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Relationship Between RNA-directed DNA Polymerase (Reverse Transcriptase) from Human Acute Leukemic Blood Cells and Primate Type-C Viruses

(human leukemia/antibody to virus polymerase)

R. E. GALLAGHER*, G. J. TODARO†, R. G. SMITH*, D. M. LIVINGSTON†‡, AND R. C. GALLO*

* Laboratory of Tumor Cell Biology, † Viral Leukemia and Lymphoma Branch, National Cancer Institute, Bethesda, Maryland 20014

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ABSTRACT **An RNA-directed DNA polymerase was isolated from the peripheral blood leukocytes of a patient with acute myelomonocytic leukemia by successive purification of a particulate cytoplasmic fraction with endogenous, ribonuclease-sensitive DNA polymerase activity. Like RNA-directed DNA polymerase from mammalian type-C virus, the human leukemic cell enzyme efficiently utilized $(A)_n \cdot (dT)_{12-18}$ and $(C)_n \cdot (dG)_{12-18}$ and had an approximate molecular weight of 70,000. Further, the leukemic cell enzyme was strongly inhibited by antisera to RNA-directed DNA polymerase of primate type-C virus in a fashion similar to that noted with an extensively purified RNA-directed DNA polymerase from a person with acute myelogenous leukemia [Todaro, G. J. & Gallo, R. C. (1973), *Nature* 244, 206]. By these biochemical and immunological results the leukemic cell enzyme could be differentiated from all other known cellular DNA polymerases but could not be distinguished from RNA-directed DNA polymerase of primate type-C virus. We interpret these data, combined with observations published elsewhere, to indicate that human acute myelogenous leukemia cells contain components related to primate type-C virus. The parameters used in this study may provide the specificity and sensitivity required for determining the presence or absence and (if present) the relatedness of RNA-directed DNA polymerase in other cases and types of human leukemia.**

RNA tumor viruses morphologically classified as type-C viruses have been associated with leukemia/lymphoma in birds, rodents, cats (1), and subhuman primates (2, 3). In human leukemia, RNA tumor viruses have yet to be convincingly demonstrated by either morphological identification or the transmission of biological activity. However, in the past three years human leukemia cells have been demonstrated to contain an RNA-directed DNA polymerase (4, 5) and RNA molecules (6, 7) with marked similarity to analogous components from RNA tumor viruses. In leukemic cells the virus-like DNA polymerase and RNA may be complexed, as determined by the endogenous synthesis of DNA with the RNA template (4–7), and they can be found in a cytoplasmic "particle" that has the density (1.14–1.17 g/ml) characteristic of RNA tumor viruses (5–7). These associated physical properties were used in the present study to separate leukemic cell RNA-directed DNA polymerase from other cellular DNA polymerases, rather than using more conventional procedures for purifying polymerase enzymes. This leukemic cytoplasmic enzyme was identical in its biochemical properties to RNA-directed DNA polymerase of mammalian type-C viruses, and as in our recent patient with acute myelogenous leukemia (5), it was selectively inhibited by antisera to RNA-directed DNA polymerase of certain primate type-C viruses. Further, such antisera have failed to inhibit the two major DNA-directed DNA polymerases and the so-called "R-DNA" polymerase (8) from normal and leukemic human leukocytes (5 and see *text*). These findings suggest that antisera to RNA-directed DNA polymerase of certain primate type-C viruses represent specific and potentially sensitive probes for detecting and for determining the cellular distribution of RNA-directed DNA polymerase of human leukemic cells.

‡ Present address: Children's Cancer Research Foundation, 35 Binney Street, Boston, Mass. 02115.

MATERIALS AND METHODS

Source of Cells. Patient C.P. (designated HL-8) is a 69-year-old caucasian male with a diagnosis of acute myelomonocytic leukemia. Presenting signs included gingival hypertrophy and hepatomegaly. He was anemic, and his leukocyte count was markedly increased to 250,000 per mm^3 (normal range 5,000 to 10,000 cells per mm^3). More than 70% of the leukocytes were immature myeloid elements, although substantial numbers of mature polymorphonuclear leukocytes and occasional basophils and eosinophils were noted. The peripheral leukocytes, separated from whole blood as described (9), were used before therapy.

