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Detection of Reverse Transcriptase Activity in Human Cells[1]

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ABSTRACT

Samples of three nonmalignant and seven leukemic human cells were examined for DNA polymerase activity that could be identified as RNA tumor virus reverse transcriptase. Experiments on virus-infected model animal cells provided the basis for cell fractionation procedures, and reconstituted systems of known virus, added to human cells, established a threshold of virus detection by enzyme assay at 1 to 10 particles/cell. DNA polymerase activity with some properties similar to a reverse transcriptase was detected in some of the human leukemic cells. However, parallel analyses of nonmalignant cells showed sufficient similarities to raise serious questions about the specificity of the criteria. Reverse transcriptase activity has been reported to be present in white blood cells from a proportion of cases of leukemia; however, it is concluded from the present study that the usual enzymatic criteria using synthetic template primers, which were used in most of the studies reported, are not sufficient to identify a DNA polymerase activity as viral reverse transcriptase.

INTRODUCTION

The unique properties of RNA tumor virus DNA polymerase ("reverse transcriptase") (47), especially its ability to transcribe natural and synthetic RNA's, have suggested the possibility that this enzyme activity would provide a sensitive assay for RNA tumor viruses. For this reason, much effort has been directed toward detecting human tumor viruses by assaying malignant cells for reverse transcriptase activity (1, 3, 5, 12–21, 25, 26, 28, 29, 32, 36, 37, 48). However, the difficulties inherent in detecting a viral enzyme expected to be present in low concentrations in malignant cells have been compounded by the presence of cellular DNA polymerases that can also respond to certain assays that were earlier thought to be specific for viral reverse transcriptase (4, 8, 11, 27, 39, 40, 46). A variety of criteria have been proposed to minimize confusion of viral enzyme with normal cellular enzymes, including specific responses by viral DNA polymerase to particular templates or divalent metal ions (13, 16, 18–20) and antigenic similarity of virus enzyme to primate virus reverse transcriptases (9, 12, 48).

This communication describes studies to determine the usefulness of some of these criteria in detection of viral reverse transcriptase in human leukemic cells. Animal cells infected with tumor viruses provided the positive controls necessary to establish guidelines for cell fractionation and assay of the human malignant cells. Reconstituted models prepared by adding known quantities of MuLV[4] to human nonleukemic or leukemic cells prior to cell disruption and enzyme assay were used to determine sensitivity for detection of virus in human cells. Control cells used for comparison with malignant cells were from normal human placenta and spleen, a cultured human lymphocyte cell line (8866), and HeLa, a line that has been found to be negative for reverse transcriptase (11, 46).

MATERIALS AND METHODS

Viruses and Virus-infected Animal Cells

AMV and AMV-infected chick myeloblasts were gifts of Dr. George Beaudreau, Oregon State University. MuLV was grown and harvested from infected 3T3 cells (clone 1), a gift originally from Dr. Hung Fan, Salk Institute. Details of purification and virus titration were as previously described (23). Virus particle counts were determined by electron microscopy by Dr. Peter Kiessling, University of California, San Diego.

Human Cells and Human Tissues

Human leukemia cells were obtained from peripheral blood with sodium citrate or heparin as anticoagulant and harvested by gravity sedimentation after addition of 1% dextran. The buffy coat was washed twice with 5 to 10 volumes of minimum essential medium, pelleted at 800 × *g* for 10 min, and rewashed with homogenization buffer (Buffer H), which contained 0.05 M Tris-HCl (pH 7.4), 0.1 M KCl, 0.25 M sucrose, 0.005 M $MgCl_2$, 0.001 M EDTA, and 0.002 M DTT. The cells were homogenized either fresh, or following storage as packed cell pellets at −70° or in liquid nitrogen banks. HeLa and nonleukemic human lymphoblasts (8866 cells) were propagated as suspension cultures in minimum essential medium with 10% fetal calf serum. The cells were harvested and washed as described for the human leukemic cells. Human placental tissues were obtained as aborted early products of conception, or from cesarean sections. Human spleens were obtained as a fresh surgery specimen from a patient with nonmalignant disease (hereditary spherocytosis) or as fresh autopsy specimens from patients with no known cancers. The solid tissues were rendered single-cell suspensions by removing fascia, suspending in 2 volumes of Buffer H, and homogenizing in 15-sec bursts in a Sorvall Omnimix 4 to 6 times, followed by filtration through 3 to 4 layers of gauze to remove connective tissue fibers. The resulting cell suspension was homogenized in the same manner as for the other cells.

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[4] The abbreviations used are: MuLV, Moloney murine virus; AMV, avian myeloblastosis virus; DTT, dithiothreitol; poly(mC)·oligo(dG), poly(2′-*o*-methylcytidylate)·oligodeoxyguanylate; poly(dA)·oligo(dT), polydeoxyadenylate·oligodeoxythymidylate; poly(C)·oligo(dG), polycytidylate·oligodeoxyguanylate; poly(A)·oligo(dT), polyadenylate·oligodeoxythymidylate. dNTP, deoxyribonucleoside triphosphate; CML, chronic myelogenous leukemia; SiSV, simian sarcoma virus; AMML, acute myelomonocytic leukemia; AML, acute myelogenous leukemia.

