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# Search for nucleic acid sequences complementary to a murine oncornaviral genome in poly(A)-rich RNA of human leukemic cells

**[human leukemia/poly(A)-RNA/mouse sarcoma virus (Moloney)/viral cDNA/RNA·DNA hybridization]**

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**ABSTRACT** **The presence of viral-like sequences in the RNA of various types of leukemic cells was investigated by hybridizing cellular poly(A)-containing RNA with cDNA synthesized in an endogenous system of purified Moloney murine sarcoma virus [M-MSV-(MLV)]. Poly(A)-RNA·cDNA hybrids were detected by assaying their resistance to $S_1$ nuclease. Hybrids were found in 22 out of the 46 leukemias that were tested. None of the controls, including material obtained from buffy coats, bone marrow cells, and a continuous human cell line, was positive. Positive cases were found in all the different categories of leukemias with the exception of chronic myelogenous leukemias. There was no definite correlation between the category of leukemia and positivity. A few cases contained a very high proportion of poly(A)-RNA·cDNA hybrid.**

Suggestive evidence for the presence of viral constituents, even virus-like particles, in human leukemic cells or in plasma pellets from leukemic blood has been presented in the last 4 years [see for example Gallo and Gallagher (1)]. In 1971, Hehlmann *et al.* (2) detected nucleotide sequences homologous to the RNA of the murine Rauscher leukemic virus in the polysomal RNA of 89% of different leukemic cell samples. These included virtually all types of acute and chronic human leukemias. Similar results were obtained by Gallo's group, which found sarcoma-related sequences (Kirsten) in the RNA of subcellular fractions of some leukemic cells (3). No such sequences could be characterized in normal cells when *the same experimental conditions were used.* The authors advanced the hypothesis that some viral sequences are present only in leukemic cells or that these sequences are not expressed in normal cells.

In order to characterize those leukemic cell RNA sequences which hybridize to murine cDNA, experiments similar to those of Hehlmann *et al.* (2) were undertaken with the following modifications: (*i*) The probe was synthesized in an endogenous system with murine sarcoma virus (Moloney); (*ii*) The cellular RNA was fractionated to enrich for poly(A) sequences by adsorption on a cellulose column (4, 5); (*iii*) hybrids were detected by $S_1$ nuclease assay. Among the 46 leukemias which were tested, 22 gave a positive result. Nonleukemic controls tested in parallel did not contain viral sequences.

A preliminary study of this work performed on 15 cases has been recently presented elsewhere (6).

## MATERIALS AND METHODS

**Cells.** All the blood samples were obtained from the department of hematology of the Hôpital Saint-Louis (Director: Prof. J. Bernard). They were recovered either through IBM separator, or by direct bleeding. The white cells were immediately separated from the red cells by adding Plasmagel to ⅕th of the final volume. After 2 hr at 37°, the supernatant was decanted and the white cells were pelleted by sedimentation. They were immediately treated for RNA isolation.

**RNA Isolation.** Cells were homogenized in 0.02 M Na acetate, pH 5.0, buffer containing 20 μg/ml of potassium poly-(vinyl sulfate) and lysed by addition of 0.2% sodium dodecyl sulfate. An equal volume of redistilled phenol was slowly added and the final mixture was shaken at 4° for half an hour. After centrifugation at 8000 rpm, the aqueous phase was decanted and treated twice more in the same way with half volume of phenol. It has been shown that this treatment extracts more than 95% of the cellular RNA, including rapidly labeled RNA (7). When sufficient amounts of cells were available, polysomes were first prepared, according to Galibert *et al.* (8). RNA was isolated as previously described. Finally total RNA was precipitated by 2 volumes of absolute ethanol and 0.1 M NaCl, washed twice with 66% ethanol, and redissolved in H buffer (see below).

**Fractionation of Poly(A) RNA on Cellulose Columns.** Columns were prepared by suspending the appropriate quantity of cellulose (Macherey and Nagel, MN 300, 0.5 g/mg of RNA) in 0.01 M Tris·HCl, pH 7.6, 0.5 M KCl buffer (H buffer). RNA was usually loaded onto the column at a concentration of 50 μg/ml. Columns were washed until absorbance at 260 nm was near zero. Tris·HCl, 0.01 M, pH 7.6 (L buffer), was then passed through the column in order to elute poly(A)-RNA. Under these conditions, the poly(A)-RNA was enriched at least 40- to 70-fold and constituted 70% of the eluted RNA. It was dissolved in hybridization buffer (see below) and kept frozen at −20°.