Biochemical procedures

Preparation of the Cytoplasmic Pellet Fraction. The patient's leukocytes (10 g) were suspended in five volumes of buffer A (50 mM Tris·HCl, pH 7.5–5 mM $MgCl_2$–20 mM dithiothreitol–0.5 mM EDTA) containing 0.1 M sucrose and were homogenized to approximately 75% cytoplasmic rupture in a tight-fitting Dounce homogenizer. The nuclei and mitochondria were successively removed by centrifugation for 10 min at 1,000 and 12,000 × *g*, respectively, in a Sorvall centrifuge. The supernatant was layered over a 10-ml column of buffer B (50 mM Tris·HCl, pH 7.5–5 mM $MgCl_2$–0.1 M KCl–1 mM dithiothreitol–0.5 mM EDTA) containing 25% (w/w) sucrose in an SW27 rotor and centrifuged at 98,000 × *g* for 1 hr. The supernatant was removed, and the pellet was evenly suspended in buffer B and used for further studies.

Sucrose Gradient Analysis and Sepharose 4B Chromatography. The cytoplasmic pellet fraction was centrifuged to equilibrium through a linear 20–50% (w/w) sucrose gradient in buffer B. Fractions of the following densities were combined: >1.19, 1.17–1.19, 1.15–1.17, 1.13–1.15, and <1.13 g/ml; they were pelleted by centrifugation at 100,000 × *g* for 1 hr (after dilution of the sucrose concentration to 10%) and

TABLE 1. *Template-primer specificity of the HL-8 leukemic cytoplasmic RNA-directed DNA polymerase after Sepharose 4B purification**

Additions	[^{3}H]Deoxyribonucleotide triphosphate	Enzyme activity (pmol incorporated/100 μl)
$(A)_n \cdot (dT)_{12-18}$	dTTP	7.56
$(dA)_n \cdot (dT)_{12-18}$	dTTP	0.17
$(dT)_{12-18}$	dTTP	0.36
	dGTP	0.01
$(C)_n \cdot (dG)_{12-18}$	dGTP	3.02
$(dG)_{12-18}$	dTTP	0.06
	dGTP	0.08

* Reactions were performed under standard reaction conditions as detailed in *Methods*.

resuspended in buffer B. Each was applied to a 0.9 cm × 30 cm Sepharose 4B column (Pharmacia, Uppsala, Sweden) equilibrated in buffer B. Fractions eluted from the Sepharose column were assayed for DNA synthesis in the absence (endogenous DNA synthesis) or presence of added synthetic homopolymeric-oligomeric hybrids, as described below. Ribonuclease-sensitive, endogenous DNA synthesis (4) with this patient's cells was noted only in Sepharose eluates derived from sucrose gradient cuts with a density less than 1.15 g/ml, particularly in the 1.13–1.15 g/ml density cut. This activity was localized in the void volume of the Sepharose eluate, indicating that the DNA polymerase catalyzing this reaction is present in a particle with a minimum molecular size of 20 × 10^6. The post-Sepharose void-volume fractions derived from the 1.13–1.15 g/ml sucrose density cut were combined, concentrated 5-fold by dialysis against buffer B containing 30% polyethylene glycol, dialyzed against buffer B without polyethylene glycol, and stored at −20°C in 50% glycerol. This is the enzyme preparation that was used for determining template-primer specificity and size, and for immunological studies.

Assays with Homopolymeric-Oligomeric Template/Primers. $(dG)_{12-18}$ and $(C)_n \cdot (dG)_{12-18}$ were obtained from Collaborative Research (Waltham, Massachusetts); $(dT)_{12-18}$, $(dA)_n \cdot (dT)_{12-18}$ were obtained from P. L. Biochemicals (Milwaukee, Wisconsin). Enzyme was activated by treatment with 0.1% Triton X-100 at 37°C for 15 min. Reactions were performed at 37°C for 30 min in a final reaction volume of 0.05 ml, containing 50 mM Tris · HCl, pH 8.3, 100 mM KCl, 1 mM $MnCl_2$, 3 mM dithiothreitol, 16 μM[^{3}H]dTTP (Amersham, 6600 cpm/pmol) or 16 μM[^{3}H]dGTP (Schwarz-Mann, 4356 cpm/pmol), 10 μl of enzyme, and 3860 pmol (expressed as pmol of mononucleotide) of oligomer [$(dG)_{12-18}$ or $(dT)_{12-18}$] with or without an equimolar amount of complementary polymer [$(C)_n$, $(A)_n$, or $(dA)_n$]. Under these conditions, incorporation of deoxynucleoside monophosphate was proportional to enzyme concentration.

