Electron Microscope Study of Ribonucleic Acid of Myxoviruses

KWOK-KWONG LI¹ AND J. T. SETO

Department of Microbiology, California State College, Los Angeles, California 90032

Received for publication 14 December 1970

Intact ribonucleic acid (RNA) molecules in an extended form were extracted from purified influenza virus and observed in the electron microscope. For this study, the RNA extraction procedure and the Kleinschmidt protein monolayer technique were modified. The mean lengths of RNA from X7, X7-F1, and WSN strains of influenza virus were found to be 2.69, 2.55, and 2.37 μm , respectively. From these measurements, the corresponding estimated molecular weights would be 2.9, 2.8, and 2.5×10^6 daltons. X7 and WSN RNA preparations were exposed to pH 3 to disrupt intact molecules. Histograms of length measurements showed five peaks, which were interpreted to represent the five pieces of RNA reported to exist in the influenza virion. X7 RNA appeared to be more stable than WSN RNA when stored at 4 C. The profiles of histograms of incomplete virus RNA suggest that the high molecular-weight component is missing. In preliminary experiments on Newcastle disease virus RNA, molecules of various lengths were observed.

Influenza virus ribonucleic acid (RNA) has been shown to be heterogeneous in size and to consist of five discrete pieces (1, 2, 11, 12). To characterize further viral RNA, an electron microscope study of isolated RNA was carried out. The protein monolayer method, developed by Kleinschmidt (9), has been shown to be most useful for morphological analysis of deoxyribonucleic acid (DNA) molecules and more recently for RNA molecules (5, 6, 8, 9, 16). In a preliminary report, we presented evidence of the demonstration of intact X7 virus RNA molecules (K. K. Li and J. T. Seto, *Bacteriol. Proc.*, p. 182, 1970). The present studies were extended to include examination of incomplete virus RNA, some physicochemical properties of the viral genome, and determination of length measurements for molecular-weight estimations of RNA molecules from X7-F1 and WSN strains of influenza virus and from a paramyxovirus, Newcastle disease virus (NDV).

MATERIALS AND METHODS

Virus. Influenza strains X7, X7-F1, and WSN and the Milano strain of NDV were used. Viruses were propagated and assayed for hemagglutinating activity (HA titer) and egg infectivity (EID₅₀) as described previously (18). Infected embryonated eggs were incubated for 20 to 24 hr, and seed virus was stored

at -60 C. Stock NDV virus was prepared in a similar manner, except that the eggs were incubated for 48 hr. Incomplete virus (von Magnus virus, VM) was prepared as described by Pons and Hirst (13). Concentration and purification of influenza viruses were by the method of Nayak and Baluda (11) and of NDV by a modified method of Duesberg and Robinson (3).

Extraction of viral RNA. Usual precautions against nuclease contamination were taken. The general experimental procedure for the extraction and isolation of RNA from highly purified virions was the same as reported previously (11) with the following modifications. Highly purified virus was diluted 15- to 20-fold with NT extraction buffer [0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris-hydrochloride), pH 7.4]. Virus was treated with deoxyribonuclease in TM buffer (0.01 M Tris-hydrochloride, pH 7.4; 0.002 M MgCl₂) and ribonuclease in 2× SSC (reference 11; SSC = 0.15 M NaCl plus 0.015 M sodium citrate; Worthington Biochemical Corp. Freehold, N.J.), each at a final concentration of 10 $\mu\text{g}/\text{ml}$ for 30 min at room temperature. Action of deoxyribonuclease was inhibited by the addition of ethylenediaminetetraacetate (EDTA) at a final concentration of 0.001 M (7) and 1% 2-mercaptoethanol. RNA was isolated by sodium dodecyl sulfate (SDS)-phenol extraction method (11). Excessive and vigorous shaking was avoided, and the reaction tube was allowed to roll back and forth on a towel. RNA was precipitated by the addition of 2 volumes of cold absolute ethanol to the aqueous phase made to contain 1.6% sodium acetate and stored for 48 hr at -20 C. "Pelleted" RNA was resuspended either in NTE buffer (0.1 M NaCl; 0.01 M Tris-hydrochloride, pH 7.4; 0.001 M EDTA) or TE

¹ Recipient of special Graduate Research Award from Associated Students, California State College, Los Angeles. Present address: Department of Bacteriology, University of California, Los Angeles, Calif. 90024.

buffer (0.01 M Tris-hydrochloride, pH 7.4; 0.001 M EDTA). Purity of RNA preparations was analyzed at 260 and 280 nm and assayed for protein by the method of Lowry et al. (10). Extraction of RNA preparations was repeated when the protein concentration exceeded 3 to 5%.

