Serological Analysis of Cellular and Viral DNA Polymerases by an Antiserum to DNA Polymerase γ of Human Lymphoblasts†

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ABSTRACT: An antiserum has been prepared against a highly purified DNA polymerase γ from NC37 cells, a normal human lymphoblast cell line. The antiserum does not possess enzyme neutralizing activity, but does bind specifically to DNA polymerase γ. When tested in a double antibody immunoprecipitation assay, the antibody does not cross-react with DNA polymerases α or β, purified from NC37 cells, or with reverse transcriptases of avian, murine, or primate RNA tumor viruses. Antisera prepared against purified reverse transcriptases similarly do not recognize DNA polymerase γ, either in an enzyme neutralization assay or in the more sensitive double antibody immunoprecipitation assay. The availability of an antiserum to DNA polymerase γ will allow the further characterization of enzyme activities isolated from cellular material and suspected of being related to viral reverse transcriptases. In those cases where such activities do not immunologically resemble known viral DNA polymerases, the anti-DNA polymerase γ will help determine the viral or cellular nature of the unknown activity.

The history of DNA polymerase γ (Weissbach et al., 1975) has been interwoven from the beginning with the history of RNA tumor virus reverse transcriptase. The initial report of DNA polymerase γ (Fridlender et al., 1972) as an RNA-directed DNA polymerase activity of normal cells was striking because it seemed to negate the importance of a viral-like reverse transcriptase activity which had been found in human leukemic cells (Gallo et al., 1970). In time, careful studies showed that DNA polymerase γ was a distinct cellular enzyme which could be distinguished both biochemically and immunologically from reverse transcriptase (Weissbach, 1975; Lewis et al., 1974a, b; Spadari and Weissbach, 1974; Gerard, 1975; Matsukage et al., 1975). Similarly, the human leukemic enzyme has been well investigated and its biochemical (Sarngadharan et al., 1972; Bhattacharyya et al., 1973; Witkin et al., 1975; Chandra et al., 1975) and immunological (Todaro ard Gallo, 1973; Gallagher et al., 1974; Gallo et al., 1974; Steel, L. K., Laube, H., and Chandra, P., submitted for publication) similarity to reverse transcriptase has been substantiated. In spite of these and other investigations, the identification of DNA polymerase γ and reverse transcriptase in cellular extracts continues to be difficult. The nature of DNA polymerase γ, its relationship to other cellular DNA polymerases and to reverse transcriptase, and its function in normal cells remain unknown. With regard to human leukemic cells, reverse transcriptase cannot be routinely isolated, and it is possible that when reverse transcriptase is detected with high frequency the activity could be due rather to DNA polymerase γ.

Because of our interest in both enzymes, in conjunction with our studies on DNA polymerase γ we attempted to prepare an antiserum to the enzyme. Such an antiserum would allow us to address several questions: (1) a more complete evaluation of the relationship of DNA polymerase γ to reverse transcriptase, (2) the distinction of DNA polymerase γ and reverse transcriptase in cell extracts, and (3) the relationship of DNA polymerase γ to other cellular DNA polymerases. Earlier studies had suggested in addition to an immunological distinction between DNA polymerase γ and reverse transcriptase (Lewis et al., 1974a; Spadari and Weissbach, 1974; Matsukage et al., 1975) an immunological distinction between polymerase γ and other cellular DNA polymerases (Lewis et al., 1974a; Spadari et al., 1974). It was desirable to test these preliminary observations further.

We report here the successful preparation of an antiserum to highly purified DNA polymerase γ of human lymphoblasts, and its initial use in serological analyses of cellular and viral DNA polymerases. Future uses will involve the antiserum in investigations of human leukemic cells as well as in basic studies concerned with the role of DNA polymerases in DNA synthesis.

Experimental Procedure

Materials

NC37 cells from a line of normal human lymphoblasts (Gallo and Pestka, 1970) were supplied by Associated Biomedic Systems, Inc., Buffalo, N.Y. as frozen cell pellets, or by Pfizer, Inc., Maywood, N.J., as 50% cell suspensions frozen in 1640 media containing 50% fetal calf serum and 5% dimethyl sulfoxide. PL-Biochemicals, Milwaukee, Wisc., supplied $(dT)_{12-18}\cdot(A)_n$ and $(dT)_{12-18}\cdot(dA)_n$. [^{3}H]dTTP was a product of New England Nuclear, Boston, Mass. Unlabeled deoxynucleoside triphosphates were purchased from Sigma Chemical Co., St. Louis, Mo. BPA[1] was obtained from Armour Pharmaceutical Co., Chicago, Ill. Rabbit antisera to goat IgG and rat IgG, and goat antiserum to rabbit IgG were obtained from Cappel Laboratories, Inc., Cochranville, Pa., or from Miles Laboratories, Inc., Elkhart, Indiana. AMV was generously supplied by Dr. Joseph Beard, Life Sciences, Inc.,

