Deoxyribonucleic Acid Synthesis in Bacteriophage SP01-infected Bacillus subtilis

II. PURIFICATION AND CATALYTIC PROPERTIES OF A DEOXYRIBONUCLEIC ACID POLYMERASE INDUCED AFTER INFECTION*

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CLIFFORD O. YEHELE and A. T. GANESAN

From the Department of Genetics, Lt. Joseph P. Kennedy, Jr., Laboratories for Molecular Medicine, Stanford University Medical School, Stanford, California 94035

SUMMARY

A new DNA polymerase has been purified 1000-fold from Bacillus subtilis infected with bacteriophage SP01. It consists of a single polypeptide chain of approximately 122,000 molecular weight. The enzyme requires deoxyribonucleoside 5'-triphosphates, Mg2+, β-mercaptoethanol, and single stranded DNA templates for activity. Bihelical DNA which has been partially denatured serves as the preferred template in in vitro reactions. Although 5-hydroxymethyluracil replaces thymine in SP01 DNA, deoxynucleotide analogues of 5-hydroxymethyldeoxyuridine 5'-triphosphate can serve as efficient substrates for the phage polymerase. The purified enzyme is free of endonuclease activity.

DNA polymerases have been isolated and extensively characterized from a number of biological systems. However, semiconservative DNA replication has not been accomplished by these enzymes in vitro. Bacillus subtilis and some of its phage systems offer a novel approach in that template DNA and product can be biologically characterized by means of the transformation and transfection systems. Phage systems are less complex because of their smaller chromosome size, number of DNA polymerase activities coded by the genome, and thus provide a better opportunity to examine the mechanism of DNA replication in vitro.

Earlier studies (1) provided evidence that a new DNA polymerase activity is induced upon infection of B. subtilis by the virulent phage SP01. Further evidence that this new polymerase activity is coded by the phage genome requires purification and characterization of the new activity and comparison of its properties with those of the host DNA polymerases (2). The present report describes the purification of a new DNA polymerase which occurs in cells upon infection with SP01 and which differs in many aspects from those of the host. These studies also reveal some similarities to the properties of other enzymes induced by virulent phages of Escherichia coli.

MATERIALS

Cell Growth and Strains—Procedures for the growth of bacteria and the preparation of phage stocks are described in the first paper of this series (1). B. subtilis strain SB1060 (trpC hisB pheA polA5), a DNA polymerase-I-deficient mutant (3), was used in these experiments.

Nucleotides and Polymers—Unlabeled deoxynucleoside 5'-triphosphates, [8-3H]dAMP, [8-3H]dATP, [methyl-3H]dTTP, and [5-3H]dCTP were purchased from Schwarz-Mann. 5-Bromodeoxyuridine 5'-triphosphate was supplied by Terra-Marine Bioresearch. Poly[d(A-T)] was a product of Miles Laboratories. [3H]Poly[d(A-T)] and P22 [3H]DNA used for exonuclease assays were the gifts of Dr. P. Modrich of the Department of Biochemistry and Dr. V. Saramella of the Department of Genetics, respectively. Salmon sperm DNA was obtained from Calbiochem.

Enzymes and Proteins—DNase I (1 time crystallized), RNase (crystallized from ethanol), snake venom phosphodiesterase, and β-galactosidase were obtained from Worthington Biochemical Corporation. Pyruvate kinase from rabbit muscle was purchased from Sigma. Egg white lysozyme, bovine serum albumin fraction V, and bovine γ-globulin fraction II came from Armour and Company. Human hemoglobin was prepared by osmotic shock of red blood cells. DNA polymerase I (4) and exonuclease III (5) from E. coli were provided by Drs. D. Brutlag (Department of Biochemistry) and P. Modrich, respectively.

Miscellaneous—DEAE-Sephadex A-50, Sephadex G-200, and Dextran T500 were purchased from Pharmacia Fine Chemicals. Whatman No. 3MM and DE81 chromatography papers and cellulose phosphate P11 were supplied by Recco Angel Company. Phosphoenolpyruvate, p-hydroxymercuribenzoate, and chloramphenicol were obtained from Sigma. N-Ethylmaleimide
came from Nutritional Biochemicals, and diethiothreitol was a product of Calbiochem. Polyethyleneimine-impregnated cellulose sheets (Polygram, Cel 300 PEI) were purchased from Brinkman Instruments. Aquosol is a product of New England Nuclear. 6-(p-Hydroxyphenylazo)-uracil was the generous gift of Dr. Bernard Langley of the Imperial Chemical Industries, Ltd. Stock solutions in 50 mM NaOH were reduced immediately before use by incubation at 30° for 10 min with 20 mM dithiothreitol (6).

