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Further Evidence for Oncornaviruses in Human Milk: The Production of Cores

(RNA-instructed DNA polymerase/60–70S RNA)

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ABSTRACT **Cores, or nucleoids, have been isolated from particles in human milk. The cores have a density of 1.26–1.27 g/ml and contain a 60–70S RNA in association with an RNA-instructed DNA polymerase. The data offer further evidence of similarity between human milk particles and animal RNA tumor viruses. In addition, core isolation provides a new method for detection of these particles by minimizing the difficulties generated by the presence of cell-associated debris often found in the density region (1.16–1.19 g/ml) characteristic of the intact virion. The procedures described now make available preparations of purified subviral components of a putative human RNA tumor virus.**

Certain human milk samples contain particles possessing biochemical and biophysical properties diagnostic of the known RNA tumor viruses. In particular, these human particles have a density of 1.16–1.19 g/ml and, as demonstrated by the simultaneous detection test (2), contain a 60–70S RNA template physically associated with an RNA-instructed DNA polymerase (1–9). Further, the 60–70S RNA of the human milk particles contains stretches of polyadenylic acid about 200 nucleotides in length (10), another characteristic common to the RNA tumor viruses (11–14).

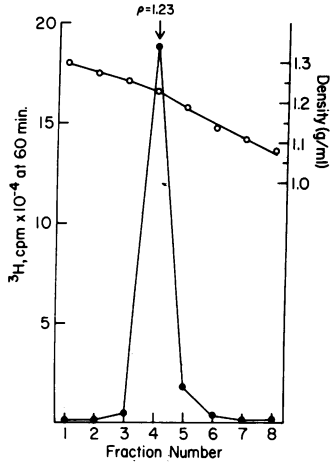

FIG. 1. Production of cores from mouse mammary tumor virus. Mouse mammary tumor virus was purified from 10 ml of RIII mouse milk and was treated with phospholipase C–ether as described in *Methods*. The preparation was then layered on a 25–65% linear sucrose gradient and centrifuged at 98,000 × *g* for 16 hr at 4° (Spinco SW-27). Eight equal fractions of the gradient were collected, diluted, and pelletted at 98,000 × *g* for 30 min at 4°. Each fraction was assayed for endogenous DNA polymerase activity in the presence of actinomycin D (100 μg/ml).

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It is the purpose of the present paper to present evidence that the human particles share yet another feature of the oncogenic RNA viruses, i.e., the presence of a nucleoid, or core, possessing a density of 1.26–1.27 g/ml and containing a 60–70S RNA in association with an RNA-instructed DNA polymerase.

Cores from avian tumor viruses (15–18) and from murine lukemia (19–22) and murine mammary (23, 24) tumor viruses have been produced by the use of surfactants and ether. These cores contain the viral 60–70S RNA (17) and a DNA polymerase activity (15–18, 20).

We report here a technique, using phospholipase C (EC 3.1.4.3), for preparation of cores from the mouse mammary tumor virus and from human milk particles. In addition to offering further evidence of similarity between the human milk particles and the RNA tumor viruses, core isolation obviates certain technical difficulties. Because of their uniquely higher densities, cores, unlike complete virions, band in a region comparatively free of cellular contaminants. This minimizes the problems generated by enzyme inhibitors and by the presence of cellular debris found in human milk. Under these circumstances the assays for particles become more sensitive and certain.

MATERIALS AND METHODS

Purification of Mouse Mammary Tumor Virus. 10 ml of RIII (Institute of Cancer Research Colony) mouse milk, and an equal volume of 0.15 M EDTA (pH 7.5) were mixed and centrifuged at 1500 × *g* for 10 min at 4°. The clear "milk–plasma" zone between the lipid and precipitated casein layers was removed and centrifuged at 10,000 × *g* for 10 min at 4°. The resulting clear "milk–plasma" zone was then removed, layered on an 8-ml column of 20% glycerol in TNE buffer [10 mM Tris·HCl (pH 8.3)–150 mM NaCl–2 mM EDTA] resting on a 6-ml 100% glycerol cushion (Beckman SW-27 cellulose nitrate tube), and centrifuged at 98,000 × *g* for 1 hr at 4°. The material on the 100% glycerol cushion was removed in 1 ml of 0.01 M Tris·HCl (hydroxymethyl) aminomethane HCl (pH 8.3) and kept at 4° for immediate core preparation.