Velocity sedimentation centrifugation. X7 RNA (0.2 ml) in TE buffer was layered on a 5.0-ml linear (5 to 20%) sucrose gradient (11) in a low-salt NTE buffer (0.01 M NaCl; 0.001 M Tris-hydrochloride, pH 7.4; 10^{-4} M EDTA). After centrifugation at 49,000 rev/min for 4.5 hr in a SW50L rotor at 4°C, 0.25-ml fractions were collected. Effluent fractions were read at 260 nm with a microcuvette in a Beckman DB-G spectrophotometer. Fractions containing RNA were immediately "spread" for electron microscopic examination as described below.

pH 3.0 treatment of viral RNA. RNA sample (0.2 ml) in 0.01 M Tris-hydrochloride buffer (pH 7.4) was diluted to 1.0 ml, and 0.05 ml of 0.35 N HCl was added to adjust the pH of the reaction mixture to 3.0. After 30 sec at room temperature, the pH 3 treatment was stopped by the addition of 0.05 ml of 0.5 M EDTA (7). The RNA (0.3 ml) of the reaction mixture was "spread" for electron microscopic examination.

Electron microscopy. A modified Kleinschmidt method (9) was used. Formvar-carbon-coated 200- or 400-mesh copper grids were used, and they were prepared not more than 72 hr ahead of time.

Viral RNA samples were diluted with NTE or TE buffer to a final concentration of 2 to 5 $\mu\text{g/ml}$; 0.2 to 0.3 ml of RNA solution was added to an equal volume of filtered cytochrome *c*, 1.0 mg/ml in 0.5 M ammonium acetate. In some preliminary experiments, either 4 to 8 M urea or 25 to 60% formamide was added to RNA-cytochrome *c* mixture before 0.1 ml of the specimen was gently dropped onto a clean glass slide to form the protein monolayer. This was facilitated by spreading the sample onto the surface of the ammonium acetate (0.02 M, pH 8.0) in a sterile plastic petri dish (90 mm). Immediately after spreading, the RNA molecules were "picked up" on specimen grids, rinsed with distilled water for 20 to 30 sec, stained with 10^{-4} M uranyl acetate in acid-ethanol (5×10^{-4} N HCl) for 30 sec, and immersed in 2-methylbutanol for 10 sec to dry the sample. Specimens were examined at a magnification between 8,000 and 12,000 $\times$ with an RCA-EMU-3G electron microscope at 50 kv. Magnifications were determined with the aid of a carbon grating replica (Ernest F. Fullan Inc.). The lengths of the RNA molecules were measured, either on prints or on tracings obtained by projecting the photographic plates onto a piece of paper, at a final magnification of 46,500 to 125,000 $\times$, with a map measurer. Molecular weight estimations were calculated as described by Granboulan and Scherrer (7).

RESULTS

Analysis of X7 virus RNA. X7 virus RNA molecules, spread with or without urea or formamide, were linear, noncyclic, and non-branched. An intact molecule is illustrated in

Fig. 1. Rosette-like structure was noted on occasions at one end of the molecule (Fig. 2). This was believed to be attributed to the use of aged cytochrome for spreading. Approximately 25 to 35% of the molecules observed were in an aggregated form in the absence of urea or formamide. Resuspension of the "pelleted" RNA preparations in NTE or TE buffers did not induce any detectable morphological alterations of the molecules. Only molecules which could be readily traced were randomly selected and measured. Seven experiments were carried out, and the average mean length measurement of the RNA molecules was 2.69 μm . A representative histogram of the length distribution of 300 X7 virus RNA molecules is presented in Fig. 3. Standard X7 RNA has a modal length of 2.57 μm and a mean length of $2.69 \pm 2.0 \mu\text{m}$. The molecular weight estimation from the mean length measurement was calculated to be 2.9×10^6 daltons. RNA of another recombinant X7-F1, derived from X7 virus (19), was examined. X7-F1 RNA had a mean length of $2.55 \pm 1.95 \mu\text{m}$, which corresponds to an estimated molecular weight of 2.8×10^6 daltons. The molecules were similar in morphology to X7 RNA.

