POLY(A)-CONTAINING MOLECULES IN HETEROGENEOUS NUCLEAR RNA: DIFFERENCES BETWEEN NORMAL LYMPHOCYTES AND ACUTE LEUKEMIA BLAST CELLS

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Rapidly labeled nuclear RNA from mammalian cells can be divided into two major classes. The first class is formed by the 45S ribosomal precursor RNA and its products of cleavage 41S, 36S and 32S RNA (1, 2). Experiments with HeLa cells have shown that these molecules are synthesized and processed in the nucleolus (3). The base composition of these molecules is similar to that of ribosomes, and they are further characterized by the presence of methylated bases and sugar moieties (4) and by the absence of polyadenylic acid sequences (5).

The second type of nuclear RNA consists of molecules ranging in size up to 120S. The base composition of these molecules resembles that of DNA (6), and they are further characterized by the absence of methylated bases and sugar moieties and by the presence of polyadenylic acid sequences (7). These molecules are generally considered to be completely heterodisperse, so that this class of RNA is called heterogeneous nuclear RNA. However, fractionation of these molecules by gel electrophoresis as performed in our laboratory, has shown that, at least in human blood cells, the pattern is more complex than previously considered (8, 9).

As shown in fig. 1, by analyzing nuclear RNA from human small lymphocytes several discrete fractions were resolved behind the 28S ribosomal RNA component. Some of them may be identified with the already mentioned short-lived products of cleavage of the 45S ribosomal precursor RNA, but several of these discrete fractions run more slowly than this latter molecules. Evidence has been presented (9) that they are not technical artifacts and that they can also be observed in PHA-stimulated lymphocytes, provided that the period of incubation with the labeled precursor was not longer than 15 minutes (Fig. 2 and 3).

One of the most recently demonstrated characteristics of heterogeneous nuclear RNA is the presence, in this RNA, of polyadenylic acid sequences covalently linked. In a group of experiments carried out in our laboratory we have assayed the proportion of poly(A)-containing molecules in electrophoretically separated fractions of nuclear RNA obtained from unstimulated and PHA-stimulated normal human lymphocytes. The assay was carried out by measuring: 1) the amount of radioactivity bound to nitrocellulose filters after filtration of radioactive RNA dissolved in buffer with high salt concentration; 2) the amount of radioactivity bound to glass fiber filters containing poly(U).

