Title

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Authors

Libertini, Louis J.
Adams, Jesse.

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Louis J. Libertini and Jesse Adams

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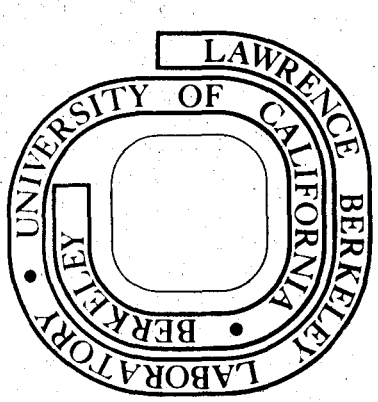

DISCLAIMER

POLY(rA):OLIGO(dT)-DIRECTED DNA POLYMERASE ACTIVITY IN CARCINOGEN INDUCED RAT MAMMARY TUMORS AND IN NORMAL TISSUES*

Louis J. Libertini and Jesse Adams

Chemical Biodynamics Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

Received

Mammary tumors induced in Sprague-Dawley Rats by the carcinogen 7,12-dimethylbenz(a)anthracene contain a DNA polymerase similar to that found in RNA tumor viruses. It has a molecular weight of 105,000 daltons and is active on the synthetic templates poly(rA):oligo(dT) and poly(rC):-oligo(dG) but is inactive on poly(dA):oligo(dT). This polymerase may be purified more than 300 fold with a 25% yield by ammonium sulfate precipitation, phosphocellulose chromatography and hydroxyapatite chromatography. A similar polymerase is also found in lactating normal rat mammary tissues.

Studies of the polymerases found in RNA tumor viruses have shown a consistent pattern of activity on synthetic templates. Activity is obtained on poly(dA):oligo(dT) and on poly(rC):oligo(dG) while the activity on poly(dA):oligo(dT) is very low or zero in comparison (1). It has been suggested that this pattern of response to synthetic templates might serve to distinguish the viral polymerase from cellular polymerases (1). Recent work, however, indicates that several types of cells of tumor origin contain a polymerase with similar characteristics even when no virus is present or clearly implicated (1-4). Reports also suggest its presence in WI-38 cells, a normal human diploid fibroblast cell line (2), and even in calf thymus (5).

In a study of the DNA polymerases of carcinogen induced rat mammary tumors, we have found a polymerase which resembles that from RNA tumor virus in its synthetic template specificity and molecular weight. The partial purification and characterization of this polymerase is reported here. Evidence that the same polymerase is present in normal lactating rat mammary tissue is also given (6).

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MATERIALS AND METHODS.

Tritium labelled dTTP and dGTP were products of New England Nuclear; they were received in 50% ethanol which was evaporated to about 20% of the original volume with a stream of dry nitrogen before dilution with unlabelled dNTP to a specific activity of 1 Ci/mmole. The unlabelled dNTP's were obtained from P-L Biochemicals as were poly(dA) and poly(dA-dT):poly(dA-dT). Poly(rA):oligo(dT), poly(rC):oligo(dG) and oligo(dT) were obtained from Collaborative Research. The oligo(dT) and oligo(dG) were 12-18 nucleotides in length while the homopolymers and the alternating copolymer were specified as > 100 nucleotides. Poly(dA):oligo(dT) was formed by mixing the two components. The base ratio for each of the polymer:oligomer combinations was 1:1. Activated DNA was prepared from calf thymus DNA by incubation with Worthington pancreatic deoxyribonuclease I as described by Ross et al. (7).

Phosphocellulose (Whatman fibrous P11) was obtained from Reeve Angel while hydroxyapatite (Bio-Gel HTP) and beaded 10% agarose (Bio-Gel A0.5M, fractionation range for proteins: 10,000-500,000 daltons) were purchased from Bio-Rad Laboratories. Triton X-100 and Brij-35 were obtained from Sigma. Triton DN-65, a nonionic detergent with properties similar to Triton X-100 but containing no aromatic group, was a gift from Rohm and Haas.

Mammary tumors were obtained from Sprague-Dawley rats (Holtzman) which had been treated with 7,12-dimethylbenz(a)anthracene as described (8). Necrotic portions were discarded and the tumors quickly frozen in liquid nitrogen and stored in liquid nitrogen until needed. Levels of DNA polymerase activities in crude extracts were equivalent whether the tumor was obtained fresh and extracted or had been frozen. Tumors stored in liquid nitrogen for eighteen months showed activities more than half those of fresh tissues.

