

## RNA Related to That of a Murine Leukemia Virus in Burkitt's Tumors and Nasopharyngeal Carcinomas

D. KUFÉ, R. HEHLMANN, AND S. SPIEGELMAN

Institute of Cancer Research and Department of Human Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

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**ABSTRACT** RNA homologous to that of the Rauscher leukemia virus has been detected in Burkitt's lymphomas and nasopharyngeal carcinomas. Earlier excellent experimental evidence has linked these two human tumors with the Epstein-Barr virus, a DNA-containing agent.

The detection of viral-specific RNA in tumors (1) by molecular hybridization (2) has revealed that certain human neoplasias exhibit a specific pattern of viral-related RNAs remarkably similar to that observed in the corresponding viral-induced tumors of mice. Thus, human breast carcinomas contain an RNA possessing homology to that of the mouse mammary tumor virus. The breast tumor RNA exhibited no sequence relatedness to RNA of the Rauscher leukemia virus, an agent possessing no homology to mouse mammary tumor virus (manuscript in preparation). On the other hand, RNAs from human leukemic cells (3), human sarcomas (4), Hodgkins' disease, and other human lymphomas (5) were all unrelated to that of the breast tumor virus, showing instead unique homology to the RNA of the leukemogenic Rauscher virus.

The pattern of specificities exhibited by the human tumors examined are in accord with the experience accumulated with the mouse experimental system. It was of obvious interest to subject this intriguing parallelism to further scrutiny by a similar analysis of Burkitt's lymphoma and nasopharyngeal carcinoma, both of which have been experimentally related to the DNA-containing Epstein-Barr virus.

Burkitt's disease, a malignant lymphoma, is the most common cancer found in children living in a geographical belt extending across Central Africa. Epidemiological (6, 7), serological (7, 8), and nucleic acid hybridization (9) studies have linked this disease to the herpes-like Epstein-Barr virus detected in (10) and isolated (11) from Burkitt's lymphoma cells grown in culture. Nasopharyngeal carcinoma, a tumor containing both epithelial and lymphoid elements (12), is prevalent in certain groups in the Far East. Serological (8, 13) and hybridization (9) evidence has similarly implicated the Epstein-Barr virus in this disease.

Though arresting, such correlative data cannot by themselves permit a definitive decision on whether Epstein-Barr virus is causative or contributory in these diseases (14). Even finding sequences from this virus in the relevant human cancer cells is not conclusive, and particularly so until other types of "oncogenic" information have been sought for and eliminated as possible participants in the carcinogenic process. To shed light on these and related issues, we address ourselves to the following question: Do Burkitt's lymphomas

and nasopharyngeal carcinomas contain RNA homologous to that of the RNA tumor viruses found in our studies (1-5) of human lymphomas and other neoplasias?

The data obtained establish that African Burkitt's tumors and nasopharyngeal carcinomas do contain RNA homologous to that of the Rauscher leukemia virus.

### METHODS AND RESULTS

Annealing reactions between [<sup>3</sup>H]DNA of Rauscher virus and RNA from polysomes of tumors and normal tissues were performed in 0.4 M NaCl-50% formamide at 37° for 18 hr. These conditions were chosen because they (unpublished observations) minimize thermal degradation of RNA during the incubation. Although initial rates of hybridizations can be increased by elevation of the temperature, the extent of hybridization reached at 37° after 18 hr was the same as at higher temperatures.

Annealing reactions were analyzed in Cs<sub>2</sub>SO<sub>4</sub> gradients. This method was chosen because of its low background and superior sensitivity to other methods—such as hydroxylapatite chromatography and the use of nucleases specific for single-stranded nucleic acid. The specificity of positive reactions in Cs<sub>2</sub>SO<sub>4</sub> gradients with tumor RNA was internally monitored by parallel tests with RNA from normal tissues, as well as by challenging positive tumor RNA with DNA homologous to unrelated RNA from mouse mammary tumor virus and avian myeloblastosis virus.

