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Particles Containing RNA-Instructed DNA Polymerase and Virus-Related RNA in Human Breast Cancers

(70S RNA/mouse mammary tumor virus/hybridization)

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ABSTRACT **Human breast cancers contain an RNA related to that of mouse mammary tumor virus. In 79% of the breast malignancies examined, this type of RNA is a 70S-component encapsulated with RNA-instructed DNA polymerase in a particle possessing the density characteristics of RNA tumor viruses. Further, the DNA synthesized by the human RNA enzyme complex hybridizes specifically with the RNA of mouse mammary tumor virus. Thus, four features diagnostic of agents similar to mouse mammary tumor virus are also exhibited by a particle found with high frequency in human breast cancers. The accumulating evidence for the involvement of RNA tumor viruses in at least some human neoplasias is becoming increasingly compelling.**

Human breast adenocarcinomas have been shown (1) by molecular hybridization (2, 3) to contain RNA possessing sequences homologous to those of mouse mammary tumor virus (MMTV). This type of RNA could not be detected in normal tissue or in samples derived from such benign conditions as fibrocystic disease or fibroadenomas. The breast cancer studies were paralleled by similar hybridizations with RNA from human leukemias (4), sarcomas (5), and lymphomas (6). These experiments culminated in the demonstration that all of these human malignancies contained an RNA related to that of Rauscher leukemia virus, an agent belonging to a group of viruses known to cause similar neoplasias in mice.

The presence of the appropriate virus-related RNA in the corresponding human tumors supports, but does not establish, the causative involvement of a viral agent in these diseases. The RNA molecules detected could represent transcripts of DNA segments possessing some sequences in common with those of the homologous murine viruses. Any attempt to decipher the etiologic implications of these findings for human cancer requires the performance of experiments designed to answer the following questions concerning the virus-related RNA found in human breast cancer: (*a*) How large is the RNA being detected? (*b*) Is it associated with an RNA-instructed DNA polymerase (7, 8)? (*c*) Are the two found in a structure possessing a known physical characteristic of an RNA tumor virus? Finally, if a large RNA is found in association with a RNA-instructed DNA polymerase (reverse transcriptase), is the DNA synthesized by the two in concert complementary to the RNA of the corresponding tumor virus?

The experimental resolution of these questions was made feasible by the development (9) of a technique for the simultaneous detection in biological fluids of RNA-instructed DNA polymerase and high molecular weight (70S) RNA, two diagnostic features of the RNA tumor viruses. This procedure was used to reveal (10) the presence of 70S RNA and RNA-instructed DNA polymerase in human milk particles.

The detailed procedure required to apply these techniques to tissue extracts was evolved with mouse mammary tumors (11) as a model system. The method has been successfully applied (12) to demonstrate complexes of 70S RNA and RNA-instructed DNA polymerase in leukocytes from the peripheral blood of 95% of leukemic patients examined.

We report here the identification of a 70S-RNA-instructed DNA polymerase in extracts of human breast adenocarcinoma. We show further that the enzyme and its RNA template are localized in a particle possessing a density characteristic of the RNA tumor viruses and that the DNA synthesized hybridizes specifically to the RNA of MMTV.

METHODS AND RESULTS

Detection of 70S RNA-instructed DNA polymerase in tissue extracts

Attempts to find RNA-instructed DNA polymerase associated with 70S RNA in tissue extracts are inevitably complicated by the presence of cellular nucleases, other polymerases, and massive amounts of ribosomes. The fractionation method developed (11) was designed to minimize these sources of confusion. Possible virus particles were enriched by disruption of the cells in the presence of EDTA to destroy ribosomal structures and by the prior removal of nuclei and mitochondria.