METHODS

DNA Templates for DNA Polymerase Reaction—DNA preparations were purified as described previously (1). DNA was "activated" by limited digestion with pancreatic DNase I (7), and portions were further digested until 5 to 10% of the material became acid-soluble with E. coli exonuclease III to produce molecules containing gaps. Denatured DNA was prepared either by boiling for 5 min and fast cooling or by alkali (0.1 × NaOH) treatment for 5 min and neutralization.

Preparation of Nucleotide Kinase—A partially purified enzyme extract of SP01-infected cells catalyzes the phosphorylation of hmdUMP, with the use of ATP, to its triphosphate. SB19 (3 g), collected at 15 min after infection with SP01, was suspended in 30 ml of 0.02 M Tris, pH 7.5, 0.01 M β-mercaptoethanol, 0.1 mM EDTA and treated with lysozyme (200 μg per ml) at 37° for 5 min. The lysate was sonicated at the maximum output of a Branson Sonifier for 5 min, centrifuged at low speed to remove cell debris, and followed by further centrifugation at 100,000 × g for 60 min. The protein concentration of the 100,000 × g supernatant was adjusted to 10 mg per ml, and 0.2 ml of freshly prepared 5% streptomycin sulfate per ml was added slowly while the solution was stirred. The suspension was centrifuged at 14,500 × g for 20 min. The supernatant was preincubated with a saturating amount of (NH₄)₂SO₄, centrifuged, and the precipitate dissolved in 0.04 M Tris, pH 7.6, 0.1 M KCl, 0.01 M β-mercaptoethanol, 1 mM MgCl₂, and 0.1 mM EDTA, dialyzed against the same buffer and stored at −15°. Combined nucleoside kinase and nucleoside diphosphokinase activity was measured by the conversion of [3H]dAMP to [3H]-dATP. DNA was "activated" by limited digestion with pancreatic DNase I and venom phosphodiesterase, and the resulting deoxynucleoside triphosphate band eluted with 0.1 mM EDTA. The lane in which the material was chromatographed.

Synthesis of 5-Hydroxymethyldeoxyuridine 5'-Triphosphate—SP01 DNA was enzymatically digested with pancreatic DNase I and venom phosphodiesterase, and the resulting deoxynucleoside monophosphates were separated by paper chromatography. hmdUMP, recovered from the paper, was used as a substrate for the nucleotide kinase from SP01-infected cells to yield the corresponding deoxynucleoside triphosphate. SP01 DNA (4400 nmoles) in 2.0 ml of 0.02 M Tris, pH 7.5, 0.02 M NaCl, 0.01 M MgCl₂ was hydrolyzed with crystalline pancreatic DNase I (2 mg) for 3 hours at 37°. The pH was raised to 8.6 by the addition of NH₄OH, and the deoxynucleotidylate was digested to mononucleotides by treatment with venom phosphodiesterase (1 mg) at 37° for 3 hours. Conversion of mononucleotides to deoxynucleosides by contaminating 5'-nucleotidase was minimal. The digestion mixture was heated in a boiling water bath for 2 min, chilled, and the flocculent precipitate was removed by centrifugation. The supernatant was applied in a band to Whatman No. 3MM paper and developed 10 to 20 hours by descending chromatography in a solvent of isobutyric acid-1 N ammonia-0.2 M EDTA (100:80:0.8 parts, respectively). hmdUMP was eluted from the paper with 0.1 M EDTA, pH 7.0. Concentration was determined by optical density measurement at 264 nm with a ε₉ = 10.2 × 10³ (8). A total of 920 nmoles of hmdUMP was recovered following chromatography which represented a 77% yield.

hmdUMP was incubated with the nucleotide kinase from SP01 infected cells to prepare the corresponding 5'-triphosphate. The incubation mixture contained in a final volume of 0.4 ml: 50 mM Tris, pH 7.6; 31 mM MgSO₄; 3 mM KCl; 1 mM ATP; 0.3 mg of bovine serum albumin; 12.5 mM phosphoenolpyruvate; 3.5 units of pyruvate kinase; 0.5 mM hmdUMP; and 0.8 mg of the SP01 nucleotide kinase preparation. After 60 min of incubation at 37°, the reaction mixture was heated at 80° for 2 min and centrifuged at 15,000 × g for 15 min. The supernatant was chromatographed as described above and the resulting deoxynucleoside triphosphate band eluted with 0.1 mM EDTA. hmdUTP was obtained in approximately 50% yield.

