Human placentas contain a specific inhibitor of RNA-directed DNA polymerase

(reverse transcriptase/development/retrovirus/RNA-dependent DNA nucleotidyltransferase)

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ABSTRACT Human placental extracts contain a specific inhibitor of mammalian retroviral RNA-directed DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) activity. This inhibitor copurifies with retrovirus-like particles in human placental extracts. The inhibitor can be removed from these particles by salt extraction, which leads to the recovery of the polymerase activity. Thus, the inhibitor does not irreversibly inactivate the particle-associated RNA-directed DNA polymerase activity. The inhibitory preparation contained no nuclease, protease, or phosphatase activity. Because its inhibitory action can be eliminated by the addition of more virus to the reaction, nonspecific inactivation of enzyme substrate has been ruled out. A partial characterization of the inhibitor indicates that it is (i) insensitive to ether, trypsin, and phospholipase C; (ii) stable to heat and pH 2-12; and (iii) nondialyzable.

Major efforts have been made to isolate retroviruses from normal and malignant human tissue (for review, see refs. 1 and 2). In normal tissues, retroviruses have been detected most frequently in the placenta (for review, see ref. 3), and the best evidence for their presence comes from the electron microscopic examination of placental tissue. These studies demonstrate particles resembling type C or D retroviruses budding from the syncytiotrophoblast layer (4-8). These observations are highly suggestive of the existence of a human retrovirus, but conclusive evidence awaits its isolation and growth in culture. We and others have been investigating the expression of retroviral proteins in human placentas and have detected virus-associated RNA-directed DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) in more than 80% of tissues examined (9). Other investigators have not found this enzyme (1, 10, 11). This discrepancy and our inability to find the enzyme in all placentas (9) led us to look for an inhibitor of the polymerase in placental extracts.

We report here the isolation from human placentas of an inhibitor specific for mammalian retroviral RNA-directed DNA polymerase. This inhibitor copurifies with a polymerase-like activity in human placentas and can be removed from the particle-associated enzyme by salt extraction. The polymerase activity is subsequently detected in the placental extract. Thus, this inhibitor does not irreversibly inactivate the particle-associated RNA-directed DNA polymerase activity in human placenta.

MATERIALS AND METHODS

Reagents and Materials. Trypsin recrystallized from bovine pancreas and trypsin inhibitor prepared from soybean were purchased from Sigma. Phospholipase C was obtained from Worthington. [8-³H]dGTP (tetrasodium salt) was purchased

from ICN. The template-primers (C)_n·(dG)₁₂₋₁₈ and (dC)_n·(dG)₁₂₋₁₈ were obtained from P-L Biochemicals. Trasolyl was purchased from FBA (New York). Azocoll was obtained from Calbiochem. Spectrapor 2 dialysis membrane was purchased from VWR Scientific (Portland, OR). Immunodiffusion plates containing antisera to the human gamma globulins IgG and IgM were purchased from Hyland Laboratories (Costa Mesa, CA), a division of Traverol Laboratories. Ethylene glycol bis(β-amino-ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was purchased from Sigma.

Viruses and Enzymes. Calf thymus DNA polymerases α and γ from fetal calf liver were purchased from Worthington. Mouse mammary tumor virus, Rauscher murine leukemia virus (Rauscher MuLV), simian sarcoma virus (SSV), Mason-Pfizer virus, baboon endogenous virus (BaEV), Prague B Rous sarcoma virus, and the RD114 cat virus were obtained through the assistance of Jack Gruber (Biological Carcinogenesis Branch, National Cancer Institute), the Frederick Cancer Research Center, and the John L. Smith Memorial for Cancer Research.

Virus and Polymerase Assays. DNA synthesizing activity in retroviruses, purified DNA polymerases, and samples from placental preparations were assayed in 100-μl reactions as described with (C)_n·(dG)₁₂₋₁₈ (9).

Extraction of Placental RNA-Directed DNA Polymerase and Inhibitor. Full-term human placentas were obtained from Good Samaritan Hospital (Corvallis, OR) and Albany General Hospital (Albany, OR). The placentas were processed within 1 hr after birth. All procedures were done at 4°C. Approximately 100 g of tissue from the trophoblast layer was processed as described (9). The postmitochondrial placental extract was layered onto a gradient consisting of 2 ml of 15% sucrose on a 7-ml linear gradient of 20-65% sucrose (wt/vol) in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.4/0.001 M EGTA. After centrifugation in the Beckman SW41 rotor at 150,000 × g at 4°C for 12 hr, 4 ml was removed, and then the gradient was fractionated into 25 equal aliquots. These fractions were assayed for RNA-directed DNA polymerase activity with the template-primer (C)_n·(dG)₁₂₋₁₈ as described (9).

