Studies on the Activity of the A Particle-associated DNA Polymerase

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SUMMARY

The DNA polymerase activity in preparations of A particles from mouse myeloma has been further characterized. Most of the radioactive product from reactions containing poly(rA)·(dT)12-18 and dTP·3H was composed of dTMP·3H in the form of nuclease-sensitive macromolecules with an average density of 1.428 g/ml, determined in cesium sulfate gradients at neutral pH; the product therefore possessed characteristics of poly(dT). Approximately 50% of this product was similar in size to the template, poly(rA), while the remainder was of lower molecular weight. Studies on the kinetics of the poly(rA)·(dT)12-18-dependent incorporation revealed that enzyme activity was optimal at 37°C, with an apparent activation energy of 25.2 kcal/mole, and was higher at the optimal concentration of Mg2+ than with Mn2+ even at Mn2+ concentrations below 0.2 mM. As observed previously, poly(rA)·(dT)12-18 was the most effective template-primer for promoting deoxynucleotide incorporation. However, incorporation was also observed in reactions containing poly(rI)·(dC)12-18 and dCTP·3H, but not in reactions either without template-primer or in those containing the other combinations of deoxynucleotide and template-primers tested. The optimal ratio of µg poly(rA) to µg (dT)12-18 was 6 to 1, and higher levels of (dT)12-18 inhibited dTMP incorporation approximately 50%. In the presence of a 1:1 ratio of poly(rA) to (dT)12-18, highest activity was observed in the range of 0.5 to 6 µg each per reaction.

Studies on the specificity of an enzymatic assay for A particle DNA polymerase revealed that a crude homogenate from myeloma exhibited activity, and mixing of this homogenate with A particles resulted in no change in the activity of the A particle preparation. In contrast, a crude homogenate from myeloma exhibited activity, and mixing of this particle DNA polymerase revealed that a crude homogenate of mouse intracisternal type A particles contained DNA polymerase activity and the reaction product was analyzed. In addition to providing more information about the reaction properties of the A particle DNA polymerase, these studies were undertaken in order to find reaction conditions suitable for its quantitative assay in crude tissue fractions without interference from other DNA polymerases and enzymes.

INTRODUCTION

Previous studies have demonstrated (13) that preparations of mouse intracisternal type A particles contained DNA polymerase activity. On the basis of the reaction properties and template-primer preference of the DNA polymerase activity, it was suggested that the enzyme was distinct from previously recognized DNA polymerases. In this study, additional reaction properties of the A particle DNA polymerase activity were determined and the reaction product was analyzed. In addition to providing more information about the reaction properties of the A particle DNA polymerase, these studies were undertaken in order to find reaction conditions suitable for its quantitative assay in crude tissue fractions without interference from other DNA polymerases and enzymes.
DNA Polymerase Assay

DNA polymerase activity was a measure of the capacity of preparations to incorporate tritium-labeled deoxynucleoside-5'-triphosphate into cold acid-insoluble material. A particles and other enzyme preparations were thawed immediately before use, mixed with 1 volume of BPA, 20 mg/ml, and then 1 volume of this extract: BPA solution was mixed with 9 volumes 60% glycerol. Then 9 volumes of the extract: glycerol: BPA solution was mixed with 1 volume of Solution A (1.25 mM Tris-HCl, pH 8.3, at 37°, 1.0 mM KCl, 313 mM magnesium acetate, 25 mM dithiothreitol, and 0.25% Nonidet P-40).

Unless otherwise specified, reactions contained (in a final volume of 0.025 ml): 50 mM Tris-HCl buffer, pH 8.3, at 37°, 18.7% (v/v) glycerol; 0.01% (v/v) Nonidet P-40; 250 mM KCl; 12.5 mM magnesium acetate; 1 mM dithiothreitol; BPA, 360 mg/ml; 0.5 mM ATP; 10 μg nucleoside diphosphokinase; poly(rA) and (dT)12-18, 230 μg/ml each; 1 to 3 μg enzyme extract protein; and 0.5 mM dTTP-3H (205 to 310 cpm/pmol). All reaction components except nucleotides and nucleoside diphosphokinase were preincubated at 37° for 20 min in silicon-treated soft glass tubes (10 x 75 mm). Reaction mixtures were then transferred to a 0 to 1° ice-water bath, and the remaining components were added. Incubations were then carried out at 37° for 75 min. Reactions were terminated by transferring the tubes to Dry Ice and then to a 0 to 1° ice-water bath, where 100 μg of BPA and then 1.5 ml of Solution B [100% trichloroacetic acid: 10 mM thymidine: saturated sodium pyrophosphate: saturated sodium orthophosphate (1:1:4:4)] were added at 4°. Contents of each tube were mixed and, after 15 min at 0°, tubes were centrifuged at 7000 x g for 10 min and the supernatant was carefully withdrawn and discarded. The precipitate was suspended in approximately 1 ml of Solution B at 4° with vigorous mixing, and then insoluble material was collected on a Millipore nitrocellulose filter (0.45-μm pore size) and washed with approximately 40 ml of 10% trichloroacetic acid at 4°. The filters were transferred to a scintillation vial and dissolved in 1 ml of methyl cellosolve (11) at 24°. Ten ml of Triton X-100: toluene: Permafluor (32:64:4) (Packard Instrument Co., Downers Grove, Ill.) scintillation mixture were then added and, after thorough mixing, radioactivity was determined at a counting efficiency of 15 to 17% in a liquid scintillation counter.

