RNA TUMOR VIRUSES AND HUMAN LYMPHOMAS

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We have used molecular hybridization to detect virus-specific RNA in tumors (1), and have found that corresponding neoplasias of murine and human origin exhibit remarkable similarities. Thus, human breast carcinomas contained (2) RNA possessing sequence homology to that of mouse mammary tumor virus (MMTV). This type of RNA was unique to the malignant adeno- and medullary-carcinomas, being undetectable in normal breast tissue and in such benign pathologies as fibrocystic disease and fibroadenoma. In keeping with the known unrelatedness of the murine leukemogenic and mammary tumor viruses, we found that breast cancer RNA did not hybridize to DNA complementary to the RNA of Rauscher leukemia virus (RLV). Finally, and more compelling, was the demonstration that human leukemic cells (3) and human lymphomas (4), including Burkitt's disease (5), contain RNA showing homology to that of Rauscher leukemia virus and not to that of mouse mammary tumor virus.

An Analysis of the Significance of Viral-related RNA in Human Neoplasia

The existence of viral-related RNA in human neoplasias does not of course establish a viral etiology for this disease. One must now perform experiments designed to answer the following questions: (1) How large is the RNA being detected? (2) How much homology does it in fact have to the RLV RNA? and (3) Is the viral-related RNA associated with a reverse transcriptase (RNA-instructed DNA polymerase) and is it located in structures characteristic of incomplete or complete virus particles? The requisite techniques resolving these issues were developed (6–9) and were applied to extracts of human tumors and normal tissues in a search for evidence of oncogenic RNA viruses.

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 X 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 a standard endogenous reverse transcriptase reaction to generate [^{3}H]DNA after 15 min of synthesis at 37°. The reaction product was freed of protein by treatment with SDS and phenol and subjected to sedimentation analysis in a 10 to 30 % glycerol gradient with suitable markers to determine the apparent

size distribution of the DNA synthesized. In a reaction mediated by a B- or C-type virus, [^{3}H]DNA will sediment in a 70S region of the gradient representing the 70S RNA : [^{3}H]DNA early reaction product.

Such features as sensitivity to ribonuclease and the requirement for all four deoxyribonucleoside triphosphates can also be used to demonstrate that the appearance of the 70S-RNA : [^{3}H]DNA complex is in fact the result of a reverse transcriptase reaction. However, due to the possibility of nontemplated end addition reactions, the most definitive proof finally demands that the [^{3}H]DNA synthesized is hybridizable to an RNA derived from a known oncogenic virus.

The successful use of the simultaneous detection technique to detect RNA viruses in mouse and human milk (7, 8) led to the demonstration of complexes of 70S RNA and reverse transcriptase in peripheral white blood cells of 95 % of leukemic patients (10) and in 79 % of human breast cancers examined (11). In both of these malignancies, high molecular weight RNA has been found to be encapsulated with reverse transcriptase in a particle possessing the density characteristic of the RNA tumor viruses.

The Simultaneous Detection of 70S RNA and Reverse Transcriptase in Human Lymphomas

Figure 1A shows a representative outcome of a 70S RNA : DNA complex synthesized by the pellet fraction from an involved spleen of a patient with Hodgkin's disease. In certain samples additional peaks have been observed at positions of 52S and 35S. As shown in Fig. 1B, peaks are detected at both the 70S and 52S positions. It is further demonstrated that these complexes are due to an RNA-dependent reaction, as prior treatment with RNase eliminates both of the peaks. When equivalent quantities of either a normal spleen or an uninvolved spleen from a patient with Hodgkin's disease are analyzed by the same technique, no incorporation of [^{3}H]TTP is detected in a rapidly sedimenting structure (Figs. 1C and 1D).

Simultaneous detection assays were performed on 36 human lymphomas, which include 28 Hodgkin's disease specimens, 6 lymphosarcomas, and 2 reticulum cell sarcomas (12). The control series consisted of 14 uninvolved spleens and 5 cases of hypersplenism. The number of cpm in the 70S region of the glycerol gradient as determined by external size markers was taken as a measure of the presence and extent of the reaction. The average cpm in the 70S region for the control series was 14, whereas in contrast, the malignant tissues yielded an average of 302. In view of the low value of the controls, we have arbitrarily assigned any reaction yielding more than 30 cpm in the 70S region as being positive. With these criteria, all of the control samples were negative and 80.6 % of the malignant tissues were positive. Fifteen of the positive tumors were tested for ribonuclease sensitivity of the 70S-DNA complex, and in each of them the complex was degraded. It should be noted that 3 cases of hypersplenism yielded positive responses, which were sensitive to ribonuclease. The significance of this and its possible relation to premalignant conditions requires further investigation.