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[1] Abbreviations used are: BPA, bovine plasma albumin; DTT, dithiothreitol; AMV, avian myeloblastosis virus; RLV, Rauscher murine leukemia virus; SSV, woolly monkey (simian) sarcoma virus; GaLV, gibbon ape leukemia virus; BaEV, baboon endogenous virus; RD114, endogenous feline virus grown in human rhabdomyosarcoma cells; DAI, double antibody immunoprecipitation; SSA, activated salmon sperm DNA; RNase, ribonuclease; DEAE, diethylaminoethyl; EDTA (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Gulfport, Fla. RLV was obtained from the Frederick Cancer Research Center, Frederick, Md. RD114 and SSV were purchased from Electro-Nucleonics, Inc., Bethesda, Md. Pfizer, Inc., Maywood, N.J., supplied GaLV and BaEV. Poly(U)-Sepharose was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

Methods

Enzyme Purification. DNA polymerase γ was purified from NC37 cell extracts as described (Robert-Guroff et al., 1977). In some cases, the majority of the DNA polymerase α activity was first removed in the soluble cytoplasmic fraction following lysis of the cells in the hypotonic buffer. The standard cell extraction, including Triton X-100 and KCl, was then continued. In these cases, a step elution of the hydroxylapatite column could be used instead of a gradient elution. DNA polymerase γ activity was eluted from hydroxylapatite between column washes of 0.15 and 0.4 M potassium phosphate and was further purified on DNA-cellulose. These procedural alterations did not adversely affect the purification. *DNA polymerase α* was purified from the hypotonic extract of NC37 cells. The soluble fraction from 60 g of cells was dialyzed against 50 mM Tris-HCl, pH 7.9, 1 mM DTT, 50 mM KCl, and 20% ethylene glycol, and applied to a 2.5 × 24 cm column of phosphocellulose equilibrated with the same buffer. The column was washed with 1 column volume of equilibrating buffer containing 0.1 M KCl, and then eluted with 3 column volumes of equilibrating buffer containing 0.4 M KCl. This high-salt eluate containing the DNA polymerase α activity was concentrated by dialysis against 40 mM potassium phosphate buffer, pH 7.2, containing 1 mM DTT, 20% ethylene glycol, and 30% polyethylene glycol (buffer C). Following dialysis against 40 mM potassium phosphate, pH 7.2, 1 mM DTT, and 20% ethylene glycol (buffer P), it was loaded at 7.5 mL/h onto a 1.5 × 15 cm column of hydroxylapatite. The column was washed successively with 3 column volume portions of buffer P, buffer P containing 0.15 M potassium phosphate, buffer P containing 0.3 M potassium phosphate, and buffer P containing 0.4 M potassium phosphate. DNA polymerase α activity eluting at the 0.3 M potassium phosphate step was pooled and concentrated by dialysis against buffer C and then dialyzed against buffer P. The DNA polymerase α was divided into small portions and stored at −70 °C. DNA polymerase α from 8402 cells (Huang et al., 1974) was purified as described (Smith et al., 1975) and similarly stored at −70 °C. *DNA polymerase β* was purified simultaneously with DNA polymerase γ through the microgranular DEAE-cellulose and phosphocellulose chromatography steps as described (Robert-Guroff et al., 1977). It was then concentrated by vacuum dialysis against 50 mM Tris-HCl, pH 7.9, 1 mM DTT, 50 mM KCl, and 20% ethylene glycol, and applied to a 2.5 × 30 cm column of Sephadex G-100 equilibrated in the same buffer. The DNA polymerase β activity eluted as a single peak and was concentrated by vacuum dialysis against the same Tris buffer. The enzyme was divided into small portions and stored at −70 °C. *Viral DNA polymerases* were purified as follows. AMV reverse transcriptase was purified as described (Robert-Guroff et al., 1977). SSV and RD114 reverse transcriptases were purified according to Abrell and Gallo (1973). GaLV and BaEV reverse transcriptases were purified similarly to SSV and RD114 but, in addition, they were further applied to a 2 × 6 cm column of poly (U)-Sepharose equilibrated in 10 mM Tris-HCl, pH 7.9, 1 mM DDT, 20% glycerol, 0.05% Triton X-100, 1 mM $MnCl_2$. The column was washed with 3 column volumes of equilibrating buffer and then eluted with a 500-mL linear gradient of 0 to 0.8 M KCl in equilibrating buffer. The reverse transcriptase activity which eluted at 0.33 M KCl was concentrated by vacuum dialysis against 50 mM Tris-HCl, pH 7.9, 1 mM DTT, 0.1 mM EDTA, 20% glycerol. One-hundred percent glycerol was added to bring the final glycerol concentration to 50% prior to storage. RLV reverse transcriptase was purified by the method of Wu and Gallo (1974). All purified reverse transcriptases were stored in buffer containing 50% glycerol at −20 °C.

Assays of DNA Polymerase Activities. During enzyme purifications, standard assay systems described for DNA polymerase α, β, γ, and reverse transcriptases were used (Robert-Guroff et al., 1977). GaLV, RD114, and BaEV reverse transcriptases were assayed using Mn^{2+} as described for RLV and SSV. In immunological assays, reaction conditions were adjusted as indicated below. One unit of DNA polymerase activity is defined as that amount causing the incorporation of 1 pmol of labeled dNMP into acid-insoluble material per hour under the various conditions described.

