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NOTES

Evidence that the Genome of Hepatitis A Virus Consists of Single-Stranded RNA

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Nucleic acid was extracted from purified hepatitis A virus, radiolabeled with ^{125}I, and shown to consist of single-stranded RNA which sediments at 35S and contains sequences of polyadenylic acid. These findings are consistent with hepatitis A virus being a member of the genus *Enterovirus* within the family Picornaviridae.

Since the discovery of hepatitis A virus (HAV) by Feinstone et al. in 1973 (8), studies of its biochemical characterization have been hampered by the difficulty in obtaining large quantities of the virus. To date, HAV has proved difficult to isolate in vitro (18), and relatively small quantities of virus have been recovered from the feces of hospitalized patients (14) or experimentally infected animals (15). The development of techniques for purifying HAV (12) and the availability of several high-titer fecal specimens (5) have provided sufficient material to enable us to study both the structural polypeptides and the biophysical characteristics of the virus. We now report an extension of these studies for the characterization of the viral genome, which is shown in the present paper to consist of single-stranded RNA with a sedimentation coefficient of approximately 35S and to contain sequences of polyadenylic acid [poly(A)]. These findings are compatible with those expected of a member of the genus *Enterovirus* within the family Picornaviridae (2).

HAV was purified from feces by differential centrifugation and solvent extraction, followed by agarose gel filtration and two cycles of isopycnic ultracentrifugation in cesium chloride (3, 12). The pellets from all centrifugation and extraction steps were reprocessed to obtain additional virus. To exclude the possibility that other cytopathic agents were present, samples of each purified preparation were inoculated into primary cynomolgus monkey embryo kidney and HeLa cell cultures and examined for the presence of cytopathic effects. None were noted. Each purified preparation (Fig. 1) was estimated to contain more than 10^4 HAV particles per electron microscope 400-mesh grid square (at least 5×10^9 physical particles per ml) (14). No other virus-like particles were apparent, and the identity of each preparation was established by immune electron microscopy (13) with human pre- and postinfection hepatitis A sera. The presence of HAV-specific antigen was monitored at all stages of the purification process by solid-phase radioimmunoassay (13).

Nucleic acid present within the virus was released after four extractions by the phenol-chloroform method (17). The material present within the extract was then iodinated by a modification of the chloramine-T oxidation procedure described by Shaposhnikov et al. (19) which did not involve a heating step to remove unstable iodine intermediates; when another RNA iodination procedure involving such a step on the extracted nucleic acid of poliovirus type 1 was used (22), a range of low-molecular-weight RNAs was always produced, suggesting that scission of the larger RNA molecule had taken place. Labeled nucleic acid present in the preparation was initially characterized by sucrose gradient centrifugation. Fractions from the gradients were then collected, and each fraction was divided into three equal volumes. The first volume per fraction was spotted onto a Whatman no. 1 filter paper (3 by 4 cm). All papers were then dried and acid washed by the method of Dalgarno et al. (6). Each of the remaining volumes was then treated with either 1 μg of pancreatic RNase A (type IIIA; Sigma Chemical Co., St. Louis, Mo.) or 1 μg of DNase I (Sigma Chemical Co.) for 1 h at 25°C. Each incubation mixture was placed onto separate filter papers, which were dried and acid washed, and the radioactivity present was determined in a

