Simultaneous Detection of Reverse Transcriptase and High Molecular Weight RNA Unique to Oncogenic RNA Viruses

Abstract. *The simultaneous assay of the reverse transcriptase and 60S to 70S RNA of oncogenic RNA viruses is described. The virus can be detected at low concentrations in biological fluids containing enzymatically active cell fragments or other contaminants. The fact that two features diagnostic of the oncornaviruses are used in the tests increases the certainty with which a positive outcome can be interpreted.*

The search for enzymatic evidence of oncogenic RNA viruses in human milk, plasma, or tumor extracts constitutes a major endeavor of present-day cancer research. These efforts are often made difficult by low viral content compounded by massive contamination with enzymatically active cell debris that can be obscured by and confused with irrelevant reactions. Ideally the detection method should be simple, sensitive, and sufficiently discriminating so that a positive outcome can be immediately taken as an acceptable signal for the presence of the viral agent being sought. It is obvious that certainty can be increased by devising a test that simultaneously identifies two diagnostic features.

The oncogenic RNA viruses, or oncornaviruses (*1*), exhibit two biochemical properties unique to them as a group. They possess a large single-stranded RNA molecule with a sedimentation coefficient of 60*S* to 70*S* (*2*), and thus it is often referred to as high molecular weight (HMW) RNA. They also contain reverse transcriptase, an enzyme capable of using the viral RNA as a template to generate a DNA complementary copy (*3*, *4*). We now describe a procedure for detecting RNA tumor viruses that identifies both these properties simultaneously.

The possibility of a concomitant test for 70*S* RNA and reverse transcriptase was suggested by (*4–6*) studies of the early reaction intermediates. The initial DNA product was found complexed to the 70*S* RNA template. The structure could be detected by the unusual position of the newly synthesized small [^{3}H]DNA products in cesium sulfate equilibrium and glycerol velocity gradients. That these complexes were held together by hydrogen bonds was demonstrated by mild denaturation (heating for 10 minutes at 68°C in 50 percent formamide), following which the [^{3}H]-DNA was found in its expected positions in both the cesium sulfate and glycerol gradients (*4*, *5*).

It seemed probable that these features of the early stages of the reaction could be used to design the assay required by subjecting isotopically labeled products to sedimentation analysis. If the labeled DNA product is complexed to a 70*S* RNA molecule, evidence is provided for the presence of a reverse transcriptase that uses a 70*S* RNA template and, hence, for the presence of an oncornavirus.

Our procedure has been carried out with biological material containing B-type and C-type viruses of avian, rodent, feline, and primate origin. We illustrate the procedure principally by data obtained with the mouse mammary tumor virus (MMTV) found in the milk of the RIII strain of mice. This virus is extremely difficult to propagate in tissue culture and at present can only be detected in milk, which is grossly contaminated with cells and cell fragments. Further, the MMTV is very similar to particles observed by Moore *et al.* (*7*) in the milk of Parsi women and in that of American women with family histories of mammary neoplasia. Finally, we have shown (*8*) that the particles in certain human milks contain the reverse transcriptase characteristic of the oncogenic RNA viruses. The mouse mammary tumor virus serves, therefore, as an excellent experimental model for perfecting more certain and sensitive methods for detecting virus particles in human milk as well as in other biological fluids.

It is obviously desirable to eliminate any superfluous purification steps prior to the assay for viral enzyme activity. We have found that the commonly used sucrose gradient separation of virus is not necessary. For most fluids, the only

