JOURNAL OF VIROLOGY, Apr. 1978, p. 48-53
0022-538X/78/0026-0048$02.00/0

Vol. 26, No. 1
Printed in U.S.A.

Characterization and Classification of Virus Particles Associated with Hepatitis A

II. Type and Configuration of Nucleic Acid

GÜNTER SIEGL[1]* AND GERT G. FRÖSNER[2]

Institute of Hygiene and Medical Microbiology, University of Bern, CH 3010 Bern, Switzerland,[1] and Max von Pettenkofer-Institute of Hygiene and Medical Microbiology, D-8000 Munich 2, Federal Republic of Germany[2]

Received for publication 17 October 1977

Virus particles banding at 1.34 g/ml in CsCl and sedimenting at 160S in sucrose gradients were isolated from fecal specimens of patients suffering from hepatitis. In the presence of 4 M urea and about 90% formamide, these particles released linear nucleic acid molecules of the kinked appearance characteristic of single-stranded RNA or single-stranded DNA. They could be distinguished from the nucleic acid of phage λ added to the preparation as a marker for double-stranded configuration. Experiments in which the virus particles under investigation were incubated at pH 12.9 at 50°C for 30 min revealed that their nucleic acid molecules were hydrolyzed as readily as the RNA genome of poliovirus type 2 analyzed in parallel. Both the single-stranded DNA of phage φX174 and that of parvovirus LuIII, however, proved unaffected by this treatment, and the double-stranded DNA of phage λ was denatured to single-stranded molecules. It was concluded, therefore, that the virus of human hepatitis A contains a linear genome of single-stranded RNA and has to be classified with the picornaviruses.

In the accompanying paper (12), we reported on the purification of virus-like particles from fecal specimens collected during an outbreak of hepatitis A in Germany. The majority of the isolated particles were shown to band at 1.34 g/ml in CsCl and to sediment at 160S in linear sucrose gradients. These physicochemical characteristics, as well as a diameter of 27 to 29 nm, were found congruent with those of poliovirus type 2 used as a marker picornavirus and incompatible with the size, density, and sedimentation behavior of LuIII, a marker parvovirus analyzed in parallel. Additional particles, banding in the density range of between 1.38 and 1.44 g/ml and assumed to favor the classification of hepatitis A virus (HAV) with the parvoviruses (4), were shown to behave like the so-called "dense component" of several picornaviruses (10). Upon reisolation from CsCl, they disintegrated into three particle species sedimenting at 160, 90 to 100, and about 50S. These observations strongly supported the earlier conclusion of Provost et al. (8) that the causative agent of human hepatitis A is a picornavirus rather than a parvovirus.

In the present paper we report on the isolation and characterization of the nucleic acid contained within the 160S fecal particles; control experiments including LuIII as a marker parvovirus and poliovirus type 2 as a picornavirus marker were run in parallel. The data obtained provide reliable evidence that the virus particles under investigation have a genome of single-stranded RNA and, therefore, should be classified with the picornaviruses.

(A preliminary report of part of this work was presented at the 10th International Congress of Chemotherapy, Zurich, Switzerland, September 1977).

MATERIALS AND METHODS

Origin and purification of the viruses. The origin of the fecal specimens used as a source of HAV, the techniques applied for purification, and the characteristics of the isolated particles have been described in detail (5, 12). Moreover, the HAV samples used in the following experiments are identical to the preparations characterized in the previous paper (12). Both the hepatitis-associated particles and the two marker viruses were stored in 50 mM Tris-hydrochloride (pH 7.4) at −20°C in siliconized glass tubes. λ phage was a gift of M. Fürst, Institute of Hygiene and Medical Microbiology, Bern, Switzerland, and single-stranded, circular DNA of phage φX174 *am*3 was generously supplied by R. H. Dennin, Klinikum der Medizinischen Hochschule, Lübeck, Germany.

