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Virion Nucleic Acid of Ebola Virus

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The virion nucleic acid of Ebola virus consists of a single-stranded RNA with a molecular weight of approximately 4.0×10^6. The virion RNA did not bind to oligodeoxythymidylic acid-cellulose under conditions known to bind RNAs rich in polyadenylic acid and was not infectious under conditions which yielded infectious RNA from Sindbis virus, suggesting that Ebola virus virion nucleic acid is a negative-stranded RNA.

Ebola virus can cause lethal hemorrhagic fever in humans. This virus was first isolated in 1976 (5, 8, 16), and its natural history remains obscure (6, 22). The virus is taxonomically ungrouped and has a distinctive morphology shared only with Marburg virus (13), but it apparently has no antigens in common with Marburg virus (8).

To better understand the nature of this formidable disease agent, we have examined the Ebola virion nucleic acid. At the outset of this project, no data were available on the nature of the Ebola virus genome, and our analysis considers whether (i) the virion nucleic acid is RNA or DNA, (ii) the genome is single or double stranded, (iii) the genome is single or multipartite, (iv) the genome nucleic acid is infectious, (v) the genome is rich in polyadenylic acid, and (vi) the molecular weight of the nucleic acid is similar to that of any other virus group.

MATERIALS AND METHODS

Special containment facilities. Because Ebola virus disease can be fatal and is transmitted directly from person to person, all work was done in the Maximum Containment Laboratory at the Center for Disease Control, Atlanta, Ga. Personnel were isolated from exposure to infectious material by wearing positive-pressure encapsulating plastic suits (ILC Industries, Dover, Del.).

Ebola virus propagation and purification. The Ebola virus strain used was the Mayinga strain recovered from the blood of a patient in Zaire during the 1976 outbreak. The virus had been isolated in Vero cell cultures and passaged twice in these cells to prepare the seed stock used in all experiments.

For the experiments described in this report, Ebola virus was grown in roller bottle cultures of Vero cells. Vero cell monolayers were grown in Eagle minimum essential medium with 10% fetal calf serum and were maintained after infection in Eagle minimum essential medium with 1% fetal calf serum.

Roller bottles of Vero cells were infected with virus at a multiplicity of infection of approximately 0.1 50% tissue culture infective dose per cell. Virus was labeled with 10 μCi of [5-^{3}H]uridine (Moravek Biochemicals, City of Industry, Calif.) per ml between 3 and 6 days postinfection. Virus was purified by a modification of the method of Obijeski et al. (15). Infected-cell medium was clarified by centrifugation at $740 \times g$ for 5 min. Additional NaCl was added to the clarified medium to achieve a final concentration of 0.53 M, and polyethylene glycol was added to a final concentration of 7% (wt/wt). After a minimum of 2 h at 4°C, the now insoluble virus particles were collected by centrifugation at $10,000 \times g$ for 20 min. This pelleted material was suspended in TNE (0.01 M Tris [pH 7.4], 0.15 M NaCl, 0.002 M EDTA) and layered onto a 0 to 40% potassium tartrate gradient (made in TNE). The potassium tartrate gradient was centrifuged at $160,000 \times g$ overnight at 5°C in an SW40 rotor. The virion band that formed in the gradient was collected, diluted with an equal volume of TNE, and applied to a 20 to 70% sucrose gradient (made in TNE) and rebanded after centrifugation at $160,000 \times g$ for 30 min in an SW40 rotor. For most experiments, the sucrose was dialyzed away.

Reference viruses and nucleic acids. Vesicular stomatitis virus, (VSV), Indiana strain (obtained from J. Obijeski), and Sindbis virus (obtained from H. Lindsey-Regnery) were purified as described above for Ebola virus, although the incubation times before virus harvest were shorter. Reovirus III (obtained from E. Palmer) was grown in L cells and purified in potassium tartrate gradients ranging from 0% to saturated potassium tartrate.

Unless otherwise specified, all nucleic acids used in this study were purified by the addition of sodium dodecyl sulfate (1% [wt/vol]), followed by threefold extraction with equal volumes of phenol-chloroform-isoamyl alcohol (50:48:2) and precipitation with 2.5 volumes of absolute ethanol overnight at −20°C. Cell nuclei and cytoplasm were fractionated before extraction of DNA and rRNA by adding 0.75% Triton X-100 and 0.75% sodium deoxycholate to cells, followed by centrifugation at $740 \times g$ for 5 min.