Preparation of Antisera. An antiserum to DNA polymerase γ was prepared in a goat. To obtain enough antigen it was necessary to use three separate preparations of polymerase γ; each enzyme preparation exhibited properties similar to the preparation described (Robert-Guroff et al., 1977). The goat was initially bled to obtain control serum and then inoculated with 250 μg of purified enzyme, half emulsified in an equal volume of complete Freund's adjuvant and inoculated subcutaneously and the other half simply injected intravenously. Two additional 250-μg boosters of antigen were given at 3-week intervals in a similar fashion. One month later, a third booster of 140 μg of enzyme was emulsified in complete Freund's adjuvant and inoculated subcutaneously. A final booster of 260 μg of enzyme was given 6 months later, emulsified in incomplete Freund's adjuvant and inoculated subcutaneously. Test bleeds were routinely taken on days 4, 7, 11, and 14 after each boost. The last bleed following each boost consisted of approximately 100 mL.

Antisera against purified viral reverse transcriptases were prepared in rabbits. Initial subcutaneous inoculations consisted of 50–100 μg of antigen emulsified in an equal volume of complete Freund's adjuvant. Subsequent booster doses of similar amounts of antigen were emulsified in an equal volume of incomplete Freund's adjuvant and administered subcutaneously. The booster doses were given approximately every 3 weeks. Rabbits were routinely bled on the 7th and 14th days following a boost of antigen. Sera with good enzyme neutralizing activity were generally obtained following the fifth inoculation.

The antiserum to DNA polymerase α has been described (Smith et al., 1975).

All sera were stored at −70 °C and thawed once prior to preparation of IgG fractions.

Purification of IgG. Purified IgG fractions of all antisera were used in immunologic assays, as use of whole sera often resulted in nonspecific inhibition of DNA polymerase activities. IgG fractions were prepared essentially as described by Reif (1969), except the amount of DEAE-cellulose necessary to obtain more than 99% pure IgG was packed in a column, rather than used batchwise. Thus, a 30-mL column of DEAE-cellulose was used for each 2 mL of whole sera. IgG was eluted from the cellulose with 3 column volumes of 0.01 M potassium phosphate buffer, pH 8.0. The IgG was concentrated by precipitation with an equal volume of saturated ammonium sulfate. Following centrifugation at 30 000*g* for 15 min, the IgG was dissolved in 0.1 M Tris-HCl, pH 8.0, and

dialyzed extensively against the same buffer. The final IgG fractions were divided into small portions and stored at −70 °C.

Immunologic Assays. The enzyme neutralization assay has been previously described (Smith et al., 1975; Robert-Guroff et al., 1977). The original DAI assay (Smith et al., 1975) has been modified (Wheeler, Robert-Guroff, and Gallo, manuscript in preparation), as well as expanded, to include titration curves. The first step consists of incubating DNA polymerase plus primary antibody for 16–20 h at 4 °C in a 20-μL mixture containing 25 mg/mL BPA, 150 mM KCl, 50 mM Tris-HCl, pH 8.0, and 0.2 to 1 unit of enzyme activity. The second step consists of precipitating the primary antibody plus any bound DNA polymerase by adding 20 μL of a secondary antibody with specificity to the primary antibody. Following 24 h at 4 °C to ensure complete precipitation, the mixture is centrifuged for 5 min in a Beckman microfuge B. The amount of enzyme activity remaining unbound in the supernatant is determined by withdrawing 25-μL aliquots for standard DNA polymerase assays. The amount of enzyme bound to the primary antibody is expressed as percent of control activity, based on the amount of DNA polymerase activity remaining in the supernatant when the primary antibody consists solely of nonimmune IgG. For all titration curves, dilutions of immune IgG were made in nonimmune IgG so that a constant amount of primary IgG was always available for precipitation. In practice, the amount of primary IgG which could be used in the assay was limited by the titer of the secondary antiserum. Thus, for each new lot of primary immune or nonimmune IgG prepared, a titration was carried out to determine the point of maximal precipitation. One further dilution of both primary immune and nonimmune IgG past the maximum precipitation point was then used in the DAI assay. This meant that a slight excess of precipitating antibody was therefore present, thus ensuring complete precipitation of all primary antibody. In the case of the DNA polymerase γ system, the primary antibody was a goat IgG, and the secondary antibody was the IgG fraction of a rabbit-anti-goat IgG antiserum. Anti-DNA polymerase α was a rat IgG, and the secondary antibody was the IgG fraction of a rabbit-anti-rat IgG antiserum. All other primary antibodies used were rabbit IgGs and the secondary antiserum was the IgG fraction of a goat-anti-rabbit IgG antiserum. None of the IgG fractions were nonspecifically toxic to any of the DNA polymerases used. The nonimmune IgG for the DNA polymerase γ system was prepared from serum drawn from the goat prior to its immunization. Nonimmune IgG's used as controls in the other antibody systems were prepared from sera of unimmunized animals.