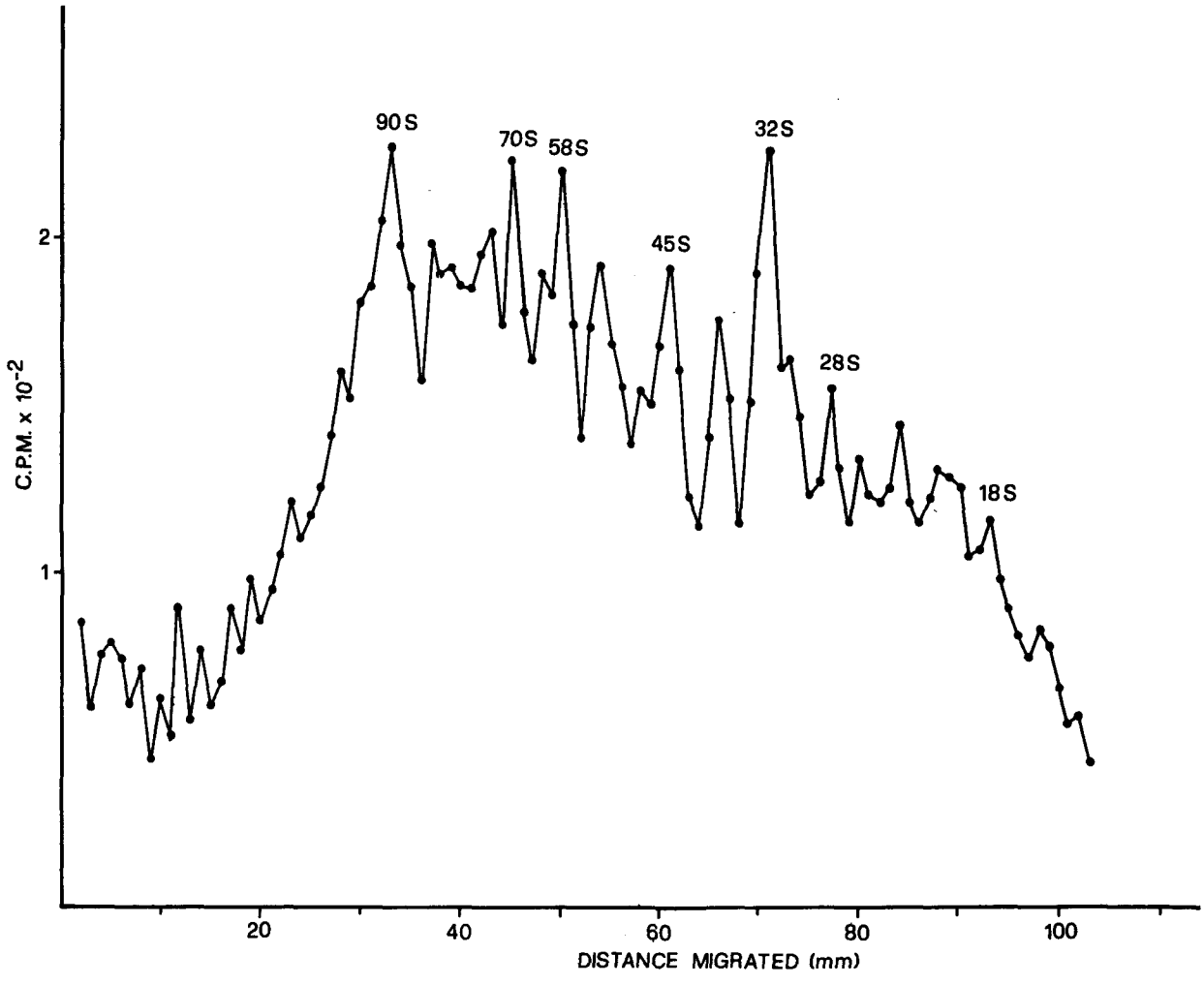

Fig. 1: Electrophoretic profile of newly synthesized nuclear lymphocyte RNA. The cells were incubated with ^{3}H-uridine (10 μc/ml) for 6 hours. Nuclei were separated and extracted together with an amount of unlabeled KB cells sufficient to give enough 28S and 18S RNA as markers. Electrophoresis was für 180 minutes at 3 mA/gel. After the electrophoretic run the gel was scanned at 260 mμ and sliced in 1 mm slices. Nominal S values were assigned on the basis of the linear relationship between the logaritm of the molecular weight of a given RNA component and its relative electrophoretic mobility. The S values were therefore calculated from the known S values of the two RNA components of the ribosomal subunits (18S and 28S).

To evaluate the binding of labeled RNA to nitrocellulose filters, 2 ml of buffer (0.01 M Tris, pH 7.4, 0.5 M KCl, 1 mM $MgCl_2$) containing the RNA recovered from the gel were diluted to 10 ml with the same buffer and then filtered at room temperature through Millipore filters (HA 0.45 μm, Millipore Filter Corp., Bedford, Mass.) previously soaked in the same salt solution. The filters were then washed twice with 10 ml of this salt solution, dried and counted in toluene scintillation mixture. Input values were obtained by drying 0.5 ml aliquots of the RNA solution on Millipore filters previously soaked in high salt buffer, and counting. To evaluate the binding of labeled RNA to glass fiber filters containing poly(U), RNA in gel slices was solubilized in 3 ml of binding buffer (0.01 M Tris. KCl (pH 7.5), 0.12 M NaCl). 2 ml of this solution were brought to 10 ml with the same buffer, then filtered at 2 ml per min through glass fiber filters (Whatman, GF/C 2.5 cm) on which poly(U) had been previously immobilized by the technique of Sheldon et al. (10).

The filters were washed with 20 ml of binding buffer at 25 °C, followed by 20 ml of ice cold 5 % TCA and 10 ml of 95° ethanol, then dried and counted. Table I shows that an average 20 % of the labelled molecules in the size range between 49S and 120S bind to nitrocellulose filters, whereas a reduced amount of filter-bound radioactivity was found in fractions corresponding to S values lower than 48. This may be explained by the observation that ribosomal RNA and its precursors lack poly(A) segments (5). Little differences were found between RNA from unstimulated and PHA stimulated lymphocytes. The proportion of poly(A)-rich molecules was a littel lower in the former cells, at least in the fractions corresponding to S values from 16 to 60. The average proportion of labeled molecules bound to glass fiber filters containing poly(U) was about 11 %, as shown in table II. The poly(A)-rich molecules of total nuclear RNA were also analyzed by gel electrophoresis. The results show that whereas total nuclear RNA was resolved in several discrete peaks, the poly(A)-rich molecules of the same RNA were heterogeneously distributed through the gel (Fig. 4).

