Studies on the Synthesis of Deoxyribonucleic Acid by Mammalian Enzymes

II. AN INVESTIGATION OF THE PRIMER REQUIREMENTS OF PARTIALLY PURIFIED REGENERATING RAT LIVER DEOXYRIBONUCLEIC ACID NUCLEOTIDYLTRANSFERASE*

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ROGER MANTSAYNOS† AND BENJAMIN MUNSON‡

From the Department of Biochemical Pharmacology, School of Pharmacy, and the Department of Biochemistry, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214

SUMMARY

A study has been made of the deoxyribonucleic acid primer requirements of partially purified deoxyribonucleic acid deoxynucleotidyltransferase fractionated from regenerating liver of the adult rat. Native, double stranded DNAs derived from either viral, bacterial, or mammalian sources serve more effectively as primers than either single stranded or heat-denatured DNAs for the incorporation of deoxyribonucleoside 5'-triphosphates into polydeoxyribonucleotides. The highly ordered, helical, alternating, copolymer consisting of deoxyadenylate and deoxythymidylate residues was found to be the best primer tested. Destruction of the secondary structure of native DNA primers by either controlled acid or alkaline denaturation or by thermal means decreases priming activity. Additionally, changes in the primary structure of native DNA primers by limited enzymic treatment also altered their priming abilities. Crystalline bovine pancreatic deoxyribonuclease, which cleaves DNA to yield 3'-hydroxyl and 5'-phosphoryl end groups, produced stimulation in priming activity, whereas bovine spleen and micrococcal endonucleases, which produce 3'-phosphoryl and 5'-hydroxyl termini, decrease the priming activity of native DNA primers. Heating and rapid cooling of the primers partially degraded enzymically results in a further reduction of priming activity.

In a previous publication from this laboratory the basic properties and requirements of a partially purified deoxyribonucleic acid polymerase (deoxynucleoside triphosphate : DNA deoxynucleotidyltransferase, EC 2.7.7.7) from the regenerating liver of the adult rat were described (1). As in the case of the DNA polymerases purified from normal mammalian sources, calf thymus DNA polymerase (4, 5), and the enzyme from regenerating liver of the rat (1), this polymerase requires magnesium ions, a DNA primer, and the 5'-triphosphates of deoxymethylidine deoxyadenosine, deoxycytidine, and deoxyguanosine for activity (1). The DNA primer is an important component of enzyme systems involved in DNA synthesis since it determines the chemical composition and base sequence of the newly synthesized DNA. Most DNA polymerases can utilize double stranded, single stranded, or denatured DNA primers in vitro although the actual physicochemical state of the DNA primer during the process of replication in vivo is unknown. Presently, two DNA polymerases have been partially purified from normal mammalian sources, calf thymus DNA polymerase (4, 5), and the enzyme from regenerating liver of the rat (1). In this communication, evidence is presented showing that the DNA deoxynucleotidyltransferase from regenerating rat liver preferentially utilizes native, double stranded DNA as a primer much more effectively than either single stranded or denatured DNAs and that changes in either the secondary or primary structure of helical primers, by chemical, physical, or enzymic means, alters their priming activity in vitro. In contrast, purified calf thymus DNA polymerase has an absolute requirement for single stranded or denatured DNA primers (4, 5).

EXPERIMENTAL PROCEDURE

Materials

Nonradioactive deoxyribonucleoside 5'-triphosphates were purchased from P-L Biochemicals. 8-¹⁴C-Labeled dATP was purchased from Schwarz BioResearch. Procedures for the preparation of other ¹⁴C-labeled deoxyribonucleotides have been described previously (1). Crystalline pancreatic DNase, micrococcal nuclease, and bovine spleen DNase were acquired from Worthington.

We wish to thank Dr. A. Bendich for samples of phage T4 DNA and bull sperm DNA; Dr. N. Straus for samples of E. coli and Bacillus subtilis DNA; Dr. A. Mittleman for pleuropneumonia-like organisms (mycoplasmataceae); Dr. G. Patel for phage T2 DNA; Dr. S. Spiegelman for φX DNA and replicating form of φX DNA; Dr. A. Kornberg for poly dAT; and Dr.

