BIOCHEMICAL EVIDENCE FOR THE PRESENCE OF RNA TUMOR VIRUSES IN HUMAN LEUKEMIC PLASMA

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Recent biochemical techniques have enabled the detection of murine RNA tumor virus-related particle in human malignancies. Molecular hybridization has shown that human breast tumors contain RNA homologous to murine mammary tumor virus (1, 2). Similar analysis of human leukemias (3), sarcomas (4), and lymphomas (5) showed the presence of RNA related to the Rauscher leukemia virus.

Finding the appropriate oncornavirus-related RNA in the corresponding human neoplasia stimulated the search for RNA tumor viruses in human malignancies. The presence of 70S RNA and RNA-instructed DNA polymerase in the RNA tumor viruses provided a sensitive technique for the detection of such viruses (6). The possibility of a concomitant test for 70S and reverse transcriptase was suggested by the nature of early reaction intermediates (7–9). The initial DNA product was found to be hydrogen-bonded to the 70S RNA template and the structure could be detected due to the co-sedimentation of newly synthesized small [^{3}H]-DNA product with the 70S RNA. The procedure revealed the presence of 70S RNA and RNA-instructed DNA polymerase in human milk particles (11). Extension of the simultaneous detection assay to tumor tissue (12) showed the presence of particles containing 70S RNA and RNA-instructed DNA polymerase in human leukemia (13, 14), human breast tumors (15), Hodgkin's disease (16), and Burkitt's lymphoma (17). The particles were also shown to possess the density (1.17 g/ml) characteristic of the RNA tumor viruses (14–17). The presence of virus particles in plasma of Rauscher leukemia virus-infected mice (18) and in chickens infected with avian myeloblastosis virus (19) suggested a search for RNA tumor viruses or their components in the plasma of human leukemic patients. Electron microscopy has revealed electron-dense particles in leukemic patients' plasma (20, 21), and, further, work of Kiessling *et al.* (22) has shown the presence of a DNA polymerase. Gallo and his colleagues have partially purified RNA-directed DNA polymerase from leukemic cells (23) and have recently confirmed (24) the sequence relatedness of animal tumor viruses to the RNA of human leukemic particels (3, 13, 14).

Further work has shown the presence of 70S RNA and RNA-instructed DNA polymerase in the plasma of 74 % of the patients examined (25).

Examination of leukemic plasma for 70S RNA-instructed DNA polymerase.

The plasma is enriched for virus-like particles by pelleting at 100,000 X g and the pellet is washed with EDTA-containing buffers and centrifuged at 150,000 X g for 1 hr to yield a pellet (p-100). The p-100 is solubilized with 0.1 % NP-40 and a standard DNA polymerase reaction is then performed. The DNA synthesis is monitored by the incorporation [^{3}H]TTP into acid-insoluble products. Figure 1 shows the kinetics of DNA synthesis from the p-100 pellet of a patient with chronic lymphatic leukemia. For simultaneous detection of 70S RNA and RNA-directed DNA polymerase, the standard DNA polymerase reaction is deproteinized after 20 min of incubation at 37°, and the nucleic acids are subjected to sedimentation analysis in glycerol gradients with external size markers.

Figure 2 shows the results of simultaneous detection assays on the plasma of two patients with acute lymphatic (ALL-Bl) and acute myelogenous (AML-Kl) leukemia. The DNA synthesized is seen travelling with a sedimentation coefficient of 70S. The 70S-DNA complex observed with p-100 pellet from a leukemic patient's plasma is due to a complex involving a 70S RNA, because a prior treatment with RNase eliminates the [^{3}H]DNA from the 70S region of the gradient (Fig. 3).

Table 1 summarizes our findings with plasma specimens from 19 leukemic patients all in the active phase of their disease, and 13 normal blood bank donors. Included, where available, are the peripheral leukocyte counts (WBC) at the time of sampling. P-100 pellets (Fig. 2) were prepared from the plasma specimens and assayed for their ability to synthesize 70S RNA : DNA complex. The cpm in the 70S region of the glycerol gradient were taken as a measure of the presence and extent of the reaction. The reaction was termed positive if the cpm in the 70S region of the gradient exceeded 30. On this basis, 14 out of 19 leukemic plasmas examined were positive. The leukemic specimens produced an average of 184 cpm in the 70S region, with a few producing several hundreds of cpm (Table 1). Unfortunately, in many cases the amount of [^{3}H]DNA found in the 70S region was not sufficient to permit a concomitant assay for ribonuclease sensitivity. However, 4 of the 19 plasmas could be checked and in all cases the reaction was sensitive to ribonuclease, as exempliefied by Fig. 2. Similar analyses of 13 normal blood bank donors produced an average of 8 cpm in the 70S region (Table 1). One plasma produced 100 cpm, but on examination of a parallel aliquot, the cpm in the 70S region were insensitive to ribonuclease and the significance of this reaction remains obscure. There is no evidence in Table 1 of a relation between the level of leukocytosis and the activity found by the simultaneous detection test. However, the numbers available are too small to permit any definitive statements.