### Annealing reactions with RNA from tumors

Fig. 1 shows Cs<sub>2</sub>SO<sub>4</sub> gradient profiles that result from annealing reactions between [<sup>3</sup>H]DNA from Rauscher virus and polysomal RNA isolated from several Burkitt's lymphomas and a nasopharyngeal carcinoma. The responses seen are comparable in magnitude to those obtained with the same [<sup>3</sup>H]DNA annealed to polysomal RNA preparations derived from human sarcomas and leukemic cells. In general, 1-5% of the input [<sup>3</sup>H]DNA is hybridized to the polysomal RNA from the Burkitt and nasopharyngeal carcinoma tumors. Their position in the density gradient implies that RNA in the complexes is much larger than the DNA in the complexes and, therefore, determines the density of the DNA-RNA hybrid structure.

A more extensive examination of the annealing reaction is shown in Fig. 2, in which a fixed amount of [<sup>3</sup>H]DNA from Rauscher virus is annealed to different concentrations of polysomal RNA, and the % of [<sup>3</sup>H]DNA hybridized is determined by isopycnic separation in Cs<sub>2</sub>SO<sub>4</sub> gradients. The Burkitt's

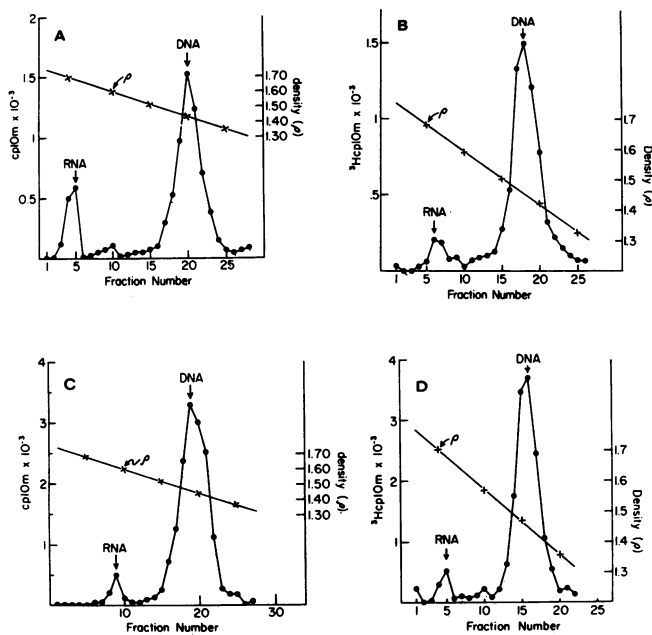


FIG. 1.  $\text{Cs}_2\text{SO}_4$  density profiles of Rauscher leukemia virus [ $^3\text{H}$ ]DNA hybridized to polysomal RNA obtained from Burkitt's tumors NA, 11719, and 12079 (A,C,D) and from nasopharyngeal carcinoma 15903 (B). [ $^3\text{H}$ ]DNA was synthesized as follows: A 1-ml reaction mixture, containing 100  $\mu\text{g}$  of viral protein purified from plasma, 50  $\mu\text{mol}$  of Tris·HCl (pH 8.3), 40  $\mu\text{mol}$  of KCl, 6  $\mu\text{mol}$  of  $\text{MgCl}_2$ , 2.5  $\mu\text{mol}$  of  $\text{S}_2$  threitol, 0.00125% NP-40 (Shell Chemical Co.), 0.8  $\mu\text{mol}$  of dGTP, dATP, and dCTP, and 50 nmol of [ $^3\text{H}$ ]TTP (8000 cpm per pmol), was incubated at 37° for 180 min. After addition of 0.5% Na dodecyl $\text{SO}_4$  and extraction with an equal volume of phenol-cresol, the [ $^3\text{H}$ ]DNA product was purified by Sephadex G-50 chromatography and treated with 0.5 M NaOH at 43° for 24 hr to hydrolyze any viral RNA present.

In studies such as those contemplated here, it must be shown before use that the radioactive DNA product bands solely in the DNA density region of a  $\text{Cs}_2\text{SO}_4$  gradient and that it hybridizes with homologous RNA, and not to normal cellular RNA.

