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Evidence for Translation of Viral-Specific RNA in Cells of a Mouse Mammary Carcinoma

(RNA–DNA hybridization/[^{3}H]DNA/nuclear RNA/mRNA/polysomes)

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ABSTRACT A procedure is described permitting the detection of viral-specific RNA in a mouse mammary tumor. The method involves molecular hybridization with radioactively labeled DNA complementary to the RNA of the mouse mammary-tumor virus. RNA homologous to that of the mammary agent has been found in both the nuclear and polyribosomal fractions of tumor cells. The results imply that the oncogenic information is serving as messenger RNA that directs the synthesis of proteins required for virus production, and perhaps for the maintenance of the neoplastic state. The technology developed is immediately applicable to tumors of human origin.

Particles similar to the mouse mammary-tumor virus have been frequently observed in milk from women with familial histories of breast cancer (1). Further, it has been shown (2) that the particles of human origin exhibit RNA-dependent DNA polymerase ("reverse transcriptase") activity and possess the 70S RNA (manuscript in preparation) characteristic of the RNA tumor viruses of animals. These observations clearly warrant the performance of experiments designed to elucidate the possible significance of the virus-like particles to human breast cancer.

Ultimately, one would like to discover a susceptible animal in which the human milk particles induce the appearance of mammary tumors. However, it is impossible to predict how long it will take to attain this definitive experimental situation. For the present, other experimental alternatives must be explored to obtain relevant information.

An accessible approach would use RNA–DNA hybridization (3) to detect the presence of viral-specific RNA in human tumors. Analogous experiments have been performed with tissue culture cells with the oncogenic DNA viruses (4, 5). Similarly, viral-specific RNA has been detected (6) in both the nuclear and cytoplasmic fractions of mouse cells infected with the Moloney sarcoma–leukemia RNA-virus complex.

The demonstration by molecular hybridization of viral-specific RNA in tumor cells is clearly of great interest. However, more revealing information could be obtained by a search for viral RNA in the polysomes, the ribosome fraction actively engaged in protein synthesis. Since our ultimate aim is to apply these methods to human cancer, it was necessary to perfect the experimental details with actual tumor material, rather than with transformed cells in tissue culture.

In addition to the presence of similar particles in the milk, other parallels can be drawn between murine mammary tumor and human adenocarcinoma of the breast. The murine system was therefore adopted as an experimental model to develop procedures permitting the detection of viral-specific RNA in tumor tissue.

This paper details the feasibility of this approach and demonstrates that viral information can be located in the polysomal fraction of a mouse breast tumor. The results provide the technology required to examine tumors of human origin.

MATERIALS AND METHODS

Virus and Cells. Mouse mammary-tumor virus was obtained from milk of the Paris RIII strain of mice, which have a high incidence of mammary tumors (7), and purified as described (2, 8). The preparation and purification of Rauscher murine leukemia virus have been detailed (8).

Breast tumors were excised from tumor-bearing Paris RIII mice. Tumors with extensive areas of necrosis and those with large cystic areas were discarded. Normal breast tissue was obtained from tumor-free lactating C57 mice. Control samples of liver were excised from female C57 mice.

DNA Polymerase Reaction. Synthesis of [^{3}H]DNA homologous to mammary-tumor virus RNA was performed as follows: purified virus (1 mg of protein/ml final reaction) was incubated in 0.2% NP-40 detergent (Shell) with 80 μmol of dithiothreitol at 0°C for 10 min. The reaction mixture was then brought to a final volume of 1 ml by the addition of 50 μmol of Tris·HCl (pH 8.3), 10 μmol KCl, 8 μmol $MgCl_2$, 40 μmol (each) of dGTP, dCTP, dATP, 8 μmol of [^{3}H]dTTP × (5000 cpm/pmol). The reaction was then incubated for 120 min and stopped by the addition of 0.1 volume of 10% sodium dodecyl sulfate and 4 M NaCl. The [^{3}H]DNA product was purified by phenol extraction, followed by Sephadex G-50 chromatography to remove labeled nucleoside triphosphates and treatment with 0.4 M NaOH at 37°C for 18 hr to hydrolyze any viral RNA present.

Nucleic Acid Preparation. Nucleic acid was extracted from purified virions (7).

