UTILIZATION OF MAMMALIAN 70S RNA BY A PURIFIED REVERSE TRANSCRIPTASE FROM HUMAN MYELOCYTIC LEUKEMIC CELLS

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SUMMARY

A DNA polymerase activity present in the 60,000 x g pellet of human acute myelocytic leukemic blood cells catalyzes an endogenous RNA-dependent (RNase sensitive) DNA synthesis. After purification, this enzyme transcribes heteropolymeric regions of 70S RNA from various RNA tumor viruses but prefers mammalian over avian viral RNA. DNA transcribed from the viral RNA is, like that from RNA tumor virus reverse transcriptase, hydrogen bonded and covalently attached to the RNA template-primer. The DNA transcript, after alkaline hydrolysis, hybridizes specifically to the template-primer RNA. This activity is not decreased in the presence of DNA polymerases from normal cells, suggesting that the inability of the latter polymerases to catalyze transcription of viral RNA is not due to the presence of inhibitor(s). This DNA polymerase is easily distinguished from a terminal addition deoxynucleotidyl transferase and has previously been shown to be immunologically closely related to primate type-C virus reverse transcriptase.

In establishing whether or not type-C RNA tumor virus information is present in human leukemic cells, one approach is to look for viral-related proteins in apparently virus-free cells. One viral protein, RNA-dependent DNA polymerase (1,2), can be distinguished from the major DNA polymerases from normal mammalian cells by biochemical (3-6) and immunological (7) techniques. The isolation from cells of a DNA polymerase activity which could transcribe RNA was first reported by this laboratory using fresh human acute leukemic blood cells (8). Subsequently, this enzyme was traced to a cytoplasmic particulate fraction where it was shown to catalyze DNA synthesis dependent on RNA† (9-11). The relative utilization of various synthetic

†Nomenclature used to describe these activities is sometimes confusing. The terms as used in our laboratory are defined here. RNA-dependent refers to an endogenous DNA polymerase activity which is sensitive to RNase. RNA-directed DNA synthesis indicates any RNA-dependent DNA polymerase activity for which RNA-DNA hybrid intermediate products have been identified and/or the DNA product has been shown to hybridize back to the template RNA.

template-primers by the DNA polymerase purified from this particulate fraction from acute lymphoblastic leukemic cells (9,10) is also similar to viral DNA polymerases. In addition, the human enzyme catalyzed transcription of heteropolymeric regions of 70S RNA isolated from avian myeloblastosis virus (AMV) (9). Baxt, Hehlmann, and Spiegelman (11) also report that the endogenous DNA product of the crude particulate fraction is associated with a high molecular weight RNA and the DNA associated with this RNA is complementary to 70S RNA from Rauscher leukemia virus (RLV), suggesting that the template RNA is viral-related. We have recently confirmed and extended this observation and find that the DNA product of the endogenous RNA-directed DNA polymerase reaction from the post-mitochondrial pellet of fresh human myeloblastic leukemia cells is more than 50% complementary to murine and primate type-C sarcoma virus RNA (12).

An endogenous RNA-dependent DNA polymerase activity has also been described in a high speed cytoplasmic pellet fraction from phytohemagglutinin (PHA)-stimulated normal human blood lymphocytes (13). However, this reaction is similar to that of the mitochondrial fraction (manuscript in preparation), in that both appear to be RNA-primed and DNA directed (13) rather than RNA directed. Moreover, the DNA polymerase activity purified from this fraction and two other major DNA polymerases purified from soluble fractions of normal white blood cells (14), as well as the "R-DNA polymerase" (15, 16; also, our unpublished observations) are, unlike the viral enzyme and the leukemic pellet DNA polymerase, apparently unable to transcribe heteropolymeric regions of viral 70S RNA and show a different response to some synthetic template-primers under identical conditions of assay. Finally, antibody against reverse transcriptase from type-C mouse and primate leukemia virus, while cross reacting significantly with this leukemic enzyme (17, 18) shows no cross reaction with the major DNA polymerases purified from normal mammalian cells (7, 13, 14) including normal human blood leukocytes (13, 14) and only a trivial cross reaction with the "R-DNA polymerase" (G. Todaro and

R. C. Gallo, unpublished results). In addition, antibody against DNA polymerase I from normal human lymphocytes does not inhibit the leukemic pellet DNA polymerase (R. G. Smith, R. Nowinski, and R. C. Gallo, unpublished results).