DNA polymerase assays were done in a volume of 100 ul. Standard assay conditions are defined as 0.1 M Tris (pH 7.8 at 20°C), 0.1 M KCl, 0.1% 2-mercaptoethanol, 0.3 mM manganese acetate, 10 ug/ml poly(rA):oligo(dT) and 0.02 mM tritiated-dTTP (1 Ci/mmole). Except for activated DNA which was used at 100 ug/ml, the other templates were also used at 10 ug/ml with appropriate changes in the substrates (e.g., use of activated DNA also implies addition of 0.02 mM each of dATP, dCTP and dGTP). Deviations from these standard conditions will be specified as required. Each reaction was initiated by the addition of enzyme and incubated at 37°C for 30 minutes after which carrier RNA (250 ug) was added and the products precipitated with 10% trichloroacetic acid. After at least 15 minutes in ice, the acid precipitable counts were collected on nitrocellulose filters, washed and counted in a toluene-based scintillation fluid. Quenching of counts due to the sample not being dissolved in the fluid was estimated by oxidizing several samples (after they had been counted on the filter) on a Packard Tri-Carb sample oxidizer and the results used to correct for such quenching. One unit of activity is defined as 1 pmole/hr of labelled nucleotide monophosphate incorporated into acid precipitable material.

RESULTS AND DISCUSSION.

Subcellular fractionation of the tumor tissues (Table I) demonstrated that nearly 90% of the activity on poly(rA):oligo(dT) was found in the clarified cytoplasmic fraction with essentially all of the remaining activity in the microsomal fraction. This observation is in contrast

with results on activated DNA of which only 30% was in the cytoplasmic fraction while 60% was in the nuclear fraction and the remainder was in the microsomal fraction.

The clarified cytoplasmic extract showed a self-inhibitory quality which resulted in a very nonlinear relationship of activity to volume of crude extract added to the assay. The inhibition was nonspecific, also inhibiting a partially purified DNA polymerase from another source. Characterization of this property by preincubation of the extract with template, substrate, etc. demonstrated that the inhibition results from an effect on the substrate, making it unavailable to the DNA polymerase. This effect is probably due to a nucleotide triphosphatase in the extract. Birnie and Fox (9) observed a similar effect in extracts of mouse embryo and showed that addition of ATP could be used to diminish the self-inhibition. For the tumor extracts, addition of up to 0.2 mM ATP increased the observed activity while higher concentrations were inhibitory. Either Triton X-100 or Triton DN-65 at a concentration of 0.05% or higher in the assay also decreased the self-inhibitory effect in a manner partially additive to the effect of ATP. Interestingly, Brij-35, another nonionic detergent, did not show the same ability. This self-inhibitory property is retained after ammonium sulfate precipitation but is completely eliminated by phosphocellulose chromatography.

Purification of the poly(rA):oligo(dT)-directed DNA polymerase is described in the caption of Figure 1. The figure shows the results of phosphocellulose chromatography which gives a peak of activity on poly(rA):oligo(dT) near 0.5 M KCl. A separate experiment showed a coincident peak of activity on poly(rC):-oligo(dG) while no activity on either poly(dA):oligo(dT) or poly(dA-dT):-poly(dA-dT) was observed in this same region. Activity on activated DNA was demonstrable and at times formed a peak coincident with that on poly(rA):-oligo(dT).

The molecular weight of the poly(rA):oligo(dT)-directed DNA polymerase was estimated by gel chromatography on beaded 10% agarose (Bio-Gel A0.5M). The result is shown in Figure 2. A value of 105,000 daltons was obtained by comparison with proteins of known molecular weight. This value compares well with the value of 100,000 obtained for the mouse mammary tumor virus (10) and also with the result of 110,000 determined for the Mason-Pfizer monkey tumor virus (11) which was isolated from a monkey breast tumor. It is also in agreement with the 110,000 daltons found for the "R-DNA" polymerase in HeLa cells (2).

The optimal pH on poly(rA):oligo(dT) for the purified polymerase was in the range 7.6-7.9. Greatest activity was obtained with manganese at 0.1-0.2 mM while magnesium gave only 15% of the activity with manganese. No activity is obtained without divalent cation. A nearly linear increase in activity with KCl concentration up to 0.15 M was observed. Between 0.15 and 0.3 M KCl the activity dropped only slightly. In the absence of KCl (0.1 M Tris, pH 7.8) the activity was less than 10% that at 0.15 M KCl.

Activity on poly(rC):oligo(dG) and some activity on activated DNA were retained throughout purification of the polymerase (each ranged from 5-20% of the maximum activity on poly(rA):oligo(dT)). However, maximum activity on each of these templates was observed with magnesium acetate--about four times greater than with manganese for poly(rC):oligo(dG). No activity on poly(dA):oligo(dT) was observed with either divalent cation beyond the phosphocellulose chromatography step.

