The Genome of Equine Arteritis Virus

B. A. M. VAN DER ZEIJST, M. C. HORZINEK

Institute of Virology, Veterinary Faculty, State University Utrecht, Yalelaan 1, Utrecht, The Netherlands

AND

V. MOENNIG¹

Federal Research Institute for Animal Virus Diseases, 74 Tübingen, West Germany

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Equine arteritis virus (EAV) contains an infectious RNA. [³H]uridine-labeled RNA was released from purified virus (density, 1.155 g/ml in sucrose; $s_{20,w}$, 224 ± 8 S) with sodium dodecyl sulfate and 2-mercaptoethanol. An $s_{20,w}$ value of 48 S was found in isokinetic sucrose gradients in 0.1 M saline. In 1 mM saline, sedimentation was slower (33 S), in 0.1 M saline plus 1 mM MgCl₂, a value of 56 S was measured. A molecular weight of 4.0×10^6 was determined by polyacrylamide-agarose-gel electrophoresis. Heating of purified RNA in the presence of 1.1 M formaldehyde and subsequent centrifugation in gradients containing formaldehyde did not result in degradation to smaller RNA's. From this procedure a molecular weight of 4.1×10^6 was calculated. Buoyant density in Cs₂SO₄ of the RNA was 1.65 g/ml. In most experiments Semliki forest virus RNA was taken as a reference. It behaved almost indistinguishably from the RNA of EAV. In conclusion EAV contains an infectious, colinear molecule of single-stranded RNA with a molecular weight of about 4 million. These data justify a definite inclusion of this virus in the family Togaviridae.

INTRODUCTION

Equine arteritis virus (EAV) was isolated in 1956 in the United States (Doll *et al.*, 1957) and in 1964 in Europe (Bürki, 1965). A number of properties of the virion, such as buoyant density, sensitivity to organic solvents and detergents, size of the virion and the presence of an isometrical core, have led to the proposal to include the virus in the togavirus family, being a nonarthropod-borne member (see Horzinek, 1973, for a review). However, until now the nucleic acid of the virion has not been characterized. Radwan *et al.* (1973) reported the incorporation of [³H]uridine into the virion, proving that the virus contains RNA.

The present paper reports some physicochemical properties of EAV-RNA which

was studied in a comparative way, the RNA of a well-known togavirus, Semliki forest virus (SFV), serving as a reference.

MATERIALS AND METHODS

Buffer solutions. Hypertonic buffer I: 10 mM Tris-Cl, 0.6 M NaCl, 20 µg/ml of polyvinyl sulfate (Sigma Chemical Co., St. Louis, Mo.), pH 7.5. Hypertonic buffer II: same as hypertonic buffer I except that the NaCl concentration was raised to 1.1 M. Buffer TES: 0.02 M Tris-Cl, 1 mM EDTA, 0.1 M NaCl, pH 7.4. PBS-DEAE is phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) containing 50 µg/ml of DEAE-dextran (Pharmacia, Uppsala, Sweden).

Cells and virus. BHK-21 cells were grown in Earle's salt solution containing twice the amount of Eagle's vitamins, 0.1 g/liter of yeast extract (Difco, Detroit,

¹ Present address: Institute of Virology, Veterinary School, 3 Hannover, West Germany.

Mich.), 5.0 g/liter of lactalbumin hydrolysate (Nutritional Biochemical Co., Cleveland, Ohio), 50 mg/liter of glutamine, 10% calf serum and penicillin/streptomycin (100 IU/ml and 100 μ g/ml).

The Bucyrus strain of EAV (Doll *et al.*, 1967) was obtained from Dr. F. Bürki, Vienna. A virus stock was prepared by infecting monolayers of BHK-21 cells in Roux bottles with virus (no more than five passages from the original stock) diluted in PBS-DEAE to give an m.o.i. of 0.03. After 2 hr at 37° the inoculum was removed and replaced by 50 ml of Vm3a salt solution (Schwöbel and Siedentopf, 1961) containing 137 mM NaCl, 4 mM KCl, 1.6 mM CaCl_2 , 1 mM MgCl_2 , 23.8 mM NaHCO_3 , 10 mM glucose and 20 mg/liter of phenol red, pH 7.2. Virus harvested 40 hr after infection, titering 2.1×10^6 PFU/ml, was kept frozen in small aliquots at -70° and served as seed stock for all the experiments described below.