The detailed procedures for the isolation and assay of the P-100 pellet fraction are described in the legend to Fig. 1. After 15 min of synthesis at 15°, the reaction mixture is deproteinized and subjected to sedimentation analysis in glycerol gradients with external size markers. Fig. 1*A* shows a typical result observed with a P-100 fraction prepared from a human breast carcinoma. The DNA synthesized is seen traveling with a sedimentation coefficient of 70 S. Occasionally, peaks at the positions of 52 S and 35 S, as shown in Fig. 1*B*, are also observed. When an equivalent quantity of either fibroadenoma or normal human breast tissue is processed to yield an identical P-100 fraction, no incorporation of [^{3}H]TTP into a rapidly sedimenting structure is detectable (Figs. 1*C* and 1*D*).

Fig. 2 demonstrates that the 70S DNA complex observed with P-100 preparation from a malignant tumor is due to an

Abbreviation: MMTV, mouse mammary tumor virus.

TABLE 1. *Test for 70S RNA–[³H]DNA in human breast tissues*

Tissue	cpm 70S	Reaction
Malignant adenocarcinoma		
17-28-FSP	584	+
40-22-FSP	3010	+
40-35-FSP	500	+
17-39-FSP	30	−
25-7-FSP	36	+
40-19-FSP	1256	+
9-141-FSP	156	+
Pool A	271	+
26-4-FSP	6	−
26-2-FSP	632	+
Pool B	205	+
9-163-FSP	880	+
383-FSP	484	+
14-37-FSP	210	+
17-34-FSP	26	−
17-32-FSP	36	+
9-131-FSP	0	−
38-2-FSP	131	+
26-8-FSP	335	+
26-9-FSP	17	−
T255T	308	+
40-42-FSP	485	+
38-4-FSP	22	−
40-24-FSP	776	+
Pool C	43	+
37-108-FSP	550	+
C22482	113	+
40-38-FSP	34	−
T310T	655	+
RMSK-1	206	+
T287T	19	−
MHSK	1032	+
38-10-FSP	1912	+
756-5-72	2508	+
38-12-FSP	875	+
24-53-FSP	785	+
25-57-FSP	2270	+
25-22-FSP	76	+
	Average = 540 cpm	Average of positives = 668 cpm Average of negatives = 20 cpm
Normal breast		
C14701	10	−
62-19-17	20	−
FED-SK	0	−
AS-SK	4	−
	Average = 8 cpm	
Fibroadenoma		
14-31-FSP	6	−
7-24-FSP	0	−
F-1	0	−
MA-SK	6	−
JR-SK	7	−
F-2	21	−
	Average = 7 cpm	

Preparation of P-100 fractions and assay for synthesis of [³H]-DNA–70S RNA complex are as described in the legend to Fig. 1.