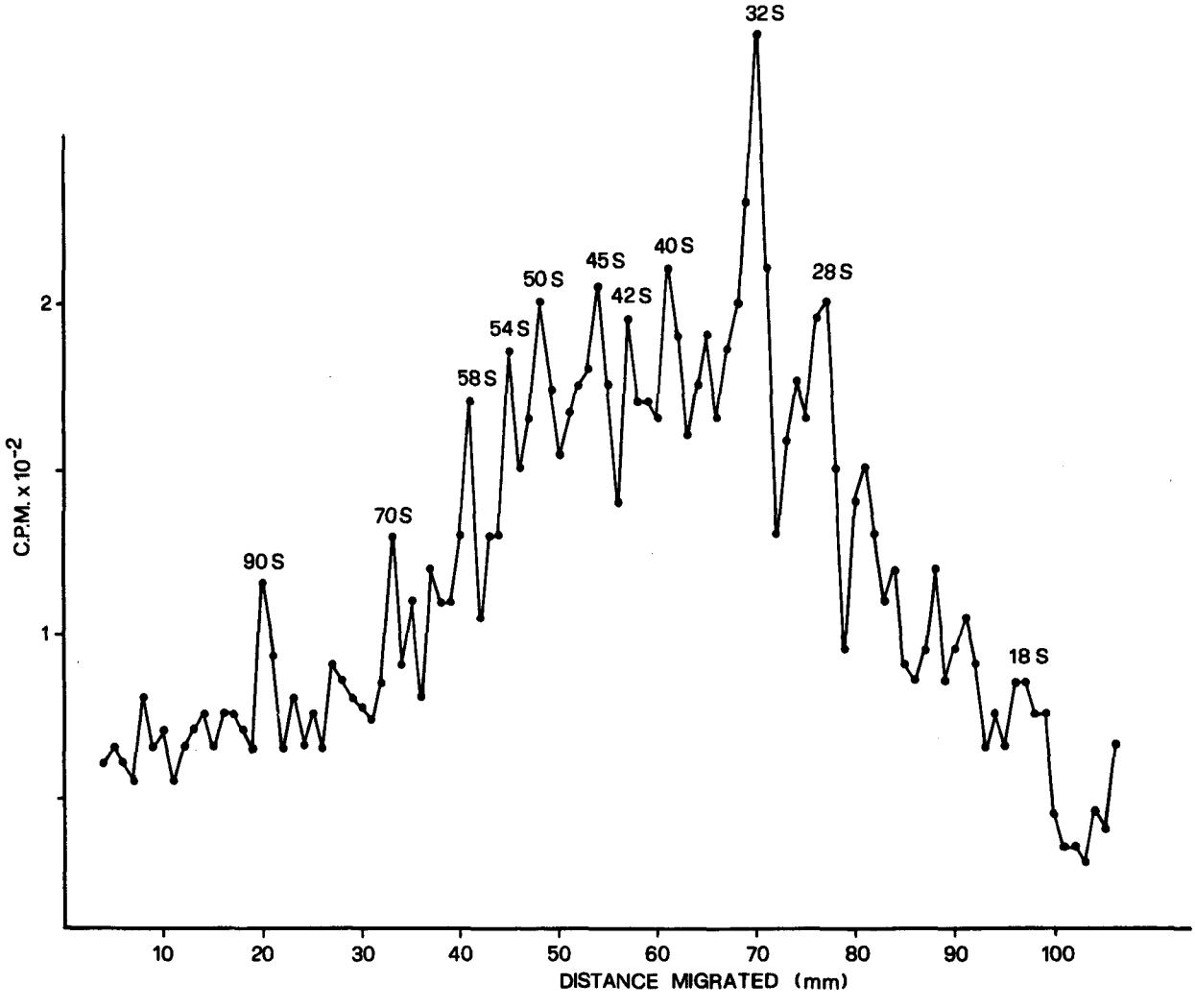

Fig. 2: Electrophoresis of labeled whole cell RNA extracted from PHA-stimulated lymphocytes incubated for 12 minutes with ^{3}H-uridine (20 µc/ml). Run for 200 minutes at 3 mA/gel.