Protease and Nuclease Assays. The general proteolytic substrate Azocoll was utilized to determine protease activity in the inhibitor fraction. The assay consisted of incubating the sample in 0.1 M potassium phosphate, (pH 7.0), with 50 mg of Azocoll at 37°C for 15 min. The sample was filtered and the A₅₂₀ was determined for the filtrate on a Perkin-Elmer spectrophotometer. Trypsin was used as a positive control.

Assays for the presence of RNase and DNase were performed

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; MuLV, murine leukemia virus; BaEV, baboon endogenous virus; SSV, simian sarcoma virus.

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with [^3H]-RNA (5×10^4 cpm/ μg) isolated from baby hamster kidney/21 cells and with [^3H]-DNA (6×10^3 cpm/ μg) extracted from *Bdellovibrio bacteriovorus*. After incubation at 37°C with various dilutions of the fractions containing the inhibitor in the buffer used to assay inhibitor activity against RauscherMuLV [40 mM Tris-HCl, pH 7.8 at 22°C /0.12 mM EGTA/3.9 mM dithiothreitol/37 mM glutathione (reduced form)/5% glycerol/6 mM NaCl], the acid-precipitable radioactivity was determined at the indicated intervals. DNase I and RNase A ($1 \mu\text{g}/100 \mu\text{l}$; Worthington) were utilized as positive controls.

Treatment with Trypsin and Phospholipase C. A 100- μl vol of the inhibitor fraction that contained 0.4 mg of protein per ml was incubated with 10 μl of trypsin (10 mg/ml) at 37°C for 15 min. The trypsin was inactivated by the addition of 10 μl of a soybean trypsin inhibitor (2 mg/ml). Phospholipase C treatment was conducted in the presence of 6 mM CaCl_2 at 22°C . Controls for these experiments utilized 0.15 M NaCl/0.01 M Tris-HCl, pH 7.4/0.001 M EGTA instead of the inhibitor preparation. After each treatment, the inhibitor fraction or control saline was tested for activity against RauscherMuLV as described.

Treatment with Acid and Base. The pH of the inhibitor fraction was adjusted to 2 or 12 by the addition of 1 M HCl or 1 M NaOH, respectively. Samples were incubated for 1 hr at 4°C and neutralized to pH 7. Samples with NaCl/Tris/EGTA were treated as controls.

Extraction with Ether, Ethanol/Ether, and Chloroform/Methanol. A 200- μl vol of the placental inhibitor fraction was extracted with an equal vol of peroxide-free diethyl ether buffered in NaCl/Tris/EGTA for 10 min at 0°C with shaking. After removal of the ether phase, both phases were evaporated and resuspended in 200 μl of NaCl/Tris/EGTA. The inhibitor preparation also was extracted with 20 vol of ethanol/ether, 3:1 (vol/vol) or chloroform/methanol, 2:1 (vol/vol) at 4°C for 1 hr as described (12). All precipitates were resuspended in NaCl/Tris/EGTA.

Immunodiffusion Tests. Radial immunodiffusion tests were performed with prepared immunodiffusion plates containing antisera to either human IgG or IgM. Wells were loaded with varying amounts of placental inhibitor fraction and incubated at 4°C . Plates were checked for lines of precipitation every day for 7 days. These plates detect up to 0.1 μg of immunoglobulin.

Determination of Protein Concentration. The protein content of extracts containing inhibitor was determined by a Coomassie blue G-250 protein assay (Bio-Rad). Although the inhibitor has not been clearly identified as a protein nor is it pure in these extracts, this measurement does permit some quantitation of the inhibitor present in each extract.

RESULTS

Extraction of Inhibitor from Placental Extracts. We have reported the presence of RNA-directed DNA polymerase activity in the microsomal fraction of extracts of the trophoblast layer of human placentas (9). This activity was found at a density of 1.15 g/ml after equilibrium centrifugation in sucrose. Additional peaks of enzymatic activity were observed in some placentas at a density of 1.12 g/ml and at the density of viral cores, 1.24 g/ml (9). In some placental extracts after equilibrium centrifugation, the RNA-directed DNA polymerase activity was very low or absent throughout the gradient. We suspected that this apparent absence was caused by an enzyme inhibitor present in the placental extracts.