Physicochemical properties of X7 virus RNA. To establish the nature and identity of X7 RNA preparations, they were treated with ribonuclease and deoxyribonuclease. No RNA molecules could be seen if the sample was treated with ribonuclease (10 $\mu\text{g/ml}$) for 30 min at room temperature. But RNA molecules treated similarly with deoxyribonuclease (10 $\mu\text{g/ml}$) were unaffected. The histogram profile of the length distribution of X7 RNA molecules after deoxyribonuclease treatment was similar to that of the control (Fig. 3). RNA of the treated preparation had a modal length of 2.58 μm and a mean length of $2.65 \pm 1.9 \mu\text{m}$.

X7 RNA preparations, resuspended in TE buffer, were stored in 0.2-ml samples at 4°C for 3, 5, and 7 days to determine their stability and if intact molecules may fragment into pieces. RNA molecules examined were similar in morphology to the freshly prepared control samples. Smaller pieces were observed with increasing frequency in samples stored for 7 days as compared to those stored for 3 days. Molecules whose length ranged between 2.1 and 2.5 μm were still seen but less frequently.

To determine the nature of linkers which hold together pieces of RNA, preparations were exposed to pH 3. At this pH, noncovalent bonds (hydrogen bonds) are completely suppressed (7). Length measurements ranged from 0.18 to 0.96 μm , except for about 4% of the molecules observed. The molecules were linear and well-

FIG. 1. Standard X7 virus RNA molecule spread in the absence of urea or formamide. $\times 46,500$.

FIG. 2. Standard X7 virus RNA with rosette-like structure shown at one end of the molecule. $\times 60,000$.

FIG. 3. Length distribution of 300 standard X7 virus RNA molecules with a modal length of $2.57 \mu\text{m}$ and a mean length of $2.69 \pm 2.0 \mu\text{m}$.

extended. Five distinct peaks, with mean lengths of 0.27 , 0.42 , 0.59 , 0.74 , and $0.88 \mu\text{m}$, are evident in the histogram of 296 RNA molecules (Fig. 4). The modal length is $0.29 \mu\text{m}$ and the mean length is $0.48 \pm 0.46 \mu\text{m}$. Three preparations of RNA

FIG. 4. Length distribution of 296 standard X7 virus RNA molecules after pH 3 treatment. The modal length is $0.29 \mu\text{m}$ and the mean length is $0.48 \pm 0.46 \mu\text{m}$.

were examined, and the profiles of the histograms were similar.

X7 RNA preparations were centrifuged in a linear sucrose gradient in an attempt to isolate pieces of molecules of similar size. Three resolved

peaks were obtained. Fractions from each peak were "spread" for observation in the electron microscope. RNA molecules were seen in fractions from peaks I and II, but none from peak III, since the molecules in peak III were too small to be examined. The average length measurement of RNA filaments from a peak I fraction was $0.66 \mu\text{m}$. Molecules were well-extended and no intact genomes were observed.

Analysis of third-passage VM virus X7 RNA.

It has been postulated that incomplete virus genome is lacking in one of its pieces (15). RNA was isolated from third passage VM virus which had an EID_{50} to HA ratio of 3.2, whereas standard X7 virus had a ratio of 6.1. Length distribution of incomplete virus molecules is presented in Fig. 5. Four peaks are evident with length measurements of 0.26, 0.43, 0.66, and $0.85 \mu\text{m}$. The modal length was found to be $0.44 \mu\text{m}$ and the mean length to be $0.59 \pm 0.45 \mu\text{m}$; the average of three separate experiments was 0.43 and $0.59 \mu\text{m}$, respectively. Among 965 molecules, only one had a length measurement that exceeded $2.0 \mu\text{m}$. Thus, a qualitative difference is apparent in the histogram profiles between standard X7 RNA (Fig. 3) and VM virus RNA (Fig. 5).