The RNA (designated as polysomal RNA) used in the hybridizations is derived from a cytoplasmic pellet consisting of a mixture of monosomes and polysomes. Suitable care must be exercised to insure the complete removal of protein contaminants. For the preparation of polysomal RNA, the tumors were disrupted by a Silverson homogenizer at 4° in two volumes of 5% sucrose in TNM buffer [0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–2 mM  $\text{MgCl}_2$ ]. The suspension was centrifuged at 20,000  $\times g$  for 15 min at 0°. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM buffer containing 200  $\mu\text{g}$  of polyvinyl sulfate per ml and spun for 180 min at 180,000  $\times g$  in a 60 Ti rotor (Spinco). The pellet was resuspended in TNM buffer with 0.5% Na dodecyl $\text{SO}_4$  and the RNA was extracted twice with an equal volume of cresol-phenol (pH 8.0). Nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol and 0.1 volume of 4 M NaCl. The polysomal RNA was redissolved in 50% formamide–50% 5 mM EDTA. The [ $^3\text{H}$ ]DNA was denatured by incubation at 80° for 15 min in 70% formamide and subsequent quick chilling. An average of 350  $\mu\text{g}$  of polysomal RNA was hybridized to 2000 cpm of Rauscher virus- $^3\text{H}$ ]DNA in 60  $\mu\text{l}$  (0.4 M NaCl–50% formamide). The reaction was incubated for 18 hr at 37°, mixed with 11 ml of half-saturated  $\text{Cs}_2\text{SO}_4$  (starting density 1.52), and centrifuged at 44,000 rpm for 60 hr at 15° in a 50 Ti rotor (Spinco), and 0.4-ml (8-drop) fractions were collected through an 18-gauge needle from the bottom of the tube and assayed for  $\text{Cl}_3\text{CCOOH}$ -precipitable radioactivity.

lymphoma polysomal RNA (Fig. 2A) shows no evidence of saturation at 5.8 mg/ml, at which point 4.75% of the [ $^3\text{H}$ ]DNA from Rauscher virus has been involved in the complex. Similarly, the nasopharyngeal carcinoma polysomal RNA has not reached saturation at 11.3 mg/ml when 2% of the [ $^3\text{H}$ ]DNA has been hybridized.

As in previous studies, a method of recording our findings has been adopted that avoids the inconvenience of presenting the  $\text{Cs}_2\text{SO}_4$  gradient of every tissue examined. After correction for background counts, the sum of the tritium counts in the RNA density region (density 1.62–1.69 g/ml) was used to measure the amount of DNA complexed to RNA. To achieve the accuracy desired, 10-min counts (cp10m) were taken on each sample. An operational mean background and its standard deviation ( $S$ ) were empirically determined for individual machines from the total cp10m of three tubes in the negative region (i.e., tubes 2, 3, and 4) of each of 60 gradients. The convention was adopted that specimens showing a cp10m of less than three standard deviations of the background mean were considered negative.

Tables 1 and 2 record the outcomes of the annealing reactions with polysomal RNA from Burkitt's lymphomas and nasopharyngeal carcinomas. The results are recorded as the total cp10m (corrected for background) in the RNA density region, and as multiples of the mean background standard deviation. Not detailed in either table, but included in the graphical summary of Fig. 3, are the control hybridizations with polysomal RNA from various normal adult and fetal tissues. Of the 21 Burkitt's lymphomas examined, 76% yielded positive responses with Rauscher virus [ $^3\text{H}$ ]DNA, whereas 50% of the 20 nasopharyngeal carcinomas were positive. In contrast, none of the polysomal RNA preparations from the 52 normal adult and fetal tissues responded with a reaction that was unambiguously positive. The Burkitt's lymphoma clinical data presented in Table 1 demonstrate that positive

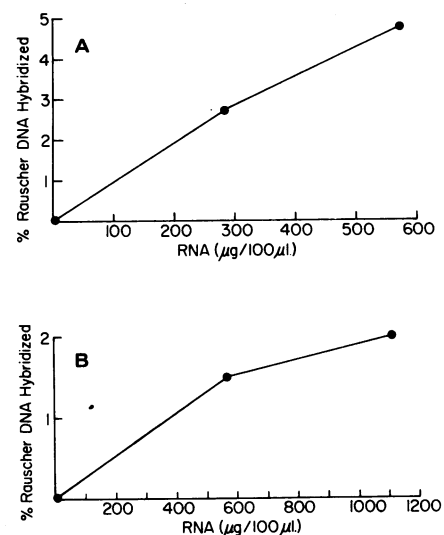


FIG. 2. Hybridization reactions between Rauscher virus- $^3\text{H}$ ]DNA and polysomal RNA from Burkitt's tumor 11719 (A) and from nasopharyngeal carcinoma 15505 (B) at different RNA concentrations. The individual annealing reactions were analyzed by  $\text{Cs}_2\text{SO}_4$  density centrifugation, and the % DNA hybridized determined by the number of [ $^3\text{H}$ ]DNA-cp10m (corrected for background) banded in the RNA region (density 1.62–1.68) of the gradients.

responses were obtained in primary cases, as well as recurrences, and that prior therapy did not significantly influence the results.

