Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3304–3308, August 1974

Evidence of Particle-Associated RNA-Directed DNA Polymerase and High Molecular Weight RNA in Human Gastrointestinal and Lung Malignancies

(C-type virus/simultaneous detection test/molecular hybridization/homology)

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Contributed by S. Spiegelman, May 20, 1974

ABSTRACT Previous communications have demonstrated that neoplastic cells of human breast cancers, leukemias, lymphomas, sarcomas, and brain tumors contain particles with similar diagnostic attributes as those found in RNA oncornaviruses. The present paper concerns malignancies of the gastrointestinal and pulmonary systems for which, like brain tumors, no suitable animal model or corresponding virus exists. By means of the simultaneous detection assay, these tumors have been found to contain 70S RNA and RNA-directed DNA polymerase encapsulated in particulate components possessing densities of 1.16–1.17 g/ml. Twelve out of 17 (70%) colon carcinomas, three out of five (60%) gastric carcinomas, all of three rectal carcinomas, and seven out of ten (70%) lung carcinomas contained detectable levels of these virus-like entities. None of the corresponding normal tissues was positive.

Molecular hybridization (1) with radioactive DNA probes synthesized with the aid of appropriate murine RNA tumor viruses has demonstrated the presence of viral-related RNA in human neoplasias (2–6). The application of the simultaneous detection test (7) to these tumors revealed that the RNA so detected was 70S in size and was encapsulated with an RNA-dependent DNA polymerase (8–10) in particles of a density characteristic of the animal oncornaviruses (9), Thus, there is evidence that some human neoplasias possess particles with properties that satisfy three of the criteria diagnostic of the viral etiologic agents identified with homologous diseases in animal models.

Initially, [^{3}H]DNA complementary copies of the RNA from animal tumor viruses were used as probes for homologous information in the RNA of corresponding human tumors. Subsequently, after the particles were identified and isolated, [^{3}H]DNA was synthesized with the particle RNA from human neoplastic tissues serving as templates. These probes were then used to determine sequence relationships between the RNA of the human particles and the RNAs of the corresponding animal oncornaviruses. When [^{3}H]DNAs synthesized with particles isolated from human breast cancer and from human leukemias were challenged with various RNAs from both murine and avian oncornaviruses, the [^{3}H]-DNA probes derived from the different human malignancies hybridized uniquely to the RNAs of the viruses that cause the corresponding neoplastic diseases in mice (9, 10).

Attention was then directed (11) towards human neoplasms of the central nervous systems for which no corresponding viral agent or animal models existed. The results demonstrated that human central nervous system neoplasms contain particulate entities possessing diagnostic characteristics of RNA tumor viruses. The RNAs of these central nervous system tumor particles show sequence homology to the RNA extracted from human brain tumors. There was, however, no detectable sequence relation to the RNAs of the avian or murine agents known to be causative of leukemias, lymphomas, sarcomas, and mammary tumors in animal models.

We describe here the results of similar studies of malignancies of the gastrointestinal tract (both upper and lower) and of the lungs. As in our previous studies the experiments were designed to answer the following questions: (*1*) Do these human malignancies contain particulate elements encapsulating a high-molecular-weight RNA with an associated RNA-directed DNA polymerase (reverse transcriptase)? (*2*) Does the DNA synthesized by these particles share sequences with the RNA from the homologous tumor or with the RNAs of known animal RNA tumor viruses?

MATERIALS AND METHODS

Tissue Samples. All malignant and normal samples used were surgical specimens that were frozen and stored at −70° within 1 hr after removal. The samples included 25 gastrointestinal (GI) tract tumors (both upper and lower) and 10 lung tumors. Histologically normal tissues were obtained from the contiguous GI tracts of the majority of the patients harboring GI tumors. In six instances of patients with lung tumors, histologically normal lung tissue samples were obtained for controls.