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† Deceased, March 5, 1966.
‡ Predoctoral Research Fellow of the National Institutes of Health, United States Public Health Service.
from Worthington. It was dissolved in 0.01 M KCl, and a solution containing 0.5 mg per ml had an extinction coefficient of approximately 7.0 at 260 mμ. Solutions of heat-denatured calf thymus DNA were prepared by heating 1.0-ml portions of this solution contained in conical centrifuge tubes in a boiling water bath for 15 min and by cooling the tubes rapidly in an ice bath. Under these conditions, a hyperchromic shift of approximately 25 to 30% was attained.

Heat-denatured samples of DNAs were prepared in a similar manner after dialysis against 0.01 M KCl at 0-4°. The hyperchromic shifts observed after heating the samples of DNA provided to us varied from 5 to 35%. In each case, however, heat denaturation resulted in less effective DNA primers.

Preparations of DNA not subjected to any denaturation, chemical, or enzymic treatments are referred to as native DNA.

The 3²P-labeled DNA used in the deoxyribonuclease studies (an 0.5-ml sample containing 200 μg per ml) was diluted with 3.0 ml of a solution containing 1.0 mg of calf thymus DNA per ml of 0.01 M KCl and the DNA was precipitated by the addition of 2.5 volumes of ice-cold 95% ethanol. It was subsequently dried and redissolved in 0.01 M KCl at a final concentration of 0.5 mg per ml.

The enzyme preparation used throughout this investigation was Fraction 4, purified from the regenerating liver of the adult rat according to the fractionation procedure described in detail previously (1) but with certain exceptions. Dialysis in 0.001 M potassium phosphate buffer, pH 8.0, containing 0.001 M 2-mercaptoethanol, rather than Sephadex G-25, was used for desalting purposes, and the hydroxylapatite was prepared in our laboratory (6). Analysis of Fraction 4 for endogenous nucleic acid content revealed that it contains 11.0 μg of RNA per ml of protein and essentially no DNA. Fraction 1 refers to the initial crude cell-free extract from 36-hour regenerating adult rat liver from which Fraction 4 was prepared (1).

Methods

DNA Deoxyribonucleotidyltransferase Assay—Details of the standard assay procedure measuring the conversion of radioactive substrates into acid-insoluble products were described before (1). Radioactivity was measured with a Nuclear-Chicago windowless gas flow counter. Experimental evidence indicating that radioactive deoxyribonucleoside 5'-triphosphate substrates are being incorporated into a product possessing several properties characteristic of DNA has been presented previously (1).

Treatment of Primer DNA with Various Deoxyribonucleases—Calf thymus DNA was subjected to a limited digestion with crystalline pancreatic DNase for 5 min according to the procedure reported by Aposhian and Kornberg (7) for preparing activated calf thymus DNA.

Partial digestion with micrococcal nuclease (8) to produce 3'-phosphoryl-terminated DNA was performed according to the method described by Richardson and Kornberg (9). The reaction mixture was terminated after 30 min and dialyzed against 4 liters of 0.01 M KCl at 2° for 24 hours. Approximately 30% of the DNA was degraded to dialyzable fragments after this treatment.

Calf thymus DNA bearing 3'-phosphoryl terminal groups was also prepared with bovine spleen deoxyribonuclease (10) essentially in the manner described by Richardson and Kornberg (9) and dialyzed against 0.01 M KCl at 2° for 24 hours.

Alkaline and Acid Denaturation of Primer DNA—Alkaline-denatured calf thymus DNA was prepared by the method described by Ehrlich and Doty (11) with slight modifications. A solution containing 1.0 mg of calf thymus DNA per ml of 0.01 M KCl, 2 ml, was mixed with 2.0 ml of 0.1 M potassium phosphate, pH 12.3, and allowed to stand at 0° for 1 hour in a glass-stopped bottle. The solution was then dialyzed at 2° for 36 hours against three 2-liter changes of 0.01 M KCl. Acid-denatured DNA was prepared by a similar procedure with the exception that the initial calf thymus DNA solution was added to an equal volume of 0.1 M potassium phosphate, pH 1.8.

Analytical Methods—Protein concentration was determined either by the method of Lowry et al. (12) or by the spectrophotometric method of Warburg and Christian (13) with the use of the empirical relationship given by Taney (14). RNA was measured by the orcinol reaction (15), and DNA was estimated by the diphenylamine reaction (16).