**Preparation of Murine Sarcoma Virus [M-MSV(MLV)].** Murine sarcoma virus (Moloney) was continuously produced by a chronically infected cell line: 78 $A_1$ strain initially obtained by Bernard *et al.* (9). Stocks of purified virus were prepared as previously described (10). Tritium-labeled cDNA was synthesized with endogenous RNA template and polymerase according to Tavitian *et al.* (10). Specific activity of the product approximated 10,000 cpm/pmol. Usually $10^3$ to $2 \cdot 10^3$ cpm were used for each test.

**Purification of *Aspergillus* Single-Strand-Specific Nuclease ($S_1$ Nuclease).** The enzyme was prepared according to Sutton (11). It was specific for single-stranded DNA and did not degrade double-stranded DNA or RNA·DNA hybrids.

**Hybridization Procedures.** The tests were performed in a final volume of 60 μl in sealed capillary pipets or small rubber-stoppered tubes. The mixtures contained 40% (vol/vol) redistilled formamide, 0.1% sodium dodecyl sulfate, appropriate amounts of poly(A)-RNA, and 1000–2000 cpm of cDNA in hybridization buffer (0.015 M Tris, pH 7.4, 0.25 M NaCl). The tubes were incubated for 72 hr at 41°. Contents

Abbreviations: M-MSV(MLV), murine sarcoma virus (Moloney); poly(A)-RNA, polyadenylylated RNA.

of capillary tubes were then poured into tubes containing 0.5 ml of $S_1$ buffer (0.025 M K acetate, pH 4.5, $10^{-4}$ M $ZnSO_4$) plus 10 $\mu$g of denatured salmon sperm DNA and a sufficient amount of $S_1$ nuclease to digest more than 95% of the denatured [$^3$H]DNA. Samples were incubated for 40 min at 45° and precipitated with trichloroacetic acid and filtered through nitrocellulose membranes (Millipore, 0.22 $\mu$m). Membranes were immersed in 5 ml of scintillation fluid and counted in a Packard 3390 scintillation counter.

For calculation, the background of blanks processed without RNA was substracted from each sample. This amounted to 5–7% of the cDNA input. Proportions of hybrids were expressed as the percentage of radioactivity resistant to $S_1$ nuclease and normalized against values given by poly(A)-RNA of M-MSV(MLV)-producing cells ($78A_1$).

**Centrifugation of Hybrids in Cesium Sulfate.** The solutions containing RNA·DNA hybrids were analyzed according to the conditions of Benveniste and Scolnick (12). The solutions were centrifuged for 56 hr at 40,000 rpm at 10° in a type 40 fixed-angle rotor. Fractions were collected with the aid of an ISCO gradient fractionator. Density along gradient was determined by weighing 50 $\mu$l fractions in a Levy capillary pipet. After addition of 10 $\mu$g of salmon sperm DNA, fractions were precipitated by 10% trichloroacetic acid and filtered through Millipore membranes which were dried and counted.

## RESULTS

### Validity of the probe

It was previously shown that M-MSV(MLV) cDNA synthesized in the endogenous system contained 90% single-stranded chains (10). Moreover, the product represented an extensive copy of the viral genome, since three molar equivalents of cDNA expressed as monomers were sufficient to protect labeled 70S M-MSV(MLV) RNA against pancreatic RNase digestion (10). When the cDNA was hybridized under standard conditions (see Fig. 1) against increasing quantities of poly(A)-RNA from $78A_1$ M-MSV(MLV)-producing cells, a saturation level was attained at a RNA/DNA ratio of $10^4$. No more than 52% of the input cDNA was protected by the cellular RNA and this value was identical to that obtained in the experiments where 70S viral RNA was used instead of the cellular RNA. Since cDNA represented most of the viral sequences, one explanation for this discrepancy could be some instability of the hybrids under the conditions of the assay. The cDNA used was heterogenous in size and some short hybrids were presumably eliminated during the incubation period or the $S_1$ treatment. Therefore, the value of the plateau obtained in each series of experiments was used to normalize each assay between human leukemic RNA and viral cDNA.

