Separation of DNA Polymerase from Rat Liver and Hepatomas

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SUMMARY

Sephadex G-200 column chromatography of hepatoma DNA polymerase yields two peaks of enzyme activity. Peak I contains enzyme having a marked preference for denatured DNA, and the levels of this enzyme increase in proportion to tumor growth rate. Peak II contains the enzyme fraction having a moderate preference for native DNA. Normal and regenerating rat liver have a predominance of Peak II, in contrast to hepatomas. Peak I has been described as the major peak in fetal rat liver, and the present results are discussed in terms of repression of Peak I in adult liver and derepression which occurs as part of the process of malignant transformation.

INTRODUCTION

Rat hepatomas have been shown to have increased DNA polymerase activity (DNA nucleotidyl transferase, EC.2.7.7.7) when compared with normal liver (13). Both the polymerase activity preferring native DNA primer and that preferring denatured DNA primer are increased in rough proportion to the rate of tumor cell growth. In hepatomas, in contrast to normal or regenerating liver, the enzyme preferring denatured DNA primer has a greater activity than that preferring native DNA primer. This paper reports our attempts to separate these two enzyme activities and suggests the presence of a major DNA polymerase component in hepatomas which is barely detectable in normal liver.

MATERIALS AND METHODS

Materials

Thymidine 5'-triphosphate-3H, deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and deoxyctydine 5'-triphosphate were purchased from Schwarz BioResearch, Inc. Sephadex G-200 was a product of Pharmacia Fine Chemical, Inc., and calf thymus DNA was from Sigma.

Preparation and Source of pH 5.0 Protein Fraction

The pH 5.0 protein fraction was prepared by the method of Mantsavinos (6) from normal rat liver, regenerating rat liver, and tumor tissue. Partial hepatectomy (removal of 67% of the liver) was performed according to the method of Higgins and Anderson (3). Tumor-bearing rats were shipped by air express from Howard University, Washington, D. C., to Duke University, Durham, North Carolina. Animals were kept for at least 48 hours after arrival before the tumors were removed. The hepatomas, which were transplanted intramuscularly in male Buffalo strain rats, included 9633, 5123-D, and 7777. All rats were fed ad libitum, and they weighed 200–280 gm at the times of the experiments. Rats were killed by cervical dislocation, and the tumors were freed of necrotic and nontumorous tissue prior to homogenization. Regenerating rat liver was removed 24 and 48 hours after the operation. Experiments were timed so that tissues were removed at 10:00 A.M. in order to minimize diurnal variations. Enzyme prepared from the liver of normal rats gave the same results on Sephadex G-200 chromatography as did enzyme prepared from host liver.

Sephadex G-200 Gel Filtration

A Sephadex G-200 column was prepared according to Bellair (1). The pH 5.0 protein fraction was centrifuged at 5000 X g for 10 minutes to remove insoluble material and the supernatant adjusted to 1 M NaCl and 10% sucrose. After standing at 4°C for two hours, a sample containing 50–80 mg protein was applied to a 2.5-cm diameter column, packed with 150 ml Sephadex G-200. The column had been equilibrated with 0.02 M potassium phosphate buffer, pH 8.0, containing 1 M NaCl, and the same buffer was used for elution. The flow rate was approximately 15 ml per hour and 5-ml fractions were collected. Absorbance was determined at 260 and 280 nm, and the fractions were dialyzed against 0.02 M potassium phosphate buffer, pH 9.0, for four hours. Protein determinations were done by the method of Lowry (5), and the samples were assayed for DNA polymerase activity.

DNA Nucleotidyltransferase

The assay procedures were the same as previously described (13). The protein concentration of the enzyme fraction added varied between 0.05 and 0.200 mg. The assay was found to be

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proportional with respect to time up to 60 minutes and with respect to enzyme concentration over the range used in these experiments. Deoxyribonucleoside triphosphate concentrations were at saturating levels throughout.

Terminal Transferase Assay

The assay conditions were as described for the DNA nucleotidytransferase determination except that deoxyguanosine \(5'-\text{triphosphate} \) and deoxycytidine \(5'-\text{triphosphate} \) were omitted from the incubation mixture.

RESULTS

Sephadex G-200 chromatography of the pH 5.0 protein fraction resulted in a typical elution pattern shown in Chart 1. The ratio of A\(260 \, \text{mg} / A\(280 \, \text{mg} \) is about 1 in the first peak, <1 in the trough, and >1 in the second peak. This chart also shows the protein concentration of the fractions as measured by the Lowry method. An early protein peak appears, coinciding with the first peak of absorbance; the following fractions show a steady decline in protein concentration and have no correlation with the second absorbance peak. This elution pattern is in agreement with that reported by Bellair (1) and remained essentially constant for all other samples tested.

