Isolation of Single Stranded DNA from Purified Hepatitis A-Virus

Bertram Flehmig *, Hans Dieter Royer ** and Hans-Joachim Gerth

Z. Naturforsch. **33 c**, 594–597 (1978);
received February 7/May 2, 1978

Hepatitis A-Virus- Single-Sranded DNA, Electron Microscopy, Length Measurements, Parvovirus

Hepatitis A-virus was purified from human stools by three purification steps. Virus was identified by radioimmunoassay and purity monitored with immune electron microscopy. Virus particles, serologically and morphologically identical, banded in CsCl in two density ranges at 1.31–1.34 g/cm³ and at 1.41–1.43 g/cm³. Virions of density 1.31–1.34 g/cm³ were shown to contain single-stranded DNA of different size classes. Class I 1.33 kb, class II 4.61 kb in addition a small amount of molecules was detected with lengths up to 15 kb.

In 1973 in stools of volunteers infected with the hepatitis A-strain MS-1, 27 nm viruslike particles were visualized [1] and in several reports these particles could be shown to be hepatitis A-virus (HAV) [2–6]. The classification of hepatitis A-virus is open and up to now only indirect and conflicting evidence regarding the nature of the nucleic acid has been published [7, 8]. In this preliminary communication we report on the purification of hepatitis A-virus strain GBG and the demonstration of single stranded DNA as viral nucleic acid. During an outbreak of hepatitis A in Southern Germany in 1975 stools from healthy contact persons were collected. One of them, patient GBG, developed an acute hepatitis. Stool of this patient, collected 6 days before the onset of jaundice served as a source of HAV. Stools were examined for the presence of HAV by immune electron microscopy (IEM) and radioimmunoassay (RIA). IEM and RIA were performed as described previously [6, 9]. The identity of HAV strain GBG with MS-1 HAV reference strain was proven by immune agglutination with MS-1 reference antiserum, and the HAV-IgG agglutinates were visualized with the electron microscope [6]. This result proves that the isolated particles are hepatitis A-virus. Hepatitis A-virus strain GBG was isolated by three purification steps. Each step was examined for contaminants by IEM, and HAV was identified by RIA. A 10% suspension of stool GBG in 0.9% NaCl, 4 mM Na_2HPO_4, 1 mM KH_2PO_4 (pH 7.2), containing 0.1% NP_{40} was clarified by low speed centrifugation. HAV was sedimented by centrifugation with 40 000 rpm for 2 hours, pellets were dissolved with the same buffer and banded in a preformed CsCl gradient, density 1.1–1.5 g/cm³ (purification step 1) the centrifugation time was 20 hours at 35 000 rpm in a SW 40 rotor. Two discrete peaks are found, peak 1 at density 1.31–1.34 g/cm³ and peak 2 at density 1.40–1.43 g/cm³ (Fig. 1 a). The greater portion of HAV is found in peak 1. Because of the low concentration of HAV in peak 2, we used only HAV from peak 1 for further purification. After dialysis against PBS, HAV containing fractions were layered onto a 10–30% sucose gradient and spun for 70 min at 204 000 × g (purification step 2). Fig. 1 b shows the sedimentation profile of HAV. HAV was then agglutinated with Anti-HAV IgG prepared from GBM convalescent serum and the agglutinates were collected by centrifugation with 10 000 × g for 2 hours (purification step 3). Fig. 2 shows ag-

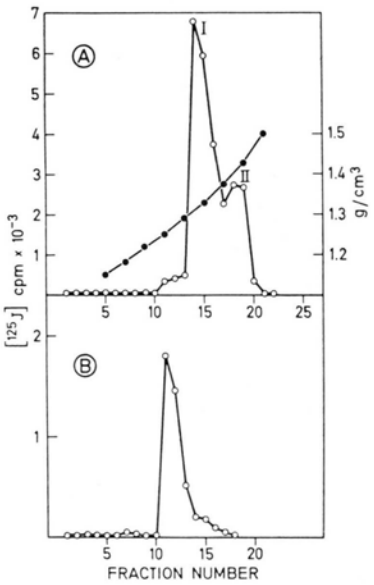

Fig. 1. a) CsCl density gradient of HAV, showing two peaks at density 1.31–1.34 g/cm³ (I) and at density 1.40–1.43 g/cm³ (II). HAV was banded in a preformed CsCl gradient ranging from 1.1–1.5 g/cm³, centrifugation time 20 hours at 35 000 rpm in a SW 40 rotor.
b) Velocity sedimentation of HAV, density 1.31–1.34 g/cm³ in a 10–30% sucrose gradient for 70 min at 204 000 × g, with rotor SW 40.
From both gradients, fractions were taken from the tube, starting at the top.

