RNA TUMOR VIRUSES AND HUMAN LEUKEMIAS

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Considerable information is available concerning the etiological role of RNA tumor viruses in leukemias, lymphomas, sarcomas, and breast cancer in a variety of animals, e. g., chicken (1), mice (2), cats (3), etc. In animals, causative proof has been achieved by inoculation experiments that satisfy Koch's postulates. In man, such experiments obviously cannot be undertaken, and other more indirect evidence has to be assembled to clarify the etiology of human malignancies with its implications for prophylaxis and therapy.

The data reported here on the presence of viral-like particles in human leukemias are based on the technique of molecular hybridization of nucleic acids (4—10) and of the simultaneous detection of high molecular weight RNA associated with a reverse transcriptase at physical densities characteristic of oncogenic RNA viruses (11—17).

The general biochemical and physical properties of the RNA tumor viruses include a characteristic density of 1.16 g/cm^3. Inside an outer shell they contain nucleoids with a density of 1.23 g/cm^3. As genetic material they possess a high molecular weight, with 60 to 70S-sedimenting RNA, which can dissociate into 35S subunits. The RNA is associated with an RNA-dependent DNA polymerase (reverse transcriptase) (18, 19). With this enzyme, a radioactively labeled DNA can be synthesized that is complementary to the tumor virus RNA (5).

Several considerations (5, 7) led us to the assumption that the RNAs of different mammalian leukemia viruses might be related to each other with regard to their base sequence. To test this assumption, radioactive DNA , which had been synthesized on RNA of a leukemia virus of a mouse (RLV), was hybridized to RNA isolated from leukocytes of a leukemic rat (7). After a positive outcome of this comparison involving two closely related animal hosts, a similar experiment was undertaken by hybridizing RLV-^{3}H-DNA to RNA isolated from the cytoplasm of human leukemic cells (7). In Fig. 1 the density profiles are shown of hybridizations with RNAs from four human leukemias. The ^{3}H-DNA mainly bands in the DNA density region of the gradient (ρ = 1.45 g/cm^3), but some of the radioactive DNA can be seen banding in the RNA density region of the gradient (ρ = 1.65 g/cm^3) complexed to the denser RNA. Homologies to mouse leukemia virus RNA were found, whether

