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Burkitt's Tumors Contain Particles Encapsulating RNA-Instructed DNA Polymerase and High Molecular Weight Virus-Related RNA

(Epstein–Barr virus/Rauscher leukemia virus/70S RNA)

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ABSTRACT **Burkitt's lymphomas, linked by previous studies with the DNA-containing Epstein–Barr virus, contain an RNA related in sequence to that of Rauscher leukemia virus. The present study establishes that the viral-related RNA found in Burkitt's tumors is a 70S component encapsulated with RNA-instructed DNA polymerase in a particle possessing a density characteristic of RNA tumor viruses. Further, the DNA synthesized by the Burkitt particles hybridizes specifically to the RNA of Rauscher leukemia virus. Thus, four features characteristic of a known oncogenic RNA agent are also exhibited by particles found with a high (87%) frequency in Burkitt's tumors. The relation between the RNA particle and the Epstein–Barr virus and their etiological roles remains to be elucidated. However, relevant to these issues is the finding reported here that the presence of Epstein–Barr virus information in nonneoplastic cells does not lead to the production of the RNA particles that have been detected now in three different human neoplasias, including leukemias, breast cancer, and Burkitt's disease.**

We have shown by RNA–DNA hybridization (1) that human leukemias (2), sarcomas (3), and lymphomas (4) all contain RNA related in sequence to that of a murine leukemogenic agent. More recently, we have demonstrated (5) that a similar situation obtains in Burkitt's disease, a malignant lymphoma found in certain African children. In many ways this last observation was most arresting since epidemiological (6, 7), serological (7, 8), and nucleic acid hybridization studies (9) had already linked Burkitt's lymphoma to the DNA-containing herpes-like Epstein–Barr virus detected in (10) and isolated from (11) Burkitt's tumor cells grown in culture.

We emphasized that finding the appropriate viral-related RNA in human neoplasias, including breast cancer (12), did not establish the presence, let alone the involvement, of a viral agent. To shed further light on these and related etiologic issues required the performance of experiments designed to answer the following questions concerning the tumor-specific RNA: (*a*) How large is the RNA being detected? (*b*) Is it associated with an RNA-instructed DNA polymerase? (*c*) Are the two found in a structure possessing a known physical characteristic of an RNA tumor virus? (*d*) Finally, if a large RNA is found in association with RNA-instructed DNA polymerase, is the DNA synthesized by the two in concert complementary to the RNA of the corresponding animal tumor virus?

Abbreviations: RLV, Rauscher leukemia virus; AMV, avian myeloblastosis virus.

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The experimental resolution of these questions was made feasible by the development (13–15) of a technique for the simultaneous detection of RNA-instructed DNA polymerase and high molecular weight (70S) RNA, two diagnostic features of the RNA tumor viruses. This method has been successfully applied to demonstrate complexes of 70S RNA and RNA-instructed DNA polymerase in the peripheral leukocytes of all types of leukemic patients (16) and in human breast cancers (17). In all the human malignancies examined, the high molecular weight RNA has been found to be encapsulated with its unique DNA polymerase in a particle possessing the density characteristic of the RNA tumor viruses.

We report here the detection of a 70S RNA-instructed DNA polymerase in 87% of the Burkitt's lymphomas examined. We further show 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 Rauscher leukemia virus. Finally, this activity was not detectable in any of five lymphoblastoid cell lines known to contain the genome of the Epstein–Barr virus.

METHODS AND RESULTS

The Detection of 70S RNA-Instructed DNA Polymerase in Tumor Tissue and Cells. The enrichment of possible virus particles is accomplished by disruption of the cells in the presence of EDTA to destroy the ribosomal structures. After removal of nuclei and mitochondria, the cellular supernatant is centrifuged at 98,000 × *g* through 20% glycerol in TNE [0.01 M Tris·HCl (pH 8.3)–0.15 M NaCl–0.01 M EDTA]. The cytoplasmic pellet is then treated with NP-40 to disrupt possible virus particles and used in an assay of endogenous RNA-instructed DNA polymerase run in the presence of 50 μg/ml of actinomycin D to inhibit DNA-directed DNA synthesis. After 15 min of synthesis at 37°, the reaction mixture is deproteinized and analyzed on a glycerol sedimentation velocity gradient with appropriate size markers. The detailed procedures for the isolation and assay of the cytoplasmic pellet are described in the legend to Fig. 1.

