Electron Microscope Study of Double-stranded Ribonucleic Acid of Influenza Virus Infected Cells

By SHIRLEY S. CHANG AND J. T. SETO*

Department of Microbiology and Public Health, California State University, Los Angeles, California 90032, U.S.A.

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SUMMARY

Virus-specific double-stranded RNA molecules extracted from x7 strain influenza virus infected cells were characterized by electron microscopy. Molecules 0.33, 0.67, and 1.0 μm in length were frequently observed, and possibly five or more subunits could be identified.

INTRODUCTION

Influenza virus ribonucleic acid (RNA) and virus-specific double-stranded RNA of infected cells have been reported to consist of five and possibly as many as 10 polynucleotide species (Duesberg, 1968; Pons & Hirst, 1968; Content & Duesberg, 1971; Lewandowski, Content & Leppla, 1971; Skehel, 1971; Young & Content, 1971). These studies involved analysis of RNA by polyacrylamide gel electrophoresis and analysis of the 3' and 5' termini of RNA subunits. However, in the characterization of the RNA-genome by electron microscopy, molecules were illustrated with length measurements which would correspond to estimated mol.wt. of 3 to $5 \times 10^6$ (Li & Seto, 1971). The study was extended to include examination of the virus-specific double-stranded RNA of influenza virus infected cells. For small RNA viruses the double-stranded molecules of infected cells and single-stranded RNA isolated from purified virus were reported to be identical in length (Granboulan & Franklin, 1966; Granboulan & Girard, 1969). In this paper we will present evidence of the observation of several species of double-stranded RNA. The predominating length measurements of the double-stranded RNA molecules correspond to the RNA subunits isolated from influenza virus particles (Skehel, 1971).

METHODS

Virus. Influenza x7 strain was used, and it was propagated and assayed for haemagglutinating activity (HA titre) as described previously (Li & Seto, 1971). x7 virus was concentrated and purified by the method of Nayak & Baluda (1967).

Extraction of virus RNA. The general experimental procedures for the extraction and isolation of RNA from highly purified virus (Nayak & Baluda, 1967; Li & Seto, 1971) and from infected tissue and cell cultures were as reported previously (Nayak & Baluda, 1968). Infected cells, stored at $-60^\circ$C, were thawed in a 37°C water bath and resuspended in pH 8.5 extraction buffer. For each ml of packed cells the final volume of buffer was 20 ml; RNA was extracted by the sodium dodecyl sulphate (SDS) phenol method. RNA was

* At present on leave at: Institute for Virology, University of Giessen, Schuberstrasse 1, 6300 Giessen, W. Germany.
precipitated by the addition of sodium acetate and 2 vol. of ethyl alcohol and stored overnight at $-20^\circ$C. The DNA-rich fraction of RNA was not pooled with RNA flocculus after treatment of the two components separately with deoxyribonuclease. To isolate double-stranded RNA from infected cells, RNA flocculus was dissolved in pH 7.4 RNA buffer and an equal volume of 4 M-LiCl was added to give a final concentration of 2 M-LiCl. The mixture was stored at 4 $^\circ$C for 4 to 5 h and centrifuged for 30 min at 17000 rev/min in an RC-2B Sorvall centrifuge equipped with an SS-34 rotor. Double-stranded RNA of host cell origin was precipitated by 2 M-LiCl(Kimball & Duesberg, 1971). Soluble virus-specific double-stranded RNA was diluted in pH 7.4 RNA buffer and precipitated with ethanol. Some LiCl soluble double-stranded RNA preparations were purified in a CF-11 cellulose column as described by Pons (1967). Preliminary experiments were carried out, with [14C]-uridine labelled RNA from control and x7 virus infected cells, to make certain the method was effective for our purposes. RNA preparations were analysed at 260 and 280 nm and all preparations had $E_{260}/E_{280}$ ratios of greater than two.

Tissue and cell cultures. Secondary chick embryo tissue and clone 1-5C-4 Chang conjunctival cell cultures and media used were as reported before (Seto & Chang, 1969). Adsorption period of x7 virus was 1½ to 2 h and at an input multiplicity of 10.