DNA Polymerase Assay—The assays measured the incorporation of labeled deoxynucleoside monophosphates from triphosphate substrates into acid-insoluble material. The reaction mixture (100 μl) contained 100 mM Tris, pH 7.5; 50 mM NaCl; 10 mM β-mercaptoethanol, 15 mM MgCl₂; 0.1 mM EDTA; 15 μM (each) of dCTP, dGTP, dATP, dTTP, one of which contained 1H label with a specific activity of 20 to 40 cpm per pmole; 75 μM DNA template; and enzyme. When poly[d(A-T)] was used as template, dCTP and dGTP were eliminated from the reaction mixture. Incubations were at 37° for 30 min, and 1 unit of enzyme is defined as the incorporation of 10 nmoles of total nucleotide into acid-precipitable product under these conditions (7).

Endonuclease Assay—Endonuclease activity was estimated by measuring the decrease in genetic linkage of closely associated markers on the B. subtilis chromosome by transformation. The aromatic linkage group carries at least nine genes specifying the biosynthesis of aromatic amino acids and includes a histidine marker. SB202, the strain used in this assay, carries four widely distributed markers of this linkage group (9). Single strand breaks in the DNA cause a loss of linkage of two markers when they occur within the DNA segment containing these markers (10), thus providing a very sensitive assay for endonucleolytic cleavage. The efficiency of a DNA preparation from wild type cells to transform all four markers following exposure to the DNA polymerase fractions was tested by using nonsaturating concentrations of the standard DNA. SB19 DNA (1 n mole per ml) was incubated under assay conditions in the presence of enzyme fractions at 30°. Competent cells of SB202 (1 × 10⁶ cells per ml) in minimal medium plus 0.5% glucose were added, and the mixtures were incubated further at 30° for 30 min. Samples were diluted and Trp⁺ recombinants were selected. A minimum of 300 Trp⁺ recombinants were counted on nutrient agar plates. These were replica plated to appropriately supplemented media to determine the remainder of the genotype.

Exonuclease Assay—Exonuclease activity in the absence of measurable endonuclease activity was assayed by the production of acid-soluble radioactivity with the use of [H]poly[d(A-T)] as substrate. Incubation mixtures (100 μl) contained 35 mM Tris, pH 8.0; 3 mM EDTA; 1 mM diethiothreitol; 14 μM [H]poly[d(A-T)] (92.1 × 10⁴ cpm per pmole); and enzyme. Following incubation at 37° for 30 min, the reaction mixtures

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1 The abbreviations used are: hmdUMP and hmdUTP, 5'-monophosphate and 5'-triphosphate of 5-hydroxymethyldeoxyuridine, respectively; SDS, sodium dodecyl sulfate.
were chilled, supplemented with 200 μg of salmon sperm DNA, and made 5% in trichloroacetic acid. The resulting precipitate was sedimented at 25,000 × g for 10 min, and the supernatant was counted in 10 ml of Aquasol by liquid scintillation spectrometry.

Alternatively, exonuclease activity was measured by the release of mononucleotides from P22 phage [3H]DNA. Native or denatured DNA (12 nmoles, 1.2 × 10^6 cpm per n mole) was incubated with enzyme under the conditions of the DNA polymerase assay, except that the deoxynucleoside triphosphates were omitted. Aliquots of the mixture were spotted on strips (1.4 × 9 inch) of DE81 paper, and mononucleotides were eluted from the origin by chromatography with 0.35 M ammonium formate (pH 8). The dried strips were cut into 1-cm segments, and the radioactivity of each was determined by liquid scintillation counting. The amount of exonuclease activity was determined from the proportion of radioactivity left at the origin as unhydrolyzed polynucleotide compared to the radioactivity which chromatographed as mononucleotide.

**Gel Electrophoresis**—Polyacrylamide die gel electrophoresis was carried out as described by Davis (11). The procedure of Weber and Osborn (12) was used for electrophoresis in the presence of SDS. Samples were heated in a boiling water bath for 2 min in the presence of 1% each of SDS and β-mercaptoethanol prior to SDS gel electrophoresis. Molecular weight standards included bovine serum albumin (68,000) and β-galactosidase (130,000). Cells were scanned at 660 nm after staining with Coomassie blue with the use of a Gilford model 2000 recording spectrophotometer equipped with a linear transport mechanism.

**General**—Protein concentration was determined by the method of Lowry et al. (13). DNA concentration was estimated by the method of Burton (14). Sucrose gradient analyses were performed as described by Martin and Ames (15).

## Results

### Purification

Extracts of SP01-infected *B. subtilis* display a significant increase in DNA polymerase activity over that observed in uninfected cell extracts (1) and which is efficiently blocked by chloramphenicol (Fig. 1). This observation suggested that a new polymerase might be induced by the phage which required protein synthesis. Phage-infected cell extracts were subjected to further purification in an attempt to characterize this enzyme. All procedures were carried out at 4°C, and all buffers contained 1 mM dithiothreitol or 10 mM β-mercaptoethanol unless otherwise indicated. A summary of the purification is presented in Table I.

