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Detection of RNA-Instructed DNA Polymerase and High Molecular Weight RNA in Malignant Tissue

(70S RNA/mouse mammary carcinoma/oncornaviruses)

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ABSTRACT **An experimental procedure is detailed that permits the detection of 70S RNA-directed DNA synthesis in mouse mammary carcinomas. The DNA synthesized is complementary to the RNA of the mouse mammary tumor virus by molecular hybridization, thus, completing the proof that an RNA-instructed DNA polymerase has been identified. Further, RNA-instructed DNA polymerase and its 70S RNA template are physically associated in a particle that has a density characteristic of oncornaviruses. These experiments now provide the technology required to perform similar examinations of human neoplasias.**

Using the mouse mammary tumor as a model, we recently designed an experimental procedure that permits the detection of viral-specific RNA in cancerous tissue (1). The method depends on molecular hybridization between cytoplasmic RNA and radioactively labeled DNA synthesized with RNA-instructed DNA polymerase with the relevant viral RNA as a template. Hybrids are detected by equilibrium centrifugation in Cs_2SO_4 density gradients.

Extension of this technique to human malignancies revealed the existence, in breast carcinomas, of RNA possessing homology to that of mouse mammary tumor virus (MMTV) (2). This type of RNA was not detected in tissues derived from normal breasts or breasts with benign lesions. These observations gained further significance when we turned our attention to human neoplasias unrelated to breast cancer. We found that human leukemic cells (3), sarcomas (4), and lymphomas (5) contained RNA that was not homologous to MMTV-RNA but rather exhibited homology to the RNA of the murine leukemogenic agent, Rauscher leukemia virus (RLV). Thus, with all four human neoplasias examined, the pattern of specific viral-related RNA they contain mirrors precisely the experience accumulated with the corresponding murine malignancies, all of which are inducible by known and well characterized viral agents.

To further illuminate the etiologic implications of these findings for human cancer, it was necessary to perform experiments designed to ask the following questions concerning the viral-related RNA found in human tumors: (*a*) How large is the RNA being detected? (*b*) Is it associated with an RNA-instructed DNA polymerase? (*c*) Is it found in a particulate structure possessing an identifiable physical parameter characteristic of an RNA tumor virus?

Abbreviations: MMTV, mouse mammary tumor virus; RLV, Rauscher leukemia virus.

The experimental resolution of such questions was made feasible by the recent development (6) of a technique for the simultaneous detection in comparatively crude biological fluids of RNA-instructed DNA polymerase and high molecular weight (70S) RNA, the two identifying biochemical features of the oncornaviruses. This procedure was successfully used (7) for demonstration of the presence of 70S RNA and RNA-instructed DNA polymerase in particles present in human milk.

The present paper shows that this same general approach is applicable to tumor tissue. The data demonstrate the feasibility of identifying 70S RNA associated with an RNA-instructed DNA polymerase in extracts of a mouse mammary carcinoma. It was further possible to show that the enzyme and the RNA are both localized in a particle possessing the density characteristic of RNA tumor viruses. The results presented provide the technology required for a similar examination of human tumors.

MATERIALS AND METHODS

Virus and Cells. MMTV was obtained from milk of the Paris RIII strain of mice with high incidence of mammary tumors (8) and purified as described (9). The preparation and purification of RLV have been detailed (9).

Breast tumors were excised from tumor-bearing Paris RIII mice. Normal breast tissue was obtained from tumor-free, lactating NIH mice. Control samples of liver were excised from female NIH mice.

Assay of 70S RNA and DNA Polymerase. Tissue was finely minced and disrupted with a Potter–Elvehjem homogenizer at 4° in two volumes of TNE buffer [0.01 M Tris·HCl (pH 8.3)–0.15 M NaCl–0.01 M ethylenediamine tetraacetate (EDTA)]. The tissue homogenate was centrifuged at 4000 × *g* for 10 min at 0°, and the supernatant was centrifuged at 10,000 × *g* for 10 min. The resulting post-mitochondrial supernatant fluid was then layered on a 15-ml column of 20% glycerol in TNE and spun for 60 min at 100,000 × *g* and 4° in a Spinco SW27 rotor. The resulting pellet was suspended in 0.01 M Tris·HCl (pH 8.3) (50 μl of 0.01 M Tris per g of tumor tissue). Insoluble debris was removed from this suspension by centrifugation at 4000 × *g* for 10 min. The suspension (50 μl) was incubated at 0° in 0.33% Nonidet (NP-40) detergent (Shell Chemical Co.)–25 mM dithiothreitol, and then added to a standard reaction mixture of RNA-instructed DNA polymerase (125 μl final volume) containing 6.25 μmol of

