Immunological Assay of Double-Helical Segments in RNA Fractions of Different Molecular Size Extracted from Acute Myeloid Leukemia Blast Cells

Umberto Torelli, Sergio Ferrari, Giuliano Montagnani, Giuseppe Torelli, Ruggero Cadossi, Stefano Ferrari, and Franco Narni

Laboratory of Molecular Hematology, Institute of Medical Pathology, University of Modena, 41100 Modena, Italy

SUMMARY

Whole-cell RNA, extracted from acute myeloid leukemia blast cells, was fractionated by sedimentation through sucrose gradients. The proportion of double-helical segments present in each fraction was then determined by a quantitative microcomplement fixation assay that specifically measures double-helical RNA. Sizable amounts of double-helical segments were detected in all fractions of cellular RNA corresponding to S values higher than approximately 20. In all cell populations examined, the highest proportion of double-helical segments was found in RNA fractions sedimenting faster than the 45 S ribosomal precursor RNA, i.e., in fractions including only heterogeneous nuclear RNA.

INTRODUCTION

In 1968, Montagnier (10) discovered in animal cell RNA a structure showing double-helical properties. The significance of this observation became clear only some years later, when Jelinek and Darnell (5) and Ryskov et al. (16, 17) demonstrated that heterogeneous RNA of animal cells contains double-helical regions that arise by base pairing of complementary sequences that exist as parts of the same molecule (intramolecular base pairing). According to these authors, most animal cell RNA involved in Watson-Crick base pairing is composed of slightly different lengths of "hairpin-like" structures, i.e., loops that form between 2 portions of a single covalently linked strand of RNA. Other authors (6, 9, 13) were able to confirm that the RNA with double-helical properties that may be extracted from animal cell nuclei is principally a part of much larger heterogeneous nRNA molecules. Besides heterogeneous nRNA, 45 S ribosomal precursor RNA and its products of cleavage also appear to have hairpin loops reproducibly located along the length of each molecule. A "secondary structure map" of HeLa cell rRNA and its precursors has been drawn by Wellauer and Dawid (27).

The functional significance of these structures is still quite obscure. According to some authors, they might function as "signals" for posttranscriptional processing of messenger and ribosomal precursors (4, 12). In light of the observation that part of the nRNA synthesized in AML blast cells shows double-helical properties (24), we have attempted the quantitative assay of the double-helical segments present in different RNA fractions of AML cells. The methods commonly used to detect and characterize double-helical RNA, such as stepwise chromatography on cellulose columns (3) or protein synthesis inhibition (15), are unsuitable for the quantitative measurement of material of this type present in the rapidly sedimenting RNA. This tiny fraction of cell RNA, in fact, includes most of the ribosomal and messenger precursors in animal cells. We have thus tackled the problem of the quantitative assay of the double-helical RNA segments by using the microcomplement deviation method of Wassermann and Levine (26), with the modifications of Stollar and Stollar (22). This immunocchemical method takes advantage of the opportunity, confirmed by several authors (11, 14, 19), to obtain an antibody that reacts specifically with double-helical ribopolynucleotide structures.

The results we report here indicate that this method, which responds to double-helical RNA concentrations below 5 ng/ml, allows the detection of sizable amounts of double-helical segments in all fractions of cellular RNA corresponding to S values higher than approximately 20. In all cell populations examined, the highest proportion of double-helical segments was found in RNA fractions sedimenting faster than the 45 S ribosomal precursor, i.e., in fractions formed only by heterogeneous nRNA.

MATERIALS AND METHODS

Preparation and Purification of the Antibody. Double-helical homopolyribonucleotides poly(I)·poly(C) and poly(A)·poly(U) of molecular size corresponding to a sedimentation value greater than 8 and methylated bovine serum albumin were purchased from Serva, Heidelberg, West Germany. Complete Freund's adjuvant was obtained from Difco Laboratories, Detroit, Mich. Each immunizing dose was formed by 150 μg of copoly-
mer mixed with an equal weight of methylated bovine serum albumin in 1 ml of 0.9% NaCl solution emulsified in the same volume of Freund’s adjuvant. Three doses were given s.c. to male rabbits at weekly intervals. An i.v. injection without adjuvant was given 1 week later, and serum was obtained after 10 days.