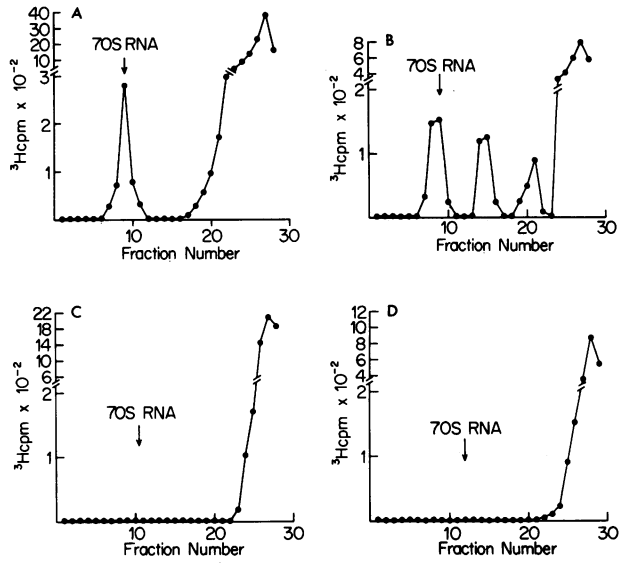

FIG. 1. Simultaneous detection of 70S RNA–[³H]DNA complex in human breast cancer tissue. (*A*) and (*B*) Human breast tumor, adenocarcinomas 38-3-FSP and 40-19-FSP, respectively, (*C*) fibroadenoma of the breast (17-24-FSP), and (*D*) normal breast (FE-1). 8 g of human breast tissue was finely minced and disrupted with a Silverson homogenizer at 4° in 0.01 M Tris·HCl (pH 8.3)–0.15 M NaCl–0.01 M EDTA. The suspension was centrifuged at 4000 × *g* for 10 min at 0°, and the supernatant was centrifuged again at 10,000 X *g* for 10 min. The resulting supernatant fluid was then layered on a 15-ml column of 20% glycerol in Tris–NaCl–EDTA buffer and spun at 98,000 × *g* for 1 hr at 4° in an SW27 rotor (Spinco). The resulting pellet was suspended in 0.01 M Tris·HCl (pH 8.3) (50 μl of 0.01 M Tris·HCl/g of tumor tissue). Insoluble debris was removed from this suspension by centrifugation at 4000 × *g* for 10 min. The suspension (50 μl) was preincubated at 0° in 0.33% NP-40 detergent (Shell)–25 mM dithiothreitol, then added to a standard endogenous RNA-instructed DNA polymerase reaction mixture (125 μl final volume) containing 6.25 μmol Tris·HCl (pH 8.3), 1 μmol of $MgCl_2$, 1.25 μmol of NaCl, 0.2 μmol each of dGTP, dCTP, dATP, and 0.2 mCi [³H]-TTP (50 Ci/mmol). After 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 (0.2 g/100 ml) of mixture was added and the final mixture was shaken at 25° for 5 min. The aqueous phase was then layered over a linear glycerol gradient (10–30% in Tris–NaCl-EDTA buffer) and centrifuged at 40,000 rpm for 210 min at 4° (Spinco SW41 rotor). External marker was [³H]70S RNA from avian myeloblastosis virus. Fractions were collected from below, and portions were assayed for acid-precipitable radioactivity.

RNA-dependent reaction, since prior treatment with RNase eliminates the DNA–RNA complex.

Table 1 summarizes our findings with 38 adenocarcinomas and 10 nonmalignant controls. P-100 fractions were prepared and assayed at the same input levels for the ability to synthesize a 70S RNA–DNA complex as described in the legend to Fig. 1. The cpm in the 70S region of the glycerol gradient was taken as a measure of presence and the extent of the reaction. A background of 12 cpm was subtracted in all

The sum of the cpm in the 70S position, monitored by external size markers, is recorded, and corrected for a background of 12 cpm. A reaction is designated as positive if the cpm exceed 35.

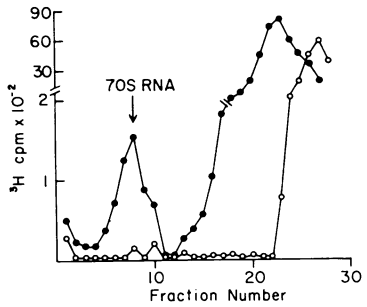

Fig. 2. Effect of ribonuclease on the detection of the high-molecular-weight RNA–[^{3}H]DNA complex. Breast tumor tissue (adenocarcinoma 756-5-72) was processed as described in the legend to Fig. 1. The viral pellet (P-100) was suspended in 0.01 M Tris·HCl (pH 8.3) and divided into two equal parts. A standard RNA-instructed DNA polymerase reaction was performed on one part of the P-100 fraction; after incubation for 15 min at 37°, the nucleic acid complex was extracted with phenol–cresol and was sized on a 30–10% linear glycerol gradient (●——●). After disruption with detergent, the other half of the P-100 fraction was incubated in the presence of RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 25°. A standard RNA-instructed DNA polymerase reaction was then performed (○——○).

cases. The average cpm in the 70S region for the control series was 8 cpm for normal tissues, and 7 cpm for benign fibroadenomas. In contrast, the malignant tissues yielded an average of 540 cpm in the 70S region. In view of the background count and the low values of the controls, we have assigned positive diagnosis to any reaction yielding 35 or more cpm in the 70S region. On this basis, the control samples were all negative and 79% of the malignant samples were positive, with many giving hundreds, and some thousands, of cpm in the 70S region.

