RAT HEPATOMA DNA POLYMERASE:

PARTIAL ANALYSIS OF THE IN VITRO DNA PRODUCT

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SUMMARY

Earlier reports from this laboratory have demonstrated differences in primer preference for DNA polymerase isolated from normal rat liver vs. hepatoma. This study reports the initial analysis of DNA products formed following a two hour in vitro incubation of hepatoma DNA polymerase with native or denatured DNA. The radioactive products were analyzed by the nitrocellulose filter technique and by chromatography with hydroxylapatite and Bio-Gel A5. With native DNA primer the product had the properties of high molecular weight (ca. $4x10^6$) double-stranded DNA. With denatured primer the product had properties intermediate between native and denatured DNA with both high and lower molecular weight fragments.

Normal or regenerating rat liver and hepatomas differ in that DNA polymerase (DNA nucleotidyl transferase (E.C. 2.7.7.7)) activities of the former have a preference for native DNA primer, whereas the latter will utilize either native or denatured DNA primer. In a series of hepatomas showing various degrees of malignancy, there is a direct correlation between the rate of growth of the tumor and its DNA polymerase activity measured with denatured DNA as primer; the activity of the enzyme was 60-fold higher in the most rapidly growing hepatomas than in normal liver (1).

Determination of the significance of the preference by the polymerase for native or denatured DNA primer depends upon product analysis as well as purification and characterization of the enzyme(s). This paper reports initial steps in the analysis of DNA products formed in vitro by the reaction of hepatoma DNA polymerase with native and denatured DNA as primers.

Morris hepatoma 7777 was transplanted intramuscularly in male, Buffalo strain rats at Howard University, Washington, D.C., and the animals were shipped by air express. The rats were fed ad libitum and housed for at

least 48 hours before removal of the tumors.

DNA polymerase from hepatoma tissue was prepared, as described previously (2), by the Mantsavinos (3) method for rat liver. Purification of DNA polymerase was carried to the state of precipitation at pH 5.0. The assay conditions were similar to those described by Mantsavinos (3) and ^{3}H-deoxycytidine triphosphate was used as the radioactive substrate. Radioactivity was determined in a Beckman scintillation spectrometer.

The products formed during a two hour incubation of tumor polymerase with native or heat denatured DNA were analyzed by the nitrocellulose membrane filter technique of Nygaard and Hall (4). Following incubation the sample was deproteinized by extraction with an equal volume of a chloroform:isoamyl alcohol mixture (24:1 v/v) and the aqueous layer was dialyzed for 12 hours against 0.001 M phosphate buffer at 4° C. One-half of the dialyzed sample was used to measure the adsorption of the product to the nitrocellulose filter and the remainder of the sample was used to determine the TCA-insoluble radioactivity by precipitation with 10% TCA and filtration onto glass fiber filters (Whatman GF/C). The data in Table 1 show that when native DNA was used as primer most of the labeled product behaved like native DNA and was not adsorbed to the filter. However, heating this product at 90° C for 5 minutes increased the adsorption of labeled product from 7% to 65%. When heat-denatured DNA was used as the primer about 55% of the labeled product behaved like denatured DNA and was adsorbed to the nitrocellulose membrane.

The products of the two hour incubation of hepatoma pH 5.0 fraction with native and heat-denatured DNA primers were also analyzed by chromatography on hydroxylapatite columns according to the procedure of Bernardi (5). As shown in Figure 1A, with native DNA as primer all of the TCA-precipitable radioactivity and most of the OD_{260} absorbing material eluted as a narrow peak in fractions 18-21. Figure 1B shows that when heat-denatured DNA was used as primer most of the OD_{260} absorbing material eluted

Table 1. ANALYSIS OF PRODUCT BY NITROCELLULOSE FILTERS

	Retained by filter		Not retained by filter	
	Counts/min.	% of Applied counts	Counts/min.	% of Applied counts
Product of native primer	1,185	7	14,485	93
Product of heat-denatured primer	12,185	55	9,840	45

The incubation mixture contained 0.02 μmoles ^{3}H-deoxycytidine triphosphate (5μCi/μmole), 0.05 μmole each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate, 8 μmoles of MgCl, 20 μmoles of glycine buffer, pH 8.0, 0.5 μmoles of 2-mercaptoethanol, 100 μg calf thymus DNA and 0.2 mg pH 5.0 protein fraction in a total volume of 0.5 ml. Deoxynucleoside triphosphates were at saturating concentrations and the reaction was linear for at least 1 hour at 37° C with the addition of up to 0.4 mg of the pH 5.0 protein fraction. The amount of primer DNA added was calculated from the absorbance at 260 mu of native DNA. DNA was denatured by heating at 90° C for 5 minutes in the reaction tubes before addition of the enzyme and deoxynucleoside triphosphates. Denaturation resulted in a 34% increase in absorbance. Radioactivity was measured in a spectrafluor scintillator (4 g. 2,5-diphenyloxazole, 50 mg 1,4-bis (5-phenyloxazolyl-a) benzene per liter of toluene).