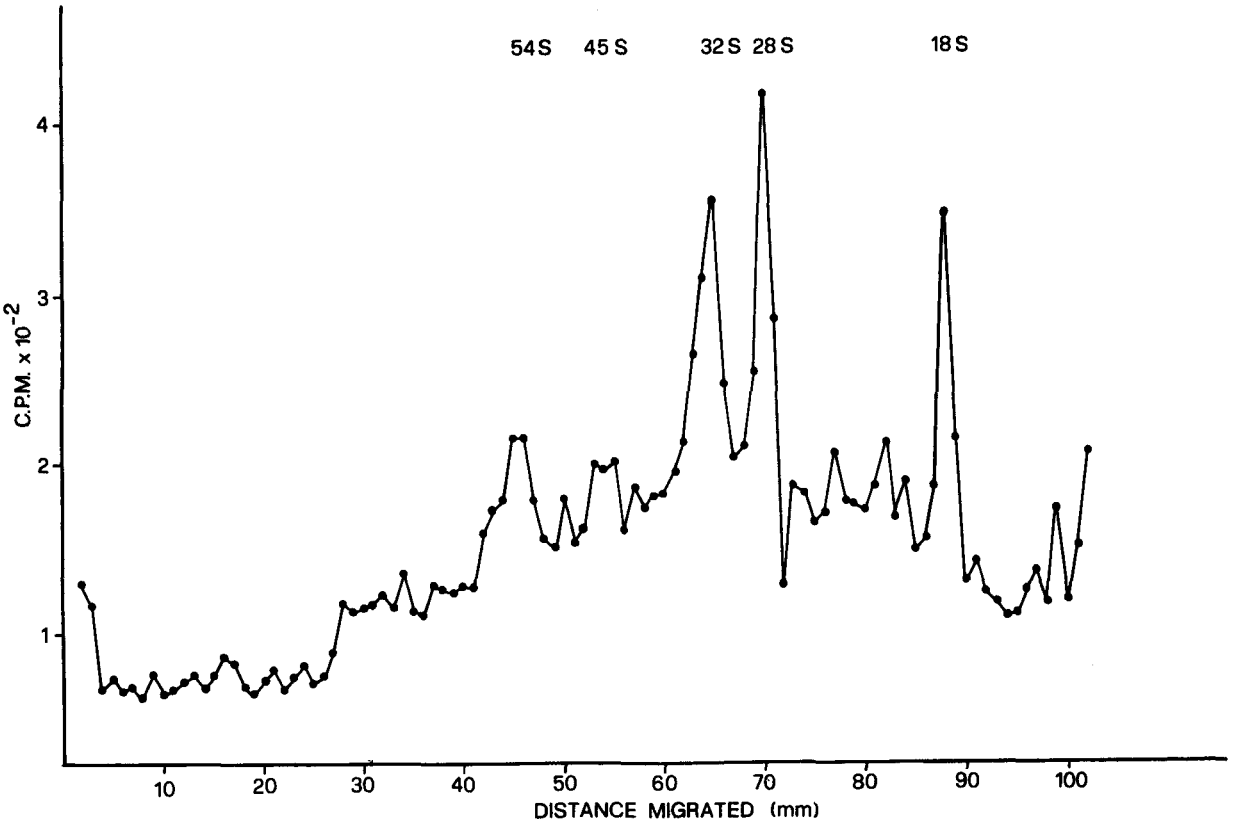

Fig. 3: Labeling pattern obtained by electrophoresis (200 minutes, 3 mA/gel) of whole cell RNA extracted from PHA stimulated lymphocytes incubated with ^{3}H-uridine (10 μc/ml) for 45 minutes.

Table I Binding of labelled nuclear RNA from unstimulated and PHA-stimulated normal lymphocytes to nitrocellulose filters

RNA fraction size range (S values)	Percent of input radioactivity bound to filters	
	unstimulated lymphocytes	PHA-stimulated lymphocytes
16 – 20	9	10
21 – 25	7	11
26 – 30	8	10
31 – 37	7	10
38 – 42	9	13
43 – 48	11	16
49 – 53	15	21
54 – 59	15	20
60 – 65	22	23
66 – 73	21	23
74 – 82	22	22
83 – 89	20	21
90 – 98	23	21
99 – 120	23	22

Several different techniques were employed in the past three years in our laboratory to detect possible differences between heterogeneous nuclear RNA of normal lymphocytes and that of acute leukemia blast cells. No difference between normal and leukemic RNA was observed by gel electrophoresis of whole cell RNA and nuclear RNA (8). The same discrete fractions resolved in RNA of normal small lymphocytes were found in RNA of acute leukemia blast cells.

More revealing findings were obtained in experiments carried out to compare nuclear RNA from normal PHA-stimulated lymphocytes and acute leukemia blast cells by competition hybridization techniques. The results of a first group of experiments have been already reported (11). Further studies were performed in our laboratory to extend our earlier findings. RNA extracted from isolated nuclei of blast cells from ALL patients and of PHA-stimulated lymphocytes was tested in competition hybridization assays. The results have confirmed that unlabeled nuclear RNA from normal lymphocytes is unable to inhibit hybridization of nuclear leukemic RNA with leukemic cell DNA. These results add further evidence to our previous data suggesting that leukemic blast cells are transcribing RNA sequences which are not transcribed in normal lymphocytes (11).

To obtain a better characterization of the heterogeneous nuclear RNA of leukemic cells we have assayed the proportion of poly(A)-containing RNA molecules in different electrophoretic fractions of nuclear RNA obtained from leukemic blast cells. Six cases of acute leukemia have been so far examined. In most fractions, the