Figs. 1*A*, *B*, and *C* show representative 70S RNA–DNA complexes synthesized by the pellet fractions of biopsy specimens of Burkitt's tumors. In certain samples, additional peaks have been observed at positions of 35 S. It is further demonstrated, as exemplified in Fig. 1*C*, that these complexes contain a 70S RNA molecule since prior treatment with RNase eliminates the peak. When equivalent quantities of peripheral leukocytes of a patient with infectious mononucleosis were

analyzed by the same technique, no incorporation of [^{3}H]TTP into a rapidly sedimenting structure was detected (Fig. 1*D*).

Table 1 summarizes the findings with 11 Burkitt's tumors and includes the modality of chemotherapy and the titer for viral capsid antigen. The 70S region of the glycerol gradient was located with the aid of external size markers and the number of cpm included in it was taken as a measure of the presence and extent of the reaction. The Burkitt's tumors as a group yielded an average of 304 cpm in the 70S region compared with an average of 11 cpm for non-Burkitt control material listed in Table 2. On the basis of the low average for negatives, we have arbitrarily designated any reaction yielding more than 30 cpm in the 70S region as being positive. With these criteria, two of the Burkitt's tumors listed in Table 1 were negative, yielding an average of 14 cpm, and nine were positive with an average of 364 cpm in the 70S region. Six of the positive tumors were tested for ribonuclease sensitivity of the 70S RNA–DNA complex, and in every instance the complex was destroyed. It should further be noted that four more Burkitt's tumors were examined (Table 4) and found to be positive. Thus, 87% of the Burkitt samples included in the present survey gave clear positive responses.

