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Sequence Homology of Nucleic Acids from Human Breast Cancer Cells and Complementary DNA's from Murine Mammary Tumor Virus and Mason-Pfizer Monkey Virus[1]

Mukkattu Ramachandra Das,[2] and Michael M. Mink

Department of Biology, Michigan Cancer Foundation, Detroit, Michigan 48230

ABSTRACT

Simultaneous presence of murine mammary tumor virus- and Mason-Pfizer monkey virus-specific sequences has been detected in nucleic acids isolated from some human breast tumors and from MCF-7 cells, a well-characterized human breast cancer cell line. Carefully characterized long complementary DNA transcripts were used in the molecular hybridization experiments. From the data that are presently available, it would appear that when homology is detected with one of the mammary tumor probes the other also generally shows homology. Among all the complementary DNA-RNA hybrids only three, all murine mammary tumor virus hybrids, show T_m values close to 80°. The rest of the hybrids are low melting with shallow slopes for their C_rt curves, indicating partial and imperfect hybrids in the majority of cases. Low levels of weak hybrid formation are also detectable with the tumor DNA's. The present experiments cannot ascertain whether the hybridizing sequences from Mason-Pfizer monkey virus and murine mammary tumor virus code for specific viral functions in their natural hosts. Annealing experiments using gene specific cDNA's would be required for fully characterizing these sequences.

INTRODUCTION

Molecular hybridization experiments have demonstrated the presence of RNA sequences in human malignant breast tumors that anneal to specific sequences present in radioactive DNA transcripts from MuMTV[3] (1-3, 9, 30, 32) and MPMV (7). In addition, Ohno and Spiegelman (25) have shown that the reverse transcriptase present in particle isolates obtained from human breast cancer tissues cross-reacts immunologically with the purified reverse transcriptase from MPMV.

However, several questions about the commonality of biological information suggested by these results remain poorly understood. Thus, despite the demonstration that there are nucleic acid sequences present in human breast tumor RNA's that show homology independently to MuMTV and MPMV, no reports are available where RNA from the same malignant tumor has been challenged with both MuMTV and MPMV cDNA's. In the absence of demonstrable homology to any appreciable extent between MPMV and MuMTV viral genomes, as reported by earlier workers (22) and on the basis of our own results, it was of interest to determine whether simultaneous homology to these viral sequences exists or whether they would fall into 2 classes, one showing sequence homology to MuMTV-specific sequences and the other to MPMV sequences. The nature of the breast tumor RNA sequences that show homology and their origin in the tumor tissues are important in understanding and evaluating the significance of the presence of sequences in human breast tumor tissues. We were interested also in examining viral specific gene expression in MCF-7 cells, a well-characterized human breast cancer cell line developed at the Michigan Cancer Foundation (6, 14, 18, 20, 21, 29). Most human breast tumors obtained at surgery usually are small and even larger ones produce only a limited amount of nucleic acids from an individual tumor. The MCF-7 cell line provides a convenient system where detailed studies can be made using uniform populations of malignant human breast cancer cells.

In the present communication, we report our results of hybridization experiments using RNA samples from malignant human breast cancer cells and well-characterized cDNA's from both MPMV and MuMTV. The largest available tumors representing different pathological types were chosen for these experiments. The selection of large tumors ensured sufficient RNA or DNA for carrying out different types of investigations on the same sample. Initially, we examined a number of tumor RNA's for homology using the Cs_2SO_4 method of analysis. Samples that showed significant hybridization with both MPMV and MuMTV were used for further experiments. Hybridization analysis using S_1 nuclease were carried out, and C_rt curves were constructed using these RNA samples. The DNA from the same samples was also challenged with cDNA's from MPMV and MuMTV. The quality of the hybrids formed in both cDNA-RNA hybridization and cDNA-tumor DNA hybridization was tested by T_m measurements.

MATERIALS AND METHODS

Chemicals

Cold deoxynucleoside triphosphates were obtained from P. L. Biochemicals, Milwaukee, Wis., and *methyl*-^{3}H-labeled TTP was obtained from New England Nuclear, Boston, Mass. DNA grade hydroxylapatite (Bio-Gel HTP) and agarose were from Bio-Rad Laboratories, Richmond, Calif. DNA markers for gels were from Bethesda Research Laboratories, Rockville, Md. ACS reagent was obtained from Amersham/Searle, Chicago, Ill. RNase-free sucrose was obtained from Schwartz/Mann, Orangeburg, N. Y. S_1 nuclease was purchased from Miles Laboratories, Elkhart, Ind.

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[2] To whom reprint requests should be addressed, at Centre for Cellular and Molecular Biology, Regional Research Laboratory, Hyderabad 500 009, India.

[3] The abbreviations used are: MuMTV, murine mammary tumor virus; MPMV, Mason-Pfizer monkey virus; TNE, 0.1 M Tris (pH 7.4):0.1 M NaCl:2 mM EDTA; AMV, avian myeloblastosis virus; PCC, phenol:*m*-cresol:chloroform, 12:1:13 containing 50 mg of 8-hydroxy quinoline per 100 ml of the mixture equilibrated with 0.01 M Tris, pH 7.5; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; SSC, standard saline citrate.

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Biologicals

Tissues were obtained through the Biological Resources Unit of the Michigan Cancer Foundation. After surgical removal, tumors were placed on ice, and within 1 hr, they were frozen and stored at −70°. Placental tissues were stored in the same manner. MPMV was grown in CMMT cells at the John L. Smith Memorial Hospital for Cancer Research, Pfizer Inc., N. J. The virus had already been banded once in sucrose density gradient and analyzed for reverse transcriptase activity, nucleic acid size and content, and protein content. Samples received in dry ice were thawed in ice, diluted with TNE and rebanded in 15 to 65% sucrose density gradient (TNE) before use. MuMTV was obtained from 2 sources. Tissue-cultured virus grown in MM5MT/Cl line, purified by zonal centrifugation, and resuspended in TNE was obtained from Frederick Cancer Research Center Viral Resources Laboratory, Fort Detrick, Md. The virus had been characterized by electron microscopy and tested for group-specific antigen and reverse transcriptase activity. Data on protein content were also available. As in the case of MPMV, the material was rebanded in sucrose density gradients before use. MuMTV was also isolated from RIII mouse milk (Meloy Laboratories, Springfield, Va.) using already established methods (10, 23, 28). Purified AMV reverse transcriptase was from Dr. J. Beard. Tissue-cultured MPMV and MuMTV, RIII mouse milk, and AMV reverse transcriptase were obtained through the Office of Resources and Logistics of the Virus Cancer Program. MCF-7 cells were grown in roller bottles in our laboratories using methods described previously (21, 29).