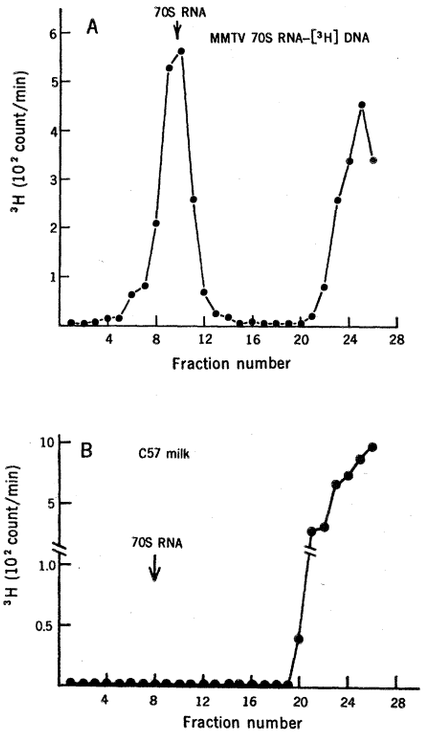

Fig. 1. Detection of the HMW-RNA–[^{3}H]-DNA complex of MMTV in mouse milk. One milliliter of milk and 1 ml of 0.15*M* EDTA (*p*H 7.5) were mixed and centrifuged at 3000*g* for 10 minutes. The clear "milk-plasma" zone between the lipid and precipitated casein layers was removed and placed on a 20 percent glycerol column resting on a 100 percent glycerol cushion and centrifuged at 98,000*g* for 1 hour at 4°C. The material on the glycerol cushion was removed, diluted, and centrifuged at 98,000*g* for 30 minutes at 4°C. (An alternative procedure is the direct centrifugation of the "milk plasma" at 98,000*g* for 30 minutes at 4°C.) The resulting pellet was resuspended in 45 μl of 0.01*M* tris-(hydroxymethyl)aminomethane (*p*H 8.3) containing 0.33 percent NP-40 and 0.1*M* dithiothreitol (DTT) and kept at 4°C for 10 minutes. This suspension was then added to a standard reverse transcriptase reaction mixture (125 μl final volume) containing 6.25 μmole of tris-HCl (*p*H 8.3), 1 μmole of $MgCl_2$, 1.25 μmole of NaCl (instead of KCl to avoid precipitation when SDS is added in a subsequent step), 0.2 μmole each of unlabeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate and [^{3}H]deoxythymidine triphosphate to a final specific activity of 8900 count min^{-1} pmole^{-1}. All reactions were carried out at 37°C for 15 minutes; they were terminated by the addition of NaCl and SDS to final concentrations of 0.2*M* and 1 percent, respectively. After addition of an equal volume of a mixture of phenol and cresol (7 : 1, *p*H 7.6) containing 8-hydroxyquinoline (3.7 g per 100 ml of the mixture), the final mixture was shaken at 25°C for 5 minutes and centrifuged at 5000*g* for 5 minutes at 25°C. The aqueous phase was then layered over a linear glycerol gradient (10 to 30 percent) and centrifuged at 40,000 rev/min for 3 hours at 4°C in a Spinco SW-41 rotor. External markers used were avian myeloblastosis virus 70*S* [^{3}H]RNA and 28*S* and 18*S* [^{3}H]RNA from NC-37 cells. Fractions were collected from below and portions were assayed for acid-precipitable radioactivity (*4*). (A) RIII milk, (B) C57 milk.

purification steps retained are a low-speed centrifugation (1,000*g* for 5 minutes) to remove cells, and a second centrifugation at 16,000*g* for 10 minutes. The virus in the supernatant is then collected by centrifugation at 98,000*g* for 30 minutes at 4°C.

In concentrating virus from milk, it is desirable to first remove the casein and lipid components, a step readily accomplished by adding an equal volume of 0.15*M* ethylenediaminetetraacetic acid (EDTA) and by centrifuging at 3000*g* for 15 minutes at 4°C. The middle layer of clear "milk plasma" is then separated from the top lipid layer and the pellet (precipitated casein). The virions are then concentrated by centrifugation at 98,000*g* for 30 minutes at 4°C. Since our intent is to assay enzyme, glycerol or sucrose pads need not be used, so that the loss of virus that often results is avoided.

The viral pellets are resuspended in tris buffer containing 0.33 percent of the detergent Nonidet P-40 (NP-40) and 0.1*M* dithiothreitol (DTT); the suspensions of viruses were incubated for 10 minutes at 4°C to disrupt the virus. A portion is then subjected to a standard 15-minute, reverse transcriptase reaction (*4*) in which [^{3}H]thymidine triphosphate (TTP) is used to follow the course of the synthesis. After the synthesized product is freed of protein by treatment with sodium dodecyl sulfate (SDS) and phenol, it is subjected to a sedimentation analysis in a 10 to 30 percent glycerol gradient with suitable size markers to determine the apparent size distribution of the DNA synthesized (Fig. 1).