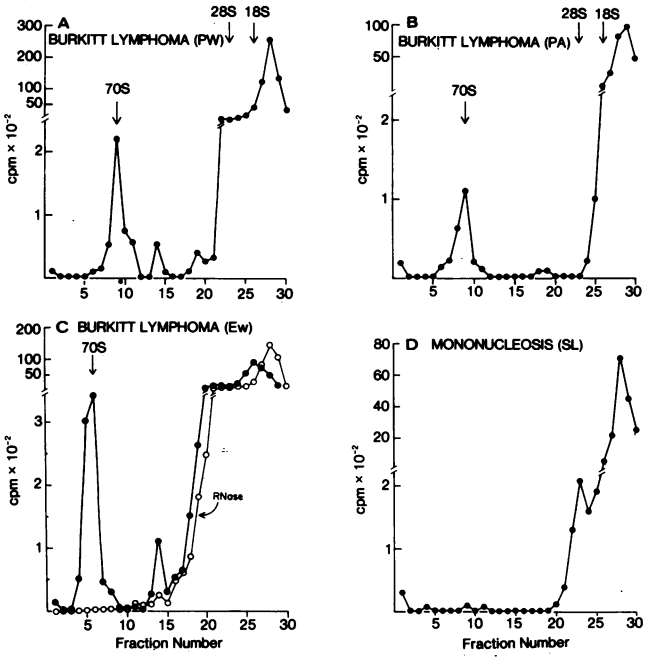

FIG. 1. Detection of 70S RNA–[^{3}H]DNA in Burkitt's lymphoma tissue: (*A*), (*B*), and (*C*), and in peripheral leukocytes from a patient with acute mononucleosis (*D*). 1–2 g of tissue was finely minced and disrupted with a Silverson homogenizer at 4° in TNE buffer [0.01 Tris·HCl (pH 8.3)–0.15 M NaCl–0.01 M EDTA]. This suspension was centrifuged at 4000 × *g* for 10 min at 2°, and the supernatant was recentrifuged at 10,000 × *g* for 10 min at 2°. The resulting supernatant fluid was then layered on a 13-ml column of 20% glycerol in TNE and spun at 98,000 × *g* for 1 hr at 4° in a SW-27 rotor (Spinco). The resulting pellet was resuspended in 0.25 ml of 0.01 M Tris·HCl (pH 8.3) brought to 0.1% Nonidet P-40 (Shell Chemical Co.) and incubated at 0° for 15 min. DNA was synthesized in a RNA-instructed DNA polymerase reaction mixture (final volume 0.5 ml) containing: 50 μmol of Tris·HCl (pH 8.3), 20 μmol of NaCl, 6 μmol of $MgCl_2$, 100 μmol each of dATP, dGTP, dCTP, and 50 μmol of [^{3}H]dTTP (50 Ci/mmol). 50 μg/ml of actinomycin D was added to inhibit DNA-instructed DNA synthesis. After incubation at 37° for 15 min, the reaction was adjusted to 0.2 M NaCl and 1% sodium dodecyl sulfate. 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 for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10–30% in TNE) and centrifuged at 40,000 rpm (200,000 × *g*) for 180 min at 2°. Fractions were collected from below and assayed for Cl_3CCOOH-precipitable radioactivity. In (*C*), one aliquot of the product was run directly on the glycerol gradient, while the other aliquot was incubated in the presence of RNase A (50 μg/ml) and RNase T1 (50 μg/ml) for 15 min at 37° before sedimentation analysis.

Table 2 lists a set of six non-Burkitt samples examined by the simultaneous detection test. These were chosen as controls for examination since they are particularly relevant to the question of the relation of the Epstein–Barr virus to the RNA particles being detected in the Burkitt's tumors. The first one is derived from the peripheral leukocytes of a patient with infectious mononucleosis, a self-limiting nonneoplastic condition in which prospective studies (18, 19) have strongly implicated the Epstein–Barr virus. The second is a cell line derived from the lymphocytes of an infectious mononucleosis patient. This line and the remaining four lymphoblastoid lines listed are known to contain the Epstein–Barr virus genome on the basis of the presence of virus-associated antigens (20). All of these lines were negative for the 70S RNA-directed

TABLE 1. *Simultaneous detection of 70S RNA and RNA-instructed DNA polymerase in Burkitt's lymphomas**

Name	Treatment	Epstein–Barr virus (VCA) titer	cpm 70S	RNase sensitivity	Reaction
1. An	None		1239	+	+
2. Na	None		196	+	+
3. Ew	CTX, MTX, VCR	320	786	+	+
4. Lu	CTX	640–1280	243	+	+
5. Ir	None	80–160	100	+	+
6. Ke	None	640	11		−
7. Am	CTX	640	16		−
8. Al	None	320–640	52	+	+
9. Pa	VCR, ORT	320	224	NT	+
10. Pw	None	NT	430	NT	+
11. Na	None		48	NT	+

Avg. = 304 cpm
Avg. of positives = 369 cpm
Avg. of negatives = 14 cpm

* 9 pos/11 = 82%; 2 neg/11 = 18%.

NT: not tested; CTX: cyclophosphamide; MTX: methotrexate; ORT: orthomerphalan; VCR: vincristine; VCA; viral capsid antigen. Preparation of tumor pellet fractions and assay for synthesis of 70S RNA–[^{3}H]DNA complex is as described in the legend to Fig. 1. The sum of the cpm in the 70S position monitored by external size markers is recorded, and is designated as positive if the cpm exceed 30. RNase sensitivity depicts abolition by RNase A and T1 of acid-precipitable radioactivity in the 70S region of the glycerol gradients, as described in the legend to Fig. 1. The clinical data, including modality of chemotherapy and Epstein–Barr virus VCA titer at the time of biopsy, are self explanatory.