RESULTS

Comparison of Priming Abilities of Native and Heat-denatured DNAs—The incorporation of ³²P-dATP into native and heat-denatured calf thymus DNA is illustrated in Fig. 1. It may be noted that the native DNA primer possesses a different optimal concentration than heated DNA for this incorporation reaction. Saturation with respect to primer is achieved with heat-denatured DNA at approximately one-fourth the concentration observed for the underdenatured primer. That the decreased priming activity of the heated DNA was due, at least in part, to a collapse in the secondary structure of the native DNA was apparent by the hyperchromic shift observed (30%) after heat denaturation and quenching of the sample in an ice bath. Similar curves were obtained for salmon sperm DNA. In order to show further that native DNAs with substantial secondary structure are more efficient primers than heated DNAs, preparations from a variety of sources were tested in their native and heat-denatured states. The results of these experiments are summarized in Table I and illustrate that in each case heat...
TABLE I
Relative effectiveness of various native and heat-denatured DNAs as primers

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Priming activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Heat-denatured</td>
</tr>
<tr>
<td>E. coli</td>
<td>2.03</td>
<td>1.83</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>3.32</td>
<td>1.03</td>
</tr>
<tr>
<td>T2 phage</td>
<td>4.54</td>
<td>1.83</td>
</tr>
<tr>
<td>T4 phage</td>
<td>2.60</td>
<td>1.85</td>
</tr>
<tr>
<td>Pleuropneumonia-like organisms (mycoplasmataceae)</td>
<td>2.18</td>
<td>1.43</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>2.97</td>
<td>1.11</td>
</tr>
<tr>
<td>Bull sperm</td>
<td>6.16</td>
<td>4.06</td>
</tr>
</tbody>
</table>

Effects of Various Treatments of Priming Activity of Calf Thymus DNA—The effects produced on the priming activity of helical, native, calf thymus DNA by altering either its primary or secondary structure with various enzymes or by chemical means are shown in Table III. After each specific enzymic treatment each sample was also heat-denatured in order to ascertain whether or not any additional changes in priming activity would occur. Since the “end addition” reaction, and very likely the “replication” reaction catalyzed by DNA polymerases initiate at the ends of polydeoxyribonucleotide chains (see Reference 17), the influence of the terminal groups of primer molecules on the incorporation of 3H-DATP into DNA was investigated by treating native DNA with various deoxyribonucleases.

TABLE II
Hydrolysis of 32P-DNA by purified and crude enzyme

<table>
<thead>
<tr>
<th>Time of Incubation</th>
<th>32P rendered acid-soluble %</th>
<th>2.04 M KOH added to the solution</th>
<th>2.04 M KOH added to the solution</th>
<th>2.04 M KOH added to the solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min</td>
<td>0.03</td>
<td>0.12</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>30 min</td>
<td>0.24</td>
<td>0.36</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>60 min</td>
<td>0.61</td>
<td>0.83</td>
<td>12.8</td>
<td></td>
</tr>
</tbody>
</table>

Thus making heated DNA less available as a primer. As an indication of the nucleolytic activity of Fraction 4, the rate and extent of hydrolysis of native and heat-denatured 32P-labeled DNAs to acid-soluble radioactive products were measured. The data presented in Table II show that major differences exist in the hydrolysis of native and heated DNA by Fraction 4, as measured by the assay procedure described in Table II. In a control experiment, 1.0 μg of crystalline pancreatic DNase rendered at least 50% of the 3P-labeled DNA to acid-soluble radioactive fragments under identical experimental conditions. On the other hand, Fraction 1, the initial crude enzyme extract from which Fraction 4 was fractionated, readily attacks native as well as heat-denatured DNA (Table II). Hence, it does not appear likely that interfering deoxyribonucleases in Fraction 4 decrease the priming activity of heat-denatured DNA although such a possibility cannot be excluded completely on the basis of these assays alone. Moreover, it is conceivable that specific DNases in Fraction 4 may be activating native DNA primers to a greater extent than heated primers, thus producing more effective primers.