Product Identification

Chromatographic Analysis of Digested Reaction Products. The cold acid-insoluble product, obtained from reactions containing dTTP-3H as substrate and poly(rA). (dT)12-18 as the template-primer was collected and then treated with DNase I, 100 μg/ml, for 1.5 hr at 37° in 50 mM potassium phosphate buffer, pH 7, containing 10 mM magnesium acetate. Then snake venom phosphodiesterase I (EC 3.1.4.1) (VPH; Lot No. 1G4; Worthington Biochemical) was added to a final concentration of 1 mg/ml, and the mixture was incubated at 37° for an additional hr. The reaction was terminated by the addition of 2 volumes of 100% methanol. The digested (6) product was then characterized by means of thin-layer chromatography. A portion of the digest was applied to polyethyleneimine cellulose 300 (Brinkman Instruments Co., Westbury, N. Y.) and developed successively in 0.5 M LiCl for 5 min, 1 M LiCl for 10 min, and 1.5 M LiCl for 35 min (System I)(10). Thymidine, TMP, and TTP, lithium salts, were run as markers. In the other 3 solvent systems used, portions of the digested product were applied to cellulose on glass plates (Brinkman), and TMP and TTP, lithium salts, were run as markers. For the solvent systems consisting of ethanol:1.0 mM ammonium acetate, pH 7.85 (7:3) (System II) (9), and ethanol: glacial acetic acid: 2 M ammonium acetate, pH 3.8 (7:1.5:1.5) (System III), chromatograms were developed for 10.5 hr. In System IV, the 1st dimension was developed in 2-propanol: 1% ammonium sulfate (2:1) (7) for 1.5 hr, and the 2nd dimension was developed in 60% ammonium sulfate in 0.1 M potassium phosphate buffer: 2-propanol, pH 6.8 (100: 2) (3) for 9 hr. Following each of the 4 chromatographic separations, the markers were located with the use of UV. Chromatograms were then cut into 1 x 2-cm pieces, each was placed in a glass scintillation vial, and radioactivity was determined after the addition of 10 ml of Triton X-100: toluene: Permafluor scintillation mixture.

Rate-Zonal Sedimentation of NaOH-treated, SDS-treated Reaction Products

A reaction containing A particles, poly(rA). (dT)12-18, and dTTP-3H, as described above, was incubated for 75 min at 37°. A 10-μl portion containing 2300 cpm of cold acid-insoluble material was mixed with 0.5 ml of a solution containing 100 mM NaCl: 1 mM EDTA: 50 mM Tris-HCl buffer: 0.2% SDS: 0.5 mM NaOH (final pH 12) and then was incubated at 80° for 60 min. The solution was then layered over a 4.6-mL 5 to 25% linear sucrose gradient at pH 11.8 containing 100 mM NaCl: 1 mM EDTA, 0.2% SDS, 50 mM Tris-HCl buffer, and approximately 0.2 mM NaOH. Gradients were centrifuged at 50,000 rpm for 135 min at 17° in an SW 50.1 rotor. Twenty gradient fractions of equal volume were collected from below, and cold acid-insoluble radioac-
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activity was measured as described above. For analysis of poly(rA), a sample containing 100 μg was mixed with 0.5 ml of a solution containing 100 mM NaCl:1 mM EDTA:50 mM Tris-HCl buffer, pH 7.2:0.2% SDS, and the resulting solution was layered over a 4.6-ml, 5 to 25% linear sucrose gradient and centrifuged as described above except that the gradient solutions contained no NaOH and were at pH 7.4. Absorbance of fractions at 260 nm was determined after the addition of 400 μl H2O.

RESULTS AND DISCUSSION

Studies on the relation between time of incubation and incorporation of radioactivity revealed an initial linear phase of incorporation, similar to that observed previously (14), and no significant change in the amount of radioactive product during the period of 3 to 10 hr of incubation. Incorporation of radioactivity during the 1st 2 hr of incubation was equal to 0.4% of the amount of dTTP-3H and 0.5% of the amount of poly(rA) present at the beginning of reactions. The relationship between temperature of incubation and rate of incorporation is shown in Chart 1. Activity was maximal at 37°. Analysis of these data by an Arrhenius plot revealed a linear relationship for the values obtained at temperatures higher than 0° and an activation energy of 25.2 kcal/mole.

