IDENTIFICATION OF VIRUS-SPECIFIC RNA IN CELLS INFECTED WITH ROUS SARCOMA VIRUS

A.C. Garapin, J. Leong, L. Fanshier, W.E. Levinson, and J.M. Bishop
Department of Microbiology, University of California
San Francisco, California 94122

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SUMMARY: The cytoplasm and nuclei of cells infected with Rous sarcoma virus contain RNA which can be annealed to single-stranded DNA synthesized by the RNA-dependent polymerase of the virus. Formation of DNA:RNA hybrids was detected by equilibrium centrifugation in Cs_2SO_4, and by step-wise elution from hydroxyapatite. RNA which contains nucleotide sequences identical to those of the viral genome constitutes approximately 0.5% of the total RNA extracted from infected cells.

Previous efforts to detect virus-specific RNA in cells infected with and transformed by RNA tumor viruses have generally been unsuccessful (1). Consequently, little is known regarding the mechanism by which the genomes of these viruses are replicated. The discovery of DNA polymerases within the virions of RNA tumor viruses (2-6) has provided a new approach to this problem. The DNA synthesized by these enzymes contains two classes of nucleotide sequences: those which are complementary to viral RNA (7,8,9), and those which are identical to viral RNA (9). These DNA's constitute useful reagents for the detection of virus-specific RNA by the use of nucleic acid hybridization. This report concerns the preliminary results of such experiments with Rous sarcoma virus (RSV): RNA containing nucleotide sequences identical to those of the viral genome has been identified in both cytoplasmic and nuclear fractions isolated from virus-infected (and transformed) cells. The size and secondary structure of this RNA, and our efforts to detect RNA which is complementary to the viral genome, will be the subjects of subsequent communications.

MATERIALS AND METHODS

The propagation and purification of RSV (Schmidt-Ruppin strain), and the extraction of RNA from virus and infected cells, have been described previously (10). Infected cells were trypsinized in suspension (0.05% trypsin, 37°, 10 minutes) and washed by centrifugation prior to extraction in an effort to remove

virions still associated with cell surfaces. Cytoplasmic and nuclear RNA's were prepared according to the procedure of Penman (11). Nuclear RNA was considered to be free of contamination with cytoplasmic RNA if it contained no detectable 18S ribosomal RNA when analyzed by electrophoresis in gels of polyacrylamide (11). Virus-specific DNA was synthesized in vitro with virion polymerase (12) and extracted as described previously (13). The conditions of the reaction were such that all of the enzymatic product is complementary to viral RNA (9). DNA was treated with 0.6N NaOH for one hour at 37° (9), then annealed to RNA under the following conditions: 0.2M NaCl-0.01M EDTA-0.02M Tris:HCl, pH 7.4 - 50% (v/v) formamide, 37°, 24 hours (7,9). The nucleic acids were analyzed for hybridization by equilibrium centrifugation in Cs_2SO_4 (9), and by step-wise elution from hydroxyapatite (13).

RESULTS

The single-stranded DNA synthesized in vitro by RSV polymerase can be annealed with the 70S RNA of the viral genome to form DNA:RNA hybrids which have a buoyant density identical to that of single-stranded RNA (7,8,9). Formation of such hybrids is readily detectable by equilibrium centrifugation in Cs_2SO_4, because the hybridized enzymatic product bands at the density of single-stranded RNA rather than in the region of DNA (7,8,9). Using this criterion for hybridization, we have found that virus-specific DNA will anneal to RNA extracted from infected cells (Fig. 1b). Virus-specific RNA, identified in this manner, is present in both cytoplasm and nuclei (Fig. 1c and d). No hybrids are formed if either the DNA is incubated with RNA from uninfected cells (Fig. 1a) or a mixture of enzymatic product and RNA from infected cells is analyzed in Cs_2SO_4 without prior incubation at 37°. These observations demonstrate the specificity of hybridization, and indicate that the presence of DNA at the density of RNA is not simply due to entrapment by a precipitated band of RNA (14).

