Properties and Mode of Action of a Partially Purified Deoxyribonucleic Acid Polymerase from the Mitochondria of HeLa Cells*

(Received for publication, August 25, 1972)

CLARK J. B. TIBBETTS† AND JEROME VINOGRADE§

From the Division of Biology and the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91109

SUMMARY

A DNA polymerase has been partially purified from the mitochondria of HeLa cells. Properties, including low levels of nuclease activity and a preference for native duplex DNA templates, were favorable for the study of in vitro DNA synthesis using circular duplex DNA templates. Initiation of DNA synthesis occurs predominantly at sites of single strand scissions with covalent addition of nucleotides to the priming template strand. Centrifugation and electron microscopy have established that the template DNA strand ahead of the growing point is displaced rather than degraded. Hairpin structures are not formed in the course of DNA synthesis on duplex DNA templates.

Circular species of DNA have been identified as a genetic component in many systems, including the mitochondria of animal cells. Mitochondria isolated from several animal cells and tissues have been shown to incorporate deoxyribonucleotides into mitochondrial DNA in situ (1-8). Kalf and Chi'ih (9) and Meyer and Simpson (10) described the isolation and partial purification from rat liver mitochondria of a DNA polymerase with fractionation and reaction properties that differ from DNA polymerase activity associated with the cell nucleus. The mitochondria of animal cells are thus a source of DNA polymerase(s) which operate on circular duplex DNA in vivo.

Current understanding of in vitro DNA replication at the level of enzyme action is largely speculative. In vitro studies of DNA polymerase activity from animal cell mitochondria were carried out with the object of extending our understanding of the process of circular DNA replication. We have isolated and partially purified a DNA polymerase-containing fraction from sonic extracts of HeLa cell mitochondria and have investigated the mechanism of DNA synthesis on circular DNA templates.

EXPERIMENTAL PROCEDURE

Materials

Nucleic Acids and Precursors—Unlabeled nucleotides were purchased from Sigma. 5-Bromodeoxycytidine 5'-triphosphate and 5-bromodeoxyuridine 5'-triphosphate were prepared as described under “Methods.” [aH]dTTP was obtained from Schwarz BioResearch and New England Nuclear. Other 3H-labeled nucleotides were obtained from Schwarz. Labeled and unlabeled nucleotide solutions were mixed to give samples with lower specific activity and higher concentration. The mixtures were analyzed by Dowex 1 chromatography (0.2 ml of approximately 1 mM nucleotide applied to 0.3 x 3.0 cm column; 60-ml linear gradient elution, from 10 mM Tris, pH 7.5, to 0.2 M HCl, 0.5 M LiCl) to determine the resultant specific activity and assess the purity of the nucleotide preparation. In each case, more than 85% of the applied radioactivity and optical density at the wave length of the nucleotide's maximum absorbance were recovered as the deoxynucleoside triphosphate with constant specific activity across the peak.

Circular calf thymus DNA was purchased from Sigma. T7 phage DNA was a gift of Dr. R. W. Hyman. SV40 viral DNA and SV40 intracellular DNA were prepared in the manner of Rush et al. (11). HeLa cell closed circular mitochondrial DNA was extracted from mitochondria prepared in the manner described under “Methods,” omitting the two buoyant sucrose gradients. 32P-labeled SV40 circular DNA was a gift of Dr. R. Eason. Further samples were prepared as needed according to Rush et al. (11). [3H]Thymidine-labeled ΦX174 single-stranded circular DNA was extracted from a phage preparation supplied by R. Benbow.

DNA preparations were treated with 20 mg per ml of SDS† at room temperature for 10 min prior to banding in a CaCl equilibration density gradient. The gradients for the isolation of closed circular DNA contained ethidium bromide (12). The dye was removed with a Dowex 50 column (0.3 x 3.0 cm) in the presence of 4 M NaCl or by extraction with isomyl alcohol.

† The abbreviation used is: SDS, sodium dodecyl sulfate.
DNA samples were then dialyzed exhaustively against 10 mM Tris, 1 mM EDTA, pH 7.5. The integrity of the DNA samples was checked by analytical band velocity centrifugation.

Enzymes—Escherichia coli exonucleases I and III were stored frozen at 15,000 units per ml and 490,000 units per ml, respectively. The preparations were gifts of D. Brutlag, Stanford University.

Other Materials—Dowex 1 × 8, Dowex AW50 × 8, and Bio-Gel P-150 were purchased from Bio-Rad. CsCl was purchased from Harshaw Chemical Company. Bovine serum albumin, fraction V (crystalline), was from Miles Laboratories. Ethidium bromide was donated by Boots Pure Drug Co. Ltd., Nottingham, England.

Methods

Chromatography—Ion exchange resins were washed with 0.5 M NaOH, 0.5 M HCl, 0.1 M EDTA, 4 M NaCl, and then washed repeatedly with glass-distilled water to pH equivalence. Washed resins were stored in 4 M NaCl. After packing, at least 20 bed volumes were eluted from columns before application of a sample. Bio-Gel P-150, 100 to 200 mesh, was prepared by swelling in 10 mM Tris, 10 mM 2-micropercaptoethanol, pH 7.5, at least 8 hours before packing. One bed volume of elution buffer was passed through the column before application of the sample. The bed volume of the gel filtration columns was 100 to 200 ml and sample volume was less than 5 ml.

Spectrophotometry—Absorption spectra were recorded using a Cary model 14 spectrophotometer. Optical density was measured as the difference in absorbance of the sample and blank solutions determined separately in the same quartz cell with an open reference beam path.

Protein concentration was assessed by absorbance at 280 nm and 260 nm using the method of Warburg and Christian (13). DNA concentration was measured at 260 nm using a value of 1.0 absorbance unit for a solution of 50 μg per ml of native duplex DNA.

Fluorescence measurements of ethidium bromide solutions were performed with quanta cells in a Hitachi Perkin Elmer MFP 2A fluorescence spectrophotometer. The excitation and emission wave lengths were 380 nm and 590 nm. Slit widths were varied according to the needs of each experiment. A 300- to 400-nm bandpass filter was placed between the sample cell and the entrance of the emission monochromator. Measurements were recorded in the ratio mode to correct for fluctuations in lamp intensity.

Centrifugation—Preparative ultracentrifugation studies were performed in Beckman model L or L2-65B centrifuges with SW 50.L or 50.1 rotors. The CsCl self-generating density gradi-ents was used for band sedimentation analysis. Samples (less than 0.2 ml) were layered through Bayol onto at least 3.0 ml of 1.70 g per ml CsCl solution prior to centrifugation at 35,000 rpm. Gradients for alkaline sedimentation velocity analysis contained 0.1 M KOH. Buoyant equilibrium CsCl density gradients were run at 35,000 rpm, 20 to 25°, for at least 36 hours. Initial solution density was 1.70 g per ml for neutral CsCl gradients, 1.74 g per ml for alkaline CsCl gradients, and 1.56 g per ml for neutral CsCl gradients containing ethidium bromide (250 μg per ml). Alkaline buoyant gradients contained 80 μl K2PO4 from a 0.25 M solution of K2HPO4 adjusted to pH 12.5 by addition of KOH. Cellulose nitrate and polyallomer tubes were used for centrifugation at neutral and alkaline pH, respectively. Velocity and buoyant gradients were punctured at the bottom of the tubes and 10-drop (about 100 μl) fractions were collected for analysis.

Liquid Scintillation Counting—Samples were applied to Whatman No. 3MM filter papers. Thoroughly dried filters were placed into plastic scintillation vials and covered with toluene-based cocktail containing 5.0 g per liter of 2,5-diphenyloxazole (PPO) and 0.10 g per liter of 1,4-bis[2-(4-dimethyl-5-phenyloxazolyl)]benzene. The volume added was 5 ml for counting 3H, 10 ml for 3H and/or 14C, and 15 ml for 3H and/or 3P. Counting efficiency and channel spillover in the Packard Tri-Carb liquid scintillation spectrometer were determined with standards applied to paper filters. Double-label experiments were analyzed using the 3H channel exclusion method.