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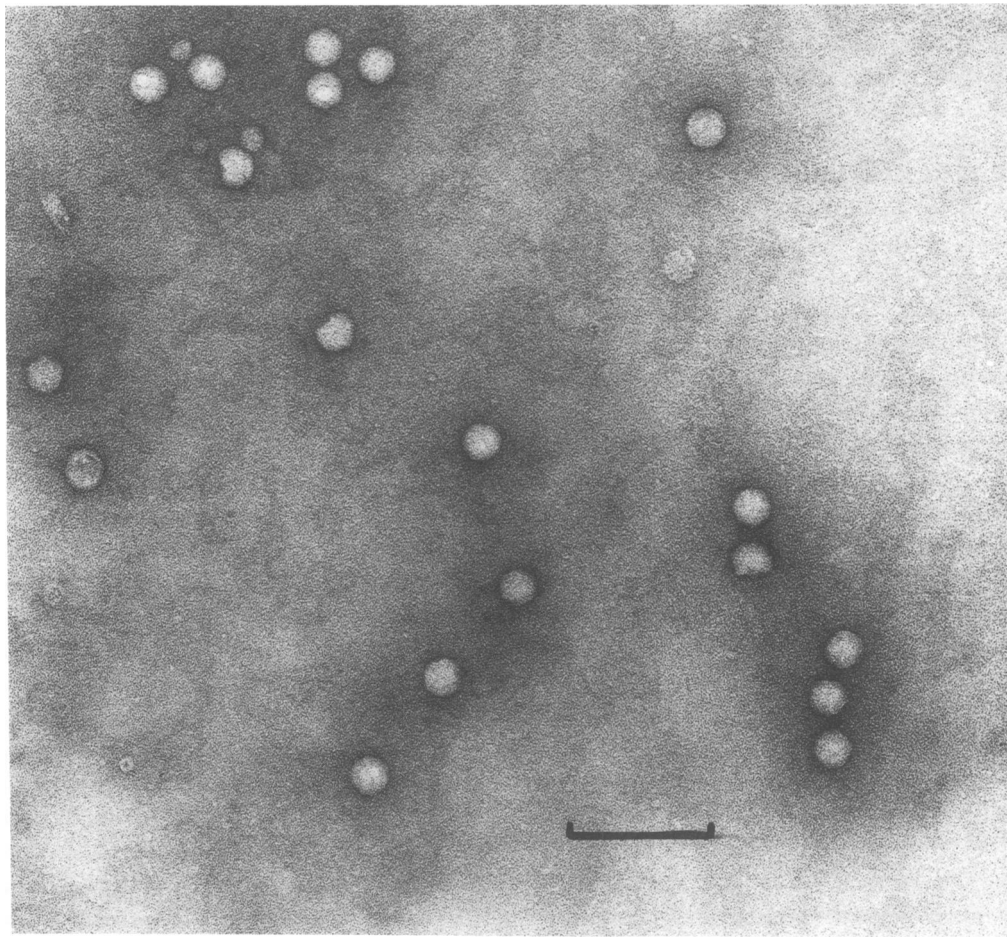

FIG. 1. *Electron microscopy of purified HAV which was used for nucleic acid characterization. A direct electron micrograph of highly purified HAV negatively stained with 4% (wt/vol) phosphotungstic acid (pH 7.4) is shown. This preparation shows negligible background debris which was essential for the efficient iodination of the HAV genome. Bar = 100 nm.*

gamma counter. The radioactive profile in Fig. 2 indicates the presence of two peaks. From the position of other RNAs centrifuged in parallel (arrows), the high- and low-molecular-weight peaks have sedimentation coefficients of approximately 35S and 4S, respectively. The 35S peak is fully susceptible to RNase but only slightly reduced by DNase digestion, whereas the 4S peak is resistant to DNase and slightly reduced by RNase. These findings suggest that the 35S peak contains single-stranded RNA with a sedimentation coefficient similar to that of picornaviral RNA, since the use of similar gradients has previously been shown to conserve double-stranded RNA structures while rendering single-stranded RNA susceptible to RNase digestion (1). The small reduction in size of the 35S peak after treatment with DNase is possibly due to RNase contamination of DNase.

The 4S peak probably consists largely of small amounts of iodinated protein which were released from the RNA but remained in the aqueous phase after phenol-chloroform extraction; the small reduction in size which occurs after treatment with RNase probably indicates the presence of a minor RNA degradation product from the 35S peak. Similar findings were also made in studies with purified poliovirus. Further evidence for the identity of the 4S material is provided by an additional study in which individual fractions were incubated with 5 μg of pronase for 1 h at 25°C. A reduction in acid-insoluble radioactivity of approximately 90% for the 4S peak and 40% for the 35S peak occurred, implying that the 4S material is largely protein and that some RNase activity is associated with the pronase preparation.

To further characterize the 35S peak, fractions

4 through 7 of a similar peak from another gradient were pooled and centrifuged to equilibrium in a cesium sulfate gradient (Fig. 3). Fractions were collected, and the buoyant density of each was determined from its refractive index. The radioactivity present in samples of each was