The demonstration of 70S RNA : DNA complex that is sensitive to ribonuclease already argues for the presence of an RNA-dependent reaction. However, due to the

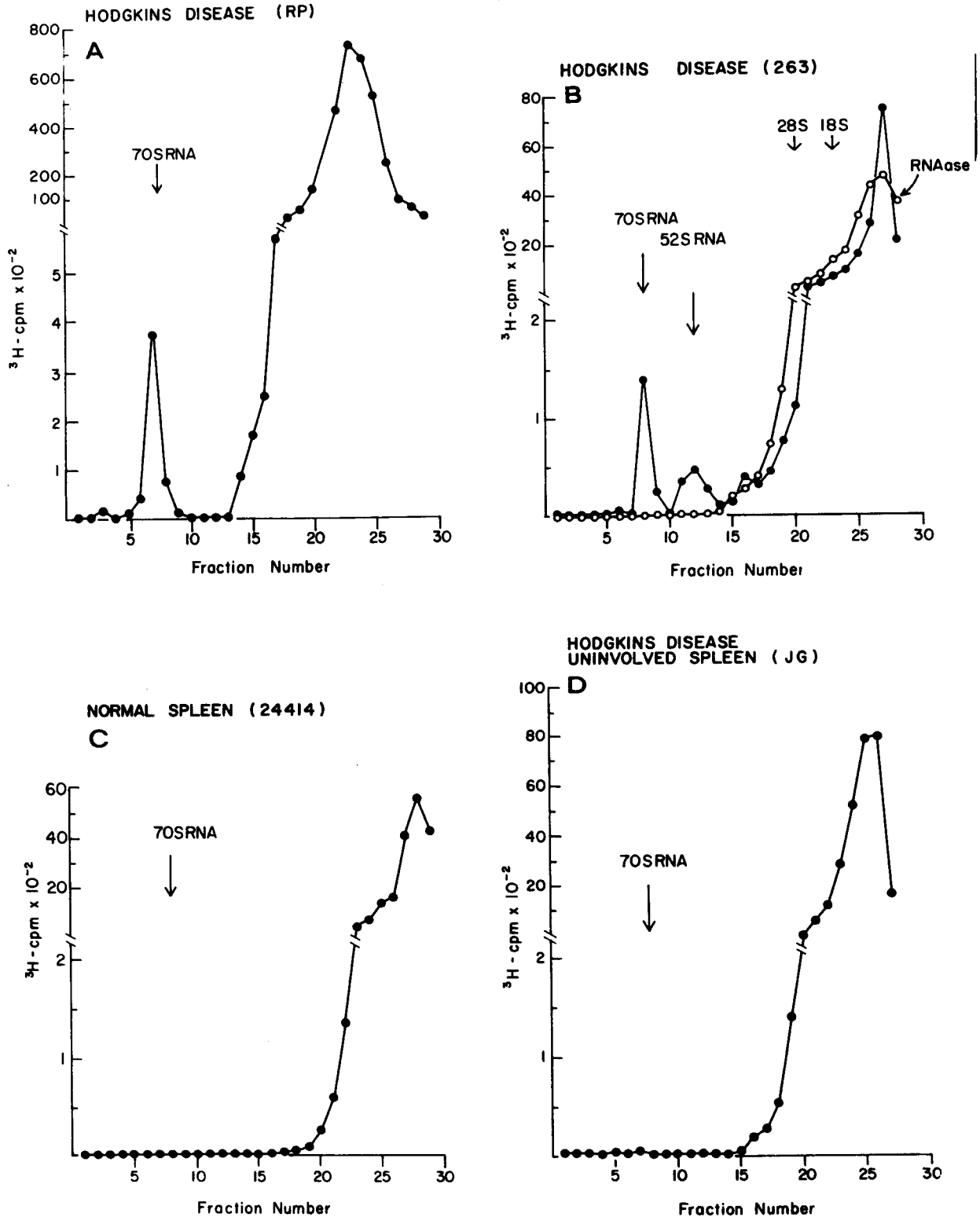

Fig. 1: Detection of 70S RNA-[^{3}H]DNA in human lymphoma tissue: A), and B), Hodgkin's disease; C), normal spleen; D), uninvolved spleen from a Hodgkin's disease patient. Five g of tissue were finely minced and disrupted with a Silverson homogenizer at 4° in TNE buffer (0.01 Tris-HCl, pH 8.3, 0.15 M NaCl, 0.01 M EDTA). This suspension was centrifuged at 4000 X 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 X g for one hr at 4° in an SW-27 rotor (Spinco). The resulting pellet was resuspended in 0.5 ml 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 reverse transcriptase reaction mixture (final volume 1 ml) containing: 50 μmol of Tris-HCl, pH 8.3, 20 μmol NaCl, 6 μmol $MgCl_2$, 100 μmol each of dATP, dGTP, dCTP, and 50 μmol-[^{3}H]dTTP (50 curries/mmole). 50 μg/ml 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 % SDS. An equal volume of a phenol-cresol (7 : 1) mixture containing 8 hydroxyquinoline (0.2 g per 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 for 180 min at 2°. Fractions were collected from below and assayed for TCA-precipitable radioactivity. In Fig. 1B, 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 T_1 (50 μg/ml) for 15 min at 37° prior to sedimentation analysis.

possibility of nontemplated end addition reactions, a more definitive proof demands that the [^{3}H]DNA synthesized is hybridizable to the relevant "oncogenic" RNA template. One approach is to hybridize the [^{3}H]DNA with 70S RNA prepared from Rauscher leukemia virus (RLV). A positive outcome would be expected from the earlier demonstration that human lymphoma pRNA hybridizes to synthetic DNA complementary to RLV-RNA (4, 5). If the hybridization to RLV-RNA is specific, the lymphoma [^{3}H]DNA should not hybridize to the 70S RNA of the avian myeloblastosis virus (AMV).

Figure 2A shows a Cs_2SO_4 equilibrium gradient profile of an annealing reaction between [^{3}H]DNA from Hodgkin's disease spleen (#211) and the RLV-70S RNA. It is clear that approximately 10 % of the [^{3}H]DNA has shifted to the RNA region of the gradient. Upon annealing an equivalent amount of AMV-70S RNA to the same [^{3}H]DNA, no significant shift to the RNA or hybrid region is observed (Fig. 2B). Note that this last result is complementary to and completes the logic of our earlier experiments in which the DNA synthesized on RLV-RNA was used as a probe to detect the virusrelated information in lymphoma cells.

The Simultaneous Detection of 70S RNA and Reverse Transcriptase in Burkitt's Tumors

Figures 3A, 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 35S. It is further demonstrated, as exemplified in Fig. 3C, that these complexes contain a 70S RNA molecule since prior treatment with RNase eliminates the peak. When equivalent quantities of peripheral white cells of a patient with infectious mononucleosis were analyzed by the same technique, no incorporation of [^{3}H]TTP into a rapidly sedimenting struktur was detected (Fig. 3D).

Of the 15 Burkitt's tumors examined, 13 or 87 % gave unambiquous evidence for the presence of 70S RNA : RNA-instructed DNA polymerase complexes (13). 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. Six non-Burkitt samples were 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 detec-