TABLE 2. *Control lymphoblastoid cells*

Cells	Donor	cpm 70S	Reaction
1. SL	Infectious mononucleosis	11	−
2. 833-L	Infectious mononucleosis	12	−
3. F265	Normal	6	−
4. L33-1-16-19	Normal	8	−
5. 303-L	Normal	7	−
6. NC37	Normal	19	−
		Avg. cpm = 11	

Simultaneous detection of 70S RNA and RNA-instructed DNA polymerase in peripheral leukocytes from a patient (*SL*) with infectious mononucleosis and in lymphoblastoid cell lines. The lymphoblastoid cell lines have been shown to contain the Epstein–Barr virus genome on the basis of expression of early antigen, viral capsid antigen, or membrane antigen (20). Preparation of cellular pellet fractions and assay for synthesis of 70S RNA–[^{3}H]DNA complex is as described in the legend to Fig. 1. The sum of the cpm in the 70S position, determined by external size markers, is recorded, and is designated as positive if the cpm exceed 30.

DNA polymerase. It is clear that the existence of Epstein–Barr virus information is not mandatorily linked to the detectable presence of the RNA particles found in the Burkitt's tumors.

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 Burkitt's tumors. However, due to the possibility of nontemplated end addition reactions, more definitive evidence demands that the [^{3}H]DNA synthesized be hybridizable to its presumed RNA template.

One approach for resolving such issues is to compare the annealing of the [^{3}H]DNA with cytoplasmic polysomal RNA isolated from tumors and normal tissue. If the [^{3}H]DNA synthesized by the Burkitt's particles is tumor-specific, then Burkitt's tumors should, and normal tissue should not, possess hybridizable RNA. Another pathway depends on our previous (5) findings that Burkitt's tumors contain RNA homologous to that of the Rauscher leukemia virus. Consequently, if the [^{3}H]DNA synthesized by the Burkitt's tumor particles is instructed by an RNA related to that of Rauscher leukemia virus, hybridization should occur with Rauscher leukemia virus (RLV) RNA and not with the unrelated avian myeloblastosis virus (AMV) RNA.

[^{3}H]DNA product complexed to 70S RNA was prepared by performing an endogenous RNA-instructed DNA polymerase reaction with the pellet fraction from a Burkitt's tumor. After velocity centrifugation of the reaction product, the [^{3}H]-DNA–RNA complex sedimenting in the 70S region of the glycerol gradient was isolated by pooling and alcohol precipitation and then analyzed on a Cs_2SO_4 gradient with results as shown in Fig 2*A*. It is seen that some DNA is released during the manipulation, but most of the product bands in the RNA density region, indicating that most of the DNA molecules remain complexed to large RNA molecules. After alkali digestion to remove the RNA, the resulting purified [^{3}H]DNA was then annealed to 70S RNAs prepared from Rauscher leukemia virus and avian myeloblastosis virus. The outcomes of the annealing reactions were analyzed in Cs_2SO_4 gradient as shown in Fig. 2. It is clear from Fig. 2*B* that the Burkitt's [^{3}H]DNA is unable to hybridize to AMV-RNA, but does complex to RLV-RNA (Fig. 2*C*). Table 3 summarizes the results of annealing reactions with [^{3}H]DNAs synthesized by particles from three different Burkitt's tumors. In all cases, specific hybridizations occur only with RLV-RNA or Burkitt's tumor RNA. This outcome is logically consistent with our earlier demonstration (5) that Burkitt's tumor RNA hybridizes to DNA complementary to RLV-RNA but not to DNA complementary to AMV-RNA.

TABLE 3. *Annealing reactions of [^{3}H]DNA from Burkitt's tumors*

Burkitt [^{3}H]DNA	Burkitt pRNA	Lymph node pRNA	RLV-RNA	AMV-RNA
1. An	An+ Na+	−	+	−
2. Na	NT	NT	+	−
3. Ny	NT	NT	+	−

Hybridization of Burkitt's tumor [^{3}H]DNA product to 300 μg of Burkitt's tumor pRNA, to 300 μg of normal lymph node pRNA, to 0.1 μg of RLV-RNA, and to 0.1 μg of AMV-RNA. The hybridization reaction (50 μl) was performed in the presence of 50% formamide and 0.4 M NaCl. Annealing conditions and Cs_2SO_4 gradient analysis is described in the legend to Fig. 2.

pRNA, polysomal RNA.