Twenty-five microliter aliquots of supernatants from the DAI assays were brought to 50-μL volumes and the following final concentrations of reagents for assay of unbound DNA polymerase activities. *For DNA polymerase γ:* 50 mM Tris-HCl, pH 7.9; 1 mM DTT; 60 mM $(NH_4)_2SO_4$; 37.5 mM KCl; 0.2 mM $MnCl_2$; 24 μM [^{3}H]dTTP (40–50 Ci/mmol); 80 μM dATP; 50 μg/mL $(dT)_{12-18}\cdot(A)_n$; 10 mg/mL BPA. *For DNA polymerase α:* 50 mM Tris-HCl, pH 7.9; 1 mM DTT; 37.5 mM KCl; 0.5 mM $MgCl_2$; 24 μM [^{3}H]dTTP (40–50 Ci/mmol); 80 μM each dATP, dCTP, and dGTP; 75 μg/mL SSA; 10 mg/mL BPA. *For DNA polymerase β:* 50 mM Tris-HCl, pH 7.9; 1 mM DTT; 50 mM KCl; 1 mM $MnCl_2$; 24 μM [^{3}H]dTTP (40–50 Ci/mmol); 80 μM dATP; 50 μg/mL $(dT)_{12-18}\cdot(dA)_n$, 10 mg/mL BPA. *For reverse transcriptases:* 50 mM Tris-HCl, pH 7.9; 1 mM DTT; 50 mM KCl; 10 mM $MgCl_2$ for AMV or 1 mM $MnCl_2$ for the other reverse transcriptases; 24 μM [^{3}H]dTTP (40–50 Ci/mmol); 80 M dATP; 50 μg/mL $(dT)_{12-18}\cdot(A)_n$; 10 mg/mL BPA. Reaction mixtures were incubated for 1 h, those containing Mn^{2+} at 30 °C and those containing Mg^{2+} at 37 °C, and the assays were terminated by the addition of 50 μg of yeast tRNA, and approximately 2 mL of 10% Cl_3AcOH containing 0.2% sodium pyrophosphate. The acid precipitates were filtered through Reeve Angel glass-fiber filters (934AH) and washed with approximately 100 mL of 5% Cl_3AcOH each. The filters were washed with a small amount of 75% ethanol, dried, and counted in a Packard scintillation counter in a toluene–liquifluor cocktail. The extensive washing procedure was necessary in order to obtain reliable determinations in the presence of the high amounts of both BPA and [^{3}H]dTTP in the assays. In addition to careful washing, triplicate assays were routinely carried out.

Additional Methods. Enzyme protein was determined by the method of Lowry et al. (1951). Protein concentrations of IgG fractions were calculated from their absorbance at 280 nm taking the value of the extinction coefficient ($E_{1cm}^{1\%}$) of IgG as 14.2 (Little and Donahue, 1968). SSA was prepared by the method of Schlabach et al. (1971). The pH of all Tris buffers was adjusted at 4 °C.

Results

Assay for DNA Polymerase γ Antibody by Enzyme Neutralization. Test samples of sera from the goat immunized with DNA polymerase γ were initially purified to obtain IgG fractions and tested in the enzyme neutralization assay for inhibition of DNA polymerase γ activity. Beginning with sera taken following the first booster of antigen, none of the immune IgG samples showed any antibody activity in this assay system when compared with preimmune goat IgG as control. This was the case even when extremely high IgG concentrations were included in the assay.

As such a large amount of antigen was used to immunize the goat, it seemed likely that some immunologic response should have been elicited, particularly in view of positive results obtained in our laboratory with DNA polymerase α and with reverse transcriptases. Although antibody was evidently not made to DNA polymerase γ sequences surrounding the active site of the enzyme, it was possible that antibody was made to other regions of the molecule. Therefore, the DAI assay was used to test the possibility that a binding antibody, rather than an enzyme neutralizing antibody, had been made with specificity for DNA polymerase γ. Other binding assays could have been used. The DAI assay, however, has two advantages. First, one is always assured that all immune IgG, and hence any bound enzyme, is precipitated. This eliminates the necessity for numerous controls when testing various enzymes for cross-reactions. Second, many samples can be processed simultaneously.

Assay for DNA Polymerase γ Binding Antibody. Figure 1 shows the results of the initial DAI assays. The immune goat IgG clearly binds two different preparations of DNA polymerase γ. Only 7 μg of immune IgG was necessary to bind 80 to 90% of the DNA polymerase γ molecules. For these and subsequent experiments, the 2-week serum following the final booster of antigen was the source of immune IgG. It has not been determined whether serum samples obtained at other times during the immunization schedule possess higher titers of DNA polymerase γ binding antibody.

Figure 1 also indicated that the goat antibody is specific for DNA polymerase γ with regard to other cellular DNA polymerases. Neither DNA polymerase α or β, purified from NC37 cells, was bound by the anti-DNA polymerase γ IgG.