The results presented in Chart 2 (A, B, and C) show the DNA polymerase activity of Sephadex G-200 elution patterns of the pH 5.0 protein fractions prepared from normal rat liver and 24-hour and 48-hour regenerating rat liver. There was little detectable activity in the region of the first peak. The second peak shows DNA polymerase activity with native and denatured primer, with a preference for native primer over denatured primer of 2:1, 1.5:1, and 1.3:1 for normal rat liver, 24-hour regeneration, and 48-hour regeneration respectively. Chart 2 also shows a progressive increase in DNA polymerase activity with native and denatured primer (Peak II) from normal rat through 24 and 48 hours of liver regeneration, a finding that was to be expected from our previous results (11).

DNA polymerase activity in the Sephadex G-200 chromatography fraction of three transplantable hepatomas is shown in Chart 2 (D, E, and F). The polymerase activity of the pH 5.0 protein fraction for each of these hepatomas was listed in the preceding paper (13). The hepatomas were of the slow (D), medium (E), and fast (F) growing types. The charts show an early peak (I) of DNA polymerase activity preferring denatured primer. This peak increases markedly with increasing growth rate of the tumors and was much less pronounced in the chromatography patterns of either normal rat liver or regenerating liver pH 5.0 protein fraction. The second peak of DNA polymerase activity, which contains the bulk of activity in normal and regenerating rat liver, is also present in hepatoma samples. This peak has a preference for native vs denatured primer at approximately the same ratio as that determined for normal and regenerating liver. This second peak also increases with increasing growth rate of the hepatomas. Neither of the two peaks of DNA polymerase activity coincide with the peaks of ultraviolet absorbance, the peak absorbance fractions being found in Tubes 8 and 17 and the peak fractions for DNA polymerase activity occurring in tubes 10 and 16 respectively. Minimal absorbance and no DNA polymerase activity were found in Fractions 1-5 and in fractions beyond Tube 20. Chart 3 shows DNA polymerase activities expressed as cpm/hour rather than \(\mu\)g per mg protein/hour, to compare with data previously presented by Bellair (1) for regenerating liver. The results obtained for DNA polymerase activity with native primer are given in Chart 3B; those showing DNA polymerase activity with denatured primer are in Chart 3A. Bellair found considerable DNA polymerase activity with denatured primer in the first peak in a sample from regenerating rat liver. As can be seen from Chart 3A, we found little of this activity in peak 1 in normal rat liver and regenerating liver preparations.

The pH optima for both fractions with native and denatured primer are shown in Chart 4. The pH optimum is the same for both fractions but differs with respect to primer. Denatured primer has a pH optimum of 8.0 as against an optimum of pH 7.0 with native primer. The pH indicated is the actual pH of the reaction mixture, which is about 0.5 pH units lower than the pH of the buffer added. For routine assays we used a buffer at pH 8.0 which resulted in a pH of 7.5 for the final reaction mixture. The optimal magnesium requirement for both enzyme fractions is 12-16 millimoles.

As can be seen from Table 1, fraction I has a low (less than 10%) terminal transferase activity. Fraction II incorporates a substantial amount (50%) of thymidine \(5'-\text{triphosphate}^-{\text{3H}} \) if deoxyguanosine \(5'-\text{triphosphate} \) and deoxycytidine \(5'-\text{triphosphate} \) are omitted. Whether this represents terminal transferase

DNA Polymerase Separation

Chart 1. Chromatography of a normal rat liver pH 5.0 protein fraction on Sephadex G-200. Protein concentration is expressed as mg protein per ml.
activity exclusively is not known at present. Contrary to reports by Bellair, we still find deoxyribonuclease activity in almost all fractions if denatured DNA is used as a substrate. Very little detectable activity is found with native DNA as a substrate. As was the case with the pH 5.0 protein fraction (11, 13), there is more of this activity associated with column fractions from normal and regenerating rat liver than with preparations from hepatomas. The deoxyribonuclease activity was found in most fractions and did not increase in the two peaks of DNA polymerase activity.

DISCUSSION

The initial observations of high DNA polymerase activity in a large series of hepatomas were performed on crude enzyme preparations in the presence of other enzymes, including deoxyribonucleases (13). The present study demonstrates two separate peaks of DNA polymerase activity following the chromatographic separation of extracts obtained from hepatomas. The first peak had a marked preference for denatured DNA, the second a moderate preference for native DNA.
Normal rat liver had a predominance of the second peak with only small amounts of activity in the region of the first peak. During regeneration of normal rat liver there is a marked increase in DNA polymerase activity (2, 11). However, this increased activity resembles that of normal rat liver and not that of hepatomas. Thus, although total DNA polymerase activity of regenerating liver far exceeds that of slow-growing hepatomas, the enzyme separation patterns are qualitatively distinct. It seems likely, therefore, that the increased presence of enzyme having the characteristics of Peak I is not merely a nonspecific change attributable to a rapidly growing (DNA synthesizing) tissue. Peak I is increased, in fact, in several slow-growing renal tumors and human chronic lymphocytic leukemic cells in which the rate of thymidine incorporation into DNA is normal or even less than normal (unpublished data).