Purification of Human Milk Particles. The majority of human milk samples tested were generously supplied by Drs. S. Albert, M. Rich, and M. Brennan of the Michigan Cancer Foundation, Detroit.

10–15 Individual samples of human milk (10–100 ml each) were pooled, mixed with one-quarter volume of 0.5 M EDTA (pH 8.3), and centrifuged at 2500 × *g* for 10 min at 4°. The

clear "milk–plasma" zone was removed, mixed with glycerol to a final concentration of 20%, and centrifuged at 30,000 rpm for 2 hr at 4° in a Spinco Ti 15-batch rotor. The fluid was aspirated, and the outer wall of the rotor was gently scraped with a spatula and washed with 0.01 M Tris·HCl (pH 8.3). The resulting suspension was divided into twelve 1-ml aliquots and stored at −70°. Each 1-ml aliquot represented about 80 ml of the original milk pool. Several different pools of milk as well as individual milk samples were prepared and tested.

Preparation and Purification of Cores. 250 μl of 1 M dithiothreitol were mixed with an equal volume of polyvinylsulfate (1 mg/ml) in a 15-ml corex tube at 4°. 1 ml of purified milk concentrate (mouse or human) was then added to the tube followed immediately by 2 μl of phospholipase C (Sigma Chemicals Type I; 10 mg/ml in 1% bovine-serum albumin), giving a final concentration of 0.01 mg/ml. Phospholipase C was stored as a dry powder at 4°. The mixture was placed in a 37° water bath, swirled gently for 30 sec, kept at 25° for 10 min with gentle swirling for 10 sec every 2 min, and then placed in an ice bath. 1 ml of cold ether (Fisher, Anhydrous) was added, and the tube was swirled gently for 10–20 sec to allow mixing. The resulting emulsion was then centrifuged at 1500 × *g* for 10 min at 4°. A lower aqueous phase, an upper ether phase, and an interphase that contained some gelatinous material resulted. The lower aqueous phase was removed with a Pasteur pipette and transferred to a 30-ml corex tube at 4°. A stream of nitrogen was blown over the surface for 1–2 min to aid removal of ether.

The resulting solution was layered on a 25–65% linear sucrose gradient (in TNE buffer) and centrifuged for 16 hr at 40,000 rpm at 4° (Spinco SW-41 rotor). All sucrose solutions were prepared on a weight/weight basis. 10 Fractions of equal volume were collected from below and the density of each fraction was determined (Zeiss refractometer). Each fraction was then diluted in 0.01 M Tris·HCl (pH 8.3), pelleted at 98,000 × *g* for 30 min, and resuspended in 60 μl of 0.01 M Tris·HCl (pH 8.3) containing 0.2% NP-40 (a Shell nonionic detergent) and 0.1 M dithiothreitol for simultaneous detection analysis.

All reagents were dissolved in 0.01 M Tris·HCl (pH 8.3), unless otherwise specified.

Simultaneous Detection Test. Concentrated core preparations, resuspended in 0.2% NP 40 and 0.1 M dithiothreitol (60 μl volume), were kept at 4° for 10 min.

Actinomycin D was added to a final concentration of 100 μg/ml and oligo $(dT)_{12-18}$, functioning as a primer, was added to a final concentration of 0.32 mg/ml.

This suspension was then added to a standard simultaneous detection (2) reaction mixture (125 μl final volume) containing 6.25 μmol of Tris·HCl (pH 8.3), 1 μmol of $MgCl_2$, 1.25 μmol of NaCl, 0.2 μmol each of unlabeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and 0.02 μmol of [^{3}H]deoxythymidine triphosphate (New England Nuclear Corp.) to a final specific activity of 24,000 cpm per pmol. After a 15-min incubation at 37°, the reaction was terminated by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.2 M and 1%, respectively. After addition of an equal volume of a chloroform–phenol–cresol (8:7:1) mixture containing 8-hydroxyquinoline (0.185 g per 100 ml of mixture), the mixture was shaken at 25° for 15 sec (Vortex) and centrifuged at 3000 × *g* for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10–30%) and centrifuged at 40,000 rpm for 3 hr at 4° (Spinco SW-41 rotor). 28S and 18S [^{3}H]RNA from NC-37 cells were used as external size markers. Fractions were collected from below, and portions of each fraction were assayed for acid-precipitable radioactivity (25).