Electron microscopy of nucleic acid. The nucleic acid of the particles was spread for electron microscopy by the methods of Robberson et al. (9). Briefly, 500 μl of a 4 M solution of solid urea (Mann Research Laboratories, ultrapure) in analytical-grade

formamide (Merck) was mixed with 5 μl of a solution containing 4 mg of cytochrome *c* (Sigma Chemical Co., type VI) per ml of bidistilled water and 5 μl of 3 M Tris-hydrochloride (pH 8.5)–0.1 M EDTA. A total of 100 μl of the resulting solution was added to 10 μl of the concentrated, purified virus particles and kept at room temperature for 10 to 30 min. Finally, 30 μl was spread onto a hypophase of bidistilled water in a 30-cm^2 rectangular Teflon tray. Protein films spread in this manner were picked up on Parlodion-coated, carbon-enforced, 400-mesh copper grids, stained with 10^{-4} M uranyl acetate for about 30 s, and rinsed in isopentane. After the grids were rotary shadowed at an angle of 6 to 10° with Pt-Pd (8:2), they were examined in a Philips EM 300 electron microscope at 60 kV with a 30-μm objective aperture. Micrographs were taken on plates (3.25 by 4 inches [ca. 8.25 by 10.16 cm]) at an instrumental magnification of 10,000 to 16,000. The actual magnification was determined and controlled with a diffraction replica (Ladd). Negatives were projected onto tracing paper, and the molecules were measured with a map measurer.

Strict precautions were taken to minimize the loss or degradation of nucleic acid molecules. Both particle suspensions and spreading mixtures were kept in siliconized glass tubes. Calibrated micropipettes, as well as the Teflon tray, were cleaned in chromate-sulfuric acid, and glass slides were stored in nitric acid-sulfuric acid-water (1:1:2) for several days before use. Buffers and bidistilled water were autoclaved, and glassware was sterilized.

Alkaline hydrolysis of nucleic acid. Parvovirus LuIII labeled with [^{3}H]thymidine and poliovirus type 2 labeled with [^{3}H]uridine as described previously (12) were diluted in 50 mM Tris-hydrochloride (pH 7.4) to a final concentration of about 20,000 acid-precipitable cpm per 100 μl. Part of the suspension was removed to serve as an untreated control, whereas the final volume was mixed with 0.08 volume of 1 N NaOH to bring the pH to 12.9. Both the control samples and the alkaline samples were then incubated at different temperatures for various periods of time as given in Fig. 2. At the indicated times, 100-μl portions were removed and trichloroacetic acid-insoluble radioactivity was determined (13).

For electron microscopy, 20 μl of virus particles or pure nucleic acid was mixed with 2 μl of 1 N NaOH, sealed in a siliconized glass capillary, and heated for 30 min to 50°C. The reaction mixture was then transferred to a siliconized glass tube containing 2 μl of 3 M Tris-hydrochloride (pH 7.0)–0.1 M EDTA to readjust the pH to 8.5. A total of 250 μl of the urea-formamide-cytochrome *c* solution was added, and the NaOH-treated molecules were spread as described. Controls received 50 mM Tris-hydrochloride (pH 7.4) instead of 1 N NaOH, but otherwise were treated the same way.

RESULTS

Size and configuration of nucleic acid molecules. Control experiments with purified LuIII and poliovirus type 2 revealed that virions of both the parvovirus and picornavirus families, although of quite different stability, released their genome in the presence of 4 M urea and about 90% formamide. The single-stranded RNA of the poliovirus and the single-stranded DNA of the parvovirus and of phage φX174 were well extended in the cytochrome *c* films and could be traced readily for length measurements. Moreover, the double-stranded DNA released by λ phages appeared largely undenatured, with only occasional single-stranded regions.

With a mean length of 1.7 μm, the single-stranded DNA of parvovirus LuIII proved to be in the size range determined for these molecules by different methods in previous experiments (11). Most of the single-stranded circles of φX174 DNA measured 1.5 to 1.8 μm (Fig. 1), and poliovirus RNA showed at least a bimodal size distribution, with peaks at 1.2 and 2.3 to 2.4 μm (Fig. 1).

The purified virus of hepatitis A used in these experiments was isolated from the 1.34-g/ml peak fraction after isopycnic banding of 160S particles in CsCl (see Fig. 4 of reference 12). When spread under identical conditions, they released nucleic acid molecules similar in appearance to the single-stranded DNA of parvovirus LuIII and to the single-stranded RNA of poliovirus type 2 (Fig. 2 and 3). All molecules were linear. They varied in size from small pieces of less than 0.5 μm to rather long structures of about 3.5 μm in length. However, two size classes of about 1.2 and 1.7 μm were predominant (Fig. 1).

Determination of the type of nucleic acid.