Electron Microscopy—Specimens were prepared using the formamide modification of the Klompischmidt procedure (14). The DNA samples were first dialyzed against 50% formamide, 100 mM Tris, 10 mM EDTA (pH 8.5). Cytochrome c added to 50 μg per ml prior to casting on a hypophase containing 17% formamide, 10 mM Tris, 1 mM EDTA (pH 8.5). Grids were shadowed on a rotating platform 8 degrees below and 5 cm from the point of evaporation of a Pt-Pd wire. Electron micrographs were recorded on 35 mm film using the Philips 300 electron microscope.

Preparation of Brominated Nucleotides—5-Bromodeoxyuridine 5’-triphosphate was prepared as described by Bessman et al. (15), by bromination of dCTP in formamide and subsequent deamination of the 5-bromodeoxyxycytidine 5’-triphosphate with nitrous acid. The deoxynucleoside triphosphates were isolated from reaction mixtures by adding Daalig to 0.25 M and precipitating with ethanol. The precipitates were washed in ethanol, dried in an airstream, and resuspended in 10 mM Tris (pH 7.5). The suspended nucleotides were metathesized to soluble potassium salts with Dowex 50. Ultraviolet absorption spectra and Dowex 1 chromatography were used to check the quality of the preparations.

Tissue Culture—HeLa cells were maintained in suspension culture using the Dulbecco modification of Eagle’s phosphate medium supplemented with 5% calf serum. Spinners were inoculated at 3 × 10⁶ cells per ml, 72 hours prior to the harvest of cells for the preparation of mitochondria. BSC-1 cells were maintained on plastic dishes and SV40 infections were carried out as described by Rush et al. (11).

Preparation of HeLa Cell Mitochondria—At the time of harvest the concentration of cells was 3 to 4 × 10⁶ cells per ml. Cells were recovered from suspension by centrifugation in batches for 3 min each at 1400 × g in the International PR-6, and in the latter part of the work at 3000 × g in the Sorvall RC2 using the Szent-Gyorgyi and Blum continuous flow system. A preparation of 20-liter suspension can be processed by continuous flow in the Szent-Gyorgyi and Blum continuous flow system. 2 The aid and advice of Dr. P. A. Sharp and Dr. D. L. Robberson are gratefully acknowledged.
swell in this hypotonic buffer for about 30 min. Each batch was homogenized using two strokes of the 40-ml glass Dounce homogenizer (Kontes) with tight-fitting pestle. One-third volume of 1.1 M sucrose, 10 mM Tris, 1 mM EDTA, (pH 7.5) was added to the homogenate followed by an additional stroke of the homogenizer for mixing. Phase contrast microscopy confirmed greater than 90% disruption. The free nuclei appeared intact. Cells, nuclei, and large debris were removed by centrifugation three times for 5 min at 2000 × g, discarding the pellets each time. The resulting supernatant was layered onto 1.5 M sucrose, 10 mM Tris, 1 mM EDTA (pH 7.5) in six tubes containing 25 ml and 10 ml of the supernatant and 1.5 M sucrose stages, respectively. Centrifugation followed for 30 min in the SW27 rotor at 25,000 rpm. Mitochondria were recovered from the interface and resuspended in 90 ml of Buffer C (210 mM mannitol, 70 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5). The mitochondrial sample in Buffer C was layered onto six preformed, two-step gradients containing 10 ml of 1.1 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5, over 10 ml of 1.5 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.5. Centrifugation followed for 30 min at 25,000 rpm as before. Mitochondria were recovered at the interface between the 1.1 and 1.5 M sucrose solutions, resuspended in Buffer C, and pelletted for 15 min at 12,000 rpm in the Sorvall RC2-B with an SS-34 rotor. The mitochondrial pellet was resuspended in 2% SSS, 0.1 mM EDTA, pH 7.5, for extraction of mitochondrial DNA, or 10 ml Tris, 10 mM 2-mercaptoethanol, pH 7.5, for extraction of soluble mitochondrial proteins by sonication.

**Enzyme Assays**—DNA polymerase was assayed using a modification of the procedure described by Bollum (16) based on the incorporation of labeled deoxyribonucleotides into acid-precipitable material retained on paper filters through a batch wacking procedure. A typical reaction mixture contained 10 ml Tris (pH 7.5), 3.3 mM 2-mercaptoethanol, 33 mM NaCl, 3.3 mM MgCl₂, 0.33 mM EDTA, 170 μM dGTP, 155 μM dCTP, 310 μM dTTP, 84 μM [3H]dATP (specific activity, 250 Ci per mole), 20 μg per ml of native calf thymus DNA, and enzyme protein in the range of 50 to 200 μg per ml. Prior to incubation, materials were kept at 4°C. After zero, 30, and 60 min at 37°C, 50-μl aliquots were withdrawn from the incubation mixture, with the same micropipette, onto two numbered paper filters, one of which was immediately placed in a beaker containing 500 ml of cold 5% trichloroacetic acid, 1% sodium pyrophosphate. Thirty minutes after the addition of the last filter to the bath, the filters were washed with cold 5% trichloroacetic acid three times for 20 min and then with 95% ethanol, followed by ethyl ether and drying. Incorporation of [3H]dATP was assessed in two ways. The 3H counts per min retained on the washed filter divided by the specific activity of the [3H]dATP was the amount of deoxyadenylate (nanomoles) in the 50-μl aliquot rendered insoluble by action of the enzyme system. The ratio of 3H counts per min on the washed and unwashed filters corresponds to the fraction of deoxyadenylate in the reaction which is acid-precipitable. This fraction and the initial concentration of dATP were used to calculate the total amount of deoxyadenylate polymerized by the enzyme system. The two methods gave the same result. Linear incorporation kinetics (Figs. 4 and 5) justified the calculation of the rate of incorporation from a single determination of the amount of nucleotide rendered acid-precipitable.

One unit of DNA polymerase activity is defined as the conversion of 1 nmole of labeled nucleotide into acid-precipitable material after 60 min at 37°C. Specific activity of enzyme preparations is expressed as units per mg of protein.

Levels of deoxyribonuclease activity were so low that the Kunitz assay (17) and attempts to demonstrate the solubilization of radioactive-labeled, acid-precipitable DNA samples by the action of the enzyme system were not satisfactory. A more sensitive endonuclease assay developed by Paolleti et al. (18) and based on the conversion of closed circular DNA to nicked circular DNA (or to linear DNA) was used. On binding to DNA, the fluorescence of ethidium bromide is enhanced. Endonuclease action releases a restriction on the amount of dye which can bind to closed circular DNA. The increase in fluorescence of an ethidium bromide solution containing samples of initially closed circular DNA can be related to the extent of endonuclease action. Equal aliquots were taken at 5-min intervals from a reaction mixture containing initially closed circular SV40 DNA, and diluted 25-fold in 1 ml EDTA, 1 μg per ml of ethidium bromide, pH 7.5. The fluorescence enhancement, $E$, is the measured increase in the fluorescence of the ethidium bromide solution after adding the sample. The fraction of closed circular molecules surviving in a given sample was calculated from the observed fluorescence enhancement using the linear relation

$$E_{\text{25}} - E_{\text{10}} = E_{\text{10}} - E_{\text{0}}$$

where $E_{\text{0}}$, $E_{\text{10}}$, and $E_{\text{25}}$ are the fluorescence enhancements of solutions of SV40-I and SV40-II, respectively, at the same concentrations as the DNA in the sample used to measure $E$.

**RESULTS**

**Extraction and Partial Purification of Mitochondrial DNA Polymerase**—Freshly prepared HeLa cell mitochondria were suspended in 3 to 5 ml of 10 mM Tris, 10 mM 2-mercaptoethanol (pH 7.5) and subjected to vigorous sonication. The tip of the horn of the Model S-125 Branson sonifier was immersed directly into the suspension during three 20-s bursts at level six. The 12-ml conical test tube was chilled in ice after each burst. The turbid suspension clarified. Following sonication, addition of NaCl to 1 M and extraction for up to 12 hours did not increase the yield of protein or DNA polymerase activity. Addition of salts prior to sonication greatly interfered with the extraction.