Simultaneous Detection of 70S RNA and Reverse Transcriptase. Tissues (1–4 g) were finely minced and disrupted with a Silverson homogenizer at 4° in TNE buffer (0.01 M Tris·HCl, 0.15 M NaCl, 0.01 M ethylenediaminetetraacetate). This tissue homogenate was then centrifuged at 4000 × *g* for 10 min at 0° and the supernatant was recentrifuged at 10,000 × *g* for 10 min at 0°. The resulting supernatant was then layered on a 15-ml column of 20% glycerol in TNE and spun at 100,000 × *g* for 1 hr at 1° in a Spinco SW-27 rotor. The resulting pellet (P-180) was resuspended in 0.01 M Tris·HCl, pH 8.3 (100 μl of 0.01 M Tris·HCl/g of tumor tissue). The suspension was preincubated at 0° in concentrations of 1.0% NP-40 detergent (Shell), 0.1 M dithiothreitol for 10 min. Actinomycin D (5 mg/ml) 1/10 volume and 4 M NaCl 1/20 volume were added, and these were then added to a standard endogenous RNA-instructed DNA polymerase re-

Abbreviations: GI, gastrointestinal; TNE, Tris-saline-ethylenediaminetetraacetate; pRNA, polysome RNA; CA, cancer.

action mixture containing 10 mM Tris·HCl, pH 8.3, 8 mM $MgCl_2$, 1.7 mM each of dGTP, dCTP, dATP, and 1 mCi of [^{3}H]dTTP per 200 µl volume of suspension. After 7 min of incubation at 37°, the reaction was terminated by chilling to 0° and adding 0.1 volume of 4 M NaCl and 10% sodium dodecyl sulfate. Extraction was achieved by the addition of an equal volume of a phenol–cresol (7:1) mixture containing 8 hydroxyquinoline (0.2 g/100-ml mixture). This was shaken in a vortex at 25° for 3 min and centrifuged at 16,300 × *g* for 10 min at the same temperature (in a Sorvall centrifuge). The aqueous phase was then layered over a linear glycerol gradient (10–30% in TNE) and centrifuged at 41,000 rpm for 210 min at 4° with a Spinco SW-41 rotor. External size markers were 70S [^{3}H]RNA from avian myeloblastosis virus (AMV). Fractions were collected from below and 50-µl aliquots assayed for trichloroacetic acid-precipitable radioactivity. If the 50-µl aliquots proved to contain enough 70S RNA·[^{3}H]DNA to warrant further characterization, then the corresponding portions were pooled, LiCl was added to a final concentration of 0.4 M, and then precipitated with 2 volumes of absolute ethanol. After alkaline destruction of the complexed RNAs, the resulting [^{3}H]DNA was then used for hybridization studies.

Preparation of Cytoplasmic (P-180) RNA. Malignant and normal tissues were minced and then disrupted with a Silverson 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 then spun at 4000 × *g* for 10 min at 0° and the resulting supernatant was centrifuged at 10,000 × *g* for 10 min at the same temperature. This latter supernatant was layered on 15 ml of 25% sucrose in TNM and centrifuged at 180,000 × *g* for 180 min at 4° in a Spinco 60 Ti rotor. The resulting pellet (P-180) was suspended in TNM, 0.4 M NaCl and 1% sodium dodecyl sulfate and the RNA was extracted three times with an equal volume of phenol–cresol (pH 8.4). The nucleic acids were precipitated by the addition of 2 volumes of ethanol and 0.1 volume of 4 M LiCl.

Hybridization. The [^{3}H]DNA (550–1800 cpm) was annealed to cytoplasmic RNAs of the tumors of origin where possible and, in some instances, to RNAs of the same type of neoplasia. The [^{3}H]DNA was first melted in a 50% formamide concentration at 80° for 10 min. After quick chilling to 0°, the RNAs were added and the mixture was brought to 0.4 M NaCl, 50% formamide, and 0.05 M ethylenediaminetetraacetate, in a total volume of 100 µl. This was then incubated for 24 hr at 37°. Thereafter the reaction mixture was added to 5.4 ml of 5 mM ethylenediaminetetraacetate with an equal volume of saturated Cs_2SO_4 to yield a starting density of 1.52. This was centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15°. Fractions of 0.4 ml were collected and assayed for acid-precipitable radioactivity.

Similar hybridizations were also carried out between tumor [^{3}H]DNA, viral RNAs, normal tissue polysomal RNAs (pRNAs), and tumor pRNAs, which were prepared as described previously (9).