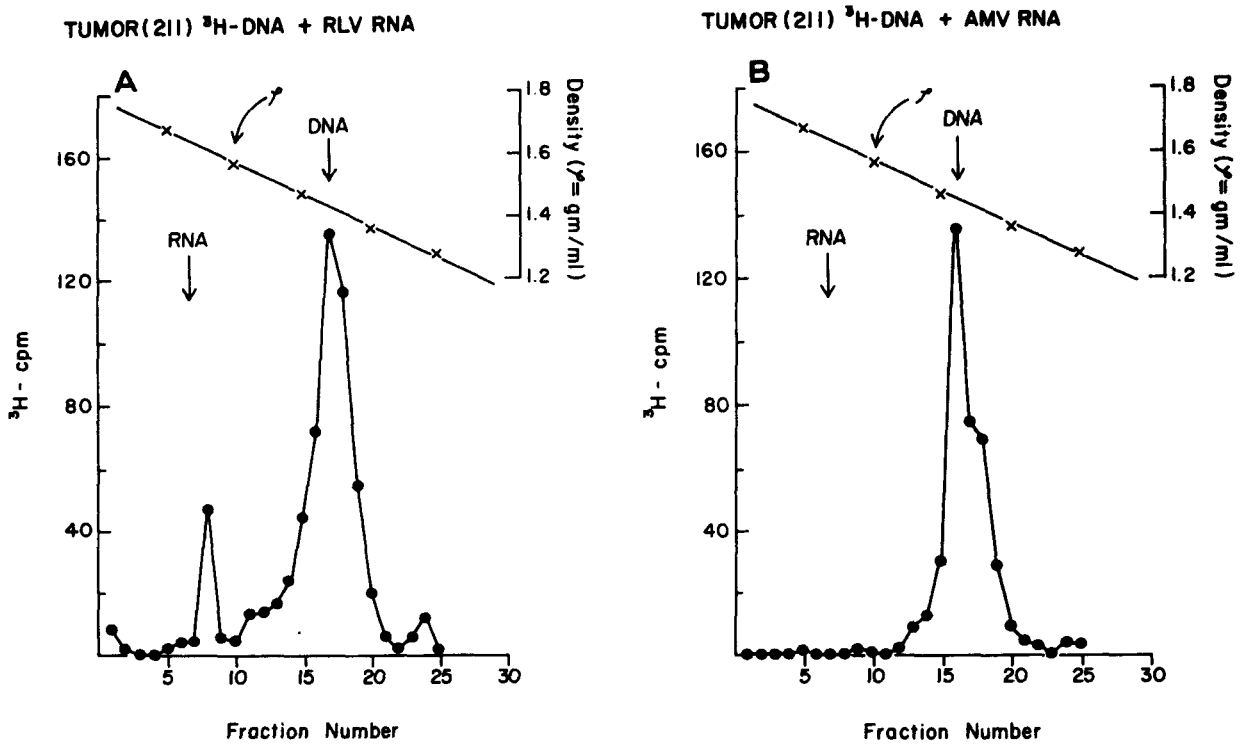

Fig. 2: Cs_2SO_4 density profiles of annealing reactions of human lymphoma #228 [^{3}H]DNA to A), RLV-70S RNA and B), AMV-70S RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legend to Fig. 1, except that the glycerol gradient sedimentation step was omitted. The aqueous phase was instead subjected to Sephadex G-50 column chromatography and the [^{3}H]DNA isolated and precipitated with two volumes of ethanol. The precipitate was digested in 0.4 M NaOH for 24 hr at 43° and neutralized. The [^{3}H]DNA product was then annealed to 1 μg of RLV-RNA and 1 μg AMV-RNA. The hybridization reaction (50 μl) was performed in the presence of 50 % formamide and 0.4 M NaCl. After annealing for 24 hr at 37°, the reaction mixture was subjected to Cs_2SO_4 density analysis.

ted in the Burkitt's tumors. One control is derived from the peripheral white blood cells of a patient with infectious mononucleosis, a self-limiting non-neoplastic condition in which prospective studies (14, 15) have strongly implicated the Epstein-Barr virus. Another control is a cell line derived from the lymphocytes of an infectious mononucleosis patient. This line and four other lymphoblastoid lines used are known to contain the Epstein-Barr genome. All the controls were negative for the 70S RNA-directed DNA polymerase. If a more extensive study confirms this pattern in infectious mononucleosis, evidence would be provided identifying the RNA particles as unique components of neoplastic tissues. In any case, it is clear that the existence of EBV information is not mandatorily linked to the detectable presence of the RNA particles found in the Burkitt's tumors.

The data thus far described indicate that Burkitt's tumors contain particles that encapsulate reverse transcriptase 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 to 55 % sucrose. The gradient was then divided into 10 equal fractions that were diluted to 15 % sucrose and again spun at 100,000 X g for 1 hr.