* Abteilung für Medizinische Microbiologie und Epidemiologie der Viruskrankheiten, Hygiene-Institut der Universität Tübingen, D-7400 Tübingen.

** Max-Planck-Institut für Biologie, Abt. Beermann, Spemannstraße 34, D-7400 Tübingen.

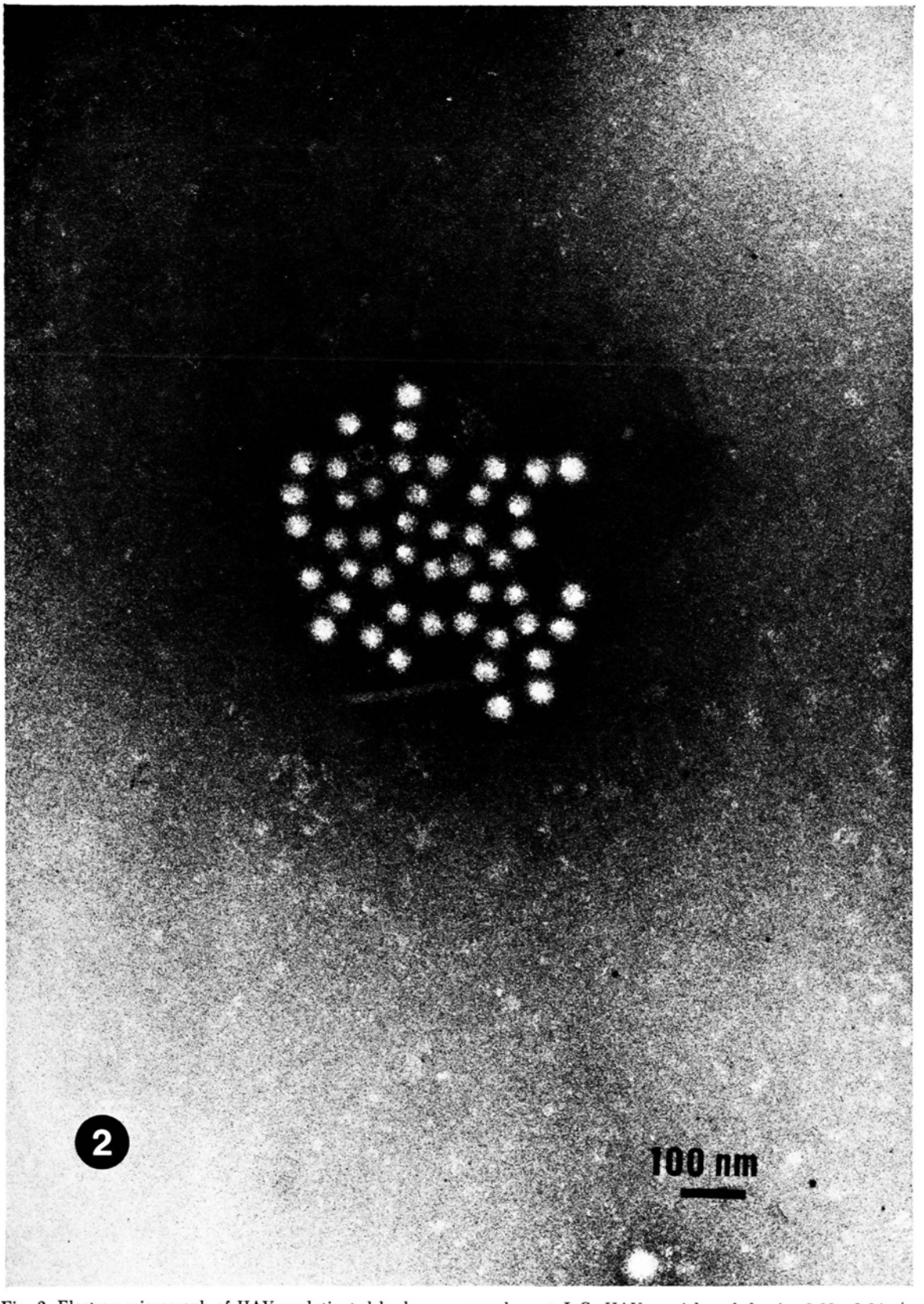

Fig. 2. Electron micrograph of HAV agglutinated by human convalescent IgG. HAV particles of density 1.31—1.34 g/cm^3.