Density of Particles Containing 70S RNA and RNA-Instructed DNA Polymerase. A P-180 fraction was prepared from tumor tissues as described above. This was then suspended in TNE buffer and layered on a linear gradient of 25–55% sucrose in TNE buffer and centrifuged at 27,000 rpm in a Spinco SW-27 rotor at 2° for 210 min. Six to ten equal fractions were gathered from below, their refractive indices were determined, and the fractions were then diluted with TNE to a sucrose concentration of less than 10%. They were pelleted through 100,000 × *g* spins. Simultaneous detection for 70S RNA and RNA-instructed DNA polymerase was then carried out on each fraction. The amount of [^{3}H]DNA synthesized and complexed to 70S RNA by an endogenous RNA-directed DNA polymerase was determined by glycerol velocity centrifugation.

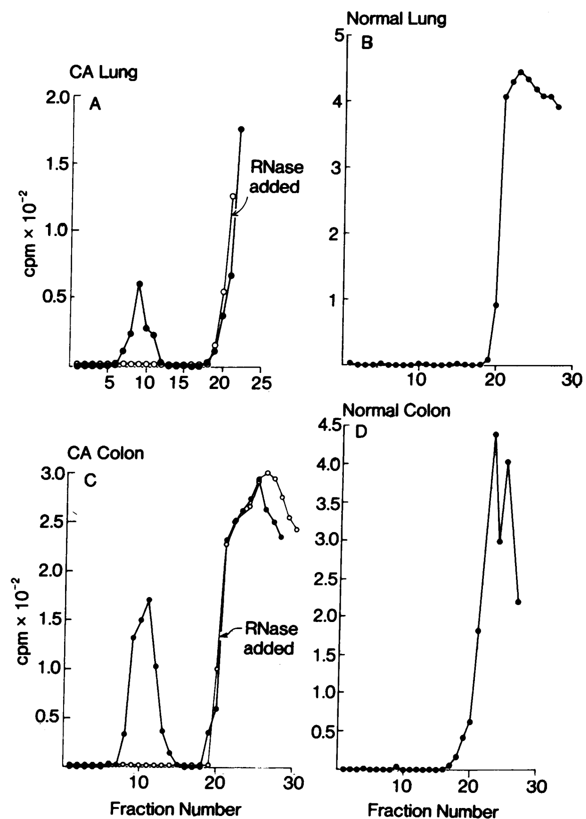

FIG. 1. Simultaneous detection of the 70S RNA·[^{3}H]DNA complex in colon and lung malignancies as well as normal tissues. Effect of ribonuclease on the high-molecular-weight RNA·[^{3}H]DNA complex. The P-180 pellet was resuspended in 0.01 M Tris·HCl, pH 8.3, buffer and divided into two equal parts. A standard RNA-directed DNA polymerase reaction was performed on one part (●). After incubation for 15 min at 37° the nucleic acid complex was extracted with phenol–cresol and sized on a 10–30% linear glycerol gradient, shown here. The other half was incubated in the presence of RNase A (50 µg/ml) for 15 min at 37° (○). A similar RNA-directed DNA polymerase reaction and centrifugation was then performed. CA, cancer.

RESULTS

Fig. 1 gives examples of simultaneous detections for RNA-dependent DNA polymerase and 70S RNA on malignant and normal samples from lung and colon. It is evident from Fig. 1*A* and *C* that tumors of both the lung and the colon show evidence of [^{3}H]DNA peaks in the 70S position of the glycerol velocity gradient. Further, in both instances pretreatment with ribonuclease leads to the disappearance of the [^{3}H]DNA from the 70S region, demonstrating its association with a large RNA molecule. Finally, no such DNA peaks are observed when simultaneous detection tests are carried out with normal samples of either lung (Fig. 1*B*) or colon (Fig. 1*D*).