Density of the Particle Containing 70S RNA and RNA-Instructed DNA Polymerase. The data thus far described indicate that Burkitt's tumors contain particles that encapsulate RNA-instructed DNA polymerase and a 70S RNA related in sequence to that of the Rauscher leukemia virus. It was of interest to see whether the particles possessed the density characteristic of an RNA tumor virus. To this end, a pellet fraction was prepared from a Burkitt's tumor and subjected to equilibrium centrifugation in a linear gradient of 15–55% 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.

Simultaneous detection tests were then performed on the pellet from each fraction to determine the distribution in the density gradient of 70S RNA-instructed DNA-synthesizing activity. It can be seen from Fig. 3 that the particles possessing 70S RNA-instructed DNA polymerase localize within a density of 1.16–1.19, the density range characteristic of the oncogenic RNA viruses. As summarized in Table 4, three Burkitt's tumors and one African histiocytic lymphoma were analyzed in a similar manner, and all of them gave the same results.

DISCUSSION

As in our previous studies with human leukemia (16) and breast cancer (17), the experiments described here were performed to elucidate the possible etiologic significance of our earlier (5) detection in Burkitt's tumors of RNA uniquely homologous to that of the Rauscher leukemia virus. The data obtained here show that at least a portion of the RNA we were

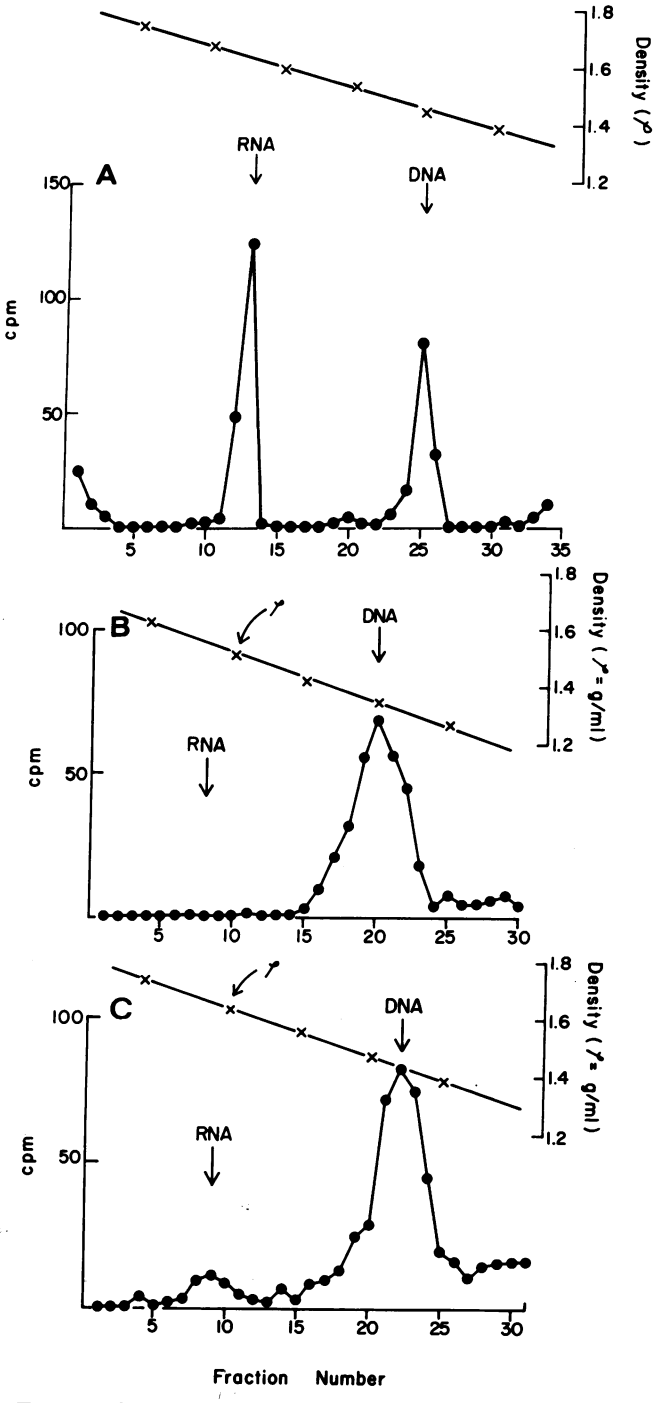

FIG. 2. Characterization of 70S RNA–[^{3}H]DNA complex from extracts of Burkitt's tumors. A simultaneous detection assay was performed on the pellet fraction from a Burkitt's tumor (*An*) and analyzed by glycerol velocity centrifugation as described in the legend to Fig. 1. Aliquots of the glycerol gradient fractions were assayed for Cl_3CCOOH-precipitable counts and the 70S RNA–[^{3}H]DNA complex was pooled and precipitated with two volumes of ethanol in the presence of yeast carrier RNA (10 μg/ml final concentration). After centrifugation at 10,000 × *g* for 30 min at −20°, the precipitate was resuspended in TNE and divided into three aliquots. Aliquot 1 (*A*) was added directly to 11 ml of half-saturated cesium sulfate (Gallard–Schlessinger) in 3 mM EDTA (ρ = 1.52 g/ml) and centrifuged in a 50 Ti rotor (Spinco) at 44,000 rpm for 60 