of specific antigens) and concomitantly prevented the establishment of CPE.

Upon extensive purification, the infectivity and the cytopathogenicity were systematically associated with the same sort of particles. Fingerprint analysis revealed a substantial homogeneity of the nucleic acids extracted from the standard-density virions, the denser and the lighter components, and even from the aggregates that occasionally pelleted in the CsCl gradients. If a contaminant was present in either the cultures or the viral preparation, it is difficult to visualize how could it have passed totally undetected. Moreover, the inability of the fast-growing strain of HHA virus to induce CPE in cells other than Frp/3 tends to exclude the possibility of trivial contamination with a cytopathogenic virus and provides additional indication for the involvement of a more specific virus-host cell interaction.

The pioneer studies of Plagemann and Swimm (23, 24) underlined the role of the host cell in the establishment of the shutoff and subsequent development of CPE. The same strain of mengovirus (a picornavirus of the cardiovirus group) that caused a strong inhibition of host cell RNA and protein synthesis in Novikoff hepatoma cells did not interfere noticeably with the host macromolecular synthesis when it replicated in another nutritional derivative of the same cell line. While the development of CPE in Frp/3 cells upon infection with the fast-growing strain of HHA virus is reminiscent of the mengovirus-hepatoma cell situation, the molecular basis for an unequal susceptibility of different cell

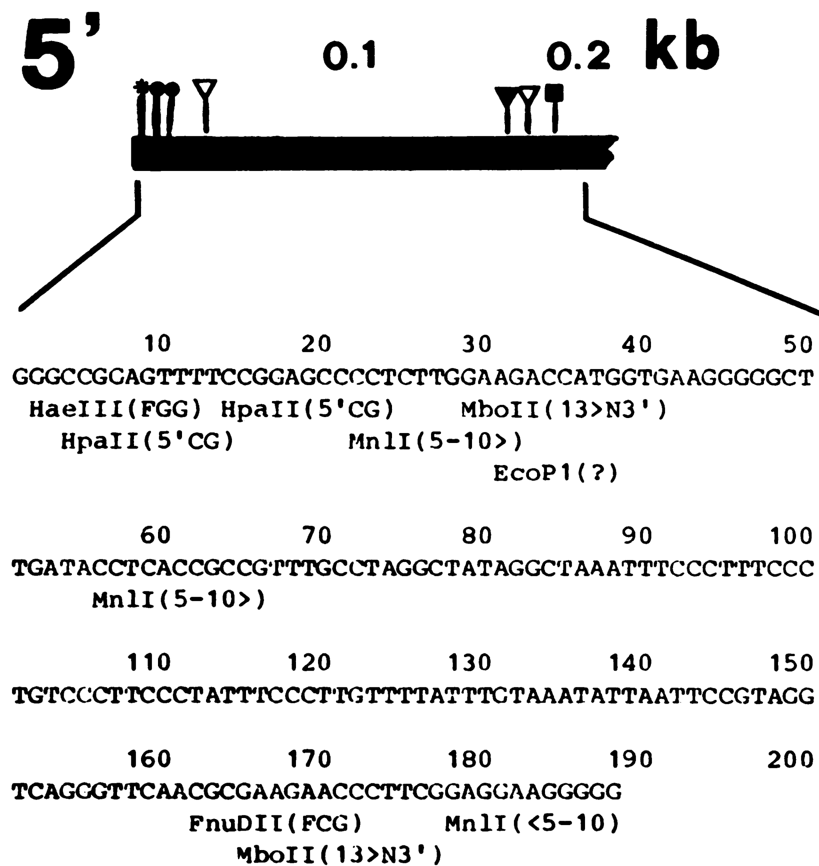


FIG. 7. Restriction map and nucleotide sequence of the 5′ end of the fast-growing strain of HHA viral cDNA. Symbols: ↑, *Hae*III; ↑, *Hpa*II; ↑, *Mnl*I; Y, *Fnu*DII; Y, *Mbo*II.

lines still remains unclear. Differences in the synthesis (or processing) of virus-specific macromolecules in different cell lines cannot be excluded a priori.