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at a lower phosphate concentration in fractions 11-17, and that most of the TCA-precipitable radioactivity eluted in a broad peak in fractions 11-21. Chromatography of control native and denatured radioactive DNA separated similar to that indicated by the optical density of calf thymus DNA used in the above experiment. Thus, it would appear from repeated experiments that the product from a two hour incubation of hepatoma pH 5.0 fraction with native DNA primer chromatographed on hydroxylapatite like native DNA, whereas that formed from incubation with heat-denatured DNA primer had properties intermediate between native and denatured DNA.

Gel filtration on Bio-Gel A5 (Bio-Rad, 100-200 mesh, 15 x 10^{6} m.w. exclusion limit) was employed to compare the size of the products formed during the two hour incubation of hepatoma pH 5.0 fraction with native and heat-denatured DNA primer. The data in Figure 2A show that when native DNA was used as primer, symmetrical, coincident peaks were obtained for TCA-precipitable radioactivity (product) and OD_{260} absorbing material (primer). When the material from the two hour incubation with native DNA

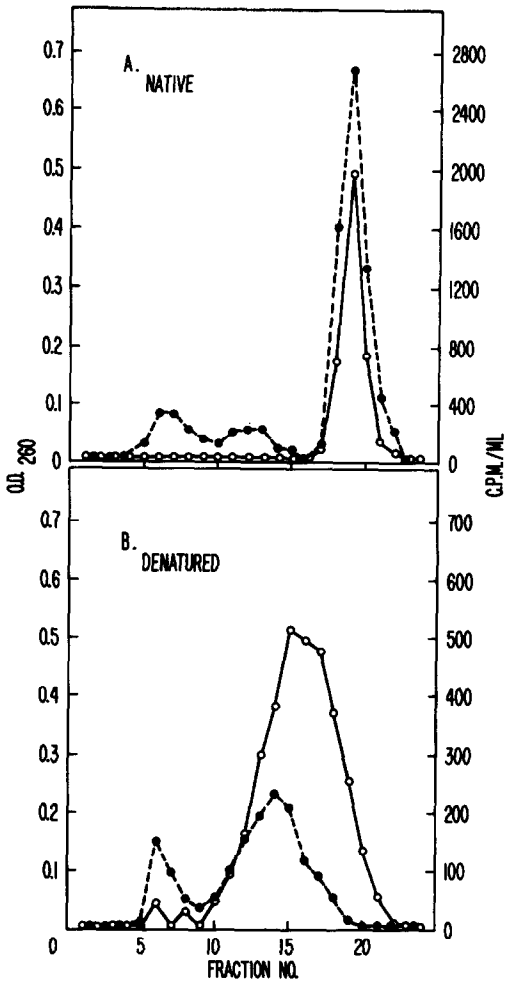

Fig. 1. Hydroxylapatite chromatography of the products from 2 hour incubations of hepatoma pH 5.0 fraction with (A) native and (B) heat-denatured calf thymus DNA primers. Incubation conditions were as described in Table 1. The incubated material was extracted with an equal volume of chloroform: isoamyl alcohol (24:1 v/v), dialyzed at 4° C for 16 hours against 0.001 M sodium phosphate buffer, pH 6.8. After washing with the equilibration buffer, the column was eluted with a linear gradient of 0.07 M - 0.03 M sodium phosphate at pH 7.0 and 1 ml fractions were collected. The OD_{260} was monitored and fractions were precipitated with 10% TCA, filtered on glass fiber filters and the radioactivity measured. Corrections for radioactivity due to terminal transferase were made by performing identical assays except that dGTP and dCTP were omitted from the incubation mixture. The pH 5.0 fraction did not significantly change the OC_{260}. Open circles, TCA-precipitable cpm/ml; closed circles, OD_{260}.