Immunological procedures

Various viral RNA-directed DNA polymerases were isolated, antisera were prepared, and IgG fractions were purified as described (5). Activated enzyme (5 μl) was first incubated with water or various concentrations of IgG purified from immune or nonimmune antisera (10 μl) at 4°C for 10 min. This mixture was then added to a reaction mixture in a final volume of 0.05 ml, containing standard reagents for assay of $(A)_n \cdot (dT)_{12-18}$ with [^{3}H]dTTP (Amersham, 23,760 cpm/pmol). The reaction was incubated at 37° for 60 min. Values are presented as the percent of inhibition compared to the control reaction containing no IgG. Incorporation of radiolabel was 4,000 to 20,000 cpm in the absence of added IgG.

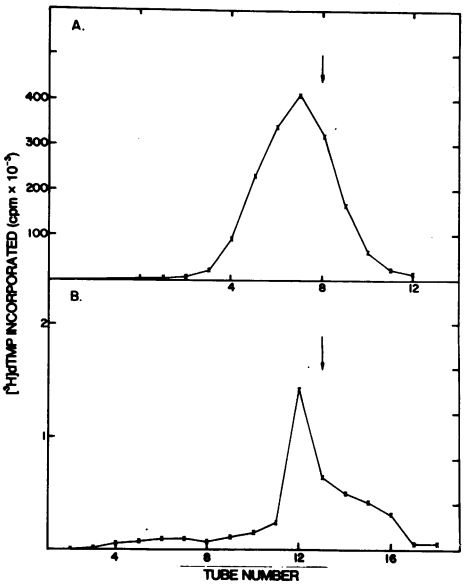

FIG. 1. Glycerol gradient sedimentation analyses of RNA-directed DNA polymerase from murine leukemia virus and acute myelogenous leukemia cells (HL-8). 0.05 ml of the viral enzyme and of the leukemic cell enzyme were each mixed with bovine-serum albumin (1 mg), and each enzyme sample was applied to a 5–25% glycerol gradient. The glycerol gradients were prepared, centrifuged, and analyzed as described (10). After centrifugation the 280-nm absorbance of each gradient fraction was determined; the *arrow* marks the bovine-serum albumin peak for each gradient. Aliquots (0.03-ml) were assayed in 0.10-ml reaction mixtures for 60 min at 37°C with $(A)_n \cdot (dT)_{12-18}$ as template (10). RNA-directed DNA polymerase of (*A*) murine leukemia virus; (*B*) HL-8.

RESULTS

Biochemical properties of the leukemic RNA-directed DNA polymerase

The preparation of the leukemic cell enzyme from the post-mitochondrial, 98,000 × *g* cytoplasmic pellet fraction by sucrose equilibrium density gradient centrifugation and Sepharose 4B chromatography is described in *Methods*.

Template-Primer Properties and Ionic Requirements. The enzyme preferred $(A)_n \cdot (dT)_{12-18}$ to $(dA)_n \cdot (dT)_{12-18}$ by a factor of greater than 40:1 (Table 1). Divalent cation was required for the reaction with $(A)_n \cdot (dT)_{12-18}$, and Mg^{++} was a poor substitute for Mn^{++}, producing no more than 12% of the Mn^{++}-stimulated activity. Under all reaction conditions tested, $(dA)_n \cdot (dT)_{12-18}$ was *not* an efficient template-primer. Since cellular DNA-directed DNA polymerases utilize $(dA)_n \cdot (dT)_{12-18}$ efficiently, this indicates that the leukemic cytoplasmic enzyme is relatively free of these DNA polymerases. In addition $(C)_n \cdot (dG)_{12-18}$ was utilized by the leukemic enzyme from 30 to 50% as efficiently as $(A)_n \cdot (dT)_{12-18}$. This level of activity for $(C)_n \cdot (dG)_{12-18}$ has previously been observed only with known viral RNA-directed DNA polymerases (5, 10, 11). This enzyme activity is not

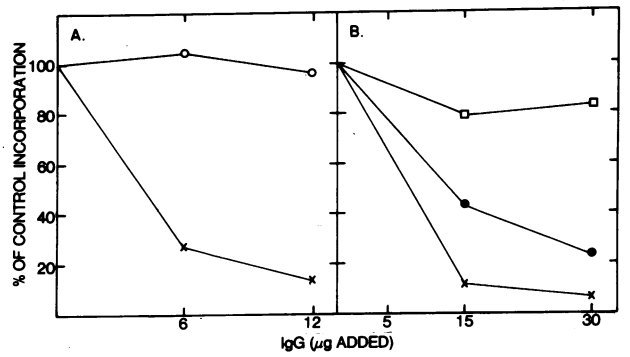

FIG. 2. The effect of various concentrations of IgG from nonimmune serum and from antisera to RNA-directed DNA polymerase from primate and mouse RNA tumor viruses on the leukemic cell RNA-directed DNA polymerase. The preparation of antisera and viral enzymes has been described (5). (*A*) (O), nonimmune IgG; (x), simian sarcoma virus anti-polymerase IgG. (*B*) (x), simian sarcoma virus anti-polymerase IgG; (●), gibbon ape lymphosarcoma virus anti-polymerase IgG; (■), murine leukemia virus (Rauscher) anti-polymerase IgG.