Characterization of the [^{3}H]DNA product. Synthesis of the ribonuclease-sensitive 70S RNA : [^{3}H]DNA complex in the p-100 pellet of leukemic plasma suggests the presence of an RNA-instructed DNA polymerase. However, definitive proof requires a demonstration that the [^{3}H]DNA synthesized is in fact complementary to the 70S RNA template. To this end, high molecular weight RNA : [^{3}H]DNA complex was pooled, alcohol-precipitated, and extensively treated with alkali to digest the RNA. [^{3}H]DNA was then used to show its specificity to its presumed template. RNA was extracted from the p-100 pellet obtained from leuke-

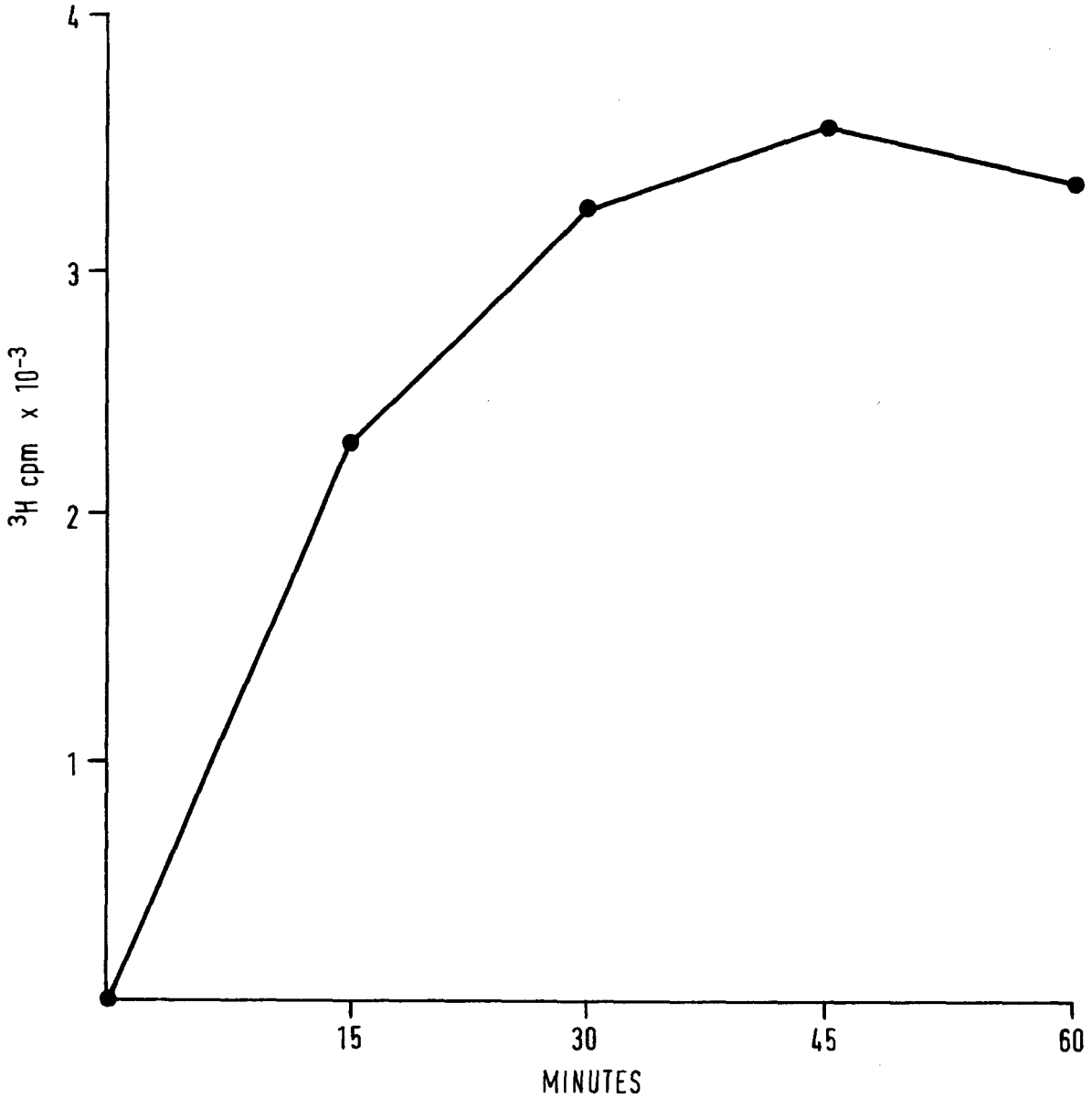

Fig. 1: Kinetics of an endogenous reaction in a pellet fraction isolated from the plasma of a chronic lymphatic leukemia (CML) patient. Plasma was centrifuged at 10,000 X g for 10 min at 3°. The clear zone between the lipids and the precipitate was processed as described (25). A 125 µl reaction containing 6.25 µmol of Tris-HCl (pH 8.3), 1 µmol $MgCl_2$-1.25 µmol NaCl-0.2 µmol each of dGTP, dCTP, dATP, and 0.2 mCi of [^{3}H]TTP (50.1 Ci/mmol) was performed. At different times, 20-µl aliquots were withdrawn and assayed for acid-precipitable radioactivity.

mic and normal blood bank donors' plasma. These RNAs were then annealed to the [^{3}H]DNA product. After 24 hr of annealing, the reaction mixtures were analyzed on cesium sulphate density equilibrium gradients. Figures 4A and B show that 92 % of the DNA hybridizes specifically to the leukemic RNA and not to the RNA isolated from normal blood bank donors.

Table 1. Simultaneous Detection of 70S RNA and Reverse Transcriptase in Leukemic Plasma Pellets

Leukemias	Volume Ml	WBC	Simultaneous Detection 70S-cpm	Reaction