DNA was labeled with [*methyl*-^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.), and RNAs were labeled with either [5-^{3}H]uridine (Moravek Biochemicals) or [^{14}C]uridine (New England Nuclear Corp.).

Nucleic acid sensitivity to sodium hydroxide. Nucleic acid from virions disrupted with 1% sodium dodecyl sulfate was treated with 0.3 N NaOH over-

night at room temperature. Control samples received no NaOH. After incubation, trichloroacetic acid was added to the samples in excess at a concentration of 5% (wt/vol), and the trichloroacetic acid-precipitable materials were collected on glass fiber disks for liquid scintillation counting.

Nucleic acid nuclease sensitivity. The conditions for nuclease digestion were standardized for both pancreatic RNase-A and DNase (Sigma Chemical Co., St. Louis, Mo.). Sindbis and Ebola viruses were disrupted with 1% sodium dodecyl sulfate before nuclease treatment. Reovirus III virion RNA, Vero cell rRNA, and Vero cell DNA were extracted and precipitated as described above and suspended in digestion buffer. Sodium dodecyl sulfate was removed from the nucleic acid solutions by adding KCl (0.15 M) and centrifuging immediately before digestion. The final digestion buffer contained 0.01 M Tris (pH 7.4), 0.3 M NaCl, 0.15 M KCl, 0.01 M $MgCl_2$, and 50 μg of either RNase or DNase per ml. Digestion was at room temperature for 30 min before addition of trichloroacetic acid to a final concentration of 5% (wt/vol) and collection of acid-precipitable material on glass fiber disks for scintillation counting.

Oligodeoxythymidylic acid-cellulose chromatography. Virion RNA was tested for the presence of polyadenylic acid-rich regions by differential elution from oligodeoxythymidylic acid-cellulose columns with buffers of different ionic strength (1). [^{3}H]uridine-labeled VSV, Sindbis, and Ebola virions were disrupted before application to the oligodeoxythymidylic acid-cellulose columns (Collaborative Research, Inc., Waltham, Mass.) with application buffer (high ionic strength) containing 0.5% lithium dodecyl sulfate. Column eluate was collected as 1-ml fractions, and the column was washed extensively with additional application buffer. Elution buffer (low ionic strength) was applied, and additional fractions were collected. Trichloroacetic acid was added to all fractions to a final concentration of 5% (wt/vol), and the acid-insoluble material was collected on glass fiber disks with vacuum for liquid scintillation counting.

Infectious nucleic acid assay. Infectivity of Ebola virion RNA was tested by a method similar to that reported by Mandel (11). Sindbis virus was used as a positive control for infectious RNA. Purified virions were disrupted with sodium dodecyl sulfate (0.01%). The sodium dodecyl sulfate was then removed by precipitation at 4°C and centrifugation (10,000 × *g* for 1 min). The supernatant, containing free RNA, was diluted in a high-salt buffer (0.8 M NaCl, 0.01 M Tris [pH 8.1], 0.005 M EDTA, and 500 mg of DEAE-dextran per ml) and inoculated onto Vero cell monolayers that had been prerinsed in the high-salt buffer. To establish the efficiency of infection by RNA, infectivity of intact virions was determined; virus dilutions were made in phosphate-buffered saline containing 0.75% bovine albumin before inoculation.

Infectivity of Sindbis virus RNA and whole virions was measured by plaque assays under medium containing 1% agar and stained with neutral red 48 h postinfection. Infectivity of Ebola virus was measured by a fluorescent focus-forming assay, developed at the Center for Disease Control by Alan Truant (unpublished data). In this assay, Ebola virus-infected cell monolayers were overlaid with medium containing 0.25% agar, incubated for 48 h at 35°C, and stained by sequential reaction with human anti-Ebola virus antiserum and fluorescently labeled sheep anti-human immunoglobulin G (Wellcome Research Laboratories, Beckenham, England). Tissue culture infectivity was then expressed as fluorescent focus-forming units per milliliter. To insure that infectivity after disruption of virions was due to free RNA and not to a subpopulation of still-intact virions, pancreatic RNase (10 μg/ml) was added to check that infectivity was, in fact, RNase sensitive. In an attempt to amplify possible infectious Ebola RNA and, hence, lower the limits of detection, Ebola virus RNA was also inoculated onto cell monolayers that were subsequently maintained under liquid medium for 7 days, thereby permitting a generalized infection. These cultures were then subjected to indirect fluorescent staining as described above and simply scored as either positive or negative.

