Identification of Self-complementary Virus-specific Ribonucleic Acid in Chick Kidney Cells Infected with Chicken Embryo Lethal Orphan Virus

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SUMMARY

RNA was extracted from primary chicken embryo kidney (CEK) cells infected with chicken embryo lethal orphan (CELO) virus and exposed to a pulse of [5-3H]-uridine late in infection. When this RNA was self-annealed, 4-5% became resistant to pancreatic ribonuclease digestion. The ribonuclease-resistant RNA was isolated by chromatography on Sephadex G-100, and the RNA was found to have the characteristics of a double-stranded molecule of sedimentation coefficient 8S. Half of the column-isolated RNA hybridized to CELO DNA with equal amounts of virus RNA binding to the heavy or light stands of the CELO DNA, indicating the presence of complementary RNA species late in the infectious cycle of CELO.

Self-complementary RNA has been found to be transcribed from the DNA genome of both bacteriophage and animal viruses. For example it has been identified in cells infected with phage λ (Bovre & Szybalski, 1969), phage T4 (Jurale, Kates & Colby, 1970), vaccinia virus (Colby & Duesberg, 1969; Colby, Jurale & Kates, 1971), simian virus 40 (Aloni, 1972, 1973), polyoma virus (Aloni & Locker, 1973) and type 2 adenovirus (Lucas & Ginsberg, 1972). The role of complementary RNA is not clear but it has been suggested that complementary RNA arises from regions of convergent overlapping transcription (Aloni, 1972, 1973; Bovre & Szybalski, 1969; Jurale et al. 1970). Avian adenoviruses have been reported not to induce virus-specific double-stranded RNA in chicken cells (Bakay & Burke, 1972; Markovits & Coppey, 1972). We report in this communication that pulse-labelled RNA isolated from cells infected with CELO contained self-complementary virus-specific RNA.

The preparation of chicken kidney cells from 18 day chicken embryos, the growth of CELO and extraction of CELO DNA was described previously (Laver, Younghusband & Wrigley, 1971; Younghusband & Bellett, 1971). Separation of the heavy and light strands of CELO virus DNA was accomplished by the method of Robinson & Bellett (1974).

Confluent monolayers of CEK cells in 60 mm Petri dishes (40 dishes) were infected with CELO at a multiplicity of 20 and after 19 h the medium was replaced with fresh medium (1-5 ml per plate) containing 20 μCl/ml of [5-3H]-uridine (25 Ci/mmol) for 1 h. Cells were lysed and phenol extracted essentially as described by Somers, May & Kit (1973) but without using chloroform. The final ethanol precipitate was resuspended in 4 ml of 0-01 M-tris-HCl buffer, pH 7.4, 0-5 M-NaCl, 0-002 M-MgCl2 and incubated with ribonuclease-free deoxyribonuclease for 1 h at 37 °C. (The batch of deoxyribonuclease used was free of detectable ribonuclease as shown by sucrose gradient sedimentation of labelled ribosomal RNA treated under the same conditions.) The incubation mixture was made 0-5% (w/v) with sodium dodecyl sulphate and extracted with phenol as described above. The ethanol precipitate was resuspended in and dialysed against 2× SSC (0-3 M-NaCl, 0-03 M-sodium citrate, pH 7-0). Samples at this stage were taken and treated with 20 g/ml of pancreatic ribonuclease resistant RNA prior to annealing. The dialysed RNA was self-annealed (in a
Fig. 1. (a) Chromatography of ribonuclease-resistant RNA on Sephadex G-100, from uninfected (▲—▲) and CELO-infected (●—●) CEK cells, as described in the text. Acid-insoluble counts are indicated. (b) Pancreatic ribonuclease resistance of CELO-infected RNA (from the void volume of Sephadex G-100) heated for 6 min in 1 × SSC (●—●) (sealed tubes used) or 0.1 × SSC (■—■). After heating, the RNA (in a vol. of 0.6 ml) was quickly cooled and adjusted to 2 × SSC then incubated with 20 μg/ml of pancreatic ribonuclease at 37 °C for 30 min. Acid-insoluble counts are indicated. (c) Caesium sulphate equilibrium sedimentation analysis of CELO-infected ribonuclease-resistant RNA (from the void volume of Sephadex G-100) which is either unheated (●—●), or heated in 0.1 × SSC for 6 min and quickly cooled (■—■). The equilibrium sedimentation was carried out in 1 × SSC (total vol. 30 ml) at 20 °C and 33 000 rev/min for 72 h in an SW 50.1 rotor. The densities of chicken cell RNA and CELO DNA are indicated. Acid-insoluble counts are indicated. (d) Velocity sedimentation of self-annealed ribonuclease-resistant RNA from CELO-infected (●—●) or uninfected (▲—▲) chicken cells in neutral sucrose. Sedimentation was in 5 to 20 % sucrose in 1 × SSC at 44 000 rev/min for 4 h in an SW 50.1 rotor (4°C). The positions of 8 S and 4 S RNA are indicated. Acid-insoluble counts are indicated.

vol. of 20 ml) in 40 % (v/v) formamide, 1 × SSC at 37 °C for 18 h (Friedrich & Feix, 1972) and again dialysed against 2 × SSC. The RNA solution was treated with 20 μg/ml pancreatic ribonuclease for 1 h at 37 °C and the percentage ribonuclease resistant RNA estimated. The treated RNA was twice phenol extracted as before. To the aqueous layers 100 μg yeast RNA was added and the mixture was ethanol precipitated. The RNA precipitate was collected, and resuspended in 0.1 ml of 0.01 m-tris-HCl buffer, pH 7.4 containing 0.25 m- NaCl and applied to a Sephadex G-100 column (column size 60 cm x 1.1 cm). The RNA was eluted with the same buffer and 2.1 ml fractions were collected. A 0.05 ml portion of each fraction was used for determination of acid-insoluble radioactivity. RNA-DNA
Table 1. *Hybridization of denatured double-stranded RNA to CELO DNA*