In this report, we show: (1) the identification of this enzyme from human acute myelocytic leukemic white blood cells; (2) transcription by this purified pellet DNA polymerase of heteropolymeric regions of 70S RNA from RLV and feline leukemia virus (FeLV) as well as AMV (but preference for the mammalian 70S RNA's over avian); and (3) evidence that the inability of the major DNA polymerases from normal human lymphocytes to transcribe heteropolymeric regions of 70S RNA (13, 14) is probably not due to the presence of inhibitors. In this communication, the RNA-dependent DNA polymerase is referred to as the leukemic pellet DNA polymerase.

MATERIALS AND METHODS

The particulate pellet fraction with endogenous RNase-sensitive DNA polymerase activity was isolated from 20 g of fresh white blood cells from a patient with acute myelocytic leukemia and the DNA polymerase purified as described previously (9). (We find it necessary that no less than 5 g, i. e., about 5×10^9 leukocytes, be used for isolation of this pellet polymerase.)

FeLV and RLV were obtained from Electro-Nucleonics, Incorporated (Bethesda, Maryland) and AMV was generously supplied by Dr. Joseph Beard (Duke University, Durham, North Carolina). 70S RNA from AMV, RLV, and FeLV (Rickard) was isolated by incubation of virus with 0.5 mg/ml nuclease-free pronase (Calbiochem) for 30 minutes at 37°C, addition of sodium dodecyl sulfate to a concentration of 1% plus 2 mg/ml washed bentonite followed by a further 15 minute incubation at 37°C, and successive extractions with phenol:cresol:H_2O (50:7:5) 0.1% in 8-quinolinol (twice) and $CHCl_3$ (twice). Total viral nucleic acid was precipitated with 67% EtOH-0.3 M NaCl, then with cetyltrimethylammonium bromide (CTAB) and ethanol-salt as described previously (19). The nucleic

acid pellet was suspended in 0.25 ml TNE buffer (0.01 M Tris HCl, pH 7.2; 0.1 M NaCl; 0.001 M EDTA) and layered on a 10-30% glycerol gradient (10 ml). The gradients were centrifuged four hours at 40,000 rpm in a SW-41 rotor and fractionated. Fractions in the 70S area were pooled, again precipitated in ethanol-salt, and redissolved in TNE buffer at a concentration of 0.25 mg/ml.

Cs_2SO_4 density gradient analysis were performed as described before (9, 19) except each gradient was made 0.001 M in diethyl oxydiformate (Eastman). Cs_2SO_4 (optical grade) was obtained from Schwarz-Mann. Single strand-specific (S1) nuclease from Aspergillus oryzae (20) was purified as described by Sutton (21) and pancreatic RNase A was purchased from Worthington Biochemicals. Unlabelled deoxyribonucleoside triphosphates were obtained from P-L Biochemicals and ^{3}H-deoxynucleoside triphosphates were from New England Nuclear Corporation. All labelled substrates were flash evaporated just before use.

RESULTS AND DISCUSSION

Transcription of Viral 70S RNA by the Purified Leukemic Pellet DNA Polymerase

DNA polymerase activity was isolated from the cytoplasmic 60,000 x g pellet from human acute myelocytic leukemic cells by extraction with Triton X-100 and 0.8 M KCl, and purified by successive column chromatography as described previously (9, 10). This DNA polymerase activity is able to transcribe heteropolymeric regions of viral 70S RNA (see below) and shows salt elution and template-primer preference characteristics similar to that of the previously described pellet DNA polymerase from acute lymphoblastic leukemic cells (9, 10).

Poly (A) regions of 70S RNA (22-24) appear to be transcribed to some extent by DNA polymerases from normal animal cells (12, 13, 25) and from E. coli (26; M. Robert-Guroff, unpublished results). To minimize ambiguous results from transcription of the poly (A) regions of 70S RNA we used ^{3}H-dCTP as the only labelled substrate in all experiments. The "pellet" DNA polymerase

catalyzes the incorporation of ^{3}H-dCTP into acid-insoluble material in the presence of 70S RNA from AMV, RLV, and FeLV; this reaction is completely destroyed by preincubation of the RNA with RNase (Table 1). The DNA polymerase preparation is free from any contaminating terminal deoxynucleotidyl transferase activity (see legend to Table 1).