Analysis of WSN virus RNA. WSN RNA was examined to determine whether any differences might exist between strains and to compare the results with data reported on the characterization of RNA by other physicochemical procedures (12). The morphology of RNA molecules, extracted from standard virus and "spread" in the presence or absence of urea or formamide, was similar to X7 RNA. An electron micrograph is presented in Fig. 6, and a representative histogram of one preparation of RNA is presented in

FIG. 5. Length distribution of 395 von Magnus X7 virus RNA molecules with a modal length of $0.44 \mu\text{m}$ a mean length of $0.59 \pm 0.45 \mu\text{m}$.

FIG. 6. Standard WSN virus RNA molecule spread in the absence of urea or formamide. $\times 46,500$.

Fig. 7. WSN RNA has a modal length of $2.57 \mu\text{m}$ and a mean length of $2.37 \pm 1.60 \mu\text{m}$. The profile of the histogram (Fig. 7) is qualitatively different from that of X7 RNA (Fig. 3); the mean lengths of X7 RNA and WSN RNA suggest that WSN RNA molecules might be shorter.

Physicochemical properties of WSN RNA.

RNA preparations were treated with deoxyribonuclease and ribonuclease, and no molecules of WSN RNA were observed after ribonuclease treatment. Deoxyribonuclease treatment had no detectable effect on WSN RNA, and the molecules were similar in morphology to untreated control preparations. WSN RNA molecules, resuspended in TE buffer, were stored at 4°C in 0.2-ml samples. Samples were examined in the electron microscope after 3, 5, and 7 days of storage. Molecules were linear and well-extended. The length distribution of WSN RNA molecules after 1 week of storage is shown in the histogram presented in Fig. 8. The existence of five major peaks is evident, with size distributions of 0.17, 0.29, 0.45, 0.58, and $0.73 \mu\text{m}$, respectively. The modal length is $0.31 \mu\text{m}$ and the mean length is $0.48 \pm 0.33 \mu\text{m}$.

When samples of fresh RNA preparation were submitted to pH 3 treatment, over 95% of the

FIG. 7. Length distribution of 140 standard WSN virus RNA molecules with a modal length of 2.57 μ m and a mean length of $2.37 \pm 1.60 \mu$ m.

FIG. 8. Length distribution of 334 standard WSN virus RNA molecules after storage for 7 days at 4°C. The modal length is 0.31 μ m and the mean length is $0.48 \pm 0.33 \mu$ m.

molecules examined in the electron microscope were linear and ranged from 0.15 to 0.74 μ m in length. Molecules greater than 1.2 μ m in length were seldom noted. Five prominent peaks were evident at 0.19, 0.28, 0.44, 0.58, and 0.72 μ m, with a mean length of $0.45 \pm 0.36 \mu$ m (Fig. 9). The data on WSN RNA preparations after 7 days of storage (Fig. 8) and pH 3 treatment (Fig. 9) were tabulated in terms of length meas-

urements and molecular-weight estimations. Comparative data on WSN RNA and X7 RNA together with reported results (12) are presented in Table 1. Peaks II to VI (12) correspond to pieces I to V, respectively (Fig. 4, 8, and 9). The molecular weights of the sum of the pieces of WSN RNA storage material and of pH 3-treated X7 RNA are in general agreement with their respective untreated controls.

Analysis of NDV RNA. For comparative purposes, NDV RNA preparations were examined in the electron microscope. NDV RNA preparations, prepared in a similar manner as

FIG. 9. Length distribution of 154 standard WSN virus RNA molecules after pH 3 treatment. The modal length is 0.29 μ m and the mean length is $0.45 \pm 0.36 \mu$ m.

TABLE 1. Comparison of molecular weight estimations of the various pieces of viral RNA extracted from WSN and X7 strains of influenza virus

Peak	WSN ^a	WSN ^b		X7 ^c after pH 3.0 treatment
		Storage for 7 days at 4°C	pH 3.0 treatment	
I	1.82 ^d			
II	2.72	1.84	1.91	2.72
III	3.52	3.36	3.06	4.45
IV	4.65	4.86	4.56	6.05
V	5.80	6.43	6.10	7.48
VI	6.70	8.00	7.60	9.07
Total	25.21	24.49	23.23	29.77

^a Taken from Pons and Hirst (12).