**Preparation of SP01-infected Cells**—Strain SB1060 (*trpC hisB phaA polA*) was grown in 20 liters of minimal medium (16) supplemented with 0.1% casein hydrolysate and 20 μg per ml each of the required amino acids at 37°C to a cell concentration of 4 × 10^9 per ml. The culture was divided. SB1060 cells were infected with SP01 at a multiplicity of 5 and the culture was divided. One culture was made 150 μg per ml in chloramphenicol and the other served as control. At various periods aliquots were removed, and cells were collected by centrifugation and lysed. The lysates were assayed for DNA polymerase activity using the endogenous DNA template. No chloramphenicol (O--O); chloramphenicol (●--●).

**Preparation of Cell Extract**—The phage-infected cells were suspended in 0.25 M sodium phosphate buffer, pH 6.8, to a final volume of 240 ml. The suspension was then incubated at 37°C for 30 min in the presence of 50 μg per ml of egg albumin lysozyme and 20 μg per ml of ribonuclease. The viscosity was reduced by brief treatment in a Waring Blender. The lysate was then brought to a final volume of 530 ml by the addition of more buffer (Fraction I).

**Phase Extraction**—Nucleic acids were removed from Fraction I by extraction in a polyethylene glycol-dextran phase partition system (17). To Fraction I was added 120 g of 30% (w/w) polyethylene glycol and 72 g of 20% (w/w) dextran T500 to give final concentrations of 5% and 2%, respectively. The homogenate was stirred for 2 hours and then centrifuged at 500 × g for 15 min. The resulting top phase was removed and replaced by an equal volume of upper phase from a previously prepared mixture. NaCl (61.2 g) was slowly added to the solution to give a final concentration of 1.5 M, mixed, and centrifuged as above. NaCl was removed from the resulting upper phase by extensive dialysis against 0.1 M sodium phosphate buffer, pH 7.6, giving a final volume of 670 ml (Fraction II).
Ammonium Sulfate Precipitation—Solid ammonium sulfate (134 g) was slowly added to Fraction II with continuous stirring, and the mixture was allowed to stand in a separatory funnel. After 8 hours, a lower phase containing the enzyme separated from the upper polyethylene glycol phase. Further ammonium sulfate (0.3 g per ml) addition resulted in the formation of a precipitate which was removed by centrifugation at 13,260 × g for 90 min. The precipitate was dissolved in 90 ml of 0.1 M Tris, pH 7.6, containing 1 mM MgCl₂ (Fraction III).

DEAE-Sephadex Chromatography—A column of DEAE-Sephadex (5 cm² × 15 cm) was prepared and equilibrated with 0.1 M Tris, pH 7.6, containing 1 mM MgCl₂. Fraction III was diluted with buffer and absorbed to the column at a rate of 0.5 ml per min. The column was then washed with 100 ml of the above buffer and eluted with a 450-ml gradient of NaCl from 0 to 0.5 M final concentration. The flow rate was adjusted to 0.5 ml per min, and 5-ml fractions were collected and assayed for enzyme activity. The enzyme eluted at 0.35 M NaCl, and all of the active fractions were pooled and concentrated on an Amicon apparatus equipped with a XM-50 (50,000 molecular weight cut-off) filter. The concentrated enzyme solution was dialyzed for 6 hours against 0.1 M potassium phosphate buffer (pH 7.6) containing 20% glycerol and stored at −15° until used (Fraction IV).

Phosphocellulose Chromatography—A portion of Fraction IV (7.5 ml) was dialyzed against 0.1 M potassium phosphate buffer (pH 6.5) and applied to a phosphocellulose column (0.6 cm² × 15 cm) previously equilibrated with the same buffer. The column was then washed and a linear gradient (80 ml) of 0.1 to 0.7 M potassium phosphate buffer was applied. Two-milliliter fractions were collected, and the DNA polymerase was eluted at a buffer concentration of 0.48 M. The fractions containing polymerase activity were pooled (Fraction V). This fraction contained 63% of the DNA polymerase activity present in the crude extract and represented a 1040-fold purification. Fraction V was concentrated by the addition of (NH₄)₂SO₄ (0.5 g per ml) and dissolving the precipitate collected by centrifugation in 0.1 M Tris, pH 7.6, containing 1 mM MgCl₂. Fraction III was concentrated by the addition of ammonium sulfate (0.3 g per ml) and centrifugation in the presence of 10% glycerol, stored at −15° until used (Fraction IV).