Cell Fractionation Procedures

Cells were suspended in 3 to 5 volumes of Buffer H and homogenized in a tight-fitting Dounce homogenizer until less than 10% whole cells remained (50 to 60 strokes for normal cells, 20 to 40 strokes for malignant cells).

Nuclei were removed by 2 centrifugations at 800 × *g* for 10 min. Mitochondria were removed by 2 centrifugations at 10,000 × *g* for 10 min. For some experiments, the postmitochondrial supernatant was layered directly over 10 to 70% sucrose density gradients in Buffer A [0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, and 0.001 M DTT] containing 0.1 M KCl, and centrifuged to equilibrium. The gradients were fractionated, and each fraction was assayed in System 1 or 2 (see below). In other experiments, particularly with large initial volumes such as the spleen or placenta preparations, membrane fractions were obtained from postmitochondrial supernatants by centrifugation at 30,000 rpm in the SW40 rotor (Beckman, Palo Alto, Calif.) for 3 hr, or 40,000 rpm in the SW50 rotor for 2 hr through 20% (w/v) sucrose in Buffer A. The membrane fraction thus obtained was resuspended by gentle sonication in Buffer A containing 5% sucrose, layered over a continuous 10 to 70% sucrose density gradient as above, or over a discontinuous 20 to 40% sucrose gradient in Buffer A, centrifuged to equilibrium as described in the text, fractionated, and assayed in System 1 or 2 (see below).

Enzyme Assays

DNA was prepared from normal human spleen according to the method of Marmur (34) and maximally "activated" by treatment with pancreatic DNase (17 to 19% solubility) (23, 24). Poly(mC)·oligo(dG)$_{12-18}$ (1:1, w/w) and poly(dA)·oligo(dT)$_{12-18}$ (1:1, w/w) were obtained from P-L Biochemicals (Waltham, Mass.). Poly(C)·oligo(dG)$_{12-18}$ was either from P-L Biochemicals (1:1, w/w) or from Collaborative Research-(Milwaukee, Wis) (2:1, w/w) as was poly(A)·oligo(dT)$_{12-18}$ (2:1, w/w). Nonradioactive dNTP's and DTT were obtained from P-L Biochemicals. [^{3}H]dGTP (20 Ci/mmol) was from New England Nuclear Corp. (Boston, Mass.), [^{3}H]dTTP was prepared from [^{3}H]thymidine (60 Ci/mmol) as previously described (23). Radioactive dNTP's were used without dilution of the specific activity unless otherwise stated.

Enzyme aliquots were adjusted to 0.25% Nonidet P-40 (Shell Oil Co., Houston, Texas) and 5 mM DTT and kept at 0° for at least 10 min prior to addition of assay mixtures, which resulted in a 5-fold dilution of enzyme-detergent.

System 1. DNA-dependent polymerase was assayed by incubation for 20 min at 37° of a mixture (50 to 150 μl) containing 30 mM Tris-HCl (pH 8.3), 20 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mM concentrations of each nonradioactive dNTP, activated DNA (500 μg/ml) (31), bovine plasma albumin (1 mg/ml), and [^{3}H]dTTP (2 μM), except as in Table 1 (reaction with activated DNA for AMV myeloblasts, HeLa, and soluble fraction of AML-2 for which [^{3}H]dGTP (5 μM) was used).

System 2. Poly(A)·oligo(dT)-stimulated DNA polymerase activity was assessed by incubation for 20 min at 30° in a mixture (50 to 150 μl) containing 30 mM Tris-HCl (pH 8.3), 80 mM KCl, 0.5 mM $MnCl_2$, 2 mM DTT, 0.2 mM dATP, poly(A)·oligo(dT) (40 μg/ml), bovine plasma albumin (1 mg/ml), and 2 μM [^{3}H]dTTP.

Endogenous Reactions. System 1 minus activated DNA was used for assay of endogenous reactions, with [^{3}H]dTTP (2 μM) in all cases. Where indicated, actinomycin D was added at 100 μg/ml and pancreatic RNase was added at 20 μg/ml.

Other Template Primers. Activities with poly(C)·oligo(dG) and poly(mC)·oligo(dG) were assayed in System 2 minus KCl, substituting dCTP for dATP and [^{3}H]dGTP for [^{3}H]dTTP. These assays were incubated at 37° for 20 min. In the experiments described in Charts 1 and 2, using poly(C)·oligo(dG), $MgCl_2$ (5 mM) was substituted for $MnCl_2$. Activity with poly(dA)·oligo(dT) was assayed in System 2 substituting 5 mM $MgCl_2$ for 0.5 mM $MnCl_2$.