A full-term placenta was obtained after normal delivery from a woman who had four previous spontaneous abortions and was extracted for RNA-directed DNA polymerase activity. Initial screening of the placental extracts after equilibrium centrifugation revealed no apparent activity at densities of 1.15 or 1.12

g/ml (Fig. 1A). Gradients were pooled and sequentially extracted with 0.5 M, 1.0 M, and 1.5 M NaCl in 0.01 M Tris-HCl, pH 7.4. After centrifugation, the pelleted materials from the 0.5 M and 1.5 M NaCl extraction were banded to equilibrium on sucrose. The supernatant fluids from each of the extraction concentrations were dialyzed against either 0.15 M NaCl/0.01 M Tris-HCl, pH 7.4/0.001 M EGTA or 0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4 and stored at -30°C . After extraction with 0.5 M NaCl, no change in the polymerase activity profile was observed in the material after centrifugation to equilibrium in sucrose (Fig. 1B). The pellet remaining after the 1.5 M NaCl extraction did produce polymerase activity at densities of 1.15 and 1.19 g/ml (Fig. 1C). Thus, the 1.5 M NaCl extraction removed the substance that inhibited the placental RNA-directed DNA polymerase activity.

Analysis of five other full-term placentas, which initially contained no polymerase activity, revealed the presence of a similar inhibitory substance. After extraction with 1.0–1.5 M NaCl, extracts of these placentas exhibited normal RNA-directed DNA polymerase activity profiles on recentrifugation to equilibrium in sucrose. The amount of inhibitory activity found in these placentas was less than that found in the extract of the placenta from a woman with a history of spontaneous abortions. Therefore, we selected the inhibitor fraction from this latter placenta for further studies.

Specificity of the Inhibitory Factor. The dialyzed supernatants from salt extractions of the placental extract were tested for inhibitory activity against Rauscher MuLV RNA-directed DNA polymerase activity. The 1.5 M NaCl extraction released an inhibitor into the supernatant that decreased the Rauscher-MuLV reaction by 88%. The 0.5 M and 1.0 M NaCl extracts did not remove the inhibitory factor. Instead, the supernatant fluids from these extractions contained factors that stimulated rather than inhibited the polymerase reaction. These observations indicated that the inhibitory factor was bound tightly to the placental extract and high-salt concentrations were necessary for its removal. A stimulatory factor for the polymerase reaction appeared to be released at the lower salt concentrations.

FIG. 1. The RNA-directed DNA polymerase activity (●—●) before and after salt extraction. The placenta from a woman with a history of four previous spontaneous abortions was extracted and subjected to sucrose gradient centrifugation. After equilibration, 25 equal fractions were collected and 20- μl samples from each fraction were assayed for RNA-directed DNA polymerase activity (A). These gradients were pooled and sequentially extracted with 0.5 M NaCl and 1.5 M NaCl in 0.01 M Tris-HCl, pH 7.4. The NaCl-extracted material was concentrated by centrifugation. The pellets from 0.5 M and 1.5 M NaCl were resuspended in NaCl/Tris/EGTA, homogenized through an 18-gauge needle, and resedimented to equilibrium in a 20–65% sucrose gradient in a Beckman SW50.1 rotor. Each gradient was fractionated and 20- μl samples were assayed for polymerase activity after 0.5 M NaCl extraction (B) and 1.5 M NaCl extraction (C). The densities were determined by refractometry (○—○).

FIG. 2. Effect of increasing concentrations of inhibitor. Increasing amounts of inhibitor preparation were added to constant amounts of placental particle-associated enzyme, BaEV, SSV, and MuLV. Each 100- μ l reaction contained 135 μ g, 4.5 μ g, 14.8 μ g, or 12.4 μ g of protein from a placental enzyme preparation (A), BaEV (B), SSV (C), and MuLV (D), respectively. The mixture contained the indicated amounts of protein in the inhibitor preparation and standard reaction components. The reactions were preincubated with inhibitor on ice for 30 min before the addition of [3 H]dGTP and (C) $_n$ (dG) $_{12-18}$. After incubation for 30 min at 37°C, the reactions were terminated by the addition of an equal volume of 10% (vol/vol) trichloroacetic acid and 0.01 M sodium pyrophosphate. The specific activity of the [3 H]dGTP was 5280 cpm/pmol.