Assays of this nature were carried out on milks from RIII mice, which carry the MMTV, and from C-57 mice, which are free of this agent (Fig. 1, A and B). Only the milk known to contain the MMTV (Fig. 1A) shows evidence of tritiated DNA in the 70*S* position of the gradient. However, both gradients contain tritiated DNA sedimenting at 6*S* or less, ascribable to the considerable content of cellular fragments, a constant feature of all milks. These fragments are invariably associated with cellular DNA polymerases as well as with template DNA, a combination that can, and does, lead to the synthesis of radioactively labeled DNA during the assay (*9*). However, this introduces no ambiguity in this assay since the DNA synthesized by a contaminating cellular enzyme is readily distinguished by its gradient position from the DNA synthesized on the viral RNA template.

A number of simple experiments can be performed to establish that labeled DNA in the 70*S* region is in that part of the gradient because it is hydrogen-bonded to a large RNA molecule and that it is formed as a result of the reverse transcriptase reaction.

The 70*S* region of the gradient in Fig. 1A is pooled, precipitated, and redissolved. Half the sample serves as a control and the other is exposed to

Table 1. Assay of oncornaviruses for 70*S* RNA–[^{3}H]DNA.

Agent	Source	70*S* RNA–[^{3}H]DNA
	Oncornavirus	
Mouse leukemia	Plasma	+
Feline leukemia	Tissue culture	+
Avian leukosis (RAV)	Tissue culture	+
Avian myeloblastosis (AMV)	Tissue culture	+
Avian myeloblastosis	Plasma	+
Mouse mammary tumor	Milk	+
Mouse mammary tumor	Blood	+
Mason-Pfizer monkey	Tissue culture	+
	Other	
Vesicular stomatitis virus		−
Cell culture supernatant		−
C57 milk		−
Mycoplasma 359, human		−
Mycoplasma 360, human		−
Mycoplasma 358, human		−

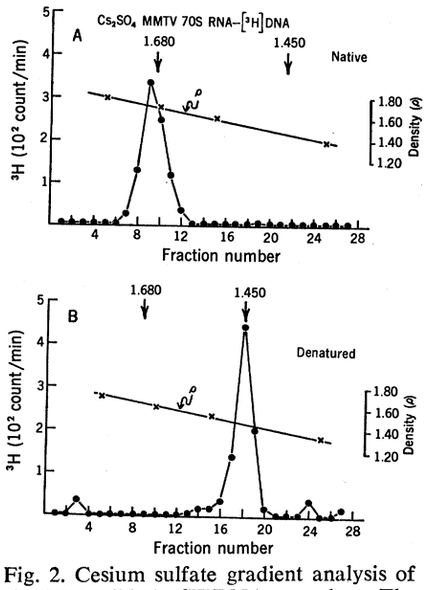

Fig. 2. Cesium sulfate gradient analysis of the HMW-RNA–[^{3}H]DNA complex. The material in the 60*S* to 70*S* region of Fig. 1A was pooled, and treated with 2 volumes of ethanol and yeast RNA (15 μg/ml final concentration) and stored at −20°C overnight; and the precipitate was removed by centrifugation at 16,000*g* for 20 minutes at 0°C. The pellet was suspended in 0.002*M* EDTA, and the suspension was divided in half. (A) One part was kept at 4°C for 10 minutes (native); (B) the other part was heated to 68°C for 10 minutes in the presence of 50 percent formamide (denatured). To each part, 0.002*M* EDTA and saturated cesium sulfate were added to give a final density of 1.550 g/cm^3; after centrifugation at 44,000 rev/min in a 50 Ti rotor (Spinco) for 60 hours at 20°C, fractions were collected and processed for acid-precipitable radioactivity (*4*).