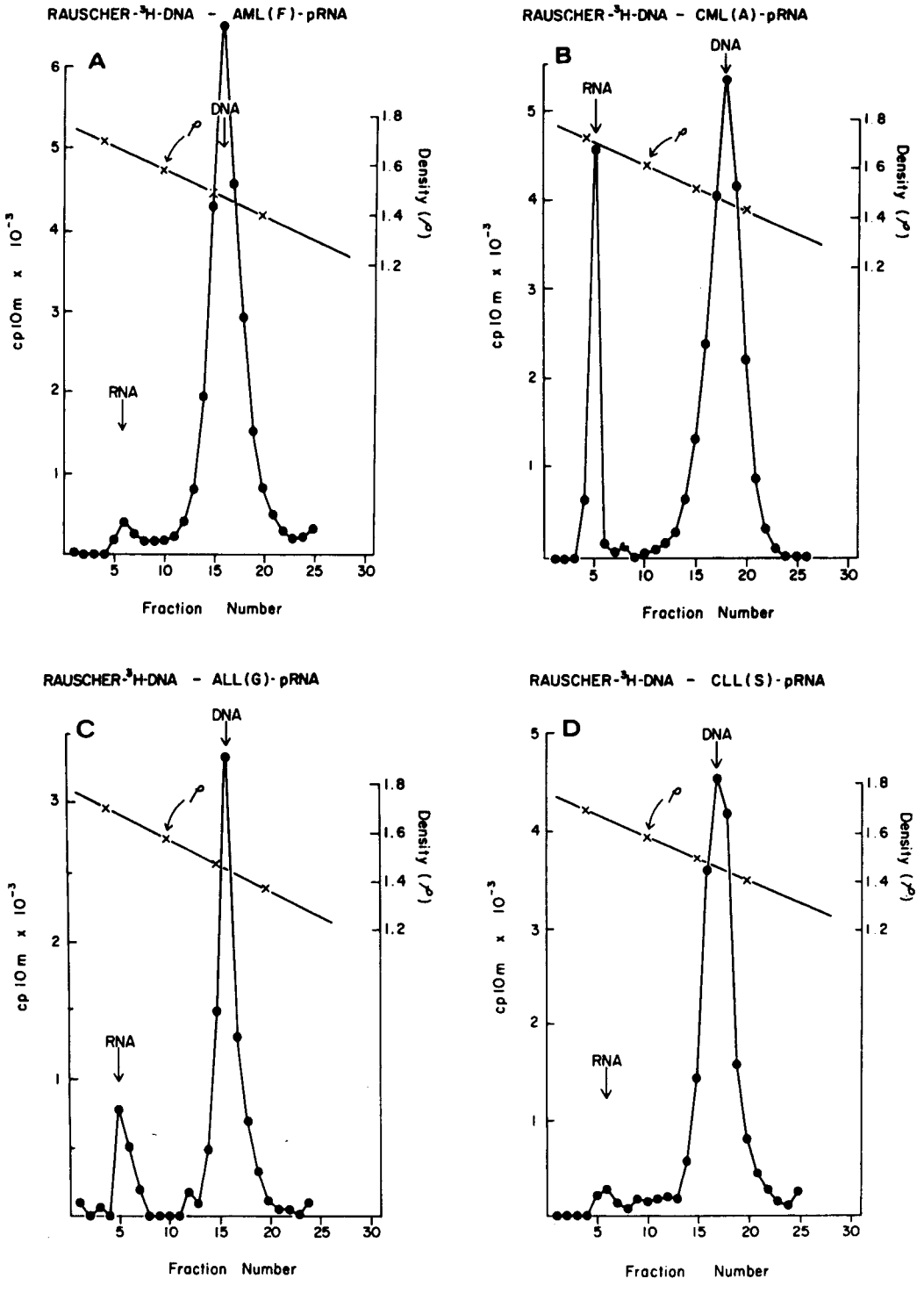

Fig. 1: Cs_2SO_4 density profiles of hybridization reactions between ^{3}H-RLV-DNA and cytoplasmic RNA isolated from human leukemic white blood cells. ^{3}H-RLV-DNA and cytoplasmic RNA were prepared as described (7). About 350 µg RNA was hybridized to 2000 cpm of purified, heat denatured ^{3}H-RLV-DNA (10,000 cpm/pmol) for 18 hr at 37 °C in the presence of 0.4 M NaCl and 50 % formamide (total vol 60 µl). After incubation, the reaction mixture was added to 10 ml half-saturated Cs_2SO_4 (ρ = 1.52 g/cm^3) in 5 mM EDTA and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15 °C. Fractions of 0.4 ml were collected from below and assayed for TCA-precipitable activity. The cytoplasmic RNA was derived from A) AML (F), B) CML (A), C) ALL (G), D) CLL (S).

the human leukemic RNA was derived from lymphoblastic or myelogenous, acute, or chronic leukemias. Positive outcomes were observed in 28 out of 31 leukemic patients, i. e., in more than 90 % the RNA isolated from leukemic leukocytes showed homology to the RNA of a mouse leukemia virus. In contrast, more than 50 normal control tissues, including normal leukocytes, did not show this kind of response.

The position of the RNA-DNA-hybrids in the Cs_2SO_4 density gradients indicates that the human leukemia-specific RNA determines the density of the hybrids and therefore is considerably larger than the 5–8S large radioactive DNA. It was logical to assume that this large RNA is of the 60–70S type and possibly associated with a reverse transcriptase in a complete viral particle.

The resolution of these and related questions was made feasible by the apllication of a technique for the simultaneous detection of a high molecular weight RNA associated with a reverse transcriptase (11, 12). The basis for the simultaneous detection of 60–70S RNA and reverse transcriptase stemmed from the observations (20–22) that the initial DNA product is found complexed to its 70S-RNA template. If early DNA product is found on sedimentation analysis to travel as if it were a 70S molecule, and if supplementary evidence is provided that its apparent size is due to its being complexed to a 70S-RNA molecule, evidence is provided for the presence of reverse transcriptase that uses a 70S-RNA template.