To obtain high-titered virus stocks, BHK-21 clone 13 cells grown in suspension in modified Eagle's medium containing 10% calf serum (Barteling, 1972) were used. Cells (10^6 /ml) were infected with virus at an m.o.i. of 0.1. A maximum virus yield of 2.5×10^7 PFU/ml was obtained when about 30% of the cells were killed. After removal of the cells by low speed centrifugation, virus was pelleted at 100,000 g for 2 hr and resuspended in TES buffer. This suspension was frozen and stored at -70°C.

Plaque assay of virus and RNA. The method was essentially that of Hyllseth (1969): Confluent BHK-21 cell cultures in 5-cm petri dishes were washed once with 4 ml of PBS-DEAE and 0.2 ml of the virus suspension, diluted in 10-fold steps in PBS-DEAE containing 2% calf serum, was added. After 30 min at 37° the unadsorbed virus was aspirated and the cells were overlaid with 4 ml of 1% agar (Bacto-Agar, Difco Laboratories, Detroit, Mich.) in Eagle's medium containing 2% calf serum. Plaques were counted after 4 days.

For the assay of the infectivity of EAV-RNA, the same procedure was employed with the following modifications. After

washing with PBS, treatment with hypertonic buffers I and II ensued; after 5 min of incubation, hypertonic buffer II was aspirated and 0.2 ml of RNA diluted in this buffer was inoculated. After a 15-min adsorption period at 22° the monolayer was washed with hypertonic buffer I and the overlay was applied.

Radioactive virus. BHK-21 cells were trypsinized and diluted in Dulbecco's modification of minimal essential medium (DMEM) containing 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) instead of bicarbonate and supplemented with 10% foetal calf serum. All experiments were performed in vials containing 5-ml quantities (3×10^6 cells/ml) and 1 μ g/ml of actinomycin D (Serva, Heidelberg, Germany). Virus and [^3H]uridine ([5- ^3H]uridine, 29 Ci/mmol, The Radiochemical Centre, Amersham, England) were added (10–50 μ Ci/ml). The vials were incubated in a shaker bath at 37°. The culture fluid was harvested 13 hr after infection and cells were removed by low speed centrifugation. After the addition of 2×10^8 PFU/ml of carrier EAV, virus was pelleted at 100,000 g for 2 hr. Pellets resuspended in 0.2–0.5 ml of TES buffer were centrifuged in a 20–50% (w/w) sucrose gradient in the same buffer in a Spinco SW 50L rotor at 50,000 rpm at 5° for 3 hr.

Preparation of EAV and marker RNAs. Gradient fractions of EAV were diluted with TES buffer. Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol to make a 1% solution of each, and 0.1 mg/ml of carrier yeast RNA (Boehringer, Mannheim, Germany), purified by phenol extraction and ethanol precipitation, were added. The solution was extracted with an equal volume of buffer-saturated phenol. After shaking for 10 min, the layers were separated (10 min centrifugation at 10,000 g) and the aqueous layer was removed. The phenol phase was reextracted once, and the combined water layers were extracted eight times with ether. Residual ether was evaporated by nitrogen bubbling. The RNA was precipitated by addition of 0.1 volume of 1 M ammonium acetate buffer (pH 5.0) and two volumes of ethanol (2 hr at -20°).

RNA was spun down (10 min, 50,000 g), dried and dissolved in H₂O to a final concentration of about 1 mg/ml.