The virions produced by $78A_1$ cells are a mixture of MSV pseudotypes and MLV (13). According to results of the XC test and the focus-forming assay, the ratio of leukemogenic to sarcomatogenic viruses was estimated to be at least 100/1. This indicates that the cDNA which was used as a probe contained mostly nucleotide sequences complementary to the leukemogenic virus genome.

### Investigation of leukemic cell poly(A) RNA

Table 1 recapitulates the results with the different leukemic cell RNAs which hybridized M-MSV(MLV) cDNA. Two types of assays were performed according to the relative amount of poly(A)-RNA used in the assay. In the first one, three different RNA/DNA ratios, $10^3$, $10^4$, and $5{\cdot}10^4$, expressed as moles of monomer, were used. Thus, it was possible to follow the percentage of hybrids obtained as a function of the RNA input. The second way consisted of adding a large amount of RNA (e.g., 60 $\mu$g). It should be noted that poly(A)-RNA constitutes no more than 2% of the total RNA, and therefore 60 $\mu$g are equivalent to approximately 2.4 mg of total RNA.

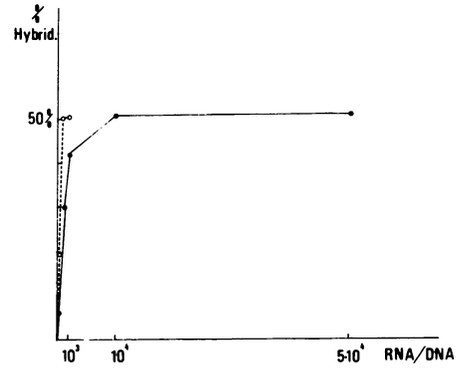


FIG. 1. Hybridization of poly(A)-RNA from $78A_1$ cells (●—●) and MSV(MLV) 70S RNA (O - - O) with [$^3$H]cDNA synthesized in an endogenous reaction. 1000 cpm of cDNA were used in each assay. RNA/DNA concentrations were expressed as picomoles of monomer. At the end of the incubation period (72 hr), contents of the tubes were poured off in 0.5 ml of $S_1$ nuclease buffer (0.03 M sodium acetate, pH = 4.5, 0.15 M NaCl). $S_1$ enzyme was added and the mixture was digested at 45° for 50 min. Trichloroacetic acid was added to a 10% final concentration and the samples were filtered through Millipore membranes (0.22 $\mu$m). After drying, the filters were transferred into vials containing 5 ml of scintillation solution. Counting took place in a Packard 3390 counter.

Fig. 2 summarizes the highest values found in Table 1. From this figure, it can be seen that: (*1*) The values obtained with the different controls (see Table 1) never exceeded 1.2%. All the leukemic RNA samples which fell within this range were considered as negative and values exceeding this level were scored as positive. (*2*) According to these criteria some of the leukemic cases studied were negative, others positive, and a few highly positive.

Because of the limited number of cases available in the various cytological types, it was not possible to establish a correlation between the cytological types and the outcomes of the test. However, summing positive cases of acute granulocytic leukemias (acute myelogenous and monocytic leukemias) gave a 12/20 ratio, which was higher than that resulting from the sum of positive lymphoblastic cases (6/14). Conversely, it was interesting to note the overall negativity of the five chronic myelocytic cases.

(*3*) Three cases presented an extremely and surprisingly high percentage of hybridization (nos. 13, 63, and 89). A second assay with the poly(A)-RNA of case no. 13 and also its poly(A)-minus RNA confirmed this result. These three samples came from acute lymphoblastic and myeloblastic leukemias.