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was heated at 90° C for 5 minutes and quickly cooled in an ice bath before gel filtration, there occurred a slight shift of the peak for TCA-

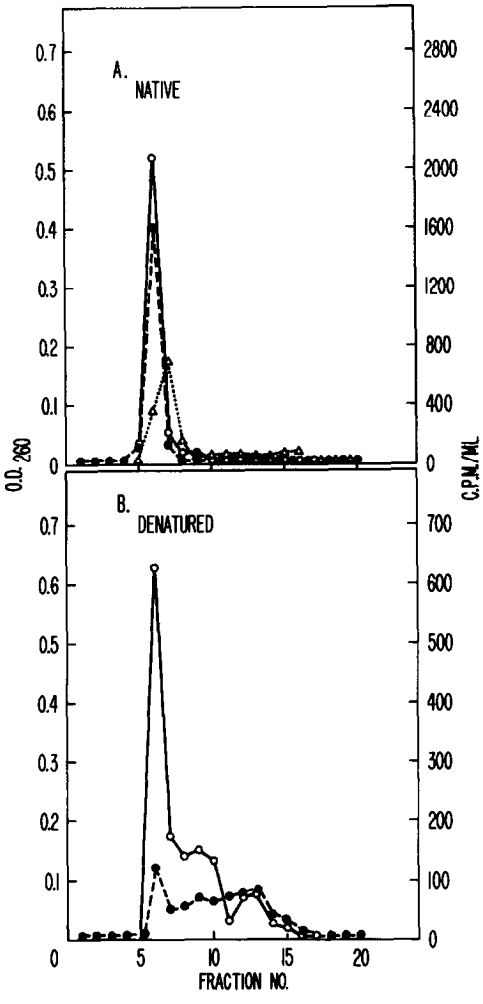

Fig. 2. Gel filtration on Bio-Gel A5 of the products from a 2 hour incubation of hepatoma pH 5.0 fraction with (A) native and (B) heat-denatured calf thymus DNA primer. The incubation conditions were identical to those described in Table 1. The incubated material was extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and dialyzed for 16 hours against SSC/10 (0.015 M NaCl - 0.0015 sodium citrate) at 4° C. Sample volumes of 0.3 ml were loaded onto 1 x 18 cm columns of Bio-Gel A5 (Bio-Rad, 100-200 mesh). SSC/10 was the equilibration and filtration buffer and 1 ml fractions were collected. The columns were run at room temperature, the OD_{260} monitored, fractions precipitated with 10% TCA and the radioactivity measured and corrected for terminal addition as described in Fig. 1. The above patterns were readily reproducible. Open circles, TCA-precipitable cpm/ml; closed circles, OD_{260}; triangles, TCA-precipitable cpm/ml for material heated at 90° C for 5 minutes following the regular 2 hour incubation.

precipitable radioactivity toward a lower molecular weight. Gel filtration of product and primer following a two hour incubation with heat-denatured

DNA demonstrated considerable trailing of lower molecular weight, TCA-precipitable radioactivity and OD_{260} absorbing material, in addition to the major peak of higher molecular weight material (Fig. 2B, fractions 5-7).

The molecular weight range of the gel filtration fractions was estimated by filtration of various substances of known molecular weights such as Blue Dextran, catalase, bovine serum albumin and deoxynucleoside triphosphates. The material giving rise to the peak of radioactivity in fractions 5-7 in Figure 2A was estimated to have a minimum molecular weight of 4×10^6. The TCA-precipitable radioactive material forming the shoulder between fractions 8 and 10 in Figure 2B was estimated to be 100-250,000 m.w. Thus, relatively high molecular weight products were obtained from the incubation of the hepatoma pH 5.0 fraction with native or heat-denatured DNA primer, although some lower molecular weight (100-250,000 m.w.) product was present when heat-denatured DNA was used as primer. The TCA-precipitable radioactivity of each fraction was corrected by subtracting the contribution caused by terminal nucleotide addition.

The results reported here indicate that a high molecular weight product is formed during a two hour period of in vitro synthesis with a DNA polymerase fraction from hepatoma 7777 and native DNA primer. Most of the product with heat-denatured DNA as primer is also high molecular weight, although some lower molecular weight DNA (100-250,000 m.w.) is produced. Hydroxylapatite chromatography indicates that the product synthesized with heat-denatured DNA as primer has properties intermediate to native and denatured DNA, a finding consistent with the nitrocellulose filter data. The observations are of interest in view of the evidence from bacterial studies that support a discontinuous synthesis of DNA (6,7). In interpreting these data the effect of low activities of deoxyribonucleases should be considered, particularly in that they might give rise to underestimation of product size. A possible contributory role of DNA repair in causing apparent DNA synthesis can not be excluded. We are currently further

purifying the DNA polymerase from rat liver and hepatoma in order to obviate these problems and also to determine the basis for the difference in primer preference by the polymerases obtained from normal and malignant tissues.

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