Isolation of RNA's

Viral RNA's. Initially, 3 different methods for isolating 70S viral RNA from MuMTV and MPMV were compared. The final method adopted for viral RNA extraction for use as templates in *in vitro* reverse transcriptase reactions is similar to the method described by Drohan *et al.* (11). A viral pellet corresponding to 10 mg of protein was resuspended in 1 ml of TNE, and the suspension was made 1% with respect to lithium dodecyl sulfate. The clear lysate was treated with self-digested pronase at a concentration of 200 μg/ml for 20 min at 37°. It was then extracted 2 times at room temperature with equal volumes of PCC. The combined PCC layer was back extracted with one-half its volume of TNE. The aqueous layers were combined, adjusted to 0.4 M with LiCl, and mixed with 2 volumes of chilled absolute ethanol (−20°). The mixture was kept overnight at −20°. The RNA was pelleted by centrifugation at 10,000 rpm in siliconized centrifuge tubes in an HB-4 rotor using a Sorvall centrifuge at −10° for 30 min. The supernatant was drained off, and the pellet was taken up in 0.1 ml of TNE. The RNA solution was layered on a 10 to 30% preformed glycerol gradient (TNE; 4°) and centrifuged at 200,000 × *g* for 3 hr at 4° in a Spinco SW41 rotor. The tubes were bottom punctured and 0.4-ml fractions were collected. The position of RNA was monitored by absorbance at 260 nm. Fractions containing the 60 to 70 S component were pooled, and the pool was made 0.4 M with LiCl. The RNA was isolated by ethanol precipitation as before. After draining the supernatant, the RNA pellets were dried in a vacuum dessicator at room temperature for 15 min. The pellet was taken up in 0.001 M Tris (pH 8.3) for use in reverse transcriptase reactions. ^{3}H-Labeled 18 and 28S mouse ribosomal RNA were used as markers in parallel gradients.

We have also used 2 other methods: direct banding of viral RNA on glycerol gradients after lysing the virus with SDS (26) and the CsCl-method of isolation (13). Although both these methods give comparable yields of viral RNA, the phenol-extracted RNA gave the best yield of long cDNA in reverse transcriptase reactions (see "Results").

Tumor RNA's. Tumors stored at −70° were allowed to thaw on ice. Polysomal RNA was isolated from tissues according to methods described elsewhere (2, 9). Nuclear RNA was isolated from nuclear pellets that were separated during polysomal RNA preparations. The pellets were resuspended in TNE and lysed with 1% SDS, and PCC-extracted as described for viral RNA extractions. The extractions were carried out 3 times. Before separating the aqueous layer, the mixture was shaken at room temperature for 20 min. No back extraction of the phenol layer was done, and the aqueous layer was extracted once with water-saturated ether to remove phenol before ethanol precipitation. The DNA was removed by winding, and the RNA left behind was pelleted by centrifugation at 10,000 rpm in an HB-4 rotor for 30 min at −10° after keeping the suspension overnight at −20°. The RNA pellet was taken up in TNM (0.01 M Tris (pH 7.4), 0.15 M NaCl, and 0.005 M $MgCl_2$) and treated with RNase-free DNase at 20 μg/ml concentration for 1 hr at 37°. The solution was PCC-extracted 3 times, as described above. The RNA was isolated by ethanol precipitation followed by vacuum drying. The residue was taken up in 0.005 M EDTA (pH 7.4) and was adjusted to 10 mg/ml concentration. Total RNA was extracted from tissues by the method described for nuclear RNA using minced virtis-homogenized tissues without effecting the separation of nuclear and cytoplasmic fractions.

DNA Extractions

DNA was extracted and purified from tissues by a modification of the method described by Drohan *et al.* (11). After the first PCC extraction, the pooled DNA was dissolved in 0.01 (SSC) (1 × SSC = 0.15 M NaCl:0.0015 M sodium citrate). After the DNA was in solution, it was adjusted to 1 × SSC and sonicated with a Branson sonifier using a macro tip at half-maximum output at a 30% pulse frequency for 3 min at a DNA concentration of 1 mg/ml. The sonicated solution was made 0.3 M with KOH and incubated at 37° overnight. The solution was neutralized with 0.3 M HCl and was PCC-extracted 3 times. Two volumes of cold ethanol (−20°) were added to the aqueous extract, and the suspension was kept at −20° for 2 hr. The DNA was precipitated by centrifugation, and the contents were taken up in 0.005 M EDTA at a concentration of 10 mg/ml. The material obtained in this manner banded in alkaline sucrose gradients with S values from 5 to 8 and had an A_{260}:A_{280} ratio of greater than 1.8. Occasionally some samples showed lower A_{260}:A_{280} ratios. Such samples were reextracted with PCC and ethanol precipitated until the ratio was greater than 1.8.

Synthesis of [^{3}H]cDNA

The [^{3}H]cDNA syntheses were carried out in 0.1 ml of reaction volume under conditions similar to those described by Myers *et al.* (24). The reaction mixture contained 50 mM Tris (pH 8.3); 40 mM KCl; 8 mM $MgCl_2$; 1.6 mM dATP, dCTP, dGTP; and 0.15 mM [^{3}H]dTTP (0.5 mCi), 10 mg/ml of $(dT)_{10}$, 4 mM

each of dithiothreitol and sodium pyrophosphate, 0.01% NP40, 30 to 50 μg viral RNA, and 100 units of purified AMV reverse transcriptase (5542 units/ml). All reactions contained 50 μg/ml of Actinomycin D. The reactions were carried out at 37° for 2 hr in a closed container. To terminate the reaction, the contents were diluted to 0.5 ml with 0.4 M NaCl and brought to 0.5% with lithium dodecyl sulfate and 5 mM EDTA. Purified yeast RNA (50 μg) was added as a carrier, and the contents were extracted twice with PCC. The combined PCC layer was back extracted with 0.5 ml TNE and mixed with the original aqueous extract. Unincorporated radioactivity was separated by Sephadex G-50 chromatography, and the nucleic acids were recovered by ethanol precipitation. The residue was taken up in 0.1 ml of 0.3 M NaOH and kept at 100° in a water bath for 5 min. After cooling, the solution was carefully neutralized with HCl and dialyzed extensively against 0.003 M EDTA. The dialysed material was lyophylized in a small plastic container, kept in a vacuum dessicator, and was taken up in 100 μl distilled deionized water.