Acute lymphatic				
Bl	60	900,000	1780	+
Du	62	27,500	148	+
Acute Myelogenous				
#196	6	–	32	+
Ra	30	26,800	56	+
Si	40	50,000	193	+
Ba	45	7,800	135	+
Gu	75	40,000	171	+
El	45	77,000	50	+
McL	100	60,000	285	+
Kl	115	16,500	110	+
Chronic Lymphatic				
Mil	75	150,000	61	+
Sc	120	9,300	0	–
Chronic Myelogenous				
Mi	28	80,000	0	–
To	100	–	80	+
Co	10	62,000	0	–
Te	1.5	–	100	+
Mo	37	114,000	300	+
Acute Myelomonocytic				
Be	72	52,000	0	–
Acute Leukemia (non-sp.)				
#379	9	–	0	–
Non-leukemic				
Le (Polycythemia)	50	60,000	0	–
Fi (Polycythemia)	90	9,500	20	–
Za (High WBC Counts)	90	82,000	15	–

Summary of simultaneous detection assay for 70S RNA and reverse transcriptase in plasma pellets. The sum of cpm in the 70S position monitored by external size marker is recorded (Methods) and is designated as positive if the cpm exceeds 30, which is three times the background count. All of the leukemic patients examined were in the active phases of their disease. Peripheral leukocyte counts (WBC) at the time of blood sampling are indicated where available.

Table 1. Continued

Leukemias	Volume Ml	WBC	Simultaneous Detection Reaction 70S-cpm	
Normal Pooled Plasma	75	–	0	–
Normal Plasma A	75	–	0	–
" " B	75	–	0	–
" " C	75	–	0	–
" " D	75	–	4	–
" " E	75	–	0	–
" " F	75	–	0	–
" " G	75	–	100	? *
" " H	75	–	14	–
" " I	75	–	2	–

* = Ribnuclease-insensitive as determined on another aliquot of the same sample.

We have previously shown [3] that the DNA product of virus-like particles in human leukemia shows homology to the RNA from Rauscher leukemia virus. It was therefore of obvoius interest to test the homology to the RNA from Rauscher leukemia virus of endogenous DNA product synthesized in the presence of actinomycin D by the p-100 pellet of leukemic plasma.