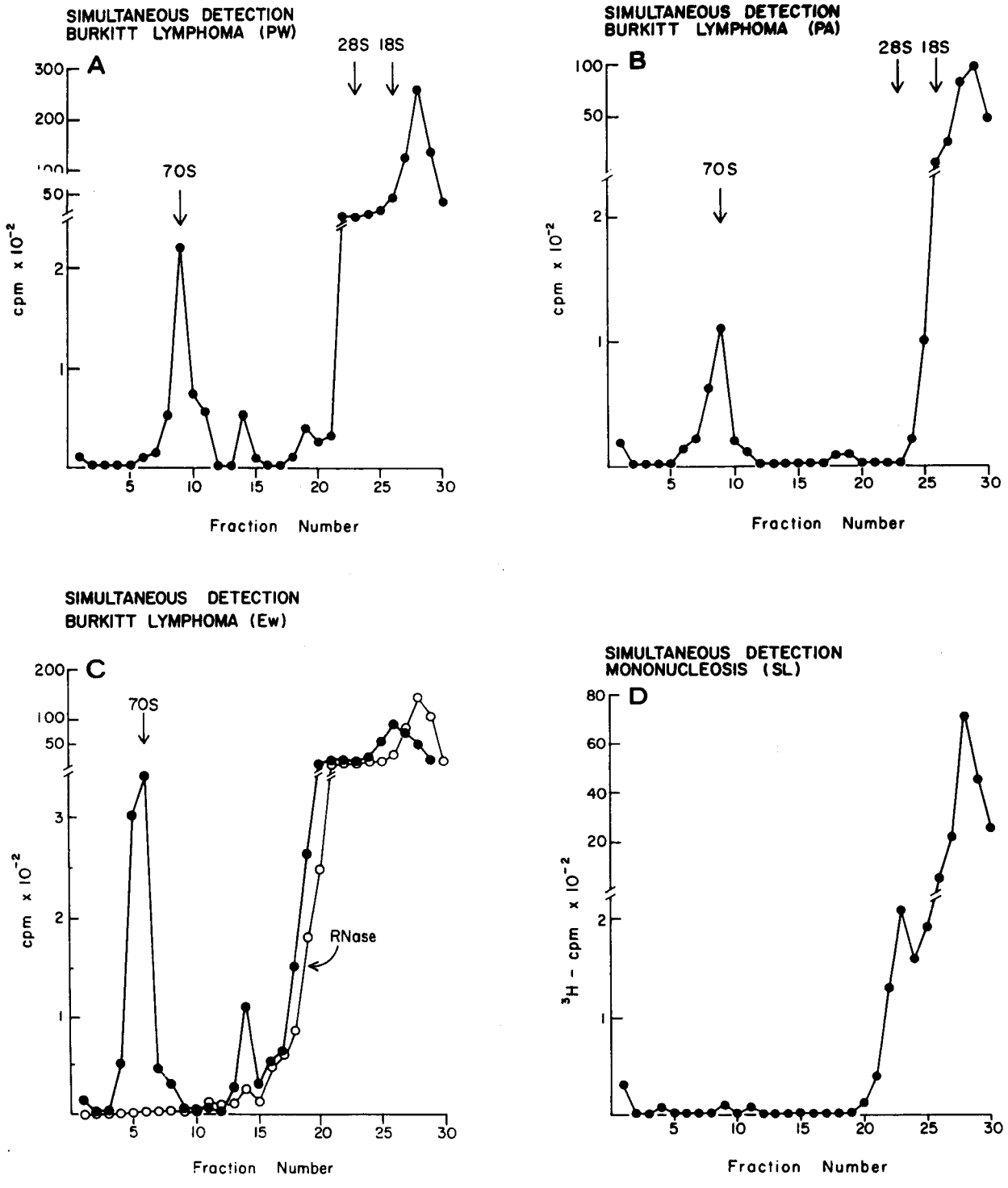

Fig. 3: Detection of 70S RNA-[^{3}H]DNA: in Burkitt's lymphoma tissue, (A), (B), (C); and (D), peripheral white blood cells from a patient with acute mononucleosis. The procedure employed is as described in the legend to Fig. 1. In Fig. 3C, one aliquot of the product was run directly on the glycerol gradient, while the other aliquot was incubated in the presence of RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37° prior to sedimentation analysis.