The enzyme gives 4-fold higher transcription of RNA from the mammalian viruses than that from the avian (Table 1). This observation was repeatedly made with several different viral RNA preparations; however, we do not know the basis for this preference. As also shown in Table 1, a DNA polymerase purified from the cytoplasmic high speed pellet of PHA stimulated lymphocytes (13) by identical procedures and tested under identical conditions does not transcribe heteropolymeric regions of 70S RNA as measured by incorporation of ^{3}H-dCTP. When these normal and leukemic enzymes are mixed, transcription of 70S RNA is equal to that with the leukemic enzyme alone, suggesting that these DNA polymerases from normal lymphocytes do not contain inhibitors. Similar mixing experiments with DNA polymerase I (6-8S enzyme) and II (3-4S enzyme) of normal lymphocytes (14) did not interfere with the ability of the leukemic enzyme to transcribe viral RNA (unpublished results). The enzyme from normal lymphocytes also responds quite differently to synthetic template-primers (13) compared to the DNA polymerases from oncornaviruses and from the subcellular cytoplasmic pellet of human leukemic cells.

Analyses of the Reaction Products of the Leukemic Pellet DNA Polymerase and 70S RNA

^{3}H-DNA was prepared from a reaction of the leukemic pellet DNA polymerase and RLV 70S RNA and purified as described in the legend to Figure 1. As shown in Figure 1A, the DNA product bands predominantly in the RNA region of a Cs_2SO_4 density gradient. Since alkali treatment (Figure 1B) but not heat treatment (100°, 10 minutes; not shown) moved the radioactivity from the RNA to the DNA density region, it appears that the labelled

Table 1. Comparison of the Transcription of Viral 70S RNA by DNA Polymerases of Human Leukemic Cells and Phytohemagglutinin-Stimulated Normal Human Lymphocytes

Addition	pmoles of ^{3}H-dCTP incorporation /0.1 mg of protein		
	AMV 70S RNA	FeLV 70S RNA	RLV 70S RNA
Human Leukemic "Pellet" enzyme#	3.5	12.9	13.6
Human Leukemic "Pellet" enzyme plus $(dT)_{12-18}$	12.6	N.T.	N.T.
Human Leukemic "Pellet" enzyme plus RNase	<0.5	<0.5	0.7
Human Normal Lymphocyte "Pellet" enzyme	<0.5	<0.5	<0.5
Human Normal Lymphocyte "Pellet" enzyme plus RNase	<0.5	<0.5	<0.5
Human Leukemic "Pellet" enzyme plus Normal Lymphocyte Pellet enzyme	3.5*	13.1*	N.T.

The incubation mixture contained (in 0.05 ml) 50 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaCl, 1 mM dithiothreitol, 5 mM NaF, 80 µM each of dATP, dGTP, dTTP, and 28.6 µM ^{3}H-dCTP (28 C/mmole). Concentrations of 70S RNA were 50 µg/ml. For RNase sensitivity the RNAs were incubated with RNase (20 µg/ml) for 30 min at room temperature (25°C) prior to the addition of the enzyme. Reactions were stopped after 30 min. at 37° by adding 2 ml of 10% trichloroacetic acid containing 0.2 mM sodium pyrophosphate. The samples were filtered on millipore filters, washed with 5% TCA, and counted in a liquid scintillation counter.

#This enzyme utilized oligo (dG).poly (rC) as a template-primer, incorporating ^{3}HdGTP, (34.6 pmoles/0.1 mg protein), whereas with $(dG)_{12-18}$· $(dT)_{12-18}$· and $(dC)_{12-18}$ as primers, only 0.6, 1.2, and 0.6 pmoles/0.1 mg protein, respectively, of ^{3}H-dGTP was incorporated. These results show that the polymerase prepartion contains insignificant, or no, terminal transferase activity. ^{3}H-dGTP (7.8 C/m mole) concentration used in the assay was 12 µM. The enzyme also showed a 5:1 preference for $(dT)_{12-18}$·rA over $(dT)_{12-18}$·dA. This ratio varies with different purified enzyme preparations. In some cases it approaches 100:1 (Gallagher, R. G., unpublished results).