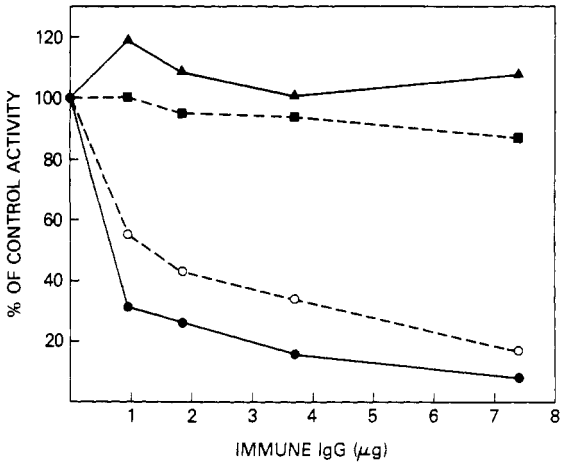

FIGURE 1: DAI assay of anti-DNA polymerase γ serum vs. cellular DNA polymerases. DAI assay points were always carried out in triplicate. Each point represents the mean of three to five separate experiments. Approximately 0.4 unit of enzyme activity was used for each point. For NC37 DNA polymerase α (■), this meant 1 to 2 μg of protein; for NC37 DNA polymerase β (▲), 0.1 to 0.3 μg of protein; for two preparations of NC37 DNA polymerase γ (●, ○), 0.1 to 0.5 μg of protein.

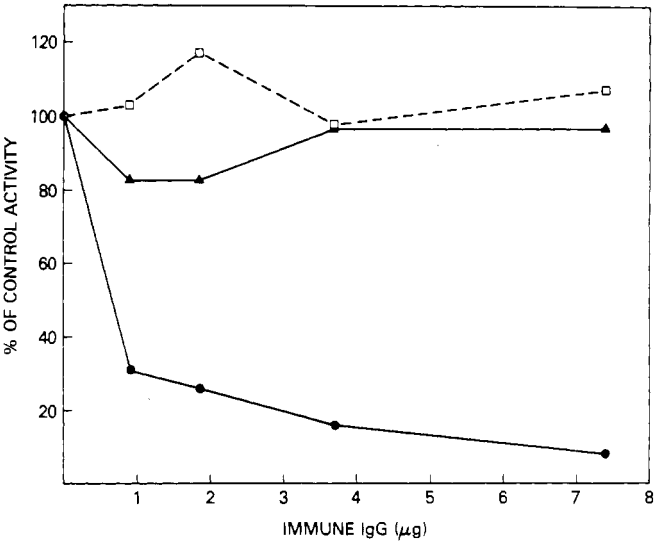

FIGURE 2: DAI assay of anti-DNA polymerase γ serum vs. avian and murine reverse transcriptases. Assay points were carried out in triplicate. Each point represents the mean of two to three separate experiments. The protein concentrations of AMV (▲) and RLV (□) reverse transcriptases were not measured as too great a proportion of the preparations would have had to be sacrificed. Approximately 0.4 unit of enzyme activity was used for each point. (●) DNA polymerase γ.

Lack of Immunological Relationship of DNA Polymerase γ with Reverse Transcriptase. The possibility that the DNA polymerase γ binding antibody might detect relationships between DNA polymerase γ and viral reverse transcriptases was next investigated. Figure 2 shows the results of DAI assays in which avian and murine RNA tumor virus DNA polymerases were tested. Neither AMV nor RLV reverse transcriptase showed any binding by the polymerase γ antiserum. Titrations similar to those shown in Figure 2 were carried out with the reverse transcriptases of RD114, BaEV, and GaLV. In no case was a cross-reaction detected. Under conditions where the percent of control activity for DNA polymerase γ was 7 to 16%, corresponding values for the primate virus DNA polymerases were 104%, 97%, and 128% for RD114, BaEV, and GaLV, respectively. It should be pointed out that in the DAI system, a result of 0 to 85% of control activity would indicate a cross-reaction between the enzyme in question and polymerase γ. Whether data falling between 85 and 99% of control activity reflect minor cross-reactions or merely experimental variation cannot be determined until an even more sensitive assay is developed. Similarly, values greater than 100% of control reflect the variability of the assay and cannot be interpreted any further at this point. To overcome these limitations of the system, we have always carried out the DAI assay using several concentrations of immune IgG in order to detect any trends toward cross-reaction.

Previous data (Todaro and Gallo, 1973; Gallo et al., 1974; Lewis et al., 1974a; Spadari and Weissbach, 1974; Matsukage et al., 1975) showed that antisera made against purified viral reverse transcriptases do not inhibit DNA polymerase γ in an enzyme neutralization assay. In general, these studies used single-point antibody inhibition determinations and/or short preincubation times. It has been demonstrated that a relatively long preincubation time is often necessary for an antibody-enzyme neutralization reaction to reach completion (Smith et al., 1975). It is also possible that when testing less pure enzyme preparations slight immunological cross-reactions between enzymes could be missed in a neutralization reaction due to masking by contaminating enzyme activities. It therefore seemed important to retest the relationship of the highly purified DNA polymerase γ to viral reverse transcriptases by also using antisera to the purified viral enzymes. This was done in enzyme neutralization assays in which the sensitivity was increased by extending the preincubation period of enzyme with antibody to 20 h. The results of these experiments are shown in Figure 3. While in the systems studied, including AMV, RLV, SSV, and RD114, all the reverse transcriptases showed significant inhibition by their homologous antisera, in no case, was DNA polymerase γ inhibited by antisera to viral reverse transcriptase.