Phosphocellulose Chromatography—A portion of Fraction IV (7.5 ml) was dialyzed against 0.1 M potassium phosphate buffer (pH 6.5) and applied to a phosphocellulose column (0.6 cm² × 15 cm) previously equilibrated with the same buffer. The column was then washed and a linear gradient (80 ml) of 0.1 to 0.7 M potassium phosphate buffer was applied. Two-milliliter fractions were collected, and the DNA polymerase was eluted at a buffer concentration of 0.48 M. The fractions containing polymerase activity were pooled (Fraction V). This fraction contained 63% of the DNA polymerase activity present in the crude extract and represented a 1040-fold purification. Fraction V was concentrated by the addition of (NH₄)₂SO₄ (0.5 g per ml) and dissolving the precipitate collected by centrifugation in 0.1 M potassium phosphate buffer (pH 7.6) containing 20% glycerol. The concentrated solution had a protein concentration of 1.2 mg per ml and was stable upon storage at −15° for several months.

The characteristics of the enzyme activity in Fractions IV and V studied in the following sections were similar, and several experiments that required large amounts of enzyme were performed with the use of Fraction IV as indicated. The enzyme showed linear incorporation over a 30-min period, leveling off after 60 min. Incorporation was linear over a range of 0.04 to 0.5 unit of polymerase. Polyacrylamide gel electrophoresis of samples of Fraction V (Fig. 2) revealed the presence of three visible bands. The SP01 DNA polymerase band, located by the dye marker is described by the peak on the right side of the top profile.

**Fig. 2.** Polyacrylamide gel electrophoresis profiles of SP01 polymerase. Aliquots (20 μg) of Fraction V were subjected to electrophoresis in the absence (top) and presence (bottom) of 0.1% SDS as described under “Methods.” The dye marker is described by the peak on the right side of the top profile.

**Table II**

**Requirements of SP01 polymerase for deoxyribonucleotide incorporation**

<table>
<thead>
<tr>
<th>System</th>
<th>Relative activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Omit dATP, dCTP, or dGTP</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Omit Mg²⁺</td>
<td>1</td>
</tr>
<tr>
<td>Omit Mg²⁺, plus Mn²⁺</td>
<td>26</td>
</tr>
<tr>
<td>Omit β-mercaptoethanol</td>
<td>60</td>
</tr>
<tr>
<td>Omit β-mercaptoethanol, plus p-hydroxymercuribenzoate</td>
<td>4</td>
</tr>
<tr>
<td>Omit β-mercaptoethanol, plus N-ethylmaleimide</td>
<td>13</td>
</tr>
<tr>
<td>Omit EDTA</td>
<td>97</td>
</tr>
<tr>
<td>Omit DNA</td>
<td>1</td>
</tr>
</tbody>
</table>

The complete DNA polymerase assay mixture contained 10 μmol of denatured salmon sperm DNA as template and 0.04 unit of Fraction IV. Where indicated, the concentration of Mn²⁺ was 0.3 mM, p-hydroxymercuribenzoate was 1 mM, and N-ethylmaleimide was 4 mM.

**Properties of SP01 DNA Polymerase**

**Reaction Requirements**—As in the case of other reported DNA polymerases, omission of one of the deoxyribonucleoside triphosphates, MgCl₂, or template DNA reduces the level of incorporation to negligible amounts (Table II). Absence of β-mercaptoethanol in the reaction reduced the extent of incorporation by half, whereas lack of EDTA had no effect. When the reaction was carried out in the absence of β-mercaptoethanol and in the presence of either p-hydroxymercuribenzoate, or N-ethylmaleimide, it was inhibited to an extent of 96% and 87%, respectively. The Mg²⁺ requirement could be partially replaced by MnCl₂. Optimal activity at a Mn²⁺ concentration of 0.3 mM decreased to less than 1% with increasing or decreasing concentrations. The reaction was not inhibited by the presence of 200 μM 6-(p-hydroxyphenylazo)-uracil in the assay mixture. The optimal pH range for the enzyme was 7.3 to 7.5 with either sodium or potassium phosphate and Tris buffers (Fig. 3). The enzyme was similarly active with all buffers.
Fig. 3 (left). pH dependence of SP01 polymerase. Assays containing 0.04 unit of enzyme and denatured salmon sperm DNA template were performed under standard conditions, except that Tris (---) or potassium phosphate (O---O) buffers of the indicated pH values replaced Tris at pH 7.4. pH was determined at 0.05 M concentrations and at room temperature. Values are plotted relative to the optimum in Tris, pH 7.5.