Pure IgG was obtained by chromatography of the serum on DEAE-cellulose columns that had been previously equilibrated with 0.2 M Tris and 0.5 M glycine buffer, pH 8. The resulting fraction was dialyzed against microcomplement fixation diluent buffer, without bovine serum albumin, and the purity of the IgG solution was tested by agar gel immunoelectrophoresis and double immunodiffusion with goat antiserum against rabbit IgG. IgA, IgM, and total serum protein content of the solution was determined by the method of Lowry et al. (7).

Preparation and Purification of Nucleic Acids. Whole-cell RNA was extracted from circulating blast cells of AML patients, as previously described (25). Samples of RNA were applied to 5 to 20% sucrose gradients that were centrifuged at 15,000 rpm for 13 to 16 hr and were fractionated in 1-mI fractions. Each fraction was dialyzed to eliminate sucrose, and total RNA was determined by measurement of absorbance at 260 nm. Undiluted and diluted fractions were then assayed immunochromically for double-helical RNA, as described below. DNA was extracted from leukemic cells by the method of Marmur (8).

Escherichia coli tRNA was purchased from Calbiochem, San Diego, Calif. Labeled double-helical segments were isolated from whole-cell RNA extracted from leukemic cells by the following method.

The RNA was dissolved in 5 ml of buffer (50 mM NaCl-2 mM MgCl₂-10 mM Tris-HCl, pH 7.4), and DNase (Worthington Biochemical Corp., Freehold, N. J.) was added at a final concentration of 20 µg/ml. After 30 min of incubation at room temperature, the solution was brought to 0.25 M NaCl by the addition of 5 M NaCl. Thirty µg of pancreatic RNase per ml (Sigma Chemical Co., St. Louis, Mo.) and 20 units of T1 RNase per ml (Worthington Biochemical Corp.) were then added, and the solution was incubated 30 min at 37°C. Fifty µg of subtilisin were then added, and the reaction was terminated 30 min later by extraction twice with phenol-m-cresol and precipitation of the nucleic acid by ethanol.

To isolate and purify double-helical RNA from single-helical RNA, tRNA, and DNA, we used chromatography on cellulose, according to the method of Franklin (3). The nucleic acid was dissolved in 0.5 ml of buffer (0.1 M NaCl-0.05 M Tris-HCl-0.001 M sodium-EDTA, pH 6.9) and brought to 35% with ethanol. The sample was applied to a cellulose column (1.5 x 10 ml), and the column was washed with the same solution. Under these conditions, DNA and tRNA failed to remain on the column but were eluted directly by buffer with 35% ethanol. Single-helical RNA remained on the column but could be eluted with buffer containing 15% ethanol. Double-helical RNA was finally removed with buffer alone.

Gel Electrophoresis. Radioactive double-helical RNA eluted from the column in buffer alone was precipitated with the use of trNA as carrier and was then dissolved in electrophoresis buffer (0.05 M NaCl-0.001 M sodium-EDTA, pH 6.2). Gel electrophoresis was carried out with 15% polyacrylamide gels, 8 cm long, at 5 mA/gel for 120 to 180 min. After the run was over, the gels were extruded and scanned at 260 nm in a Joice and Loebl apparatus. The gels were then cut into 1-mm slices, which were solubilized and counted.

Quantitative Microcomplement Fixation. Lyophilized guinea pig complement (Microbiological Associates, Bethesda, Md.) and rabbit anti-sheep erythrocyte stroma serum (Cordis Laboratories, Miami, Fla.) were reconstituted to original volume in diluent supplied by the producer. Small aliquots of reconstituted complement were stored at −70°C to minimize the loss of activity that accompanies thawing. Sheep erythrocytes were stored in sterile Al-server’s solution at 4°C and were used 10 days after bleeding for a maximum period of 2 months. Poly(l)·poly(C) and poly(A)·poly(U) were dissolved in 0.01 M Tris buffer, pH 7.4, containing 0.14 M NaCl, 0.0005 M MgCl₂, 0.00015 M CaCl₂, and 0.1% bovine serum albumin (Biotest, Frankfurt, West Germany). This buffer was used as diluent for all reagents. A solution of pure IgG at an original concentration varying from 2.5 to 5 mg/ml was used as antibody.

Sheep erythrocytes were washed 3 times in diluent buffer by centrifugation for 10 min at 2000 rpm, and the concentration was adjusted to 1 x 10⁸/ml. One-tenth ml of this suspension was lysed in 1.3 ml of distilled water, and absorbance of the resulting hemoglobin solution was determined at 413 nm. The cell suspension was then adjusted to give an absorbance value of 0.420 after lysis.