hr at 15°. Aliquots 2 and 3 were digested in 0.4 M NaOH at 43° for 24 hr, neutralized with HCl in the presence of 0.01 M Tris·HCl (pH 7.4), and brought to 50% formamide (Eastman). After heat denaturation for 10 min at 80°, aliquot 2 was hybridized to 0.1 μg of AMV-RNA (*B*), and aliquot 3 to 0.1 μg of RLV-RNA (*C*) in the presence of 50% formamide and 0.4 M NaCl for 18 hr at 37°. The annealing reactions were then analyzed by Cs_2SO_4 density analysis.

TABLE 4. *Density localization of simultaneous detection activity in Burkitt's tumors*

Name	Treatment	cpm 70S	Density region	Reaction
1. Na	CTX	1321	1.177	+
2. Il	None	601	1.19	+
3. Ny	None	6347	1.177 1.188	+
4. Oy*	None	633	1.18	+

Sucrose gradient localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of Burkitt's tumors. The amount of 70S RNA–[^{3}H]DNA synthesized by an endogenous RNA-instructed DNA polymerase was determined by glycerol velocity centrifugation for each of 10 sucrose density fractions, as described in the legend to Fig. 3. The density fraction or fractions yielding the maximum number of cpm in the 70S region are listed. The reaction is designated as positive if the cpm in the 70S region exceed 30. The modality of chemotherapy at the time of biopsy is included.

* Histiocytic lymphoma.

finding exists in the form of a 70S RNA 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 the Burkitt's tumors have at least three of the biochemical and physical features diagnostic of the RNA tumor viruses.

Of particular interest was the demonstration with the particle fraction from the Burkitt's tumors that the DNA synthesized on its own RNA hybridizes specifically to RNA from Burkitt's lymphomas and to RLV-RNA. The lack of response to the unrelated AMV-RNA (Fig. 2*B*) eliminates the possibility that complexing with tumor and RLV-RNA is due to the poly(A) stretches identified in RNA tumor viruses. The successful specific hybridization to RLV-RNA of the DNA synthesized by the Burkitt's tumor enzyme and its RNA template is complementary to and completes the logic of our earlier experiments (5), in which DNA synthesized under the direction of RLV-RNA was used as a probe to find viral-related information in Burkitt's tumors. At the same time, this finding establishes that the template RNA associated with Burkitt's tumor particles is related in sequence to a known RNA oncogenic agent.