Fig. 4 (right). Sucrose gradient sedimentation of SP01 polymerase. Fraction IV (3.8 units) was sedimented for 29 hours at 35,000 rpm in 5 ml of 5% to 20% linear sucrose gradients. A control tube contained 70 units of Escherichia coli DNA polymerase I (Pol I). Three hundred micrograms of human hemoglobin (Hb) were added to each gradient as a standard. After centrifugation, 200-μl fractions were collected and SP01 polymerase (O---O) was assayed using denatured salmon sperm DNA template and E. coli polymerase was assayed using poly[d(A-T)]. Exonuclease (E---E) was assayed by the acid solubilization of 3H-poly-[d(A-T)].

Table III
Relative efficiency of SP01 polymerase with DNA templates

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Relative activity (% of SP01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denatured SP01</td>
<td>100</td>
</tr>
<tr>
<td>Native SP01</td>
<td>5</td>
</tr>
<tr>
<td>Native B. subtilis</td>
<td>1</td>
</tr>
<tr>
<td>Denatured B. subtilis</td>
<td>69</td>
</tr>
<tr>
<td>“Activated” salmon sperm</td>
<td>2</td>
</tr>
<tr>
<td>Exonuclease III-treated salmon sperm</td>
<td>4</td>
</tr>
<tr>
<td>Denatured salmon sperm</td>
<td>92</td>
</tr>
<tr>
<td>Poly[d(A-T)]</td>
<td>18</td>
</tr>
</tbody>
</table>

Polymerase Efficiency with Various DNA Templates—The SP01 polymerase, when separated from the endogenous phage DNA in cell-free extracts, exhibited a preference for single stranded DNA (Table III). Native DNA templates are utilized very poorly as compared to denatured SP01 or salmon sperm DNA preparations. Neither DNase I “activation” of native DNA nor digestion with exonuclease III produced a significant increase in its template efficiency. Poly[d(A-T)] is capable of directing incorporation with the phage polymerase in spite of the presence of exonuclease activity in this fraction. Addition of increasing amounts of poly[d(A-T)] did not lead to increased incorporation. Similar results were obtained with Fraction V. Recently we discovered that DNA templates which have been partially denatured by heating below the thermal denaturation temperature are 2- to 3-fold more efficient in synthesis than completely denatured templates.

Molecular Weight Determinations—The molecular weight of the phage polymerase was estimated by three methods. First, the enzyme was subjected to zone sedimentation on 5 to 20% linear gradients of sucrose as described by Martin and Ames (15). The sucrose solutions contained 50 mM Tris, pH 7.6, 10 mM β-mercaptoethanol, and 0.1 mM EDTA. The samples were centrifuged at 35,000 rpm in the SW 39 rotor of a Spinco L2-65 preparative ultracentrifuge for 29 hours at 5°C. E. coli DNA polymerase I and human hemoglobin served as standards. As shown in Fig. 4, the polymerase activity presented a unimodal distribution, sedimenting as a globular protein of 129,000 daltons. The enzyme was also chromatographed on Sephadex G-200 along with bovine γ-globulin and human hemoglobin. A molecular weight of 123,000 was calculated in this case. Again, the enzyme activity eluted in an unimodal fashion. Thirdly, the enzyme migrated as a single band during electrophoresis in the presence of 0.1% SDS (Fig. 2) with a mobility slightly greater than that of β-galactosidase. Based on a molecular weight for β-galactosidase of 130,000 (12), the SP01 DNA polymerase has a molecular weight of 122,000.

Deoxynucleotide Analogue Incorporation—A number of deoxynucleotide analogues of hmdUTP serve as efficient substrates for the SP01 DNA polymerase as shown in Table IV. The ability of these analogues to substitute for the natural substrate is determined by their hydrogen-bonding specificities. Addition of increasing amounts of hmdUTP to reaction mixtures in which the 3H label is contained in dTTP resulted in reduced incorporation.

Endonuclease Activity—When SB19 DNA was exposed to 0.2 unit of SP01 DNA polymerase (Fraction V), no decrease in the
subsequent assay for Trp\(^+\) transforming activity was observed. When these recombinants were examined for the cotransfer of all four markers of the linkage group using SB202, again there was no loss of linkage (Table V). In fact, the linkage analyses showed an increase compared with that of the control.

**Exonuclease Activity**—When Fraction IV was assayed for exonuclease activity on \(^{3}H\)poly[d(A-T)], an appreciable level was found. However, the majority of this activity failed to sediment with the phage polymerase in sucrose gradients (Fig. 4). Only a minor portion of the activity coincided with the DNA polymerase profile. Addition of deoxynucleoside triphosphates to the reaction mixture produced an appreciable decrease in activity. Exonuclease activity was also found in Fraction V (Table VI). In this case, hydrolysis was measured by DE81 paper chromatography of the reaction products. The maximal rate of hydrolysis of the substrates examined was obtained with denatured DNA.