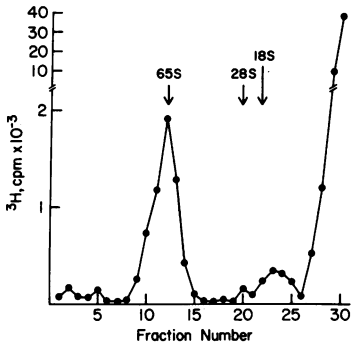

FIG. 2. Application of simultaneous detection test to mouse mammary tumor virus cores. Mouse mammary tumor virus cores were prepared as described in the legend of Fig. 1. Particles banding at a density of 1.23–1.24 g/ml were assayed by the simultaneous detection test (see *Methods*) for [^{3}H]DNA·60–70S RNA complexes.

RESULTS

Cores from mouse mammary tumor virus

Mouse mammary tumor virus was purified from RIII mouse milk and treated with phospholipase C–ether as described in *Methods*. The preparation was then subjected to equilibrium density gradient analysis, and each resulting density region was assayed for endogenous DNA polymerase activity in the presence of actinomycin D. A linear rate of DNA synthesis, checked at 10-min intervals for 60 min, was observed in the 1.23–1.24 g/ml fraction (Fig. 1). All other density fractions were negative, including the 1.16–1.19 g/ml region, the density of mouse mammary tumor virus before core generation. DNA polymerase activity in the presence of actinomycin D is indicative, but not definitive, that the DNA synthesis is RNA-directed. To demonstrate this latter property of viral cores we ran a simultaneous detection assay (2) on the 1.23–1.24 g/ml density fraction. As seen in Fig. 2, DNA has been synthesized with the endogenous viral 60–70S RNA as a template.

Cores from human milk particles

The technology developed for the preparation of purified cores from mouse mammary tumor virus was applied to particles from human milk. 1 ml of a milk pool concentrate, representing 80 ml of starting milk, was treated with phospholipase C–ether and subjected to equilibrium density gradient analysis.

The resulting density regions were concentrated and assayed for particles with 60–70S RNA and RNA-instructed DNA polymerase by the simultaneous detection test. A positive response was obtained (Fig. 3) only in the 1.26–1.27 g/ml density region. All other regions of the gradient, ranging from densities 1.12–1.30 g/ml, were negative. To further indicate that the DNA (acid-precipitable [^{3}H]TTP) peak in the 60–70S region of the gradient was due to its complexing with

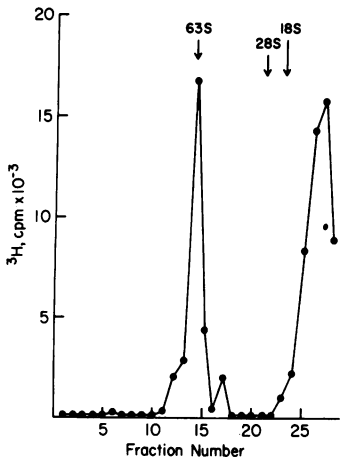

FIG. 3. Application of simultaneous detection test to cores of human milk particles. Several human milk samples were pooled, and particles were concentrated as described in *Methods*. 1 ml of milk pool concentrate, representing 80 ml of starting milk, was treated with phospholipase C–ether as described, and was layered over a 25–65% linear sucrose gradient. After centrifugation at 40,000 rpm for 16 hr at 4° (Spinco SW-41), the 1.26–1.27 g/ml density region was diluted in 0.01 M Tris·HCl (pH 8.3) and pelletted at 40,000 rpm for 30 min at 4° (Spinco SW-41). The resulting pellet was assayed for [^{3}H]DNA·60–70S RNA by the simultaneous detection test.