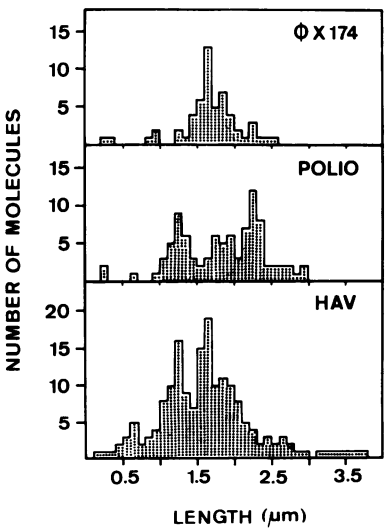

FIG. 1. *Length distribution of circular, single-stranded DNA molecules of phage φX174 (n = 56), linear single-stranded RNA of poliovirus type 2 (n = 93), and linear nucleic acid molecules released from HAV particles (n = 175).*

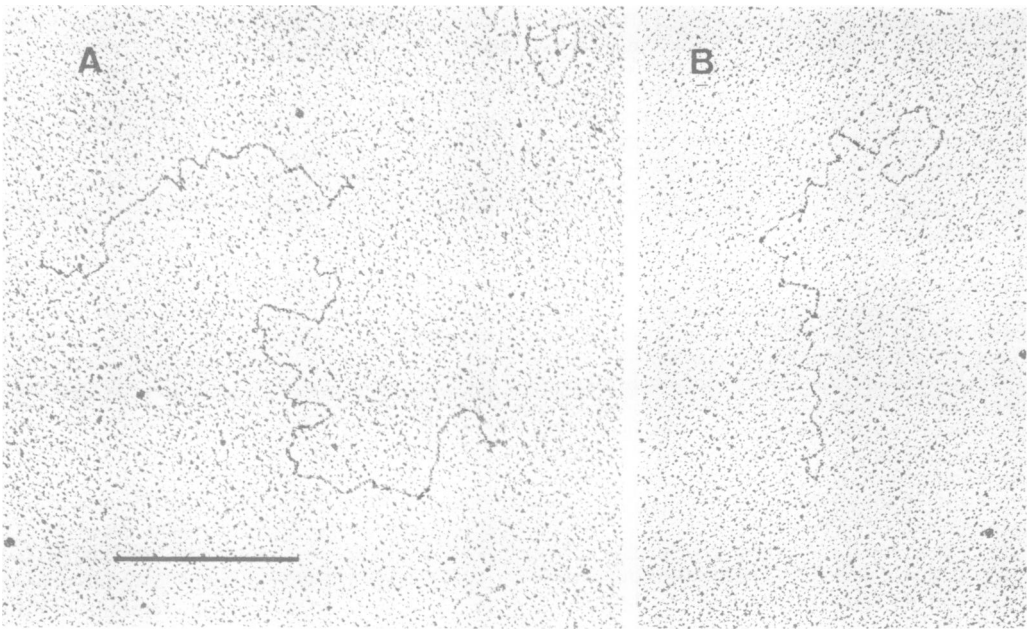

FIG. 2. *Nucleic acid molecules of various lengths released from 160S HAV particles. Bar = 0.5 μm and is valid for (A) and (B).*

In contrast to DNA, RNA is readily hydrolyzed in alkaline solutions. The degree of hydrolysis depends on the concentration in OH^-, the temperature, and the length of time the reaction is allowed to take place. The influence of two of these variables on the integrity of single-stranded DNA and of single-stranded RNA was studied in a pilot experiment shown in Fig. 4. It is evident that detectable quantities of the single-stranded DNA of parvovirus LuIII are converted into acid-soluble products only after prolonged incubation at 80°C. Under the same conditions, more than 90% of the single-stranded RNA of poliovirus type 2 was rendered acid soluble within only 5 min. At 50°C, the total quantity of radiolabeled DNA remained acid insoluble throughout the tested period of time, whereas more than 90% of RNA could be hydrolyzed within 30 min. The milder treatment was expected to result in small fragments of RNA that might be visible in the electron microscope. The detection of these residual small pieces, rather than the complete loss of all molecules, would exclude the possibility that the absence of molecules from electron microscope grids resulted from artifacts during spreading or from inappropriate adsorption of nucleic acid/cytochrome *c* films onto the grids. Consequently, all further experiments were run at 50°C for 30 min.

The crucial experiment—incubation of purified HAV particles with and without NaOH—was accompanied by a series of controls. First, parvovirus LuIII, poliovirus type 2, and pure DNA of φX174 were treated in the same manner as HAV particles. Second, λ phages were added to the HAV particles before incubation with NaOH. The experiments were repeated three times, and at least 10 grids per nucleic acid sample were examined in the electron microscope.