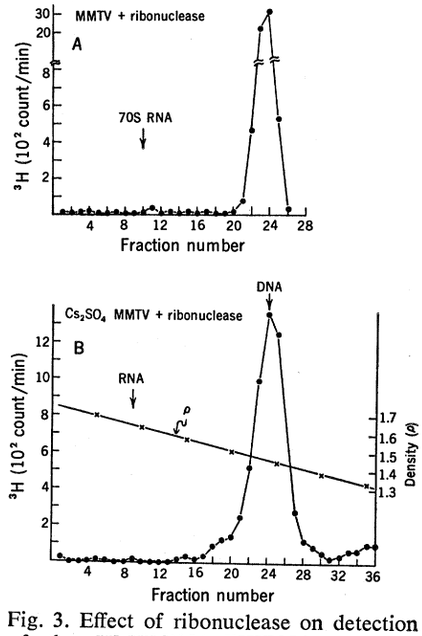

Fig. 3. Effect of ribonuclease on detection of the HMW-RNA–[^{3}H]DNA complex. (A) One milliliter of RIII milk was processed as described (Fig. 1). The viral pellet was resuspended in 0.01*M* tris (*p*H 8.3) containing 0.33 percent NP-40 and 0.1*M* DTT and kept at 4°C for 10 minutes. Ribonuclease A was then added to a final concentration of 50 μg/ml, and the mixture was incubated at 25°C for 10 minutes. The subsequent reverse transcriptase reaction, nucleic acid extraction, and velocity sedimentation were as described (Fig. 1). (B) Portions from the fractions (21 to 24) of A, containing acid-precipitable radioactivity (count/min), were pooled and subjected to cesium sulfate equilibrium gradient centrifugation as described (Fig. 2).

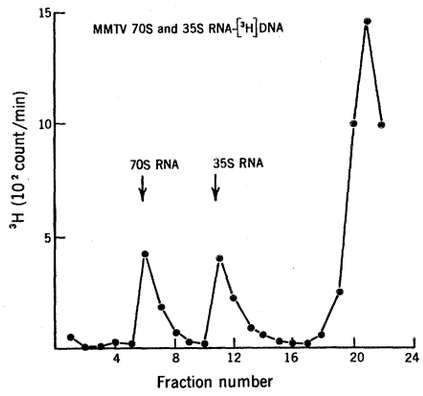

Fig. 4. Detection of 35*S* and 70*S* RNA–[^{3}H]DNA complexes in MMTV. One milliliter of RIII milk was processed as described (Fig. 1).

mild denaturation to rupture hydrogen bonds (incubation at 68°C for 10 minutes in 50 percent formamide). The control and the treated samples are then subjected to cesium sulfate equilibrium centrifugation (Fig. 2, A and B). The [^{3}H]DNA of the untreated portion (Fig. 2A) resides in the RNA region (1.650 to 1.680) of the density gradient as would be expected if small DNA pieces are hydrogen bonded to relatively large RNA molecules. On the other hand, the profile of the denatured sample (Fig. 2B) shows that all the [^{3}H]DNA has shifted to the DNA region (1.420 to 1.450) of the equilibrium gradient. This demonstrates that mild denaturation can completely remove the newly synthesized DNA from the complex in which it was originally found, a result observed in previous studies on such early intermediates (*4*).

Such features as sensitivity to ribonuclease and the requirement for all four deoxyribonucleoside triphosphates can also be used to demonstrate that the appearance of the 70*S* RNA–[^{3}H]-DNA complex is in fact the result of a reverse transcriptase reaction. If the reaction described in Fig. 1 is carried out after prior incubation of the enzyme preparation with ribonuclease, the glycerol velocity gradient shows no evidence of any tritiated DNA in the high molecular weight region of the gradient (Fig. 3A). However, there is still considerable synthesis of DNA not complexed to RNA, as might be expected because ribonuclease would not inhibit the cellular DNA-directed DNA polymerase activity.

The same conclusion can be drawn by subjecting the product from the ribonuclease-treated enzyme preparation to cesium sulfate centrifugation as shown in Fig. 3B. Here it is seen that none of the newly synthesized DNA is complexed to RNA. All the materials synthesized are in the DNA region of the density gradient. Furthermore, we have found that, if one of the deoxyribonucleoside triphosphates is omitted, there is no DNA in the 70*S* position in a velocity gradient.