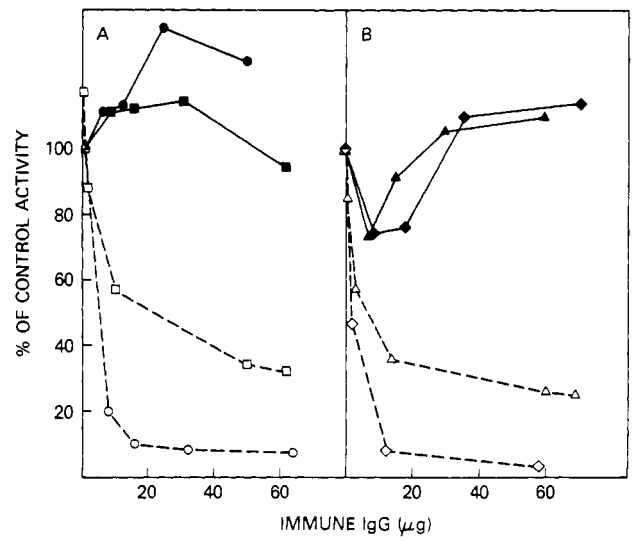

FIGURE 3: Failure of antisera to viral reverse transcriptase to inhibit DNA polymerase γ. The enzyme neutralization assay used is described under Methods. Closed symbols with solid lines represent the reaction with DNA polymerase γ. Open symbols with dashed lines indicate reaction with the homologous viral reverse transcriptase. The antisera tested were prepared against SSV (●, ○), RD114 (■, □), AMV (♦, ◇), and RLV (▲, △).

While a cross-reaction was not observed between DNA polymerase γ and reverse transcriptase when the enzymes were tested against antisera to reverse transcriptases in the enzyme activity neutralization assay, experience with the antibody to

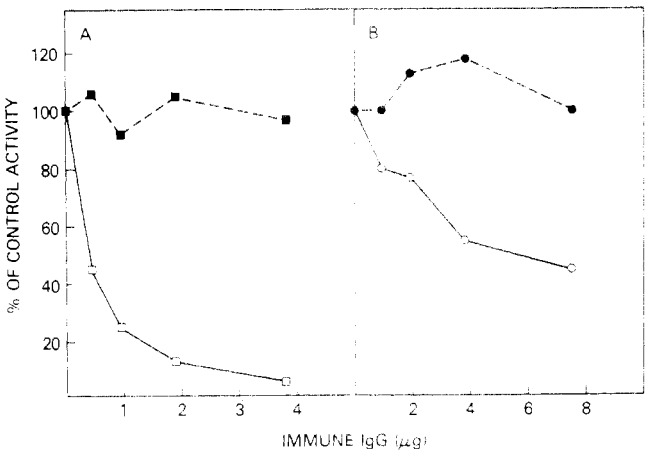

FIGURE 4: DAI assay of anti-reverse transcriptase sera vs. homologous enzymes and DNA polymerase γ. (A) Anti-SSV reverse transcriptase vs. SSV reverse transcriptase (□) and DNA polymerase γ (■). (B) Anti-RD114 reverse transcriptase vs. RD114 reverse transcriptase (○) and DNA polymerase γ (●).

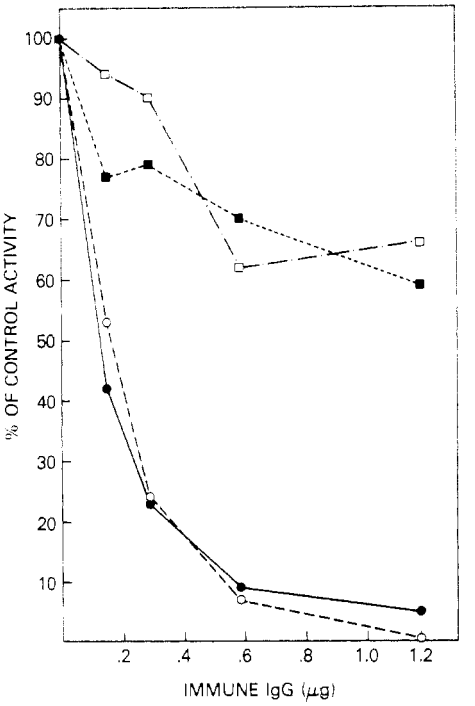

FIGURE 5: DAI assay of anti-DNA polymerase α serum vs. DNA polymerase γ. Assay points were carried out in triplicate. The following amounts of enzyme were used; NC37 DNA polymerase α (●), 1 μg; 8402 DNA polymerase α (○), 0.1 μg; NC37 DNA polymerase γ, 0.1 to 0.5 μg, assayed with either $(dT)_{12-18}\cdot(A)_n$ (■) or SSA (□) as template.