We have also been able to detect DNA:RNA hybridization with a simple procedure which utilizes step-wise elution from hydroxyapatite (13). This procedure facilitates rapid analysis of large numbers of samples, and can be used to obtain

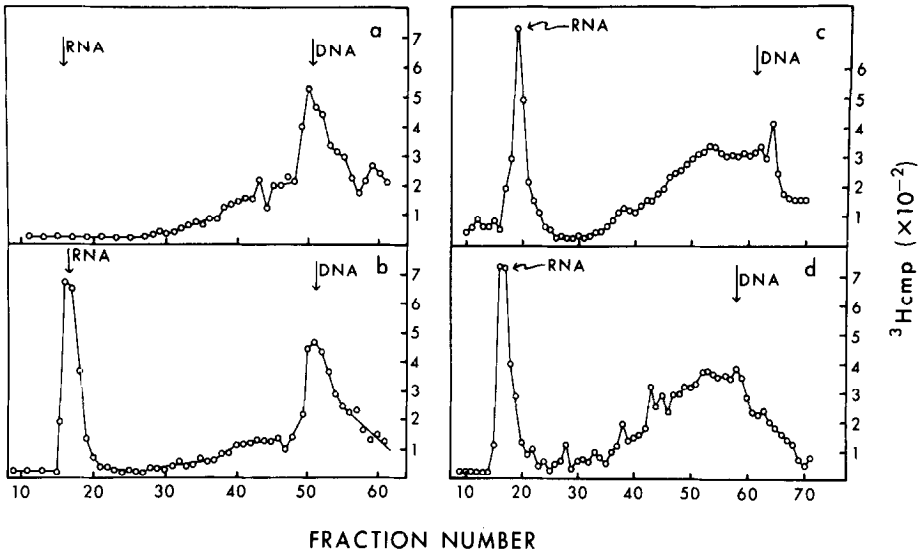

Fig. 1. Analysis of hybridized nucleic acids by equilibrium centrifugation in Cs_2SO_4. The following combinations of nucleic acids were incubated under conditions for hybridization, then analyzed by equilibrium centrifugation in Cs_2SO_4; (a) ^{3}H-labeled single-stranded DNA (0.005 μg) and RNA (50 μg) from uninfected cells; (b) ^{3}H-labeled single-stranded DNA (0.005 μg) and RNA (50 μg) from infected cells; (c) ^{3}H-labeled single-stranded DNA (0.005 μg) and RNA (50 μg) from the cytoplasm of infected cells; (d) ^{3}H-labeled single-stranded DNA (0.005 μg) and RNA (20 μg) from the nuclei of infected cells. The arrows indicate the locations of ^{32}P-labeled single-stranded bacterial RNA and lambda phage DNA.

data which are quantitatively more useful than those provided by equilibrium centrifugation. Single-stranded enzymatic product elutes from hydroxyapatite at a low concentration of sodium phosphate (Fig. 2a). By contrast, 70S RSV RNA has a relatively high affinity for hydroxyapatite (Fig. 2b). Consequently, hybridization of product DNA to viral RNA should alter the elution properties of the DNA. This expectation is based on the previous demonstration (7,8,9) that, under the conditions used here, only small amounts of DNA anneal to any given RNA molecule. Thus, elution of the hybrid molecules from hydroxyapatite should be determined primarily by their RNA components. This supposition has been confirmed previously for the native DNA:RNA hybrids isolated from enzyme reaction mixtures (13). We now present data which demonstrate that the same is true for the hybrids formed by annealing single-stranded enzymatic product to viral RNA.