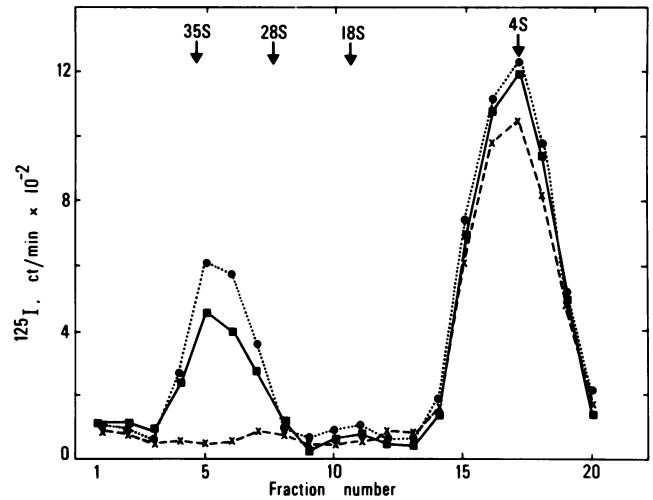

FIG. 2. *Sucrose gradient analysis of nucleic acids released from HAV by phenol-chloroform extraction. Nucleic acid was released from purified virus after four extractions by the phenol-chloroform method. To the extract was added 10 μl of 1 M NaH_2PO_4 buffer (pH 7.4), 100 μg of chloramine-T dissolved in 25 μl of 50 mM NaH_2PO_4, and 15 μl of $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.; specific activity, 119 mCi/mmol). The mixture was shaken for 25 s at 25°C, and the reaction was stopped by the addition of 100 μl of 5 mM $Na_2S_2O_5$ and 200 μl of 100 mM KI. The iodinated nucleic acid was then separated from the excess iodine by passage through a Sephadex G-25 column equilibrated with NET and precipitated with ethanol in the presence of 100 μg of yeast carrier RNA. After the precipitate was washed twice with ethanol, it was resuspended in ice-cold NET. A 500-μl sample was centrifuged at 310,000 × g through 4.0 ml of a 15 to 30% (wt/vol) sucrose gradient prepared in NET for 2.33 h at 7°C. Fractions were collected and treated as described in the text. The profiles of acid-insoluble radioactivity for untreated (●) fractions and fractions treated with RNase (×) and DNase (■) are shown. The arrows represent the positions of 35S poliovirus RNA and 28S, 18S, and 4S rRNA's, which were determined in parallel gradients.*

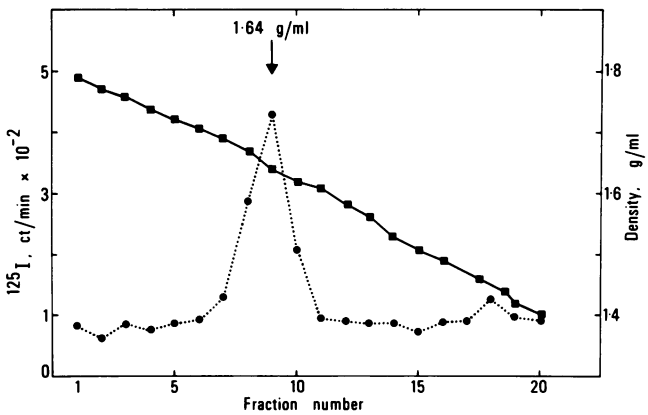

FIG. 3. *Buoyant density of 35S iodinated nucleic acid prepared by sucrose gradient centrifugation. Fractions 4 through 7 from the lower peak of a sucrose gradient (Fig. 2) were pooled, precipitated with 100 μg of yeast carrier RNA, and suspended in 400 mM NaCl-10 mM EDTA-10 mM Tris (pH 7.4). A total of 500 μl of the solution was layered onto 2.5 ml of a solution of cesium sulfate prepared in the same buffer which had been adjusted to a density of 1.64 g/ml and placed in a Spinco SW50.1 tube. The tube was topped with paraffin oil and centrifuged at 100,000 × g for 65 h at 7°C. Fractions were then collected, and the density of 10-μl samples of each was determined from their refractive indices (■). The radioactivity present in the remainder of each sample was determined in a gamma counter (●). The arrow at 1.64 g/ml represents the position of [^{3}H]uridine-labeled poliovirus RNA in a parallel gradient.*

then determined, and the results for the gradient are shown in Fig. 3. Only a single peak of radioactivity was obtained with a density of 1.64 g/ml, which was identical to that obtained with poliovirus in a parallel gradient, thereby providing strong confirmatory evidence that the 35S peak in Fig. 2 contains single-stranded RNA (7). These findings preclude the possibility that either double-stranded RNA (density, 1.61 g/ml) (9) or single-stranded DNA (density, 1.71 to 1.72 g/ml) (16) is present within the HAV genome.