The linear single-stranded DNA of LuIII and the circular single-stranded DNA of φX174 proved unaffected by incubation at pH 12.9. The single-stranded RNA molecules of poliovirus, on the other hand, were almost completely lost. Occasionally, a few molecules in the size range of between 0.3 and 0.6 μm could be found on the respective grids. Almost identical results were obtained when HAV particles were treated with NaOH. Individual squares of the 400-mesh grids showed on an average two to four rather short molecules, 0.2 to 0.6 μm in length. The largest molecule found measured 0.9 μm. Particles incubated in the presence of 50 mM Tris (pH 7.4), however, yielded grids with about 30 to 40 normal-sized nucleic acid molecules per square. In control samples to which λ phages had been added before incubation at pH 12.9, the originally double-stranded DNA proved completely denatured. Nevertheless, the resulting single-stranded molecules were clearly visible. Struc-

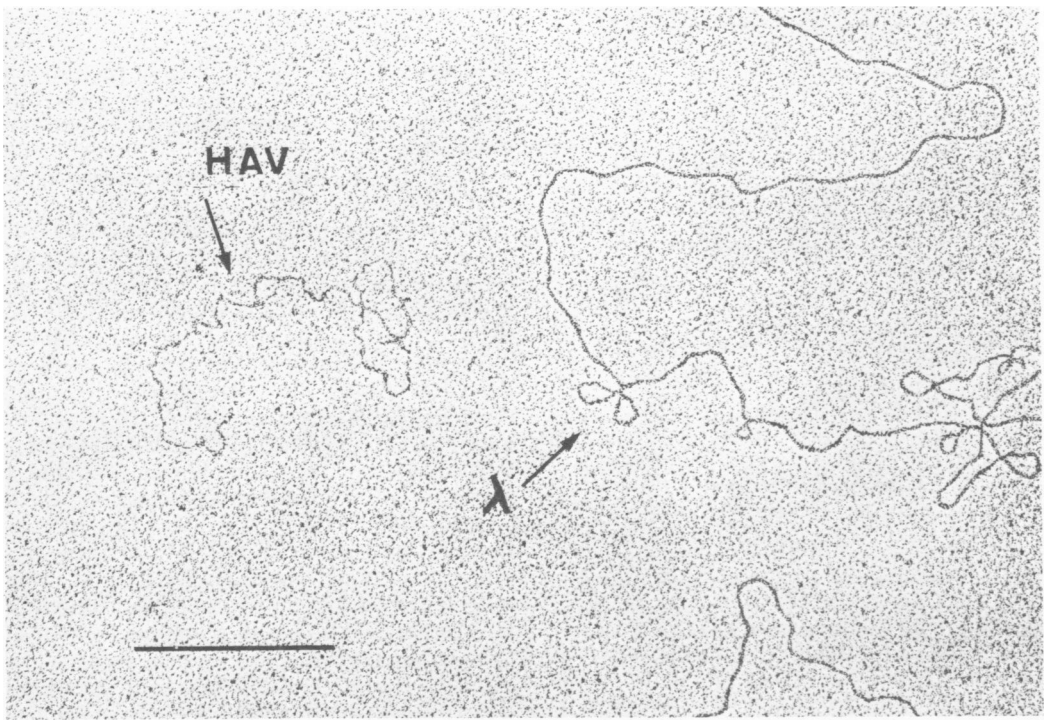

FIG. 3. *Double-stranded λ DNA and the probably single-stranded nucleic acid released from HAV particles. Note the difference in both contrast and folding of the molecules. Bar = 0.5 μm.*

tures in the size range of HAV nucleic acid, however, could not be detected. It was concluded, therefore, that the nucleic acid of virus particles banding at 1.34 g/ml in CsCl and sedimenting at 160S contained a nucleic acid of the RNA type.

DISCUSSION

The technique described in this paper offers the possibility for determining both the type and the configuration of the nucleic acid of virus particles isolated in limited quantity from clinical specimens. The method per se is easy to perform; yet several precautions must be taken to avoid misleading results. First, the virus suspension should be homogeneous in the sense that it contains only one type of particle. Second, no free, unencapsulated nucleic acid should be present. Third, it is essential to include marker DNA molecules into the samples during hydrolysis. The detection of single-stranded DNA of known length after NaOH treatment excludes the possibility that the absence of the nucleic acid molecules of interest results from anything except a preparational artifact.