Measurement of Deoxyribonuclease Activity—One possible explanation for the results presented in Fig. 1 and Table I is that the partially purified enzyme preparation used throughout this investigation contains contaminating nucleases that hydrolyze heat-denatured DNA at a faster rate than native DNA,
Limited digestion of the primer with trace amounts of crystalline bovine pancreatic DNase, an endonuclease that cleaves phosphodiester bonds of DNA to form 3'-hydroxyl and 5'-phosphoryl termini ("activated DNA") (18, 19), produced some stimulation in the rate of "C-dATP incorporation, whereas bovine spleen DNase, an endonuclease (20) that splits DNA to produce 3'-phosphoryl and 5'-hydroxyl groups (10) presumably by cleaving simultaneously both strands of the DNA at or near the same level (18), and micrococcal nuclease (8), an endonuclease (21) that attacks phosphodiester bonds of DNA in the same manner as the splenic enzyme, markedly reduced priming activity (Table III). These results show that partial degradation of the primer with enzymes that produce 3'-phosphoryl and 5'-hydroxyl termini inhibits the incorporation reaction whereas enzymes that yield 3'-hydroxyl and 5'-phosphoryl end groups enhance the incorporation of deoxyribonucleotides into DNA. Keir (22) has made a similar suggestion to explain analogous findings in soluble enzyme extracts prepared from Landschutz ascites carcinoma cells. The above findings compare favorably with the results obtained with highly purified E. coli DNA polymerase (17, 23) and show that the introduction of specific types of terminal groups on a DNA primer by enzymes can either increase or reduce priming activity. We found that, in each case, heat denaturation of the enzymatically treated primer results in a decrease in priming activity (Table III). Additionally, controlled acid or alkaline denaturation of a DNA primer (see "Methods") is similarly effective in reducing its priming activity as shown in Table III. The denaturation of native DNA by treatment with acid or base generally resembles heat denaturation and results in the destruction of the characteristic helical structure of native DNA. Hence, both the secondary and primary structures of the DNA primer influence the catalytic properties of DNA nucleotidyltransferase fractionated from adult regenerating rat liver.

Comparison of Priming Activities of Various Polydeoxyribonucleotides—In addition to the DNA preparations discussed above, several other polydeoxyribonucleotides were tested for their ability to serve as primers. The results of these experiments are shown in Table IV. It may be noted that replicating form of φX DNA, which is circular (24) and double stranded (25), is a more effective primer for the incorporation of "C-dATP than φX DNA, which is also circular (26) but single stranded (27). The increase in the amount of "C-dATP incorporated with the replicating form of φX DNA as primer to φX DNA is approximately of the same order of magnitude as that seen when native and heat-denatured calf thymus DNAs are used as primers at the same concentration (Fig. 1). Under conditions of the standard assay, the poly dAT copolymer (28) produced the maximal rate of incorporation for a given amount of enzyme and was found to be the most effective primer for the regenerating rat liver enzyme (Table IV). Experiments with poly dAT as primer provide the most convincing evidence obtained so far that polydeoxyribonucleotides possessing a helical double stranded configuration are superior to single stranded or denatured DNA primers for this enzyme. A nearly quantitative incorporation of both dATP and dTTP into poly dAT has been achieved with some of the most active enzyme preparations. It may also be seen that a limited incorporation of single triphosphates occurs with poly dAT as primer (Table IV). The incorporation of a single triphosphate to the primer molecule may be similar to the "limited reaction" observed with E. coli DNA polymerase (29) or may be owing to a contaminating "terminal" deoxyribonucleotidyltransferase enzyme (6, 30, 31). The incorporation of either dATP or dTTP is not affected substantially by the presence of either dGTP or dCTP, neither of which are incorporated to any appreciable extent into the poly dAT copolymer (Table IV).^2

**DISCUSSION**

The reactions catalyzed by DNA nucleotidyltransferase enzymes are critically dependent on the primary and secondary structures of the DNA primer. The physicochemical state of the primer not only determines the base composition of the newly synthesized DNA but also the extent to which the reaction proceeds. The results of this investigation have shown that DNA nucleotidyltransferase partially purified from crude extracts of regenerating liver of the adult rat as described previously (1) preferentially utilizes native DNAs possessing a helical structure much more effectively as primers than either denatured or single stranded DNAs at the same concentration (Table IV).^2

<table>
<thead>
<tr>
<th>Primer and substrates</th>
<th>&quot;C-Deoxyribonucleotide incorporated</th>
<th>μmole/kr</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;C-dATP + dTTP + dGTP + dCTP... 9.32</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dATP... 8.79</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dGTP + dATP + dTTP... 8.26</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dGTP + dATP + dTTP + dGTP... 8.26</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dATP + dTTP + dGTP... 8.10</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dATP + dGTP + dCTP... 8.03</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dTTP + dGTP + dCTP... 8.03</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dTTP + dGTP + dCTP... 8.03</td>
<td>0.08</td>
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<tr>
<td>&quot;C-dATP + dTTP + dGTP + dCTP... 8.03</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>&quot;C-dATP + dTTP + dGTP + dCTP... 8.03</td>
<td>0.08</td>
<td></td>
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</table>