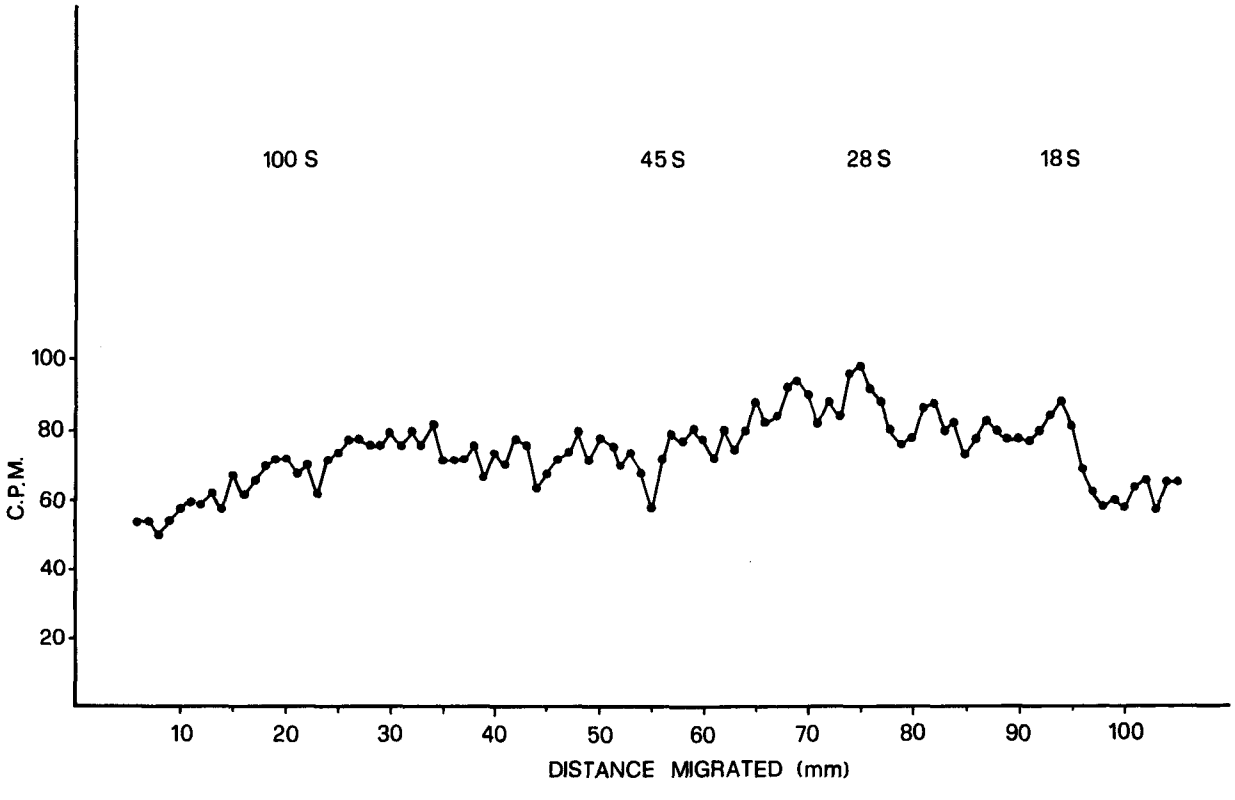

Fig. 4: Electrophoretic profile of the poly(A)-containing fraction of the nuclear RNA extracted from small unstimulated lymphocytes. The RNA was filtered through a Millipore filter, then 2 small discs, 6 mm in diameter, were cut from the filter and put on top of a gel column. The discs were then covered with electrophoresis buffer containing unlabeled KB cell RNA as a carrier. Run was prolonged for 4 hours at 3 mA/gel.

Table II Binding of labelled nuclear RNA from unstimulated and PHA-stimulated normal lymphocytes to Poly (U)-containing glass-fiber filters

RNA fraction	Percent of input radioactivity bound to filters	
	unstimulated lymphocytes	PHA-stimulated lymphocytes
16 – 20	5	6
21 – 25	4	5
26 – 30	4	5
31 – 37	5	6
38 – 42	5	7
49 – 53	8	10
54 – 59	10	13
60 – 65	11	11
66 – 73	11	11
74 – 82	11	10
83 – 89	12	12
90 – 98	11	12
99 – 120	12	10

Table III Binding of labelled nuclear RNA from acute leukemia blast cells to nitrocellulose and Poly (U)-containing glass fiber filters

RNA fraction	Percent of input radioactivity bound to filters	
	Millipore filters	glass fiber filters
16 – 20	17	14
21 – 25	19	25
26 – 30	20	12
31 – 37	25	11
38 – 42	26	11
43 – 48	24	28
49 – 53	36	36
54 – 59	34	39
60 – 65	26	29
66 – 73	31	18
74 – 82	28	12
83 – 89	26	11
90 – 98	20	12
99 – 120	22	10