Tris·HCl (pH 8.3)–1 μmol $MgCl_2$–1.25 μmol NaCl–0.2 μmol each of dGTP, dCTP, dATP, and 0.2 mCi of [^{3}H]TTP (50.1 Ci/mmol). After a 15-min incubation at 37°, the reaction was terminated by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.4 M and 1%, respectively. An equal volume of a phenol–cresol (7:1) mixture containing 8-hydroxyquinoline (3.7 g/100 ml of mixture) was added, and the final mixture was shaken at 25° for 5 min and centrifuged at 5000 × *g* for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10–30% in TNE) and centrifuged at 40,000 rpm for 210 min at 4° (Spinco SW41 rotor). External marker was ^{3}H-labeled 70S RNA from avian myeloblastosis virus. Fractions were collected from below, and portions were assayed for acid-precipitable radioactivity (9).

Nucleic Acid Preparations. Nucleic acid was extracted from purified virions as described (9).

For polysomes, tissue was disrupted with a Potter–Elvehjem homogenizer at 4° in 2 volumes of 5% sucrose in TNM buffer [0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–5 mM $MgCl_2$]. The suspension was centrifuged at 15,000 × *g* for 40 min at 0°. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM and spun for 180 min at 180,000 × *g* in a Spinco 60 Ti rotor. The pellet (P-180) was suspended in TNM and 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 2 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° for 10 min in 50% formamide to denature the DNA. After quick chilling to 0°, 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°.

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; the mixture was centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 20°. 0.4-ml Fractions were collected and assayed for Cl_3-CCOOH-precipitable radioactivity.

RESULTS

Detection and characterization of the 70S RNA–DNA complex

Attempts to detect RNA-instructed DNA polymerase and 70S RNA in tumor extracts will inevitably be complicated by the presence of cellular nucleases, polymerases, and RNA. The fractionation procedure developed was designed to minimize these sources of confusion by disruption of the cells in the presence of EDTA to destroy ribosomal structures and by the prior removal of nuclei and mitochondria. The resulting supernatant fluid was then layered on a 20% glycerol column and centrifuged at 100,000 × *g* for 1 hr, to yield a pellet (P-100) that should contain principally membrane structures and viral particles.

Assay of DNA synthesis by the P-100 fraction is preceded by prior incubation at 0° for 10 min in the presence of 0.3% NP-40. A standard RNA-instructed DNA polymerase reaction (see *Methods*) is then performed, monitored by the incorporation of [^{3}H]TTP into acid-insoluble DNA. After incubation at 37° for 15 min, the nucleic acids are separated from the proteins in the reaction mixture and subjected to sedimentation analysis in a glycerol gradient. A distinct peak of acid-precipitable [^{3}H]TTP is observed (Fig. 1*A*) with a sedimentation coefficient of 70 S. When the pellet (P-100) obtained from mouse mammary carcinoma extracts is treated with ribonuclease after disruption by NP-40, no evidence of activity is present in the 70S region of the gradient (Fig. 1*B*). Further, when an equivalent quantity of normal lactating breast tissue from tumor-free NIH Swiss mice is processed to yield similar P-100 fraction, it exhibits no ability to incorporate [^{3}H]TTP into rapidly sedimenting structures (Fig. 2).

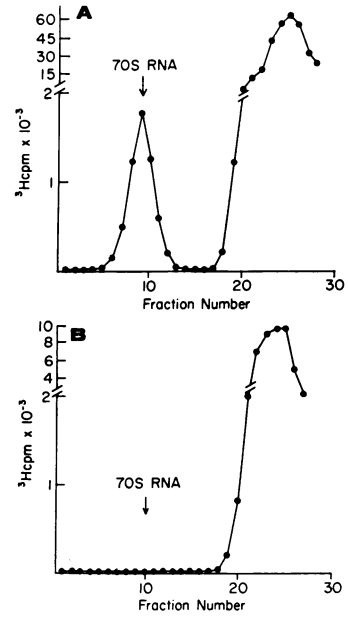

FIG. 1. Detection of 70S RNA–[^{3}H]DNA complex in mouse mammary carcinoma tissue. (*A*) A standard 125-μl RNA-instructed DNA polymerase reaction was performed on the P-100 fraction derived from a mouse mammary tumor (see *Methods*). After incubation for 15 min at 37°, the nucleic acid complex was extracted with phenol–cresol (pH 8.6) and the aqeuous phase was layered over a linear glycerol gradient (10–30% glycerol in TNE buffer) and centrifuged for 210 min (4°) at 40,000 rpm in a Spinco SW41 rotor. 0.4-ml Fractions were collected and assayed for acid-precipitable radioactivity. (*B*) After disruption by detergent, the P-100 fraction derived from mouse mammary tumor was incubated in the presence of RNase A (50 μg/ml) and RNase 1 (50 μg/ml) for 15 min at 25°. A standard RNA-instructed DNA polymerase reaction was then performed as described above.