The molecular cloning of the genome of a picornavirus from its ds RF offered distinctive advantages over conventional approaches based on the reverse transcription of the virion RNA. The ds RF of picornaviruses, in the first place, contains a full-length equivalent of the viral genome, and a complementary strand of opposite polarity, a structure that, properly modified by the addition of poly(A) sequences, proved to be perfectly suited to transcribe cDNA copies in a reaction that was primed from the 3′ ends of each strand and proceeded in both directions. Moreover, since the capsid proteins are coded for by the portions of the genome close to the 5′ end of the virion RNA (11, 20), i.e., 5,000 to 6,000 bases apart from the area of the RNA molecule where the process of reverse transcription must begin, this new procedure involving the transcription of the 3′ areas of the negative strand provided a most effective way to enrich the sequences required for studies of the structural proteins. Originally, we designed this procedure (i) to circumvent the difficulties involved in obtaining cDNA copies of the 5′-distal sequences of a 7.5- to 8-kilobase genome and (ii) to ensure that a fair representation of the areas of the genome that code for the capsid protein would be found in the population of recombinant plasmids. Ultimately, it was also hoped that the procedure would facilitate the construction of full-length cDNA copies of the genome. While the first two expectations were amply fulfilled, the last was not. Whether the shorter cDNA transcripts had an advantage over longer ones during the tailing and hybridization steps or whether the recombinant plasmids carrying shorter foreign sequences were preferentially amplified is a matter of speculation at present.

Restriction analysis of the recombinants allowed us to assign the HHA virus-derived sequences to defined areas of the viral genome, whose 5′-terminal region was then sequenced by current procedures (16). Two remarkable features emerged from these studies.

(i) The untranslated 5′-terminal region of the fast-growing strain of HHA virus presented large areas of homology with the sequences of at least two other isolates (1, 18), suggesting that the conservation of the structures preceding the initiation codon may offer a defined selective advantage.

(ii) The restriction pattern of the fast-growing strain of HHA virus used in these studies presented minor differences with the map of the isolate of HHA virus cloned by Ticehurst et al. (28). In view of the remarkable immunological homogeneity of isolates of HHA virus collected in widely distant geographical areas, the minor differences observed in the restriction pattern of these two isolates underlines the need to investigate at the molecular level the degree of homology or divergence of viral genomes. The possibility must be entertained that new, still undetected serotypes of HHA virus may be identified in pathological specimens probed with the cloned sequences.

ACKNOWLEDGMENTS

We are indebted to F. Amaldi (2nd University of Rome) and Milton W. Taylor (Indiana University, Bloomington, Ind.) for discussions

and to Irene Bozzoni, whose constant help and advice were instrumental in the starting of this project.