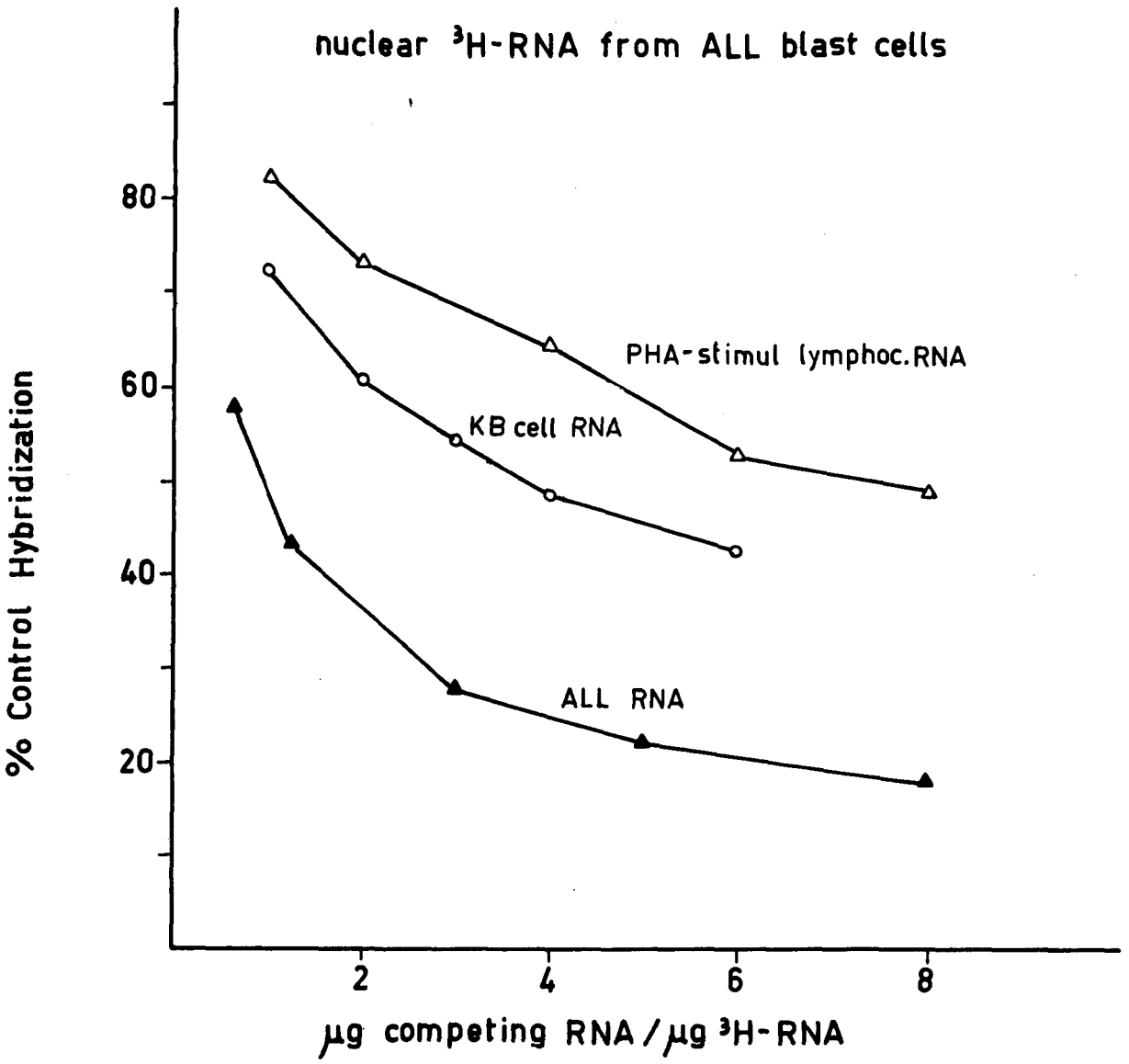

Fig. 5: The competition of unlabeled nuclear RNA from ALL, KB cells and PHA stimulated lymphocytes in hybridization reaction of labeled nuclear RNA from ALL cells with ALL DNA. Filters loaded with DNA were incubated with unlabeled RNA for 18 hr at 67 °C, drained, soaked twice for 30 min in 20 ml of 2 x SSC at 65 °C and then returned to vials for further incubation with labeled RNA.

proportion of poly(A)-containing molecules was definitely larger than that of the corresponding fractions of normal lymphocytes (Fig. 6). The largest proportion was found in all cases in the fractions formed by molecules in the size between 49 and 59S. In many fractions, corresponding to S values lower than 60, the proportion of poly(A)-containing molecules was twice as much as that of the corresponding normal fractions (table III). Furthermore, in normal RNA the proportion of poly(A)-containing molecules was strictly similar in all fractions corresponding to S values greater than 49, whereas leukemic RNA was characterized by a marked variability in the occurence of poly(A)-containing molecules in the different fractions.

It is difficult at present to offer any explanation of the increase in proportion of poly(A)-containing molecules in heterogeneous nuclear RNA of acute leukemia

cells. It is well known that the polyadenylic acid sequences have been considered a necessary "ticket" for those nuclear RNA sequences which become cytoplasmic messenger sequences (7). However, the ability to process heterogeneous nuclear RNA in poorly differentiated, slowly growing cells such as the leukemic cells is presumably lesser than normal. Furthermore, the results of our competition hybridization experiments suggest that in heterogeneous RNA of leukemic cells are transcribed "new" sequences which are not transcribed in normal lymphocytes. We must therefore seek an explanation for two facts which may be related, the transcription of new sequences and the increase in the proportion of poly(A)-containing molecules. Although no definite evidence has been so far obtained, we feel justified to postulate that at least part of the new sequences carry poly(A) segments.