Escherichia coli (strain MRE600) rRNA was phenol extracted from polyribosomes prepared according to Godson and Sinheimer (1967). Semliki forest virus [³²P]RNA was isolated from virus grown in neuroblastoma cells (American Culture Type Collection, CCL 131) in phosphate-free DMEM containing 100 μ Ci/ml of [³²P]phosphate (carrier free, The Radiochemical Centre, Amersham, England). Tobacco mosaic virus (TMV) was a gift from Dr. L. van Vloten-Doting (Leiden).

Traces of ribonuclease in buffers and solutions were inactivated by the addition of 0.1% (v/v) diethylpyrocarbonate (DEP, Fluka, Buchs, Switzerland) which was removed by boiling or by autoclaving at 120° (Solymosy *et al.*, 1968).

Analytical procedures. For the determination of sedimentation coefficients, isokinetic sucrose gradients were used (Noll, 1967). The parameters for the construction of the gradients have been taken from Van der Zeijst and Bloemers (1975).

Formaldehyde-containing sucrose gradients were prepared by a method based on that of Boedtker (1968). Viral RNA and marker rRNA in 0.09 M Na₂HPO₄ and 0.01 M NaH₂PO₄ containing 1.1 M formaldehyde were kept at 63° for 15 min, cooled rapidly in ice and layered immediately onto an isokinetic sucrose gradient in the same buffer. The gradients were spun for 5 hr at 46,000 rpm and 5° in a Beckman SW 50.1 rotor.

Buoyant densities of RNA's were determined in preformed linear Cs₂SO₄ gradients (5 ml, 33–100% saturation in TES buffer), containing 1% formaldehyde (Lozeron and Szybalski, 1966). They were centrifuged for 16 hr at 40,000 rpm in a Beckman SW 50.1 rotor at 25°. The density of gradient fractions was calculated from the refractive index according to Ludlum and Warner (1965).

Polyacrylamide–agarose-gel electrophoresis of RNA was done by using a modification of the method of Bishop *et al.* (1967) in that agarose was incorporated in the gel (Peacock and Dingman, 1968). Gels (0.6 ×

10 cm) containing 1.85% polyacrylamide and 0.5% agarose were prerun at 10 V/cm for 1 hr at room temperature. After electrophoresis at the same voltage for 2 hr, the position of RNA markers was determined by uv shadowing (Hassur and Whitlock, 1974). Subsequently, the gel was fractionated in 1-mm sections (Gilson *et al.*, 1972). Gel fractions were mixed with a toluene/Triton X-100 (2/1) mixture (Patterson and Green, 1965) and counted.

To assay incorporation of [³H]uridine into RNA, radioactive samples were spotted on Whatman 3 MM filter paper disks and washed in 5% (w/v) trichloroacetic acid (once for 15 min and twice for 5 min), followed by washing in ethanol/ether (1/1, v/v) and ether for 5 min. Subsequently, the filters were dried and counted.

RESULTS

To establish optimum conditions for labeling of viral RNA, the kinetics of virus-stimulated RNA synthesis were determined. It appeared that RNA synthesis starts at 4 hr after infection. The rate of RNA synthesis increased with the multiplicity of infection until an m.o.i. of 20. The release of progeny virus started between 8 and 9 hr after infection, being complete at 13 hr after infection. On the basis of these experiments, in all experiments described below cells were infected at an m.o.i. of about 50, and virus was harvested at 13 hr after infection.

Purification of [³H]Uridine-Labeled EAV

Radioactive virus centrifuged in a 20–50% (w/w) sucrose gradient as described in Materials and Methods had reached its buoyant density position after 3 hr of centrifugation. The peak of radioactivity at a density of 1.155 g/ml coincided with the maximum of viral infectivity (Fig. 1). The small peak at a density of about 1.17 g/ml was always present. The density of the virus found is somewhat lower than that estimated by others (see Horzinek, 1973). Centrifugation of material from the peak fraction in Fig. 1 in an isokinetic sucrose gradient was employed for determining the sedimentation coefficient; a value of 224 ± 8 S was found (Fig. 2) which

FIG. 1. Isopycnic banding of equine arteritis virus in a sucrose density gradient. Virus labeled with [^3H]-uridine (from six 5-ml incubation mixtures) infected at an m.o.i. of 43 was pelleted and centrifuged in a gradient as described in Materials and Methods. Fractions were assayed for TCA-precipitable radioactivity (●—●) and infectivity (▲—▲). The density (○—○) of every third fraction was calculated from the refractive index.