^b Molecular weight estimation of standard WSN virus RNA is 2.53×10^6 daltons.

^c Molecular weight estimation of standard X7 virus RNA is 2.91×10^6 daltons.

^d Expressed as $\times 10^5$ daltons.

influenza virus RNA, were observed in the electron microscope. The molecules observed were linear, noncyclic, nonbranched, and not in a collapsed form (8). Well-extended molecules, ranging from 1.0 to 2.0 μm in length, were seen most frequently, but an occasional molecule exceeding 4.0 μm in length was observed. The longest molecule measured was 7.2 μm .

DISCUSSION

Influenza virus RNA, isolated by a modified SDS-phenol extraction method and prepared for examination by a modified Kleinschmidt protein monolayer spreading technique, was examined with the use of the electron microscope. Intact molecules isolated from X7, X7-F1, and WSN viruses were observed. The molecules were linear, noncyclic, and nonbranched, whether spread in the presence or absence of urea or formamide. Thus, single-stranded RNA molecules from animal viruses do not appear to require urea or formamide to prevent aggregation (6, 16), unlike phage R-17 RNA which aggregates without urea present during spreading (5).

The mean length measurements of X7, X7-F1, and WSN RNA molecules were 2.69, 2.55, and 2.37 μm , respectively. To estimate their molecular weights from these values, the internucleotide spacing and average molecular weight per nucleotide must be known. Base spacing of 31.7 nm and 321 to 344 as the average molecular weight of nucleotide have been reported for several viral RNA species (7). The sodium form of influenza nucleotide is 345. With these values, the corresponding molecular weights would be 2.9, 2.8, and 2.5 $\times 10^6$ daltons for X7, X7-F1, and WSN RNA, respectively. Following in the same order, the estimated number of nucleotides would be 8,400, 8,100, and 7,400 for these three RNA species. Molecular weights of 2.5 $\times 10^6$ to 3 $\times 10^6$ daltons have been reported based on *S* values (4). The base spacing value of 31.7 nm, assumed to be valid for influenza RNA, must be verified against a reference RNA such as phage R-17 under the experimental conditions of our laboratory.

Examination of the histograms of X7 and WSN RNA molecules (Fig. 3 and 7) indicates a relatively wide variation in their size distribution. Qualitatively, the profiles of histograms are similar to those of previous reports (6, 8, 16). In one report, estimation of molecular weights was based on the mean length (6), whereas in another instance it was based on the longest molecule or cluster of molecules (16). It would seem reasonable that the longest molecules may represent the native genome of influenza.

In Fig. 3, a small peak is evident at 3.44 μm for X7 RNA; this would give an estimated molecular weight of 3.6 $\times 10^6$ daltons.

The unique structure of influenza genome, the existence of five discrete pieces of RNA, has been demonstrated by examination of pH 3-treated RNA preparations (Fig. 4 and 9). It remains unclear why the pieces of RNA, based on the frequency distribution, were not present in similar proportions. The ease by which intact molecules were disrupted is consistent with the concept that the linkers which hold the pieces together have extremely weak binding forces (14). This unique property of the influenza genome has been thought to govern its high frequency of recombination, its multiplicity reactivation, the effect of chemical inactivation of viral biosynthetic activities, and the formation of incomplete virus (17). RNA of X7 appears to be more stable than WSN RNA during storage at 4 C for several days. This may account for the lower mean length value for WSN RNA molecules. In the analysis of third passage X7 VM virus, four types of RNA molecules were identified (Fig. 5). The highest-molecular-weight RNA piece found in standard X7 virus was missing or present at relatively low frequency, as previously reported for incomplete virus RNA analyzed by polyacrylamide gel electrophoresis (2, 12). The deficiency in one of the components lends support to the interpretation that each incomplete particle lacks the identical piece of RNA (15). It should be noted that nondisrupted filaments consisting of the four pieces of VM virus RNA were not observed, suggesting that the assembly of incomplete virus genome is defective or its linkers are less stable than the linkers of standard virus genome.