Alkaline Sucrose Gradients

Samples for alkaline sucrose gradients were taken soon after the stage of the alkali treatment of the cDNA. Aliquots of samples containing the desired amount of radioactivity were made 0.7 M NaCl, 0.3 M NaOH, and 0.005 M EDTA in a total volume of 0.2 ml and layered on a 12-ml preformed 15 to 30% sucrose gradient in the same buffer. The gradients contained 0.1% of sarkosyl. Centrifugation was carried out in an SW41 rotor at 40,000 rpm for 16 hr at 22°. Fractions (0.4 ml) were collected by bottom puncture, and 100-μl aliquots from the fractions were removed, trichloroacetic acid precipitated, and the distribution of acid-precipitable radioactivity was determined.

Agarose Gel Electrophoresis

Gel electrophoresis of cDNA for size determination was carried out in 0.9% (w/v) agarose gels containing 5 mM methyl mercuric hydroxide according to the procedure of Bailey and Davidson (4) as modified by Myers *et al.* (24). The gels were calibrated using DNA markers obtained from Bethesda Research Laboratories. Hae III restriction fragments of ΦX174 R_F DNA and Eco RI digest of λ-phage DNA were both used as markers. All the electrophoreses were carried out in gels of identical size (0.6 x 11 cm) in 0.7- x 12.5-cm gel tubes (Bio-Rad Laboratories) at 7 ma/gel for 4 hr at room temperature. The positions of the markers were detected after staining the gels with ethidium bromide, using UV. Gels containing radioactive samples were sliced using a Gilson gel slicer. Each gel was cut into 50 equal parts, and the fractions were placed into scintillation vials. The contents of the vials were treated with 0.5 ml formamide at 37° for 1 hr and assayed for radioactivity in ACS reagent.

Nucleic Acid Hybridizations

Cs_2SO_4 Method for cDNA-RNA Hybridizations. Initial hybridization experiments were carried out according to the methods described elsewhere (9, 31) with minor modifications. The reactions were carried out in 100-μl volumes in sealed glass containers. The incubation mixtures contained 250 to 300 μg of RNA and 5000 cpm of cDNA. NaCl was present at 0.4 M, and the reaction mixture also contained 50% formamide (v/v). Incubations were carried out at 37° to attain C_rt values of 10^4. The products were analyzed on Cs_2SO_4 gradients starting with a 50% saturated Cs_2SO_4 solution containing 0.01 M EDTA and the desired reaction products. Centrifugation was carried out in an SW 50.1 rotor at 30,000 rpm for 60 hr. Better resolution of the banding of the nucleic acids is obtained under these conditions than at higher speeds. Trichloroacetic acid-precipitable radioactivity present in 0.15-ml fractions was determined. The fractional radioactivity banding in the hybrid density region (1.55 to 1.67) in each experiment was calculated from the data.

S_1-Nuclease Analysis of DNA-RNA Hybrids. Incubation of samples for following the kinetics of DNA-RNA hybridizations using S_1-nuclease analysis was carried out in a total volume of 10 μl in glass capillary tubes at 0.6 M NaCl, 0.003 M EDTA, and 1% SDS. Incubations were carried out at 68° for 134 hr for concentration variation experiments. Each 10-μl aliquot contained 1000 cpm of cDNA and varying concentrations of RNA for the required C_rt values. The reaction products were analyzed using single-stranded specific nuclease S_1 from *Aspergillus orizae* as described by Fan and Baltimore (12). The C_rt values reported have been normalized for standard salt concentration (5). Experiments were also carried out in which different C_rt's were attained using the same RNA concentrations (4 mg/ml) and different time intervals of incubation. There was little difference between the results obtained in this manner and those obtained in the concentration variation experiments.

Hydroxylapatite Method for DNA-DNA Hybridization. The cDNA-cellular DNA hybridizations were also carried out in sealed capillaries. To obtain different C_0t values, the time interval for incubation was varied in these experiments. cDNA corresponding to 2000 cpm/point was mixed with cellular DNA in a total volume of 0.2 ml DNA (5 mg/ml) in phosphate buffer [0.05 M phosphate (pH 6.8)], 0.005 M EDTA, 1% SDS. The contents were heated at 100° in boiling water for 5 min and quick cooled in ice. The solution was brought to 0.48 M phosphate concentration (pH 6.8) at 0°. Aliquots (15 μl) were removed and sealed in separate capillaries. They were incubated at 68° to obtain the desired C_0t values. At the end of the incubation, the contents in the capillary were diluted to 2 ml with 0.14 M phosphate buffer (pH 6.8) containing 0.4% SDS. Single-stranded and hybridized DNA's were separated by hydroxylapatite chromatography (Bio-Gel HTP, DNA grade). Unhybridized material was removed by 0.14 M phosphate (0.4% SDS) elution at 60°. The hybridized molecules were recovered by elution using the same buffer at 100°. The hydroxylapatite columns were 1 x 3.5 cm. At each temperature, elutions were carried out by 3 separate 6-ml aliquots of the buffer equilibrated at the desired temperature. The eluates at the 2 different temperatures were collected in separate sets of 2-ml fractions. Each 2-ml sample was mixed with 6 ml of ACS reagent (Amersham) and the radioactivity eluting as single-stranded and hybrid material was determined using a Beckman Scintillation Counter. The results were normalized for standard salt concentration values (5).

Thermal Stability of Hybrids

Samples for the determination of T_m's were hybridized in separate experiments. Sonicated DNA (600 μg) and 5000 cpm

of cDNA were hybridized as described above for a C_0t value of 10^4 in 100-μl volume of 0.48 M phosphate buffer. After incubation, the material was diluted with 10 ml of 0.14 M phosphate buffer and applied to a 1- x 5-cm column of DNA-grade hydroxylapatite. The column was washed twice with 10 ml of 0.14 M phosphate buffer at 50°. The elution was repeated at 5° increments up to 100°. At each temperature, two 10-ml elutions were carried out. The radioactivity eluting at the different temperatures was determined as described above. The fraction remaining bound to the column as a function of temperature was evaluated, and the temperature at which 50% of the total counts are eluted (T_m) was obtained graphically. The conditions of hydroxylapatite chromatography described here are within the appropriate window conditions of Martinson and Wagenaar (19). The conditions were checked in separate experiments using labeled nucleic acids isolated from *Escherichia coli*.