Of the 15 Burkitt's tumors examined, 13, or 87%, gave unambiguous evidence for the presence of 70S RNA–RNA-instructed DNA polymerase complexes. Our previous studies (16, 17) have shown that normal tissues have thus far not exhibited this specific type of particle or RNA-instructed activity. Of particular interest are the equally negative results with the five types of cells reported here in Table 2. Numbers 2–6 are known to contain Epstein–Barr virus information on the basis of hybridization or the possession of early, virus coat, or membrane antigens associated with Epstein–Barr virus (20). The first two control cells listed are derived from patients with infectious mononucleosis, a non-neoplastic disease implicated with (18, 19) the Epstein–Barr virus. If a more extensive study confirms this pattern in in-

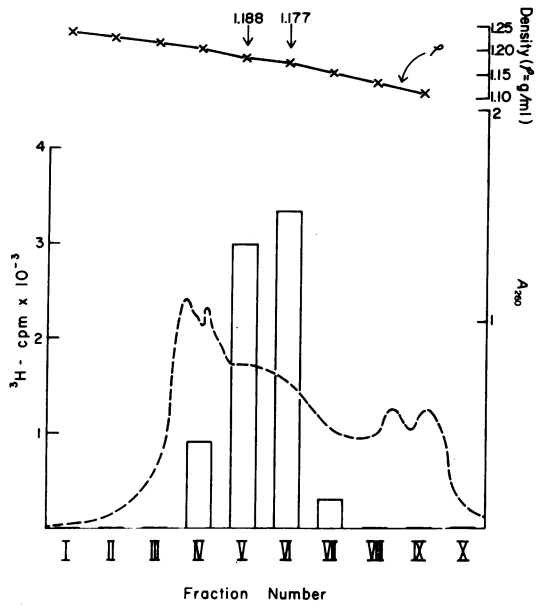

FIG. 3. Sucrose gradient localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of Burkitt's tumors. A pellet fraction was prepared from Burkitt's tumor (*Ny*) as in the legend to Fig. 1. The pellet was resuspended in TNE and layered on a linear gradient of 15–55% sucrose in TNE and spun in a SW-27 (Spinco) rotor at 4° for 210 min. The gradient was dripped from below through a recording Gilford spectrophotometer at A_{260}, and 10 equal fractions were collected. Each fraction was diluted with TNE to a sucrose concentration of less than 15% and then spun at 100,000 × *g*. The pellet obtained from each of the 10 fractions was then subjected to the simultaneous detection assay as in the legend to Fig. 1, and the amount of 70S RNA–[^{3}H]DNA synthesized from the 10 different density regions was determined by glycerol velocity centrifugation.

fectious mononucleosis, evidence would be provided identifying the RNA particles as unique components of neoplastic tissues. In any case, it is evident from the results in Table 2 that the Epstein–Barr virus information can be present in cells that lack the type of RNA particles found in Burkitt's tumors, leukemic cells, and breast cancer.

The presence in Burkitt's tumors of RNA related to that of RLV (5) was suggestive of a viral agent. The present study carries this implication much further by demonstrating that the viral-related RNA thus identified is 70 S in size and is physically associated with an RNA-instructed DNA polymerase in a particle having the density of an RNA tumor virus. The presence of these particles is obviously an observation worthy of note to those who wish to understand the biology of Burkitt's tumors. It is of central importance to determine whether the genetic information contained in the RNA of these particles is to be found in the DNA of tumor cells.

Finally, we should like to emphasize that the available information does not permit definitive conclusions concerning the etiologic roles of either Epstein–Barr virus or the RNA particles in the pathogenesis of Burkitt's disease. Both may be necessary or one may be a nonparticipating passenger. These issues will be resolved only by further investigation.

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