Figure 2 shows representative results of experiments along these lines with leukocytes of leukemic patients (13, 16). After an endogenous reverse transcriptase reaction and deproteinization of the nucleic acids a portion of the ^{3}H-DNA sediments in discrete 70S and 35S peaks in glycerol-sedimentation-gradients. After RNase digestion, the entire radioactive DNA is found in the low molecular weight region of the gradient, as exemplified in Fig. 2B. This proves that the ^{3}H-DNA was complexed to a high molecular weight RNA. In Fig. 2, experiments with cell material from patients with ALL (A) and CML (B) are shown. In total, more than 50 leukemic cell samples gave the specific DNA-synthesis resulting in ^{3}H-DNA complexed to a 70S RNA whereas only one out of 27 normal white blood cell samples yielded evidence for this type of activity. In this case, the available white blood cells did not permit further characterization by hybridization and thus an unambiguous statement as to the nature of this positive reaction cannot be made.

After extensive alkali digestion to destroy all RNA present, the ^{3}H-DNA was recovered and annealed to viral-enriched RNA of the same leukemic cells that had been used for the specific DNA synthesis. From Fig. 3A, it is clear that about 20 % of the ^{3}H-DNA is shifted from the DNA region to the hybrid and RNA regions of the gradient due to formation of RNA-DNA hybrid structures. When the same human ^{3}H-DNA is annealed with an equivalent amount of AMV-70S-RNA, no evidence of hybrid formation is seen (3B). This back hybridization completes the operational definition of a reverse transcriptase and shows that human leukemias contain 70S RNA-associated reverse transcriptase. These data are in agreement with the finding by other groups of reverse transcriptase in human leukemic cells (23, 24).

By density fractionation of the leukemic cytoplasm, it could be shown that this 70S-RNA-DNA polymerase complex has a density of 1.16 g/cm^3 (Fig. 4), which is