The isolation of intact molecules suggests that X7 RNA is a likely candidate with which infectious RNA may be demonstrated. Preliminary experiments in our laboratory have been without success (S. S. Chang and J. T. Seto, *unpublished data*). Observation of intact molecules raises the question as to possible reasons why they were not readily characterized by electrophoresis and by velocity sedimentation centrifugation. Our findings suggest that the linkers are disrupted during the analysis of RNA by these methods. For that reason, molecular weight values obtained from length measurements cannot be substantiated with values by velocity sedimentation centrifugation analysis. Thus, direct visualization of RNA molecules offers advantages not shared by electrophoretic mobility and sedimentation behavior, particularly for the characterization of myxovirus RNA species.

ACKNOWLEDGMENTS

We thank E. Kilbourne, Mount Sinai Hospital, School of Medicine of New York, for supplying X7 and X7-F1 recombinants, A. F. Rasmussen, University of California, Los Angeles, for WSN virus, and R. P. Hanson, University of Wisconsin, for the Milano strain of NDV.

LITERATURE CITED

1. Agrawal, H. O., and G. Bruening. 1966. Isolation of high-molecular weight P^{32} labelled influenza virus ribonucleic acid. *Proc. Nat. Acad. Sci. U.S.A.* 55:818-825.
2. Duesberg, P. H. 1968. The RNA's of influenza virus. *Proc. Nat. Acad. Sci. U.S.A.* 59:930-937.
3. Duesberg, P. H., and W. S. Robinson. 1965. Isolation of the nucleic acid of Newcastle disease virus (NDV). *Proc. Nat. Acad. Sci. U.S.A.* 54:794-800.
4. Fraenkel-Conrat, H. 1969. The chemistry and biology of viruses. Academic Press Inc., New York.
5. Granboulan, N., and R. M. Franklin. 1968. Replication of bacteriophage ribonucleic acid: analysis of ultrastructure of the replicative form and the replicative intermediate of bacteriophage R17. *J. Virol.* 2:129-148.
6. Granboulan, N., and M. Girard. 1969. Molecular weight of poliovirus ribonucleic acid. *J. Virol.* 4:475-479.
7. Granboulan, N., and N. Scherrer. 1969. Visualization in the electron microscope and size of RNA from animal cells. *Europ. J. Biochem.* 9:1-20.
8. Kakefuda, T., and J. P. Bader. 1969. Electron microscopic observations on the ribonucleic acid of murine leukemia virus. *J. Virol.* 4:460-474.
9. Kleinschmidt, A. K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules, p. 361-377. *In* L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 12, part B. Academic Press Inc., New York.
10. Lowry, O. H., N. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
11. Nayak, D. P., and M. A. Baluda. 1967. Isolation and partial characterization of nucleic acid of influenza virus. *J. Virol.* 1:1217-1223.
12. Pons, M. W., and G. K. Hirst. 1968. Polyacrylamide gel electrophoresis of influenza virus RNA. *Virology* 34:386-388.
13. Pons, M. W., and G. K. Hirst. 1969. The single and double stranded RNA's and the proteins of incomplete influenza virus. *Virology* 38:68-72.
14. Pons, M. W., I. T. Schultze, and G. K. Hirst. 1969. Isolation and characterization of the ribonucleoprotein of influenza virus. *Virology* 39:250-259.
15. Rott, R., and C. Scholtissek. 1963. Investigations about the formation of incomplete forms of fowl plague virus. *J. Gen. Microbiol.* 33:303-312.
16. Sarkar, N. H., and D. H. Moore. 1970. Electron microscopy of the nucleic acid of mouse mammary tumor virus. *J. Virol.* 5:230-236.
17. Scholtissek, C., and R. Rott. 1964. Behavior of virus-specific activities in tissue cultures infected with myxoviruses after chemical changes of the viral ribonucleic acid. *Virology* 22:169-176.
18. Seto, J. T., B. J. Hickey, and A. F. Rasmussen, Jr. 1959. Sialidase activity and related properties of influenza viruses. *Virology* 9:598-611.
19. Webster, R. G., W. G. Laver, and E. D. Kilbourne. 1968. Reactions of antibodies with surface antigens of influenza virus. *J. Gen. Virol.* 3:315-326.