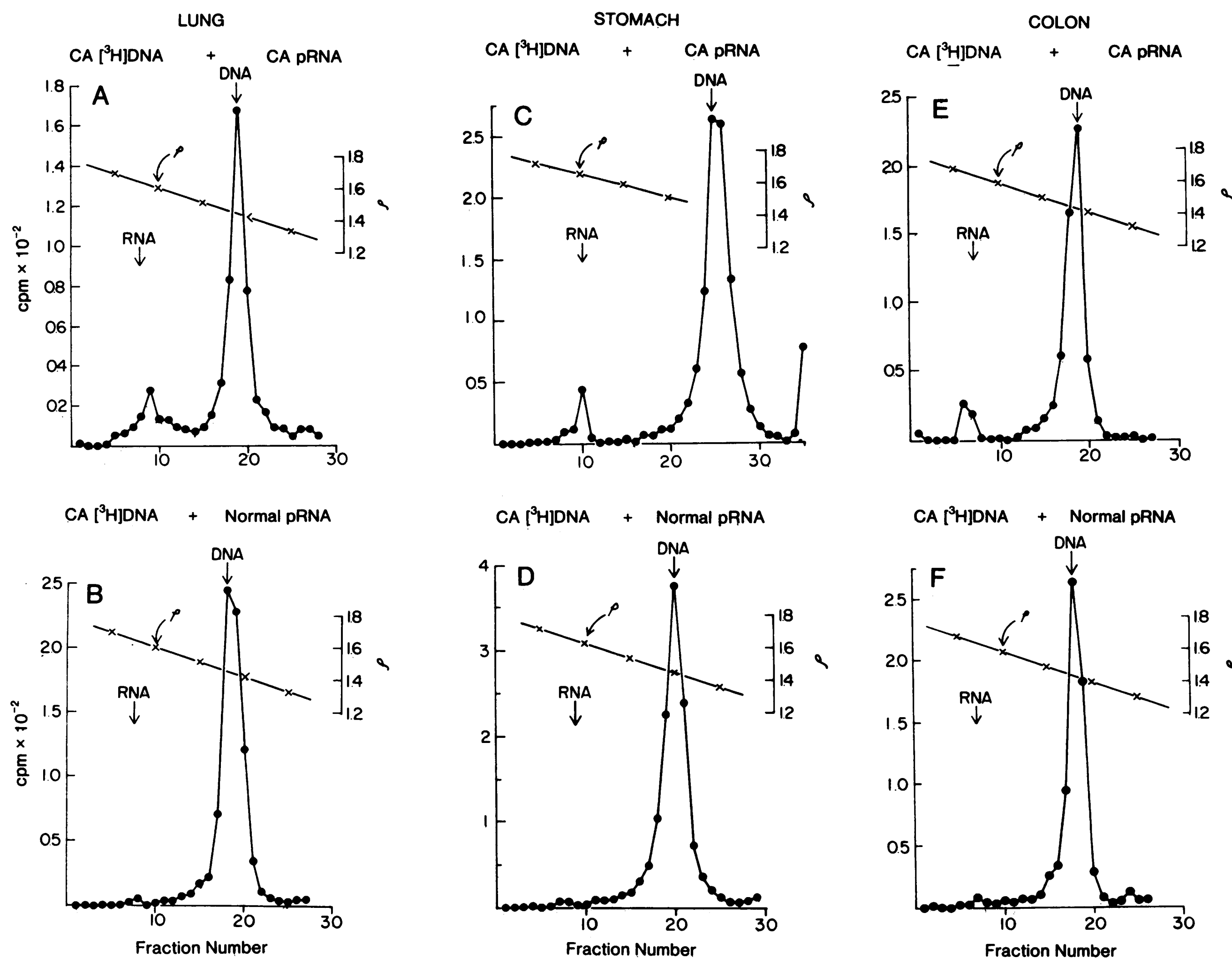

FIG. 2. Hybridization of [^{3}H]DNA made with particles from lung, stomach, and colon malignancies with their corresponding pRNAs and with their corresponding histologically normal tissues gathered from contiguous or adjacent areas. Details are described in *Materials and Methods*. ρ is density in g/ml.

Specific Nature of the [^{3}H]DNA Synthesized. The appearance of [^{3}H]DNA in the 70S region of the glycerol gradients and its elimination by ribonuclease demonstrate the existence in the malignancies examined of a ribonuclease-sensitive synthesis of an RNA·[^{3}H]DNA complex with a sedimentation coefficient of 70S. This does not of itself, however, establish that an RNA-directed DNA polymerase has been identified. Evidence must further be provided showing that the [^{3}H]-DNA synthesized can in fact be hybridized to its presumed template, which should exist in the cytoplasmic fraction. To check this, the [^{3}H]DNA synthesized by the particles derived from each tumor is annealed to the RNA (the pRNA described in *Materials and Methods*) derived from the same tumor. The outcome can then be compared with similar hybridizations to a corresponding RNA preparation from analogous normal tissue. Fig. 2 shows the results of such experiments in which the [^{3}H]DNA synthesized by tumor particles of the lung, stomach, and colon is annealed to the pRNA of the homologous tumors (Fig. 2*A*, *C*, and *E*, respectively) and to the pRNA of the corresponding normal tissues (Fig. 2*B*, *D*, and *F*, respectively). It is clear that the [^{3}H]DNA synthesized by the particles taken from each one of the tumors hybridizes back to the RNA derived from the same tumor and not to the RNA obtained from the corresponding normal tissues.