Characterization of the [^{3}H]DNA product

The behavior in glycerol gradients and the response to ribonuclease demonstrate the ribonuclease-sensitive synthesis of an RNA–[^{3}H]DNA complex with a sedimentation coefficient of 70 S in extracts of human breast carcinoma. Definitive proof of the presence of RNA-instructed DNA polymerase in human tumor extracts requires evidence that the rapidly sedimenting DNA synthesized is hybridizable to its presumed RNA template.

There are two ways of examining this question. One is to challenge the DNA to hybridize with RNA isolated from P-100 fractions derived from malignant and nonmalignant tissues. Presumably, only breast carcinomas should contain the putative template and possess RNA that can hybridize with the synthetic DNA. The other method is to use MMTV-RNA and another unrelated oncogenic RNA (e.g., Rauscher leukemia virus-RNA) in annealing reactions. Again, if the DNA synthesized by the P-100 fraction from breast cancer is instructed by an RNA related to that of the MMTV, hybridization should occur with the MMTV-RNA and not with the Rauscher leukemia virus-RNA.

RNA was prepared from malignant and nonmalignant breast tissue by layering a postmitochondrial supernatant on a discontinuous column of 25 and 50% sucrose. After centrifugation at 180,000 × *g* for 4 hr, the RNA was extracted from the material sedimenting through the 25% sucrose column, but remaining on the 50% cushion. RNA prepared in this way from extracts of murine mammary carcinoma show a significant enrichment for viral-specific RNA (unpublished observations).

Rapidly sedimenting human [^{3}H]DNA product was prepared by reaction of an endogenous RNA-instructed DNA polymerase with the P-100 fraction from human breast tumors. After velocity centrifugation analysis of the reaction product, the [^{3}H]DNA sedimenting in the 70S region of the glycerol gradient was pooled and precipitated with ethyl alcohol. The resulting nucleic acid pellet was then subjected to extensive alkali digestion to destroy all RNA present, and the DNA was recovered.

Fig. 3*A* shows a Cs_2SO_4 equilibrium gradient profile of an annealing reaction between human [^{3}H]DNA product synthesized by the P-100 fraction of a breast tumor (no. 756-5-72 of Table 1) and viral-enriched RNA isolated from the same tumor. It is clear that about 20% of the [^{3}H]DNA is shifted from the DNA region to the hybrid and RNA regions of the gradient due to formation of RNA–DNA hybrid structures. When the same human [^{3}H]DNA is annealed with an equivalent amount of RNA from the P-100 fraction from a breast fibroadenoma (Fig. 1), no evidence of hybrid formation is seen (Fig. 3*B*); all of the [^{3}H]DNA bands in the DNA region of the gradient. Similar negative outcomes were obtained with RNA from the P-100 fraction from two other fibroadenomas and with one from normal gastrointestinal epithelium.