**Enzyme Stability** Fraction IV, made 20% in glycerol and stored at \(-15^\circ\), remained quite stable over a period of several months. The enzyme rapidly lost activity when heated at temperatures above 50°C. Incubation of the enzyme in the presence of deoxynucleoside triphosphates resulted in increased stability at temperatures below 50°C.

**DISCUSSION**

Advantage was taken of the fact that the SP01-induced DNA polymerase was tightly bound to the DNA-containing fractions (1). The initial step of the polyethylene glycol-dextran phase extraction procedure was carried out at low salt concentration. Under these conditions the phage polymerase remained associated with the nucleic acid fraction and partitioned in the dextran phase. In the second step the salt concentration was increased and the enzyme became disassociated from the nucleic acid. In this case, the enzyme partitioned into the upper polyethylene glycol phase resulting in a 6-fold purification.

Earlier evidence that a new DNA polymerase is induced upon the infection of B. subtilis by SP01 is supported by a number of observations. DNA polymerase I activity disappeared following SP01 infection of wild type cells (1). The purification of the phage enzyme from a pol\^- strain suggests that DNA polymerase I of the host is not involved in phage DNA replication. Chromatography of the enzyme on DEAE-Sephadex distinguishes it from DNA polymerase II purified from SB1060 (2). DNA polymerase III from B. subtilis and the SP01 enzyme do chromatograph similarly on anion exchange columns but they differ greatly in size, stability, and cellular concentration. Both DNA polymerases II and III, having molecular weights of 140,000 to 150,000 and 160,000 to 180,000 (2), respectively, are larger than the SP01 polymerase and are much less stable upon storage than the phage enzyme.\(^2\) SP01 polymerase is also distinguished from host DNA polymerase III by nature of the 6-(p-hydroxyphenylazo)-uracil sensitivity of the latter (18) compared to the resistance of the phage enzyme.\(^3\) Both of the enzymes from SB1060 are more heat-sensitive than the phage polymerase. DNA polymerases II and III of the host and the phage enzyme are all inhibited to various extents in the presence of sulfhydryl-blocking compounds, indicating the involvement in enzymatic activity of a sulfhydryl group in each of these proteins. Finally, the template specificity of the SP01 polymerase differs greatly from those of the host enzymes. Like the DNA polymerases induced by other lytic phages (19-22), the phage enzyme shows a preference for denatured DNA over native, activated, and exonuclease III-treated DNA templates. All of the host enzymes function best on double stranded templates which have been activated by pancreatic DNase and partially digested by exonuclease III. Thus, it is unlikely that the phage DNA polymerase activity results from the modification of any of the existing host DNA polymerases but that a new enzyme is coded for by the phage genome.

Our observation that partially denatured DNA serves as a better template for SP01 DNA polymerase than completely denatured DNA is similar to results obtained by Orr et al. (21) with T5 DNA polymerase. Complete collapse of the helical DNA structure apparently results in a reduced availability of priming sites and increased difficulty of the enzyme to utilize the tightly folded template. Partial denaturation of the tem-

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\(^2\) Unpublished observations.

\(^3\) The nomenclature chosen for the DNA polymerases isolated from strain BC29(F) by Cass et al. (18) corresponds with that reported earlier by this laboratory (2).

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**Table IV**

<table>
<thead>
<tr>
<th>Deoxynucleoside triphosphate</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hmdUTP</td>
<td>100</td>
</tr>
<tr>
<td>dTTP</td>
<td>92</td>
</tr>
<tr>
<td>dUTP</td>
<td>97</td>
</tr>
<tr>
<td>5-Bromodeoxyuridine 5'-tri<strong>phosphate</strong></td>
<td>95</td>
</tr>
</tbody>
</table>

**Table V**

| SB19 DNA was incubated with 0.2 unit of SP01 DNA polymerase (Fraction V) as described under Methods for indicated periods. Control contained no polymerase. Genetic linkage is reported as the percentage of cotransfer of all four markers into SB202. |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genetic linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>37</td>
</tr>
<tr>
<td>30 min</td>
<td>46</td>
</tr>
<tr>
<td>60 min</td>
<td>49</td>
</tr>
</tbody>
</table>

**Table VI**

| Hydrolysis of mononucleotides from poly[d(A-T)] (1.4 nmoles) or P22 DNA (12 nmoles) incubated in the presence of 0.4 units of Fraction V was assayed by DE81 paper chromatography (see “Methods”). |

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Exonuclease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{3}H)poly[d(A-T)]</td>
<td>Native</td>
<td>1</td>
</tr>
<tr>
<td>P22 (^{3}H)DNA</td>
<td>Heat-denatured</td>
<td>19</td>
</tr>
</tbody>
</table>

\(^{a}\) Picomoles of nucleotide hydrolyzed per unit of enzyme per 30 min.
plate may prevent complete collapse of the single stranded portion of the molecule making this region more accessible to the polymerase molecule. Kinetic experiments show that the initial rate of synthesis on such templates is at least 2-fold greater than with completely denatured templates.