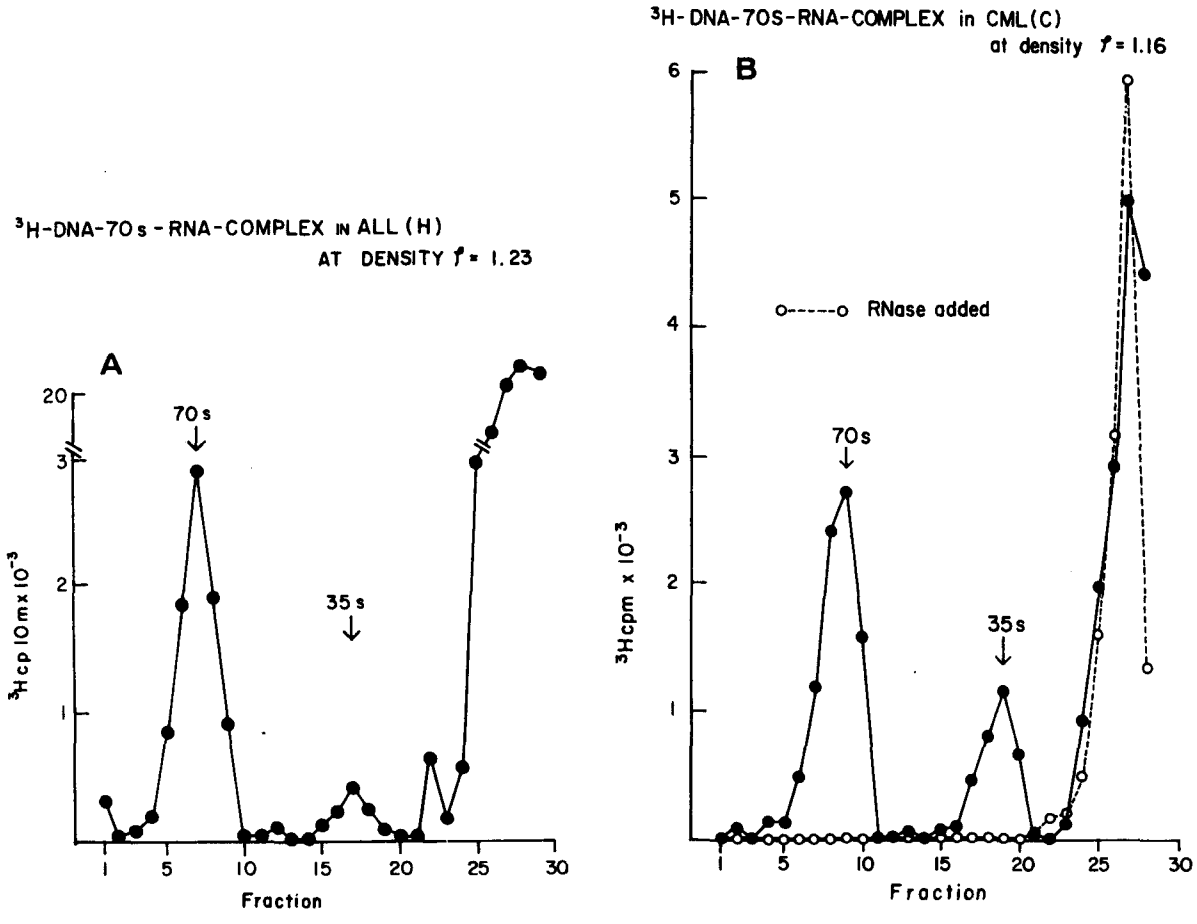

Fig. 2: Simultaneous detection of 60—70S RNA and reverse transcriptase in human leukemic white blood cells. 0.5—2 g of white blood cells were disrupted in TNE buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM EDTA) with a Dounce homogenizer. Nuclei were removed by centrifugation of the cell homogenate at 4000 X g for 10 min at 0 °C, and the supernatant was centrifuged at 10,000 X g for 10 min at 0 °C. The resulting postmitochondrial supernatant fluid was then layered on a 20-ml column of 20 % glycerol in TNE and spun at 100,000 X g for 1 hr at 1 °C in a Spinco SW-27 rotor. The resulting pellet was resuspended in TNE buffer (2 ml per 1 g of cells). Insoluble debris was removed from this suspension by centrifugation at 4000 X g for 10 min. For further purification and density determination, the suspension was then layered on several preformed 20—60 % sucrose TNE gradients and spun for 3 hr at 100,000 X g and 4 °C. Seven to 10 fractions were collected from each gradient. Each fraction was diluted in TNM buffer (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$), pelleted (100,000 X g, 1 hr, 4 °C, Spinco SW-27 rotor), resuspended in 0.3 ml of 0.1 M NaCl – 0.01 M Tris-HCl, pH 8.3, and assayed for endogenous reverse transcriptase activity: After preincubation with 0.1 % Nonidet P-40 at 0 °C for 15 min. 3 µmol $MgCl_2$, 0.4 µmol each of dATP, dGTP, dCTP, 1 mCi ^{3}H-TTP (NEN, 50.1 mCi/mmole) and 100 µg/ml actinomycin D to inhibit DNA-instructed DNA synthesis were added to give a final vol of 0.5 ml. The reaction was incubated at 37 °C for 15 min. After adjustment to 0.2 M NaCl to stabilize hybrid structures, the reaction was terminated by addition of 0.5 % SDS and subsequent deproteinization by phenol-cresol extraction. The aqueous phase was then layered on a 10—30 % glycerol in TNE gradient and centrifuged at 40,000 rpm for 3 hr at 1 °C (SW-41 rotor, Spinco). Fractions of 0.4 ml were collected from below and assayed for TCA-precipitable radioactivity. 18 and 28S r-RNA and 70S AMV-RNA served as markers. The sedimentation profiles of two representative simultaneous detection tests with the appropriate density fractions are shown. A) ALL (H) at density ρ = 1.23, B) CML (Con) at density ρ = 1.16 g/cm^3. RNase sensitivity was tested by incubating the reaction mixture with 80 µg/ml ribonuclease A (Worthington) for 15 min at 37 °C prior to sedimentation analysis.

characteristic of RNA tumor viruses (16). Further, the DNA synthesized by the leukemic cell reverse transcriptase on its own endogenous template is related in sequence to RLV RNA (13). 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 find the virus-related information in leukemic cells.

The experiments reported here demonstrate the presence in human leukemic cells of four parameters characteristic of RNA tumor viruses: 1) a high molecular weight RNA of the 60–70S type, 2) a reverse transcriptase, 3) a base sequence of the RNA, which is related to that of a mouse leukemia virus, and 4) a physical density for the 70S RNA-DNA-polymerase complex characteristic of RNA tumor viruses. The findings are individually suggestive of a viral agent, but do not prove a viral etiology of human leukemia. The data, however, do encourage further efforts along these lines.