The validity of these results was further checked by two lines of evidence. First, for several cases, the expected progression as a function of the RNA input was clearly apparent: e.g., nos. 27, 28, 33, 45, and 63 (Table 1). Second, the hybrids obtained were examined by isopycnic centrifugation. Hybridizations were conducted under the same standard conditions and the mixture was centrifuged to equilibrium in a cesium sulfate gradient (Fig. 3). While no radioactivity was present in the RNA region of the gradient from a normal control (case no. 5), a clear radioactive peak occurred in the RNA region on the profile of a leukemic sam-

Table 1. Results of hybridization reactions with M-MSV(MLV) cDNA and poly(A)-RNA from human leukemic and normal cells

| RNA samples | | | % Hybridization | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | RNA/DNA ratio* | | | RNA input | | |
| Classification | Case no. | Comments | $10^3$ | $10^4$ | $5 \cdot 10^4$ | 10 μg | 60 μg | Reaction |
| Controls | 5 | Normal leukocytes | 0.3 | — | 1 | | 0 | − |
| | 38 | Normal leukocytes | 0.5 | 0 | 0 | | — | − |
| | 43 | Normal leukocytes | 0 | — | 0 | | 0 | − |
| | 54 | Normal leukocytes | 0 | 1.1 | 1.3 | | 1.2 | − |
| | 84 | Bone marrow | — | — | — | 0 | — | − |
| | 99 | Normal leukocytes | — | — | — | | 1.2 | − |
| | 100 | Normal leukocytes | — | — | — | | 1.1 | − |
| | 92 | JIII cells | — | — | — | | 0 | − |
| | | Yeast RNA | 0 | 0 | 0 | | 0 | − |
| Acute myelogenous leukemia | 26 | | 0 | 1.3 | 2.5 | | — | + |
| | 28 | | 2.6 | 2.6 | 5 | | — | + |
| | 35 | | — | 4.8 | 4.8 | | — | + |
| | 62 | | 0 | 0 | 0 | | 6.0 | + |
| | 63 | | 13.3 | — | 27.8 | | — | + |
| | 64 | | 0 | 0 | 0.8 | | 0 | − |
| | 72 | | | | | | 0 | − |
| | 77 | | | | | | 0 | − |
| | 82 | | | | | | 0 | − |
| | 87 | | | | | | 18 | + |
| | 88 | | | | | | 0 | − |
| | 89 | | | | | | 43 | + |
| | 91 | | | | | | 18 | + |
| | 93 | | | | | | 8 | + |
| | 96 | | | | | | 0 | − |
| Acute lymphocytic leukemia | 13 | | 2 | 40 | 52 | | — | + |
| | 25 | | 5 | 7 | 7 | | — | + |
| | 39 | | — | 1.7 | 1.4 | | — | − |
| | 42 | | 0 | 1.7 | 0 | | 0 | − |
| | 45 | | 0.9 | — | 2.0 | | 3.5 | + |
| | 46 | | — | 0 | 0 | | 0.7 | − |
| | 50 | | 0 | 0 | 0 | | — | − |
| | 52 | | 0 | 0 | 0 | | — | − |
| | 53 | | 0 | 0 | 0 | | — | − |
| | 55 | | 0 | 0 | 0 | | — | − |
| | 58 | | 0.3 | 0 | 0.7 | | 4.5 | + |
| | 60 | | 0 | 0 | 0.7 | | 5.5 | + |
| | 81 | | | | | | 0 | − |
| | 86 | | | | | | 4.8 | + |
| | 13 | Second assay | 11 | 44 | 70 | | | |
| | 13 | Poly(A)-minus | 0 | 1 | 13 | | | |
| Acute monocytic leukemia | 32 | | 5.9 | 5.2 | 8.1 | | | + |
| | 36 | | 3.8 | 5 | 7 | | | + |
| | 56 | | — | — | — | | 0 | − |
| | 73 | | | | | | 8 | + |
| | 78 | | | | | | 0 | − |
| Chronic lymphocytic leukemia | 15 | | — | 0.6 | 1.2 | | 0 | − |
| | 33 | | 7.2 | 8.6 | 9.0 | | | + |
| | 67 | | 0 | 4.5 | 3.4 | | | + |
| | 17 | | 0 | 0.5 | 0.4 | | | − |
| | 15 | Poly(A)-minus | 0 | 0 | 0 | | | − |
| Chronic myelogenous leukemia | 23 | | | | | | 0 | − |
| | 24 | | | | | | 0 | − |
| | 31 | | 2.5 | 3.6 | 2.9 | | 0 | − |
| | 66 | | — | 0.2 | 0 | | 0 | − |
| | 85 | | | | | | 0 | − |
| Various | 27 | Sezary syndrome | — | 7.8 | 11 | | — | + |
| | 41 | Lymphosarcoma | 0 | 0 | 3.1 | | — | + |
| | 95 | Hodgkin's disease | — | — | — | | 0 | − |