terminal deoxynucleotidyl transferase, since no significant activity was demonstrated in the absence of appropriate template (12).

Estimation of Enzyme Size. Fig. 1 presents the glycerol gradient analysis of the leukemic enzyme compared to purified DNA polymerase from Rauscher leukemia virus. Both enzymes sediment in the same position relative to bovine-serum albumin, indicating an estimated molecular weight of approximately 70,000 (assuming a globular protein), which is in agreement with reports for other mammalian type-C polymerases (11, 13, 14).

Immunologic properties of the leukemic DNA polymerase

In order to further investigate the virus-like nature of the leukemic enzyme, it was tested for inhibition by purified IgG from antisera prepared against DNA polymerases from various RNA tumor viruses. The leukemic enzyme was markedly inhibited by anti-polymerase IgG to the two known primate type-C viruses, simian sarcoma virus, derived from a woolly monkey fibrosarcoma (15), and gibbon ape virus, derived from a gibbon ape lymphosarcoma (3) (Fig. 2). The greater inhibition of the leukemic enzyme by the anti-polymerase IgG to simian sarcoma virus compared to anti-polymerase IgG to gibbon virus is at least partially related to the greater potency of the simian sarcoma virus, antiserum for its homologous enzyme. The leukemic DNA polymerase was not inhibited by IgG specific for DNA polymerase from another primate virus which is not a type-C virus, the Mason–Pfizer monkey virus. Also, minimal or no inhibition of the leukemic polymerase was noted with potent anti-polymerase IgG to avian sarcoma virus, mouse type-C leukemia virus, or the endogenous feline type-C virus, RD-114 (Fig. 3).

As previously reported, DNA polymerases I and II from normal mouse (13) and human tissue (5) were not inhibited by antisera prepared against RNA-directed DNA polymerase of RNA tumor virus. Conversely, antisera to purified DNA polymerase I from phytohemagglutinin-stimulated lymphocytes did not inhibit RNA-directed DNA polymerase of RNA tumor virus *or* the presently reported leukemic cell polymerase (R. G. Smith *et al.*, submitted for publication).

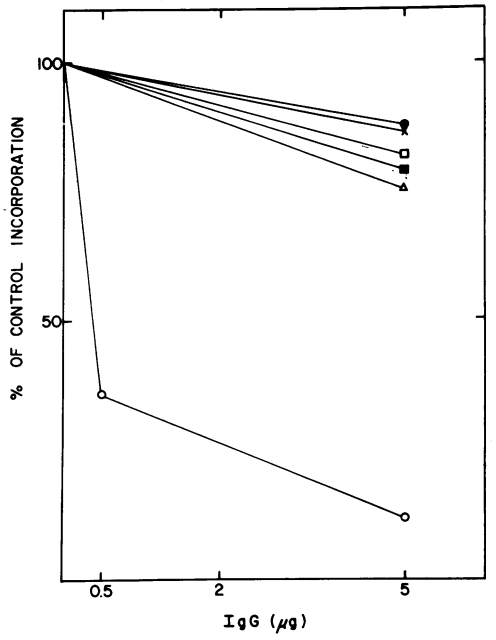

FIG. 3. Lack of significant inhibition of the leukemic cell RNA-directed DNA polymerase by IgG from antisera to RNA-directed DNA polymerase from nonprimate type-C viruses and from a primate virus that is not type C. Conditions are as indicated in the *legend* to Fig. 2. All anti-polymerase IgG produced substantial inhibition of a homologous DNA polymerase with less than 5 μg of IgG. Anti-polymerase IgG to the following viruses were tested: (X) Rous (avian) sarcoma virus; (■) mouse leukemia virus (Rauscher); (□) RD-114, Δ, Mason–Pfizer monkey virus; and (O) simian sarcoma virus. (●) nonimmune IgG.