Products were precipitated by direct absorption of assay mixtures into glass fiber papers and *in situ* precipitation in 10% trichloroacetic acid as before (23), or by addition of 50 μg carrier DNA and 5 assay volumes 10% trichloroacetic acid, 0.1 M KH_2PO_4, and 0.01 M EDTA for 10 min at 0°, followed by collection onto Whatman GF/C glass fiber filters (Whatman, Co., Clifton N. J.) under vacuum. Filters containing precipitated DNA products from both precipitation techniques were washed 3 to 5 times with cold 0.1 M HCL-0.01 M KH_2PO_4, followed by 3 to 5 washes with 0.1 M HCl containing no phosphates. DNA products were quantified on dried filters by liquid scintillation counting in a PPO-POPOP-toluene system.

All template primer-directed activities reported here have been corrected for acid-insoluble radioactivity in the absence of template primer. Tests for terminal deoxynucleotidyltransferase, by inclusion of primer without template and/or by substitution of noncomplementary dNTP, were consistently negative.

Thin-Layer Chromatography

One- to 2-μl aliquots of completed assay mixtures were chromatographed on polyethyleneimine thin-layer plates (EM Reagents, Darmstadt, Germany) for 1 hr in 1.0 M LiCl to separate dNTP's from mono- and diphosphates.

RESULTS

Animal Model Systems

Cell homogenates were prepared from AMV-infected myeloblasts and MuLV-infected 3T3 cells to establish guidelines for fractionation and enzyme assay. The 2 systems allowed comparisons to be made between avian and mammalian viruses as well as between circulating leukemic cells and tissue culture fibroblasts. The postmitochondrial supernatant fractions obtained from either the AMV- or the MuLV-infected cells yielded the same profiles of enzyme activity when centrifuged to equilibrium in isopycnic sucrose gradients (Chart 1). The same results were obtained with fresh cells, with cells subjected to 2 to 3 freeze-thaw cycles, or with cells stored at −20° for 3 years. The virus enzyme activity in both the myeloblast and 3T3 cell homogenates was equivalent to 10^3 to 10^4 virus particles/cell (23), a concentration of virus particles easily visualized in electron micrographs. However, even in this example of active viral proliferation, the virus enzyme accounted for less than 10% of total myeloblast DNA polymerase activity.

No more than 5% of the viral reverse transcriptase, as defined here as activity with poly(C)·oligo(dG), was found in the "soluble" cytoplasmic material (Chart 1, Fractions 16 to

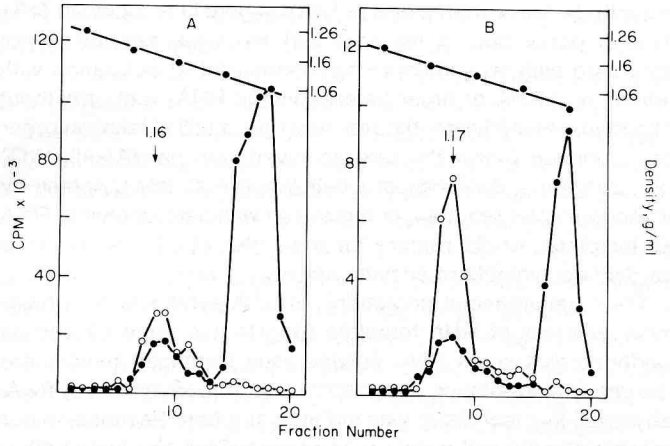

Chart 1. Isopycnic sucrose gradients of postmitochondrial cytoplasmic supernatant fractions from AMV-infected chick myeloblasts (*A*) and MuLV-infected mouse 3T3 cells (*B*). A 200-μl packed-cell volume of washed myeloblasts and a 100-μl packed-cell volume of washed 3T3 cells were processed as described in "Materials and Methods," and the postmitochondrial supernatant fractions were layered over 10 to 70% sucrose gradients in Buffer A, centrifuged to equilibrium at 25,000 rpm in a Beckman SW 40 rotor for 16 hr at 4°, and fractionated from the bottom into 0.6-ml fractions. Aliquots of 5 μl were assayed with DNA template (●) and [^{3}H]dTTP (1500 cpm/pmol), and with poly(C)·oligo(dg) (○) and [^{3}H]dGTP(2100 cpm/pmol), as described in "Materials and Methods."

20), and none of the viral enzyme appeared at the buoyant density of viral cores (1.19 to 1.21 g/ml). These results indicate that virus is either assembled on smooth membranes or readily associates with membranes having a density of 1.15 to 1.19 g/ml. The possibility of inhibitors preventing detection of virus enzymes in the nonmembrane fractions was excluded by recovery of virtually complete activity when aliquots of the gradient fractions were combined with known quantities of virus enzyme in assays containing poly(C)·oligo(dG).