The relationship between Mn2+ concentration and enzyme activity was studied in order to determine the effect of Mn2+ concentrations lower than 0.5 mM, which were not examined previously (14). As shown in Table 1, Mn2+ was less effective than Mg2+ over a wide range of concentrations, including those lower than 0.5 mM. Alteration in the order of addition of MnCl2 to the reaction mixture did not increase its effectiveness.

Properties of the Radioactive Reaction Product. Analysis of the radioactive substrate dTTP-3H by thin-layer chromatography revealed that approximately 4% of the total radioactivity was in material other than authentic dTTP. Since incorporation usually corresponded to less than 1% of the radioactive substrate present at the beginning of incubations, it was important to identify the radioactive molecule that had been incorporated. Thus, the radioactive reaction product was isolated, digested with DNase I and venom phosphodiesterase, and characterized by thin-layer chromatography. The results are shown in Table 2. In each solvent system used, most of the radioactive product recovered was chromatographically indistinguishable from authentic TMP. No other discrete components were detected.

In further experiments, the acid-insoluble dTMP-3H-containing product was rendered acid soluble by treatment with DNase I or 10% trichloroacetic acid at 95°, but was resistant to treatment with 0.5 N NaOH: trypsin, 1 mg/ml;

### Table 1

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Amount of dTMP-3H incorporated/reaction (pmoles/min x 10^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn2+</td>
<td>0.05</td>
</tr>
<tr>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>0.10</td>
<td>0.57</td>
</tr>
<tr>
<td>0.18</td>
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</tr>
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<td>0.25</td>
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<tr>
<td>0.50</td>
<td>4.97</td>
</tr>
<tr>
<td>1.00</td>
<td>2.5</td>
</tr>
<tr>
<td>Mg2+</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>66.9</td>
</tr>
</tbody>
</table>

### Chart 1

The effect of incubation temperature upon the amount of dTMP-3H incorporated. Each reaction was incubated for 75 min at the temperature indicated and contained the components described in "Materials and Methods." The amounts of radioactivity applied to the chromatograms were 12,000 cpm in System I and 2388 cpm in Systems II, III, and IV.

### Table 2

<table>
<thead>
<tr>
<th>Chromatographic system</th>
<th>% of radioactive product with Rf of authentic dTMP</th>
<th>% of applied radioactivity recovered</th>
<th>Rf dTMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>82</td>
<td>94</td>
<td>0.66</td>
</tr>
<tr>
<td>II</td>
<td>87</td>
<td>94</td>
<td>0.30</td>
</tr>
<tr>
<td>III</td>
<td>80</td>
<td>86</td>
<td>0.22</td>
</tr>
<tr>
<td>IV (1st dimension)</td>
<td>76</td>
<td>77</td>
<td>0.43</td>
</tr>
<tr>
<td>IV (2nd dimension)</td>
<td>76</td>
<td>77</td>
<td>0.50</td>
</tr>
</tbody>
</table>
and RNase A, 1 mg/ml. Rate-zonal centrifugation in alkaline sucrose gradients of the radioactive product, after it was treated with 0.5 N NaOH and with SDS, revealed 2 peaks of radioactivity of approximately equal size. The faster moving peak sedimented at 8 to 10 S, in coincidence with the sedimentation in neutral gradients of the template, poly(rA), while the slower moving peak sedimented at 2 to 4 S. Isopycnic centrifugation of the base-treated radioactive product in neutral Cs2SO4 gradients revealed a broad peak at an average density of 1.428 g/ml, a value identical to that observed with samples of authentic poly(dT). Thus, the dTMP-3H-containing reaction product possessed chemical and physical properties of poly(dT) of molecular weight not greater than the poly(rA) template.

Some Properties of the Poly(rA)-(dT)$_{12-18}$-dependent dTMP-3H Incorporation. The A particle-associated DNA polymerase activity was dependent upon the presence of the sulfhydryl reducing reagent in reaction mixtures; 2 mM glutathione and 5 mM 2-mercaptoethanol were less effective than 1 mM dithiothreitol. Enzyme activity was completely inhibited in the presence of 2 mM N-ethylmaleimide.

No incorporation of dTDP was observed in reactions that did not contain the nucleoside triphosphate regenerating system, ATP and nucleoside diphosphokinase. However, incorporation was observed in reactions containing TDP when the regenerating system was included. The effect of antiserum directed against Rauscher MuLV upon A particle activity was assessed previously (14) under reaction conditions more favorable for MuLV activity where A particle activity was very low (<5% of optimal). In the current study, the maximal A particle activity was also found to be uninhibited. Under the reaction conditions used for this experiment, activity of MuLV was inhibited by 89%.