<table>
<thead>
<tr>
<th>Source of double-stranded RNA</th>
<th>CELO DNA (µg)</th>
<th>% RNA hybridized</th>
</tr>
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<tbody>
<tr>
<td>CELO-infected cells</td>
<td>10</td>
<td>52.1</td>
</tr>
<tr>
<td>infected cells</td>
<td>5</td>
<td>51.3</td>
</tr>
<tr>
<td>infected cells</td>
<td>2</td>
<td>45.1</td>
</tr>
<tr>
<td>infected cells (only heavy strand)</td>
<td>1</td>
<td>21.0</td>
</tr>
<tr>
<td>infected cells (only light strand)</td>
<td>1</td>
<td>18.1</td>
</tr>
<tr>
<td>infected cells</td>
<td>0</td>
<td>1.2</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td>5</td>
<td>1.3</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>Uninfected cells</td>
<td>0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

RNA from the void volume of the Sephadex G-100 column (see Fig. 1) was denatured by heating for 6 min at 100 °C in 0.1 x SSC, rapidly cooled, and 10 µg yeast (to stop rapid re-association of double-stranded RNA) per fraction was added. In the experiment 950 ct/min of infected cell RNA and 1050 ct/min or uninfected RNA was used; the conditions of hybridization are described in the text.

hybridizations with the DNA immobilized on nitrocellulose filters were carried out for 18 h in 1.0 ml of 2 x SSC, 0.1 % (w/v) sodium dodecyl sulphate essentially as described by Gillespie & Spiegelman (1965).

Pulse-labelled RNA from CELO-infected or uninfected CEK cells was found to be 2.5 % and 1.4 % resistant to pancreatic ribonuclease digestion respectively. However, upon self-annealing the amount of ribonuclease-resistant RNA increased both in the uninfected (2 % now resistant) and CELO-infected (4.5 % now resistant) cell extracts. This self-annealed RNA was chromatographed on a column of Sephadex G-100 and the results are shown in Fig. 1 (a). Labelled RNA eluted with the void volume in both the infected cell RNA (22 % of loaded RNA; range of 20 to 33 % in several experiments) and uninfected cell RNA (7 % of loaded RNA; range of 5 to 10 % in several experiments) with the CELO-infected cell RNA containing a threefold higher proportion of the loaded sample in the void volume This column procedure allowed for the selection of the higher mol. wt. RNA from low mol. wt. RNA pieces with a sedimentation coefficient of 2 S which survived the ribonuclease digestion. Only fractions 7 and 8 of the column were used for the following procedures. To establish whether the RNA eluting in the void volume of the column was indeed double-stranded RNA the following experiments were done. The RNA was 100 % resistant to 20 µg/ml pancreatic ribonuclease digestion in the presence or absence of 20 µg/ml deoxyribonuclease in 0.2 M-NaCl, 0.01 M-tris-HCl buffer, pH 7.4 (0.002 M-MgCl2 when deoxyribonuclease was present). However, after heating in 0.1 x SSC at 100 °C for 6 min, 96 % of the labelled RNA became susceptible to pancreatic ribonuclease digestion. Further, the transition of RNA from a ribonuclease-resistant form to a ribonuclease-sensitive form had a sharp melting profile with a Tm of 90 °C in 0.1 x SSC and 105 °C in 1 x SSC (Fig. 1b). Also the double-stranded RNA from the CELO-infected cells had a density of 1.60 g/ml in caesium sulphate (identical to that of double-stranded RNA from uninfected cells isolated in the same manner; J. T. May, unpublished data) but on heating, the labelled RNA changed its density to 1.66 g/ml (Fig. 1c). Both the melting profile and density in caesium sulphate were characteristic of double-stranded RNA but once heated the RNA behaved like single-stranded RNA. Further, the ribonuclease-resistant RNA extracted from both uninfected and CELO-infected RNA had identical sedimentation values.
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(8 S) in sucrose Fig 1 d; see also Kimball & Duesberg, 1971). To establish whether the double-stranded RNA isolated from the CELO-infected cells included virus-specific sequences, the heat-denatured RNA was hybridized to CELO DNA on nitrocellulose filters. As shown in Table I about 50% of the CELO-infected RNA was found to anneal to CELO DNA while only 1% of uninfected RNA was bound. (Similar values were found in four separate preparations of infected and uninfected RNA). Of the RNA which hybridized to CELO DNA about equal amounts bound to heavy and light strands of the CELO DNA (Table I) further confirming the complementary nature of the virus RNA isolated.

These experiments indicate that self-complementary virus-specific RNA, which forms double-stranded RNA under self-annealing conditions, is produced late in CELO virus infection of CEK. It represents about 0.45% (i.e. half of the RNA from the column void volume which represents 22% of the 45% of the pulse-labelled RNA resistant to ribonuclease) of the RNA synthesized during a 1 h period late in infection.

It is not clear why other workers (Bakay & Burke, 1972; Markevits & Coppey, 1972) have not found virus-specific double-stranded RNA in avian adenovirus-infected cells. However they did not pulse label RNA late in the infectious cycle. This was the method used to detect double-stranded RNA in Ad2-infected cells (Lucas & Ginsberg, 1972) and their method was used here, with the addition of a self-annealing step to increase the amount of double-stranded RNA isolated.

The possible origin of self-complementary RNA from regions of convergent overlapping transcription in ‘strand switching’ regions in this adenovirus is under investigation.

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REFERENCES
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