Actinomycin D also did not inhibit the reaction of the intracellular virus enzymes with poly(C)·oligo(dG). This assay condition was applied to test fidelity of the characteristics of intracellular virus enzyme. The reaction of RNA tumor virus enzyme (purified or in detergent-disrupted virions) with poly(C)·oligo(dG) is less than 5% depressed at concentrations of actinomycin D up to 100 μg/ml, conditions that inhibit 95% of the reaction with DNA templates.[5]

Reconstruction Experiments. Purified MuLV particles were added to CML cells at proportions of 10 particles/cell and 1 particle/cell. The cell-virus mixtures were homogenized, and the cytoplasmic membrane fractions (see "Materials and Methods") were centrifuged to equilibrium in isopycnic sucrose gradients along with a similar sample from CML cells with no added virus. The recovery of virus reverse transcriptase added to the cells (appearing as a distinct peak at a density of 1.18 g/ml) was 30 to 50% from both cell-virus mixtures (Chart 2). None of the virus enzyme was found at the buoyant density of viral cores (1.19 to 1.21 g/ml) or at the top of the gradient in the form of solubilized enzyme. Aliquots of the gradient fractions did not inhibit reverse transcriptase when added to standard virus enzyme assays. If inactivation of the virus enzyme accounted for the loss of 50 to 70% of the added reverse transcriptase activity, the inactivation must require exposure to other cell fractions or exposure times that were longer than the length of incubation for enzyme activities.

[5] A. A. Kiessling, unpublished observations.

A peak of enzyme activity responding to the poly(C)·oligo(dG) template was also seen in the CML cells with no added virus (Chart 2*A*). The activity appeared in the gradient in the density region of RNA tumor viruses (1.16 g/ml) although slightly less dense than the MuLV particles (Chart 2*B*). However, the poly(C)·oligo(dG)-dependent enzyme activity in the membrane fraction of the CML cells with no added virus was 90% inhibited by actinomycin D, whereas, the poly(C)·oligo(dG)-dependent activity of virus enzyme was resistant to actinomycin D even in the cell lysates.

Similar procedures following addition of purified MuLV at the same 2 concentrations to cultured lymphocytes (8866 cells) resulted in 10% recovery of MuLV reverse transcriptase (data not shown). As was the case with the CML cells, none of the viral enzyme appeared as solubilized activity at the top of the gradient, as viral cores or with the nuclei or mitochondria, fractions. Aliquots of the sucrose gradient fractions containing the virus-lymphocyte membrane fractions were slightly more inhibitory (15 to 20%) to virus enzyme in the presence of poly(C)·oligo(dG) than were the CML cell fractions, but they did not account for the loss of the viral enzyme activity.

Sensitivity of Virus Detection by Reverse Transcriptase Assay

The reported threshold of detection of virus by enzyme assay varies from 2×10^4 particles (30) to 2×10^8 particles (43). We were able to detect 1 to 10×10^5 virus particles by DNA polymerase assay (23), in close agreement with Allaudeen *et al.* (2); *i.e.*, with poly(A)·oligo(dT) at optimal assay conditions, 10^6 particles of AMV, MuLV, feline leukemia virus, and SiSV each contain sufficient DNA polymerase to incorporate 5 fmol of dTMP (150 cpm from [^{3}H]dTTP, 60 Ci/mmol, at 20% efficiency) into product in 20 min. We have previously reported that poly(C)·oligo(dG), the more specific template primer, is

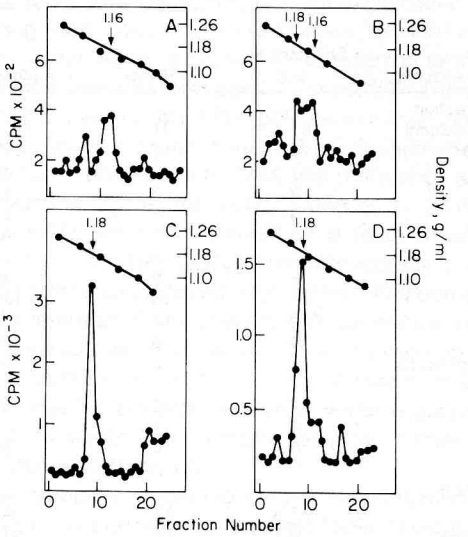

Chart 2. Isopycnic sucrose gradients of postmitochondrial cytoplasmic fractions from 2×10^9 CML cells (*A*), 1×10^9 CML cells plus 1×10^9 MuLV particles (*B*), and 1×10^9 CML cells plus 1×10^{10} MuLV particles (*D*). *C* contains 1×10^{10} MuLV particles alone as a control. Virus was added to the cells prior to disruption and separation of the cytoplasmic fraction as described in "Materials and Methods." Centrifugation was for 4 hr at 35,000 rpm in a Beckman SW 40 rotor; each gradient was separated into 0.35-ml fractions from the bottom, and aliquots of 10 μl were assayed with poly(C)·oligo(dG) and [^{3}H]dGTP (3000 cpm/pmol) (see "Materials and Methods").

twice as active with AMV DNA polymerase as is poly(A)·oligo(dT), but only one-tenth as active with the MuLV and feline leukemia virus enzymes, and at least 4 orders of magnitude less active with SiSV (23).