Nuclei were prepared from tumors and control tissue by the procedure of Busch *et al.* (9). Nuclear RNA was extracted by treatment of nuclei with DNase followed by extraction with phenol–chloroform–sodium dodecyl sulfate, by the procedure of Penman *et al.* (10).

For polysomes, tissue was disrupted with a Potter–Elvejhem homogenizer at 4°C in 2 volumes of 5% sucrose in TNM buffer (0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–2 mM $MgCl_2$).

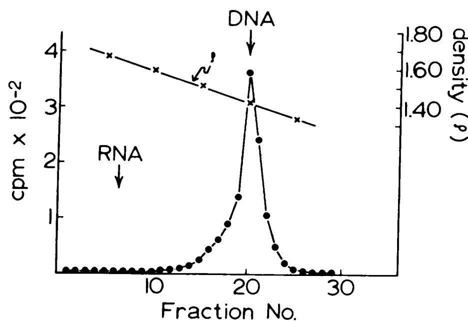

FIG. 1. Cs_2SO_4 equilibrium density-gradient centrifugation of the product of RNA-dependent DNA polymerase reaction of the mouse mammary-tumor virus. The DNA product was synthesized and purified. It was then dissolved in 5.4 ml of 5 mM EDTA, mixed with an equal volume of saturated Cs_2SO_4, and centrifuged at 44,000 rpm in a 50 Ti rotor (Beckman) for 60 hr at 20°C. 0.4-ml Fractions were collected and processed for acid-precipitable radioactivity (8).

The suspension was centrifuged at 15,000 × g for 40 min at 0°C. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM buffer and spun for 180 min at 180,000 × g in a Spinco 60 Ti rotor. The pellet (P-180) was resuspended in this buffer plus 1% sodium dodecyl sulfate, and the RNA was extracted twice with an equal volume of cresol-phenol (pH 8.4). Nucleic acid was precipitated from the aqueous phase by the addition of two volumes of ethanol and 0.1 volume of 4 M LiCl.

RNA-DNA Hybridizations. Purified [^{3}H]dTTP-labeled product (2000 cpm per reaction) was first incubated at 68°C for 10 min in 50% formamide to denature the DNA. After quick chilling of the solution to 0°C, the appropriate RNA was added and the hybridization mixture was brought to 0.4 M NaCl-50% formamide, in a total volume of 100 μl, and incubated for 18 hr at 37°C.

After incubation, the reaction mixture was added to 5.5 ml of 5 mM EDTA mixed with an equal volume of saturated Cs_2SO_4, to yield a starting density of 1.52, and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 20°C. 0.4-ml Fractions were collected and assayed (8) for Cl_3CCOOH-precipitable radioactivity.

RESULTS

We have shown (8) that the murine mammary-tumor virus contains a reverse transcriptase activity that can be used to generate radioactively labeled DNA. Before this DNA can be used as a probe for viral-specific RNA in tumor cells, it is important that it be adequately monitored for suitability in a hybridization test. In particular, it must be shown that it bands solely at the density of DNA in a Cs_2SO_4 gradient, and that it hybridizes to the homologous viral RNA and not to normal cellular RNA.

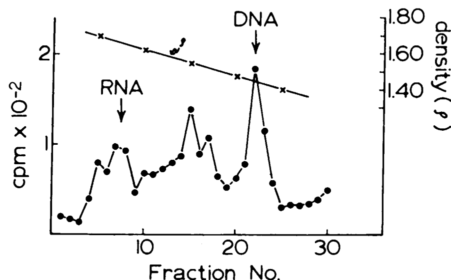

FIG. 2. Cs_2SO_4 equilibrium centrifugation of viral [^{3}H]DNA after annealing to purified mammary-tumor virus 70S RNA. The purified [^{3}H]DNA product (2000 cpm) was annealed to 2 μg of viral 70S RNA, as described in *Methods*. After annealing, the reaction was subjected to Cs_2SO_4 gradient centrifugation as described in the legend to Fig. 1.