Density of Particles Encapsulating 70S RNA and the RNA-Directed DNA Polymerase. The very fact that the P-180 fraction of the malignant tissue yielded a 70S RNA·[^{3}H]DNA complex in an endogenous reaction implies that the enzyme involved must be physically associated with its 70S RNA template. It was of interest to determine whether RNA enzyme complex was to be found in particles possessing the density characteristic of an RNA tumor virus. The procedures for doing this are detailed in *Materials and Methods* and involve subjecting the P-180 fraction to isopycnic separation in sucrose gradients and locating the peak fraction yielding positive simultaneous detection reactions. When this was done, it was found that the peak occurred at 1.168 g/ml for the lung tumor particles and at 1.17 g/ml for the colon tumor particles. Thus in both instances the particles possessing the 70S RNA and RNA-dependent DNA polymerase possess densities characteristic of the RNA tumor viruses, again in agreement with other neoplasias examined.

A Survey of Lung and GI Tumors for the Presence of Particles Containing 70S RNA and RNA-Directed DNA Polymerase. By the procedures described in *Materials and Methods* and exemplified by the results described in Figs. 1 and 2, 17 colon tumors, 5 stomach tumors, 3 rectal tumors, and 10 lung tumors

TABLE 1. *Simultaneous detection assay of 70S RNA and RNA-directed DNA polymerase in lung and GI tumors*

	Carcinoma			Normal control tissue	
Organ	70S peak cpm	Reaction	Hybridization	70S peak cpm	Reaction
Colon					
C1	4,000	+	+ (4)	14	−
C2	4,000	+	+ (3)	8	−
C3	520	+		24	−
C4	48	−		7	−
C5	38	−		31	−
C6	269	+	+ (1)	21	−
C7	4,005	+	+ (4)	21	−
C8	826	+	+ (1)		
C9	11,000	+	+ (3)	9	−
C10	3,289	+	+ (2)	16	−
C11	2,750	+	+ (2)	17	−
C12	12	−			
C13	38	−			
C14	27	−			
C15	426	+		48	−
C16	588	+	− (1)	52	−
C17	5,333	+		45	−
Rectum					
R1	353	+	− (1)	15	−
R2	353	+			
R3	2,600	+	+ (4)	18	−
Stomach					
S1	79	−		42	−
S2	5,885	+	+ (4)	20	−
S3	20	−			
S4	3,000	+	+ (2)	34	−
S5	820	+	− (1)	38	−
Lung					
L1	1,300	+	+ (1)		
L2	1,217	+	+ (4)	35	−
L3	687	+	+ (3)	18	−
L4	704	+	+ (4)	25	−
L5	600	+		29	−
L6	780	+		18	−
L7	660	+		28	−
L8	58	−			
L9	45	−			
L10	52	−			

Simultaneous detection assays and hybridizations were carried out as described in *Materials and Methods*. Reactions above 200 cpm were regarded as positive. The numbers in parentheses indicate the number of times the hybridizations were carried out.

were assayed for the presence of particulate elements containing 70S RNA and RNA-directed DNA polymerase. In 25 cases neighboring homologous normal tissue was available to serve as controls (Table 1). The average "70S" polymerase activity ranged from 16 to 23 cpm in "normal" preparations and the activity of the negatives amongst the tumors ranged from 5 to 49 cpm. In contrast, the average activity of the 70S region amongst the positive malignant samples ranged from 610 to 3235 cpm. There was no difficulty, therefore, in cataloguing reactions as either positive or negative. In 18 of the positive tumors enough [^{3}H]DNA was synthesized by the tumor particles to permit hybridization tests. In fifteen tumors, homology of the [^{3}H]DNA was observed with cytoplasmic RNA fractions from the same or similar tumor. Wherever the amount of [^{3}H]DNA available permitted several repetitive tests (indicated by the number in parentheses) concordance was obtained. In the case of the three negative outcomes there was not enough [^{3}H]DNA available to repeat the hybridization.