The sonicate was then centrifuged in polycarbonate tubes at 40,000 rpm for 1 h in the type 65 fixed angle rotor. Most of the clear, pale yellow supernatant was carefully withdrawn from the top of the tube with a Pasteur pipette. The last 0.5 to 1.0 ml of fluid gave rise to visible schlieren effects when drawn into the pipette. This material and the clear gelatinous pellet were routinely discarded. Earlier experiments in which the lower phase was included in the crude extract yielded final preparations of DNA polymerase with similar levels of activity, but with significantly lower ratios of A₂₆₀/A₂₈₀, suggesting greater nucleic acid contamination.

The crude extract, designated Fraction CE, was then applied to a Bio-Gel P-150 column for gel filtration, eluting with 10 mM Tris, 10 mM 2-mercaptoethanol (pH 7.5). Elution of the sample was monitored by absorbance at 280 nm. DNA polymerase can be located by assay of the eluted fractions, but the protein is dilute at this stage and the levels of incorporation of labeled nucleotides are low. The procedure now employed is to postpone the assay until each of the fractions in which the enzyme is routinely recovered has been individually subjected to the fractional ammonium sulfate precipitation and concentration step described next. Fig. 1 shows a typical elution profile from the Bio-Gel P-150 column and the location of DNA polymerase activity in the fractions following elution of the void volume.
Figure 1. Gel filtration of supernatant fraction following centrifugation of mitochondrial sonicate. The sample (4 ml) was applied to a Bio-Gel P-150 column having a bed volume of 170 ml. The column was pre-equilibrated with 10 mM Tris, 10 mM 2-mercaptoethanol, pH 7.5, the buffer used for elution. All column operations were performed at ambient room temperature. Fractions (4.4 ml each) were collected and chilled to 4°C. Elution of protein was monitored by absorbance at 280 nm (•—•). Aliquots from Fractions 10 through 17 were assayed for DNA polymerase activity (○—○) as described under "Methods," except that the radioactive label was 6 µM [3H]dTTP (7 Ci per mmole) and dATP was present at 175 µM. The DNA polymerase activity is represented by the radioactivity rendered acid-precipitable after incubation for 30 min at 37°C.

Selected column fractions were raised to 35% saturated ammonium sulfate by the addition of 0.54 ml of saturated ammonium sulfate per ml of sample. After 30 min at 4°C the precipitated material was removed by centrifugation at 40,000 rpm, 30 min, in the type 65 rotor. The supernatant was raised to 50% saturated ammonium sulfate by the addition of 0.30 ml of sample, chilled 30 min at 4°C, and the precipitate collected as before. The precipitated material was carefully resuspended in 0.5 ml of 10 mM Tris, 10 mM 2-mercaptoethanol, 10 mM MgCl₂, 100 µM NaCl, pH 7.5.

A typical preparation from 20 liters of suspension culture yielded 40 ml of packed cells, 1 ml of wet, purified mitochondria, about 10 mg of protein in the crude extract, and 0.5 mg of protein in Fraction AS representing the pooled fractions containing DNA polymerase activity. The expected yield of mitochondrial DNA polymerase was calculated on the basis of the ad hoc assumption of 1 to 10 molecules of the enzyme per molecule of mitochondrial DNA in HeLa cells. The yield of mitochondrial DNA from HeLa cells is about 2 µg to 2 × 10⁻¹⁸ mole per ml packed cells. If the molecular weight of the DNA polymerase in Fraction AS is 1.5 × 10⁶ g per mole (see Fig. 1), we would expect about 1 to 10 µg of pure DNA polymerase in the typical preparation of Fraction AS which leads to 500 µg of total protein derived from 40 ml of packed cells.

Attempts to purify the enzyme further by DEAE-cellulose chromatography were unsuccessful. None of the DNA polymerase activity applied to the columns was recovered in individual eluted fractions (step-gradient elution), or after pooling and concentration of the total eluted protein.

The time required for a preparation of Fraction AS is about 14 hours. The DNA polymerase activity in Fraction AS decays to about 50% in 1 week at 4°C. Reactions described in the text were performed within 48 hours of a preparation of Fraction AS.

An increase in the total DNA polymerase activity was observed at each step in the partial purification, presumably due to the removal of materials inhibiting DNA synthesis. Attempts to demonstrate DNA polymerase activity in discarded fractions of the sonicate were unsuccessful.

**Reaction Requirements**
The complete incorporation system contains 24 µg per ml of native or denatured calf thymus DNA, 210 µg per ml of Fraction CE protein or 33 µg per ml of Fraction AS protein, 2 mM 2-mercaptoethanol, 10 mM Tris (pH 7.5), 4 mM MgCl₂, 1.5 mM ATP, 150 µM dATP, 150 µM dGTP, 150 µM dCTP, and 4.6 µM [3H]dTTP, 10.35 Ci per mmole.

**Table I**

| Reaction conditions | CE | AS |
|--------------------|----|----|
| Complete system (native DNA) | 0.08 | 0.74 |
| -DNA | 0.01 | 0.00 |
| -native DNA + denatured DNA | 0.03 | 0.29 |
| -ATP | 0.14 | 0.83 |
| -MgCl₂ | 0.00 | 0.00 |
| -dATP | 0.01 | 0.00 |
| -dGTP | 0.01 | 0.00 |
| -dCTP | 0.01 | 0.00 |

**Table II**

| Monovalent cation salt | Divalent cation salt | Activity of AS |
|------------------------|---------------------|----------------|
| NaCl 32 mM | MgCl₂ 2 mM | units/mg protein |
| None | MgCl₂ | 8.0 |
| LiBr | MgCl₂ | 13 |
| NaCl | MgCl₂ | 19 |
| NaBr | MgCl₂ | 19 |
| KCl | MgCl₂ | 20 |
| KBr | MgCl₂ | 19 |
| RbBr | MgCl₂ | 31 |
| CsCl | MgCl₂ | 18 |
| NiCl₂ | MgCl₂ | 13 |
| NaCl | MgCl₂ | 19 |
| NaCl | CaCl₂ | 0.1 |
| NaCl | SrBr₂ | 0.3 |
| NaCl | BaCl₂ | 1.4 |

**Effects of monovalent and divalent cations**

Each reaction contains 12 µg per ml of native calf thymus DNA, 80 µg per ml of Fraction CE protein, 2 mM 2-mercaptoethanol, 10 mM Tris (pH 7.5), 100 µM dATP, 92 µM dCTP, 103 µM dGTP, 96 µM [3H]dTTP (0.13 Ci per mmole), and salts as specified in the table.
reaction conditions were the same except for templates, the con-

nate calf thymus DNA, T7 phage DNA, SV40 viral DNA, the ex-

incorporation of $[^3H]dTTP$ was determined for three concentra-

ions of each template. As seen in Fig. 2, the activity of Fra-

of DNA synthesis. This denatured DNA was a less

ribonucleotides to evaluate the effect of possible nucleotide

TABLE III

Table: Activity with mixed divalent cation salts

| Divalent cation salt(s) | Activity of AS (units/mg protein) |
|------------------------|----------------------------------|
| None                   | 0                                |
| 1.7 mm MgCl2           | 13                               |
| 3.4 mm MgCl2           | 17                               |
| 1.7 mm MgCl2 + 1.7 mm CaCl2 | 5                               |
| 1.7 mm MgCl2 + 1.7 mm SrCl2  | 4                               |
| 1.7 mm MgCl2 + 1.7 mm BaCl2 | 14                             |

Fig. 2 (left). DNA polymerase activity with different native duplex DNA templates. Calf thymus DNA (+), T7 phage DNA ( ), SV40 viral DNA ( ), and HeLa mitochondrial DNA ( ) were used at the indicated concentrations. The reaction mixtures contained 0.57 mg per ml of Fraction AS protein, 126 $\mu$M dATP, 126 $\mu$M dGTP, 140 $\mu$M dCTP, 65 $\mu$M $[^3H]dTTP$ (250 Ci per mole), 4 mM MgCl2, 10 mM Tris, pH 7.5, and 2 mM 2-mercaptoethanol. The extent of DNA synthesis after 60 min at 37°C was determined as described under "Methods."