Crude extracts of normal lactating mammary tissues from Sprague-Dawley rats show activity on poly(rA):oligo(dT) with a specific activity about 15% of that in the tumors. The activity precipitates between 25 and 45% ammonium sulfate but the yield of this step from the lactating tissues is generally low. Direct chromatography of the clarified crude extract on phosphocellulose results in a peak of activity on poly(rA):oligo(dT)

at 0.45 M KCl. When compared with a similarly purified polymerase preparation from tumor tissue (phosphocellulose chromatography only) on poly(rA):-oligo(dT), the polymerase from normal tissues showed the same responses to manganese, magnesium and salt concentrations as the tumor polymerase although the optimal pH appeared to be somewhat lower. The polymerase from normal tissues also showed activity on poly(rC):oligo(dG) which was greater with magnesium than with manganese as was true for the tumor-derived polymerase.

In contrast to the situation with mouse mammary tumors, a viral etiology has not been demonstrated for mammary neoplasia in the rat. Efforts to find RNA tumor virus particles in chemically induced primary tumors in rats have not succeeded (12). However, a "rat mammary tumor derived virus" has been isolated from tumors like those used here by infecting an embryo cell line with extracts of the tumor (13). It thus remains unclear whether the polymerase described here is a virus related polypeptide or a normal cellular polymerase with an unknown function. The presence of a similar polymerase in normal lactating rat mammary tissue cannot be taken as support of the latter possibility when one considers that, in the mouse, apparently normal tissues may contain and produce mouse mammary tumor virus. However, it is apparent that considerable caution should be exercised in interpreting the synthetic template responses of cellular DNA polymerases in terms of virus related information in the cells.

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TABLE I

SUBCELLULAR FRACTIONATION OF RAT MAMMARY TUMOR*

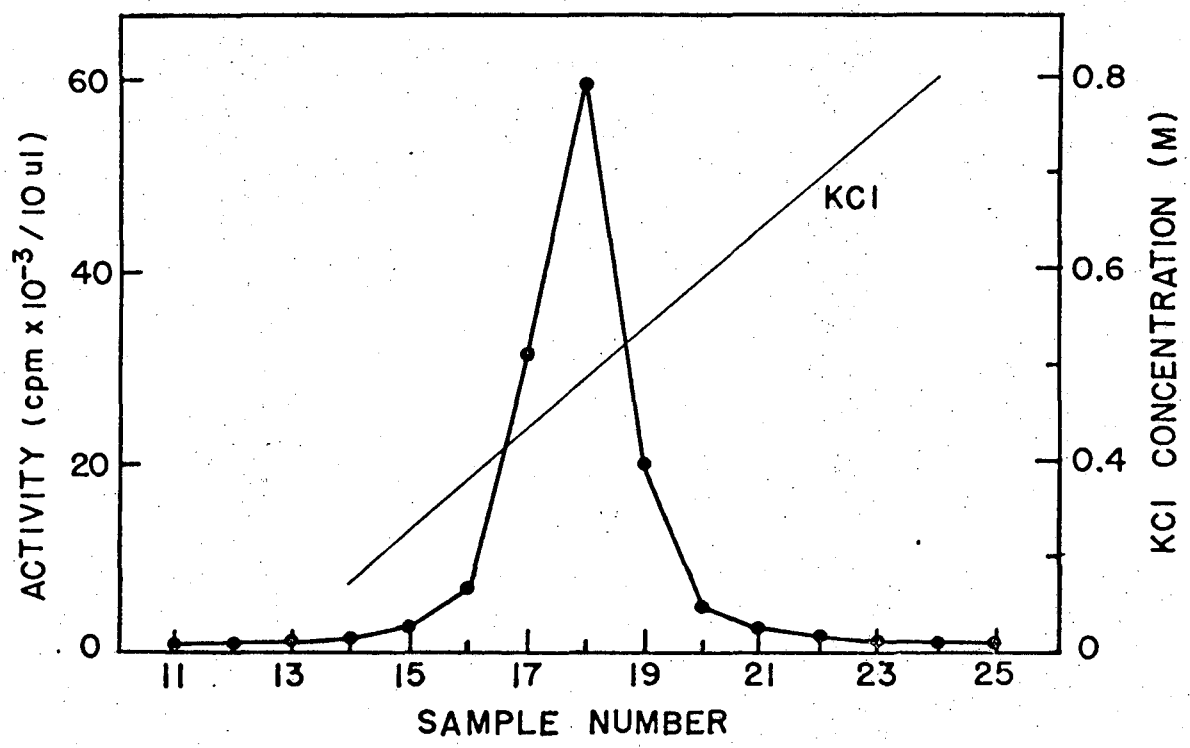
		poly(rA):oligo(dT)			activated DNA		
Fraction	total protein (mg)	specific activity (units/ug)	total activity (units)	%	specific activity (units/ug)	total activity (units)	%
Cytoplasmic	864	0.97	838,000	89	0.13	112,000	34
Nuclear	76	0.13	10,000	1	0.38	199,000	60
Mitochondrial	27	0.46	12,000	1	0.0	0	0
Microsomal	62	1.38	86,000	9	0.34	21,000	6