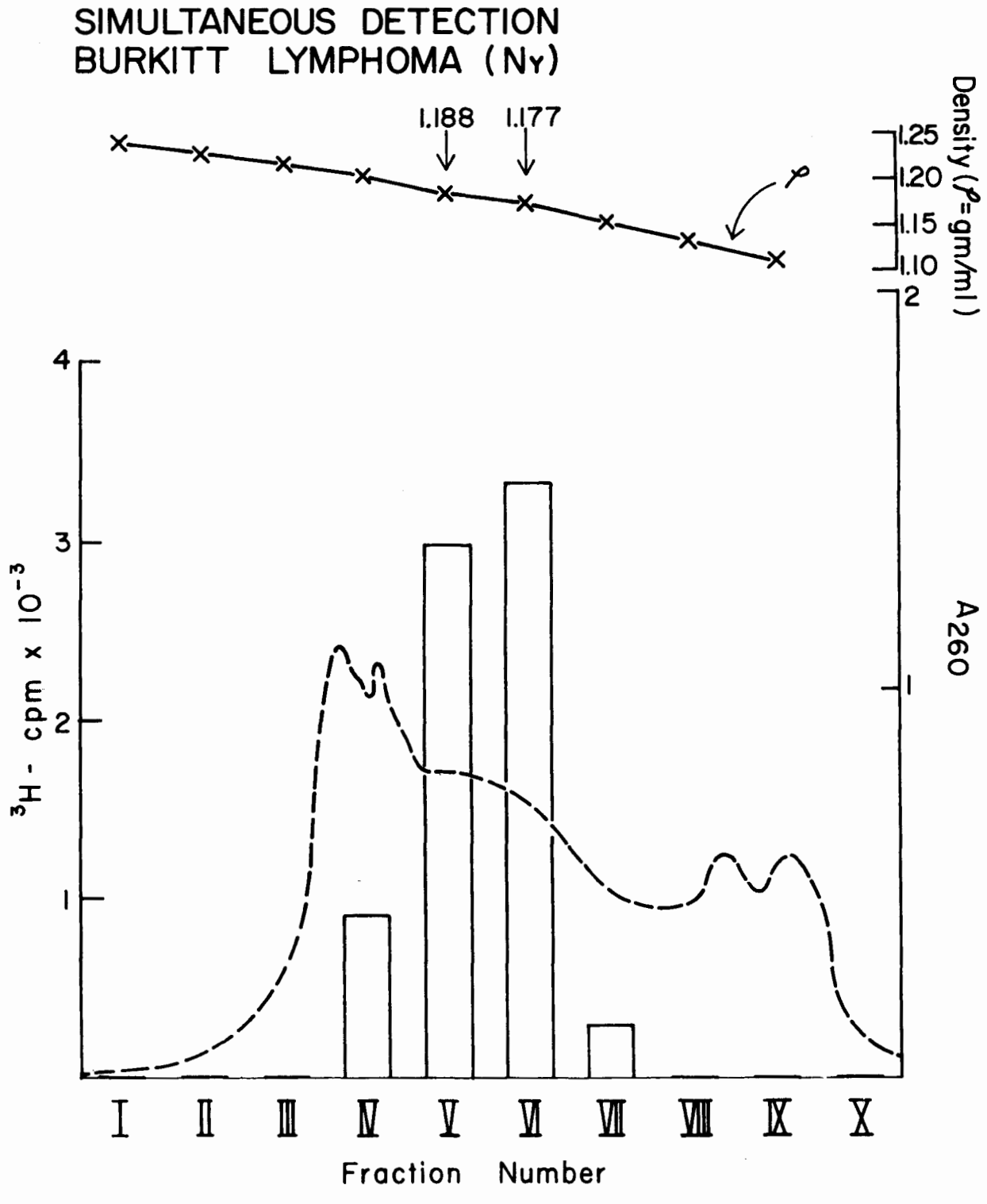

Fig. 4: Sucrose gradient localization of 70S RNA and RNA-instructed DNA polymerase activity in extracts of Burkitt's tumors. A pellet fraction was prepared from Burkitt's tumor (Ny) as in the legend to Fig. 1. The pellet was resuspended in TNE and layered on a linear gradient of 15–55 % sucrose in TNE 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 ten equal fractions were collected. Each fraction was diluted with TNE to a sucrose concentration of less than 15 % and then spun at 100,000 X g. The pellet obtained from each of the ten 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 ten different density regions was determined by glycerol velocity centrifugation.

Simultaneous detection tests were then carried out 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. 4 that the particles possessing 70S RNA-instructed DNA polymerase localize within a density of 1.16 to 1.19, the density range characteristic of the oncogenic RNA viruses. Three Burkitt's tumors and one African histiocytic lymphoma were analyzed in a similar manner and all of them gave the same results.

Particle-related Sequences in Lymphoma Nuclear DNA (16)

As in our previous studies with human leukemia (10) and breast cancer (11), the experiments described here were performed to elucidate the possible etiologic significance of our earlier (4) detection in human lymphomas, including Burkitt's tumors (5) 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 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 cells from both of these malignancies have four of the biochemical and physical features diagnostic of the RNA tumor viruses. Ultimately, final proof of the contributory or causative nature of these particles requires the demonstration that they are infectious and transforming agents. At this time, however, a logical approach in attempting to further define their involvement would be to demonstrate that the particle-related sequences are unique to the DNA of malignant cells.

These human particles can be used to generate radiocative DNA probes, which in turn could be used to probe for viral-specific information in human nuclear DNA. The requisite methodology has been developed and applied in a study of eight leukemic patients (17). In each case, the DNA of leukemic cells contained particle-related sequences undetectable in the leukocyte DNA of normal individuals.