RNA, the [^{3}H]TTP cpm in this region was shown to be eliminated when the reaction mixture was preincubated with ribonuclease (25 μg/ml), as seen in Table 1. The omission of one of the deoxyribonucleoside triphosphates (deoxyadenosine triphosphate) from the reaction mixture also eliminated the [^{3}H]DNA found complexed to the 60–70S RNA (Table 1). This is evidence that we were not observing end addition of [^{3}H]TTP but rather the synthesis of a proper heteropolymer. It should be noted that occasionally [^{3}H]DNA–35S RNA complexes were observed in human milk particles and mouse mammary tumor virus core preparations, a response also seen with animal RNA tumor viruses (2).

Conversion of activity from viral to core density

To further demonstrate that the structures banding at a density of 1.26–1.27 g/ml were human subviral cores, a "density-shift" experiment was performed. 2 ml of concentrated human milk particles (from 160 ml of milk) were subjected to equilibrium density gradient analysis. Four regions of the resulting gradient were then taken for further examination: two "core" regions (1.26–1.27 and 1.24–1.25 g/ml), the "viral" region (1.14–1.20 g/ml), and the region of protein and lipoprotein debris, i.e., a density less than 1.10 g/ml. One half of each region was pelleted and tested by the simultaneous detection test. Only the "viral" region (1.14–1.20 g/ml) (Fig. 4*C*) contained particles with 60–70S RNA and