FIG. 2. Sedimentation coefficient of EAV. Virus (20 μl) of the top fraction of Fig. 1 was diluted in order to reduce the density and layered on an isokinetic sucrose gradient in TES buffer calculated for a particle density of 1.155 g/ml (top concentration 15% (w/w)) and was centrifuged at 25,000 rpm in a Spinco SW 50L rotor for 3 hr at 5°. Samples were collected on Whatman 3 MM filters, dried and counted directly.

FIG. 3. Polyacrylamide-agarose-gel electrophoresis of EAV-RNA. [^3H]uridine-labeled RNA was released from whole virus with 2% SDS and 2% 2-mercaptoethanol (5 min at 37°) and mixed with marker RNA's. Arrows indicate the positions of TMV-RNA and *E. coli* rRNA's.

is in accordance with data obtained by analytical ultracentrifugation (Horzinek, 1971).

Molecular Weight and Sedimentation Coefficient of EAV-RNA

A molecular weight of 4.0×10^6 for EAV-RNA was determined in polyacrylamide-agarose gels by using *E. coli* rRNA's (0.55 and 1.07×10^6 daltons (Stanley and Bock, 1965)) and TMV RNA ($2.0 \times$

10^6 daltons (Boedtker, 1960)) as markers (Fig. 3). SFV-RNA run in parallel gels migrated to the same position as EAV-RNA. From independent experiments with different virus preparations values of 3.95×10^6 , 4.10×10^6 and 3.97×10^6 were calculated for the molecular weight of EAV-RNA.

The $s_{20,w}$ value of the RNA of EAV was measured in isokinetic sucrose gradients calculated for a density of 2.0 g/ml for the

RNA. The assumption of this density was shown to be right by the fact that EAV-RNA migrated at a constant velocity in gradients constructed for this particle density as predicted by the theory of isokinetic gradients. Thus a sedimentation coefficient of 48 S in 0.1 M saline was calculated. SFV-RNA sedimented to nearly the same position (Fig. 4A).

Single Strandedness of EAV-RNA

A sedimentation behavior strongly dependent on the ionic conditions in the solution is characteristic for single-stranded RNA's (Spirin, 1960; Franklin, 1967). Indeed, if the saline concentration was lowered to 1 mM, the RNA of EAV sedimented more slowly, with an apparent sedimentation coefficient of 33 S (Fig. 4B). When, on the other hand, EDTA was omitted and 1 mM Mg^{2+} ions were added, sedimentation was increased to 56 S (Fig. 4C). The RNA's of EAV and SFV behaved in the same way in these experiments.

The single-stranded nature of the RNA was confirmed by the finding that its density in Cs_2SO_4 was 1.65 g/ml at 25°. The same value was found for SFV-RNA. It agrees with other values reported for single-stranded RNA's (Szybalski, 1968).

Sucrose Gradient Centrifugation under Denaturing Conditions

In order to ascertain that the RNA of EAV consists of a continuous strand and that it is not an aggregate of smaller molecules, centrifugation was carried out in gradients containing formaldehyde. These gradients have the additional advantage that the sedimentation coefficients of a number of RNA's are not influenced by their secondary structure. An empirical relationship between molecular weight and $s_{20,w}$ value has been established (Boedtker, 1968). Figure 5 shows that the RNA of EAV sedimented as one band after formaldehyde treatment. Assuming that the gradient was isokinetic with respect to the RNA, an $s_{20,w}$ of about 22 S was determined. This value corresponds to a molecular weight of 4.1×10^6 . Therefore, the RNA of EAV has to be a