Fig. 3 (right). Effect of MgCl2 and NaCl concentrations on DNA polymerase activity in Fraction AS. Reaction mixtures contained 80 $\mu$g per ml of Fraction AS protein, 12 $\mu$g per ml of native calf thymus DNA, 10 mM Tris, pH 7.5, 2 mM 2-mercaptoethanol, and 0 mM NaCl ( ), 32 mM NaCl ( ), 100 mM NaCl ( ), and 320 mM NaCl ( ). Concentrations of nucleotides in each reaction were 100 $\mu$M dATP, 33 $\mu$M dCTP, 100 $\mu$M dGTP, and 92 $\mu$M $[^3H]dTTP$ (32 Ci per mole). The extent of DNA synthesis after 60 min at 37°C was determined as described under "Methods."

centrations of NaCl and template DNA were below optimum levels for DNA synthesis.

Salt Effects—The capacity of Fraction AS for DNA synthesis with native calf thymus DNA template was studied as a function of the concentration of MgCl2 and NaCl present in the reaction mixture. The results shown in Fig. 3 indicate that 30 mM NaCl and 3 mM MgCl2 provided maximum activity. Unless otherwise indicated, these concentrations were used in reactions described subsequently. Concentrations of NaCl higher than 70 mM were found to inhibit DNA synthesis, in distinct contrast to the optimum concentration of NaCl, 150 mM, found by Meyer and Simpson for the rat liver mitochondrial DNA polymerase (10). The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 mM, all enhance the activity of Fraction AS, but to different extents. The replacement of NaCl and MgCl2 with other salts was studied and the results are presented in Table II. The monovalent cations, added at 32 M
activity reached a plateau. Other experiments of this nature have shown that nucleotide incorporation by Fraction CE reached a maximum level followed by a gradual loss of acid-precipitable [3H]thymidine. This is presumably due to degradative activities in Fraction CE which are greatly reduced in the purification leading to Fraction AS.

Circular DNA template for DNA synthesis are an important aspect of this report. SV40 DNA was used to evaluate the dependence of incorporation kinetics on template DNA concentration. Linear incorporation kinetics over 60 min with Fraction AS was observed with different concentrations of SV40 DNA template (Fig. 5). A double reciprocal plot of the rate of DNA synthesis at different template concentrations was linear and gave values of 2.6 nmoles of deoxyadenylate per hour and 9 µg per ml for the maximum rate of incorporation and the concentration of template DNA required for half the maximum rate, respectively. The reaction mixtures contained 96 µg per ml of Fraction AS protein. At high DNA template concentrations, the specific activity approached 27 units per mg of protein.

Assuming the product contains 29.5% deoxyadenylate, in accord with the base composition of the SV40 template (19), the ratio of product DNA to initial template DNA can be calculated and used as an indication of the extent of synthesis. In the above experiment (Fig. 5) we find that after 60 min at 37°C the product to template ratio is 0.11, 0.26, and 0.48 for 40, 10, and 2.5 µg per ml of template, respectively.

Conservation of Template in DNA Synthesis and Deoxyribonuclease Activity of Fraction AS—Double label experiments were performed with Fraction AS to determine the extent of template degradation in the presence and absence of the nucleotides required for DNA synthesis. SV40 [32P]DNA, 106 cpm per µg, was used as template. The reaction in which DNA synthesis was to proceed contained [3H]dATP, 46,000 cpm per µg of product DNA assumed to contain 29.5% deoxyadenylate. The incorporation of [3H]dATP into product DNA and the fractional loss of acid-precipitable template DNA were determined in the course of 60 min at 37°C (Table IV). The template was quantitatively recoverable throughout the period of incubation. The extent of product DNA synthesis after 1 hour was approximately 10 times greater than the error associated with the template recovery analysis.

The endonucleolytic conversion of closed circular SV40 DNA to nicked circular or linear DNA, or both, was followed by the fluorometric assay (18). First order survival kinetics of SV40-I [32P]DNA (100,000 cpm per pg), 10 mM Tris (pH 7.5), 3.3 mM 2-mercaptoethanol, 33 mM NaCl, 3.3 mM MgCl2, and 0.33 mM EDTA, was used as template. The reaction mixture was incubated at 37°C. Aliquots were withdrawn at the indicated times for determination of the fraction of labeled DNA remaining circular. The sedimentation assay employed is described under "Methods." The half-life of closed circular SV40 DNA under the conditions of this experiment was 7 min.

If the endonucleolytic process is considered to be a collection of randomly placed single- or double-strand scissions, or both, with all phosphodiester bonds equally susceptible, then the average number of breaks per molecule per min may be calculated by dividing ln 2 by the measured half-life of closed circular DNA. For the experiment shown in Fig. 6, the rate was found to be 0.10 break per molecule per min for at least 20 min. We extrapolate that there would be an average of six breaks, single- or double-strand scissions, per molecule after 1 hour. No significant difference in the half-life of closed circular SV40 DNA at concentrations of 33 and 66 µg per ml was found, a result which suggests a linear dependence of nuclease activity on DNA concentration. In this system SV40 DNA sustains an average number of breaks per molecule per min which is independent of DNA concentration up to at least 66 µg per ml.

Two lines of evidence show that the endonuclease action is predominantly a single-strand scission process. A mixture of [3H]-labeled SV40-I and SV40-II was incubated at 37°C in two reactions, with and without Fraction AS. Sedimentation velocity analysis revealed that the DNA remained circular through both incubations, although the SV40-I was converted to SV40-II in the presence of Fraction AS. As seen in Fig. 7 the DNA incubated with Fraction AS sedimented as a sharp band corresponding to the position of SV40-II. Significant double-strand scission activity would have generated linear fragments. The sedimentation profile, although still peaked at 14 to 16 S, would have been skewed back to the meniscus. Electron microscope examination of a sample of initially closed circular SV40 DNA, incubated for 60 min with Fraction AS, revealed only 5% linear molecules (500 molecules counted). One might argue that the linear molecules reflect fragmentation of the more extensively nicked molecules in the sample due to proximity of single-strand scissions on opposite strands. Assuming random disposition of the breaks in the population of initially closed circular molecules, the frequency of molecules with a specified number of

| Time of incubation (min) | Template recovery | Product DNA in B (%) | Product in B/Initial template |
|-------------------------|-------------------|----------------------|-----------------------------|
| 0                       | A 85              | 100                  | 0                           |
| 20                      | 101               | 100                  | 0.13                        |
| 40                      | 100               | 98                   | 0.23                        |
| 60                      | 101               | 100                  | 0.37                        |

Fig. 6. Endonuclease assay with Fraction AS protein and SV40 DNA template under conditions of DNA synthesis. The reaction mixture contained 102 µg per ml of Fraction AS protein, 8 µg per ml of SV40 DNA (initially closed circular), 10 mM Tris, pH 7.5, 3 mM 2-mercaptoethanol, 3 mM MgCl2, and 0.33 mM EdTA. The reaction mixture was incubated for 60 min at 37°C. A aliquots were withdrawn at the indicated times for determination of the fraction of SV40 DNA remaining closed circular. The fluorescence assay employed is described under "Methods." The half-life of closed circular SV40 DNA under the conditions of this experiment was 7 min.
Fig. 7. Sedimentation analysis of a mixture of closed circular and nicked circular SV40 [3P]DNA previously incubated 60 min at 37° with (●●●●) or without (〇〇〇〇) Fraction AS protein. The incubation mixtures contained 3.6 μg per ml of SV40 [P]DNA (1,500 cpm per μg), 10 mM Tris, pH 7.5, 3 mM 2-mercaptoethanol, 33 mM NaCl, 3 mM MgCl₂, 0.3 mM EDTA, 116 μM dATP, 115 μM dCTP, 103 μM dGTP, and 307 μM dTTP. One mixture also contained 40 μg per ml of Fraction AS protein. Following incubation EDTA was added to 50 mM, SDS added to 20 mg per ml, and the samples held at room temperature for 10 min. NaCl was then added to 1 M and the samples were chilled to 4°. After 10 min the resulting precipitate was removed by centrifugation for 10 min at 10,000 × g. The supernatants were kept at 4° until the time of analysis. The samples were sedimented at 20° in two 3.0-ml self-generating CsCl density gradients (initial density, 1.40 g per ml) for 3 hours at 35,000 rpm. Fractions (100 μl each) were collected from the bottom of the tube directly onto paper filters for subsequent sedimentation analysis. Details of centrifugation, fractionation, batchwise acid-washing of filters, and liquid scintillation counting are described under “Methods.” In this and subsequent sedimentation analyses the direction of the centrifugal field is from right to left.

breaks can be predicted on the basis of the Poisson distribution. The probability that a molecule with a specified number of nicks remains circular can also be calculated with a critical number of base pairs as a variable. A molecule would not remain circular with nicks on opposite strands separated by fewer than the critical number of base pairs. An average of six single-strand scissions per SV40 molecule would linearize the molecule only if the critical separation is more than 1000 base pairs. This number of base pairs is unreasonably large. Nonrandom single-strand scissions, low double-strand scission activity, or interaction within the regions of DNA synthesis are alternative explanations of the low but significant frequency of linear SV40 molecules.