The reconstruction experiments indicated that a single assay could detect intracellular virus enzyme in as few as 1 million leukemic cells containing on the average 1 particle/cell. Based on our data from whole leukemic cells (Table 1) and isolated particles (23), at 1 particle/cell, virus enzyme would constitute approximately 0.02% of total cellular DNA-dependent DNA polymerase activity. However, assays at this level of sensitivity will also detect weak reactions of other DNA polymerases, and assay specificity for reverse transcriptases becomes a critical problem.

The Endogenous Reaction. The most specific enzymatic property of reverse transcriptase is the ability to transcribe heteropolymer RNA's. This is the basis for the "endogenous reaction" described for virus particles, *i.e.*, DNA synthesis in detergent-disrupted virions using endogenous viral RNA as template. In agreement with previous reports (22), the endogenous reactions of MuLV and AMV were found to be approximately 80% sensitive to RNase and 60 to 70% resistant to actinomycin D (data not shown).

The cell fractions containing the virus enzymes from the animal model systems were 40 to 60% resistant to actinomycin D but only 30 to 40% sensitive to RNase (Table 1). The limited sensitivity to RNase may reflect the presence of alternative templates, such as contaminating DNA fragments.

The amount of product formed in the endogenous reaction of detergent-disrupted virions is approximately 2 orders of magnitude lower than when poly(A)·oligo(dT) is included (23). This is partly due to the fact that the virus enzyme is not saturated with templates in the virions, but at saturation with added viral RNA or other heteropolymer RNA, with or without oligodeoxythymidylate, the reaction rate is still at least an order of magnitude below the rate achieved with poly(A)·oligo(dT) (22, 23). Thus, detection of a cellular reverse transcriptase by an endogenous reaction, or a reaction with heteropolymer RNA as template, would require on the order of 10^7 to 10^8 virus particle equivalents of enzyme activity.

The "simultaneous detection" of DNA synthesis on a high-molecular-weight RNA template (5, 21) has been offered as evidence that some DNA polymerases in human tumors are reverse transcriptases associated with intracellular viral RNA. However, this technique was not included here because, in our hands, only a small percentage of early DNA product synthesized by detergent-disrupted AMV or MuLV particles was associated with high-molecular-weight RNA, and the results were not reproducible with or without NaF present to inhibit the viral RNase H.

Screening Procedure for Human Cells

The data from the experiments with the animal model systems and the virus recovery experiments from human cells indicated reverse transcriptase in human malignant cells would most probably be associated with the smooth membrane fraction (1.15 to 1.18 g/ml) isolated from cytoplasm by isopyknic sucrose gradients. This is also the cell fraction in which reverse transcriptase was reported to be present in human leukemic

Table 1

DNA polymerase activities in fractions from leukemic and control cells

	Incorporation (pmol/5 × 10^7 cells/20 min[b])						
	Endogenous reaction			Exogenous template primer			
						Poly(C)·oligo(dG)	
Source of enzyme[a]	Standard assay	+ RNase	+ Actinomycin D	Activated DNA	Poly(A)·oligo(dT)	− Actinomycin D	+ Actinomycin D
Membrane fraction							
AMV myeloblasts	51	31	22	792 (10)[c]	1402	1236	1106
MuLV-3T3	19	13	12	144 (8)	1010	460	464
HeLa	11	10	4	125 (3)	75	0.4	0.1
8866	3	3	1	52 (2)	11	0.1	0.1
Placenta	4	5	1	11 (1)	2	0.1	0.1
Human spleen	13	17	9	21 (2)	2	0.1	0.1
CML-1a	8	9	3	147 (4)	37	0.1	0.1
CML-1b	21	19	12	221 (4)	123	11	0.1
CML-2	22	17	12	185 (4)	42	3	0.1
CML-3	9	9	4	98 (4)	76	1	0.1
Acute lymphocytic leukemia	4	3	2	120 (4)	22	2	0.1
AML-1	3	2	1	17 (0.3)	18	0.1	0.1
AML-2	2	2	0.4	72 (3)	18	0.1	0.2
AMML	9	14	8	232 (2)	45	8	3
Soluble fraction							
8866	37.4	NT[d]	4	2167 (97)	1	9	1
Human spleen	0.1	NT	NT	249 (15)	25	0.1	0.1
AML-2	0.1	NT	NT	257 (6)	25	0.1	0.1
CML-1a	0.8	NT	0.1	4205 (97)	5	17	2

[a] Membrane and soluble fractions, 1.15- to 1.20- and 1.07- to 1.09-g/ml fractions, respectively, from isopycnic sucrose gradient centrifugations ("Materials and Methods"). Leukemic cells are designated by diagnosis and patient number; a and b, separate samples from the same patient.

[b] Incorporation of radiolabeled deoxyribonucleoside monophosphate into product, normalized to standard assay (50 μl) containing enzyme from 5 × 10^7 cells incubated at 37° for 20 min. Actual assay conditions varied, *e.g.*, in specific activities of labeled dNTP's (see "Materials and Methods").

[c] Numbers in parentheses, percentage of total cytoplasmic (postnuclear) DNA-dependent DNA polymerase represented by that fraction.