Each reaction mixture contained, in 0.5 ml, 0.1 mM of each non-radioactive triphosphate, 10 mM MgCl₂, 1.0 mM 2-mercaptoethanol, 40.0 mM glycine buffer, pH 8.0, 350 μg of Fraction 4, radioactive substrates, 0.1 mM of each, and primers as indicated. The various primers were added in the following amounts: 5.0 μg of either φX DNA or replicating form of φX DNA; 0.1 ml of poly dAT having 3.12 optical density units per ml at 260 μm. The concentrations and specific activities of the radioactive triphosphates were as follows: 0.303 mM 8-"C-dATP (6.67 × 10⁴ cpm per μmole); 0.064 μM 8-"C-dTTP (3.37 × 10⁴ cpm per μmole); 0.040 μM 8-"C-dGTP (3.95 × 10⁴ cpm per μmole); and 0.030 μM 8-"C-dCTP (7.30 × 10⁴ cpm per μmole). After incubation at 37° for 60 min, the reaction mixtures were assayed as previously described (1).

^2 The priming activity of dGdC polymer, which consists of homopolymers of deoxyguanylate and deoxyctydylate hydrogen-bonded together (32), was not tested because of its unavailability to us at the time these experiments were conducted. The ability of this polymer, as well as other polydeoxyribonucleotides, to serve as primers for DNA polymerase of regenerating rat liver will be reported in a subsequent communication.
stranded DNAs. Alteration of the secondary structure of double stranded native DNA primer by acid, alkaline, or heat denaturation reduces priming activity to a considerable extent. These findings have been observed for several DNA preparations whether derived from viral, bacterial, or mammalian sources and do not appear to be explicable to contaminating deoxyribonucleases in Fraction 4 that hydrolyze heated DNA at a faster rate than native DNA, since both native and heat-denatured DNAs are degraded to a very small extent to acid-soluble fragments and at approximately the same rate.

Other experimental findings also support the contention that double stranded polynucleotides are preferred primers. The replicating form of 4X-DNA, which is double stranded, was shown to be more effective than single stranded 4X-DNA. Furthermore, the highly ordered, helical, poly dAT copolymer was found to be the most effective primer of the various polynucleotides tested. In this respect, the regenerating rat liver enzyme resembles the highly purified DNA polymerases fractionated from E. coli (33), and Bacillus subtilis (81), both of which utilize poly dAT with considerably greater efficiency than either single stranded or heat-denatured DNA primers. By comparison, the DNA polymerase induced by infection of E. coli with bacteriophage T2 (7), and the calf thymus enzyme (5) display specific requirements for heated or single stranded DNA primers and are practically ineffective with native DNA primers. Calf thymus DNA polymerase cannot use poly dAT or native DNA as primers unless traces of bovine pancreatic DNase are added to the reaction mixture (35). The reason for the different primer requirements for the two purified mammalian enzymes remains to be elucidated. At present, the physiological mechanism whereby native DNA is converted into an active template for DNA replication in mammalian cells remains to be delineated. Insufficient data are available to state categorically that the molecular mechanism of DNA biosynthesis is identical in all of the mammalian cells.

The catalytic activity of the regenerating rat liver enzyme can also be altered by changes in the primary structure of the DNA primer by limited treatment with specific enzymes. Pancreatic DNase, which produces 3′-phosphoryl terminal groups, enhances the incorporation reaction whereas micrococal and splenic endonucleases, which cleave the phosphodiester bonds of DNA to produce 3′-phosphoryl groups, reduce priming activity. In each case, however, heating and fast cooling of the DNA primers pretreated enzymatically further decrease their priming abilities. Nevertheless, specific endonucleases, exonucleases, and DNA-phosphatases may be important in controlling DNA synthesis in mammalian tissues, as is the case with E. coli DNA polymerase (17, 23). Current studies are directed toward studying these aspects of DNA synthesis in vitro in normal as well as cancerous liver tissues.

Acknowledgments—We are indebted to Miss Carol Schwarzott and Miss Jeanne Smith for excellent assistance during the course of this work.

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