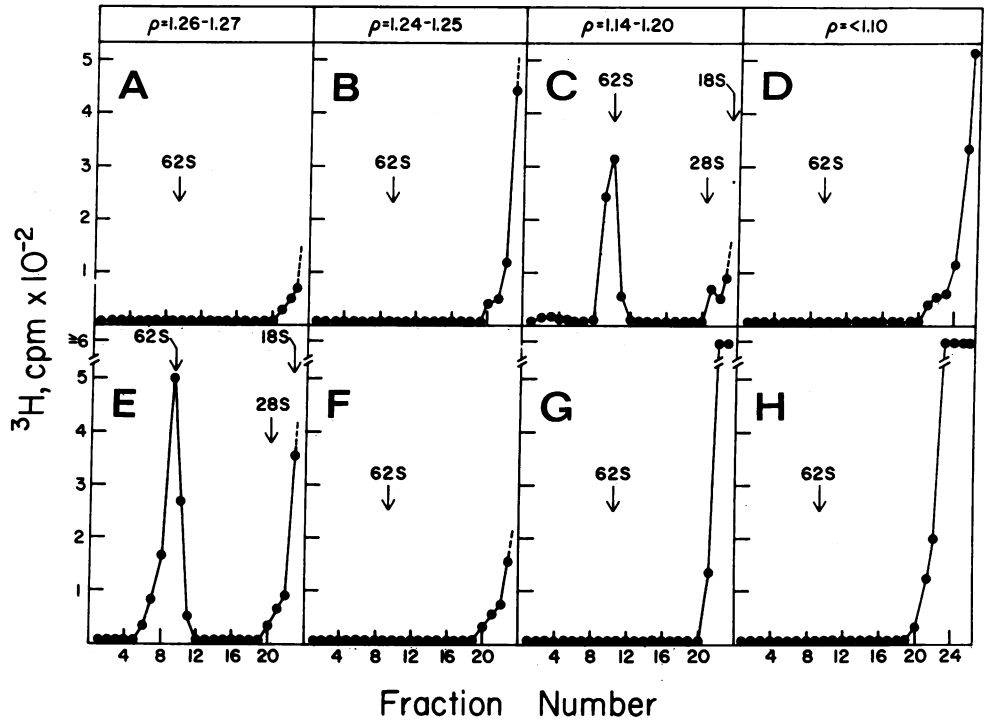

FIG. 4. Conversion of simultaneous detection activity in human milk from "viral" to "core" density region. Several human milk samples were pooled, and particles were concentrated as described in *Methods*. 2 ml of milk pool concentrate, representing 160 ml of starting milk, were layered over a 25–65% linear sucrose gradient and centrifuged for 4 hr at 98,000 × *g* at 4° (Spinco SW-27). Fractions were collected from below and the density of each fraction was taken. One-half of each of four density regions: 1.26–1.27 (*A*), 1.24–1.25 (*B*), 1.14–1.20 (*C*), and <1.10 g/ml (*D*) were diluted in 0.01 M Tris·HCl, pelletted at 98,000 × *g* for 30 min at 4°, and assayed by the simultaneous detection test. The other half of the 1.14–1.20 g/ml density region (*C*) was diluted in 0.01 M Tris·HCl, layered over 6 ml of 100% glycerol (Spinco SW-27), and centrifuged at 98,000 × *g* for 30 min at 4°. The material on top of the 100% glycerol cushion was treated with phospholipase C–ether as described in *Methods*. The resulting aqueous phase was layered over a 25–65% linear sucrose gradient and centrifuged at 98,000 × *g* for 16 hr at 4° (Spinco SW-41). The 1.26–1.27 (*E*), 1.24–1.25 (*F*), 1.14–1.20 (*G*), and <1.10 g/ml (*H*) density regions were then diluted in 0.01 M Tris·HCl, pelletted at 98,000 × *g* for 30 min at 4°, and assayed by the simultaneous detection test.

RNA-instructed DNA polymerase. The two "core" regions (Fig. 4*A* and *B*) were negative, indicating that cores are not present in this untreated milk sample. The remaining half of the "viral" region (Fig. 4*C*) was then treated with phospholipase C–ether and was again subjected to equilibrium density gradient analysis. The resulting density regions of 1.26–1.27, 1.24–1.25, 1.14–1.20, and <1.10 g/ml were then concentrated and assayed by the simultaneous detection test. As seen in Fig. 4*G*, the "viral" region (1.14–1.20 g/ml) that was positive before phospholipase–ether treatment was now negative. A positive response of 60–70S RNA and RNA-instructed DNA polymerase was now observed, however, in the 1.26–1.27 "core" region (Fig. 4*E*). In summary, concentrated human milk was shown to contain particles with 60–70S RNA and RNA-instructed DNA polymerase in the 1.14–1.20 g/ml density region. After treatment of this density region with phospholipase C–ether, the "viral" activity was shifted to the subviral or "core" density region, 1.26–1.27 g/ml.

Reduction of inhibitory factors

An advantage of the "core-isolation" technique in searching for particles with 60–70S RNA and RNA-instructed DNA polymerase is that it markedly reduces or eliminates inhibitors (possibly proteases, lipases, or nucleases) that may be present in some human milk preparations (3, 4, 26). To demonstrate this point, 2 ml of human milk concentrate, representing 160 ml of starting milk, was divided into two equal parts. One half was subjected to equilibrium density gradient analysis, and each density region of the gradient was concentrated and tested for particles with 60–70S RNA and RNA-instructed DNA polymerase by the simultaneous detection test. As can be seen in Fig. 5 (*open circles*), no activity was seen in any density region. This milk pool was assumed, therefore, to contain no particles, too few particles to detect by our assay, or to contain inhibitors of our assay. When the other half of the starting preparation, however, was first treated with phospholipase C–ether and then subjected to equilibrium gradient centrifugation, particles with 60–70S RNA and RNA-instructed DNA polymerase were detected in the "core" region, 1.26–1.27 g/ml (Fig. 5, *closed circles*). This conversion from a negative to a positive response as a result of phospholipase C–ether treatment was most probably due to shifting the viral cores away from a region containing factors (cell debris and associated enzymes) inhibitory to our assay.

TABLE 1. *Properties of the RNA-directed DNA polymerase of cores from human milk particles*

	^{3}H cpm in 60–70S region	Percent of complete reaction
Standard reaction mixture	872	100
+ RNase (25 μg/ml)	155	18
− dATP	125	14

Cores from 80 ml of human milk were purified and concentrated as described in *Methods*. The core pellet was resuspended in three 60-μl aliquots containing 0.01 M Tris·HCl, 0.2% NP-40, and 0.1 M dithiothreitol. The first aliquot was tested by the standard simultaneous detection test as described in *Methods*. The second aliquot was pretreated with RNase A (Sigma) at a concentration of 25 μl/ml. Deoxyadenosine triphosphate was omitted from the reaction mixture of the third aliquot. Results are given as ^{3}H cpm in the 60–70S region after the described glycerol velocity gradient analysis of reaction mixtures.