RESULTS

Characterization of cDNA's

The procedure for the synthesis of cDNA described in "Materials and Methods" was adopted after optimizing the experimental conditions for the synthesis of long cDNA molecules representative of the viral RNA from MPMV and MuMTV. The effect of optimal amount of detergent for disrupting the virus (15) and the use of high concentrations of either deoxynucleoside triphosphates (8, 26), ribonucleoside triphosphates, or pyrophosphate (16, 24) for the synthesis of long cDNA have been described. However, some of these methods do not seem to be applicable to MuMTV and MPMV. High concentrations of deoxynucleoside triphosphates which have been successful for making full-length probes with murine leukemia virus and Rous sarcoma virus do not yield long cDNA molecules with MPMV or MuMTV. As shown in Table 1, there is enhanced synthesis of cDNA in terms of pmol of dCTP incorporated. However, on alkaline sucrose gradients there was no difference in the size of molecules synthesized with 3 different triphosphate concentrations (Chart 1). This difference in behavior is probably due to the difference in the amounts of nuclease present in the different viruses. Even after repeatedly banding the viruses in sucrose density gradients, it has been found impossible to obtain long cDNA molecules using this procedure with our virus preparations.

We, therefore, adopted the method described by Myers *et al.* (24) making use of purified high-molecular-weight viral RNA and exogenously added purified AMV reverse transcriptase to the *in vitro* DNA-synthesizing reactions. We have extracted MuMTV and MPMV RNA's from density-banded virus using 3 different methods described in "Materials and Methods." The CsCl method is convenient for handling small quantities of biological materials, and the RNA extracted in this manner has been reported to be intact and to retain its biological activity (13). However, in reverse transcriptase reactions using exogenous AMV enzyme and purified viral RNA, RNA samples prepared by PCC extraction method resulted in better yields of long cDNA molecules. Chart 2 shows the agarose gel electrophoresis profile of a cDNA sample synthesized using purified AMV enzyme and PCC-extracted MuMTV viral RNA isolated from tissue-cultured virus. The agarose gel profile of a cDNA sample synthesized using endogenous reverse transcriptase

Table 1

MuMTV viral RNA directed DNA synthesis with varying dCTP concentrations

The reaction mixtures contained virus corresponding to 80 μg of viral protein in 100-μl volume. Cold dATP, dGTP, and TTP were present at concentrations of 4.9 mM in all reactions. The concentration of cold dCTP was varied in the different reaction mixtures. All other conditions were the same as described in the text for the synthesis [^{3}H]cDNA.

Concentration of dCTP in reaction (mM)	Specific activity (dCTP)	pmol of TCA precipitable material synthesized in 1.5 hr (5-μl aliquots)
0.1	4870	0.89
0.5	974	1.66
4.9	99	9.55

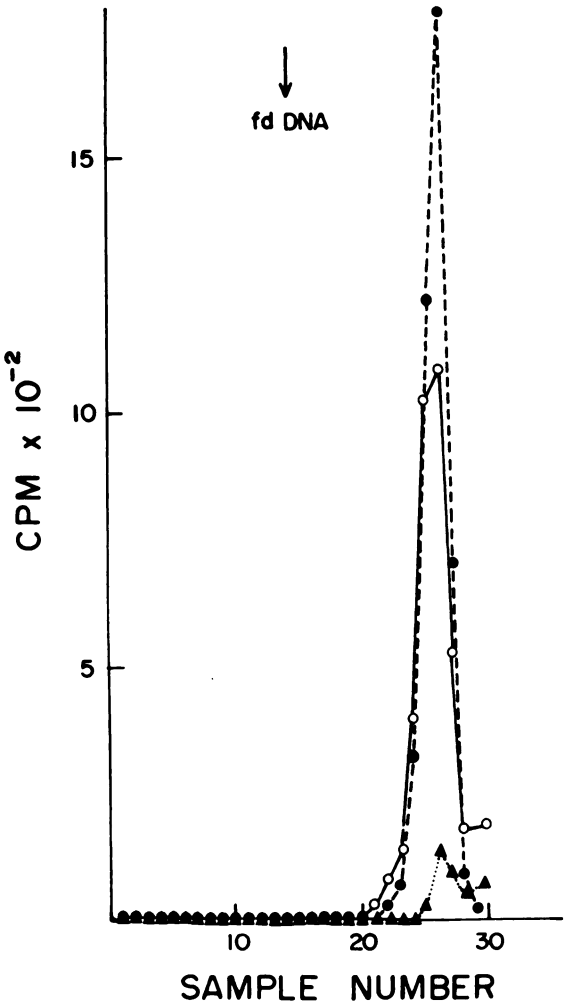

Chart 1. Alkaline sucrose gradient profile of cDNA synthesized from endogenous reverse transcriptase reactions using MuMTV. Conditions of experiments are described in the text. ▲, 4.9 mM dCTP; ○, 0.5 mM dCTP; ●, 0.1 mM dCTP. The yield of cDNA synthesized under these conditions in terms of pmol is reported in Table 1.

reaction and making use of a purified MuMTV from MM5MT/Cl cell line is also shown in Chart 2. Similar profiles are obtained in experiments making use of MuMTV isolated from the milk of RIII mice or MPMV isolated from CMMT cells. Gel electrophoretic analysis on 2.25% polyacrylamide gels also gives comparable results. The long cDNA molecules synthesized in this manner had a specific activity of 1.2×10^7 cpm/μg. The MuMTV cDNA represented 85 to 92% of the viral RNA, and