Buoyant Equilibrium Properties of Product DNA—The behavior of the ³H-labeled product DNA in buoyant equilibrium experiments was compared to SV40 [³P]DNA added as a marker prior to centrifugation. The buoyant density of the SV40 [³P]DNA under the equilibrium conditions employed is known and the density gradient in the region of the marker DNA was calculated with data given by Vinograd and Hearst (20). The magnitude of the buoyant density differences between the DNA distributions was estimated from differences in the centers of gravity of the radioactive marker and product DNA distributions.

At the end of incubations the reaction mixtures were chilled to 4° and EDTA was added to at least 10 mM. The samples were then treated with SDS (20 mg per ml) for 10 min at room temperature, followed by addition of NaCl or CsCl to 1 M, chilling to 4°, and removal of the precipitated protein and SDS. The extent of DNA synthesis was determined from the measured incorporation of ³H-labeled nucleotide.

The product DNA forms a band at the same position in a pH 7.5 CsCl density gradient as the SV40 marker [³P]DNA (Fig. 8). In a CsCl density gradient containing ethidium bromide (Fig. 9), closed circular and nicked circular SV40 DNA separate and the product DNA is found in one band, coincident with nicked circular marker DNA.

Product DNA samples were prepared using ³H]dATP, 5-bromodeoxyuridine 5'-triphosphate, and different concentrations of SV40 DNA template. At higher concentrations of template the total amount of product synthesized by Fraction AS is greater, but the amount of product relative to the template is lower. In the CsCl density gradients shown in Fig. 10, the

Fig. 8 (left). Distributions of SV40 [³P]DNA (〇〇〇〇) and ³H-labeled product DNA (●●●●) in a CsCl equilibrium buoyant density gradient, pH 7.5. The product DNA was synthesized in a reaction mixture containing 160 μg per ml of Fraction AS protein, 8 μg per ml of unlabeled SV40 DNA, 10 mM Tris (pH 7.5), 3 mM 2-mercaptoethanol, 33 mM NaCl, 3 mM MgCl₂, 0.3 mM EDTA, 65 μM [³H]dATP (250 Ci per mole), 157 μM dCTP, 124 μM dGTP, and 218 μM dTTP. Following incubation for 60 min at 37° the reaction was stopped by addition of EDTA to 50 mM and SDS to 20 mg per ml. After 10 min at room temperature CsCl was added to 1 M and the mixture chilled to 4°. The resulting precipitate was removed by centrifugation for 10 min at 10,000 × g. The supernatant was kept at 4° until the time of analysis. The sample was diluted to about 3 ml with 10 mM Tris (pH 7.5), SV40 [³P]DNA (about 4,000 cpm) was added as a marker and solid CsCl was added to raise the density to 1.70 g per ml. Centrifugation followed at 35,000 rpm, 20°, for 46 hours. Fractions (100 μl each) were collected from the bottom of the tube directly onto paper filters for subsequent batchwise acid precipitation of the DNA and analysis of the distributions of radioactivity in the density gradient. Details of these steps are described under “Methods.” The density gradient in this and subsequent equilibrium banding experiments increases from right to left and is approximately 8 mg per ml per fraction.

Fig. 9 (right). Distributions of a mixture of closed circular and nicked circular SV40 [³P]DNA (〇〇〇〇) and ³H-labeled product DNA (●●●●) in a CsCl equilibrium buoyant density gradient (pH 7.5) containing ethidium bromide. The product DNA was synthesized in a reaction mixture containing 102 μg per ml of Fraction AS protein, 8 μg per ml of unlabeled SV40 DNA (initially closed circular), 10 mM Tris (pH 7.5), 3 mM 2-mercaptoethanol, 33 mM NaCl, 3 mM MgCl₂, 0.3 mM EDTA, 165 μM dATP, 171 μM dCTP, 156 μM dGTP, and 6 μM [³H]dATP (7 Ci per mmole). After 60 min incubation at 37° the reaction was stopped by addition of EDTA to 50 mM. SV40 [³P]DNA (about 6000 cpm, a mixture of nicked and closed circular DNA) was added to the sample. The sample was diluted to about 3 ml with 10 mM Tris (pH 7.5), and ethidium bromide was added to 250 μg per ml. Solid CsCl was added to raise the density to 1.55 g per ml. Centrifugation, fractionation, and analysis of radioactive distributions were the same as described in the legend for Fig. 8. The more dense band in the [³P] distribution corresponds to the closed circular SV40 DNA.
linearly correlated with the extent of synthesis, defined as the product DNA is shifted to higher densities as the fraction of product synthesized in each of the reactions.

Samples were then treated with SDS as described in the legend. After 60 min incubation at 37°C the reactions were stopped by addition of EDTA to 50 mM. The reaction mixtures also contained 160 µg per ml of Fraction AS protein, 10 m&f Tris (pH 7.5), 3 mM 2-mercaptoethanol, 33 mM NaCl, 3 mM MgCl2, 0.3 mM EDTA, 121 µM [3H]dATP (260 Ci per mole), 140 µM dGTP, 127 µM dCTP, and 246 µM 5-bromodeoxyuridine 5'-triphosphate. After 60 min incubation at 37°C the reactions were stopped by addition of EDTA to 50 mM. The samples were then treated with SDS as described in the legend for Fig. 8. After dialysis with 10 mM Tris (pH 7.5) solid CsCl was added to raise the density to 1.72 g per ml. About 1400 cpm of SV40 [32P]DNA were added to each sample. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The amount of product synthesized in each of the reactions was evaluated from the total acid-precipitable [3H]radioactivity recovered in the density gradient and the specific activity of the [3H]dATP as described under "Methods." The extent of synthesis (product/ (product + template)) was 0.00 (top), 0.21 (middle), and 0.25 (bottom).

Product DNA is shifted to higher densities as the fraction of product in the sample increases. No peak appears at the position expected for hybrid or fully 5-bromodeoxyuridine-labeled SV40 DNA, Fractions 18 to 20 and 10 to 12, respectively. The product DNA is apparently associated with template DNA molecules, Fractions 8 to 11. The measured density shifts are linearly correlated with the extent of synthesis, defined as the ratio of product DNA to the sum of product and template DNA (Fig. 11). The point representing no density shift is taken from an experiment in which the extent of synthesis was 0.10, but the reaction contained dTTP instead of 5-bromodeoxyuridine 5'-triphosphate. The slope corresponds to 125 mg per ml density shift for unit extent of synthesis. The primary sources of error in this experiment were the estimation of the density gradient and the extent of synthesis. Combined, these amount to probably less than a 10% error in the value of the slope. The magnitude of the density shift relative to extent of synthesis will be discussed later.