DNA polymerase γ suggested that the DAI assay might uncover relationships between the enzymes not detected by neutralization assays. Thus, experiments were carried out using both anti-SSV reverse transcriptase and anti-RD114 reverse transcriptase as primary antisera in the DAI assay. Figure 4 shows the results of these studies. An antiserum to SSV reverse transcriptase bound the homologous enzyme very well, but failed to bind DNA polymerase γ (Figure 4A). A comparison of Figures 4A and 5A also shows that the DAI assay is more sensitive than the enzyme neutralization assay. Approximately fivefold less immune IgG is needed in the DAI assay for binding of 90% of homologous enzyme, compared to the amount necessary for 90% inhibition of homologous enzyme activity. Figure 4B shows that anti-RD114 reverse transcriptase also binds its homologous enzyme while not recognizing DNA polymerase γ. In this case, the sensitivity of the DAI assay was also improved compared to the enzyme neutralization assay with the same antiserum (Figure 3A) in that a lesser amount of immune IgG was needed to achieve a similar extent of reaction. With the anti-RD114 reverse transcriptase serum, it was not possible to extend either assay to obtain 90% inhibition or binding. In one case, too great a quantity of immune IgG would have been required for the enzyme neutralization assay. In the other case, the titer of the secondary antiserum used in the DAI assay did not allow the use of greater amounts of primary antiserum.

Relationship of DNA Polymerase γ to DNA Polymerase α. To this point, our relevant immunological studies indicated that DNA polymerase γ and DNA polymerase α were not related. The antiserum made against highly purified DNA polymerase γ did not bind DNA polymerase α purified from the same cells (Figure 1). Additionally, an antiserum made against a highly purified DNA polymerase α did not neutralize DNA polymerase γ activity (Robert-Guroff et al., 1977). Nevertheless, because of the greater sensitivity of the DAI assay compared to the enzyme neutralization assay, the former assay was used with anti-DNA polymerase α as primary antibody to try to detect a cross-reaction of DNA polymerase α with DNA polymerase γ. The results are presented in Figure 5. In this system, only 1 μg of immune IgG was needed to bind more than 90% of the DNA polymerase α present. However, approximately 40% of the DNA polymerase γ present was also bound. The result was the same whether the polymerase γ activity remaining in the supernatant was assayed with SSA or $(dT)_{12-18}\cdot(A)_n$ as template. The implications of this result are discussed below.

Discussion

The availability of an antiserum to human DNA polymerase γ is of immediate practical importance to those investigators concerned with viral and cellular DNA polymerases. It allows both the quick identification of DNA polymerase γ and also the ready distinction of whether a $(dT)_{12-18}\cdot(A)_n$-utilizing enzyme is polymerase γ or reverse transcriptase. Of course, biochemical criteria can also make this identification and distinction, as often can antisera to various reverse transcriptases. However, in the first case, several biochemical tests are generally necessary for convincing proof of identity of an enzyme, along with a certain degree of purification to allow the application of some of the biochemical criteria. In the second case, several antisera with specificities against a variety of reverse transcriptases must be tested. Furthermore, when the enzyme in question fails to cross-react with any of the available antisera to reverse transcriptase, it is still not certain that DNA polymerase γ is the correct identification. The polymerizing activity might rather be due to an as yet uncharacterized reverse transcriptase which does not cross-react with any of the known viral enzymes. Thus, the antiserum to DNA polymerase γ described here will be extremely useful because of its specificity for DNA polymerase γ. A positive result will convincingly identify a $(dT)_{12-18}\cdot(A)_n$-utilizing enzyme as DNA polymerase γ, as the antiserum has so far failed to cross-react with any known viral reverse transcriptases. It is not yet known whether cross-reactivity exists between γ-polymerases isolated from different species. It must be pointed out that while our results indicate that there are no cross-reactions between DNA polymerase γ and other DNA polymerases this conclusion is based merely on the immunologic assays. It is realized of course

that even more sensitive assays may detect regions of homology between the enzymes. This does not lessen the importance of the immunologic systems, which clearly can both distinguish one polymerase activity from another as well as group the enzymes according to their major cross-reactivities.

Several enzymes which resemble both DNA polymerase γ and reverse transcriptase in some respects, yet are unlike these prototypes in other respects, have been isolated from cells. The anti-DNA polymerase γ serum may help to clarify the nature of these enzymes and their relationships to other $(dT)_{12-18}$·$(A)_n$-utilizing polymerases. The enzymes in question include the RNase-sensitive DNA polymerase of mitogen-stimulated normal human lymphoblasts (Bobrow et al., 1972) which was later shown to be an RNA primed, but DNA-directed, DNA polymerase (Reitz et al., 1974), and which could be a mitochondrial DNA polymerase (Margalith et al., 1976), a similar activity present in transformed cells (Kotler et al., 1974), a RNase-sensitive endogenous DNA polymerase of normal rat tissue culture cells (Coffin and Temin, 1971), the Peak A polymerase of Balb/3T3 fibroblasts (Livingston et al., 1974), the intracisternal A particle polymerase (Wilson and Kuff, 1972; Wong-Staal et al., 1975), and the intracellular high-molecular-weight forms of human, subhuman primate, murine, and feline reverse transcriptase (Mondal et al., 1975; Wu et al., 1975; Gerwin et al., 1975).