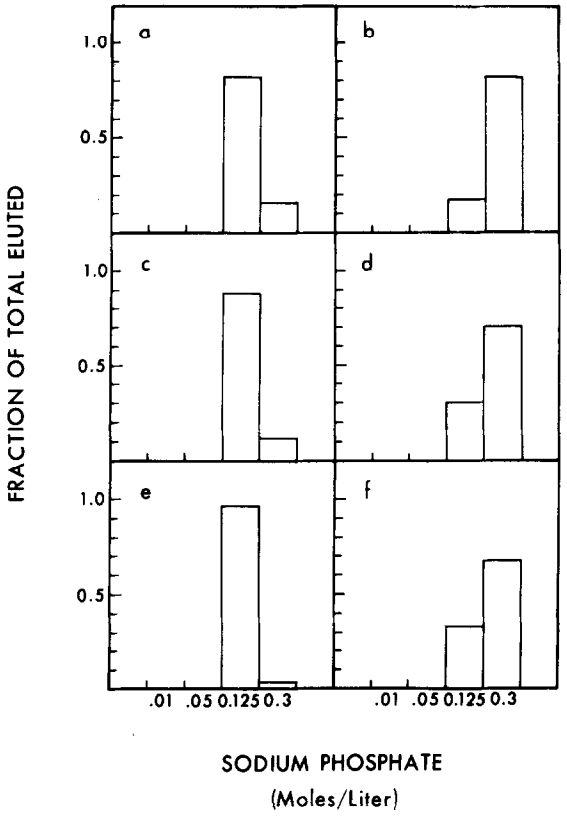

Fig. 2. Detection of hybrid formation with hydroxyapatite. Hybridization was carried out with ^{3}H-labeled single-stranded enzymatic product (0.0002 μg). Analysis of nucleic acids on hydroxyapatite was performed by stepwise elution (13) at 60°. Recovery of labeled DNA approached 100%. Samples were counted to a standard error of ± 2%. (a) ^{3}H-labeled single-stranded enzymatic product, treated with NaOH (0.4N, 12 hours, 37°) to hydrolyze RNA. (b) ^{32}P-labeled 70S RSV RNA. (c) ^{3}H-labeled single-stranded enzymatic product, carried through the hybridization reaction in the absence of viral RNA. (d) ^{3}H-labeled single-stranded enzymatic product, hybridized with 70S RSV RNA (1 μg). (e) ^{3}H-labeled single-stranded enzymatic product, incubated with poliovirus RNA (5 μg) under conditions for hybridization. (f) ^{3}H-labeled native DNA:RNA hybrid (70S), isolated from the RSV polymerase reaction as described previously (12).

Prior to hybridization, less than 20% of single-stranded DNA elutes from hydroxyapatite in 0.3M sodium phosphate (Fig. 2a). The same is true if the annealing reaction is carried out in the absence of viral RNA (Fig. 2c). Following hybridization to RSV RNA, however, 70% of the DNA elutes in 0.3M sodium phosphate (Fig. 2d). By contrast, annealing with a heterologous RNA has no effect on the elution of product DNA (Fig. 2e), a fact which again illustrates the specificity of the hybridization reaction.