*Data expressed were calculated on the basis of the protein concentration of the leukemic enzyme.

N.T. = Not tested

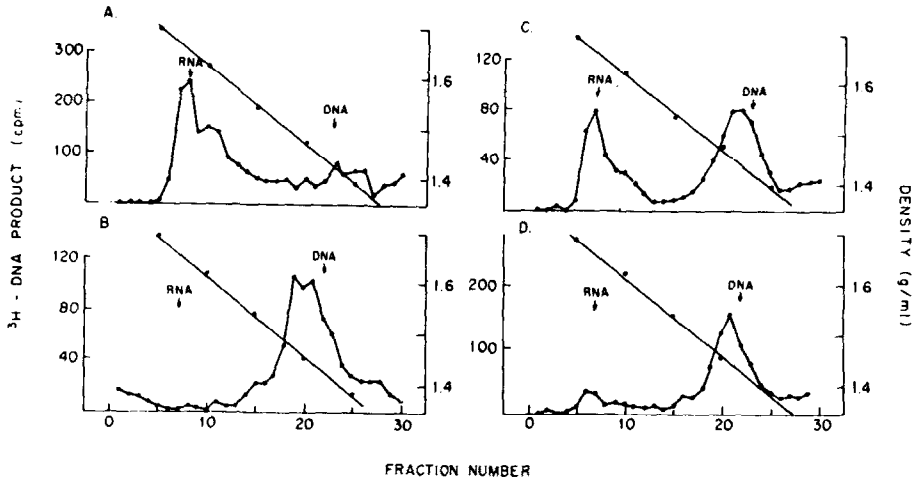

Figure 1. Cs_2SO_4 density gradient analysis of DNA transcribed by purified leukemic "pellet" DNA polymerase from RLV 70S RNA and demonstration of complementarity of DNA product to the template RNA.

A standard reaction mixture (0.14 ml) was prepared as described in Table 1 except that it contained 60 µM ^{3}H-dATP (13.2 C/mmole), 28.6 µM, ^{3}H-dCTP (28 C/mmole) and 102 µM ^{3}H-dGTP (7.8 C/mmole), 80 µM of cold TTP, and 50 µg/ml of RLV 70S RNA. It was incubated for 45 minutes at 37°C and the reaction terminated by addition of SDS to a concentration of 1% and NaCl to 120 mM. Products were then purified by extraction with phenol-cresol and precipitation with CTAB, then ethanol as described previously (19). The product was dissolved in 0.25 ml of 2 x SSC (0.01 M sodium citrate, pH 7.0; 0.15 M NaCl) and portions were analyzed by Cs_2SO_4 density gradients after the following treatments: A) no treatment; B) product was made 0.3 N in KOH and heated to 95°C for 10 minutes then neutralized with HCl; alkali treated and neutralized product was annealed in 0.2 ml 50% formamide, 3 x SSC (final concentration) for 3 days at 37°C with C) 2.5 µg of RLV 70S RNA; and D) 2.5 µg of AMV 70S RNA.

DNA is covalently attached to the primer RNA, as previously reported for DNA synthesized by AMV and RLV reverse transcriptase (27). Alkali-treated DNA product, when annealed with RLV 70S RNA, again bands in the RNA density region (Figure 1C), indicating complementarity to the template viral RNA. A much lower degree of hybridization was observed after annealing with AMV 70S RNA (Figure 1D).

DNA products from reactions with leukemic pellet DNA polymerase utilizing AMV or RLV 70S RNA as template-primers were also prepared, treated with alkali, annealed to RNA as described in the legend to Table 2, and analyzed for hybrid formation by resistance to S1 nuclease. As shown in

Table 2. Demonstration of Complementarity of DNA Product to Template RNA by S1 Nuclease Assay

Template-Primer for DNA Product	DNA Product Annealed With	Treatment	^{3}H-DNA After Treatment	^{3}H-DNA After Treatment (corrected for self-complementary ^{3}H-DNA)
			(cpm)	
RLV 70S RNA	None	None	601	441
	None	Nuclease	160	-
	AMV 70S RNA	Nuclease	151	0
	RLV 70S RNA	Nuclease	247	87
	Poly (A)	Nuclease	147	0
AMV 70S RNA	None	None	340	268
	None	Nuclease	72	-
	AMV 70S RNA	Nuclease	130	58
	RLV 70S RNA	Nuclease	90	18