Characterization of the DNA product

DNA was synthesized with a detergent-disrupted preparation of the murine mammary tumor virus. The DNA product, freed of protein and RNA (*Methods*), was examined by equilibrium centrifugation in Cs_2SO_4 with the results described in Fig. 1. It is evident that the [^{3}H]DNA synthesized bands in the expected region of the density gradient. It is important to emphasize that the alkali treatment must be rigorous enough to remove all the RNA present in the original reaction. Any residue of DNA-RNA hybrids formed during the reverse transcriptase reaction will make the product unusable as a probe for complementary RNA.

Fig. 2 shows the outcome of annealing the [^{3}H]DNA product to the 70S RNA of the murine mammary-tumor virus. As is usual in such hybridizations (8), about 50% of the DNA product is shifted to the RNA and hybrid regions of the density gradient. Except in very brief reactions not all of the DNA formed is hybridizable to the RNA, since DNA is synthesized that is identical, rather than complementary, to the template.

Comparison of Figs. 1 and 2 clearly shows that RNA molecules complementary to the [^{3}H]DNA product are readily detected by movement of the radioactive DNA towards the RNA region of the density gradient, due to the DNA-RNA hybrid complexes that form during the annealing reaction.

Detection of viral-specific RNA in mouse mammary tumors

As an initial step, it was decided to see whether viral-specific RNA could be detected in the nuclear and cytoplasmic fractions of tumor tissue. RNA was prepared from nuclei. After the removal of nuclei, the supernatant was subjected to 180,000 × g centrifugation through a 25% sucrose column, as described in *Methods*. The pellet, which would contain both monosomes and polysomes, was used as a source of what is designated as polysomal RNA.

Annealing reactions with the cytoplasmic-pellet fraction and the nuclear fraction gave results depicted in Figs. 3*A* and *B*. It is evident that both fractions contain RNA molecules complementary to [^{3}H]DNA from mammary-tumor virus. As a consequence of the annealing reaction, 30–35% of the [^{3}H]DNA has shifted to the regions of the gradient corresponding to RNA and hybrid density.

The specificity of the hybridization response

Several experiments were performed to examine the specificity of hybrid formation between polysomal RNA from tumor cells and [^{3}H]DNA from mammary-tumor virus. Fig. 4A demonstrates that polysomal RNA, prepared from normal mouse liver, exhibits no evidence of hybridization with mammary-tumor viral [^{3}H]DNA. Further, breast tumor polysomal RNA is unable to complex with [^{3}H]DNA homologous to the RNA of Rauscher leukemia virus, an oncogenic agent unrelated to the mouse mammary tumor virus (Fig. 4*B*).

Finally, the hybridizability of mammary-tumor viral [^{3}H]-DNA to breast-tumor polysomal RNA was compared with its ability to complex to polysomal RNA derived from normal breast tissue. The normal-polysome RNA was prepared from lactating C57 female mice, a strain in which mammary cancer is extremely rare. The results are shown in Fig. 5, in the form of a saturation curve. The polysomal RNA from the tumor tissue exhibits its usual ability to hybridize to tumor-virus DNA, with evidence of entering a saturation phase at about 100 μg. On the other hand, the polysomal RNA from normal breast shows no evidence of hybridizability to the same DNA within the concentration range examined.

The experiments just described support the conclusion that the reaction between breast-tumor polysomal RNA and viral DNA is specific. No complex formation is observed with polysomal RNA from normal tissue. In addition, DNA complementary to unrelated oncogenic RNA does not hybridize to polysomal RNA from mammary cancer tissue.

Localization of viral-specific RNA in the polysome fraction

The pellet (polysomal RNA) fraction used in the experiments thus far described contains both monosomes and polysomes, and was used because it is comparatively simple to prepare. We now describe experiments showing that the breast-tumor RNA hybridizable to viral DNA can be found in the polysomal fraction.

A high-speed pellet (P-180 of *Methods*) was subjected to centrifugation in a linear sucrose gradient to separate monosomes and polysomes, as shown in Fig. 6. The indicated polysomal and monosomal regions were pooled and diluted to a concentration of 10% sucrose with a 0.01 M Tris·HCl buffer containing 0.15 M NaCl plus 5 mM $MgCl_2$, then centrifuged for 12 hr at 150,000 $\times$ g. It should be noted that the diluting fluid used for the monosomes contained, in addition, 10 mM EDTA to release and eliminate fragments of messenger RNA still attached to monosomes or their subunits. The RNA in the polysomal and monosomal pellets thus obtained was purified as described in *Methods*.