Figures 4C-D show the outcomes of the annealing reaction of the [^{3}H]DNA product from a leukemic plasma pellet to 70S RNA isolated from the Rauscher leukemia virus and to that from avian myeloblastosis virus. It is clear from Fig. 4C that 20 % of the [^{3}H]DNA product anneals specifically to the 70S RNA of the RLV, whereas no hybridization occurs with the 70S RNA of AMV (Fig. 4D). This result is in complete accord with the earlier studies on particles from leukemic cells (3, 13, 14, 24, 25).

The earlier finding in human leukemia cells (3, 13, 14, 24, 25) of particles possessing four of the biochemical and physical properties of murine RNA tumor viruses suggested an analogy of the disease in humans and animals. The presence of RNA tumor viruses in the plasma of animals with neoplastic growth stimulated a search for virus-like particles or their components in the plasma from human leukemic patients.

On examining plasmas from 19 leukemic patients and 13 normal blood bank donors, 74 % of the leukemic patients showed evidence for the presence of particulate complexes containing 70S RNA and reverse transcriptase. No such complexes were identified in the plasma from normal blood bank donors.

Particularly interesting was the observation that the [^{3}H]DNA synthesized by the RNA-instructed DNA polymerase on its own endogenous RNA template hybridizes specifically to RNA from leukemic plasma (Fig. 4A) and to RLV RNA (Fig. 4C), in agreement with previous studies on particles found in the white blood cells of