The effect of increasing concentrations of inhibitor on the RNA-directed DNA polymerase reactions of the placental particle-associated enzyme, Rauscher-MuLV, BaEV, and SSV was examined (Fig. 2). All enzymes tested were sensitive to the inhibitory factor. After the addition of high concentrations of inhibitor, a resistant level of DNA-synthesizing activity was noted for placental enzyme (14.4%), SSV (13.6%), and MuLV (20.3%). However, a similarly saturating concentration of inhibitor was not attained for BaEV, even with 10 μ g of inhibitor. The BaEV concentration used was 4.5 μ g of viral protein per reaction mixture. This amount of protein is approximately one-third less than the protein present in the other viral enzyme reactions.

The specificity of the placental DNA polymerase inhibitor for several mammalian retroviruses, an avian retrovirus, and normal cellular DNA polymerases was examined also. Saturating amounts of the inhibitor were mixed with each virus or enzyme and incubated at 0°C for 30 min. The RNA-directed DNA polymerase reaction was begun by the addition of [3 H]dGTP, and samples were taken at 0, 10, 20, and 30 min after the start of the reaction. The mammalian retroviruses RD114 Cat virus (type C virus), Mason-Pfizer virus (type D virus) and mouse mammary tumor virus (type B virus) were all sensitive to the inhibitor (Fig. 3 A, B, and D). However, the RNA-directed DNA polymerase activity of Prague B Rous sarcoma virus, α DNA polymerase, and γ DNA polymerase (Fig. 3 C, E, and F) were all resistant to the inhibitory effect. These observations

suggest that the placental inhibitor specifically blocks mammalian retrovirus RNA-directed DNA polymerase activity. Again, a resistant level of enzyme activity was observed in the polymerase reactions after treatment with the inhibitor. Because the Rous sarcoma virus and the α and γ DNA polymerases are not affected by the inhibitor, these studies also suggest that RNases, DNases, phosphatases, or proteases are not present in the inhibitory extract.

Characterization of the RNA-Directed DNA Polymerase. The nature of the inhibitory factor was examined by assays for nucleases and proteases. A mixture containing 8.0 μ g of inhibitor preparation and either 10 μ g of [3 H]RNA or 11.4 μ g of [3 H]DNA was incubated for 30 min at 37°C. Aliquots were taken at 10-min intervals and measured for acid-precipitable radioactivity. No decrease in radioactivity was observed with either the labeled DNA or RNA. These observations indicated that nucleases were not present in sufficient quantity in the inhibitor preparation to account for RNA-directed DNA polymerase inhibition.

FIG. 3. Effect of inhibitor on rate of DNA synthesis by avian and mammalian RNA-directed DNA polymerase and normal cellular DNA polymerases. Standard RNA-directed DNA polymerase reaction conditions were used. Reaction mixtures of 400 μ l contained 8.0 μ g of inhibitor preparation and 22.8 μ g of RD114 cat virus (A), 37.6 μ g of mouse mammary tumor virus (B), 24 μ g of Mason-Pfizer virus (D), or 40.6 μ g of Prague B Rous sarcoma virus (C). For DNA polymerases α (E) and γ (F), a 400- μ l reaction mixture contained 8.0 μ g of inhibitor preparation, 0.096 units of DNA polymerase α or 2 units of DNA polymerase γ and 1 μ g of (dC) $_n$ (dG) $_{12-18}$. The inhibitor fraction used for the α polymerase reaction was dialyzed against NaCl/Tris buffer because polymerase is sensitive to high concentrations of salt. The inhibitor preparation in NaCl/Tris buffer was still fully active against MuLV and placental RNA-directed DNA polymerase activity (data not shown). Reaction mixes were preincubated for 30 min on ice before the addition of [3 H]dGTP and template. An 80- μ l aliquot was taken at indicated times, and the amount of radioactivity incorporated into DNA was determined. The specific activity of the [3 H]dGTP was 5280 cpm/pmol. Reactions contained NaCl/Tris/EGTA or NaCl/Tris (●—●) or inhibitor in NaCl/Tris/EGTA or NaCl/Tris (in a DNA polymerase reaction) (○—○).