FIG. 10. Distributions of SV40 [32P]DNA (O—O) and H-labeled 5-bromodeoxyuridine containing product DNA (—) in CsCl equilibrium buoyant density gradients (pH 7.5). The product was synthesized in reactions containing different concentrations of unlabeled, thymidine-containing SV40 DNA template in order to obtain samples with different extents of synthesis relative to template. Template concentrations were 160 µg per ml (top), 10 µg per ml (middle), and 2.5 µg per ml (bottom). The reaction mixtures also contained 160 µg per ml of Fraction AS protein, 10 mM Tris (pH 7.5), 3 mM 2-mercaptoethanol, 33 mM NaCl, 3 mM MgCl2, 0.3 mM EDTA, 121 µM [3H]dATP (260 Ci per mole), 140 µM dGTP, 127 µM dCTP, and 246 µM 5-bromodeoxyuridine 5'-triphosphate. After 60 min incubation at 37°C the reactions were stopped by addition of EDTA to 50 mM. The samples were then treated with SDS as described in the legend for Fig. 8. After dialysis with 10 mM Tris (pH 7.5) solid CsCl was added to raise the density to 1.72 g per ml. About 1400 cpm of SV40 [32P]DNA were added to each sample. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The amount of product synthesized in each of the reactions was evaluated from the total acid-precipitable [3H]radioactivity recovered in the density gradient and the specific activity of the [3H]dATP as described under "Methods." The extent of synthesis (product/ (product + template)) was 0.00 (top), 0.21 (middle), and 0.25 (bottom).

The nature of the association of product and template DNA was examined in alkaline CsCl density gradients (Fig. 12). Under these conditions (pH 12.5) strand separation occurs for all but closed circular DNA. The latter undergoes denaturation and is about 20 mg per ml more dense than single-stranded DNA (22). Samples of product DNA were prepared using dTTP or 5-bromodeoxyuridine 5'-triphosphate, [3H]dATP, and closed circular SV40 DNA template. Closed circular SV40 [32P]DNA was added as a marker prior to banding in alkaline CsCl density gradients. The fraction of product DNA in the two samples was 10%. In the case of thymidine incorporation the H-labeled product was found to be 20 mg per ml lighter than the [3H]labeled marker. With incorporation of 5-bromodeoxyuridine, however, the separation of the product and marker distributions was only 1.2 mg per ml. The product distribution was skewed to higher densities in this case, but very little product was found in the region expected for fully 5-bromodeoxyuridine-labeled single-stranded DNA. The very small light shoulder in the [3P]labeled marker DNA distributions indicates the limited extent of alkaline hydrolysis during centrifugation. The result of this experiment suggests that most of the product DNA is covalently bound to the template DNA strands.

It was observed that omission of the SDS treatment after incubation with the normal complement of four nucleotides led to the appearance of product DNA at slightly lower density than the marker DNA, suggesting that some protein remains bound to the DNA in untreated samples at high CsCl concentrations. This effect was not observed when untreated samples were banded in gradients with ethidium bromide or adjusted to alkaline pH.

Sedimentation Properties of Product DNA—Sedimentation
slowly sedimenting material, therefore, is not associated with template. The synthesis of this material is sedimented with and slightly ahead of the nicked circular SV40 DNA, about 7 S, was sometimes observed. When SV40 DNA was used as a template no 32P counts appeared with the 3H-labeled product DNA in this band. The slowly sedimenting material, therefore, is product DNA not associated with template. The synthesis of this material is still dependent on added template DNA and may result from a noncovalent initiation process and subsequent dissociation from the template. The amount of this material apparently depends in an unknown manner on the preparation of Fraction A8. With some preparations, the slow band was not detected. When present, the relative amount increased to as much as 30% of the product DNA. The relative amount appeared to increase when nucleotide or template concentrations were limiting.

Sedimentation studies were also performed in alkaline self-generating CsCl density gradients. 3H-Labeled φX174 single-stranded circular DNA, having approximately the same sedimentation velocity as circular single-stranded SV40 DNA, was used as a marker. The 3H-labeled product of DNA synthesis with SV40-I template sedimented with a predominant peak in the region expected for full-length linear single strands of SV40 DNA (Fig. 14). The distribution of product DNA was skewed back to the meniscus. This sedimentation behavior is consistent with an average of two to three single-strand scissions in the circular template strands and with the covalent attachment of product DNA to template DNA strands.

Susceptibility of Product and Template to Action of Escherichia coli Exonucleases I and III—The specific reactions of E. coli exonucleases I and III make them suitable for the investigation of the structure of molecules containing both template and product DNA. An incorporation system containing [32P]SV40 DNA, an incorporation system containing [32P]SV40 DNA, was used as a marker. The 3H-labeled product of DNA synthesis with SV40-I template sedimented with a predominant peak in the region expected for full-length linear single strands of SV40 DNA (Fig. 14). The distribution of product DNA was skewed back to the meniscus. This sedimentation behavior is consistent with an average of two to three single-strand scissions in the circular template strands and with the covalent attachment of product DNA to template DNA strands.

**Fig. 12.** Distributions of initially closed circular SV40 [32P]-DNA (O—O) and 3H-labeled product DNA samples (●●) after buoyant equilibrium centrifugation in alkaline CsCl density gradients. The product DNA was synthesized in two reaction mixtures, one containing 300 μM dTTP (top) and the other containing 300 μM 5-bromodeoxyuridine 5'-triphosphate (bottom). Each reaction also contained 160 μg per ml of Fraction A8 protein, 40 μg per ml of SV40 DNA (unlabeled), 10 mM Tris (pH 7.5), 3 mM 2-mercaptoethanol, 35 mM NaCl, 3 mM MgCl2, 0.3 mM EDTA, 70 μM [3H]dATP (260 Ci per mole), 175 μM dGTP, and 158 μM dCTP. After 60 min incubation at 37° the reactions were stopped by addition of EDTA to 50 mM and aliquots were removed for determination of the extent of DNA synthesis as described under "Methods." In each case the extent of synthesis (product/ template) was 0.10, indicating no change in the rate of DNA synthesis with 5-bromodeoxyuridine 5'-triphosphate instead of dTTP. The samples were then treated with SDS as described in the legend for Fig. 8. 0.05 ml of 0.25 M K2PO4 (previously adjusted to pH 12.5 with KOH) and about 200 cpm of SV40 [32P]-DNA (closed circular) were added to each sample. The volume of each sample was adjusted to 2.50 ml with distilled water and 3.30 g of solid CsCl were added to each to raise the density to 1.74 g per ml. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The differences in buoyant density of the 3H and 32P distributions were determined in the manner described in the legend for Fig. 11. The 3H-labeled products were 20 ng per ml (top) and 1.2 mg per ml (bottom) less dense than the thymidine-containing, closed circular SV40 [32P]-DNA.

**Fig. 13.** Band sedimentation analysis of 3H-labeled product DNA (●●) in a self-generating CsCl density gradient (pH 7.5). Product DNA was synthesized as described in the legend for Fig. 12 (top). SDS treatment, sedimentation, fractionation, and radioactive analysis were performed as described in the legend for Fig. 7, except that the initial density of the CsCl solution was 1.45 g per ml and the centrifugation was 21/2 hours at 35,000 rpm. About 700 cpm of SV40 [32P]-DNA (O—O, a mixture of nicked and closed circular DNA) was added to the sample prior to centrifugation.

**Methods.** In each case the extent, of synthesis (product/template) was 0.10, indicating no change in the rate of DNA synthesis with 5-bromodeoxyuridine 5'-triphosphate instead of dTTP. The samples were then treated with SDS as described in the legend for Fig. 8. 0.05 ml of 0.25 M K2PO4 (previously adjusted to pH 12.5 with KOH) and about 200 cpm of SV40 [32P]-DNA (closed circular) were added to each sample. The volume of each sample was adjusted to 2.50 ml with distilled water and 3.30 g of solid CsCl were added to each to raise the density to 1.74 g per ml. Centrifugation, fractionation, and analysis of radioactivity distributions were performed as described in the legend for Fig. 8. The differences in buoyant density of the 3H and 32P distributions were determined in the manner described in the legend for Fig. 11. The 3H-labeled products were 20 ng per ml (top) and 1.2 mg per ml (bottom) less dense than the thymidine-containing, closed circular SV40 [32P]-DNA.