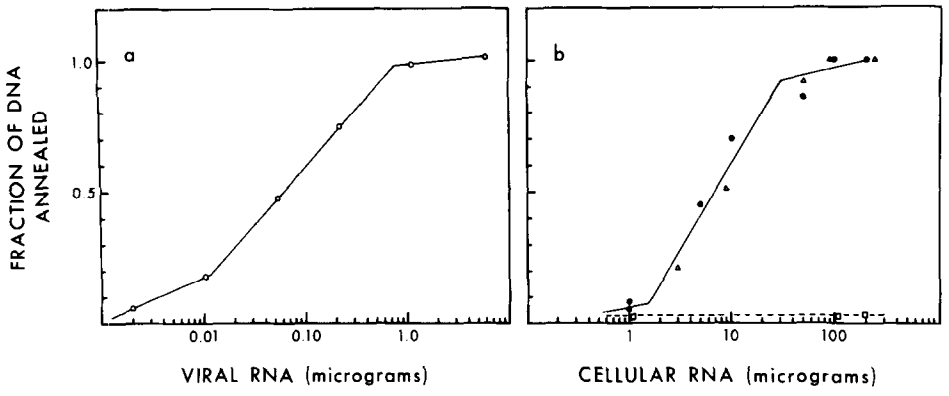

Fig. 3. Hybridization of enzymatic product with viral and cellular RNA's. Hybridization was carried out with ^{3}H-labeled single-stranded enzymatic product (0.0002 μg) and increasing amounts of unlabeled RNA in a constant volume of annealing solution. Analysis of nucleic acids on hydroxyapatite was performed as in Fig. 2. At the points of maximum hybridization, 80% of the DNA eluted in 0.3M sodium phosphate, as opposed to 15% in the case of single-stranded DNA analyzed prior to hybridization. (a) Viral RNA (70S). (b) RNA from infected cells. The data from two experiments with two separate lots of cellular RNA (● △) have been combined. □–□, results with RNA from uninfected cells.

The two concentrations of phosphate used in these experiments (0.125M and 0.3M) were chosen because they provided maximum discrimination between single-stranded and hybridized DNA (unpublished experiments). Nevertheless, 30% of the DNA still elutes in the lower phosphate concentration after hybridization (Fig. 2d). We cannot presently explain this observation although it conforms to the behavior of native DNA:RNA hybrid when analyzed in hydroxyapatite (Fig. 2f). Whatever its explanation, the elution of a fraction of the annealed DNA in 0.125M sodium phosphate does not seriously impair the utility of the hydroxyapatite assay. If a series of annealings are carried out with increasing amounts of viral RNA (and a constant amount and concentration of DNA), the point of maximum hybridization is readily determined (Fig. 3a). We have performed such calibrations repeatedly, and the results have proven to be highly reproducible: elution of 70-80% of product DNA in 0.3M sodium phosphate signifies that maximum annealing has been achieved. Similar results are obtained with RNA from infected cells (Fig. 2b), except that the quantity of RNA required for maximum annealing is much larger than in the case of viral RNA. This latter observation indicates

that virus-specific RNA is a relatively minor component of the population of cellular RNA's. By comparison of the two sets of data shown in Fig. 3, it is possible to compute the approximate amount of virus-specific RNA present in the RNA extracted from infected cells. This computation is predicated upon the fact that a standard quantity of DNA (ca. 0.0002 μg) is used at a constant concentration throughout, and the relative amount of hybridization should therefore be dependent only upon the quantity of complementary RNA present in the annealing mixture. Fifty percent of maximum annealing is achieved with 0.05 μg of viral RNA. A similar amount of virus-specific RNA must be present in the quantity of cellular RNA (8-10 μg) which hybridizes the DNA at 50% of maximum efficiency. This result indicates that virus-specific RNA constitutes approximately 0.5% of the total cellular RNA used in these experiments.

DISCUSSION

The preceding data demonstrate the manner in which DNA synthesized *in vitro* by RSV polymerase can be used to identify virus-specific RNA in both cytoplasmic and nuclear fractions of infected cells. It could be argued that this RNA is derived from virions which have not yet been released from the cell surface. Two facts mitigate against this relatively trivial explanation of our observations: 1) the cells were thoroughly trypsinized prior to extraction in an effort to release cell-associated virus, and 2) virus-specific RNA was detected in purified nuclear fractions which contained no evidence of contamination with cytoplasmic RNA. The techniques described are now being used to compare the quantities of viral RNA present in cytoplasm and nuclei, and to determine the size and secondary structure of the RNA in question.

The present report concerns only RNA which is complementary to the initial single-stranded product of RSV DNA polymerase, and which is therefore identical in nucleotide sequence to the viral genome (i.e., "plus" RNA strands) (7,8,9). However, the same techniques can be used to identify viral "minus" RNA strands. Experiments of this sort, utilizing "plus" DNA isolated from the double-stranded enzymatic product (9,13) are now in progress.

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