During the final preparation of this paper, T. Ono kindly sent us two then-unpublished manuscripts relating to our work (4, 10). Many of the results have been confirmed in both laboratories; there are, however, major discrepancies. Both laboratories show that hepatoma DNA polymerase prefers denatured primer and that this enzyme activity increases with increasing growth rate of the tumors. Both laboratories also show that at least two peaks of DNA polymerase activity can be resolved by Sephadex gel filtration. One difference in the results concerns level and preference of DNA polymerase in slow (minimal deviation) hepatomas. Iwamura and Ono found that some hepatomas have lower polymerase levels than control livers and that DNA polymerase shows the same preference for native primer as does polymerase from normal rat liver. We found some increase in DNA polymerase activity even for slow hepatomas and a definite preference for denatured primer. Ono’s group further reported that regenerating rat liver polymerase shows a preference for denatured primer (10). Several laboratories (1, 7, 8), including our own, have found that DNA polymerase from regenerating rat liver still maintains a marked preference for native primer although there is also a small increase in the enzyme preferring denatured DNA. It is difficult to compare the enzyme separations since different conditions were used in the two laboratories. Our elution pattern agrees closely with that of Bellair (1) whose methods were used. We have an internal consistency in that the relative proportion of native vs denatured polymerase activity is the same before and after column fractionation.

There are several important differences in methodology which might account for some of the discrepancies. Ono’s group began with a high speed supernatant fraction rather than the pH 5.0 preparation which contains considerably less nuclease activity. Ono’s group apparently worked with small amounts of enzyme and had low activities in their column fractions as indicated by the counts incorporated. Differences
in the amount of protein fraction assayed can alter the apparent ratio of activity with native:denatured primer, presumably due to differential instability in low protein solutions (unpublished data). With respect to the preference of regenerating rat liver DNA polymerase for native or denatured primer, the above-mentioned authors reported an enzyme preparation having a preference for denatured DNA primer after Sephadex fractionation (4) and a preference for native DNA primer when isolated on a phosphocellulose column (10). The latter procedure involves a progressive elution with phosphate buffer. Our studies show that phosphate concentration does influence the relative priming efficiency of native and denatured DNA (unpublished data). Furthermore, variations in pH optimum for the two enzyme activities, as shown above, will influence the apparent ratio of denatured to native polymerase activity. Our assays were performed at pH 7.5, intermediate between the two optima.

The distinct first peak of enzyme activity could represent a high molecular weight aggregate of DNA polymerase. This appears unlikely on the basis of preliminary determinations of molecular weights. It is also possible that the low activity in the first peak is due to the presence of a specific inhibitor present in normal and regenerating liver and absent in hepatoma. There is no evidence that bears on the possible presence of a stable inhibitor. From the differences in template preference it would appear that there are at least two distinct polymerase enzymes. Experiments are in progress which should clarify the question of whether the polymerase preferring native DNA of each peak is identical and similarly for the polymerase preferring denatured DNA.

The significance of the relatively specific increase in denatured DNA polymerase activity in hepatomas is not known. Polymerase preferring denatured DNA primer is increased in human leukemic cells (12) and in a variety of other rodent and human tumors as will be detailed separately. Its presence is independent of the gross chromosomal changes characteristic of many tumors since the enzyme peak is also found in hepatomas known to have normal karyotypes [9618A, 7800, 7794A, 9121 (9)]. Polymerase preferring denatured DNA also appears to be characteristic of fetal liver (10), a finding which has been confirmed in our laboratory. This must be interpreted with caution since fetal liver is largely a hematopoietic organ; however, similar DNA polymerase development occurs in nonhematopoietic tissues (unpublished data). It is interesting to speculate, as Potter has done for other enzymes (14), that denatured DNA-preferring polymerase is repressed and native activity is expressed in the adult rat liver and that the enzyme activity of the former is derepressed as a characteristic biochemical feature of malignant transformation. The role of these enzymes during in vivo DNA synthesis is currently under investigation.

ACKNOWLEDGMENTS

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REFERENCES


Table 1

<table>
<thead>
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<th>Sephadex G-200 fraction</th>
<th>cpm/hour</th>
<th>Native primer</th>
<th>Denatured primer</th>
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<tbody>
<tr>
<td>Fraction 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>1194</td>
<td>5227</td>
<td></td>
</tr>
<tr>
<td>Without (dGTP, dCTP)</td>
<td>114</td>
<td>495</td>
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</tr>
<tr>
<td>Fraction 2</td>
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<td></td>
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</tr>
<tr>
<td>Complete</td>
<td>1893</td>
<td>1358</td>
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<tr>
<td>Without (dGTP, dCTP)</td>
<td>788</td>
<td>611</td>
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Terminal transferase activity in Fractions 1 and 2. The three most active fractions of each peak were pooled and 0.15 mg protein from Fraction 1, or 0.10 mg protein from Fraction 2, were assayed. Hepatoma 7777 was the source of enzyme in this experiment. Counts incorporated represents total counts without subtraction of terminal transferase activity. dCTP, deoxyctydine 5'-triphosphate; dGTP, deoxyguanosine 5'-triphosphate.
DNA Polymerase Separation


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