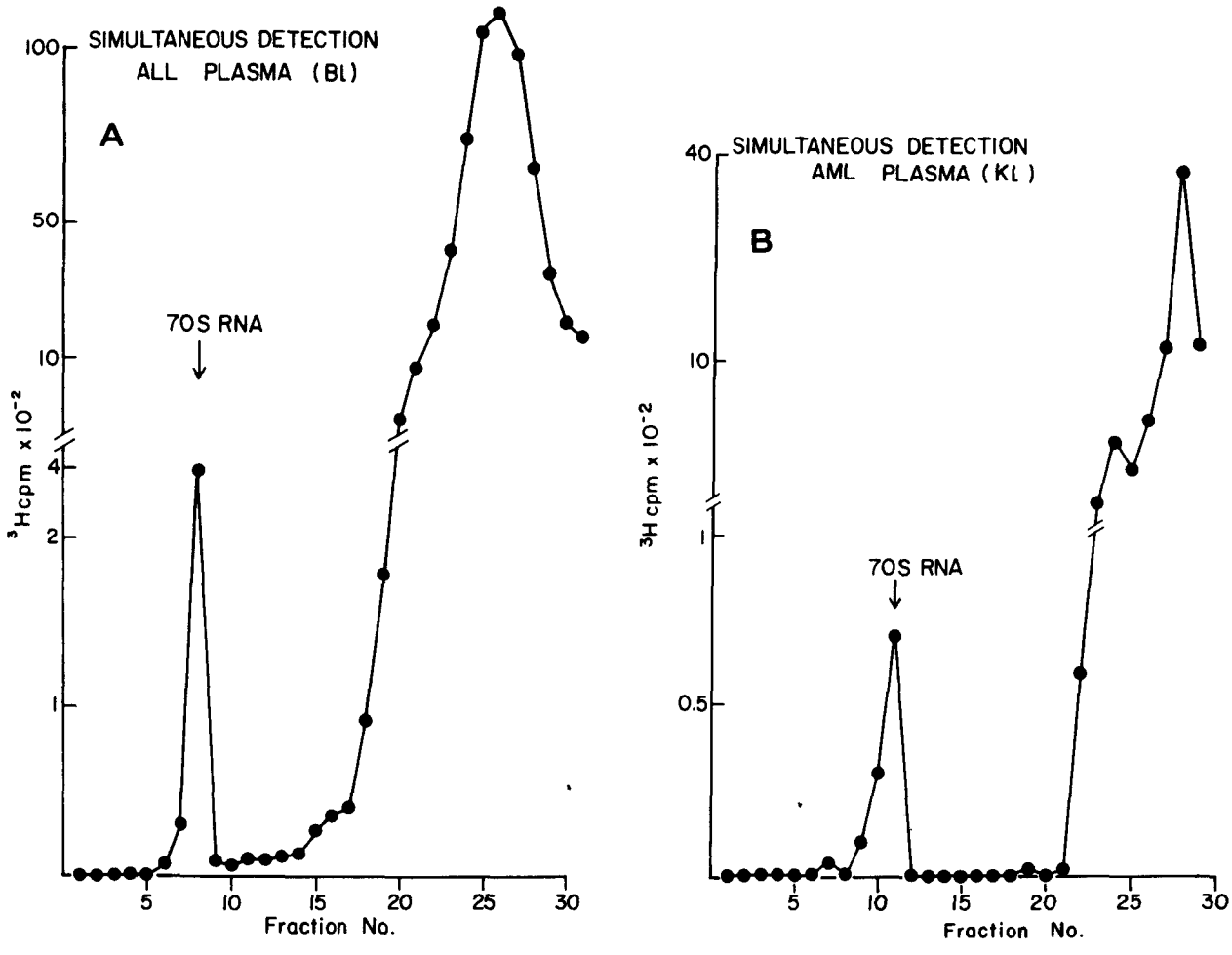

Fig. 2: Simultaneous detection of 70S RNA : [^{3}H]DNA complex in pellets of human leukemic plasma. A) acute lymphoblastic leukemia (ALL) and B) acute myelogenous leukemia (AML). Preparation of the pellet fraction from human plasma and the reagents used in a standard endogenous RNA-instructed DNA polymerase assay are described in Fig. 1. After a 20-min incubation at 37°, the reaction was terminated by the addition of NaCl and sodium dodecyl sulphate to final concentrations of 0.4 M and 1 %, respectively. An equal volume of a phenol-cresol (7 : 1) mixture containing 8-hydroxyquinoline (0.1 g/100 ml of mixture) was added, and the final mixture was shaken at 25° for 5 min and centrifuged at 5000 X g for 5 min at 25°. The aqueous phase was then layered over a linear glycerol gradient (10—30 % in TNE) and centrifuged at 50,000 rpm for 90 min at 4° (Spinco SW-50.1 rotor). [^{3}H]-labeled 70S RNA from avian myeloblastosis virus was used as an external marker. Fractions were collected from below and were assayed for acid-precipitable radioactivity.

leukemic patients (3, 13, 24). The lack of hybridizability to AMV RNA (Fig. 4D) and RNA extracted from normal blood bank donors (Fig. 4B) eliminates the possibility that complexing is due to the poly(A) stretches found in RNA tumor viruses (26—28).

Finding the 70S RNA-reverse transcriptase complexes in the leukemic plasmas is of course not unexpected in view of our previous studies of human leukemias (3, 13, 24). The nucleic acid homologies shown in Fig. 4 parallel those found for leukemic cell particles and suggest that the plasma complexes are similar to or are derived from the particles detected in the leukemic cells. Unfortunately, the amounts found in the plasmas were not sufficient for a definitive characterization with the techniques currently available.