As outlined in the studies of human leukemia, the methods used in this investigation are as follows: 1) Isolate the particles encapsulating 70S RNA and reverse transcriptase from human lymphoma specimens; 2) Use the particle fraction to endogenously synthesize [^{3}H]DNA in the presence of a high concentration of actinomycin D to inhibit host and viral DNA-directed DNA synthesis; 3) Purify the [^{3}H]DNA by Sephadex chromatography and hydroxyapatite; 4) Remove the [^{3}H]DNA sequences shared with normal DNA by exhaustive hybridization in the presence of vast excess of normal DNA followed by hydroxyapatite chromatography to separate paired from unpaired [^{3}H]DNA. 5) Test the unpaired residue for specific hybridizability to lymphoma DNA.

[^{3}H]DNA probes were synthesized from five Burkitt's lymphoma and three Hodgkin's disease specimens. In all instances, the lymphoma [^{3}H]DNA probe hybridized 35 to 40 % to normal spleen nuclear DNA. The observation that the [^{3}H]DNA synthesized by the lymphoma particles hybridized to normal DNA was not surprising in view of previous experience with murine, avian and human leukemic systems (18–25).

The sequences in [^{3}H]DNA common to normal nucelar DNA were then removed by exhaustive annealing with normal spleen DNA in vast excess. In carrying out this step,