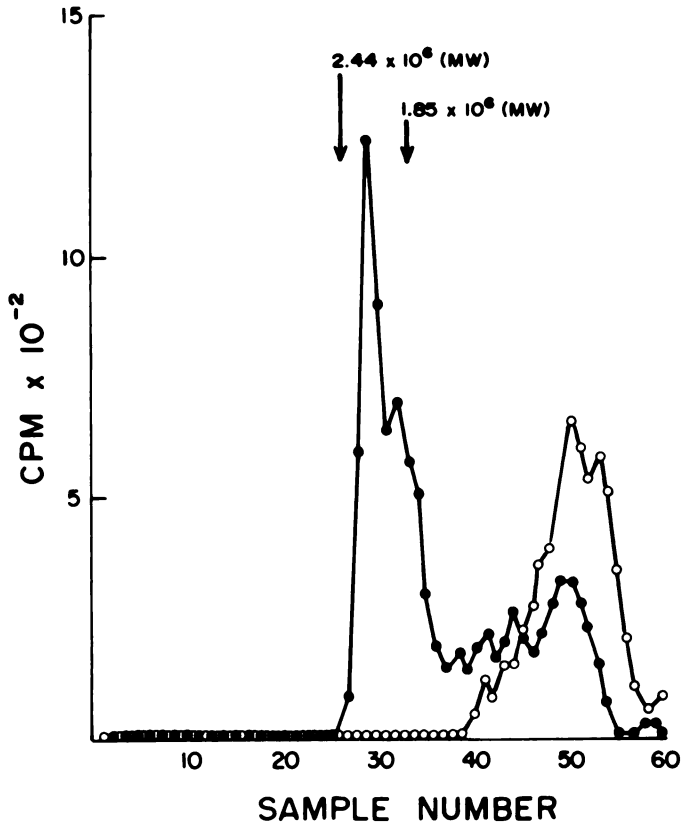

Chart 2. Agarose-methylmercuric hydroxide gel electrophoresis profile of [^{3}H]cDNA's synthesized from MuMTV and isolated from MM5 MT/Cl line (see text). The gels were run from 4 hr at room temperature at 7 ma/110- x 6-mm tubes. ●, cDNA synthesized using purified viral RNA and AMV reverse transcriptase; ○, cDNA synthesized using endogenous reverse transcriptase present in lysed virions and resident template (see text).

the MPMV cDNA represented 81 to 87% of the viral RNA at cDNA:RNA ratios of 5:1. The MuMTV cDNA hybridized to homologous viral RNA with a $C_rt_{1/2} = 4.2 \times 10^{-2}$, and the MPMV probe hybridized with $C_rt_{1/2} = 4.7 \times 10^{-2}$.

Conditions for Hybridization

Analysis of initial annealing experiments for the qualitative detection of hybrid formation with tumor cell RNA was carried out using Cs_2SO_4 gradient centrifugation. This procedure was adopted to compare the present data with the results of earlier investigations (2, 3, 7) in which human breast tumor RNA's were examined. S_1 nuclease analysis has also been used in some investigations using breast tumor RNA's (32). After obtaining hybridization results using the Cs_2SO_4 method on a number of tumors, we used the S_1 nuclease method for selected samples for a more quantitative analysis of the kinetics of hybrid formation and for the comparison of data obtained using the 2 different methods. For Cs_2SO_4 gradient analysis in most of the recent investigations (2, 3, 7, 9, 27, 31), annealings were carried out in the presence of 50% formamide at 37°. For C_rt analysis using S_1 digestion, annealing is generally carried out in aqueous medium at 68°. To determine whether the 2 types of annealing conditions make any difference in the observed extents of hybrid formation, we have used in our S_1 experiments samples annealed under the different conditions. Table 2 shows representative results where 2 of the samples showing positive hybridizations using Cs_2SO_4 analysis are analyzed using S_1 nuclease. It is clear from the data that the conditions of annealing do not make any appreciable difference in the extent of hybrid formation. The aqueous solutions, referred to in Table 2, contained 1% SDS, and the formamide solutions did not contain any SDS. The absence of SDS in aqueous solution or its presence in formamide incubation mixtures result in a wider scatter of values and irreproducible results. The small amount of formamide (5 μl) present in 10-μl reactions when diluted to 2 ml in the S_1 buffer does not affect the S_1 nuclease digestion. Based on these results, we restricted the annealing conditions to incubation at 68° in aqueous medium for all other experiments using S_1 digestion reported in this paper, although it is known that during long periods of incubation the degradation of RNA is greater at the higher temperature. On the other hand, in the aqueous condition, by avoiding the volume occupied by formamide, higher concentrations can be attained in reaction mixtures starting from stock RNA preparations which were usually made at 10-mg/ml concentration, making it possible to cut down the time required to reach any specific C_rt. Attempts to adapt the phenol emulsion reassociation technique (17) to the present experiments were unsuccessful and did not result in reproducible results.

cDNA-RNA Hybridizations

Cs_2SO_4 Method of Analysis. The results of cDNA-RNA hybridizations using radioactive cDNA's from MuMTV and MPMV and several cellular RNA's are shown in Table 3. Initial studies to detect the presence of viral sequences in tumor RNA's were carried out by the Cs_2SO_4 centrifugation method of analysis. Annealing was carried out to C_rt values of 10^4, and the reaction products were analyzed by equilibrium density gradient centrifugation as described in "Materials and Methods." In this set of experiments, the reaction products were not treated with S_1 nuclease to remove "tails" of radioactive cDNA's present in partially hybridized molecular species. These experiments were designed to select tumor RNA samples showing larger extents of homology with cDNA's for more detailed studies.

For MCF-7 cells hybridization analysis was carried out using both polysomal and nuclear RNA's; total cytoplasmic and nuclear RNA's were used for all other samples. Under identical conditions of experiments using comparable concentration of RNA's and cDNA's, nuclear RNA of MCF-7 cells shows higher homology for both MPMV and MuMTV cDNA's. It can also be seen in this set of experiments that in general MuMTV cDNA demonstrates higher homology with the human tumor RNA's.

Table 2

Comparison of hybrid formation in aqueous solution (68°) and in 50% formamide (37°)

The incubations for annealing reactions using [^{3}H]cDNA from MuMTV were carried out as described in the text. After taking the reactions to identical C_rt values in the presence and in the absence of formamide, the reaction products were treated under identical conditions with S_1 nuclease as described in the text.

Source of RNA	Log C_rt	% of cDNA protected	
		Aq (68°)	Formamide (37°)
MCF-7	1.1	9.9	9.8
MCF-7	3.1	30.1	34.6
Adenocarcinoma (1)[a]	4.1	35.3	42.1
Adenocarcinoma (1)	4.5	43.2	42.8

[a] Numbers in parentheses, numbering in Table 3 where data on several different RNA's are tested.

Three samples demonstrate appreciable homology with both MPMV and MuMTV cDNA's, 2 show homology slightly above background, and the others cannot be considered positive. It appears from the present experiments, although the sample size is small, that when homology is detected with one of the mammary tumor virus probes the other also generally shows homology. The RNA samples that showed higher values for homology were tested against AMV cDNA probes to check for sequences that might show nonspecific hybridization. No homology was found in these experiments.

S_1 Nuclease Digestion Method. Four samples that showed higher values for homologous sequences using the Cs_2SO_4 method were analyzed in greater detail. Annealing reactions were carried to different C_rt values under the aqueous reaction conditions described in "Materials and Methods", and the S_1-resistant counts were determined. The results are presented in Table 4. It may also be noticed from Table 4 that the cross-hybridization between MPMV and MuMTV is close to control values. Chart 3 shows the progress of the reaction as a function of C_rt values for representative samples. The T_m values of the hybrids are presented in Table 6. We have carried out experiments where the long cDNA probes were sonicated to 4S size before carrying out annealing reactions. This procedure does not produce any significant difference in the extent of hybridization, shape of the C_rt curves, or T_m's.