A different result than that obtained, regarding the specificity of anti-DNA polymerase γ, might have been expected. Earlier reports suggested that DNA polymerase γ and reverse transcriptase were not immunologically related (Lewis et al., 1974a; Spadari and Weissbach, 1974; Matsukage et al., 1975), although these studies were not conclusive. The enzyme neutralization assays reported here, using antisera to several reverse transcriptases and more sensitive preincubation conditions, confirmed the earlier results. Nevertheless, an antiserum against DNA polymerase γ itself might have revealed different regions of homology. Furthermore, a binding test, as the DAI assay, would look at entire enzyme molecules for homologous areas, not just regions around the active site, and the DAI assay itself was more sensitive than the enzyme neutralization assay, at least with respect to the amount of immune IgG required to produce a 90% cross-reaction in the homologous system. Thus, a greater possibility for detecting slight similarities between DNA polymerase γ and reverse transcriptase was anticipated. However, in spite of these opportunities for enhanced detection of cross-reactivity, none was observed. Thus, one can conclude that based on the more sensitive binding assay human DNA polymerase γ is not related to known viral reverse transcriptases. Whether the same is true for DNA polymerase γ's of other species remains to be determined.

The experiments reported here do not enable one to resolve the question of whether cellular DNA polymerases are interrelated. Enzyme neutralization studies with anti-DNA polymerase α (Robert-Guroff et al., 1977) and DAI assays with anti-DNA polymerase γ (Figure 1) indicate that DNA polymerases α and γ do not share common sequences. However, in a DAI assay, DNA polymerase γ was partially bound by anti-DNA polymerase α (Figure 5). This result has several interpretations. (1) DNA polymerases α and γ do, in fact, share common sequences which were antigenic in the rat which produced the anti-DNA polymerase α serum, but not in the goat which produced the antiserum to DNA polymerase γ. These common sequences are not located near the active sites of the enzyme molecules, otherwise they would have been detected in the enzyme neutralization assay. (2) DNA polymerases α and γ are not related. The result of Figure 5 stems from a contaminant in the immunizing antigen used to prepare the rat antiserum to DNA polymerase α. The resulting contaminating anti-DNA polymerase γ reactivity would only be picked up in a very sensitive enzyme neutralization assay or in a binding assay. (3) The DNA polymerase γ preparation contains a small amount of contaminating DNA polymerase α activity which is only detected in the more sensitive DAI assay using anti-DNA polymerase α. (4) The apparent binding of DNA polymerase γ by the anti-DNA polymerase α serum is merely a reflection of the inherent lack of specificity of the antiserum, detected here at low levels of immune IgG because of the greater sensitivity of the DAI assay. The third possibility actually seems the least likely. If both DNA polymerase γ and a small amount of DNA polymerase α were present in the enzyme preparation, then one would not expect the result of Figure 5. The graph shows both $(dT)_{12-18}$·$(A)_n$ and SSA templated activities being bound in parallel to the same extent. However, until either or both DNA polymerase α and γ are purified to homogeneity, this question and that of the relationship of the enzymes will remain unresolved. It should also be mentioned that none of our experiments has suggested an immunological relationship between DNA polymerases γ and β which is in keeping with the significant differences in the biochemical properties of these enzymes.

With the availability of an antiserum to DNA polymerase γ, one can envision its applicability in several areas. Aside from the aspects concerned with oncogenesis and human leukemia discussed above, these might include the role of DNA polymerases in embryogenesis and differentiation and in regulation of DNA synthesis. The antiserum may also help to elucidate the function of DNA polymerase γ in cells.

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Zinc Is Associated with the β Subunit of DNA-Dependent RNA Polymerase of *Bacillus subtilis*†

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ABSTRACT: The *Bacillus subtilis* DNA-dependent RNA polymerase holoenzyme and core enzyme each contain approximately two atoms of zinc per molecule. When the dissociated subunits of the enzyme are passed through a blue dextran–Sepharose affinity column, only the β subunit binds to the column. The total zinc content of the enzyme is tightly bound to the β subunit. Dialysis studies suggest that the two zinc ions differ in the strength of their association with the β subunit. The presence of zinc in β is consistent with several other lines of evidence which indicate that this subunit is directly involved in phosphodiester bond formation. The blue dextran–Sepharose column procedure should be useful in future studies of the dissociation and reassociation of the enzyme since the method is rapid and provides excellent recovery of the β subunit as well as the α and β' subunits of the RNA polymerase.

Several nucleotidyl transferases have been reported to contain tightly bound zinc which is essential for their activity. These include DNA-dependent RNA polymerase from *E. coli* (Scrutton et al., 1971), phage T7 RNA polymerase (Coleman, 1974), yeast RNA polymerase I (Auld et al., 1976) and B (Lattke and Weser, 1976), DNA polymerase from *E. coli* (Slater et al., 1971; Springgate et al., 1973) and from sea urchin (Slater et al., 1971), and RNA-dependent DNA polymerase from avian myeloblastosis virus (Poesz et al., 1974; Auld et al., 1974). Several studies on other nucleotidyl transferases have suggested that they may be metalloenzymes since they are inhibited by chelating agents such as 1,10-phenanthroline (Williams and Schofield, 1975; Chang and Bollum, 1970; Valenzuela et al., 1973).

Our studies were undertaken to determine whether the RNA polymerase of *Bacillus subtilis* is a zinc metalloenzyme and, if so, where the metal is located with respect to the subunits of this oligomeric protein. It appeared that the latter question might be investigated by use of a blue dextran–Sepharose affinity column. As reported by Thompson et al. (1975), the dye blue dextran forms complexes with a large number of nucleotide-binding enzymes, which possess the structural feature

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