The greatest proportion of exonuclease activity in partially purified preparations of DNA polymerase from SP01-infected cells did not further fractionate with the phage polymerase. The majority of the exonuclease activity was separated from DNA polymerase upon sucrose gradient or phosphocellulose fractionation. However, a small amount was present in Fraction V. In comparison with the exonuclease activity associated with T4 DNA polymerase (20, 23), the ratio of polymerase activity to nuclease activity, calculated from the molar incorporation of deoxynucleotides compared to acid solubilization of deoxynucleotides from labeled substrates, for the SP01 DNA polymerase is relatively high. It is not possible at this stage to conclude that the exonuclease activity is associated with the polymerase molecule.

We previously were unable to detect a nuclease activity in cell-free extracts of SP01-infected cells which would degrade poly[d(A-T)] (1). However, upon purification an exonuclease activity has been found which will cause a solubilization of counts from [H]poly[d(A-T)]. The level of exonuclease activity is not high enough to interfere with the polymerase assay when poly[d(A-T)] was used as the template. The nuclease activity was only sufficient to degrade approximately 10% of the template.

Single strand breaks in a duplex DNA molecule such as those introduced by endonucleolytic attack are capable of causing a large decrease in the cotransfer of linked markers in a transformation assay (10). Incubation of SP01 DNA polymerase from Fraction V with transforming DNA of B. subtilis led to no decrease in the linkage of markers of the aromatic amino acid group. Similar results were observed (24) in assays for loss of transforming activity upon exposure of B. subtilis DNA to the most purified fraction of E. coli DNA polymerase I. The linkage analyses performed here provide a 6- to 10-fold increase in sensitivity of this assay compared to that for single markers. Treatment of transforming DNA with 2 x 10^{-3} pg per ml of DNAse I at 0° for as little as 2.5 min is capable of reducing the linkage of these markers by 80% (10). Our failure to detect any endonuclease activity in the polymerase peak fractions does not totally eliminate the possibility that such an activity exists, but the extent of any activity associated with the phage polymerase would be extremely low.

The catalytic properties of the SP01-induced polymerase are similar to those of the enzymes induced by T2 (19), T4 (20), T5 (21), and T7 (22). The requirement for a single stranded DNA template and failure to efficiently use “activated” DNA is common to these enzymes. In contrast to the other phage polymerases, the SP01 enzyme functions optimally with its own denatured DNA template compared with salmon sperm or B. subtilis DNA denatured in a similar manner. This might be reflected by the state of collapse of the templates as discussed. Although genetic evidence was presented that these new polymerases are required for phage DNA replication, their role in replication of bivalent DNA in vivo is not understood. In addition to the known requirements for polymerase, ligase, and nucleases, it is likely that other enzymes and factors are necessary to initiate and sustain sequential replication. The gene 32 protein of T4 which binds to T4 DNA was found to stimulate synthesis with T4 single stranded DNA template (25). It was recently shown that RNA can initiate conversion of the single stranded DNA of M13 (26) or φX174 (27) to its replicative form. In addition, φX174 conversion requires the E. coli dnaE function for initiation and the dnaB function continuously. A similar reaction could be postulated to explain the mechanism by which phage DNA polymerases initiate DNA replication on a double stranded template in vivo. Accordingly, the polymerases could function at locally denatured regions by a similar mechanism and replicate discontinuously as shown by Sugimoto et al. (28).

In vivo replication in many phage systems involves the formation of DNA concatemers. To account for the formation of concatemers and Okazaki pieces, Watson has proposed a modified knife and fork model (29) based on recent studies of T7 DNA replication (30). SP01 was shown to form replication intermediates with molecular weights in excess of that of DNA from mature phage particles (31). It is thus likely that SP01 DNA replication involves the formation of concatemers and might follow a course similar to that of T7.

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Deoxyribonucleic Acid Synthesis in Bacteriophage SP01-infected *Bacillus subtilis*: II. PURIFICATION AND CATALYTIC PROPERTIES OF A DEOXYRIBONUCLEIC ACID POLYMERASE INDUCED AFTER INFECTION
Clifford O. Yehle and A. T. Ganesan


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