Hemolysis titration in a final volume of 1.4 ml was performed in complement excess with hemolysin dilution (from 1:5,000 to 1:100,000), 0.2 ml: sheep erythrocyte suspension, 0.1 ml: complement (1:30), 0.2 ml: and diluent buffer, 0.9 ml. After incubation at 37°C for 1 hr, the reaction was stopped in an ice bath for 10 min, after which the tubes were centrifuged for 10 min at 2,000 rpm.

Absorbance of the supernatant fluid was tested, and the highest dilution of hemolysin that showed complete hemolysis represents 1 unit. Two units were used in all tests.

To obtain sensitized erythrocytes, equal volumes of sheep erythrocyte suspension and diluted hemolysin were mixed at room temperature for at least 10 min before use.

For complement titration, 0.2 ml of complement dilution (from 1.60 to 1.250) was incubated with 1 ml of diluent buffer at 4°C for 18 hr. After incubation, 0.2 ml of sensitized erythrocytes was added directly to the reaction mixtures, and hemolysis was allowed to proceed at 37°C for 1 hr. The reaction was then stopped, and hemolysis was measured as described earlier. The complement dilution required to obtain 90% of hemolysis was used in quantitative assays.

For evaluation of the antibody concentration suitable for detecting antigen concentration varying from 1 to 0.0005 µg/ml, diluent buffer, 0.6 ml; complement dilution, 0.2 ml; and poly(A)·poly(U) dilution (from 1 to 0.0005 µg/ml), 0.2 ml, were mixed with 0.2 ml of varying dilutions of antibody (from 1:250 to 1:2000) and incubated at 4°C for 18 hr. 0.2 ml of sensitized erythrocytes was then added. This procedure allowed us to find the antibody dilution required to fix part of the complement present in the reaction mixture. For example, data obtained with an antibody dilution of 1:1600 in 1 experiment are shown in Chart 1. These values repre-
Identity reaction was given by precipitation lines between poly(I)·poly(C) and poly(A)·poly(U) and the reacting antibody. No precipitation was observed with E. coli tRNA and native human DNA (Fig. 1).

The results of specificity tests in which complement fixation was used are shown in Table 1. Ninety % fixation was observed with 0.05 μg of poly(A)·poly(U) per ml. A triple-stranded copolymer prepared in the proportion of 2 poly(A)·poly(U) was slightly less reactive. No complement fixation was observed with polynucleotide alone, polyadenylate alone, polyuridylate alone, E. coli tRNA, or native human DNA. The complement fixation with double-helical copolymer was measurable in the presence of a large excess of human DNA or E. coli tRNA, as these materials did not cause significant inhibition.

A possible source of error in our system was presumably represented by the formation of RNA aggregates. It was in fact observed that the RNA of some of our gradient fractions showed a significant anticomplementary activity, i.e., complement fixation in the absence of the specific antibody. This activity was attributed to the formation of aggregates of RNA macromolecules, which may occur with many extraction methods. For this reason, control of anticomplementary activity was performed on the RNA fractions before each assay. When significant amounts of complement fixation in the absence of antibody were observed in even one fraction, the RNA sample was discarded.

Reaction of the Antibody with Chromatographically Purified Human Double-Helical RNA. Microcomplement fixation was used to assay the antibody-reacting material present in 2 chromatographic preparations of human double-helical RNA. Undiluted and diluted (1:5, 1:10, 1:20) samples of the most radioactive fraction of each eluate was tested. Increasing proportions of complement fixation were obtained by increasing the dilutions, indicating that the double-helical RNA was present in relative excess in the eluates.

**RESULTS**

**Characteristics of the Antibody.** Agar-gel immunoelectrophoresis and double-immunodiffusion assay showed that chromatography on DEAE-cellulose columns allowed us to obtain a solution of pure IgG, free of any other serum fraction. Two sources of error were thus eliminated: the anticomplementary activity of whole serum and the interfering effect of nucleases able to hydrolyze double-helical RNA. The presence of such enzyme activity has been shown in the serum of some animal species (21).