Support to this hypothesis comes from the result of recent experiments carried out in our laboratory. The poly(A)-containing molecules in nuclear RNA of leuke-

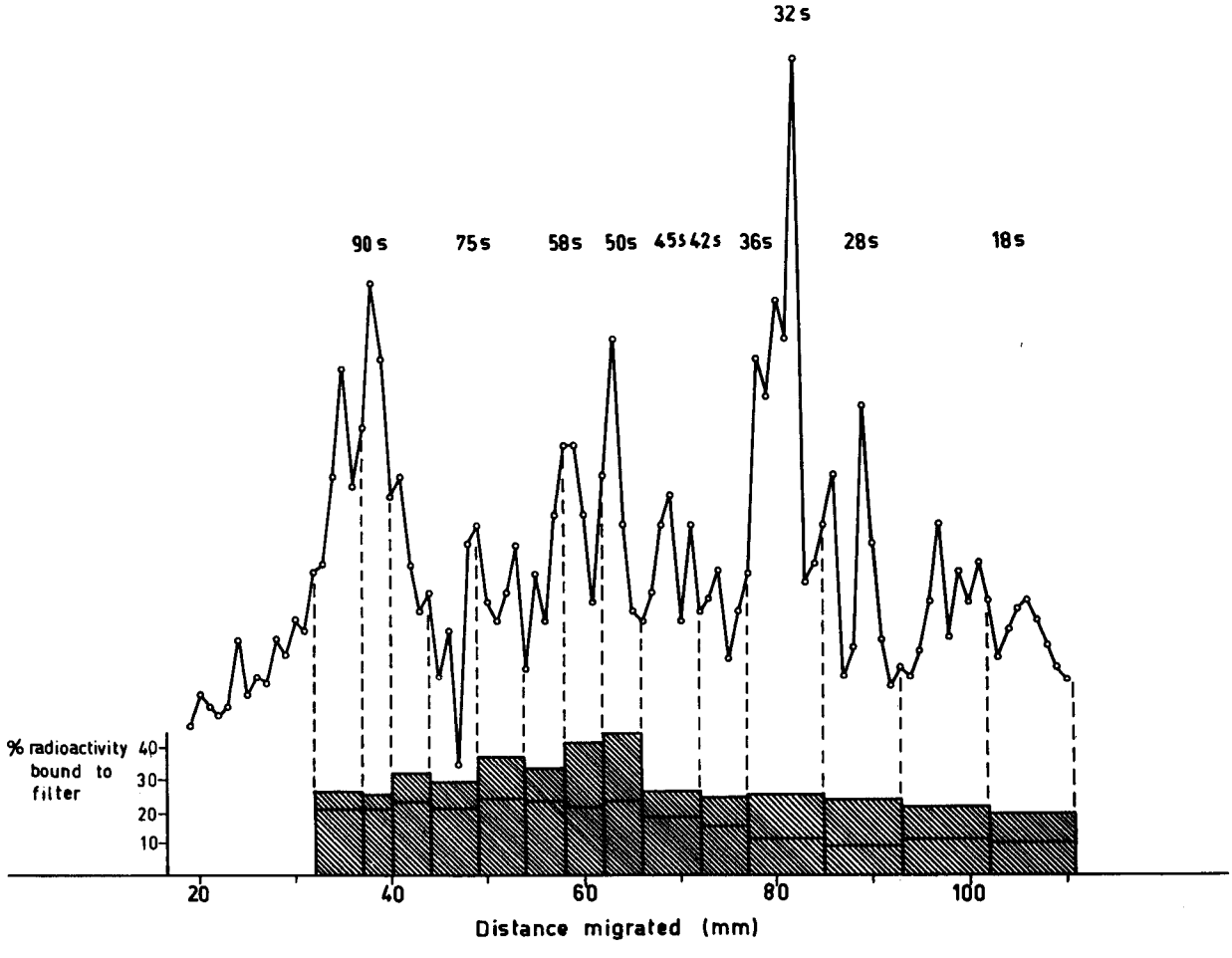

Fig. 6: Electrophoretic profiles of labeled nuclear RNA from a case of AML. Two samples of the same RNA were run on parallel columns. After scanning at 260 μm one of the columns was sliced in 1 mm slices, which were then counted. The second column was divided in fractions comprising molecules in a size range of about 5 S. The dotted lines indicate the limits of those fractions which were finely sliced to recover the RNA to be assayed for the poly(A)-containing molecules.