*(Legend appears at bottom of following page.)*

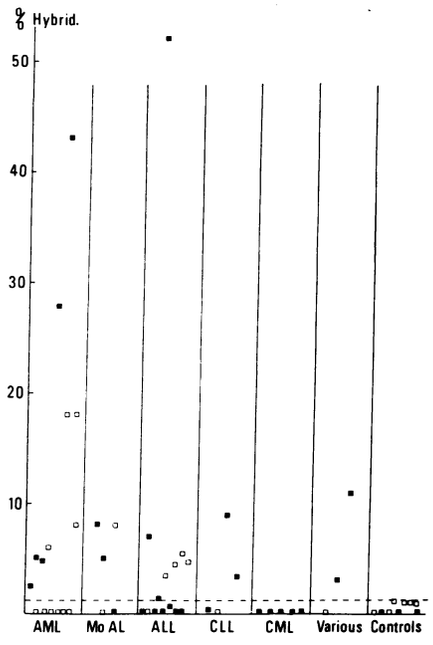


FIG. 2. Summary of hybridization reactions between M-MSV(MLV) cDNA and poly(A)-RNA isolated from human leukemic cells and normal donors' cells. All the data are recapitulated in Table 1. Filled squares represent the $5 \cdot 10^4$ RNA/DNA ratio of the serial tests. Open squares correspond to the assays made with 60 $\mu$g of RNA input. Abbreviations: AML, acute myelogenous leukemia; MoAL, acute monocytic leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia.

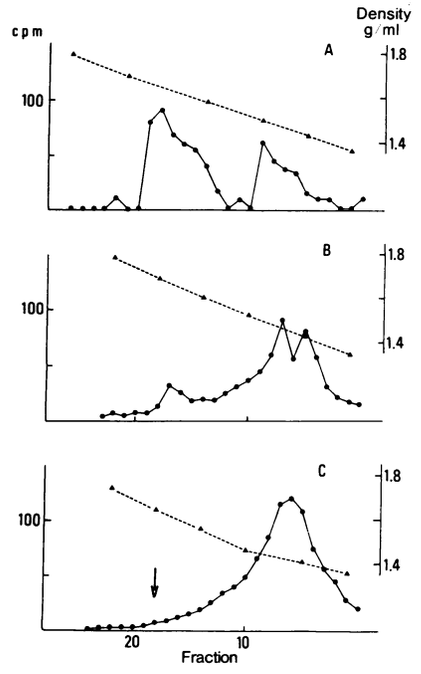


FIG. 3. $Cs_2SO_4$ centrifugation of M-MSV(MLV) cDNA hybridized with poly(A)-RNA of: (A) $78A_1$ virus-producing cells; (B) leukemic sample no. 33; and (C) normal white buffy coat, case no. 5. All the reactions were processed as described in *Materials and Methods*. The $S_1$ nuclease step was omitted; fractions were collected with an ISCO gradient fractionator and treated as described. A 40 cpm background was subtracted from each point. Arrow indicates RNA region of the gradient.

ple (no. 33). This substantiates the validity of the results obtained with the $S_1$ nuclease assays. It is worthwhile to note, however, that even under saturating conditions, $78A_1$ cell poly(A)-RNA did not displace all the DNA radioactivity. This fact could mean that some of the hybrids were not stable under our reacting conditions.