[d] NT, not tested.

cells by Baxt *et al.* (5) and Gallo *et al.* (13, 14). An additional advantage of initially isolating the membrane fraction was that only a small proportion (5 to 10%) of cellular DNA-dependent DNA polymerase remained associated with this fraction.

The assay in which poly(A)·oligo(dT) with Mn^{2+} was used was selected to provide maximum sensitivity for initial detection. Although this system will also detect cellular DNA polymerases β and γ, it is the most stimulatory template primer for the mammalian virus enzymes (23). DNA polymerase activity detected by poly(A)·oligo(dT) in the membrane fraction was further characterized by: (*a*) response to poly(C)·oligo(dG) in the presence and absence of actinomycin D; (*b*) the effect of actinomycin D and RNase on the endogenous reaction; (*c*) the activity with poly(A)·oligo(dT) and poly(dA)·oligo(dT) in the presence of Mg^{2+}; and (*d*) a test for inhibitors by measurement of the activity of MuLV or AMV virus enzymes when mixed with the human leukemic cell fractions.

Characteristics of the Enzymes in Human Leukemic and Nonleukemic Cells. Total cellular DNA-dependent DNA polymerase was greater in the malignant cells than in the tissue culture cells; the normal tissues, placenta or spleen, contained even less DNA-dependent DNA polymerase than either malignant or tissue culture cells (Table 1). All membrane-associated enzyme activities (indicated as a percentage of total cellular DNA-dependent DNA polymerase in Table 1), were stimulated at least 50% by the addition of Nonidet P-40, but this was not the case for enzyme in the soluble, cytoplasmic fractions.

An endogenous reaction, as measured by synthesis of DNA without added template primers, was detected in all membrane fractions examined. The endogenous reaction of membrane fractions from acute lymphocytic leukemia, AMML, and the 4 samples of CML were at least 38% resistant to the presence of actinomycin D, but the same was true for normal human spleen (Table 1). In none of these samples, *i.e.*, neither malignant nor nonmalignant, was the endogenous reaction greater than 20 to 30% sensitive to RNase treatment; in fact, most were actually stimulated by RNase. The endogenous reactions most sensitive to RNase were with membrane fractions from acute lymphocytic leukemia, AML-1, and CML-2.

Activity with poly(A)·oligo(dT) and Mn^{2+} was found in the isolated cytoplasmic membranes of all cells examined. Greater than 80% of the total cellular activity stimulated by poly(A)·oligo(dT), from either malignant or nonmalignant cells, was membrane bound and remained so in spite of repeated fractionation by isopyknic sucrose gradient centrifugation (data not shown). The values for poly(A)·oligo(dT) stimulation reported in Table 1 are with Mn^{2+} for this template primer. In general, the leukemic cells contained more poly(A)·oligo(dT)-stimulated activity than did the control cells, but the leukemic cells also contained more DNA-stimulated DNA polymerase, so that the poly(A)·oligo(dT)-stimulated activity was 10 to 30% of the DNA-stimulated activity in membrane fractions from all cells studied except 4, *i.e.*, HeLa (60%), AML-1 (100%), CML-3 (75%), and CML-1b (50%).

It has been reported that, in the presence of Mg^{2+}, DNA polymerase γ prefers poly(dA)·oligo(dT) to poly(A)·oligo(dT), whereas the reverse is true for the virus enzymes (16). For this reason, some cell fractions (AMV myeloblasts, MuLV-3T3, 8866, placenta, spleen, AML-1, AML-2, and AMML) were assayed with poly(dA)·oligo(dT) as well as poly(A)·oligo(dT), each with Mg^{2+} instead of Mn^{2+}. The animal virus enzyme in the infected model cells fulfilled the expectation, with the result that with Mg^{2+} poly(dA)·oligo(dT) was only 10 to 20% as active as was poly(A)·oligo(dT). However, the opposite results were obtained with the human enzyme samples, in that poly(dA)·oligo(dT) was a more effective template primer than was poly(A)·oligo(dT) in the presence of Mg^{2+}. The one exception to this was the AMML sample, for which poly(dA)·oligo(dT) and poly(A)·oligo(dT) were equally effective in the presence of Mg^{2+}. These results seemed to indicate that the poly(A)·oligo(dT)-Mn^{2+} assay system was detecting DNA polymerase γ in most of the membrane fraction. However, this interpretation is now open to question in light of more recent reports (27, 44) [in contrast to the earlier report (16)] that DNA polymerase γ from human lymphocytes also prefers poly(A)·oligo(dT) to poly(dA)·oligo(dT) in the presence of Mg^{2+}.