Electrophoresis of RNA on agarose gels. For analysis of size and composition of Ebola virion RNA, electrophoresis in agarose gels was done after RNA denaturation with dimethyl sulfoxide and glyoxal by the method of McMaster and Carmichael (12). Phenol-extracted, ethanol-precipitated Ebola virion RNA, VSV virion RNA, and Vero cell rRNA (see above) were suspended together in electrophoresis buffer and denatured. Samples were electrophoresed on 10-cm, cylindrical, 1.5% agarose gels (Eastman Kodak Co., Rochester, N.Y.) at 5 mA/gel for 100 min with constant recirculation of electrophoresis buffer. After completion of electrophoresis, the gels were fixed in cold 10% trichloroacetic acid and sliced on a Mickle gel slicer (Mickle Laboratory Engineering Co., Guildford, England) in preparation for liquid scintillation counting. Molecular weights for the reference RNA, used to calculate the molecular weight of Ebola virus virion RNA, were as follows: Vero cell rRNA's, 0.7×10^6 and 1.8×10^6; VSV virion RNA, 3.8×10^6 (17). The molecular weight for Ebola virion RNA was calculated by assuming the linear log of molecular weight relationship to electrophoretic mobility (3).

RESULTS

Preliminary experiments demonstrated that when Ebola virus-infected Vero cells were exposed to either [5-^{3}H]uridine or [*methyl*-^{3}H]thymidine for short time periods, only uridine was incorporated into purified virions.

Treatment of Ebola virion nucleic acid with 0.3 N NaOH, under conditions able to digest RNA but not DNA, reduced the acid-precipitable material, relative to untreated controls, by 97% (control, 3,938 cpm/100 μl; NaOH-treated nucleic acid, 106 cpm/100 μl).

Samples of virion RNA containing 13,000 cpm of tritium were treated with RNase and DNase as described above. After RNase treatment, 605 cpm remained (4.6% resistant), whereas 9,900 cpm remained (76% resistant) after DNase treatment. Other single-stranded RNAs (Sindbis and Vero cell rRNA's) were as sensitive to RNase as

Ebola virus RNA, whereas reovirus RNA was 10 times more resistant.

Ebola virion RNA was not retained on an oligodeoxythymidylic acid-cellulose column under conditions which quantitatively retained the RNA of Sindbis virus, a reference RNA known to possess significant amounts of polyadenylic acid (7). VSV virion RNA did not bind to the oligodeoxythymidylic acid column and served as a negative control.

Ebola virion RNA was noninfectious under conditions that were conducive to infection of cells with naked Sindbis virus RNA (Table 1). Infectious Ebola virion RNA was not detected in these tests, and if Ebola virion RNA is infectious, its infectivity must be less than 0.0005% of that of the whole virus particle. Sindbis virion RNA was 0.024% as infectious as whole Sindbis virions, and RNase rendered Sindbis RNA noninfectious.

When electrophoresed on agarose gels, Ebola virion RNA migrated as a single distinct peak (Fig. 1); when the virion RNA was denatured and coelectrophoresed with reference RNA, it consistently migrated slower than the virion RNA of VSV. From the data shown in Fig. 1, the molecular weight of denatured Ebola virion RNA was calculated to be approximately 4.0×10^6 to 4.2×10^6. When the same RNA was electrophoresed in the absence of the glyoxal denaturant, estimates for the molecular weight of Ebola virion RNA were larger (approximately 4.5×10^6); however, under nondenaturing conditions, the RNA did not adhere to a strictly linear relationship between the log molecular weight and the distance migrated (data not shown).

DISCUSSION

Previous reports for Marburg virus have suggested that this virus is an RNA virus, based on its insensitivity to 5-bromo-deoxyuridine, 5-iododeoxyuridine, and actinomycin during replication (4, 9, 10, 19). Our studies with the morphologically similar Ebola virus show nearly complete susceptibility of the virion nucleic acid to digestion by NaOH and by pancreatic RNase; therefore, we conclude that the genome of Ebola virus is single-stranded RNA.

From the electrophoretic mobility of the Ebola virion RNA, under both nondenaturing and denaturing conditions, it is clear that the Ebola genome is a single RNA species. This observation has been supported by subsequent

TABLE 1. *Infectivity of Ebola and Sindbis virus RNAs*

Virus	Whole virions (per ml)	Free RNA (per ml)	$\frac{\text{Free RNA}}{\text{Whole virion}} \times 100$
Sindbis	1.35×10^6 PFU	3.2×10^2 PFU	0.024
Ebola (with agar overlay)	7.97×10^6 FFFU[a]	$<8.1 \times 10^2$ FFFU[b]	<0.013
Ebola (with liquid overlay)	7.97×10^6 $TCID_{50}$[c]	$<4 \times 10^1$ $TCID_{50}$[b]	<0.0005

[a] FFFU, Fluorescent focus-forming unit.
[b] Limit of resolution.
[c] $TCID_{50}$, Fifty percent tissue culture infective dose; 1 $TCID_{50}$ = 1 FFFU (unpublished data).