Electron microscopy. Virus RNA samples were diluted with pH 7.4 RNA buffer (final concentration varied from 10 to 100 $\mu$g/ml) and prepared for examination by a modified Kleinschmidt method (Li & Seto, 1971). Samples treated with ribonuclease (10 $\mu$g/ml) were diluted in 2 x SSC (SSC = 0.15 M-NaCl and 0.015 M-sodium citrate). Nuclease treatment was for 10 to 15 min and RNA was ‘spread’ immediately on a protein monolayer. Specimens were shadowed with uranium at an angle of 6 to 8° in two directions and examined at a magnification of 7700 $\times$ with an RCA-EMU-3G electron microscope at 50 kv. Lengths of RNA molecules were measured on prints, at a final magnification of 30000 $\times$, with a map measurer.

RESULTS

Analysis of x7 virus RNA

x7 virus RNA molecules were examined in the electron microscope. Molecules with a mean of 2.53 $\mu$m, mode of 2.58 $\mu$m, a minor peak at 3.51 $\mu$m and a few molecules as long as 5 $\mu$m in length were observed, similar to previous findings in this laboratory (Li & Seto, 1971). Two preparations containing purportedly the intact virus-genome were assayed without success for infectious RNA, most likely because of the lack of the presence of a transcriptase.

![Length distribution of 366 LiCl soluble double-stranded RNA molecules isolated from conjunctival cells infected with x7 virus for 18 h.](image)
Fig. 2. (a) $x7$ virus-specific double-stranded RNA molecules, and (b) single-stranded RNA molecule from control cells.

**Analysis of double-stranded RNA**

RNA was extracted from $x7$ influenza virus infected conjunctival cells, 18 h after infection (p.i.). In order to isolate the virus-specific double-stranded RNA molecules, the RNA solution was made to contain 2 M-LiCl. Salt soluble molecules were reprecipitated with ethanol and resuspended in pH 7.4 RNA buffer prior to spreading on a protein monolayer.
A histogram showing the distribution of 366 molecules is presented in Fig. 1. Three or more subunits of RNA are evident, with molecules 0.33, 0.67, and 1.0 μm in length appearing most frequently. Morphology of the molecules is illustrated in Fig. 2; the molecules are relatively rigid in contrast to single-stranded host RNA.

To further characterize LiCl soluble double-stranded RNA, a preparation was purified by chromatography in a CF-11 cellulose column. A histogram of an RNA preparation treated in this manner is presented in Fig. 3. The length distribution profile of 251 molecules is qualitatively similar to that shown in Fig. 1, with three predominating sizes of molecules.
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Fig. 5. Length distribution of 237 LiCl soluble double-stranded RNA molecules isolated from chick tissue cultures infected with x7 virus for 4 h.

Fig. 6. Length distribution of 171 LiCl soluble double-stranded RNA molecules purified in a CF-11 cellulose column. RNA was isolated from chick tissue cultures infected with x7 virus for 4 h.

0.33, 0.67, and 1.0 μm long. Molecules longer than 1.3 μm were not observed. Another preparation of LiCl soluble double-stranded RNA molecules from x7 virus infected cell cultures was resuspended in 2 × SSC buffer and treated with pancreatic ribonuclease for 10 min immediately before ‘spreading’ on the protein monolayer. A histogram illustrating the distribution of 134 LiCl soluble and RNase resistant molecules is presented in Fig. 4. In this experiment five to six types of molecules, 0.27, 0.33, 0.47, 0.67, 0.87, and 1.0 μm in length, were identified. Molecules were not observed in a preparation of double-stranded RNA in pH 7.4 RNA buffer of low ionic strength which was treated with RNase.