Enzyme activity with poly(C)·oligo(dG) was found in the membrane fractions from some cells (malignant and nonmalignant) but, in contrast to the virus-infected cells, this activity was sensitive to actinomycin D in all cases but one (Table 1). The one exception was the AMML sample, for which the activity with poly(C)·oligo(dG) was 38% resistant to actinomycin D. Experiments in which various cell fractions were added to viral DNA polymerase with poly(C)·oligo(dG) did not reveal significant inhibitors, and the poly(C)·oligo(dG) stimulation of the viral enzymes was not depressed by actinomycin D in the presence of aliquots of the leukemic cell fractions. In addition, [^{3}H]polycytidylate, with or without oligodeoxyguanylate primer, was degraded less than 10% by enzyme fractions from the leukemic cells, and at least 70% of the initial [^{3}H]dGTP [as well as [^{3}H]dTTP from assays with poly(A)·oligo(dT)] was recovered at the termination of the assay. Thus, degradation of neither template primer (13, 16) nor substrates (19) was a significant problem with any of the postmitochondrial supernatant cell fractions obtained by these procedures.

Of the samples (placenta, spleen, AML-1, AML-2, and AMML) that were also tested with poly(mC)·oligo(dG), only one had activity greater than 1% of the DNA-dependent activity. In this sample, which again was the AMML referred to above, the activity was the same as with poly(C)·oligo(dG) (Table 1).

The principal cytoplasmic DNA polymerase from one sample of CML cells, which was sensitive to *N*-ethylmaleimide and had chromatographic properties of DNA polymerase α, was found to be stimulated by either poly(C)·oligo(dG) (Chart 3) or poly(A)·oligo(dT) (data not shown) at a level of 1% of the stimulation by activated DNA. These 3 activities remained associated at the same ratio through DEAE-cellulose and DNA-agarose chromatography, eluting at salt concentrations different from the oncornaviral enzymes. Thus, it would appear that, at detection sensitivities of 1% or less of reactions with DNA template, it may be possible to detect weak reactions of the cellular enzymes with the synthetic template primers, as has been indicated previously (8).

The 4 cell samples containing enzyme that satisfied the most criteria for a reverse transcriptase (see Table 1) were leukemic cells, *i.e.*, AML-1, CML-1b, CML-3, and AMML, with HeLa cells being not too dissimilar. Only the AMML cells contained a poly(C)·oligo(dG)-stimulated enzyme that was resistant to actinomycin D. The AML and CML samples contained a high ratio of poly(A)·oligo(dT) to DNA-stimulated activity but exhibited no reaction with poly(C)·oligo(dG) that was resistant to actinomycin D. However, the properties of SiSV DNA polymerase

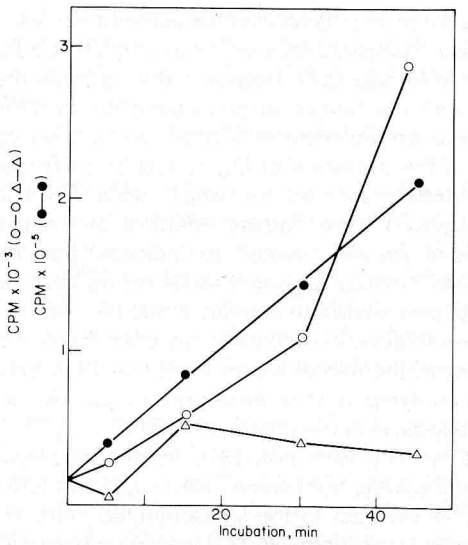

Chart 3. Kinetics of response of DNA polymerase from CML cells with no added template primer (△) or in the presence of DNA (●), and poly(C)·oligo(dG) (○). Soluble cytoplasmic DNA polymerase from CML cells was separated from nucleic acids by passing through DEAE-cellulose equilibrated with 0.3 M KCl in Buffer B (Buffer A plus 10% glycerol; see "Materials and Methods"). The passthrough solution containing enzyme was dialyzed against 0.05 M KCl in Buffer B and adsorbed to a DEAE-cellulose column equilibrated with the same buffer. Enzyme was eluted with a linear gradient of 0.05 to 0.5 M KCl in Buffer B, and the peak fraction was further chromatographed twice on DNA-agarose (42) with a linear gradient of 0.03 to 0.8 M KCl in Buffer B. The peak fraction eluting at 0.2 M KCl was added to assay mixtures containing [^{32}P]dGTP (100,000 cpm/pmol) and the indicated templates as described in "Materials and Methods."

(23) indicate that all RNA tumor virus enzymes might not be stimulated by this template primer.

DISCUSSION

Our objective was to test for the presence of reverse transcriptase in human leukemia by the most sensitive and specific enzymatic procedures available and to compare with the results in nonmalignant cells. This required an appraisal of the sensitivity and specificity of currently available assay procedures for viral reverse transcriptase in cell homogenates.

A simple cell fractionation procedure was used to enrich for viral enzyme. Our results with the animal model cells, indicating that viral enzyme remains associated with a particulate membrane cell fraction, are in agreement with previous reports from other laboratories with similar animal model systems (13, 21). The reports of detection of reverse transcriptase in human leukemia cells have also emphasized the particulate or membrane-bound nature of the enzyme (5, 14). Therefore, the detection procedure used here focused on the membrane fraction (1.15 to 1.20 g/ml) obtained by isopyknic sucrose gradient centrifugation.