It is of some interest to emphasize the increased sensitivity that derives from using the gradient analysis of the product. For example, the total reaction shown in Figs. 1A and 3A was examined for sensitivity to ribonuclease by incorporation of tritiated TTP into the acid-insoluble material, and very little difference was found. Had we depended only on this observation, we would have concluded that there was little or no reverse transcriptase activity in the material. This is not an uncommon situation when examinations are made with crude material because of the large amounts of cellular DNA polymerases and DNA templates usually present. Sedimentation analysis of the reaction product, however, quickly reveals the presence of the reverse transcriptase reaction and its sensitivity to ribonuclease.

It is well known (*10*) that the 70*S* RNA of oncornaviruses is easily converted into structures that sediment at 35*S*. It is therefore not surprising to find that occasionally, when a sedimentation analysis of a reaction is run, tritiated DNA is found complexed to molecules sedimenting at both 70*S* and 35*S*. An example of this with the MMTV is shown in Fig. 4.

In addition to the experiments described with milk containing the MMTV, we have examined a number of other biological fluids for the formation of the 70*S* RNA–[^{3}H]DNA complex. Figure 5A shows detection of the Rauscher leukemia virus in plasma. Figure 5, B and C, illustrates the method with feline leukemia and avian myeloblastosis viruses, respectively, in unconcentrated supernatant

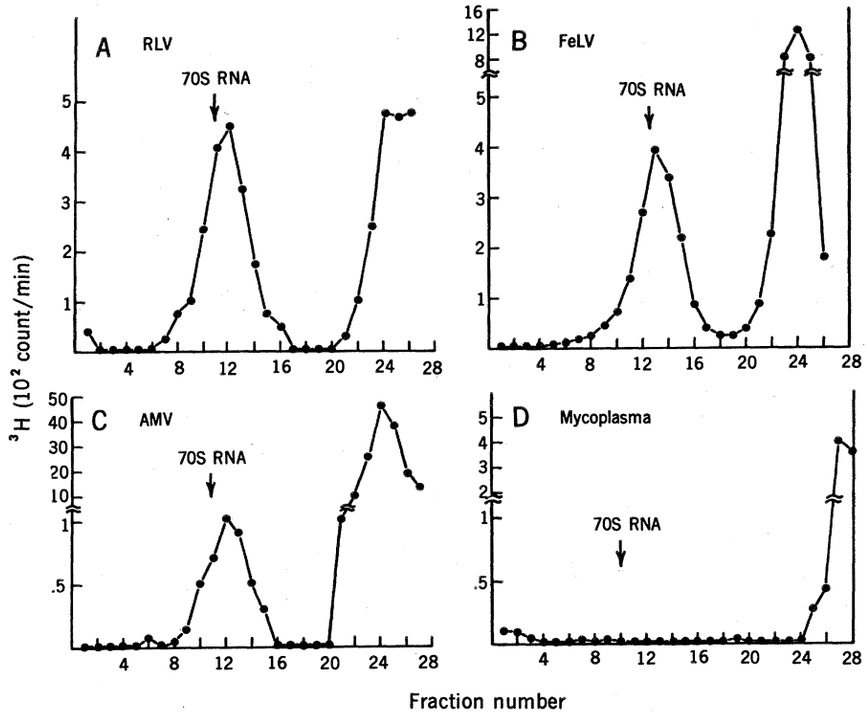

Fig. 5. Assay for 70*S* RNA–[^{3}H]DNA of (A) Rauscher murine leukemia virus (RLV) from 4 ml of mouse plasma concentrate; (B) Rickard feline leukemia virus (FeLV) from 30 ml of tissue culture supernatant (only one-tenth of each fraction was assayed); (C) avian myeloblastosis virus (AMV) from 30 ml of myeloblast cell culture supernatant; (D) mycoplasma 358 from biopsy culture of human skin. All of the above were first centrifuged at 1000*g* for 10 minutes to remove cells. After centrifugation at 16,000*g* for 10 minutes at 4°C, the resulting supernatants were centrifuged at 98,000*g* for 30 minutes at 4°C. The resulting pellets were then treated as described (Fig. 1). The pellets from the centrifugation at 16,000*g* (10 minutes) of mycoplasma were also assayed and found to be negative.

fluids of infected cells. Finally, Fig. 5D shows the negative outcome of the reaction with a purified human mycoplasma.