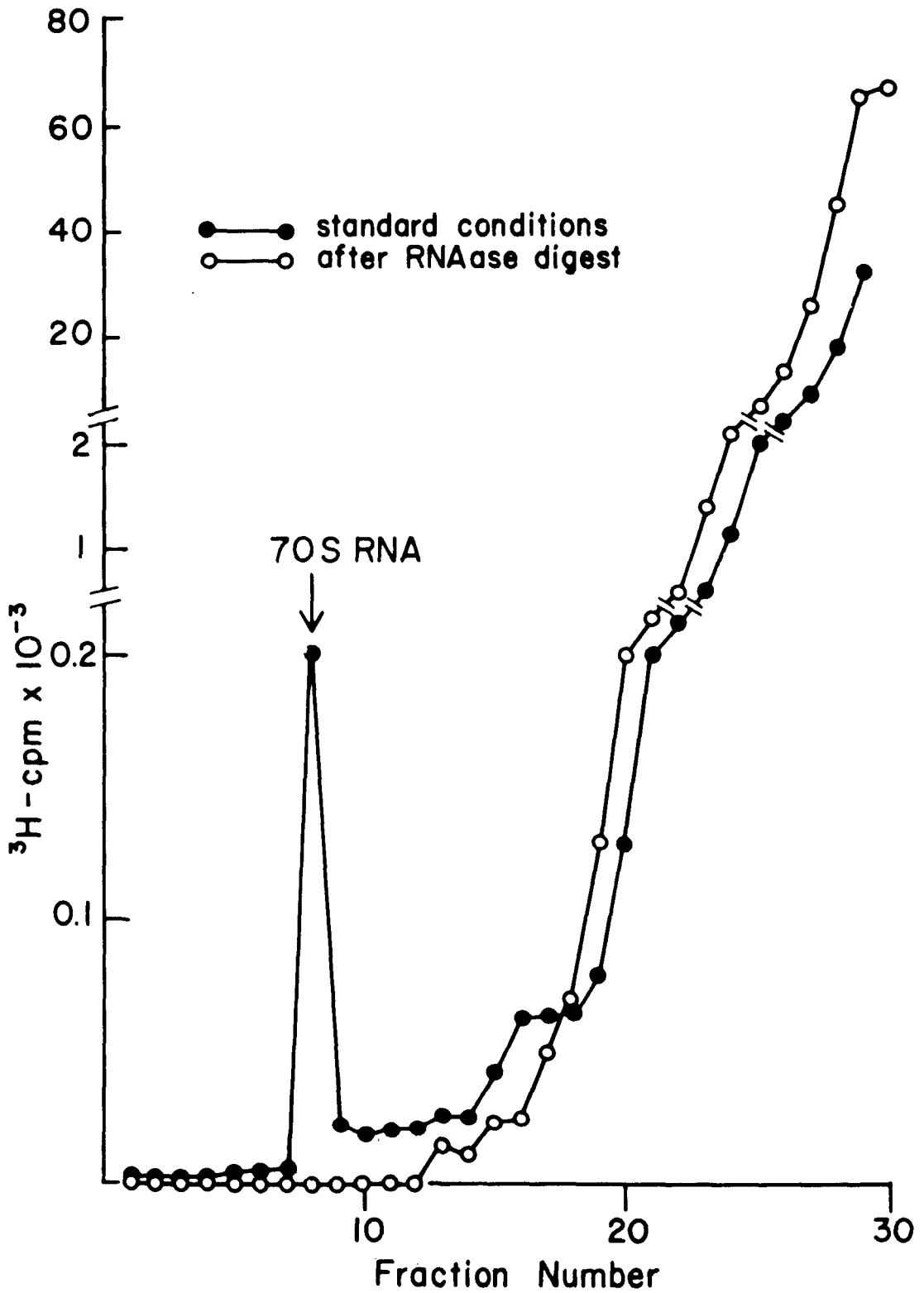

Fig. 3: Effect of ribonuclease on the 70S RNA : [^{3}H]DNA complex. Plasma pellet of a chronic myelogenous leukemic patient was processed and assayed as described. After 20 min of incubation at 37°, the nucleic acid was extracted and divided into two equal parts. Onehalf was left untreated (●——●), whereas the other half was treated with RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37° (0——0). The two parts were then layered on separate gradients and analyzed for 70S RNA : [^{3}H]DNA complex by the procedure described (25).