The strikingly different response of the malignant and normal samples of Rauscher virus- $^{3}\text{H}$ ]DNA argues strongly for the specificity of the positive reactions observed with the polysomal RNAs from lymphomas and carcinomas. Nevertheless, as in our previous studies (3-5), it is desirable to offer further support for this conclusion by use of  $^{3}\text{H}$ ]DNA complementary to the RNA of unrelated oncogenic viruses. This is particularly relevant in view of the recent findings (15-17) that the RNAs of tumor viruses contain extensive stretches of poly(A). Preferential complexing to Rauscher virus-RNA might then be explained by the presence of poly(T) stretches in the synthetic DNA probe. However, if the annealing reaction is specific for the overall sequence, one would not expect Burkitt's lymphoma or nasopharyngeal carcinoma polysomal RNA, positive for a reaction with Rauscher virus- $^{3}\text{H}$ ]DNA, to show the ability to hybridize with unrelated DNAs. Tables 3 and 4, as well as Fig. 4, show

TABLE 1. Test for viral-specific RNA in Burkitt's lymphomas

Number	RNA cp10m	RNA cp10m/S	Reaction	Recurrence*	Previous treatment
10438	397	4.61	+	+	+
11719	850	9.90	+	+	+
12079	821	9.54	+	+	+
14858	544	6.30	+	+	-
11392	391	4.55	+	+	+
12078	606	7.05	+	-	-
14857	271	3.15	+	-	-
15043	590	6.86	+	+	+
15976	541	6.29	+	+	+
697	292	3.40	+	-	-
1143	428	4.98	+	-	-
LU	463	5.38	+	-	-
NA	1211	14.08	+	-	-
AN	682	7.93	+	-	-
NK	371	4.31	+	+	+
P <sub>3</sub> HR-1†	524	6.10	+	-	-
16253	238	2.77	-	-	-
9471	31	0.36	-	-	-
6606	59	0.69	-	-	-
8176	179	2.08	-	-	-
6613	34	0.40	-	+	+

Results of hybridization reactions between Rauscher virus- $^{3}\text{H}$ ]DNA and polysomal RNA isolated from Burkitt's tumors. Between 200 and 600  $\mu\text{g}$  of polysomal RNA of each sample were hybridized to 2000 cpm of Rauscher virus- $^{3}\text{H}$ ]DNA, and the reactions were analyzed by  $\text{Cs}_2\text{SO}_4$  equilibrium centrifugation. The amount of DNA banding in the RNA region of the gradient (density 1.62-1.69) was determined. The results are expressed as cp10m (corrected for background) banding in the RNA region for each RNA sample tested, and as multiples of  $S$ , the operational standard deviation (see text and legend to Fig. 3). The annealing reaction is considered positive only if the cp10m per RNA region is greater than  $3S$ , thus providing 99.9% confidence. Columns 5 and 6 refer to clinical data.

21 tested, 16 positive; 76%.

\* (+) indicates a recurrent tumor and (-) indicates a primary tumor.

† Tissue culture line.

TABLE 2. Test for viral-specific RNA in nasopharyngeal carcinomas

Number	RNA cp10m	RNA cp10m/S	Reaction
12707	386	4.48	+
6720	403	4.69	+
13030	302	3.51	+
10924	535	6.22	+
13092	374	4.34	+
8507	297	3.45	+
15903	615	7.15	+
15803	324	3.76	+
7820	432	5.02	+
15505	559	6.50	+
13337	207	2.40	-
9423	26	0.30	-
8778	225	2.61	-
11682	228	2.65	-
9857	57	0.66	-
15434	171	1.98	-
2782	174	2.02	-
2723	136	1.58	-
719	143	1.66	-
16304	175	2.03	-

Results of hybridization reactions between Rauscher virus- $^{3}\text{H}$ ]DNA and polysomal RNA isolated from nasopharyngeal carcinomas. (See Table 1 for details.)

that these expectations are realized. None of the Rauscher virus-positive lymphoma or nasopharyngeal carcinoma polysomal RNAs annealed significantly to either unrelated viral DNA tested.