The choice of an assay system specific for virus reverse transcriptase was less straightforward. Each of the synthetic template primer/metal ion combinations previously thought to be specific for virus reverse transcriptase activity has subsequently been reported to be utilized to some extent by cellular enzymes that are not virus reverse transcriptases. Ratios of enzyme activity with one template primer relative to another, *e.g.*, poly(A)·oligo(dT) to poly(dA)·oligo(dT) in the presence of Mg^{2+} (16), or the most recently reported polydeoxythymidylate·oligoriboadenylate to polyriboadenylate·oligodeoxythymidylate in the presence of Mn^{2+}, have also been proposed (44); but the former has subsequently been shown to be the same for cellular enzymes as for viral enzymes (27, 44), and the latter will not distinguish small amounts of viral enzyme in the presence of equal or greater amounts of cellular enzymes. Poly(C)·oligo(dG) has been shown to be a template primer for cellular enzymes also (27, 46); in addition to this problem of lack of specificity, it is not a consistently effective template primer with all virus enzymes, particularly SiSV (23). The methylated derivative, poly(mC)·oligo(dG), has been used as a specific template primer for virus reverse transcriptase (18–20), but it is much less active with virus enzymes than is poly(C)·oligo(dG) (23). Moreover, it has recently been reported to stimulate some cellular enzymes at a level of 0.2% of their activity with DNA (44).

Heteropolymer RNA remains the most specific template for virus reverse transcriptase, but enzyme activity is much less with natural RNA than with the synthetic template primers, *e.g.*, by 2 orders of magnitude for a murine viral enzyme (23). In addition, it must be shown in each case that the natural RNA is, in fact, serving as template for synthesis of the product, which has been difficult to demonstrate clearly (6, 13, 16, 37, 41, 42, 45). Because of the limited amounts of clinical material ordinarily available for assay, principal reliance has been placed upon reactions with synthetic template primers for detection and identification of virus-related reverse transcriptase in human cells (1, 3, 5, 12–21, 25, 26, 28, 29, 32, 36, 37, 48). Our results indicate that this approach suffers seriously from lack of specificity.

We were able to detect virus enzyme in the membrane fraction of the cell-virus mixtures at a level as low as 1 virus particle/cell in the reconstruction experiments. This is 2 orders of magnitude more sensitive than a previous report of a reconstituted system (2). The parallel sucrose gradient from the cell fraction containing no added virus also showed a peak of enzyme activity with poly(C)·oligo(dG). Although this cellular enzyme activity was inhibited by actinomycin D, whereas the virus enzymes were not, the presence of this cellular enzyme made it difficult to interpret low levels of virus-like enzyme activity.

Enzyme activities from malignant cells with any of the properties of viral transcriptase were compared with similar fractions from nonmalignant cells. Distinct qualitative differences could not be demonstrated, however, because none of the leukemic cells examined in this study contained enzyme activities that were not also associated with control cells; nor were there any leukemic cell enzymes that satisfied all of the criteria for a reverse transcriptase. In general, the differences between malignant and control cells were quantitative, *i.e.*, the leukemic cells contained more poly(A)·oligo(dT)-stimulated activity than did the control cells, and the leukemic enzymes that responded to poly(C)·oligo(dG) exhibited higher levels of this activity than did the control cells. The fact that the malignant cells also contained a higher concentration of DNA-dependent DNA polymerase than did any of the control cells used created an additional problem in interpretation of the quantitative differences observed, due to the increased risk of detecting minor activities of cellular enzymes.

Although it is possible that one or more of the enzymes detected in this sample of leukemic cells may be virus-related reverse transcriptase, the assay techniques were incapable of

clearly distinguishing between enzyme activities found in normal and malignant cells. Enzymes with the properties of viral reverse transcriptase have been found in normal monkey and human placentas (35, 38). With the use of physical and enzymatic properties, as here, it is also possible to confuse the DNA polymerase activities of viral particles with those of mitochondria (7, 33, 42). It is apparent that none of the human leukemic cells examined contains virus reverse transcriptase approaching the level of the animal model systems. Even if all the poly(A)·oligo(dT)-stimulated activity in the leukemic cells is assumed to be virus enzyme, which seems unlikely, the human leukemic cells contain the equivalent of less than 10 particles/cell.

In a much larger series of leukemic patients than the one we report here, Gallo *et al.* (13) have reported that, by their criteria, approximately 10% of the human leukemic cells examined were positive for reverse transcriptase activity, and most of these were of myelogenous origin. The fact that 90% of human leukemias may be negative for virus reverse transcriptase indicates several possibilities, including: (*a*) most human leukemias are transformed, nonproducer cells; (*b*) most human leukemias are not associated with tumor virus infection; or (*c*) occasional tumor virus infection occurs; however it is not etiological, but secondary or even unrelated to the leukemia. At the least, the difficulty in detecting RNA tumor viruses in humans, the absence of epidemiological clustering typical of infectious agents, and the clonal nature of the human leukemias and lymphomas (10) indicate some differences between animal models and the human diseases.

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