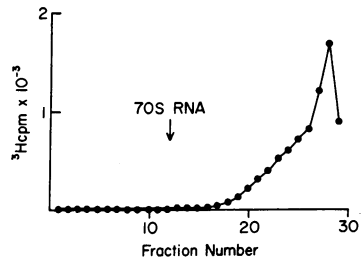

FIG. 2. Assay for 70S RNA–[^{3}H]DNA complex in lactating breast tissue. 5 g of lactating breast tissue from tumor-free, lactating NIH mice were processed for 70S RNA–[^{3}H]DNA as described in the legend to Fig. 1.

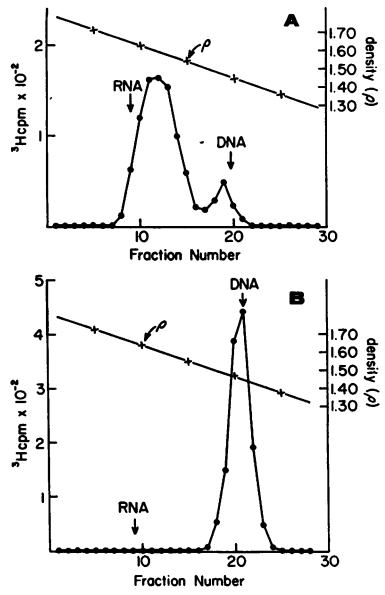

FIG. 3. Centrifugation of R-III-[^{3}H]DNA in Cs_2SO_4 equilibrium density gradient after annealing to purified MMTV and RLV 70S RNA. A standard RNA-instructed DNA polymerase reaction was performed with the P-100 fraction from mouse mammary tumors. 70S RNA–[^{3}H]DNA was obtained after velocity centrifugation and digested with 0.4 M NaOH at 37° for 18 hr to remove all RNA present. The [^{3}H]DNA product (R-III-[^{3}H]-DNA) was then annealed to (*A*) 0.2 μg of MMTV 70S RNA and to (*B*) 0.2 μg of RLV 70S RNA. After annealing, the reaction was subjected to Cs_2SO_4 gradient centrifugation as described in *Methods*.

When the rapidly sedimenting (70S) [^{3}H]DNA complex synthesized by mouse mammary tumor extract is centrifuged in a Cs_2SO_4 equilibrium density gradient, all of the [^{3}H]DNA synthesized bands at the density of RNA (1.64–1.68 g/ml). When this 70S complex is denatured by heating to 98° for 10 min to eliminate hydrogen bonding, all of the [^{3}H]DNA now bands at the density of DNA (1.44 g/ml).

Proof of RNA-instructed DNA polymerase by back hybridization to putative template

The response to ribonuclease and the behaviors in glycerol and Cs_2SO_4 gradients before and after heat denaturation all serve to demonstrate a ribonuclease-sensitive synthesis of an RNA–[^{3}H]DNA complex with a sedimentation coefficient of 70 S. These findings, however, do not yet establish that an RNA-instructed DNA polymerase has been identified in the extracts of tumor cells. Rigorous proof requires a demonstration that the DNA synthesized can back hybridize to the putative template, which in the present case is the 70S RNA of MMTV. To this end, a standard RNA-instructed DNA polymerase reaction was performed with the P-100 fraction from mouse mammary tumors. After velocity centrifugation analysis of the reaction products, the [^{3}H]DNA sedimenting in the 70S region of the glycerol gradient was pooled and precipitated with ethanol. The resulting nucleic acid pellet was then subjected to extensive alkali digestion to remove all RNA present.

Fig. 3*A* shows a Cs_2SO_4 equilibrium gradient analysis of the outcome of an annealing reaction between this [^{3}H]DNA product (R-III-[^{3}H]DNA) and 70S RNA of MMTV isolated from the milk of Paris R-III mice. It is evident that about 85% of the DNA product is shifted from the DNA region to the RNA and hybrid region of the gradient due to DNA–RNA hybrid complexes that form during the annealing reaction. When this R-III-[^{3}H]DNA is annealed with an equivalent amount of 70S RNA isolated from RLV, no evidence of hybrid formation is detected, with all of the R-III-[^{3}H]DNA remaining in the DNA region of the gradient (Fig. 3*B*).