### DISCUSSION

Of the 21 African Burkitt's lymphomas, 76% were positive, as were 50% of 20 nasopharyngeal carcinomas. The absence of positive reactions with some of the tumors examined may raise questions of universality in the minds of some. However, there are various reasons why a negative outcome cannot be accepted as definitive evidence for the complete absence of

TABLE 3

Viral $^{3}\text{H}$ ]DNA	Burkitt's tumor no.	RNA cp10m	RNA cp10m/S	Reaction
Avian myeloblastosis	11719	52	0.60	-
Avian myeloblastosis	10438	153	1.77	-
Mouse mammary tumor	11719	138	1.60	-
Mouse mammary tumor	10438	236	2.74	-
Mouse mammary tumor	14857	3	0.04	-

Results of hybridization reactions between avian myeloblastosis virus and mouse mammary tumor virus  $^{3}\text{H}$ ]DNA and polysomal RNA isolated from Burkitt's tumors. Between 200 and 600  $\mu\text{g}$  of polysomal RNA of each sample were annealed to 2000 cpm of  $^{3}\text{H}$ ]DNA, and the reactions were subjected to  $\text{Cs}_2\text{SO}_4$  equilibrium centrifugation. The data are expressed on cp10m (corrected for background) banding in the RNA region of the gradient, and as multiples of the standard deviation ( $S$ ) (as described in the legends to Table 1 and Fig. 3.)

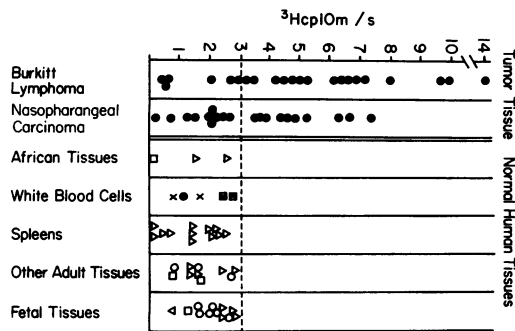


FIG. 3. Results of hybridization reactions with Rauscher virus- $^3\text{H}$ ]DNA and polysomal RNA from Burkitt's tumors, nasopharyngeal carcinomas, and normal human cells. The polysomal RNAs of normal tissues were derived from normal leukocytes ( $\times$ ), PHA-stimulated lymphocytes ( $\boxtimes$ ), a human lymphocyte cell line, NC37 ( $\otimes$ ), spleens, other adult tissues: liver ( $\Delta$ ), intestine ( $\circ$ ), striated muscle ( $\square$ ), fetal tissues: liver ( $\Delta$ ), lung ( $\nabla$ ), limbs ( $\circ$ ), placenta ( $\square$ ), and African control tissues: tonsillitis ( $\Delta$ ), benign mandibular cyst ( $\square$ ). The reactions were then subjected to  $\text{Cs}_2\text{SO}_4$  equilibrium density centrifugation as described under Fig. 1. The amount of  $^3\text{H}$ ]DNA, expressed as cp10m corrected for background banding in the density region of RNA (density 1.62–1.69), was determined for each reaction. An operational mean and standard deviation ( $S$ ) were then determined for each counter by the total cp10m of three tubes (i.e., 2,3,4) of each of 60 gradients. The number of  $^3\text{H}$ ]DNA cp10m corrected for background banding in the RNA region of the gradient was then divided by the appropriate operational standard deviation. Any reaction with cp10m in the RNA region less than  $3S$  is considered negative, thus providing 99.9% confidence that those reactions retained as positive (greater than  $3S$ ) are significant.

the relevant RNA, whether the tissue being tested is neoplastic or normal. In the first place, all of the samples tested in the present series were collected in laboratories remote from our own. Any significant delay between removal and freezing of the sample, or if it suffers more than one freezing and thawing, can lead to loss of the relevant RNA by nucleolytic degradation. The sensitivity of detection of complementary RNA is limited by the amount of polysomal RNA that can be dissolved in the annealing mixture, a difficulty that will be obviated by the development of a suitable enrichment procedure for the pertinent RNA fraction. Finally, it may be

TABLE 4

Viral $^3\text{H}$ ]DNA product	Nasopharyngeal carcinoma	RNA cp10m	RNA cp10m/ $S$	Reaction
Avian myeloblastosis	15505	146	1.69	—
Avian myeloblastosis	6720	149	1.73	—
Mouse mammary tumor	15903	175	2.03	—
Mouse mammary tumor	15505	226	2.62	—
Mouse mammary tumor	15803	207	2.40	—

Results of hybridization reactions between avian myeloblastosis virus and mouse mammary tumor virus  $^3\text{H}$ ]DNA and polysomal RNA obtained from nasopharyngeal carcinomas.