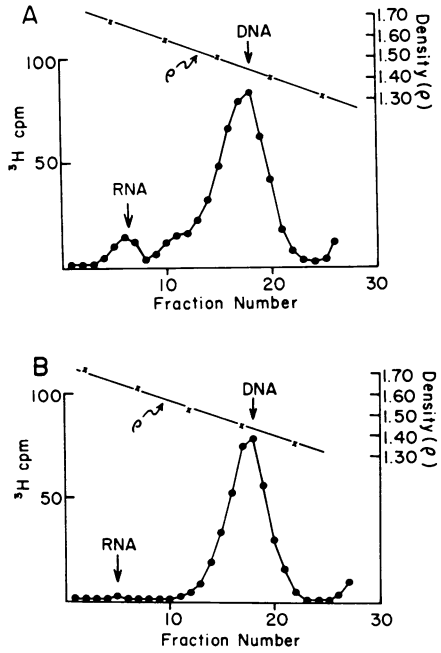

Fig. 3. Cs_2SO_4 equilibrium density gradient centrifugation of annealing reactions of human breast tumor (756-5-72) [^{3}H]DNA to (*A*) human breast tumor (756-5-72) RNA and (*B*) fibroadenoma (F-1) RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legend to Fig. 1. The 70S RNA–[^{3}H]DNA complex obtained after velocity centrifugation was digested with 0.4 M NaOH for 18 hr at 37° to remove all RNA present. The [^{3}H]DNA product was then annealed to 100 μg of RNA enriched in viral sequences (see text). The hybridization reaction (50 μl) was performed in the presence of 50% formamide and 0.4 M NaCl. After annealing for 24 hr at 37°, the reaction mixture was subjected to Cs_2SO_4 gradient centrifugation as described (11).

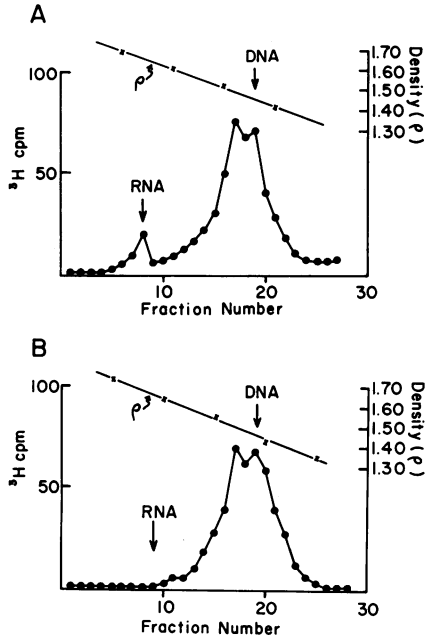

FIG. 4. Annealing reaction of breast tumor (40-19-FSP) [^{3}H]DNA to 4 μg of 70S RNA from (*A*) MMTV and (*B*) Rauscher leukemia virus. The [^{3}H]DNA was obtained and hybridized as described in Fig. 3. The annealing reactions were analyzed by Cs_2SO_4 equilibrium gradient centrifugation.

It is worth noting that the [^{3}H]DNA formed by the 756-5-72 tumor yielded significant hybrid formation not only with RNA obtained from the homologous tumor, but also with RNA prepared from the two other breast tumors tested as well (6.3% with 38-12-FSP and 12.3% with 24-53-FSP).

We have shown (1) that RNA isolated from human mammary tumors shows homology to MMTV-RNA. It was therefore of obvious interest to determine whether the rapidly sedimenting [^{3}H]DNA synthesized by extracts of human adenocarcinoma of the breast shows any evidence of complementarity to 70S RNA isolated from the MMTV. Fig. 4*A* shows the result of an annealing reaction between rapidly sedimenting [^{3}H]DNA from human breast tumor (40-19-FSP, see Fig. 1*B*) and MMTV-70S RNA. As a result of the annealing reaction, 15% of the [^{3}H]DNA has shifted to the RNA and hybrid region of the gradient. When an equivalent amount of 70S Rauscher leukemia virus-RNA is annealed to the same [^{3}H]DNA, no significant shift into the RNA and hybrid regions is observed.

Density of the particle containing 70S RNA and RNA-instructed DNA polymerase

The data thus far described indicate that human mammary carcinomas contain particles that encapsulate RNA-instructed DNA polymerase and a 70S RNA related to that of MMTV. It was of obvious interest to see whether this particle possessed the density characteristic of an RNA tumor virus. To this end, a P-100 fraction was prepared from a human breast tumor and subjected to sucrose equilibrium centrifugation on a linear gradient of 25–50% sucrose. The gradient was then divided into 10 equal fractions that were diluted to 15% sucrose and again spun at 100,000 × *g* for 1 hr.

Pellets from each fraction were tested simultaneously to determine the distribution in the density gradient of 70S RNA-instructed DNA synthesizing activity. It can be seen from Fig. 5 that the particles possessing the RNA-instructed DNA polymerase and its 70S RNA template localize at a density between 1.16 and 1.19 g/ml, the density characteristic of oncogenic RNA viruses.