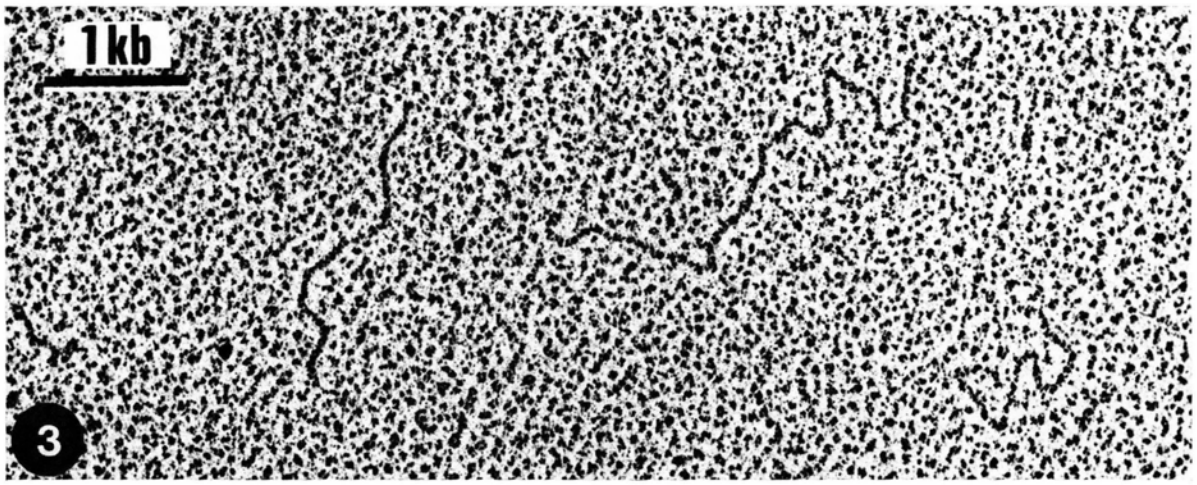

Fig. 3. Electron micrograph of single-stranded linear DNA molecules isolated from purified HAV and spread from 50% formamide.

glutinated Hepatitis A-virus particles. After CsCl-gradient centrifugation (purification step 1) besides HAV many phages, bacterial debris and viruslike particles were seen in the electron microscope, after velocity sedimentation (purification step 2) the amount of contaminants was reduced, and following immune-agglutination (purification step 3) all contaminants were removed except very few bacterial cilia and some membranous fragments. Fig. 2 shows HAV agglutinates besides very few contaminants.

With several HAV negative control stools the same purification procedure was performed and fractions of density ranges in which HAV was found in the positive stools were taken as controls for nucleic acid determinations. As a further control HAV after purification step 2 was incubated with GBG preillnes serum and nucleic acid determination was done with these samples. From HAV after purification step 3 and from controls nucleic acids were extracted by the phenol detergent method. Nucleic acids were precipitated with 2 volumes ethanol, solubilized with 10 mM Tris HCl (pH 8), 1 mM EDTA and extensively dialysed against the same buffer. For visualization of the nucleic acids in the electron microscope the formamide spreading technique was applied [10], using a modified microdrop speading procedure [11], grids were stained with uranyl acetate and shadowed with Pt/Pd/Au. Fig. 3 shows linear unfolded nucleic acid molecules of different lengths without recognizable secondary structure, which suggested the nucleic acid to be a single-stranded DNA molecule [14]. To prove this suggestion the nucleic acid preparation was incubated with DNase (20 μg/ml), or RNase (20 μg/ml). After DNase treatment no molecules could be detected, RNase had no effect. To check the RNase activity 18S rRNA from *Chironomus tentans* was included in the reaction. Before RNase addition the grids were fully covered with RNA when spread for the EM, after RNase treatment only viral DNA molecules were seen. The nucleic acid determination of controls showed only low concentrations of DS and SS DNA molecules but never the molecules found in HAV positive fractions. To exclude co-purification of free DNA molecules which may have influenced the results seen in the electron microscope, we treated purified HAV prior to nucleic acid extraction with DNase to destroy any DNA molecule, which could have been adsorbed to the surface of HAV. However, after these treatments the same length distribution of ss-DNA molecules was found. Hydrolysis with 0.4 M KOH at 50 °C for 1.5 h does not destroy the described nucleic acid molecules. These results clearly showed that HAV contains single stranded DNA.