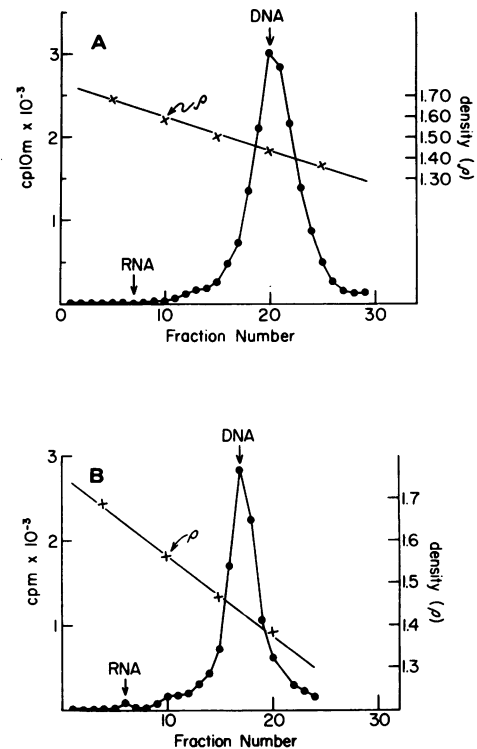


FIG. 4.  $\text{Cs}_2\text{SO}_4$  density profiles of avian myeloblastosis virus  $^3\text{H}$ ]DNA hybridized to polysomal RNA obtained from Burkitt's tumor 11719 positive in a reaction with Rauscher virus- $^3\text{H}$ ]DNA (A) and of mouse mammary tumor virus  $^3\text{H}$ ]DNA hybridized to polysomal RNA from nasopharyngeal carcinoma 15903 positive with  $^3\text{H}$ ]DNA from Rauscher virus (B). The annealing reactions were analyzed by  $\text{Cs}_2\text{SO}_4$  density centrifugation as described in Fig. 1.

worth explicitly noting an inherent difficulty in the kinds of hybridizations we can perform. The DNA probes generated by RNA-directed DNA polymerase do not constitute complete copies of the viral RNAs used as templates. Both of these features diminish the effectiveness of these probes as detecting devices. The eventual availability of proper DNA transcripts of the appropriate viral RNA will greatly improve the sensitivity and certainty of the annealing reactions. Until this result is achieved, and the other technical difficulties noted are overcome, negative outcomes must remain uninterpretable.

The fact that we find RNA that is related to Rauscher virus-RNA does not of course establish the presence of tumor viruses in these human malignancies. One must now perform experiments designed to answer the following two questions: (i) How large is the RNA in question? and (ii) Is it associated physically with a reverse transcriptase in structures characteristic of complete or incomplete virus particles? The requisite techniques to answer these questions have been developed (18, 19).

It is obviously desirable and necessary to perform similar studies on those animal neoplasias in which a DNA virus has been implicated, including the Lucké tumor of the frog, tumors induced by polyoma, and by SV-40 viruses. Even the avian Marek's disease, where the evidence for involvement of a herpes-like virus as the causative agent is impressive (20), could conceivably yield unexpected information of value.

Of particular interest is infectious mononucleosis in

humans, a self-limiting non-neoplastic condition in which prospective studies (21, 22) have strongly implicated the Epstein-Barr virus. This benign disease may well provide the opportunity to identify the type of viral-related information that is unique to malignant tissues.

Finally, we want to emphasize that we draw no conclusions on the etiology of Burkitt's lymphoma and nasopharyngeal carcinoma from the experiments described here. The presence of RNA homologous to that of Rauscher virus is obviously an observation worthy of note to those who wish to understand the biology of these tumors. The etiologic questions and implications generated by these findings will be resolved only by further investigation. There also remains the question of a possible homology between Epstein-Barr virus-DNA and Rauscher virus-RNA.

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