As a test for the presence of poly(A) sequences within the RNA, fractions 4 through 7 from the 35S peak of another sucrose gradient were pooled and applied to a polyuridylic acid-Sepharose 4B column (0.5 by 2 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The initial filtrate was collected, and nonadherent RNAs were removed in five steps with wash buffer consisting of 1 part formamide and 3 parts NET (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride [pH 7.4]). Poly(A)-containing RNAs were removed in five steps with elution buffer consisting of 9 parts formamide and 1 part NET. The profile for radioactivity in the HAV RNA filtrate and in each wash and eluate is shown in Fig. 4a, and this is compared with profiles obtained for influenza A virus RNA [containing no poly(A)] and poliovirus RNA [containing poly(A)] in Fig. 4b and c, respectively. A total of 58% of HAV RNA could be released from the column with elution buffer, indicating that poly(A) sequences are present. With influenza A virus RNA, only 9% could be eluted, but with poliovirus RNA the amount was 83%. A significant number of RNAs containing no poly(A) within the 35S peak of HAV RNA, which are evident in the washing steps, may be due to RNase activity in the fecal specimens which provided the initial source of virus. They could also be caused by oxidation steps in the iodination procedure, which resulted in RNA cleavage. Evidence that such cleavage may have occurred is suggested from the small amounts of RNase-susceptible radioactivity in the 4S peak (Fig. 2). Deficiencies in the poly(A) content of Rous sarcoma virus and human coronavirus OC-43 RNAs have been noted when these viruses were prepared under conditions which favored the activity of RNase (11, 21).

The presence of poly(A) in the 35S RNA peak is evidence that HAV possesses a genome with the same polarity as its intracellular messenger (10). This has been confirmed by demonstrating the infectivity of extracted HAV RNA in Vero cells (S. A. Locarnini, A. G. Coulepis, E. G. Westaway, and I. D. Gust, unpublished data), thereby clearly indicating that HAV is a positive-stranded RNA virus (2). This is consistent with reports from other laboratories which have identified HAV as an RNA virus (20; Y. Moritsigu, J. W.-K. Shih, T. Kakefuda, S. M. Feinstone, J. L. Gerin, and R. H. Purcell, submitted for publication). The findings reported here, together with recent evidence that four polypeptides with molecular weights of 33,000, 26,500, 22,500, and 14,000 are present within the HAV virion (4), strongly indicate that HAV is a picornavirus (2).

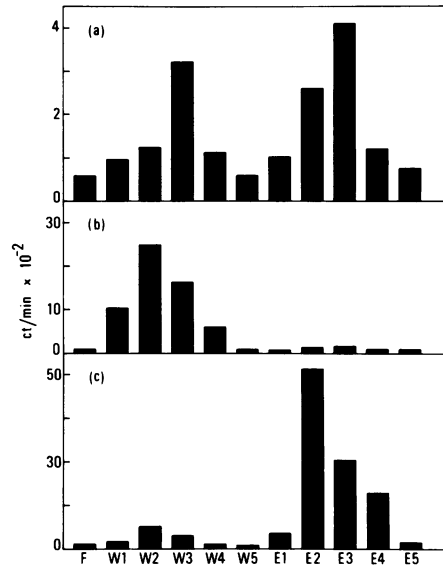

FIG. 4. *Elution profile of HAV RNA (a), influenza A virus strain PR8 RNA (b), and poliovirus type 1 RNA (c) in polyuridylic acid-Sepharose 4B columns. Fractions 4 through 7 from an HAV sucrose gradient (Fig. 2) were pooled, and the RNA present was precipitated in the presence of 100 μg of yeast carrier RNA. The RNA was suspended in 0.5 ml of NET and applied to a column (0.5 by 2 cm). The initial filtrate (F) was collected, and nonadherent RNAs were removed in five steps (W1 to W5), each with 0.5 ml of washing buffer (1 part formamide and 3 parts NET). Poly(A)-containing RNAs were then eluted in five steps (E1 to E5) with 0.5 ml of elution buffer (9 parts formamide and 1 part NET). The radioactivity in the filtrate, washes, and eluates is shown (a). A similar procedure was followed with RNAs prepared by phenol-chloroform extraction of purified influenza A virus strain PR8 (b) and poliovirus (c).*

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ADDENDUM IN PROOF

The findings in this paper have been extended by analyzing one more fecal specimen containing a high titer of HAV. The nucleic acid obtained from the preparation of purified HAV was radiolabeled with ^{125}I, and its sedimentation rate and electrophoretic mobility was compared with those of poliovirus and rRNA. The RNA genome of HAV was shown to have a sedimentation coefficient of 33S in sucrose gradients and an approximate molecular weight of 2.25×10^6 when analyzed by electrophoresis in agarose-acrylamide gels under nondenaturing conditions.

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