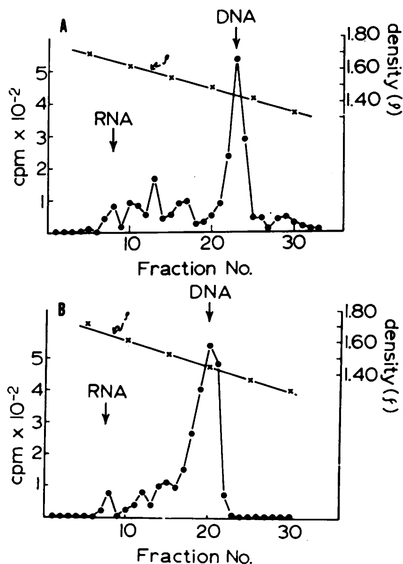

FIG. 3. Cs_2SO_4 equilibrium centrifugation of viral [^{3}H]DNA after annealing to nuclear and polysomal RNA from mouse mammary tumors. (*A*) Polysomal RNA and (*B*) nuclear RNA were isolated from mouse mammary tumors, and 250 μg was annealed to viral [^{3}H]DNA at 37°C for 18 hr. The reactions were then subjected to Cs_2SO_4 equilibrium centrifugation.

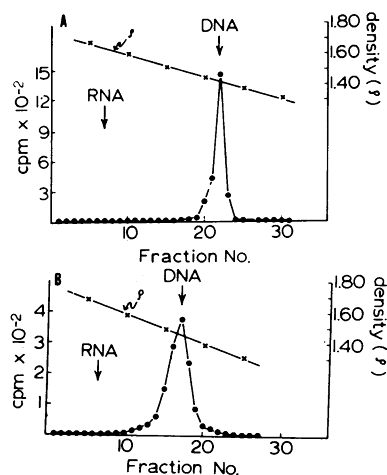

FIG. 4*A*. Cs_2SO_4 equilibrium centrifugation of viral [^{3}H]DNA after annealing it with mouse-liver polysomal RNA. Polysomal RNA was extracted from tumor-free female mice, and 250 μg was annealed to purified viral [^{3}H]DNA (4000 cpm). After annealing, the reaction was subjected to Cs_2SO_4 equilibrium centrifugation, and fractions were collected and analyzed.

4*B*. Cs_2SO_4 equilibrium centrifugation of Rauscher leukemia virus [^{3}H]DNA after annealing to mouse breast-tumor polysomal RNA. The virus was purified, and [^{3}H]DNA product was synthesized (8). 250 μg of mouse mammary-tumor polysomal RNA was then annealed to Rauscher leukemia viral [^{3}H]DNA (2000 cpm), and the reaction was subjected to Cs_2SO_4 equilibrium centrifugation.

Annealing reactions with the monosomal and polysomal RNAs gave the results shown in Fig. 6. It is clear (Fig. 6*A*) that the EDTA-treated monosomal fraction contains little or no RNA that can hybridize with viral [^{3}H]DNA. On the other hand, the polysomal RNA yields an excellent complex in the RNA region of the density gradient.

DISCUSSION

The experiments detecting RNA–DNA hybrid formation between mouse mammary-tumor viral DNA and mammary-

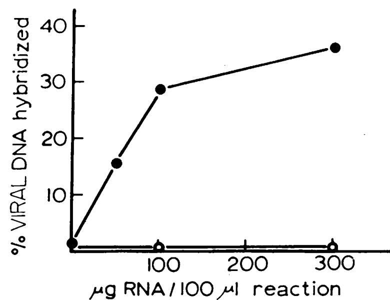

FIG. 5. Comparison of annealing reaction between mammary-tumor viral [^{3}H]DNA and mouse mammary-tumor polysomal RNA and normal-breast polysomal RNA. RIII mouse breast-tumor polysomal RNA and polysomal RNA from lactating breasts of tumor-free C57 mice were extracted and annealed to [^{3}H]DNA from mammary-tumor virus at various RNA concentrations. The individual annealing reactions were then analyzed by Cs_2SO_4 equilibrium centrifugation and the percent DNA hybridized was determined by the cpm of DNA sedimenting in the RNA and hybrid regions of the gradients. O—O normal polysomal RNA; ●—●, tumor polysomal RNA.