The specificity of the antibody was checked by both immunodiffusion and microcomplement fixation. Antibodies from all immunized rabbits precipitated with poly(I)·poly(C) but not with polynucleotide alone or polycytidylate alone. Precipitation lines were also obtained with poly(A)·poly(U), and no reaction again was obtained with polyadenylate alone or polyuridylate alone.

**Control of anticomplementary activity of antibody and RNA solutions, control of complement activity, and drawing of a reference curve had to be performed before each assay.**

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**Table 1.** Different proportions of complement (C') fixation obtained by testing a 1:1600 dilution of the chromatographic eluate containing the antibody with different dilutions (from 1 to 0.0005 μg/ml) of poly(A)·poly(U).

<table>
<thead>
<tr>
<th>Poly(A):Poly(U) μg/ml</th>
<th>C' Fixation %</th>
</tr>
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<tbody>
<tr>
<td>0.0001</td>
<td>0</td>
</tr>
<tr>
<td>0.0005</td>
<td>0</td>
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<tr>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
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<tr>
<td>50.0</td>
<td>100</td>
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<tr>
<td>100.0</td>
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**Fig. 1.** Two-dimensional immunodiffusion of anti-poly(I)·poly(C) antibody with poly(I)·poly(C) (100 μg/ml) and poly(A)·poly(U) (100 μg/ml); polycytidylate and polyadenylate (100 μg/ml); and E. coli tRNA (50 μg/ml) and human native DNA (20 μg/ml). The original chromatographic eluate containing the IgG was undiluted.
samples. Chart 2 shows that the radiolabel migrated in a gel electrophoresis experiments allow an approximate evaluation of the size of the double-helical segments isolated from those of a viral replicative form or a synthetic double helical RNA. Quantitation of the complement consumption was obtained with the sheep hemolysin assay system described in the text.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Concentration (μg/ml)</th>
<th>% complement fixation</th>
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</thead>
<tbody>
<tr>
<td>Poly(A)·poly(U)</td>
<td>0.05</td>
<td>90.2</td>
</tr>
<tr>
<td>2 Poly(A)·poly(U)</td>
<td>0.05</td>
<td>78.5</td>
</tr>
<tr>
<td>Polyinosinate</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Polyadenylate</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Polypuridylate</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>tRNA</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5</td>
<td>90</td>
</tr>
<tr>
<td>Poly(I)·poly(C) + tRNA</td>
<td>0.05 + 10</td>
<td>93</td>
</tr>
<tr>
<td>Poly(I)·poly(C) + DNA</td>
<td>0.05 + 10</td>
<td>90</td>
</tr>
</tbody>
</table>

Gel Electrophoresis of Double-Helical Segments. The gel electrophoresis experiments allow an approximate evaluation of the size of the double-helical segments of our RNA samples. Chart 2 shows that the radiolabel migrated in a rather heterodisperse fashion, penetrating the gel less rapidly than the tRNA, which was subjected to coelectrophoresis as a reference marker. This observation indicates that most of the double-helical RNA segments isolated from leukemic blast cells had a size corresponding to S values of approximately 6 to 8.

Proportion of Double-Helical Segments in Sucrose-Gradient RNA Fractions. Chart 3 shows the results obtained by assaying the immunologically reacting double-helical RNA present in different sucrose-gradient fractions of whole-cell RNA extracted from circulating blast cells of 4 AML patients. The pattern observed was remarkably similar in all the cell populations examined.

Double-helical segments were barely detectable in RNA molecules up to approximately 20 to 22 S, the average proportion of immunologically reacting substance being around 0.05%. A 10-fold higher proportion was found in fractions including molecules in a size range of 22 to 45 S, which showed an average proportion of 0.63% double-helical RNA. The highest proportion of immunologically reactive double-helical RNA was, however, found in the giant heterogeneous RNA sedimenting faster than 45 S. The average proportion of double-helical segments among these molecules was in fact 0.94%.