*Subcellular fractionation of 20 g of fresh tumor tissue followed the method described by Hogeboom (14) except that the homogenization buffer (180 ml) contained 0.025 M potassium phosphate (pH 6.8) and 0.1% 2-mercaptoethanol in addition to 0.25 M sucrose. The nuclei were further purified by suspension in homogenization buffer containing 2.0 M sucrose (50 ml) and pelleted at 100,000 g for 1 hour. The nuclear pellet was sonicated in (total volume, 30 ml) 0.2 M potassium phosphate (pH 6.8) and 0.1% 2-mercaptoethanol, kept at 0°C for 2 hours and clarified (100,000 g for 1 hr). The mitochondrial pellet was sonicated in 20 ml of 0.025 M potassium phosphate (pH 6.8), 1M KCl, and 0.1% 2-mercaptoethanol, kept at 0°C for 2 hours then clarified. The microsomal pellet was suspended in 30 ml of 25 mM potassium phosphate (pH 6.8), 0.5 M KCl, 1% Triton DN-65 and 0.1% 2-mercaptoethanol, kept 2 hours at 0°C and clarified. Polymerase assays were done under standard conditions (Materials and Methods) except that each assay also contained 0.1% Triton DN-65 and 0.2 mM ATP.

FIGURE CAPTIONS

Figure 1. Results of phosphocellulose chromatography during purification of rat mammary tumor poly(rA):oligo(dT)-directed DNA polymerase activity. All steps were done at 0-4°C. Fifty grams of tissue were minced and homogenized for 15 seconds at top speed in a Sorval blade homogenizer in 450 ml of buffer A (0.01 M potassium phosphate, pH 6.8, and 0.1% 2-mercaptoethanol). After centrifugation at 30,000 g for 15 minutes the supernatant was clarified at 100,000 g for 1 hour. The fraction precipitating between 25 and 45% ammonium sulfate was collected by adding a saturated solution of ammonium sulfate to the concentrations required, stirring for 15 minutes, and centrifuging at 30,000 g for 15 minutes. Yields from this step ranged from 65 to 85% with a 4 to 5 fold purification. The ammonium sulfate pellet was dissolved in 250 ml of buffer A containing 0.2 M KCl and applied to a 2.5 x 25 cm column of phosphocellulose equilibrated with the same buffer. The activity was eluted at 60 ml/hr with a 400 ml gradient of 0.2 to 1.2 M KCl in buffer A. Samples of 10 ml were collected and 10 ul aliquots were tested for activity on poly(rA):-oligo(dT) (filled circles in figure) under standard assay conditions except that 0.1% Triton DN-65 and 0.2 mM ATP were included. The yield from the phosphocellulose step ranged from 60 to 80% with a 20 to 30 fold increase in specific activity. The eluted polymerase was somewhat unstable unless the fractions were dialysed against buffer A. Alternately, further purification of the combined peak fractions (17-19 in figure) could be accomplished without dialysis by immediate chromatography on a 1.5 x 25 cm column of hydroxyapatite equilibrated with buffer A (results not shown). At a flow rate of 20 ml/hr, a 100 ml gradient from 0.05 to 0.5 M potassium phosphate (pH 6.8) containing also 0.1% 2-mercaptoethanol elutes the activity in a peak between 0.10 and 0.12 M potassium phosphate. A 3 to 4 fold purification is generally obtained with a yield for the step of 50-60%. The peak fractions were dialysed against buffer A containing 30% glycerol and stored at 4°C. The overall purification and yield of the three purification steps were usually 300 fold and 25%, respectively.