FIG. 4. The effect of increasing virus concentration on the inhibition of MuLV. Each 100- μl reaction mixture contained 16 μg of inhibitor preparation and the indicated amounts of MuLV. Standard RNA-directed DNA polymerase reaction conditions were employed. Reaction mixes were preincubated with inhibitor for 30 min on ice before the addition of [^3H]dGTP and (C)_n-(dG)₁₂₋₁₈. Reactions were terminated after 30 min at 37°C.

The possible presence of proteases in the extract was assessed with Azocoll at 37°C and 45°C. After 15 min with 2.5, 5.0, and 12.5 μg of the inhibitor preparation, no protease activity was detected for any inhibitor concentration. In control reactions, less than 1 μg of trypsin was detected by this method.

To determine if the inhibitor affected a substrate in the reaction mixture, we added a saturating concentration of inhibitor fraction to a predetermined amount of MuLV. Then increasing concentrations of virus were added to this reaction. If the inhibition were due to the loss of a necessary substrate in the reaction mixture, an addition of virus should not result in recovery of the polymerase activity, but Fig. 4 shows that an increase in virus led to recovery of the activity. These results suggest that the inhibitor affects the viral polymerase directly.

A summary of the characteristics of the placental RNA-directed DNA polymerase inhibitor is given in Tables 1 and 2. The preparation containing the inhibitor was exposed to varying

Table 1. Characterization of inhibitor by heat, pH, and enzymatic digestion with MuLV

Treatment	Virus inhibitor			MuLV inhibition, [†] %
	None*	Treated	Untreated	
Heat				
10', 37°C	39.94	6.32	6.06	84
10', 85°C	39.94	3.64	6.06	93
10', 100°C	48.11	45.48	23.37	5
pH				
2, 1 hr	40.20	13.31	6.06	67
12, 1 hr	55.15	11.43	6.06	79
Enzyme				
Trypsin	41.95	8.86	6.06	79
Phospholipase	24.92	9.47	4.99	62

Assays for inhibitor activity were conducted with 12.4 μg of MuLV and 20 μg of treated and nontreated inhibitor by using standard RNA-directed DNA polymerase assay conditions. The actual data is presented as pmol of [^3H]dGTP incorporated in 30 min at 37°C. The specific activity was 5280 cpm/pmol. ND, Not determined.

* As an enzyme control, a volume of NaCl/Tris/EGTA was treated similarly.

[†] Percent inhibition of the MuLV RNA-directed DNA polymerase activity with the treated inhibitor.

Table 2. Characterization of inhibitor by solvent extraction

Treatment	Virus inhibitor			MuLV activity, [†] %
	Treated	Untreated	None*	
Ether extraction				
Aqueous phase	57.85	34.17	117.49	49
Ether phase	123.49	34.17	117.49	105
Ethanol/ether				
Organic phase	33.27	6.06	42.55	78
Precipitate	2.01	6.06	51.35	4
Chloroform/methanol				
Organic phase	32.45	6.06	ND	81
Precipitate	46.79	6.06	ND	117

Assays for inhibitor activity were conducted with 12.4 μg of MuLV and 50 μl of the corresponding resuspended phases and precipitates. Standard RNA-directed DNA polymerase assay conditions were used. Each number represents pmol of [^3H]dGTP incorporated in 30 min at 37°C. The specific activity of the labeled nucleotide triphosphates was 5280 cpm/pmol.

* As a control, a volume of NaCl/Tris/EGTA equal to the inhibitor volume was treated similarly and assayed for RNA-directed DNA polymerase inhibitory activity as described.

[†] Percent of MuLV RNA-directed DNA polymerase activity remaining after exposure to the treated inhibitor versus the treated NaCl/Tris/EGTA control.

temperatures, pH conditions, enzymatic digestions, and solvent extraction. After each particular treatment, the activity of the inhibitor was tested against MuLV RNA-directed DNA polymerase. Control samples containing NaCl/Tris/EGTA alone were treated in the same manner.

The RNA-directed DNA polymerase inhibitor fraction has been stable at -30°C for more than a year. When this fraction was heated for 10 min at 37°C, 56°C, or 85°C, no change in inhibitory activity was found. However, the inhibitory factor was unstable to heating at 100°C for 10 min. It was resistant to conditions at pH 2 and pH 12 for 1 hr at 4°C and to treatments with trypsin and phospholipase C. After ether extraction the inhibitor remained in the aqueous phase albeit somewhat decreased in titer. When the factor was treated with ethanol/ether, residual inhibitory activity was found in the precipitate and not in the organic phase. Treatment with chloroform/methanol inactivated the inhibitor.