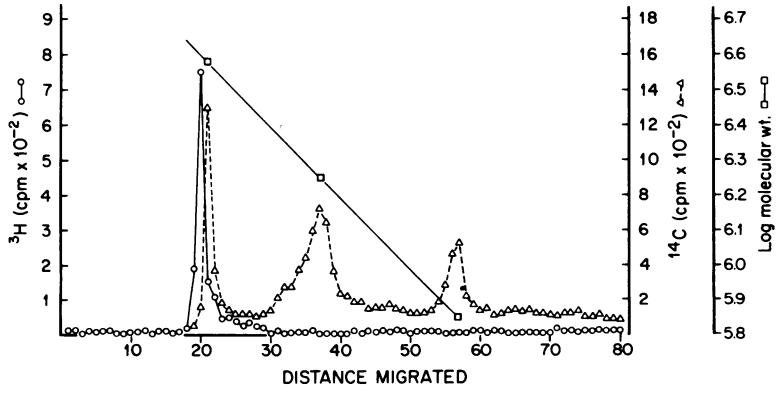

FIG. 1. *Coelectrophoresis of Ebola virion RNA and reference RNA on an agarose gel under glyoxal denaturing conditions. Symbols: ○, [^{3}H]uridine-labeled Ebola virion RNA; △, [^{14}C]uridine-labeled VSV virion RNA and Vero cell rRNA. The logs of molecular weight of reference RNAs (□) were calculated by using molecular weight values given in the text.*

analysis of the gamma radiation-sensitive target size for Ebola virus inactivation which indicates a genome size compatible with that estimated by gel electrophoresis (unpublished data).

Ebola virus virion RNA consistently demonstrated a slower electrophoretic mobility than the VSV virion marker RNA. The strict linearity of RNA mobility versus the log molecular weight of the reference RNA in the glyoxal denaturing system permits accurate extrapolation to find the molecular weight of the Ebola virion RNA, 4.0×10^6 to 4.2×10^6. No RNA species larger than 4.2×10^6 molecular weight was ever observed, and no radioactivity was ever found "trapped" at the top of the gel. Likewise, no other Ebola virion RNAs were detected which migrated faster than the 4.0×10^6 molecular weight species.

Although Ebola virus virion RNA lacks demonstrable polyadenylic acid tracks, some positive-stranded viruses also lack polyadenylic acid (14, 18, 20, 21), thus making the presence or absence of polyadenylic acid less than definitive for determination of positive or negative strandedness of virion RNA. Another indicator of strandedness is RNA infectivity. In our experiments on infectious RNA, Ebola and Sindbis virion RNAs were prepared in an identical manner at the same time. Both RNAs are approximately the same size, strongly suggesting that if both RNAs were infectious, the ratios of infectivity of naked RNA to whole virions would be comparable; these ratios, however, were not the same. Although Sindbis virus RNA was infectious, no infectivity could be detected for Ebola virion RNA. The evidence suggests that Ebola virion RNA is negative stranded. Confirmation of the negative-stranded character of Ebola virion RNA must await future analysis of possible virion-associated RNA polymerase and analysis of intracellular virus-specific polysomal RNAs.

Consideration of the features of the Ebola virus genome has led us to a new, but as yet incomplete, appreciation of the general taxonomic outline for this unusual virus. Although one can draw analogies between many of the fundamental properties of the virion RNA of Ebola virus (and by inference, Marburg virus) and the virion RNAs of Rhabdoviridae, other significant differences between these viruses exist. For example, the molecular weights of the virion structural proteins of Ebola virus are 12.5×10^4, 10.4×10^4, 4.0×10^4, and 2.6×10^4, a pattern not considered to be like that of rhabdoviruses (M. P. Kiley, R. L. Regnery, and K. M. Johnson, J. Gen. Virol., in press). Perhaps our data are most useful in indicating what Ebola virus is apparently not related to; the Ebola virion RNA is clearly unlike that of any other group of animal viruses, with the possible exception of rhabdoviruses.

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