TABLE 2. *Distribution of positive simultaneous detection assays*

Tissue	No. positive (average cpm)	No. negative (average cpm)	% Positive
		Malignant	
Colon	12 (2312)	5 (32)	70
Stomach	3 (3235)	2 (49)	60
Rectum	3 (1102)	0 −	100
Lung	7 (610)	3 (5)	70
		Normal	
Colon	0	13 (24)	0
Stomach	0	4 (33)	0
Rectum	0	2 (16)	0
Lung	0	6 (25)	0

Table 2 summarizes the distribution of the positive simultaneous detection assays amongst the various malignant tissues and corresponding normal samples. Seventy percent of the colon tumors showed clear evidence of 70S RNA in association with the RNA-directed DNA polymerase. Similarly, positive outcomes were obtained in 60% of the stomach tumors, 100% of the rectal tumors, and 70% of the lung tumors. None of the corresponding 25 normal tissues showed detectable evidence of particles containing the "70S" RNA and RNA-directed DNA polymerase. It should be noted that in all cases the "normal" samples come from non-malignant regions of the organ involved. The negative evidence does not necessarily mean an absolute absence of particles or of nonproductive transformation. Other techniques will be required to resolve these issues.

Examination of Sequence Homologies to Known Oncornaviruses. As in our studies of brain tumors (11) and other human cancers (2–6, 9, 10), it was of interest to determine whether the [^{3}H]DNAs synthesized by particles from lung and gastrointestinal malignancies would exhibit any homology to the RNAs isolated from some of the known animal oncornaviruses. Since our findings were uniformly negative, we will simply summarize them here. [^{3}H]DNAs prepared from lung and the three types of GI tumors were all challenged with RNAs derived from the avian myeloblastosis virus, simian sarcoma virus, Rauscher leukemia virus, and the mouse mammary tumor virus. The annealing reactions were carried out as described in *Materials and Methods* and examined in Cs_2SO_4 equilibrium gradients. Despite the relatively relaxed conditions employed, no homology was detected between any of the tumor RNAs and the viral RNAs tested. In this respect, the [^{3}H]DNA synthesized by particles from lung and GI tumors resemble the brain tumors, which also showed no homologies to the same group of viruses. This is in sharp contrast to breast tumors, which contained RNA homologous to that of mouse mammary tumor agent and to the leukemias, lymphomas, and sarcomas, which exhibited sequence homology to the RNA of the murine leukemia virus.

DISCUSSION

As in the case of the brain tumor studies (11), the present search for evidence of a viral agent in human lung gastrointestinal malignancies attempts to apply the methodologies of molecular biology in the absence of an available animal model involving a known viral agent. We, therefore, had to forego hybridization with a viral probe as the first step in detecting viral-related information in these tumors. Again, however, the simultaneous detection test (7) makes it possible to perform the necessary experiments despite the absence of a suitable animal oncornavirus. The simultaneous detection test can provide evidence for the existence in the tested malignancies of particulate elements possessing the telltale 70S RNA, RNA-directed DNA polymerase, and other features diagnostic of the RNA tumor viruses.

The negative reactions with some of the tumors tested may raise questions of the universality of the association. However, a negative outcome cannot be accepted as evidence for the *absence* of these particles. The presence of nucleases or the limit of sensitivity of the method could result in negative simultaneous detection tests.

With this paper and our previous work we have now shown the presence of particles possessing some of the diagnostic features of the RNA tumor viruses in the following human neoplasias: the acute and chronic lymphatic and myelogenous leukemias, the sarcomas, the lymphomas (including Hodgkin's disease and Burkitt's tumors), brain tumors, adenocarcinoma of the breast, carcinomas of the lung, stomach, colon and rectum. In all of these cases [^{3}H]DNA probes can now be synthesized with the aid of the corresponding particles found in these tumors.

We are now in a position to mount a study that should provide insight into the number of identifiably different particles found in human neoplasias in various organ sites. If even some of them are distinct, a pathway may well be opened for an organ site-specific assay that could be useful in both diagnosis and in monitoring therapy.

We thank the following for supplying tissue samples and histological diagnoses: Drs. J. Roth, Y. Fu, J. Fenoglio, F. Parker, and other members of the Department of Pathology, College of Physicians and Surgeons, and Dr. R. Lim of Santa Barbara, Calif. This research was supported by the National Institutes of Health, National Cancer Institute, Virus Cancer Program Contract no. 1-CP-3-3258 and by the National Cancer Institute Grant CA-02332.

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