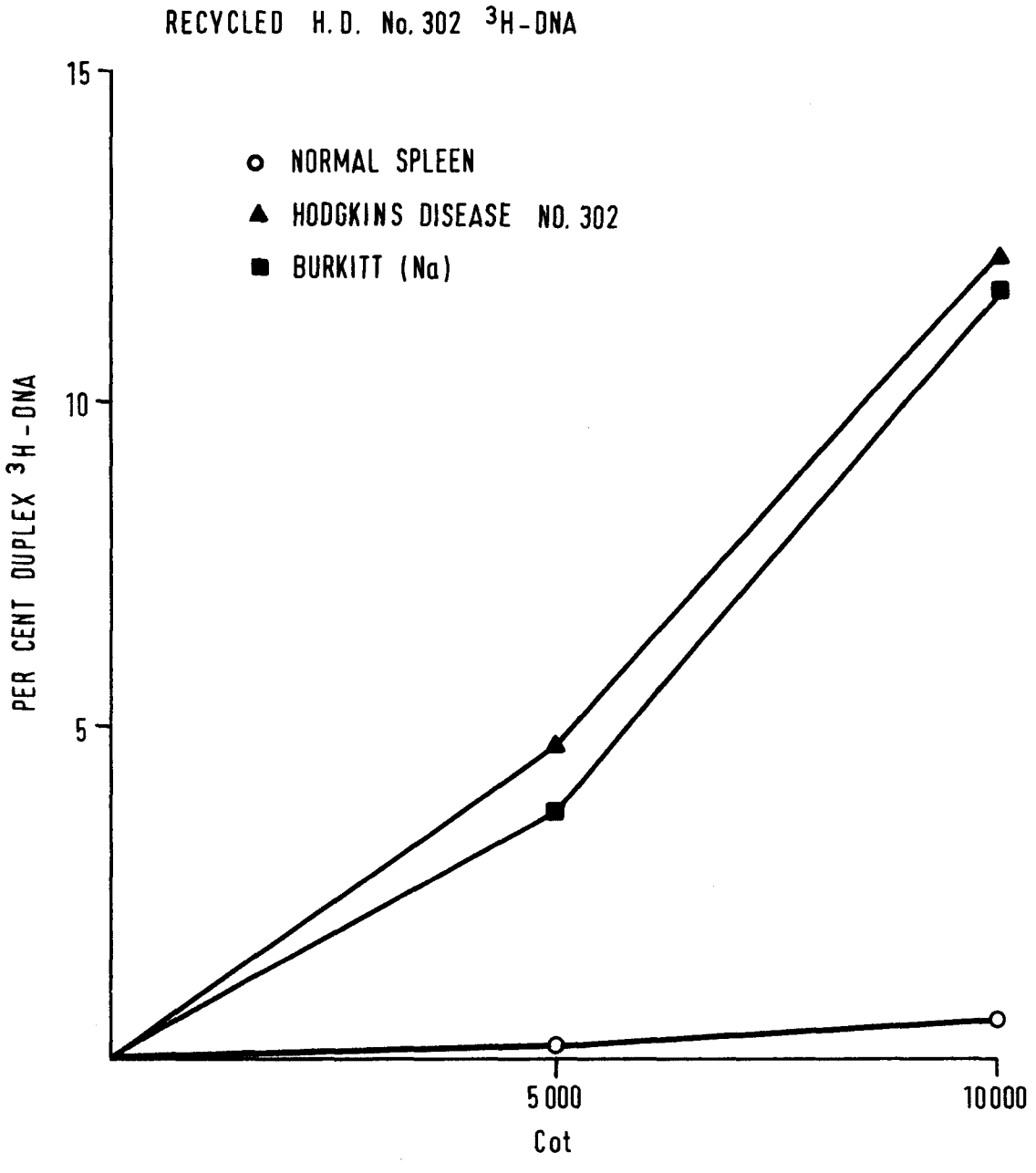

Fig. 5: Hybridization of recycled Hodgkin's disease #302 [^{3}H]DNA to nuclear DNA isolated from normal spleen (O- - - - O), Hodgkin's disease #302 (▲- - -▲), and Burkitt's lymphoma Na (■ - - ■). Tritiated DNA probe synthesis, nuclear DNA preparation, and annealing conditions are detailed elsewhere (16). Hybrid formation was analyzed by hydroxyapatite chromatography at a phosphate buffer elution concentration of 0.15 M. Five fractions of 4 ml were collected at each of four temperatures (60°, 80°, 88°, 95°) and the [^{3}H]DNA counts in each fraction were assayed by scintillation counting in 10 ml Aquasol (NEN). The method identifies unpaired strands that elute at 60° and poorly paired duplexes that disassociate at 80°. *Only the duplexes disassociating and eluting at 88° to 95° are counted here as hybridized.*

annealing reactions were set up to contain 60 A_{260} units of normal cellular DNA, 0.1 pmol of [^{3}H]DNA (1000 cpm) and allowed to anneal to a C_0t of greater than 10,000. After this reaction, the recovered unpaired strands should no longer contain sequences complementary to those found in normal DNA. Exclusive hybridizability of such recycled [^{3}H]DNA to lymphoma DNA would then establish that the genome of lymphoma cells contains specific sequences not present in normal DNA.

Figure 5 shows the outcome of hybridizing such recycled Hodgkin's disease [^{3}H]DNA to normal spleen nuclear DNA, Hodgkin's disease nuclear DNA, and to Burkitt's lymphoma nuclear DNA. The input counts for each C_0t point is 1500 to 2000 cpm. Only those stable duplexes disassociating and eluting above 88° are counted here as hybridized. Taking into account normal background on our hydroxyapatite columns, it is evident that no stable complexes are formed with normal DNA. On the other hand, over 10 % of the input recycled Hodgkin's disease [^{3}H]DNA forms well-paired duplexes