Length measurements of linear ss-DNA molecules revealed different size classes. As internal size standard ss-DNA of phage ΦX 174 was included in the spreadings. The length of this DNA was taken as 5375 bases or 5.4 kb [15]. Lengths are expressed in units of kilobases (kb). Class 1 contains small molecules with a length of 1.33 kb, these molecules might either be broken molecules or they might originate from defective HAV particles. It is known

from the Aleutian mink disease virus which is considered to be a parvovirus with unusual properties, that small sized molecules are found as well as full length genomes [13]. Class 2 molecules with a mean length of 4.62 kb have roughly the same length as DNA molecules isolated from various parvovirus (for review see [12]). In addition longer ss-DNA molecules with lengths up to 15 kb were seen in the electron microscope, 15% of the ss-DNA molecules belonged to the latter population.

Our results are in contrast to the results obtained by Siegl *et al.* which used the same stool material. Siegl *et al.* found the same densities of HAV in CsCl and extracted a nucleic acid from purified HAV which has roughly the same length distribution as class II molecules described in this paper. Siegl *et al.* treated purified HAV with NaOH, from the absence of molecules in EM preparations they comcluded that RNA has to be the nucleic acid of HAV. As a control ^{3}H labelled Poliovirus RNA was hydrolysed and hydrolysis was measured by TCA preparation. (Siegl, personal communication.)

We thank Drs. H. Frank and G. F. Meyer for providing electron microscope facilities, Dr. K. Geider for a gift of ΦX 174 DNA, Dr. C. P. Hollenberg for discussions, P. Hardy for critical reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

[1] S. M. Feinstone, A. Z. Kapikian, and R. H. Purcell, Science **182,** 1026–1028 (1973).
[2] C. C. Mascoli, O. L. Ittensohn, V. M. Villarejos, G. J. A. Arguedas, P. J. Provost, and M. R. Hilleman, Proc. Soc. Exp. Biol. Med. **142,** 276–282 (1973).
[3] S. A. Locarnini, A. A. Ferris, A. C. Stott, and J. D. Gust, Intervirology **4,** 110–118 (1974).
[4] C. R. Gravelle, C. L. Hornbeck, J. E. Maynard, E. H. Schable, and D. W. Bradley, J. Infect. Dis. **131,** 167–171 (1975).
[5] J. Rakela, O. H. Fay, D. Stevenson, I. Gordon, and W. Mosley, Bull. World Health Organ. **54,** 561–564 (1976).
[6] B. Flehmig, H. Frank, G. G. Frösner, H.-J. Gerth, Med. Microbiol. Immunol. **163,** 209–214 (1977).
[7] S. M. Feinstone, A. Z. Kapikian, J. L. Gerin, and R. H. Purcell, J. Virol. **13,** 1412–1414 (1974).
[8] P. J. Provost, B. S. Wolanski, W. J. Miller, O. L. Ittensohn, W. J. McAleer, and M. R. Hilleman, Proc. Soc. Exp. Biol. Med. **148,** 532–539 (1975).
[9] B. Flehmig, M. Ranke, H. Frank, and H.-J. Gerth, Med. Microbiol. Immunol., in press.
[10] R. W. Davis, M. Simon, and N. Davidson, Methods in Enzymology (L. Grossman, and K. Moldave, eds.), **Vol. 21,** D, pp. 413–428, Academic Press, New York 1971.
[11] R. B. Inman and M. Schnös, J. Mol. Biol. **49,** 93–98 (1970).
[12] J. A. Rose, Comprehensive Virology (H. Freankel-Conrat and R. W. Wagner, eds.), **Vol. 3,** pp. 1–61, Plenum Press, New York 1974.
[13] M. S. Shahrabadi, H. J. Cho, and R. G. Marusyk, J. Virol. **23,** 353–362 (1977).
[14] P. K. Wellauer and B. D. Dawid, Proc. Nat. Acad. Sci. **70,** 2827–2831 (1973).
[15] F. Sanger, G. M. Air, B. G. Barrel, N. L. Brown, A. R. Coulson, J. C. Fiddes, C. A. Hutchinson III, P. M. Slocombe, and M. Smith, Nature **265,** 687–695 (1977).