IDENTIFICATION OF DOUBLE-STRANDED VIRUS-SPECIFIC RIBONUCLEIC ACID IN KB CELLS INFECTED WITH TYPE 2 ADENOVIRUS

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Received August 8, 1972

SUMMARY: Using agarose gel chromatography, RNA with characteristics of double-stranded molecules was isolated from KB cells infected with type 2 adenovirus. Results of studies measuring its susceptibility to a number of nuclease activities were consistent with its identification as double-stranded RNA. The material was resistant to digestion by both pancreatic and T1 ribonuclease and by pancreatic deoxyribonuclease; and after heating at 100°C, it was much more sensitive to ribonuclease hydrolysis. Moreover, nearly 30% of the RNA formed a ribonuclease-resistant hybrid when denatured and incubated with purified type 2 adenovirus DNA, indicating that at least a portion of the double-stranded RNA in infected KB cells was transcribed from the viral genome.

Self-complementary RNA is not only synthesized in a number of cultured animal cell types, including primary cells, established cell lines and transformed cells (10), but it is also transcribed from the genomes of both bacteriophages and animal viruses. For example, it has been identified in cells infected with phage λ (1) and T4 (5,6) and vaccinia virus (2,3).

It was suggested that the synthesis of complementary RNA may merely indicate an abnormality in RNA chain termination (5). However, there is some evidence as to possible functions for double-stranded RNA in mammalian cell systems. The double-stranded RNA isolated from rabbit kidney, chick embryo and Hela cells was shown to induce interference with virus multiplication (2,7). Also, the viral double-stranded RNA found in Hela cells infected with poliovirus inhibits the initiation of protein synthesis in an *in vitro* system derived from rabbit reticulocyte lysates (4).

The identification of virus-specific complementary RNA in adenovirus-infected cells

is reported here. This finding, together with the observations that at least some cytoplasmic virus-specific RNA species may be cleavage products of larger precursor molecules in the nucleus (12), that adenovirus-specific RNA contains adenine-rich regions (13), and that the transport of virus-specific RNA from the nucleus to the cytoplasm is nonconservative (9) offers additional evidence for the concept (9) that the synthesis, processing, and transport of adenovirus-specific RNA may be similar in many ways to that of mammalian cell RNA species. The adenovirus system, then, may prove to be an appropriate model for the further study of these events in mammalian cells.

MATERIALS AND METHODS

Procedures used for tissue culture, infection of KB cells with type 2 adenovirus, purification of virus, extraction of viral DNA, isotopic labeling and extraction of RNA from KB cells, and DNA-RNA hybridization were described in a previous report (8).

Double-stranded RNA was isolated using procedures described by Oberg and Philipson (11) and Colby and Duesberg (2). RNA was prepared from whole infected KB cells as described above. After ethanol precipitation, it was dissolved in a solution containing 50 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), 2 mM $MgCl_2$, pH 7.4, and treated with pancreatic deoxyribonuclease (pDNase; at a concentration of 20 μg/ml) for 30 min at room temperature. The concentration of NaCl was adjusted to 0.2 M and pancreatic ribonuclease (pRNase) was added at a concentration of 20 μg/ml. After 30 min at room temperature, the solution was extracted three times with phenol at room temperature, and then extracted with ether. Ether was removed by evaporation and the RNA was precipitated by the addition of cold ethanol. The precipitate was dissolved in a solution containing 0.1 M LiCl, 10 mM Tris, 2 mM ethylenediaminetetracetate (EDTA), pH 7.4, and applied to a column of Sepharose 4B (column size: 63.5x1.5 cm). The column was eluted with the same buffer and the void volume was determined using blue dextran-2000. The optical density of each 4 ml fraction was determined at 260 nm, and a portion of each

fraction was used for the determination of acid-precipitable radioactivity. That is, to 3 ml of each fraction, 200 μg of yeast RNA was added as a carrier; cold trichloroacetic acid was added at a concentration of 10%; and precipitates were collected on paper filters (Whatman #3). After washing with acetone and drying, each filter was placed in 5 ml of a solution of 0.5 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 0.6 g of 2,5-diphenyloxazole per liter of toluene, and counted in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS AND DISCUSSION

RNA labeled with ^{3}H-uridine from 18 to 19 hours after infection was extracted from adenovirus-infected KB cells. After treatment with pRNase, the RNA was again extracted, ethanol precipitated and dissolved in buffer. About 10% of the RNA prepared from the cells was in an ethanol-precipitable form after the RNase treatment and subsequent extractions.

The results of chromatography of this sample on a column of Sepharose 4B is shown in Fig.1. A small amount of labeled material (about 4% of the total counts eluted) was present in the void volume, while nearly all detectable ultraviolet light-absorbing material and most of the radioactivity eluted later. The large peak presumably represents small fragments of RNA which survived the RNase treatment. Since RNA molecules are separated on agarose on the basis of both size and shape, the material eluting in the void volume could be either double-stranded RNA (which under the conditions used would be in the form of "rigid rods") or single-stranded RNA (as "random coils") with much higher molecular weight than that of the material eluting later (11). Since high molecular weight single-stranded RNA would not be expected to survive RNase hydrolysis, the latter possibility seemed unlikely.