Table 1 contains a complete summary of the preparations tested for the 70*S* RNA–[^{3}H]DNA complex and their source. Of particular interest is the detection of the DNA–70*S* RNA complex in the plasma of the RIII mouse. Infectivity experiments have shown (*11*) that there are 10^5 to 10^6 more infectivity units per milliliter in RIII milk than in RIII plasma. Indeed the amount in plasma is so low that thus far immunological assays have failed to detect the presence of the MMTV antigen (*12*). However, the gradient assay of reverse transcriptase activity revealed the presence of the agent without difficulty.

None of the three human mycoplasmas tested showed any evidence of reverse transcriptase. This is of some technical interest since mycoplasma is a common contaminant of human milk.

There are two ways in which the initial assay can be performed. First, protein-free product is analyzed in a cesium sulfate gradient. Newly synthesized DNA is then looked for in the RNA region of the cesium gradient. This will occur whether or not the RNA being used as a template is partially broken either because of processing or because of the presence of ribonucleases in the material being tested. Second, product is analyzed in a velocity gradient. The presence of DNA in the 70*S* position of the gradient with subsequent analysis on cesium sulfate shows that an RNA molecule has been used as a template and that its size is 70*S*. By these devices one can demonstrate the presence of a 70*S* RNA molecule in a viral agent, which one cannot label isotopically or obtain in sufficient amounts to prepare RNA for examination by optical density in a velocity gradient.

The sensitivity and comparative certainty of the assay described for reverse transcriptase and the HMW-RNA make it a useful tool for exploring the presence of oncogenic RNA viruses in biological material of both animal and human origin.

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26 October 1971

Activation of Spontaneous Murine Leukemia Virus–Related Antigen by Lymphocytic Choriomeningitis Virus

Abstract. *Persistent infection with lymphocytic choriomeningitis (LCM) virus activates a phenotypic expression of murine leukemia virus–related antigen. NZB and (NZB × NZW)F_1 mice, which normally carry large amounts of Gross virus, and C57BL/6 and NZW mice, which normally carry little virus, were infected with LCM virus. All had Gross soluble antigen in their plasmas at 3 months of age, while noninfected matched controls of all strains did not. This effect was seen after infection with LCM virus that was tissue passed or plaque purified. Similarly, cultures of mouse-embryo fibroblasts produced Gross soluble antigen when infected with LCM virus, but noninfected cultures failed to do so.*

In an attempt to explain oncogenesis, it has been suggested that cancer is a natural event which is determined by spontaneous or induced derepression (or both) of an endogenous viral oncogene (*1*). Accordingly, all cells carry or contain information for the activation of oncorna viruses, with cancer expression being determined by both host genetic factors and events such as irradiation, carcinogenesis, and aging (*1, 2*). In addition to the above known modifying factors, we now report that chronic infection of mice or cultured mouse cells with lymphocytic choriomeningitis (LCM) virus activates a phenotypic expression of Gross antigen or antigens determined by the viral genome.

Inbred strains of NZW and NZB were originally obtained from the Laboratory Animal Center, Medical Council, Carshalton Surrey, England. Breeding was effected by brother-sister mating in our laboratory. The SWR/J and C57BL/6 inbred mice were obtained from Jackson Laboratories, Bar Harbor, Maine. The NZB and (NZB × NZW)F_1 hybrids have a relatively high leukemia incidence and carry large amounts of Gross virus, whereas C57BL/6, SWR/J, and NZW mice have low leukemia incidence; overt expression of Gross virus is generally absent in early life and only minimal in later life. Random testing of mice indicated that they were free of LCM, polyoma, and lactate dehydrogenase

Table 1. Appearance of Gross soluble antigen in the plasma of 3-month-old mice chronically infected with LCM virus. Mice were inoculated with either 1000 LD_{50} of LCM, 0.5 × 10^6 plaque-forming units (polyoma), or a 10 percent suspension of noninfected isologous brain tissue (control) within the first 15 hours of life.

Strain	Inoculum	Mice (No.)	GSA positive (%)
NZW	Control	29	3
	Polyoma	15	0
	LCM	25	84
NZB	Control	20	5
	Polyoma	10	0
	LCM	20	90
NZB × W	Control	20	5
	Polyoma	10	5
	LCM	10	90
C57BL/6	Control	25	0
	LCM	25	60