**DISCUSSION**

Antibodies to double-helical polyribonucleotides, mainly poly(A)·poly(U) and poly(I)·poly(C), have been obtained in several laboratories (11, 14, 18, 19). Different patterns of specificity have been described. In all cases there has been extensive reciprocal cross-reactivity between poly(A)·poly(U) and poly(I)·poly(C), as well as between poly(A)·poly(U) and double-helical viral RNA (11, 18, 22). Different results have been obtained in assays of cross-reactivity with other forms of naturally occurring RNA. Nahon-Merlin et al. (11) obtained anti-poly(I)·poly(C) sera reacting with mammalian rRNA and tRNA. The ability of the sera to react with whole-cell RNA was eliminated by prior absorption with poly(I)·poly(C), so that double-helical regions in the RNA single chain were considered to be determinant structures. Anti-poly(A)·poly(U) or anti-poly(I)·poly(C) antisera obtained in other laboratories showed a narrower cross-reactivity. In fact, the antibodies still reacted with either of these synthetic helical forms but did not react with mammalian rRNA or tRNA (19), although these RNA's have a secondary structure that includes some base-paired regions. A similar cross-reactivity pattern has been shown by the antibodies obtained in our laboratory.

Clearly, the antibodies to the helical RNA do not react with a specific base or base sequence, and the determinant probably involves the backbone structure on the outside of the helix. Both strands are probably involved in forming a determinant, since single-stranded forms do not inhibit the reaction. It seems probable that the size and shape of the base-paired regions in the nonreacting RNA's are different from those of a viral replicative form or a synthetic double-helical copolymer. Tertiary structure also may mask the base-paired regions; in tRNA, the loops may fold into a more compact structure and overlie the base-paired stems (1).

Our experiments suggest that the size and shape of at least a large proportion of double-helical segments of leukemic RNA did allow the reaction of these segments with the antibody. As indicated by our experiments, a large excess of rRNA or tRNA did not inhibit the reaction. It remains clearly possible that a more- or less-significant portion of the double-helical regions of leukemic cell RNA escapes immunochromenical detection because of its size or because it is masked by tertiary structure.

The quantitative determination depends partly on the assumption that the naturally occurring double-helical RNA segments and the poly(A)·poly(U) standard require identi-
Double-Helical RNA in Human Leukemia Cells

Chart 3. Proportion of antibody-reacting double-helical segments present in different fractions of whole-cell RNA extracted from AML blast cells. RNA was extracted, applied to a linear sucrose gradient (5 to 20%), and centrifuged for 13 to 16 hr at 15,000 rpm. One-ml fractions were assayed for absorbance at 260 nm (△) and for double-helical RNA (○). The quantification of double-helical RNA was based on the total RNA required for a given level of complement fixation in comparison with the poly(A):poly(U) standard.

cal amounts of antigen for a given level of complement fixation. Although we lack definitive evidence, large differences appear unlikely here, since the naturally occurring double-helical segments and the copolymers used to obtain the antibody and to prepare the reference curves had a similar molecular size.

As previously mentioned, sizable amounts of double-helical RNA have already been detected in uninfected animal cells by several methods, including immunochemical techniques. Our experiments represent the 1st attempt to assay quantitatively the double-helical segments included, in nanogram quantities, in density-gradient fractions of whole-cell RNA obtained from leukemic blast cells. The RNA of these cells shows a definite pattern of distribution of the double-helical segments, the proportion of which apparently correlates roughly with the size of the molecules. The largest proportions were found in fractions with RNA molecules larger than the 45 S ribosomal precursor RNA, i.e., in frac-
tions formed by that portion of heterogeneous nRNA that is considered by some authors (20) as the "nascent" mRNA precursor. The lower proportions observed in fractions containing molecules in the 20 to 45 S size range may be explained by the simultaneous presence in these fractions of heterogeneous nRNA and ribosomal precursor RNA with its products of cleavage.

This latter RNA species displays a reproducible secondary structure of hairpin loops (27), but the proportion of double-helical segments is presumably lower than that of the heterogeneous nRNA. On the other hand, the molecules of this latter RNA species in the 20 to 45 S size range might well have a smaller proportion of double-helical segments, if they have to be considered, as some evidence suggests (2, 20), intermediate products of processing of the largest molecules. In fact, recognition and digestion of the base-paired regions by a specific enzyme might be an important step for the cleavage process (4, 12, 23).

The RNA molecules smaller than 20 S lack antibody-reacting segments almost completely. This does not indicate that these molecules completely lack base-paired regions, because some of these regions, as previously pointed out, might be unable to react because of their size and shape or because they are masked by tertiary structure. It must, however, be pointed out that Wellauer and Dawid (27) were able to visualize secondary structure interpreted as hairpin loops in all mRNA molecules of HeLa cells, with the exception of 18 S RNA. We thus seem justified in concluding that RNA molecules up to 20 to 22 S carry a scarcely significant proportion of double-helical segments.

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