FIG. 4. Sucrose gradient centrifugation of RNA from EAV. Isokinetic gradients (top concentration 10% (w/w)) were prepared as described in Materials and Methods. [3H]uridine-labeled RNA was released from whole virus (a diluted peak fraction from a gradient similar to Fig. 1) with 2% SDS and 2% 2-mercaptoethanol (5 min at 37°) and layered on the gradients which were spun at 5° for 1.6 hr at 50,000 rpm in a Beckman SW 50.1 rotor. Gradients were made up in (A), 0.01 M Tris-Cl, 1 mM EDTA, 0.1 M NaCl, pH 7.4; (B), 0.01 M Tris-Cl, 1 mM EDTA, 1 mM NaCl, pH 7.4; (C), 0.01 M Tris-Cl, 1 mM $MgCl_2$, 0.1 M NaCl, pH 7.4. Arrows indicate the positions of *E. coli* rRNA markers and SFV-RNA. Samples were collected on Whatman 3 MM filters, dried and counted directly.

FIG. 5. Centrifugation of EAV-RNA in a formaldehyde-containing isokinetic sucrose gradient. RNA was phenol extracted from [^3H]uridine-labeled EAV and layered on the gradient after heating in the presence of formaldehyde. Fractions were collected on Whatman 3 MM filter papers and counted directly. Values of $s_{20,w}$ were calculated and converted to molecular weights by using the relationship derived by Boedtker (1968). Arrows indicate the positions of *E. coli* marker rRNA's and SFV-RNA.

continuous strand. Identical results were obtained for SFV-RNA.

Infectivity of Viral RNA

RNA extracted from purified virus was infectious, the titer being 10^{-6} – 10^{-7} times the titer of the virus before phenol extraction. RNase treatment ($2\text{ }\mu\text{g/ml}$ for 30 min at 22°) lead to a complete abolishment of the infectivity, whereas the viral infectivity was completely resistant to RNase.

DISCUSSION

The EAV genome has been characterized as a single-stranded RNA which is infectious. Both the sedimentation behavior at different ionic strengths and the density in Cs_2SO_4 indicate the single-strandedness of the RNA. Heating in the presence of formaldehyde and subsequent centrifugation in formaldehyde-containing gradients proved that the RNA is one continuous strand and does not contain hidden breaks. The value

of 4.1×10^6 daltons obtained from sedimentation data correlates quite well with the value of 4.0×10^6 daltons calculated from experiments employing polyacrylamide-agarose-gel electrophoresis.

Criticism has arisen about the validity of molecular weight determinations employing the methods mentioned above. It has been shown in some cases, especially with RNA's of high G+C-content, that anomalous migration may occur, depending on the temperature and the ionic strength in the gel (Grivell *et al.*, 1971). Therefore pronounced secondary structure of the nucleic acid, as has been reported for togaviruses (Sprecher-Goldberger, 1967; Engelhardt, 1972), may influence the results and lead to widely differing values. In an attempt to circumvent these difficulties, methods have been developed for electrophoresis under denaturing conditions. However, these have not been tested for RNA molecules larger than 2×10^6 daltons until now (see Reijnders *et al.*, 1974, for a discussion). Also for centrifugation under denaturing conditions artifacts have been described due to incomplete unfolding of the RNA (Kolakofsky *et al.*, 1974; Simmons and Strauss, 1972).

As the purpose of this study was not to provide new methods for RNA molecular weight determination, we have confined ourselves to the comparison of EAV- and SFV-RNA's. The observation that the two RNA's have the same sedimentation coefficients both in normal sucrose gradients at different ionic strengths and in formaldehyde sucrose gradients suggests that they have comparable degrees of secondary structure and similar molecular weights.

The results presented provide additional data about the structure of EAV (Horzinek *et al.*, 1971) and justify its classification as a member of the Togaviridae family (Horzinek, 1973; Fenner *et al.*, 1974). Further support of this taxonomic position was derived from analysis of the virion polypeptides (J. J. W. Zeegers, B. A. M. van der Zeijst and M. C. Horzinek, in preparation).

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