Distinction between leukemic cell RNA-directed DNA polymerase and DNA polymerase III ("R-DNA" polymerase)§

Virus-free mammalian cells, including human leukocytes, have been reported to contain an enzyme(s) that efficiently transcribes $(A)_n$ primed with oligo(dT) in the presence of Mn^{++} (8, 11, 16). It was, therefore, important to compare this enzyme with leukemic cell RNA-directed DNA polymerase. Accordingly, DNA polymerase III was purified from HL-8 leukocytes by the procedure of Lewis *et al.* (16). Like RNA-directed DNA polymerase, this enzyme preferred $(A)_n \cdot (dT)_{12-18}$ to $(dA)_n \cdot (dT)_{12-18}$ (6:1). However, it did not utilize $(C)_n \cdot (dG)_{12-18}$. When analyzed as described in the *legend* to Fig. 1, it was determined to have a molecular weight of greater than 90,000, which is significantly larger than RNA-directed DNA polymerase and is in agreement with other size determinations of DNA polymerase III (11, 16). Further, when tested with anti-polymerase IgG to simian sarcoma virus, *no* inhibition of dTMP incorporation with $(A)_n \cdot (dT)_{12-18}$ was observed. These results, which differentiate HL-8 RNA-directed DNA polymerase and DNA polymerase III, as well as the distinguishing biochemical and immunological properties of other cellular and viral DNA polymerases, are summarized in Table 2.

§ Cellular enzymes that efficiently utilize the synthetic template-primer $(A)_n \cdot (dT)_{12}$ in comparison to appropriately primed DNA templates were first described by Fridlender *et al.* (8), who coined the term "R-DNA" polymerase. For reasons presented elsewhere (16), we prefer to call this enzyme activity DNA polymerase III, which is the term used throughout this text.

TABLE 2. *Summary of the distinguishing biochemical and immunological properties of DNA polymerases of viral and cellular origin**

Test	Classification and source of DNA polymerase						
	RNA-directed DNA polymerase				Cellular DNA polymerases		
	Primate type-C viruses		Human leukemic leukocytes		Normal and leukemic leukocytes		
	Gibbon ape virus	Woolly monkey	HL-3†	HL-8	DNA Pol I	DNA Pol II	DNA Pol III
Ratio of activity $(A)_n \cdot (dT)_{12-18}$/ $(dA)_n \cdot (dT)_{12-18}$	20–80:1‡	20–100:1 (14)	5:1 (4, 5)	40–50:1	<1:100 (5, 18)	1:12 (5, 18)	>5:1 (8, 16)
Response to $(C)_n \cdot (dG)_{12-18}$	+(20)	+(14)	+(5)	+	0 (5, 18)	0 (5, 18)	0§
AMV 70S RNA	+‡	+(14)	+(4, 5, 17)	N.T.	0 (5, 18)	0 (5, 18)	0 (11, 16)
Approximate molecular weight	70,000‡	70,000 (14)	N.T.	70,000	150,000 (18)	40,000 (18)	>90,000 (11, 16)
Inhibition by							
Primate type-C virus anti-polymerase IgG (% inhibition by 30 μg)	>90 (21)	>90 (21)	>90 (5)	>90	<10 (5)	<10 (5)	<10
Human lymphocyte anti-DNA Pol I IgG (% inhibition by 20 μg)	<10¶	<10¶	<10	<10	80¶	<10¶	<10 (16)

* The data presented in this table were derived in part from results presented in other reports, which are cited by the numbers in *parentheses*. The data presented were obtained under optimal conditions for that particular enzyme as indicated in the *text* or in the appropriate reference.

† The isolation of the HL-3 leukemic cytoplasmic pellet enzyme was first described in ref. 4, patient 3. Reports further characterizing the properties of this enzyme have subsequently appeared in refs. 5 and 17.

‡ Our unpublished data.

§ Bolden *et al.* reported very low but detectable utilization of this template-primer by one human "R-DNA" polymerase (19). We have not observed any significant incorporation of dGMP with $(C) \cdot (dG)_{12-18}$ when incubated with HL-8 R-DNA polymerase III or with other poly(A)-directed cellular enzymes (unpublished data and refs. 11 and 16).

¶ Smith, R. G., Lewis, B. L., Abrell, J. W. & Gallo, R. C., submitted for publication.

Pol, polymerase; *AMV*, avian myeloblastosis virus; *N.T.*, not tested.