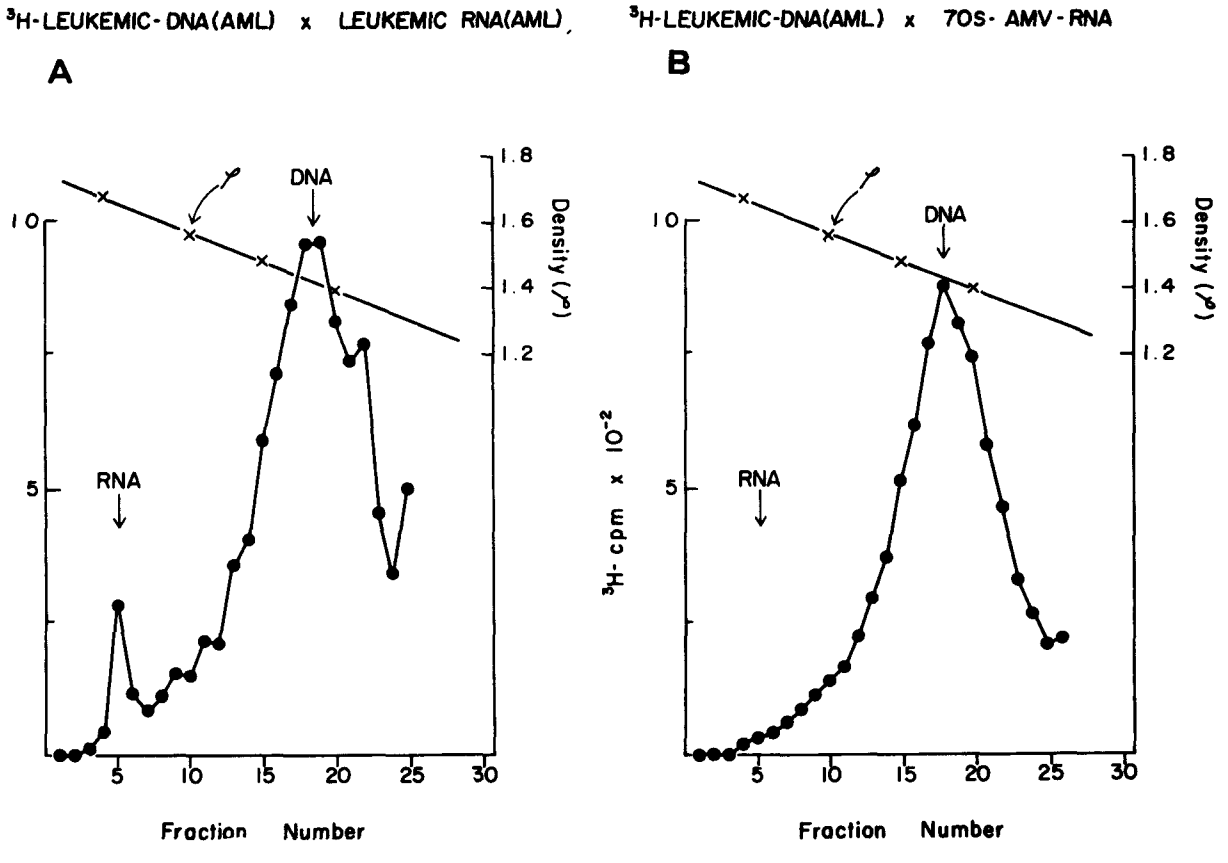

Fig. 3: Cs_2SO_4 equilibrium density gradient centrifugation of annealing reactions of human leukemic (AML [T]) ^{3}H-DNA to (A) 85 μg human leukemic (AML [T]) RNA enriched in viral sequences by prior density fractionation of cytoplasmic material and subsequent RNA extraction from the 1.10–1.26 g/cm^3 density region, and (B) 0.5 μg highly purified 70S AMV-RNA. A standard RNA-instructed DNA polymerase reaction was performed as described in the legend to Fig. 2. The high molecular weight RNA ^{3}H-DNA complex obtained after glycerol sedimentation centrifugation was digested with 0.4 M NaOH for 18 hr at 37 °C to remove all RNA present. The ^{3}H-DNA product was then annealed to the respective RNAs in a vol of 60 μl in the presence of 50 % formamide and 0.4 M NaCl. After annealing for 18 hr at 37 °C, the reaction mixture was subjected to Cs_2SO_4 gradient centrifugation as described under Fig. 1.