When hybridization is extensive, a convenient method for following the process kinetically is hydroxyapatite chromatography at room temperature (22–24°) in the presence of 50% formamide (manuscript in preparation). Fig. 4 shows such kinetic analyses of annealing reactions between R-III-[^{3}H]-DNA and 70S RNA from MMTV and RLV. While MMTV RNA reaches a saturation level of 90% hybrid structure by 9 hr, no evidence of hybrid formation is noted with RLV RNA.

Use of [^{3}H]DNA synthesized with tumor fraction as a probe for RNA in mammary tumor

We have shown (1) that MMTV [^{3}H]DNA synthesized by MMTV isolated from the milk of Paris R-III mice can be used as a molecular probe to detect the presence of viral-specific RNA in mouse mammary tumor. It was therefore of interest to determine whether the R-III-[^{3}H]DNA synthesized by mammary tumor extracts could serve as a similar probe for the detection of MMTV RNA in tumor tissue. Fig. 5*A* shows the outcome of an annealing reaction with R-III-[^{3}H]DNA and polysomal RNA (see *Methods*) isolated from mouse breast tumors. As a consequence of the annealing reaction, 70% of the [^{3}H]DNA has shifted to the RNA and hybrid region of the gradient. When a similar polysomal RNA fraction obtained from the livers of tumor-free NIH mice is annealed with R-III-[^{3}H]DNA, no evidence of hybrid formation is observed (Fig. 5*B*).

Localization of RNA-instructed DNA polymerase and viral RNA in a density gradient

The very fact that the P-100 fraction yields a 70S RNA–[^{3}H]-DNA complex in an endogenous reaction already implies that the RNA-instructed DNA polymerase must be associated with the template it uses. It was of interest to see whether this association could be identified with a particulate element possessing a density characteristic of an RNA tumor virus. At the same time, one could determine whether all the viral-related RNA was confined to one particular density fraction.

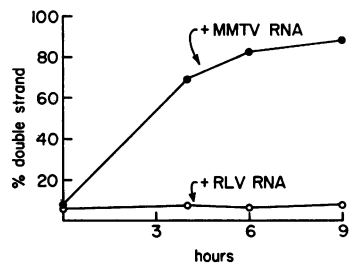

FIG. 4. Kinetics of annealing reaction between R-III-[^{3}H]-DNA and purified 70S RNA from MMTV and RLV. R-III-[^{3}H]-DNA was annealed to 0.2 μg of MMTV 70S RNA (●——●) and RLV 70S RNA (O—O). At various time intervals aliquots were removed and subjected to analysis by hydroxyapatite chromatography at room temperature in the presence of 50% formamide.

An experiment was therefore designed to permit the simultaneous detection of 70S RNA and RNA-instructed DNA polymerase and the independent identification of viral-specific RNA by molecular hybridization in a tumor extract subjected to density fractionation. A P-180 fraction was prepared from mouse breast tumor tissue in buffers that contain Mg^{++} to preserve the integrity of the polyribosomes. The pellet fraction was then centrifuged in sucrose density gradients to permit equilibrium banding of viral particles and velocity sedimentation of polyribosomes. The absorbance profile (A_{260}) of this density gradient is shown in Fig. 6. The gradient was then divided into 10 fractions. Half of each fraction was pelletted to assay for the presence of RNA-instructed DNA polymerase activity, whereas RNA was extracted from the other half for analysis by annealing reactions.

Fig. 6*A* shows the amount of [^{3}H]DNA cpm sedimenting in the 70S region of a glycerol velocity gradient after the performance of an RNA-instructed DNA polymerase reaction on each of the ten sucrose fractions. These data show that the activity of RNA-instructed DNA polymerase and 70S RNA present in the P-180 fraction of the tumor extracts localizes at a density between 1.16 and 1.19 g/ml, the density characteristic of the oncogenic RNA viruses. Fig. 6*B* shows the outcome of annealing reactions between MMTV [^{3}H]DNA and RNA extracted from an equal volume of each gradient fraction. As expected, maximum viral-specific RNA is detected at a density of 1.17 g/ml, coincident with the density of intact virions. However, it will be noted that significant viral-specific RNA is detected at densities where no viral activity is found. Whether this represents viral RNA associated with polyribosomes or some other structure cannot be determined without further investigation.