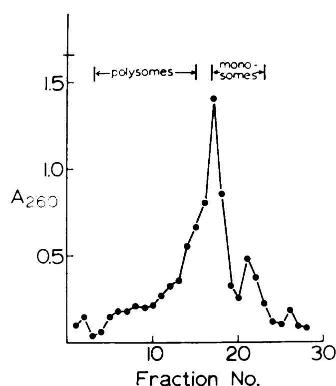

FIG. 6. Sucrose density-gradient centrifugation profile of RIII mouse breast-tumor polysomes. A polysomal fraction (P-180) was prepared from RIII mouse mammary tumor RNA, as described in *Methods*. The pellet was dissolved in 1 ml of TNM buffer, layered on a linear gradient of 7–47% sucrose in the same buffer, and spun at 26,000 rpm in a SW27 rotor at 4°C for 210 min. 1.2-ml Fractions were collected and monitored for absorbance at 260 nm.

tumor RNA clearly establishes the presence of viral-specific RNA in the nuclear and cytoplasmic fractions of tumor tissue. The negative response of breast tumor RNA to the unrelated DNA homologous to the Rauscher leukemia virus (Fig. 4*B*), and the positive reaction with murine mammary-tumor virus DNA (Fig. 3), support the conclusion that the annealing reaction is specific. This conclusion is further strengthened by the failure to find RNA complementary to viral DNA in similar fractions derived from normal liver (Fig. 4*A*) and normal breast tissue (Fig. 5).

The detection of viral-specific RNA in the polysome fraction of the mammary tumor itself lends credence to the supposition that at least some portion of the viral RNA is, in fact, serving as messenger RNA to direct the synthesis of proteins required for virus production, and perhaps for the maintenance of the neoplastic state. Further exploration of the latter speculation requires the examination of tumors that do not produce virus particles.

Molecular biological techniques have thus far been successfully used only with purified viruses and cells in tissue culture. The experiments described here demonstrate their applicability to actual tumor tissue. Because they provide us with the necessary technology to handle tumor specimens, the results have obvious implications for new experimental approaches to detect possible viral agents in human cancer.

It is now clearly technically feasible to obtain an experimental answer to the following question: "Can one find RNA molecules in the polysome fraction of a human tumor that is homologous to a putative human viral agent (e.g., the particle found in human milk) or to an analogous animal virus of proven oncogenic potential?" While a positive outcome would not constitute definitive proof, rather compelling evidence for a viral etiology would have been provided.

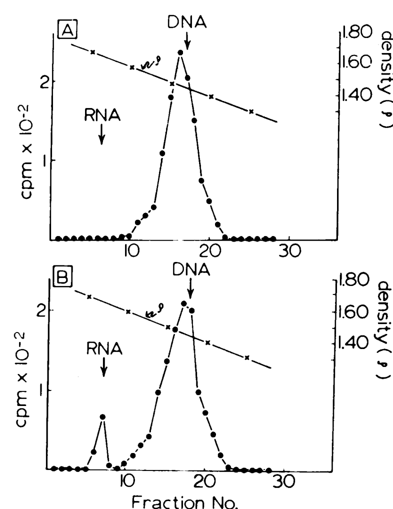

FIG. 7. Cs_2SO_4 equilibrium centrifugation of mammary-tumor viral [^{3}H]DNA after annealing to mouse mammary-tumor monosomal and polysomal RNA. Monosomes and polysomes were fractionated as described in the legend to Fig. 6. The indicated polysomal region was diluted to 10% sucrose with TNM buffer, while the monosomal region was diluted to 10% sucrose with 0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–10 mM EDTA. Both fractions were then centrifuged for 12 hr at 4°C in a 60 Ti rotor (Beckman) at 150,000 × *g*. RNA was purified from the monosomal and polysomal pellets. 250 μg of monosomal RNA (*A*) and polysomal RNA (*B*) were annealed to viral [^{3}H]DNA (2,000 cpm per reaction) and the results were analyzed by Cs_2SO_4 centrifugation.

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