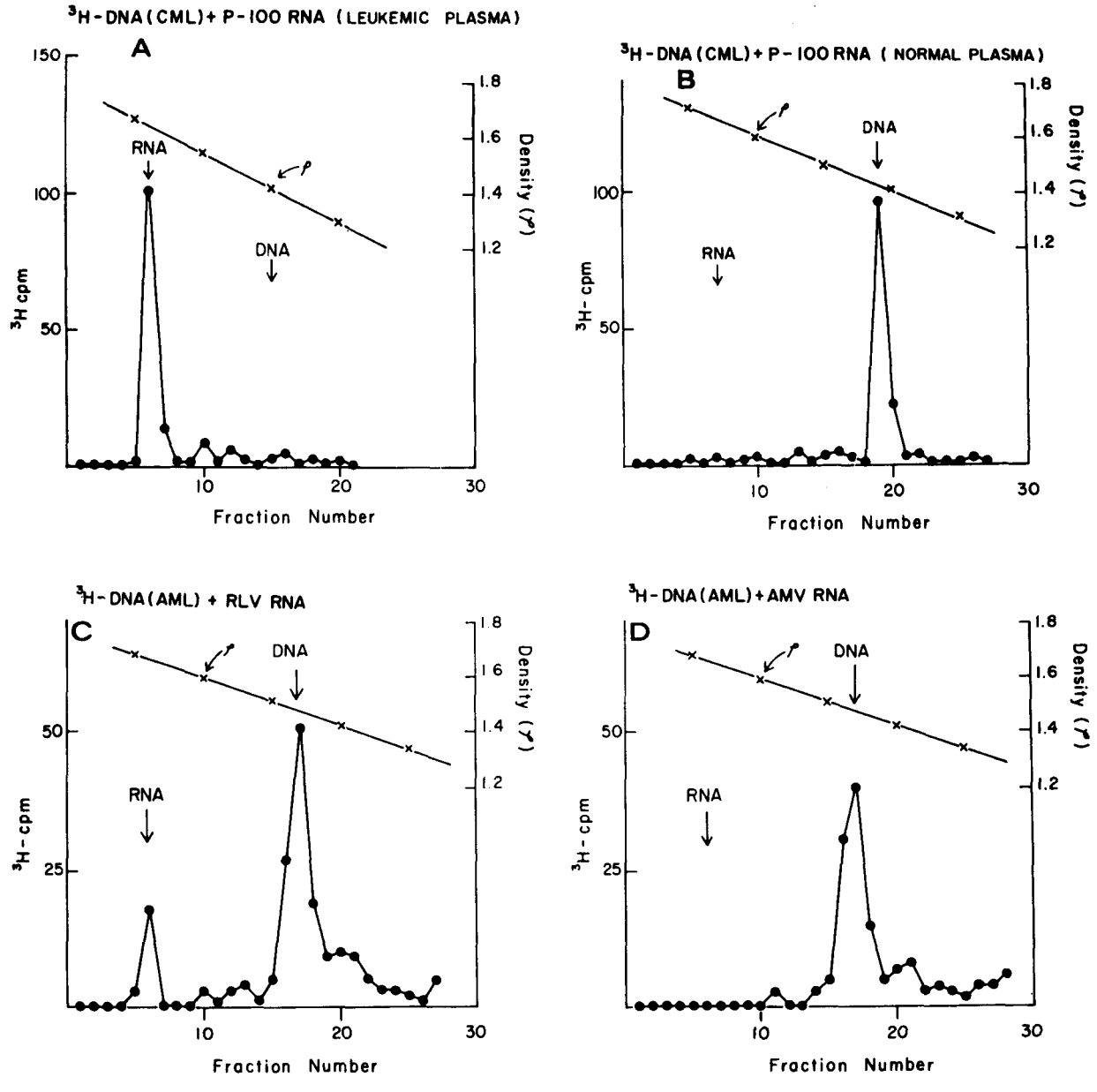

Fig. 4: Cesium sulphate equilibrium density gradient analysis of annealing reactions of leukemic plasma pellet [^{3}H]DNA to: A) RNA from leukemic plasma, B) RNA from normal plasma, C) RLV-70S RNA, and D) AMV-70S RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legends to Figs. 1 and 2. The 70S RNA: [^{3}H]DNA complex obtained following velocity centrifugation was digested with 0.4 M NaOH for 8 hr at 37° to destroy all RNA present. The [^{3}H]DNA product from a plasma pellet of a CML patient (Te) was then annealed to 12 μg of plasma pellets RNA (A, B). The [^{3}H]DNA product from an AML patient (Si) was annealed to 1 μg of 70S RNA from Rauscher leukemia virus (C) and avian myeloblastosis virus (D). After annealing for 18 hr, the reaction mixture was analyzed by cesium sulphate isopycnic gradient centrifugation (25).

The presence of these complexes in the leukemic plasmas raises a number of interesting questions and possibilities with respect to their relation to the disease and its state. It is conceivable that the levels of these complexes could be used as infor-

mative parameters in both diagnosis and treatment. The simultaneous detection test (6) and molecular hybridizations used to identify these complexes are highly informative since they focus attention on the relevant genetic information. However, it must be emphasized that these techniques are too sophisticated and laborious for the routine use demanded by extensive clinical studies. An obvious pathway for exploiting the potential usefulness of these RNA-enzyme complexes is to develop a radioimmune assay (29) for their protein components. This approach, if successful, would possess the simplicity, sensitivity, and speed required for realistic clinical applications. Attempts along these lines are in progress.

Summary. Complexes containing 70S RNA and RNA-directed DNA polymerase were detected in 74 % of the human leukemic plasmas, whereas none was observed in normal plasmas. The DNA product synthesized by these complexes hybridized to the RNA of Rauscher leukemia virus and to RNA obtained from leukemic cells and did not hybridize to the RNA of normal leukocytes or to the RNA of the unrelated avian meyeloblastosis virus.

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