Product DNA was synthesized, purified, hydrolyzed with alkali, neutralized, and annealed with 2.5 µg of the indicated 70S RNA as described in the legend to Figure 1. To 0.12 ml of annealing mixture was added 0.2 ml S1 buffer (0.025 M NaOAc, pH 4.5; 0.3 M NaCl; 0.006 M $ZnSO_4$) and 0.005 ml (approximately 30 µg purified protein) of a nuclease from Aspergillus oryzae which specifically hydrolyzes single stranded nucleic acids (20, 21), purified as described by Sutton (21). These were incubated for 60 minutes at 37°C, precipitated, and counted as described in Table 1. In each series, S1 nuclease was omitted to determine input radioactivity. Self annealed samples served to measure radioactivity due to the presence of self-complementary DNA. This value was subtracted from the values obtained from the other experiments to calculate the net hybrid formation.

Table 2, alkali-treated DNA product is largely nuclease-sensitive after self-annealing, indicating only a small amount of synthesis of a second strand of DNA. Both DNA products hybridize to template RNA to about 20-30%, while little or no hybridization is observed to heterologous 70S RNA or to poly (A). DNA product purified from a similar reaction with FeLV 70S RNA shows a similar

hybridization pattern (data not shown). It is not certain why hybridization to the template RNA does not approach 100%, but this is also our experience with murine tumor virus DNA polymerase products, both from endogenous reactions (28) and from reactions with purified DNA polymerase and endogenous 70S RNA (unpublished results). We believe it is due to portions of the products being of very small size, since the portion of DNA from endogenous reverse transcriptase reactions of murine tumor viruses which fail to hybridize to the template RNA are quite small (<75 nucleotides) relative to the hybridizable fraction (4S and larger) (28).

In summary, the pellet DNA polymerase can also be isolated from white blood cells of at least some patients with acute myelocytic leukemia. The enzyme can catalyze transcription of heteropolymeric regions of tumor virus 70S RNA and exhibits a preference for mammalian viral RNA over avian viral RNA. We also show that this DNA polymerase is free from contaminating terminal deoxynucleotidyl transferase activity. Finally, from mixing experiments it appears that the inability of a DNA polymerase purified from PHA-stimulated normal human blood lymphocytes to transcribe heteropolymeric regions of viral RNA is probably not due to the presence of inhibitor under our assay conditions.

The characteristics of the purified leukemic pellet DNA polymerase (9, 10) in transcribing heteropolymeric regions of viral 70S RNA and synthetic template-primers are similar to those of the reverse transcriptase of RNA tumor viruses. In addition, the RNase sensitivity of the leukemic endogenous DNA polymerase activity (utilizing nucleic acid native to the cytoplasmic pellet) (9, 11) and the finding that the leukemic endogenous DNA product is partially complementary to 70S RNA from murine (11, 12) and primate type-C virus (12) and is apparently originally hydrogen bonded to a high molecular weight RNA (11), are suggestive of the presence of viral-related RNA and polymerase. The biochemical observations alone do not permit a firm conclusion that this enzyme system is viral in origin. However, recent experiments have

shown that this leukemic enzyme†, but neither any of the major DNA polymerases from normal human lymphocytes (17, 18) nor the "R-DNA polymerase"†† isolated from human tissue culture cells (15, 16) are significantly inhibited by antibodies prepared against the RNA-dependent DNA polymerase from the type-C viruses of mouse and especially primate (gibbon ape leukemia virus and woolly monkey sarcoma virus). These results indicate that the white blood cells of at least some patients with acute leukemias contain one expression of type-C virus information.

Our present results indicate that this enzyme is detectable in a limited number of patients with acute leukemia. However, we believe it is too premature to estimate its distribution or whether it is present only in certain stage of the disease. We emphasize that it is preferable to use relatively large quantities of these blood cells to isolate this enzyme.

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† The enzyme preparation in this paper was from patient HL3, the same preparation used for most of the studies in References 17 and 18.

†† The so-called "R-DNA polymerase" may be slightly inhibited. The magnitude of inhibition is not comparable to inhibition of virus reverse transcriptase nor this enzyme from human leukemic cells (G. Todaro and R. C. Gallo, unpublished results).

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