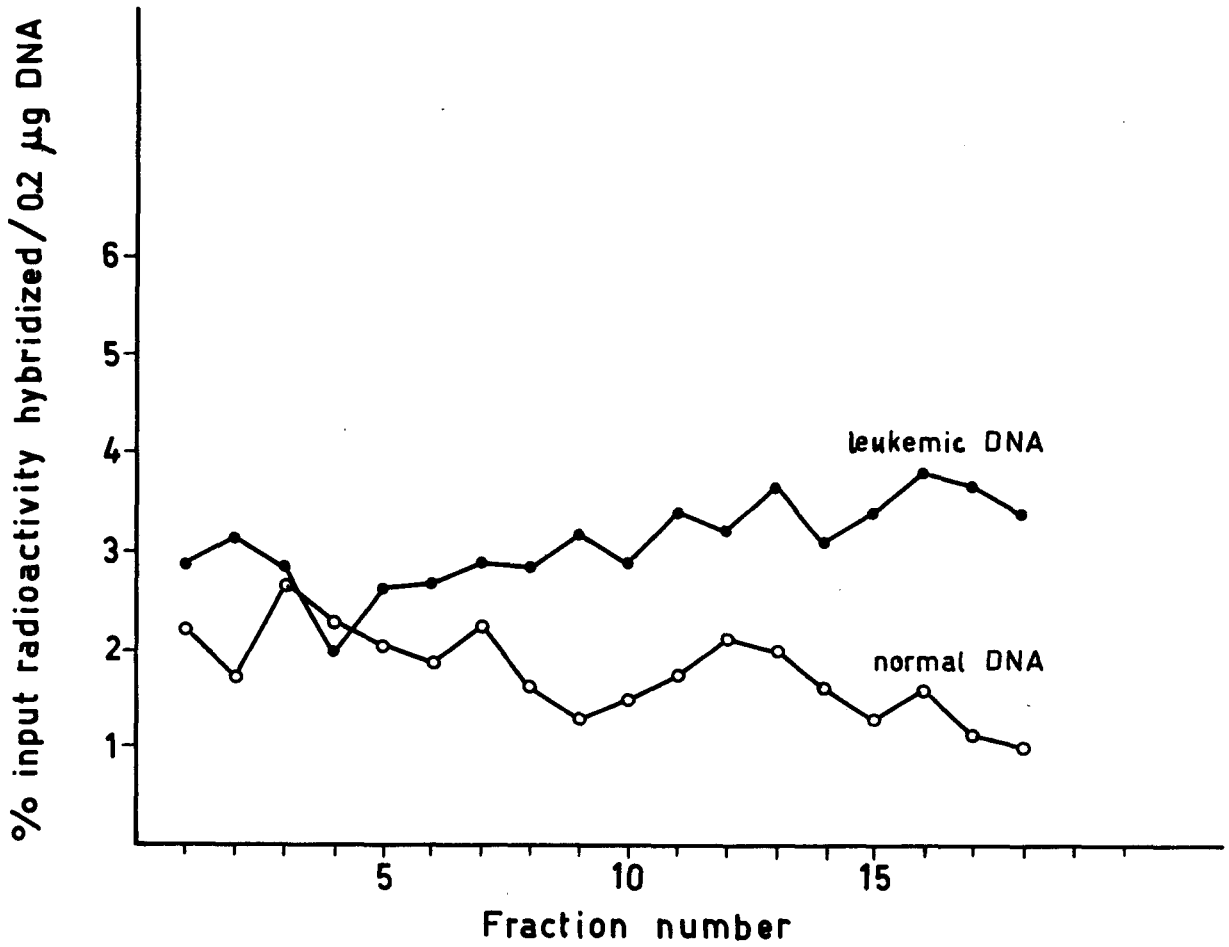

Fig. 7: Hybridization of poly(A)-containing nuclear RNA molecules from ALL blast cells to normal and leukemic DNA. The molecules were fractionated in 18 fractions by gel electrophoresis, using unlabeled KB cell ribosomal RNA as carrier.

mic cells were separated from those lacking poly(A) by hybridization with polyuridilic acid followed by chromatography on hydroxylapatite, according to the technique of Greenberg and Perry (12). The poly(A)-containing molecules were precipitated with ethanol after addition of electrophoretically purified 28S and 18S RNA from KB cells. The redissolved RNA was then fractionated by agarose acrylamide gel electophoresis and the gel column was cut in 0.3 mm slices. Groups of slices corresponding to 18 fractions were extracted with 1 ml of 2 x SSC. DNA was prepared from normal human lymphocytes and from leukemic blast cells by the method of Marmur (13). The DNA was denaturated by boiling and loaded on Millipore filters. Disks 4 mm in diameter containing 0.2 µg of DNA were punched out of each filter and placed in 1 ml vials, together with a blank filter to monitor non specific binding of RNA. Labeled RNA from each gel fraction, in 0.5 ml of 2 x SSC was placed in the vials which were sealed and incubated at 65 °C for 40 hours. The filters were then removed, washed with 2 x SSC, digested with RNAse, washed again and counted. The results of one of our experiments are shown in

fig. 7. It is apparent that the ability of poly(A)-rich nucelar RNA from leukemic cells to hybridize to leukemic DNA was always greater than the ability to hybridize to normal DNA. This result, which has been so far obtained in two cases, suggests that poly(A)-rich RNA from nuclei of leukemic blast cells contains a fairly large amount of sequences which are complementary to DNA sequences present in leukemic DNA but absent from normal DNA. These RNA sequences may then explain the inability of nuclear RNA from normal cells to inhibit the hybridization of leukemic nuclear RNA with DNA.

These investigations were supported by grants from the Consiglio Nazionale delle Ricerche.

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