Inhibitor activity was not lost after dialysis of the fraction with membranes having a molecular weight limit of 12,000-14,000. After sedimentation in a linear glycerol gradient, the inhibitor activity was found in a diffuse band in the middle third of the glycerol gradient, which contained 0.5 M KCl to prevent protein aggregation. Finally, no human IgG or IgM was found in the inhibitor preparation by radial immunodiffusion tests that had a limit of detection of 0.1 μg of gamma globulin protein.

DISCUSSION

The results presented in this paper indicate human placentas contain a factor which specifically interferes with the RNA-directed DNA polymerase activity of mammalian retroviruses. This inhibitor copurified with the RNA-directed DNA polymerase-synthesizing complex that has been isolated from human trophoblast extracts (9). Its recovery from the microsomal fraction requires a high concentration of salt. After removal of the inhibitor by salt extraction, the DNA-synthesizing complex remains active and unchanged; the particle-associated activity continues to band at a density of 1.15 g/ml after equilibrium centrifugation in sucrose. In addition, a RNA-directed DNA polymerase-like activity banding at a density of 1.19 g/ml in sucrose is also detected; it may represent partially formed viral cores or aggregates of them. This inhibitor of RNA-directed

DNA polymerase may have been responsible for the absence of detectable soluble polymerase activity in human placentas reported by others (1, 10, 11). It is most likely the cause of our inability to find the enzyme in all human placental extracts (9).

The placental inhibitor appears to be selective for mammalian RNA-directed DNA polymerases because it does not inhibit the avian retrovirus polymerases or the normal cellular DNA polymerases α and γ . Although strong inhibition of the enzyme reaction was observed for the mammalian retroviral enzymes, a resistant level of DNA synthesis was found for RD114 cat virus, Mason-Pfizer virus, mouse mammary tumor virus, SSV, and Rauscher MuLV. Despite reduced virus protein concentration in each reaction mixture, saturating concentrations of inhibitor were never reached for BaEV. This result does suggest that the inhibitor can distinguish between mammalian viral enzymes. Experiments are in progress to determine if higher concentrations of the inhibitor would result in inhibition of BaEV. However, the efficient inactivation of the RNA-directed DNA polymerase activity of RD114 cat virus (Fig. 3), whose enzyme closely resembles that of BaEV, suggests that other factors are involved. Further study with purified viral polymerases and inhibitor is required to define the specificity of this reaction.

Nonspecific inhibition by nucleases, proteases, or phosphatases present in the inhibitor preparation was ruled out by observations that avian retrovirus polymerase and cellular α and γ polymerases do catalyze DNA synthesis in the presence of the inhibitor. The absence of nucleases and proteases in the inhibitor extract was substantiated further by specific tests for these enzymes. More importantly, the specificity of this inhibitor for the mammalian and placental RNA-directed DNA polymerases was indicated by the elimination of the enzyme inhibition with increased amounts of virus. Thus, inhibition is because of a direct effect on a virus component.

At this time, the exact chemical nature of this RNA-directed DNA polymerase inhibitor in human placental extracts remains uncharacterized. Its resistance to alkaline pH and sensitivity to extraction by chloroform/methanol implies it is not a DNA or RNA molecule. Its specificity suggests that the active factor may be an immunoglobulin or neutralizing lipoprotein. Both substances have been shown to specifically inactivate retroviruses (12–18). However, the sedimentation behavior of the inhibitor in glycerol gradients then suggest that it is not a lipoprotein and immunodiffusion tests for detection of IgG or IgM were negative.

Inhibitors of DNA polymerases have been found in many other tissue preparations. A DNA molecule from the sera of tumor-bearing mice was found to inhibit ribopolymer-transcribing cellular DNA polymerases in a murine myeloma cell line (19, 20). This inhibitor had no effect on the RNA-directed DNA polymerase activity of MuLV or avian myeloblastosis virus. Moreover, a small protein from tissue extracts of bovine mammary glands was found to decrease DNA synthesis in HeLa and Ehrlich cells (21). In human milk, nonspecific inhibitors of the polymerase reaction have been found to copurify with the enzyme. Some of these inhibitors have been identified tentatively as ribonucleases (22) or phosphatases (23) which can be removed by sedimentation of the milk extract through Metrizamide. Others remain to be characterized (13, 14, 24).