DISCUSSION

Until a human viral agent is isolated and a susceptible experimental animal is identified, definitive proof of a viral etiology

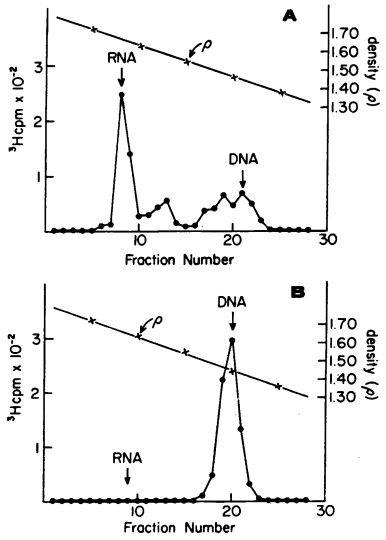

FIG. 5. Centrifugation of R-III-[^{3}H]DNA in Cs_2SO_4 equilibrium density gradients after annealing to polysomal RNA from R-III breast tumor and NIH mouse liver. Polysomal RNA was isolated from mouse mammary carcinomas (*A*), and NIH mouse liver (*B*); 100 μg of polysomal RNA from each tissue was annealed to R-III-[^{3}H]DNA. After annealing, the reaction mixtures were centrifuged in Cs_2SO_4 equilibrium gradients.

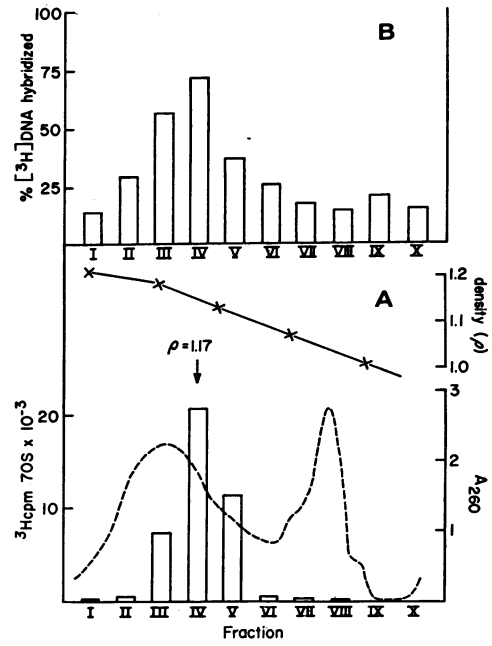

FIG. 6. Localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of mouse mammary tumors on sucrose gradients. A polysomal fraction (P-180) was prepared from mouse mammary tumors (see *Methods*). The pellet was suspended in TNM buffer and layered on a linear gradient of 7–47% sucrose in TNM buffer and spun at 25,000 rpm in a SW-27 rotor at 4° for 195 min. The gradient was dripped from below, and absorbance at 260 nm (– – –) was monitored in the flow-through cuvette of the Gilford 2400S recording spectrophotometer. 10 Equal fractions were collected and divided into two aliquots: one for assay of RNA-instructed DNA polymerase and the other for RNA extraction. The amount of 70S [^{3}H]DNA synthesized by an RNA-instructed DNA polymerase reaction performed on each fraction was determined by glycerol velocity centrifugation (*A*). RNA obtained from an equal volume of each of the 10 fractions was then annealed to R-III-[^{3}H]DNA. The annealing reactions were then analyzed by Cs_2SO_4 density centrifugation (*B*).

of human cancer will not be obtained by satisfying Koch's postulates. In the interim, one can circumvent these technical limitations by translating these postulates into their modern biochemical equivalents in the hope that the accumulated technology of molecular biology will permit the performance of informative experiments. Thus, in modern terminology, Koch's first postulate asks the following question: Does malignant tumor contain viral-related information not found in corresponding normal tissue?

To answer these and related questions, we initiated a series of experiments that culminated in the demonstration of the appropriate viral-specific RNA in human breast carcinomas (2), leukemias (3), sarcomas (4), and lymphomas (5). The significance of these findings for a viral etiology of human neoplasia depends to a large extent on the size of these viral-specific RNAs and whether they are associated with particulate elements possessing the biochemical and physical properties characteristic of the RNA tumor viruses. The aim of the present study was to devise the techniques necessary to provide data relevant to these issues.

The results described show that it is possible to identify 70S RNA-directed DNA synthesis in murine mammary tumor

tissue. Further, it was demonstrated that the RNA-instructed DNA polymerase and its 70S RNA template are physically associated in a particle possessing the density (1.17 g/ml) characteristic of the oncornaviruses. Finally, it was independently demonstrated, by molecular hybridization, that a large percentage of the viral-specific RNA present in the mouse mammary tumor is found in a particle of the same density.

It is evident that the experiments described here provide the technology required to perform similar examinations on tumors of human origin.

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