Sedimentation studies were also performed in alkaline self-generating CsCl density gradients. 3H-Labeled φX174 single-stranded circular DNA, having approximately the same sedimentation velocity as circular single-stranded SV40 DNA, was used as a marker. The 3H-labeled product of DNA synthesis with SV40-I template sedimented with a predominant peak in the region expected for full-length linear single strands of SV40 DNA (Fig. 14). The distribution of product DNA was skewed back to the meniscus. This sedimentation behavior is consistent with an average of two to three single-strand scissions in the circular template strands and with the covalent attachment of product DNA to template DNA strands.
FIG. 14. Band sedimentation analysis of \(^{3}H\)-labeled product DNA (––––) in a denaturing (alkaline) self-generating CsCl density gradient. The \(^{3}H\)-labeled product is from the same sample used in the experiment described in the legend for Fig. 13. About 500 cpm \(^{14}C\)-labeled \(\phi X174\) phage DNA (O–O) was mixed with the sample before layering onto 4.0 ml of CsCl solution, density 1.60 g per ml, containing 0.10 M KOH. Centrifugation followed for 5 hours at 35,000 rpm, 20\(^{\circ}\). Fractionation and radioactivity analysis were performed as described in the legend for Fig. 7.

The product DNA isolated and purified from standard incorporation reaction was also treated with exonuclease I. The DNA was isolated in CsCl-ethidium bromide density gradients following treatment with SDS (20 mg per ml) as previously described. After removing the dye, the DNA containing 10% product was dialyzed against 10 mM Tris, 1 mM EDTA (pH 7.5). Exonuclease I was diluted to 300 units per ml in 10 mM Tris, 0.18 M (NH\(_4\))\(_2\)SO\(_4\), 0.1% bovine serum albumin (pH 7.5). Ten microliters of 1 mM MgCl\(_2\) and 50 \(\mu\)l of either the exonuclease I solution or its dilution buffer were added to 0.5 ml of the DNA sample. After 20 min at 37\(^{\circ}\), the reactions were stopped by chilling and adding 100 \(\mu\)l of 0.1 mM EDTA (pH 7.5). Preparative velocity gradients showed no change in the sedimentation behavior of the product DNA from the action of exonuclease I. In another experiment the DNA was dialyzed against 50 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.5). The sample was denatured by heating at 90\(^{\circ}\) for 5 min, then quickly chilled to 0\(^{\circ}\). Exonuclease I was diluted to 60 units per ml in 10 mM Tris, 100 mM MgCl\(_2\), 0.05% bovine serum albumin (pH 7.5). On incubation at 37\(^{\circ}\) of a solution containing equal volumes of the denatured DNA and exonuclease I solutions, more than 95% of the \(^{3}H\)-labeled product was solubilized in 5 min. The product DNA is, therefore, rendered susceptible to the action of exonuclease I only after denaturation.

**Electron Microscopy of Template-Product DNA.** Basic protein film electron microscopy was used to visualize molecules containing regions of product and template DNA. The formamide system (14) was chosen for two reasons. The standard aqueous Kleinschmidt procedure (23) applied to samples of DNA from reaction mixtures usually led to aggregation of molecules in the film and a rather coarse contour of duplex DNA. This may have been due to persistence of contaminating protein in the samples, despite treatment with SDS and banding in CsCl density gradients. The formamide may have dissociated protein-DNA complexes. In addition, the formamide technique allows discrimination between single- and double-stranded
regions in DNA molecules. Single-stranded DNA appears with somewhat reduced contrast from background and a more kinked contour than the thicker and smoother duplex DNA regions.

The electron micrographs presented in Fig. 16 show representative molecules in a sample containing 18% product DNA in a 1-hour incubation of circular SV40 template with Fraction AS. The most apparent structures are circular duplex, SV40-length molecules with single-stranded branches. About half of the circular duplex molecules have an easily visualized single-stranded branch. Molecules with more than one branch were not uncommon. Most of the branches observed were of the size seen in the molecules of Fig. 16, A, but occasionally much larger branches were found as in the molecules of Fig. 16, B and C.

The foregoing results, together with the conclusions drawn from the centrifugation and exonuclease susceptibility studies, indicate the displacement of a template strand in the course of product DNA synthesis. The length of the displaced template strand should be the same as the associated region of product DNA, since no degradation of template could be demonstrated in the course of product DNA synthesis. The more frequent smaller branches were about 2% by mass of an SV40 molecule. The longer branches shown in Fig. 16, B and C, were 20% and 10% of SV40 molecular weight, respectively. An 18% extent of synthesis would lead to an 18% increase in the average molecular weight of the SV40 molecules in the sample. The frequency of molecules with long branches was too small to significantly affect the average molecular weight. The molecules with short branches could account for an increase in the average molecular weight of only 1 to 2%.

In another sample of DNA containing 6% product, the frequency of circular duplex molecules with small single-stranded branches was much lower, about 2 to 3%, which could account for only 1% of the expected increase in average molecular weight of the SV40 DNA. The rather low frequency of branched molecules and the small size of most of the branches suggest that product DNA synthesis also gives rise to short displaced template branches, too small to be visualized. Such a result is compatible with initiation of DNA synthesis at many of the single-strand cleavages introduced during the incubation with Fraction AS. Previous experiments indicated that approximately 10 single-stranded cleavages per molecule occurred during a 60-min incubation. A 10% extent of synthesis would represent an average of about 100 nucleotides incorporated at each nick. Displaced single-stranded template DNA branches of this average length would be hard to distinguish from the contour of otherwise duplex DNA. Occasional stretches of more extensive synthesis generate branches long enough to be observed in the electron microscope.

**DISCUSSION**

A DNA polymerase-containing fraction (AS) has been isolated and partially purified from soluble protein extracted after sonic disruption of HeLa cell mitochondria. Meyer and Simpson (10) have described DNA polymerase preparations from the mitochondria of rat liver, characterized the reaction requirements, and were able to distinguish the activity from that of rat liver nuclear DNA polymerase. Following ammonium sulfate precipitation, their mitochondrial enzyme fraction wasfree of contamination of the nuclear DNA polymerase, as judged by DEAE-cellulose chromatography. Since the purification of the rat liver mitochondria described by Meyer and Simpson (10) was less extensive than that which we have employed, the presence of the soluble HeLa nuclear DNA polymerase in Fraction AS is probably minimal. Furthermore, the soluble nuclear DNA polymerase of HeLa cells has from three to five times greater activity with heat-denatured than with native DNA templates (24), in contrast to the native template preference of the DNA polymerase activity in Fraction AS. Berns et al. (24) were also

---

**Fig. 16.** Electron micrographs of SV40 DNA following DNA synthesis with Fraction AS. The formamide spreading procedure and electron microscopy are described under “Methods.” The molecules are from a sample in which the extent of synthesis after 60 min at 37° was 0.18. The reaction mixture and SDS treatment are described in the legend for Fig. 8. The DNA was banded in a buoyant CsCl density gradient containing ethidium bromide as described in the legend for Fig. 9. The fluorescent band of DNA was collected and the dye removed using the isooctyl alcohol-extraction procedure as described under “Methods.”
able to recover the nuclear enzyme from DEAE-cellulose columns, whereas all of the activity in Fraction AS was lost during DEAE-cellulose chromatography. We therefore regard the DNA polymerase activity in Fraction AS to be of mitochondrial origin.

The preparations of rat liver mitochondrial DNA polymerase by Meyer and Simpson (10) and Kalf and CiCh'i (9) differed somewhat in their properties. These differences appear to be due to the different levels of purification. Meyer and Simpson (25) noted that sonication yielded higher activity of DNA polymerase than their preferred method of grinding frozen mitochondria with alumina. However, they were unable to recover the former activity from ion exchange columns successfully employed in the purification of the latter activity. Our attempts to utilize ion exchange columns in purification of sonic extracted DNA polymerase activity from HeLa cell mitochondria were likewise unsuccessful. It may very well be, as discussed in the following paper (21), that mitochondria contain two DNA polymerases and that the activity we have described here is functionally distinct from that described by the above investigators.