Effect of Template-Primers. The relation between poly(rA) and oligo(dT) concentrations and the rate of enzyme activity was investigated in the experiment shown in Chart 2. Activity was completely dependent upon (dT)$_{12-18}$ and increased in hyperbolic fashion between the concentrations of 0.3 and 1 μg/reaction (Chart 2A). As oligo(dT) concentration was increased above this level, however, less activity was observed. At the optimal concentration of 1 μg/reaction, the average molar ratio of (dT)$_{12-18}$ to poly(rA)

Table 3

Incorporation of deoxynucleotides in the presence of RNA templates and DNA primers

<table>
<thead>
<tr>
<th>Template-primer</th>
<th>Tritium-labeled deoxynucleotides</th>
<th>12.5 mM Mg$^{++}$; 250 mM KCl</th>
<th>0.5 mM Mn$^{++}$; 75 mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minus A particles</td>
<td>Plus A particles</td>
<td>Minus A particles</td>
</tr>
<tr>
<td>None</td>
<td>dATP</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Poly(rA)-(dT)$_{12-18}$</td>
<td>dTTP</td>
<td>0.3</td>
<td>65.6</td>
</tr>
<tr>
<td>Poly(rC)-(dG)$_{12-18}$</td>
<td>dGTP</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Poly(rU)-(dA)$_{12-18}$</td>
<td>dCTP</td>
<td>0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Amount of radioactive nucleotide incorporated/reaction (pmoles)

<table>
<thead>
<tr>
<th>Template-primer</th>
<th>Tritium-labeled deoxynucleotides</th>
<th>12.5 mM Mg$^{++}$; 250 mM KCl</th>
<th>0.5 mM Mn$^{++}$; 75 mM KCl</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Minus A particles</td>
<td>Plus A particles</td>
<td>Minus A particles</td>
</tr>
<tr>
<td>None</td>
<td>dATP</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Poly(rA)-(dT)$_{12-18}$</td>
<td>dTTP</td>
<td>0.3</td>
<td>65.6</td>
</tr>
<tr>
<td>Poly(rC)-(dG)$_{12-18}$</td>
<td>dGTP</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Poly(rU)-(dA)$_{12-18}$</td>
<td>dCTP</td>
<td>0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* No unlabeled deoxynucleotides were present in reaction mixtures.
* 0.027 mM dATP-3H, dCTP-3H, and dGTP-3H (6850 cpm/pmole); 0.1 mM dTTP present in reaction mixtures.
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was approximately 33 to 1. In reactions containing 6 µg (dT)12,18 (Chart 2B), activity was not completely dependent upon poly(rA), but did increase as the concentration of poly(rA) was increased. In the study shown in Chart 2C, activity was dependent upon poly(rA)-(dT)12,18 (1:1 by weight), was maximal between 0.5 and 6 µg/reaction, and was inhibited at concentrations higher than 6 µg/reaction.

The amount of A particle DNA polymerase activity in the presence of a set of synthetic RNA templates with complementary DNA primers was investigated in the experiment shown in Table 3. Reactions were performed under 2 types of reaction conditions, i.e., conditions optimal for A particle preparation poly(rA)-(dT)12,18-directed activity (12.5 mM magnesium acetate: 250 mM KCl) and conditions optimal for poly(rA)-(dT)12,18-directed activity of Rauscher MuLV DNA polymerase (0.5 mM MnCl₂: 75 mM KCl). Poly(rA)-(dT)12,18 was the most effective template-primer under both types of reaction conditions; however, significant deoxynucleotide incorporation was also observed in reactions containing poly(rI)-(dC)12,18. Deoxynucleotide incorporation in the presence of A particles and either poly(rC)-(dG)12,18 or poly(rU)-(dA)12,18 was not significantly higher than in reactions without A particles. Enzyme-dependent incorporation also was not observed in reactions without template-primer.

Specificity of the Assay for the A Particle-associated DNA Polymerase. The results of this study confirm and extend previous findings of DNA polymerase in A particle preparations (14, 15). In addition to characterizing the activity of the enzyme and documenting its existence, these studies were performed so that reaction conditions could be selected for its quantitative measurement in crude extracts. The specificity of the assay for the A particle DNA polymerase activity was investigated in the experiments shown in Table 4. A crude homogenate from mouse myeloma MOPC-104E possessed low enzyme activity that was proportional to the amount of homogenate in reactions, and this homogenate had little effect on the activity of A particles when the 2 samples were mixed. Activity was not detected in a crude homogenate from mouse liver, and the liver extract also had no effect on the activity of the A particle preparation. Adult mouse liver is known to be devoid of or to contain only extremely low numbers of A particles (12). The specificity of this DNA polymerase assay indicates its use in investigating both the subcellular distribution of the DNA polymerase in A particle-containing cells and its existence in various other tissues (13).

REFERENCES

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