Similar series of experiments were carried out with x7 virus infected chick embryo tissue cultures. The profile of a histogram of the LiCl soluble molecules from 18 h infected cells was identical to Fig. 1, and three possible size-classes of molecules were evident. In a preparation of LiCl soluble RNA from 4 h infected chick tissue cultures, among 237 molecules several pieces, 0.20, 0.47, 0.60, 0.73, 1.07 and 1.27 μm in length, were observed (Fig. 5). Another preparation of LiCl soluble RNA from 4 h infected cells was purified in a CF-11 cellulose column and ‘spread’ for examination in the electron microscope. Among 171 molecules, pieces 0.33, 0.67, 1.0, and 1.3 μm long were observed (Fig. 6), and fewer molecules much greater than 1.3 μm long were seen. The histograms of RNA molecules from chick
Table 1. Comparison of length measurements of single-stranded (SS) RNA molecules extracted from x7 and x31 strains of influenza virus, and double-stranded (DS) RNA molecules extracted from cells infected for 18 h with x7 virus

<table>
<thead>
<tr>
<th>RNA subunits</th>
<th>DS-RNA*</th>
<th>SS-RNA†</th>
<th>SS-RNA‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.27§</td>
<td>0.29</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>0.33</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>3</td>
<td>0.47</td>
<td>0.59</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>0.67</td>
<td>0.74</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>0.87</td>
<td>0.88</td>
<td>0.86</td>
</tr>
<tr>
<td>6</td>
<td>1.00</td>
<td>—</td>
<td>0.91</td>
</tr>
</tbody>
</table>

* Data from Fig. 4.
† Taken from Li & Seto, 1971.
‡ Taken from Skehel, 1971.
§ Length in μm.

tissue cultures (Fig. 5, 6) show size distributions similar to preparations of RNA molecules from conjunctival cells, illustrated in Figs. 1, 3, 4. The profiles of the histograms presented in Fig. 5 and 6 are symmetrical in contrast to the asymmetrical profiles illustrated in Fig. 1, 3, and 4. In the latter instances molecules 0.33 and 0.67 μm long are more frequent. Whether this might be related to the replication characteristics of x7 virus in conjunctival cells and in chick tissue cultures (Seto & Chang, 1969) remains to be investigated.

Comparative analysis of single- and double-stranded RNA

Length measurements of double-stranded RNA molecules are compared with single-stranded x7 virus RNA pieces (Li & Seto, 1971), together with other reported results (Skehel, 1970). Values from Skehel’s paper are translated into corresponding length measurements, by the method used in calculating estimated mol. wt. from length measurements of molecules (Li & Seto, 1971). The length of double-stranded molecules we observed are in general agreement with single-stranded virus-RNA length measurements (Table 1).

DISCUSSION

Influenza virus RNA molecules were isolated from virus particles and examined in the electron microscope. Previous findings from this laboratory were confirmed (Li & Seto, 1971). Molecules with a modal length of 2.5 μm, a mean of 2.58 μm, and some as long as 5 μm were observed. These length measurements correspond to estimated mol. wt. of 2.7 × 10^6, 3.0 × 10^6, and 5.0 × 10^6, respectively. Recent estimates suggest that the total mol. wt. of the RNA-genome subunits may be 5 × 10^8 (Lewandowski et al. 1971).

In this electron microscope study of double-stranded RNA isolated from influenza virus infected cells, several subunits were observed. The most prominent lengths were 0.33, 0.67, and 1.0 μm, and consisting of possibly six to eight subunits, which could be identified. Length measurements of x7 virus-specific double-stranded RNA molecules are consistent with the length of the pieces of RNA isolated from x7 virus particles, which were exposed to pH 3 treatment to fragment the genome (Li & Seto, 1971). These results (Table 1) correspond remarkably well with length measurements calculated from mol. wt. estimations of single-stranded RNA analysed by polyacrylamide gel electrophoresis (Skehel, 1971). The observation, by electron microscopy, of relatively long x7 virus RNA molecules isolated from particles and of several size-classes of x7 virus-specific double-stranded RNA extracted
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from infected cells is unlike that reported for small RNA viruses. In the latter instance the RNA-genomes are of identical length with their respective double-stranded RNA molecules (Granboulan & Franklin, 1966; Granboulan & Girard, 1969). Thus, our findings support the interpretation that influenza virus RNA is synthesized as pieces on templates of varying lengths (Pons, 1971). On the other hand, the mechanism by which the genome subunits assemble into one molecule within the particle is unknown.

REFERENCES


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