Under conditions optimized with respect to salt, Mg++, and nucleotide concentrations, Fraction AS can catalyze extensive synthesis of DNA without degradation of template. buoyant equilibrium and sedimentation velocity studies led to the conclusion that most of the product was synthesized in a covalent extension of template DNA strands. Initiation of DNA synthesis in this system is thus template-primed. The initiation sites appear to be the single-strand scissions introduced by the limited endonucleolytic activity in Fraction AS. Base sequence fidelity is suggested by the requirement of all four deoxynucleotides or appropriate analogues for DNA synthesis. To maintain fidelity of base sequence without degrading template, the template strand ahead of the point of polymerization must be displaced to allow base pairing interaction of added nucleotides with the opposing region of the complementary template strand. Electron microscopy provided evidence of such single-stranded regions in circular duplex molecules after DNA synthesis. The assignment of these single-stranded branches as displaced regions of template DNA is supported by the results of studies of the sensitivity of template and product DNA in these molecules to the action of E. coli exonucleases I and III.

The results characterize a mode of action consistent with the "rolling circle" model of Gilbert and Dressler (26). The model in Fig. 17a has a single-stranded tail with a length of about 20% of that of the duplex circle. After denaturation the product was rendered susceptible to digestion by exonuclease I. The product, therefore, does not contain short self-complementary regions at the growing point, such as in Fig. 17b. After an incubation long enough to achieve 10% synthesis, several nicks would have been introduced in most molecules. Electron microscopy examination showed that the lengths and frequency of observed single-stranded branches were too small to account for localization of product in one or perhaps two sites per molecule. The formation of a pinwheel structure (Fig. 17c) accounts for all of the results in this work.

The buoyant density of a template-product complex representing the limit of unit extent of synthesis (product/product + template), with 5-bromodeoxyuridine 5'-triphosphate instead of dTTP, on an SV40 DNA template is the same as the buoyant density of 5-bromodeoxyuridine-labeled single-stranded SV40 DNA. The density difference between such a species and thymidine-containing duplex SV40 DNA was calculated using the base composition of SV40 DNA, 41 mole per cent dG + dC (19), the 0.20 g per ml density difference between the synthetic polymers poly(dA dT) and poly(dA dBRU) (27), and the density difference between duplex and single-stranded DNA (28). We estimate the error associated with these values to be at least ±1 mole per cent dG + dC, ±0.01 g per ml, and ±1 mg per ml, respectively. The density shift expected for the mode of DNA synthesis described is 125 to 142 mg per ml for unit extent of synthesis. The value determined from the slope in Fig. 11 was 125 mg per ml.

Annealing of complementary displaced strands in the samples under investigation would have maximally lowered the slope by 16 mg per ml. The slope would represent an overestimate of the density shift per unit extent of synthesis if there were large variations in the amount of product DNA among the population of template molecules, leading to different mass distributions of product and template in the density gradients. The assumption of coincident product and template distributions is supported by the electron microscopy studies which suggest that product DNA was localized in several small regions on most of the template molecules. Molecules with long stretches of product DNA synthesis were rare.

A similar mechanism of template priming of DNA synthesis and subsequent template strand displacement was observed in recent studies with the E. coli DNA polymerase I and nicked circular duplex PM2 DNA template (29). Two important distinctions in mode of action should be noted. The HeLa mitochondrial system (Fraction AS) does not show the 5'-exonucleolytic activity of the E. coli DNA polymerase and does not appear to hairpin in the course of DNA synthesis.

Further studies with Fraction AS involved the use of the natural template for the system, HeLa mitochondrial DNA. In addition to evidence for base sequence fidelity, we have also observed a preference for the initiation of DNA synthesis on the
heavy complement of the mitochondrial DNA. These results are described in the following publication (21). Recent studies of mitochondrial DNA replication in vivo have shown that a template strand is displaced ahead of the growing point in the first stages of replication (30). This aspect of the mode of DNA replication in vivo was likewise observed with the mitochondrial DNA polymerase in vivo.

Acknowledgments We thank J. Edens and P. Koen for expert technical assistance and advice. We also thank R. Summers for preparation of the manuscript.

REFERENCES
1. Parsons, P., and Simpson, M. V. (1967) Science 155, 91
2. Brewer, E. N., Devries, A., and Rush, H. P. (1967) Biochim. Biophys. Acta 145, 686
3. Neubert, D., Oberdisse, E., Schmieder, M., and Reinsch, I. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1709
4. Neubert, D., Oberdisse, E., and Bass, R. (1968) in Biochemical Aspects of the Biogenesis of Mitochondria (Slater, E. C., Tager, J. M., Papa, S., and Quagliarello, E., eds) p. 103, Adriatica Editrice, Bari, Italy
5. Parsons, P., and Simpson, M. V. (1968) in Biochemical Aspects of the Biogenesis of Mitochondria (Slater, E. C., Tager, J. M., Papa, S., and Quagliarello, E., eds) p. 171, Adriatica Editrice, Bari, Italy
6. Neubert, D., Teske, S., Schmieder, M., Kühler, E., and Oberdisse, E. (1969) Arch. Pharmacol. Exp. Pathol. 262, 264
7. Nass, M. M. K. (1960) Science 165, 25
8. Ter Scheeget, J., and Borst, P. (1971) Biochim. Biophys. Acta 246, 239
9. Kelf, C. F., and Chin, J. J. (1968) J. Biol. Chem. 243, 4604
10. Meyer, R. R., and Simpson, M. V. (1968) Proc. Nat. Acad. Sci. U. S. A. 61, 130
11. Rush, M. G., Eason, R., and Vinograd, J. (1971) Biochim. Biophys. Acta 228, 585
12. Radloff, R., Bauer, W., and Vinograd, J. (1967) Proc. Nat. Acad. Sci. U. S. A. 57, 1514
13. Warburg, O., and Christian, W. (1941) Biochem. Z. 310, 384
14. Westmoreland, B. C., Skibalski, W., and Ris, H. (1969) Science 163, 1343
15. Beug, M. J., Liedman, J. R., Adler, J., Zimmerman, S. B., Simms, E. S., and Kornberg, A. (1958) Proc. Nat. Acad. Sci. U. S. A. 44, 633
16. Bollum, F. J. (1966) in Procedures in Nucleic Acid Research (Cantoni, G. L., and Davies, D. R., eds) Vol. 1, p. 296, Harper and Row Publishers, Inc., New York
17. Kunitz, M. (1950) J. Gen. Physiol. 33, 384
18. Paoletti, C., LePecq, J. B., and Lehman, I. R. (1971) J. Mol. Biol. 55, 75
19. Crawford, L. V., and Black, P. H. (1964) Virology 24, 388
20. Vinograd, J., and Hearst, J. E. (1962) Fortschr. Chem. Org. Naturst. 20, 372
21. Tibbetts, C. J. B., and Vinograd, J. (1973) J. Biol. Chem. 248, 3380
22. Vinograd, J., Ledowitz, J., and Watson, R. (1968) J. Mol. Biol. 33, 173
23. Kleinschmidt, A. K., and Zahn, R. K. (1959) Z. Naturforsch. 14B, 770
24. Berns, K. I., Silverman, C., and Weissbach, A. (1969) J. Biol. Chem. 244, 15
25. Meyer, R. R., and Simpson, M. V. (1970) J. Biol. Chem. 245, 3426
26. Gilbert, W., and Dressler, D. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 473
27. Wake, R. G., and Baldwin, R. L. (1962) J. Mol. Biol. 5, 201
28. Vinograd, J., Morris, J., Davidson, N., and Dove, W. F. (1963) Proc. Nat. Acad. Sci. U. S. A. 49, 12
29. Masamune, Y., and Richardson, C. C. (1971) J. Biol. Chem. 246, 3692
30. Kasamatsu, H., Robberson, D. L., and Vinograd, J. (1971) Proc. Nat. Acad. Sci. U. S. A. 68, 2252
Properties and Mode of Action of a Partially Purified Deoxyribonucleic Acid Polymerase from the Mitochondria of HeLa Cells

Clark J. B. Tibbetts and Jerome Vinograd

J. Biol. Chem. 1973, 248:3367-3379.

Access the most updated version of this article at http://www.jbc.org/content/248/10/3367

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/10/3367.full.html#ref-list-1