LITERATURE CITED

1. **Baroudy, B. M., J. R. Ticehurst, T. A. Miele, J. V. Maizel, R. H. Purcell, and S. M. Feinstone.** 1985. Sequence analysis of hepatitis A virus cDNA coding for capsid proteins and RNA polymerase. Proc. Natl. Acad. Sci. USA **82**:2143–2147.
2. **Coulepis, A. G., S. A. Locarnini, and I. D. Gust.** 1980. Iodination of hepatitis A virus reveals a fourth structural polypeptide. J. Virol. **35**:572–574.
3. **Coulepis, G. A., S. A. Locarnini, E. G. Westaway, G. A. Tannock, and I. D. Gust.** 1982. Biophysical and biochemical characterization of hepatitis A virus. Intervirology **18**:107–127.
4. **Coulepis, A. G., G. A. Tannock, S. A. Locarnini, and I. D. Gust.** 1981. Evidence that the genome of hepatitis A virus consists of single-stranded RNA. J. Virol. **37**:473–477.
5. **Daemer, R. J., S. M. Feinstone, I. D. Gust, and R. H. Purcell.** 1981. Propagation of human hepatitis A virus in African green monkey kidney cell culture: primary isolation and serial passage. Infect. Immun. **32**:388–393.
6. **Deinhardt, F., and I. D. Gust.** 1982. Viral hepatitis. Bull. W.H.O. **60**:661–691.
7. **Dienstag, J. L., A. N. Schulman, R. J. Gerety, J. H. Hoofnagle, D. E. Lorenz, R. H. Purcell, and L. F. Baker.** 1976. Hepatitis A antigen isolated from liver and stool: immunological comparison of antisera prepared in guinea pigs. J. Immunol. **117**:876–881.
8. **Flehmig, B.** 1980. Hepatitis A virus in cell culture. I. Propagation of different hepatitis A isolates in a fetal rhesus monkey cell line (FRhK-4). Med. Microbiol. Immunol. **168**:239–248.
9. **Froesner, G. G., F. Deinhardt, R. Scheid, V. Gauss-Mueller, N. Holmes, V. Messelberger, G. Siegl, and J. J. Alexander.** 1979. Propagation of human hepatitis A virus in a hepatoma cell line. Infection **7**:303–306.
10. **Gauss-Mueller, V., K. von der Helm, and F. Deinhardt.** 1984. Translation in vitro of human hepatitis A virus RNA. Virology **137**:182–184.
11. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
12. **Linemeyer, D. L., J. G. Menke, A. Martin-Gallardo, J. V. Hughes, A. Young, and S. W. Mitra.** 1985. Molecular cloning and partial sequencing of hepatitis A viral cDNA. J. Virol. **54**:247–255.
13. **Locarnini, S. A., A. C. Coulepis, E. G. Westaway, and I. D. Gust.** 1981. Restricted replication of human hepatitis A virus in cell culture: intracellular biochemical studies. J. Virol. **37**:216–225.
14. **Lucas-Lenard, J.** 1979. Inhibition of cellular protein synthesis after virus infection, p. 73–112. *In* R. Perez-Bercoff (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
17. **Mechali, M., R. Perez-Bercoff, G. Carrara, and E. Falcoff.** 1973. An improved methodology for the preparation of virus-induced double-stranded RNA. Biochimie **55**:361–363.
18. **Najarian, R., D. Caput, W. Gee, S. J. Potter, A. Renard, J. Merrywheather, G. Van Nest, and D. Dina.** 1985. Primary structure and gene organization of human hepatitis a virus. Proc. Natl. Acad. Sci. USA **82**:2627–2631.
19. **Panà, A., A. M. Patti, and E. Paroli.** 1982. Hepatitis A virus (HHA) cultivation in the cecopithecus kidney cell line RC-37. Ann. Sclavo **24**:344–351.
20. **Palmenberg, A. C., E. M. Kirby, M. R. Janda, N. L. Drake, G. M. Duke, K. F. Potratz, and M. S. Collet.** 1984. The nucleotide and deduced aminoacid sequence of the encephalomyocarditis viral polyprotein coding region. Nucleic Acids Res. **12**:2969–2985.
21. **Perez-Bercoff, R. (ed.).** 1979. The mechanism of replication of picornavirus RNA, p. 293–318. *In* The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
22. **Perez-Bercoff, R., M. Gander, and E. Preisig.** 1978. The genomic RNA of mengovirus. II. Comparative analysis of the standard and the denser virions. Virology **85**:378–386.
23. **Plagemann, P. G. W.** 1968. Mengovirus replication in Novikoff rat hepatoma and mouse L cells: effects on synthesis of host-cell macromolecules and virus-specific synthesis of ribonucleic acid. J. Virol. **2**:461–473.
24. **Plagemann, P. G. W., and H. E. Swim.** 1966. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cell. J. Bacteriol. **91**:2317–2326.
25. **Provost, P. J., and M. R. Hilleman.** 1979. Propagation of human hepatitis A virus in cell culture in vitro. Proc. Soc. Exp. Biol. Med. **160**:213–221.
26. **Siegl, G., G. G. Froesner, V. Gauss-Mueller, J.-D. Tratschin, and F. Deinhardt.** 1981. The physicochemical properties of infectious hepatitis A virion. J. Gen. Virol. **57**:331–341.
27. **Taylor, M. W., and G. Chinchar.** 1979. Host-restriction of picornavirus infection, p. 337–348. *In* R. Perez-Bercoff (ed.), The molecular biology of picornaviruses. Plenum Publishing Corp., New York.
28. **Ticehurst, J. R., V. R. Racaniello, B. M. Baroudy, D. Baltimore, R. H. Purcell, and S. M. Feinstone.** 1983. Molecular cloning and characterization of hepatitis A virus cDNA. Proc. Natl. Acad. Sci. USA **80**:5885–5889.
29. **Tratschin, J. D., G. Siegl, G. G. Frösner, and F. Deinhardt.** 1981. Characterization and classification of virus particles associated with hepatitis A. III. Structural proteins. J. Virol. **38**:151–156.