DISCUSSION

The following properties of the RNA-directed DNA polymerase isolated from leukemic leukocytes of patient HL-8 readily distinguish it from the two known DNA-directed DNA polymerases from normal leukocytes: (*1*) a strong preference for the synthetic template-primer, $(A)_n \cdot (dT)_{12-18}$ over $(dA)_n \cdot (dT)_{12-18}$; (*2*) efficient utilization of $(C)_n \cdot (dG)_{12-18}$; (*3*) inhibition by antisera to RNA-directed DNA polymerase of primate type-C virus; (*4*) lack of inhibition by anti-DNA polymerase I IgG; and (*5*) estimated molecular weight of 70,000 (Table 2). Although not available for the HL-8 enzyme, another distinguishing feature of the cytoplasmic pellet enzyme from leukemic patients is the response to 70S RNA template from RNA tumor viruses (HL-3, Table 2; refs. 4, 5, 17). The HL-8 enzyme may also be distinguished from terminal deoxynucleotidyl transferase (12), which was recently reported in the leukemic cells from one patient with acute lymphocytic leukemia (22), since the HL-8 enzyme did not significantly catalyze the incorporation of deoxynucleoside monophosphates in the absence of template (Table 1). By these same criteria the leukemic cytoplasmic enzyme is indistinguishable from RNA-directed DNA polymerase from primate type-C viruses (Table 2) and, therefore, we feel justified in referring to this enzyme as leukemic cell "reverse transcriptase."

As described in *Results* and indicated in Table 2, DNA polymerase III isolated from these same cells could be distinguished from the RNA-directed DNA polymerase of leukemic cells by criteria *2*, *3*, and *5*. We previously reported partial inhibition of "R-DNA" polymerase by primate anti-polymerase IgG (5); however, in subsequent studies, including this enzyme isolated from HL-8 leukemic cells and from replicating human lymphoblastoid (NC37) culture cells (16), inhibition has not been observed. Conversely, a poly(A)-directed polymerase recently described in nonvirus-producing mouse cells was partially inhibited by antisera to RNA polymerase of murine leukemia virus (11). Thus, although these poly(A)-directed, poly(dT)-synthesizing enzymes can be differentiated from RNA-directed DNA polymerase, they are poorly characterized at the present time and could have some relationship to viral enzyme that requires further investigation.

The studies with primate type-C virus anti-polymerase IgG in which leukemic cell RNA-directed DNA polymerase was inhibited to approximately the same extent as homologous viral RNA-directed DNA polymerase strongly suggest extensive structural homology of these enzymes. By similar reasoning, there is presumably less structural homology of the leukemic enzyme to RNA-directed DNA polymerase from lower mammalian type-C viruses or from primate non-type-C virus. This interpretation is reinforced by the following: (*1*) similar results were achieved with antisera prepared against RNA-directed DNA polymerase of simian sarcoma virus when the virus was obtained from three different types of cultured cells (human, marmoset, and rat) and when antisera were raised in two different animals (rabbit and rat); (*2*) no crossreactivity was detected with the known cellular DNA polymerases; (*3*) a similar pattern of immunological inhi-

bition of RNA-directed DNA polymerase of primate type-C virus by anti-polymerase IgG from various RNA tumor viruses was previously reported (21); and (4) a similar pattern of homology was observed by molecular hybridization between nucleic acids associated with human RNA-directed DNA polymerase and 70S RNA from various RNA tumor virus species (6).

The biochemical properties and the pattern of immunologic reactivity of the HL-8 leukemic enzyme are quite similar to those recently published for RNA-directed DNA polymerase from the leukocytes of another patient with acute myelogenous leukemia (5). In the limited time since antisera to viral RNA-directed DNA polymerases have been available, five of six biochemically-defined RNA-directed DNA polymerases from patients with acute myelogenous leukemia have been substantially inhibited by primate type-C virus anti-polymerase IgG (ref. 5 and unpublished data). The single instance in which inhibition was not observed may have been due to insensitivity of the leukemic cell enzyme to inhibition when in an aggregated form (Mondal, Gallagher, and Gallo, in preparation). These findings suggest that a common viral element exists in human myelogenous leukemia. While this could be due to activation of endogenous viral information (23), it alternatively could be due to infection by an exogenous agent in a manner analogous to that demonstrated for the horizontal transmission of leukemia in some groups of house cats (24). By applying molecular probes specific for primate type-C virus molecules, as presented here and elsewhere (4, 5), to the study of additional leukemic patients, we may be able to discriminate between these two alternatives and to further evaluate the possible etiologic role of type-C virus information in human leukemia.

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