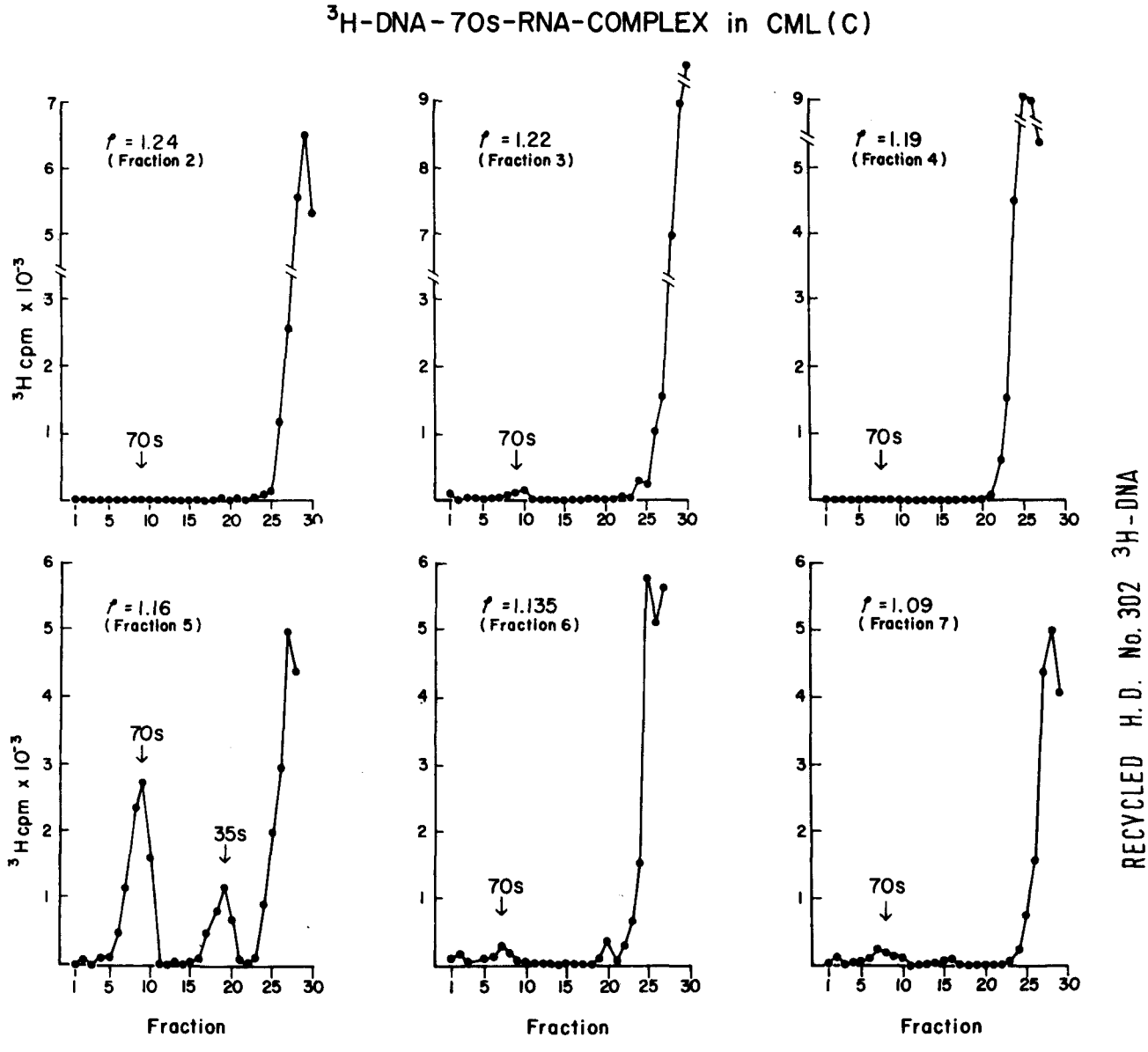

Fig. 4: Sedimentation profiles of a complete set of simultaneous detection tests after sucrose density fractionation of leukemic cytoplasm. 2 g of white blood cells from a patient with CML (Con) were prepared and examined as described in Fig. 2.

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