Table 3

Percentage of input counts in RNA density regions in Cs_2SO_4 gradients

Hybridization conditions and Cs_2SO_4 gradient analysis are described in the text. The percentage of counts reported in the table are those banding in the RNA and hybrid density regions.

	cDNA		
	MPMV	MuMTV	AMV
Source of RNA			
MCF-7 (polysomal)	35.5	41.8	Not detectable
MCF-7 (nuclear)	52.1	55.0	Not detectable
Tumor RNA's			
1. Adenocarcinoma	48.6	54.2	Not detectable
2. Adenocarcinoma	27.4	19.3	
3. Adenocarcinoma	12.2	7.5	
4. Adenocarcinoma	5.5	3.2	
5. Infiltrating ductal carcinoma	6.2	19.1	
6. Infiltrating ductal carcinoma	12.8	16.9	
7. Comedocarcinoma	7.4	5.1	
8. Comedocarcinoma	0.0	2.7	
9. Medullary carcinoma	22.1	35.5	
10. Malignant breast tumor (not characterized)	4.1	1.5	
Placenta	<5.0	<4.0	

Table 4

cDNA-RNA hybridizations (S_1 nuclease digestion method)

Hybridization reactions were carried out at 68° in aqueous condition as described in the text.

	% S_1-resistant Cpm at $C_rt = 10^{4.5}$	
Source of RNA	MPMV cDNA	MuMTV cDNA
MCF-7 cells	41.3	45.2
Adenocarcinoma (1)[a]	41.5	43.2
Adenocarcinoma (2)	32.8	26.9
Infiltrating ductal carcinoma (6)	15.2	17.8
Medullary carcinoma (9)	24.8	27.3
Placenta	7.1	5.2
Poly A	<3.0	<2.0
MPMV ($C_rt = 5$)	87.0	9.7
MuMTV ($C_rt = 5$)	12.4	92.0

[a] Number in parentheses, numbers in Table 3.

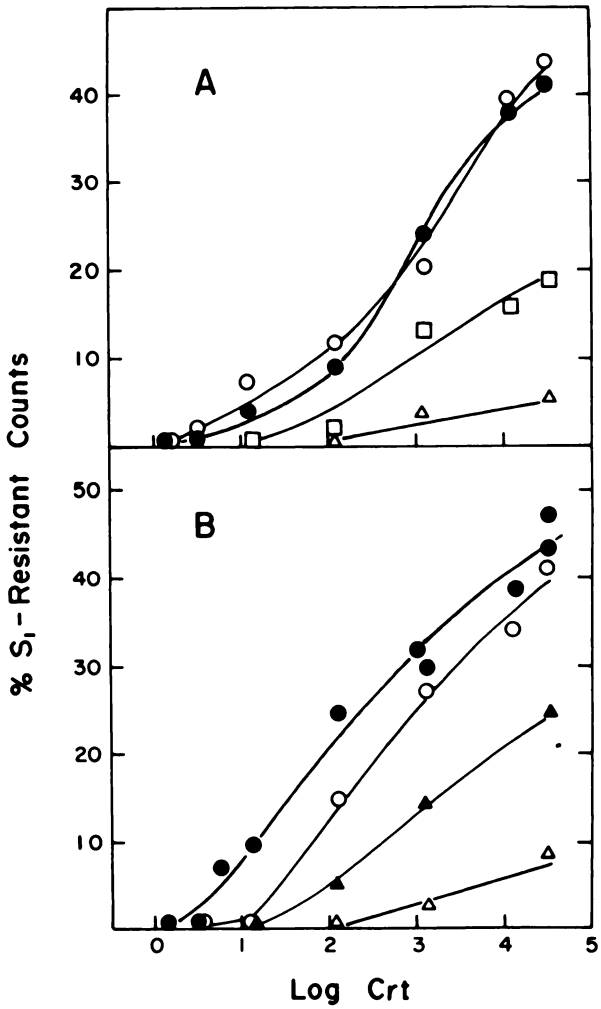

Chart 3. Percentage of S_1 nuclease-resistant counts as a function of C_rt values in hybridization experiments using RNA from breast cancer cells, MuMTV (*A*), and MPMV (B). Details of experimental conditions are described in the text. ○, RNA from MCF-7 cells; ●, Tumor 1 (Table 3); ▲, Tumor 9 (Table 3); □, Tumor 6 (Table 3); △, placental RNA.

cDNA-Cellular DNA Hybridizations

DNA from the same samples as the ones used for S_1 experiments (Table 4) was hybridized with MPMV and MuMTV cDNA's. The products of the reactions were analyzed using hydroxylapatite chromatography as described in "Materials and Methods," and the results are presented in Table 5. They do show homology, but the hybrids are imperfect as demonstrated by their low T_m values (Table 6; Chart 4). Sonicated probes did not show any significant difference in the DNA annealing experiments as in the cDNA-RNA hybridizations.

DISCUSSION

The present experiments clearly demonstrate sequence homology between some breast tumor RNA's and cDNA's from MuMTV and MPMV using either the Cs_2SO_4 centrifugation method of analysis of DNA-RNA hybrids or using the S_1 nuclease analysis. However, any comparison of the percentage of radioactivity detected as hybrids should be restricted to experiments belonging to the same class. In the Cs_2SO_4

method of analysis, as used here without treating the hybrid for removing tails of radioactive cDNA's present in partially hybridized molecular species, the percentage of radioactivity banding in the RNA and hybrid density regions gives a higher estimate. There is also one other difference in the experimental conditions used. The incubations for the Cs_2SO_4 experiments were carried out to attain C_rt values of 10^4, whereas the incubations for S_1 analysis were taken to C_rt values of $10^{4.5}$. These 2 factors would affect the observed percentages of hybrid formation in opposite directions. The Cs_2SO_4 experiments were carried out for qualitative detection of hybrid formation, whereas the S_1 analysis experiments were designed for a more quantitative comparison of virus-related information in samples showing positive hybridization using the former method.