## DISCUSSION

This work was undertaken to confirm results initially obtained by Spiegelman's group, who showed that leukemic cells contain some murine viral information that is not present in normal cells (2). We were able to confirm the results of these authors by using another murine probe and a somewhat different approach. This last point can explain the differences which occurred in the percentage of positive cases found in the two investigations (89% versus 47% in our hands). To explain this discrepancy, we have to consider several aspects of the technology we used. Our conditions of hybridization were more stringent than those of Hehlmann *et al.*, as it has been proven that $S_1$ nuclease abolishes unstable hybrid regions. Thus, it could be that short stretches of RNA·DNA hybrids were eliminated under our experimental conditions which would have given a positive result under those of Hehlmann *et al.* (2). Another difference comes from the use of poly(A)-RNA. At the beginning, we decided to fractionate cellular RNA on the assumption that poly(A)-RNA represented almost all informative RNA with very few exceptions such as histone mRNA. Since then, several authors have reported that an important part of mRNA, even when attached to ribosomes, lacks poly(A) (14). Thus, we could have missed RNA sequences that were not polyadenylylated and this fact could explain some of the negative cases observed. Accordingly, it is interesting to observe that the RNA fraction lacking poly(A) of the highly positive case no. 13 contained, at a much lesser extent, however, sequences that hybridized with M-MSV(MLV) cDNA. Alternatively, negative results can also reflect absence of any viral expression in the leukemic cells.

Another question related to the results of Hehlmann *et al.* (2) concerns the identity of sequences that were detected in their experiments and ours. As we previously mentioned, the cDNA probe that was used reflects principally murine leukemogenic sequences, as murine leukemia virus is the main component of virus stocks. Moloney and Rauscher viruses are two variants which are very closely related and this makes it likely that sequences detected by Spiegelman's group and ourselves were basically the same. Similar results were reported by Gallo *et al.*, who found that endogenous cDNA synthesized by microsomal virus-like particles of human leukemic cells hybridized to 70S RNA of murine leu-

Legend to Table 1 *(on preceding page)*. The poly(A)-RNA was separated from total RNAs by chromatography on cellulose columns (5). For the hybridization tests, poly(A)-RNA and [$^3$H]cDNA were incubated for 72 hr at 41° in 60 $\mu$l reaction mixtures containing 0.015 M Tris·HCl, pH = 7.4, 0.25 M NaCl, 40% redistilled formamide and 0.1% sodium dodecyl sulfate. At the end of the reaction, the samples were treated with $S_1$ nuclease and processed as described in Fig. 1. Two types of experiments were performed: (a) three different RNA/DNA ratios were analyzed: $10^3$, $10^4$, and $5 \cdot 10^4$ (1); (b) a unique and large amount of poly(A)-RNA was hybridized with the cDNA. All the results were normalized by taking as a 100% reference the values obtained with poly(A)-RNA of the $78A_1$ virus-producing cells which were included in each series of assays. Positive and negative results were defined as described in the *text*.

* Expressed as molar ratio of monomers.

kemia virus and woolly monkey sarcoma virus (3). It is not evident that the sequences of the human cDNA which hybridize with the murine 70S RNA on the one hand and the simian 70S RNA on the other are identical. We have cross-hybridized cDNA of M-MSV(MLV) and woolly monkey viruses with the poly(A)-RNA of the two virus-producing cells and found that there does not exist more than 10% homology between the two probes (unpublished result). Hybridizing woolly monkey cDNA with poly(A)-RNA of leukemic cells should further substantiate the extent of this relationship.

The significance of the presence of murine leukemogenic viral sequences in human leukemic cells raises several questions. The first one concerns the possible viral etiology of the disease. To date, no data are available proving this hypothesis and it is still a matter of speculation. However, the presence of these sequences and proteins which crossreact with murine p30 products as tested by radio-immunoassay (15) strongly suggests that leukemic cells contain murine endogenous nucleotide sequences and express them. That these endogenous sequences are related to a true human virus is highly questionable. In fact, it is quite possible that all the cells, normal and leukemic, contain the same information integrated in their genome but express it differently, as they are not in the same state of differentiation. Baxt and Spiegelman suggested that this is not the case since the DNA of leukemic cells contained something which is not present in the DNA of normal leukocytes (16). In fact, it was established by Baxt that these additional sequences were complementary to murine cDNA (17). In our own experiments very few differences (if any) were observed between the rates of association of M-MSV(MLV) cDNA with cellular DNA of leukemic and normal individuals (unpublished results). However, the cDNA we used for reassociation kinetics was not recycled against normal cellular DNA.

It should be emphasized that all leukemic RNA samples tested in these experiments were obtained from hyperleukocytic individuals who were as yet untreated. Therefore, our results may not reflect the average situation encountered among human leukemias.

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