Figure 2. Gel chromatography of poly(rA):oligo(dT)-directed DNA polymerase on Bio-Gel A0.5M. The column size was 1.5 x 90 cm. Blue dextran 2000 was used to determine the void volume. A 2 ml sample of polymerase (purified through the hydroxyapatite step) or of standard protein in elution buffer containing 2% sucrose was layered onto the column and eluted with 0.01 M potassium phosphate (pH 6.8), 0.1% 2-mercaptoethanol and 0.02% Triton DN-65. The flow rate was 18 ml/hr and 1.8 ml samples were collected. Aliquots of 60 ul of the indicated fractions were tested for activity on poly(rA):oligo(dT) under standard conditions except that 0.1% Triton DN-65 was included. The estimated molecular weight is 105,000 daltons.


ACTIVITY (cpm x 10^{-3} / 10 ul)
KCl CONCENTRATION (M)
KCl
SAMPLE NUMBER
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20
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60
0
0.4
0.6
0.8
11
13
15
17
19
21
23
25

Figure 1

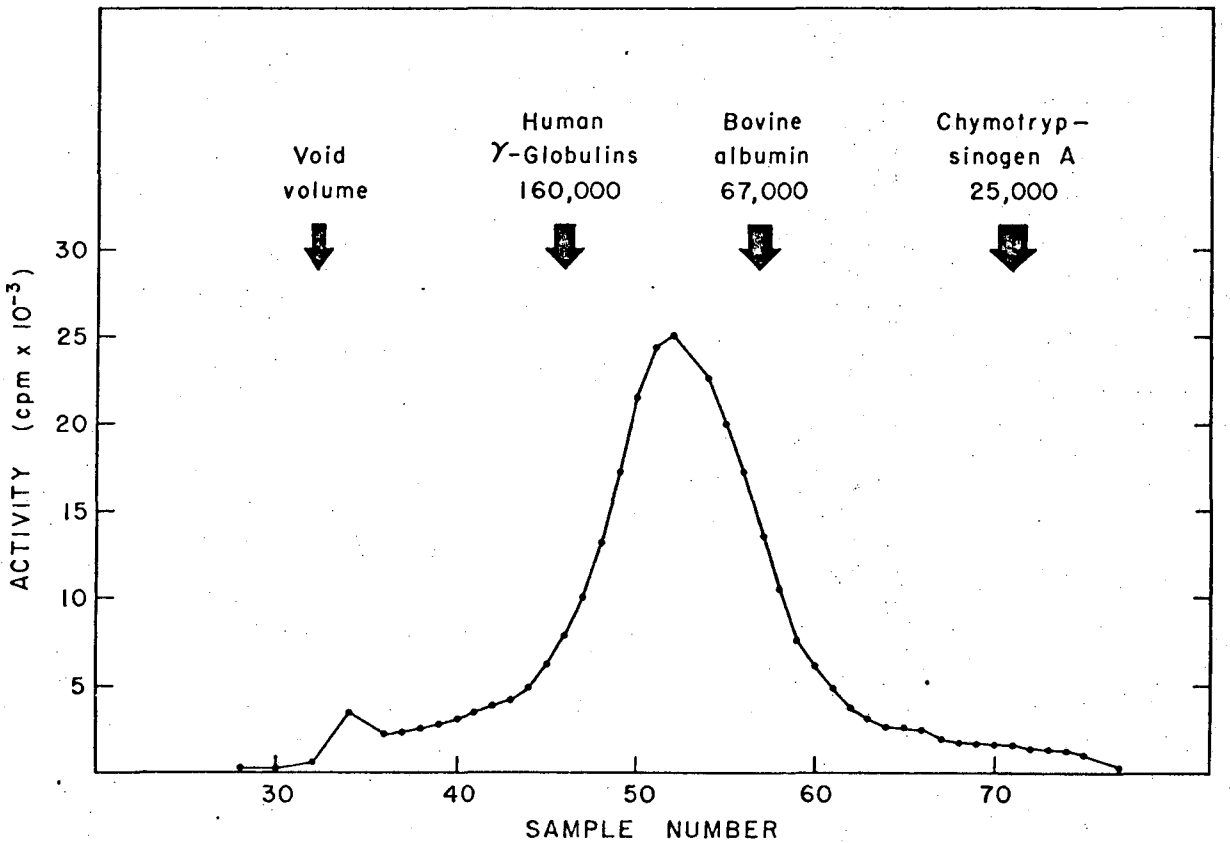
Void volume
Human γ-Globulins 160,000
Bovine albumin 67,000
Chymotrypsinogen A 25,000
ACTIVITY (cpm x 10⁻³)
30
25
20
15
10
5
30
40
50
60
70
SAMPLE NUMBER

Figure 2

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