In the evaluation of the hepatitis A-associated particles described in this report, all of these requirements were strictly fulfilled. Only particles banding at a density of 1.34 g/ml and sedimenting as a homogeneous peak at 160S were analyzed. Free nucleic acids eventually contaminating the partially purified virus suspensions had been removed by digestion with RNase and DNase, and denatured single-stranded molecules of λ DNA were readily detected on the grids after hydrolysis of HAV. The originally observed smaller molecules, however, were absent. It may be concluded, therefore, that the genome of HAV consists of RNA.

The molecules released from the 160S particles showed the kinked appearance characteristic of single-stranded RNA and, after simultaneous spreading, could be readily distinguished from double-stranded λ DNA molecules (Fig. 3). The spreading conditions applied in these experiments also have been used successfully to analyze the sequence of single-stranded and double-stranded regions in rRNA (9, 15). It is, therefore, rather unlikely that the genome of HAV in situ consists of a double-stranded RNA molecule that denatured completely into complementary single strands and that did not give rise to at least some partially double-stranded structures. At the present time, however, such a possibility

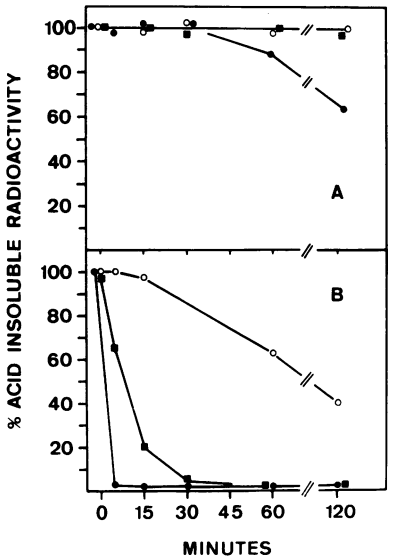

FIG. 4. *Hydrolysis of [^{3}H]thymidine-labeled parvovirus DNA (A) and of [^{3}H]uridine-labeled poliovirus RNA (B) at various temperatures. Labeled parvovirus LuIII and poliovirus type 2 were suspended in 50 mM Tris (pH 7.4), brought to pH 12.9 by the addition of 1 N NaOH, and were incubated either at ambient temperature* (○), *50°C* (■), *or 80°C* (●). *At the indicated time, 100-μl portions were removed in duplicate and assayed for acid-insoluble radioactivity.*

cannot be excluded completely. Single-stranded RNA is known to behave as a rather labile structure during preparation for electron microscopy, and many viral RNA genomes were found to disintegrate into a series of fragments (1, 3, 6, 14). For poliovirus type 1 RNA, Granboulan and Girard (6) observed molecules of between 0.5 and 4.5 μm in length, with two predominant forms accumulating around 1.1 and 2.4 μm in the histogram. In our hands, the size distribution of poliovirus type 2 RNA proved to be almost identical, and 1.2 and about 2.3 μm were recorded for the most prominent strands. Assuming that the 2.3-μm strand represents the complete genome with a molecular weight of 2.6 × 10^6 (2), a ratio of weight per length of 1.13 × 10^6 daltons per μm would then be characteristic for poliovirus RNA under the present experimental conditions.

HAV particles were shown to band at 1.34 g/ml and to sediment at 160S, as did mature virions of poliovirus type 2 (12). It might be expected, therefore, that the single-stranded RNA molecules contained in both types of particles are also comparable in size. The histograms established for the two nucleic acids, however, differ considerably. In general, HAV nucleic acid proved to be shorter than poliovirus RNA, and its main molecular form measured only about 1.7 μm. On the basis of the ratio of weight per length determined for poliovirus RNA, this size would be consistent with a molecular weight of only 1.9 × 10^6. Similarly, 1.3 × 10^6 can be calculated for the smaller molecule with a length of 1.2 μm.