The C_rt curves presented in Chart 3 do not have sharp slopes, and the majority of the hybrids, with the exception of 3 MuMTV hybrids, show low T_m values (≈70°). Although a broadening of the C_rt curves could in principle result from the absence of sufficient excess of driver RNA over the cDNA, the low-T_m values would suggest that the hybrids formed are, in general, imperfect. We have observed also that there is no appreciable difference in the T_m for an RNA-cDNA hybrid either with the long probes or with sonicated probes of 4S size. In both these sets of experiments, T_m measurements were carried out using reaction products without any nuclease treatment. The observed low T_m's, therefore, are not an artifact resulting from the long probes used in the present experiments. The existence of 3 higher-melting hybrids for MuMTV cDNA (see Table 6; Chart 4) suggest more faithful sequence homology

Table 5

cDNA-cell DNA hybridizations (hydroxylapatite analysis)

Conditions for annealing and hydroxylapatite analysis are described in the text. Incubations were carried to log C_rt = 4.5. The percentage values reported in the table correspond to the fraction of radioactive cDNA eluting at 100° with 0.14 M phosphate after a first elution with the same buffer at 60°.

	% of cDNA eluting at 100°	
Source of DNA	MPMV cDNA	MuMTV cDNA
MCF-7 cells	21.2	17.8
Adenocarcinoma (1)[a]	23.0	19.1
Adenocarcinoma (2)	15.3	13.9
Infiltrating ductal carcinoma (6)		12.6
Medullary carcinoma (9)	19.1	12.3
Human placenta	8.3	6.5
Calf thymus	5.8	3.9

[a] Numbers in parentheses, numbering in Table 3.

Table 6

T_m measurements of hybrids

The experimental conditions for T_m determinations are described in the text.

	Measured T_m°			
	cDNA-RNA hybrids		cDNA-DNA hybrids	
Source of nucleic acids	MPMV cDNA	MuMTV cDNA	MPMV cDNA	MuMTV cDNA
MCF-7 cells	72.0	81.5	70.0	70.0
Adenocarcinoma (1)[a]	68.5	80.0	67.5	68.0
Adenocarcinoma (2)	74.5	69.0	68.0	69.0
Infiltrating ductal carcinoma (6)	70.0	72.5	71.5	70.0
Medullary carcinoma (9)	70.0	79.0	70.0	75.0
MPMV-RNA	86.0			
MuMTV-RNA	87.5			

[a] Numbers in parentheses, numbers in Table 3.

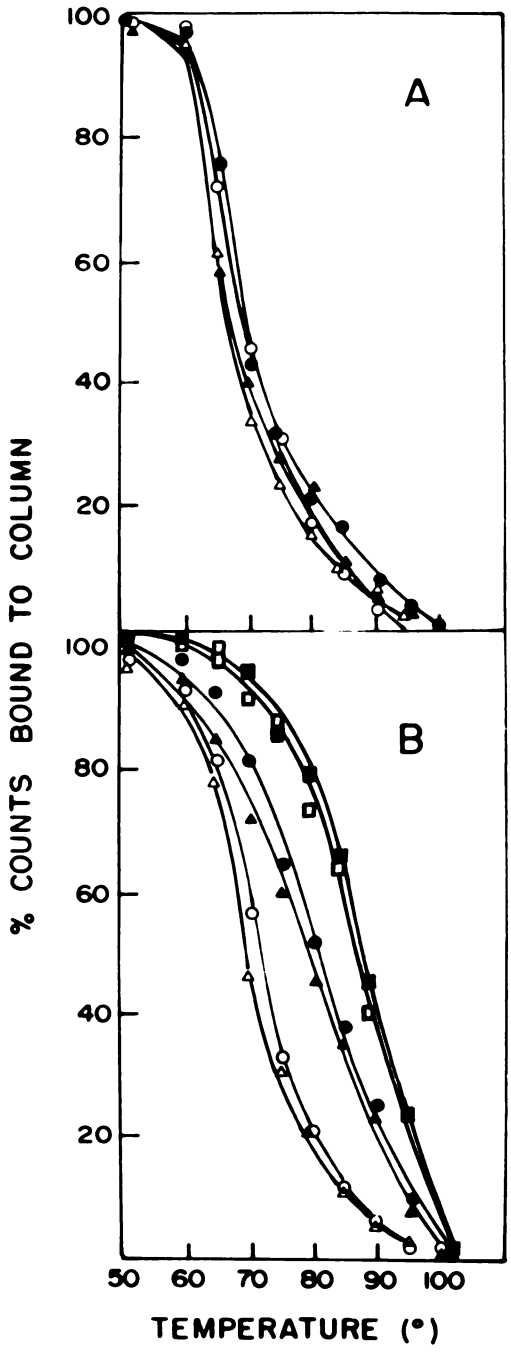

Chart 4. Thermal stability of cDNA-cellular DNA (*A*) and cDNA-RNA (*B*) hybrids using thermal elution from hydroxylapatite columns (see text). ●, nucleic acid of MCF-7 origin and MuMTV cDNA; ○, nucleic acid of MCF-7 origin and MPMV cDNA; ▲, nucleic acid from adenocarcinoma (Table 3, 1) and MuMTV cDNA; △, nucleic acid from adenocarcinoma (Table 3, 1) and MPMV cDNA; ■, MuMTV homologous duplex; and □, MPMV homologous duplex.

between the tumor RNA's and the mouse viral genome. Because MPMV was originally isolated from the breast tumor of a Rhesus monkey, one would, *a priori*, expect greater homology with the monkey virus than with the mouse virus for reasons of phylogenetic relatedness. These expectations are not borne out by our experiments. In the absence of related sequences in MPMV and MuMTV, it can be concluded that the observed hybridization from the same tumor cell RNA probably results from different sequences in the viral genomes. Annealing ex-

periments using gene specific cDNA's would be required to fully characterize these sequences. Experiments of this type are in progress.

The data presented in Table 5 indicate that both MPMV and MuMTV cDNA's demonstrate homology with human tumor DNA's above control values. However, it may be seen from the data presented in Table 6 and Chart 4 that all the hybrids are low melting, and unlike the RNA hybrids, there is no difference in the melting behavior between any set of MPMV and MuMTV hybrids. The small differences found in the percentage of double-stranded material formed while using the 2 different cDNA's may not be significant because all the values are low.

We have not attempted evaluation of viral gene copies in the human material for several reasons. Even at the highest C_0t or C_rt values, to which the annealing reactions were taken, we do not observe saturation. The large majority of the hybrids are low melting. The partial and imperfect homology suggested by these observations indicates that estimates of gene copies would not be meaningful.