The presence of a specific mammalian inhibitor of RNA-directed DNA polymerase in human placental extracts suggests that the enzyme has a role in placental development. Further

studies must be carried out to determine whether levels of the factor fluctuate during gestation. If abnormal amounts of this polymerase inhibitor are manifest at critical times during placental development, it is possible that abnormal fetal development and subsequent spontaneous abortion may result. Such a phenomenon may explain our finding of high levels of this inhibitor in extracts from a placenta of a woman with a history of several spontaneous abortions. This proposed regulation of RNA-directed DNA polymerase activity by fluctuations in inhibitor concentration resembles that suggested for the regulation of ribonuclease by a specific inhibitor present in animal tissues including human placentas (25, 26).

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- Gardner, M. B., Rasheed, S., Shimizu, S., Rongey, R. W., Henderson, B. E., McAllister, R. M., Klement, V., Charman, H. P., Gilden, R. V., Heberling, R. L. & Huebner, R. J. (1977) in *Origins of Human Cancer*, eds. Hiatt, H., Watson, J. D. & Winsten, J. A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, Press, NY), Vol. B., pp. 1235–1251.
- Hehlmann, R. (1976) *Curr. Top. Microbiol. Immunol.* **73**, 141–214.
- Panem, S. (1979) *Curr. Top. Pathol.* **66**, 175–189.
- Kalter, S. S., Helmke, R. J., Heberling, R. L., Panigel, M., Fowler, A. K., Strickland, J. E. & Hellman, A. (1973) *J. Natl. Cancer Inst.* **50**, 1081–1084.
- Vernon, M. L., McMahon, J. M. & Hackett, J. J. (1974) *J. Natl. Cancer Inst.* **52**, 987–989.
- Dalton, A. J., Hellman, A., Kalter, S. S. & Helmke, R. J. (1974) *J. Natl. Cancer Inst.* **52**, 1379–1381.
- Imamura, M., Philips, P. E. & Mellors, R. C. (1976) *Am. J. Pathol.* **83**, 383–390.
- Dirksen, E. R. & Levy, J. A. (1977) *J. Natl. Cancer Inst.* **59**, 1187–1192.
- Nelson, J. A., Leong, J. C. & Levy, J. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6263–6267.
- Kiessling, A. A. & Goulian, M. (1979) *Cancer Res.* **39**, 2062–2069.
- Krauss, S. W. & Linn, S. (1980) *Biochemistry* **19**, 220–228.
- Leong, J. C., Kane, J. P., Oleszko, O. & Levy, J. A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 276–280.
- Sarkar, N. H., Charney, J., Dion, A. S. & Moore, D. H. (1973) *Cancer Res.* **33**, 626–629.
- Kantor, J. A., Lee, Y., Chirikjian, J. G. & Feller, W. F. (1979) *Science* **204**, 511–513.
- Faulk, W. P., Jeannet, M., Creighton, W. D., Carbonara, A. & Hay, F. (1974) *J. Reprod. Fert. Suppl.* **21**, 43–57.
- Kouttab, N. M., Fowler, A. K., Strickland, J. E. & Hellman, A. (1976) *J. Immunol.* **117**, 1644–1650.
- Jurjais, A., Wheeler, D. A., Gallo, R. C. & Witz, I. P. (1979) *Arthritis and Rheum.* **22**, 1308–1313.
- Hirsch, M. S., Kelly, A. P., Chapin, D. S., Fuller, T. C., Black, P. H. & Kurth, R. (1978) *Science* **199**, 1337–1340.
- Gottlieb, A. A., Smith, A. H., Plescia, O. J., Persico, F. J. & Nicholson, D. E. (1973) *Nature (London)* **246**, 480–482.
- Gottlieb, A. A., Chang, J., Buhrer, D. P. & Brennessel, B. A. (1980) *Cancer Res.* **40**, 758–770.
- Gonzalez, R. & Verly, W. G. (1978) *Eur. J. Cancer* **14**, 689–697.
- McCormick, J. J., Larson, L. J. & Rich, M. A. (1974) *Nature (London)* **251**, 737–740.
- Sanner, T. (1976) *Cancer Res.* **36**, 405–408.
- Fieldsteel, A. H. (1975) *Cancer Res.* **34**, 712–715.
- Roth, J. S. (1967) *Methods Cancer Res.* **3**, 151–243.
- Blackburn, P. & Jaikhani, B. L. (1979) *J. Biol. Chem.* **254**, 12488–12493.