Single-stranded RNA having a molecular weight of about or even less than 2 × 10^6 is not characteristic for vertebrate picornaviruses; yet it was frequently isolated from small RNA viruses of invertebrates, as well as from RNA plant viruses (for example, see reference 2). Some of the latter viruses also were shown to contain their genetic information distributed in two or more RNA strands (2, 7). Although it is tempting to speculate that the histogram shown in Fig. 1 is suggestive of a multistrand genome, which possibly could explain the difficulties encountered in the cultivation of HAV in tissue cultures, the present results are far from allowing reliable statements on the molecular weight of HAV RNA. Thus, due to a varying content of guanine plus cytosine, it might be necessary to calculate the molecular weight with a ratio of weight to length other than the one determined for poliovirus RNA (3). Moreover, the dissociation of picornaviruses in the presence of urea is known to yield a nucleoprotein strand rather than a clean RNA molecule (16). The observed length may then be a function of the stability and mode of the nucleic acid-protein interaction. However, both the marker picornavirus and the HAV particles were spread under the same conditions. The observed differences in the size distribution therefore nevertheless suggest clear differences in the in situ conformation of the respective genomes.

ACKNOWLEDGMENTS

We are grateful to F. Deinhardt and D. Jachertz for continuous encouragement and stimulating discussions.

This study was supported by grant Fr 400/5 from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Breese, S. S., Jr.** 1976. A comparison of molecular weights of foot-and-mouth disease virus RNA fragments determined from lengths and S-rates. J. Gen. Virol. **31**:1-8.
2. **Brown, F., and R. Hull.** 1973. Comparative virology of the small RNA viruses. J. Gen. Virol. **20**(Suppl.):42-60.
3. **Chi, Y. Y., and A. R. Bassel.** 1974. Electron microscopy of viral RNA: molecular weight determination of bacterial and animal virus RNAs. J. Virol. **13**:1194-1199.
4. **Feinstone, S. M., A. Z. Kapikian, J. L. Gerin, and R. H. Purcell.** 1974. Bouyant density of the hepatitis A virus-like particle in cesium chloride. J. Virol. **13**:1412-1414.
5. **Frösner, G. G., L. R. Overby, B. Flehmig, H. J. Gerth,**

H. Haas, R. H. Decker, C. M. Ling, A. J. Zuckerman, and H. R. Frösner. 1977. Seroepidemiological investigation of patients and family contacts in an epidemic of hepatitis A. J. Med. Virol. **1**:163–173.

6. **Granboulan, N. E., and M. Girard.** 1969. Molecular weight of poliovirus ribonucleic acid. J. Virol. **4**:475–479.
7. **Newman, J. F. E., and F. Brown.** 1973. Evidence for a divided genome of Nodamura virus, an arthropod-borne picornavirus. J. Gen. Virol. **21**:371–384.
8. **Provost, P. J., B. S. Wolanski, W. J. Miller, O. L. Ittensohn, W. J. McAleer, and M. R. Hilleman.** 1975. Physical, chemical, and morphologic dimensions of human hepatitis A virus strain. Proc. Soc. Exp. Biol. Med. **148**:532–539.
9. **Robberson, D., Y. Aloni, G. Attardi, and N. Davidson.** 1971. Expression of the mitochondrial genome in HeLa cells. VI. Size determination of mitochondrial ribosomal RNA by electron microscopy. J. Mol. Biol. **60**:473–484.
10. **Rowlands, D. J., M. W. Shirley, D. V. Sangar, and F. Brown.** 1975. A high density component in several vertebrate enteroviruses. J. Gen. Virol. **29**:223–234.
11. **Siegl, G.** 1973. Physicochemical characteristics of the DNA of parvovirus LuIII. Arch. Gesamte Virusforsch. **43**:334–344.
12. **Siegl, G., and G. G. Frösner.** 1978. Characterization and classification of virus particles associated with hepatitis A. I. Size, density, and sedimentation. J. Virol. **26**:40–47.
13. **Siegl, G., and M. Gautschi.** 1976. The multiplication of parvovirus LuIII in a synchronized culture system. III. Replication of viral DNA. J. Virol. **17**:841–853.
14. **Weber, G. H., J. E. Dahlberg, M. Cottler-Fose, and U. Heine.** 1974. Electron microscopy of single-stranded RNA from vesicular stomatitis virus. Virology **62**:284–287.
15. **Wellauer, P. K., and I. B. Dawid.** 1974. Secondary structure maps of ribosomal RNA and DNA. I. Processing of *Xenopus laevis* ribosomal RNA and structure of single-stranded ribosomal DNA. J. Mol. Biol. **89**:379–395.
16. **Wiegers, K. J., U. Yamaguchi-Koll, and R. Drzeniek.** 1976. A complex between poliovirus RNA and the structural polypeptide VP-1. Biochem. Biophys. Res. Commun. **71**:1308–1312.