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REFERENCES

1. Axel, R., Gulati, S. C., and Spiegelman, S. Particles containing RNA-instructed DNA polymerase and virus-related RNA in human breast cancers. Proc. Natl. Acad. Sci. U. S. A., *69:* 3133–3137, 1972.
2. Axel, R., Schlom, J., and Spiegelman, S. Presence in human breast cancer of RNA homologous to MMTV RNA. Nature (London), *235:* 32–36, 1972.
3. Axel, R., Schlom, J., and Spiegelman, S. Evidence of translation of viral-specific RNA in cells of a mouse mammary carcinoma. Proc. Natl. Acad. Sci. U. S. A., *69:* 535–538, 1972.
4. Bailey, J., and Davidson, N. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem., *70:* 75–85, 1976.
5. Britten, R. J., and Smith, J. A bovine genome. Carnegie Institute. Wash. Year Book, *68:* 378–386, 1970.
6. Brooks, S. C., Locke, E. R., and Soule, H. D. Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. J. Biol. Chem., *248:* 6281–6283, 1973.
7. Colcher, D., Spiegelman, S., and Schlom, J. Sequence homology between the RNA of MPMV and the RNA of human malignant breast tumors. Proc. Natl. Acad. Sci. U. S. A., *71:* 4975–4979, 1974.
8. Collett, M., and Faras, A. *In vitro* transcription of DNA from 70S RNA of RSV: identification and characterization of various size classes of DNA transcripts. J. Virol., *16:* 1220–1228, 1975.
9. Das, M. R., Sadasivan, E., Koshy, R., Vaidya, A. B., and Sirsat, S. M. Homology between RNA from human malignant breast tissues and DNA synthesized by milk particles. Nat. New Biol., *239:* 92–95, 1972.
10. Das, M. R., Vaidya, A. B., Sirsat, S. M., and Moore, D. H. Polymerase and RNA studies on milk virions from women of the Parsi community. J. Natl. Cancer Inst., *48:* 1191–1196, 1972.
11. Drohan, W., Kattmann, R., Colcher, D., and Schlom, J. Isolation of the MMTV sequence not transmitted as germinal provirus in the C3H and RIII mouse strains. J. Virol., *21:* 986–995, 1977.
12. Fan, H., and Baltimore, D. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Mol. Biol., *80:* 93–117, 1973.
13. Glisin, V., Crkvenjakov, R., Byus, C. RNA isolated by CsCl centrifugation. Biochemistry, *13:* 2633–2637, 1974.
14. Horwitz, K. B., Costlow, M. E., and McGuire, W. L. MCF-7 a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. Steroids, *26:* 785–795, 1975.
15. Junghans, R. P., Duesberg, P. H., and Knight, C. A. *In vitro* synthesis of full-length DNA transcripts of Rous sarcoma virus RNA by viral DNA polymerase. Proc. Natl. Acad. Sci. U. S. A., *72:* 4895–4899, 1975.
16. Kacian, D. L., and Myers, J. C. Synthesis of extensive, possibly complete, DNA copies of poliovirus RNA in high yields and at high specific activities. Proc. Natl. Acad. Sci. U. S. A., *73:* 2191–2195, 1976.
17. Kohne, D. E., Levinson, S., Byers, M. Room temperature method for increasing the rate of DNA reassociation by many thousand fold: the phenol emulsion reassociation technique. Biochemistry, *16:* 5329–5341, 1977.
18. Lippman, M. E., and Bolan, G. Estrogen responsive human breast cancer in continuous tissue culture. Nature (Lond.), *256:* 592–593, 1975.
19. Martinson, H. G., and Wagenaar, E. B. Thermal elution chromatography of nucleic acids on hydroxylapatite. Biochim. Biophys. Acta., *474:* 445–455, 1977.
20. McCoy, J. L., Jerome, L. F., Anderson, C., Cannon, G. B., Alford, T. C., Connor, R. J. Oldham, R. K., and Herberman, H. Leukocyte migration inhibition by soluble extracts of MCF-7 tissue culture cell line derived from breast carcinoma. J. Natl. Cancer Inst., *57:* 1045–1049, 1974.
21. McGrath, C. M., Grant, P. M., Soule, H. D., Glancy, T., and Rich, M. Replication of oncornavirus-like particle in a human breast carcinoma cell line, MCF-7. Nature (Lond.), *252:* 247–250, 1974.
22. Miller, N. R., Saxinger, W. C., Reitz, M. S., Gallegher, R. E., Wu, A. M., Gallo, R. C., and Gillespie, D. Systematics of RNA tumor viruses and virus-like particles of human origin. Proc. Natl. Acad. Sci. U. S. A., *71:* 3177–3181, 1974.
23. Moore, D. H., Sarkar, N. H., Kelly, C. E., Pillsbury, N., and Charney, J. Type B particles in human milk. Tex. Rep. Biol. Med., *27:* 1027–1039, 1971.
24. Myers, J. C., Spiegelman, S., and Kacian, D. L. Synthesis of full-length DNA copies of avian myeloblastosis virus RNA in high yields. Proc. Natl. Acad. Sci. U. S. A., *74:* 2840–2843, 1977.
25. Ohno, T., and Spiegelman, S. Antigenic relatedness of the DNA polymerase of human breast cancer particles to the enzyme of the MPMV. Proc. Natl. Acad. Sci., U. S. A., *74:* 2144–2148, 1977.
26. Rothenberg, E., and Baltimore, D. Synthesis of long representative DNA copies of the murine RNA tumor virus genome. J. Virol., *17:* 168–174, 1976.
27. Schlom, J., Michalides, R., Kufe, D., Hehlmann, R., Spiegelman, S., Bentvelzen, P., and Hageman, P. A comparative study of the biologic and molecular basis of murine mammary carcinoma: a model for human breast cancer. J. Natl. Cancer Inst., *51:* 541–551, 1973.
28. Schlom, J., Spiegelman, S., and Moore, D. H. RNA-Dependent DNA polymerase activity in virus-like particles isolated from human milk. Nature (Lond.), *231:* 97–100, 1971.
29. Soule, H. D., Vasquez, J., Long, A., Albert, S., and Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst., *51:* 1409–1413, 1973.
30. Spiegelman, S., Axel, R., and Schlom, J. Virus related RNA in human and mouse mammary tumors. J. Natl. Cancer Inst., *48:* 1205–1211, 1972.
31. Spiegelman, S., Burny, A., Das, M. R., Keydar, J., Schlom, J., Travnicek, M., and Watson, K. Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. Nature (Lond.), *227:* 563–567, 1970.
32. Vaidya, A. B., Black, M. M., Dion, A. S., and Moore, D. H. Homology between human breast tumor RNA and MMTV genome. Nature (Lond.), *249:* 565–567, 1974.