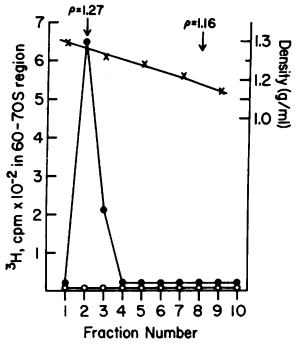

FIG. 5. Effect of generation of cores from human milk particles on the simultaneous detection of [^{3}H]DNA·60–70S RNA complexes. Several human milk samples (representing 160 ml of milk) were pooled and concentrated as described in *Methods*. One half of the pool concentrate was layered over 25–65% linear sucrose gradient and centrifuged at 98,000 × *g* for 4 hr at 4° (Spinco SW-41). 10 Equal fractions were collected from below, diluted in 0.01 M Tris·HCl, pelletted at 98,000 × *g* for 30 min at 4°, and assayed by the simultaneous detection test (*open circles*). The other half of the starting milk pool concentrate was treated with phospholipase C–ether (see *Methods*), and the aqueous phase was layered over a 25–65% linear sucrose gradient. After centrifugation at 98,000 × *g* for 16 hr at 4° (Spinco SW-41), 10 equal fractions were collected from below, diluted in 0.01 M Tris·HCl, pelletted at 98,000 × *g* for 30 min at 4°, and assayed by the simultaneous detection test. *Closed circles* represent [^{3}H]-DNA·60–70S RNA complexes found in each density region.

"Metado rapido" for core production and assay

A more rapid, but less definitive, method to isolate and assay putative viral cores is possible. Individual human milks were concentrated, treated with phospholipase C–ether as described, and layered over 0.4 ml of 25% sucrose on top of 1.5 ml of 42% sucrose in a Spinco SW-56 centrifuge tube. After centrifugation for 2 hr at 50,000 rpm at 4°, the resulting pellets were assayed by the simultaneous detection test with positive results for both RNA-instructed DNA polymerase and 60–70S RNA. Particles that go through a column of 42% sucrose must have a density of greater than 1.20 g/ml. Since positive results are obtained only after phospholipase C–ether treatment, one may assume that these particles with 60–70S RNA and RNA-instructed DNA polymerase are also putative viral cores. The equilibrium density gradient centrifugation experiments, although more tedious, are more diagnostic of "subviral" core activity since densities are defined.

DISCUSSION

These studies provide additional information leading to the conclusion that the particles found in human milk have many properties in common with known RNA tumor viruses of animals. The possession of an inner core, or nucleoid, with a density of 1.26–1.27 g/ml and containing 60–70S RNA and RNA-instructed DNA polymerase is diagnostic of this group of viruses. We have thus far refrained from calling these human milk particles viruses. In a recent report, however, Keydar *et al.* (8) have succeeded in the propagation of these particles in cultures of human embryo fibroblasts.

The use of lipoprotein solvents to generate and to characterize cores of RNA tumor viruses was first accomplished by O'Connor *et al.* (19) using the Moloney and Rauscher murine leukemia viruses. Bader *et al.* (16) used phospholipase to obtain cores at a low efficiency from Rous sarcoma virus. Bolognesi *et al.* (17) demonstrated that the core membrane could be disrupted with lipase or phospholipase A, but not with phospholipase C. In this connection, it is of interest to note that phospholipase C can be successfully used to yield enzymatically active cores from human particles.

It should be mentioned that the possibility still exists that phospholipase-C is not the active agent in core generation. For this reason, highly purified preparations of phospholipase C are being prepared and tested.

As shown in Fig. 5, core isolation is extremely useful in reducing or eliminating cell-associated debris and degradative enzymes (3, 26). Preliminary electron microscopic observations of human core preparations have revealed structures similar to the cores of mouse mammary tumor virus reported (23, 24).

The isolation and biochemical characterization of cores from human milk particles now makes available purified preparations of subviral components of putative human RNA tumor viruses.

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