DISCUSSION

The experiments reported were performed in the hope that they would provide more information about the etiologic significance of our earlier (1) findings in human breast cancer of RNA uniquely homologous to that of the MMTV. The data obtained here show that at least a portion of the virus-related RNA we were detecting in the human tumors is in the form of a 70S RNA physically associated with an RNA-instructed DNA polymerase in a particle having a density between 1.16 and 1.19 g/ml. The particles thus identified in human breast cancers possess three of the biochemical and physical features diagnostic of the RNA tumor viruses.

Of interest was the demonstration with the P-100 preparations derived from human adenocarcinomas that DNA synthesized by the RNA-instructed DNA polymerase on its own endogenous RNA template hybridizes specifically to RNA from malignant tumors (Fig. 3) and to MMTV-RNA (Fig. 4*A*). The lack of response to Rauscher leukemia virus-RNA (Fig. 4*B*) eliminates the possibility that complexing is due to the poly(A) stretches recently found in RNA tumor viruses (13–15). It should be noted that the successful specific hybridization to MMTV-RNA by the DNA synthesized by the tumor enzyme and its RNA template is complementary to and completes the logic of our earlier experiments (1) in which DNA synthesized on MMTV-RNA was used as a probe to find viral-related information in breast carcinomas.

30 Out of the 38 adenocarcinomas examined gave clear evidence that they contained the 70S RNA–RNA-instructed DNA polymerase complexes, whereas all of the 10 nonmalignant controls were negative. It need hardly be emphasized that negative outcomes, whether they occur with neoplastic or

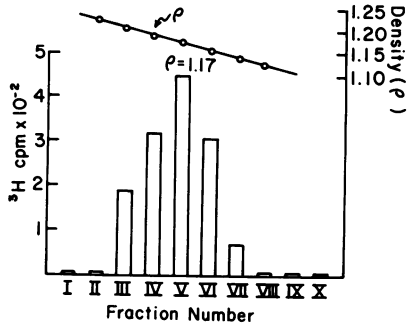

FIG. 5. Localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of human mammary tumors by sucrose gradient centrifugation. A P-100 pellet was prepared from human mammary tumor as in legend to Fig. 1. The pellet was suspended in Tris–NaCl–EDTA buffer and layered on a linear gradient of 50–25% sucrose in the same buffer and spun at 25,000 rpm in a SW27 (Spinco) rotor at 4° for 195 min. The gradient was dripped from below and ten equal fractions were collected; fractions were then diluted with Tris–NaCl–EDTA buffer to a sucrose concentration of less than 15%. Each fraction was then spun at 100,000 × *g*. The amount of 70S [^{3}H]DNA synthesized by an endogenous RNA-instructed DNA polymerase was determined by glycerol velocity centrifugation.

nonneoplastic samples, cannot be accepted as evidence for the absence of the relevant reaction. Indeed, in the course of the present study, we received one sample from a patient with benign fibrocystic disease of the breast that yielded a positive 70S reaction. Unfortunately, too little DNA was synthesized to permit further characterization. In any event, it would not be terribly surprising if more extensive studies uncover occasional healthy individuals that show evidence of 70S RNA–RNA-instructed DNA polymerase complexes. In this connection it is relevant to recall that we have reported (10) the presence of particles containing such complexes in the milk of normal cancer-free women.

The presence in human adenocarcinomas of RNA related to that of MMTV (1) was suggestive of a viral agent in this human disease. The present study carries this implication much further by showing that the virus-related RNA is 70 S in size and is physically associated with a RNA-instructed DNA polymerase in a particle having the density of a RNA tumor virus. Thus, for leukemia (12), and now for breast cancer, the evidence for the involvement of an RNA tumor virus in human neoplasia is becoming more compelling. To complete the proof it will be necessary to show that the complexes we have identified are infectious and transforming particles.

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