However, to establish whether the material in the void volume was indeed double-stranded RNA, a preparation of labeled material obtained as described above was treated with a number of nucleases, both before and after heating to 100°C for 5 min, to denature double-stranded molecules. The RNA in the void volume, fractions 8 to 10, was pooled and will be referred to as v-v RNA. Material from fraction 23 was used as a control

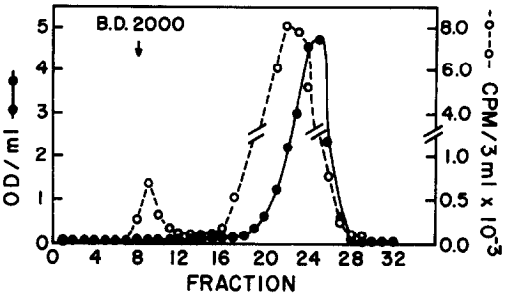

FIG. 1. Chromatography of RNA on Sepharose 4B. Eluting buffer was 0.1 M LiCl, 10 mM Tris, 2 mM EDTA, pH 7.4. RNA was prepared by the method employing hot phenol and sodium dodecyl sulfate. It was then treated with pancreatic ribonuclease (20 μg/ml), again extracted with phenol three times, precipitated with cold ethanol, and dissolved in the eluting buffer before chromatography. 200μg of yeast RNA was added to 3 ml of each fraction eluted and the RNA was precipitated with cold trichloroacetic acid. The precipitates were collected on paper filters, which were washed with acetone and dried before counting. (counts/min per 3 ml) X 10^{-3} (--0--0--); optical density at 260 nm (-●-●-).

TABLE I

NUCLEASE TREATMENTS OF SINGLE AND DOUBLE STRANDED RNA

Sample[1]	Treatment[2]	CPM resistant to hydrolysis	% Resistant
v-v RNA 1	None	1952	----
v-v RNA 2	T1 RNase;pRNase	1719	88.1
v-v RNA 3	T1 RNase;pRNase;DNase	1328	68.0
v-v RNA 4	heated at 100C;then as in 3	299	15.3
s-s RNA 1	None	10,914	----
s-s RNA 2	T1 RNase;pRNase	428	3.9
s-s RNA 3	T1 RNase;pRNase;DNase	582	5.3
s-s RNA 4	heated at 100C;then as in 3	72	0.7

1 v-v RNA refers to RNA eluted in the void volume of the Sepharose 4B column (Fig. 1). s-s RNA refers to single-stranded RNA, i.e., material from fraction 23 for the Sepharose column.

2 Final concentrations of enzymes were : pRNase (pancreatic ribonuclease), 20 μg/ml; T1 RNase, 0.5μg/ml; pDNase (pancreatic deoxyribonuclease), 20μg/ml. For Samples 4 and 8, RNA was heated at 100C for 5 min in 0.01 M NaCl, 0.01 M Tris (pH 7.4). RNase treatments were performed in 0.2 M NaCl, 0.01 M Tris (pH 7.4). When DNase hydrolysis was also done, $MgCl_2$ was added at a concentration of 2 mM.

TABLE II

HYBRIDIZATION OF DENATURED DOUBLE-STRANDED RNA WITH ADENOVIRUS DNA

Filter No.	DNA/filter (μg)	CPM input RNA[1]	CPM hybridized[2]	Corrected (CPM)	Per cent hybridization
1	10	1366	396	389	28.5
2	10	1366	400	393	28.8
3	0	1366	7	0	----

1 RNA was obtained from the void volume of a Sepharose 4B column (see Figure 1). The RNA was denatured by heating at 100C for 5 min in 0.01 M NaCl, 0.01 M Tris (pH 7.4). It was rapidly cooled and yeast RNA was added to minimize reassociation.

2 The conditions used for hybridization are described in Materials and Methods.

sample and will be called s-s RNA. As shown in Table 1, nearly 70% of the v-v RNA was resistant to the three nucleases, which together should degrade single-stranded RNA and DNA-RNA hybrids. Only about 5% of the s-s RNA was not digested by such treatment. After heating at 100°C, the material was much more sensitive to digestion.

To establish whether or not the putative double-stranded RNA included virus-specific sequences, the following experiment was performed. A portion of the RNA was denatured by heating at 100°C for 5 min at low ionic strength. It was then rapidly cooled and yeast RNA was added in an attempt to minimize reassociation. After adjusting the solution to 2 x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), hybridization with adenovirus DNA was performed (Table 2). About 30% of the RNA was found to anneal to DNA.

These experiments, then, indicate that complementary virus-specific RNA, which forms double-stranded molecules, is produced in adenovirus-infected cells. It represents about 0.12% (30% of 4% of 10%) of the RNA synthesized during a one hour period late in

infection. The finding that such small amounts of complementary RNA are transcribed, however, does not eliminate the possibility that it may play some role in viral multiplication.

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service research grants A1-03620 and 5 TI-A1-203 from the National Institute of Allergy and Infectious Diseases. Joseph J. Lucas was supported by National Defense Education Act Title IV Fellowship 66-04895 and a National Science Foundation Traineeship.

We gratefully acknowledge the excellent assistance of Joseph Higgs.

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