with both Hodgkin's and Burkitt's disease nuclear DNA. In all eight instances, the lymphoma [^{3}H]DNA hybridized to nuclear DNA from the same pathological type of lymphoma from which the probe was synthesized. In several, but not all cases, Burkitt's [^{3}H]DNA hybridized to Hodgkin's disease nuclear DNA indicating that there is some, but not complete homology between their viral-specific sequences. A similar situation was obtained in hybridizing Hodgkin's disease [^{3}H]DNA with Burkitt's lymphoma nuclear DNA. It is pertinent to point out that the lymphoma [^{3}H]DNA did not show significant levels of hybridizability with nuclear DNA from either carcinoma of the breast or colon. This indicates that the lymphoma particle information is specific for lymphomas but not for all types of malignancy. Furthermore, our particle-specific information cannot be related to the Epstein-Barr virus due to the fact that the [^{3}H]DNA does not hybridize to the nuclear DNA of NC37 cells or of infectious mononucleosis cells.

As in the experiments in human leukemia (16), the results described here for human lymphomas strongly argue against the applicability of the virogene theory to these diseases. The experiments designed here involve nuclear DNA and consequently all genes, expressed or silent. It is clear that particle-specific information, undetectable in normal nuclear DNA, is present in each lymphoma nuclear DNA examined. This implies that the addition of particle-related information was required for the conversion of the normal to the malignant cell. The virogene hypothesis on the other hand demands that the lymphoma-specific information be present in the genomes of both normal and malignant cells. The relationship of these findings to the epidemiologic evidence of time-space clustering in both Hodgkin's and Burkitt's disease remains to be determined.

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