A New Deoxyribonucleic Acid Dependent Deoxyribonucleic Acid Polymerase from HeLa Cell Mitochondria†

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ABSTRACT: A new DNA-dependent DNA polymerase has been partially purified from HeLa cell mitochondria. The mitochondrial DNA polymerase differs from the previously described nuclear and cytoplasmic HeLa cell DNA polymerases in molecular weight, sensitivity to inhibitors, primer–template utilization, and its requirements for maximal activity. The partially purified mitochondrial DNA polymerase retained some ability to copy native and mitochondrial DNA templates. The unique utilization of these templates by the mitochondrial polymerase may be explained by the presence, in the preparations, of an endonucleolytic activity which can introduce breaks into circular, double-stranded DNA.

Well-documented evidence shows that mitochondria from a wide variety of cell types possess a unique DNA which differs from nuclear DNA (see reviews by Borst, 1970; Sager, 1972). The mitochondrial DNA from mammalian cells is circular and appears in various molecular forms, *i.e.*, supercoiled double-stranded circles, nicked circles, and catenated forms (Nass, 1966; Sinclair and Stevens, 1966; Hudson and Vinograd, 1967; Clayton and Vinograd, 1967, 1969; Corneo *et al.*, 1968). The ability of mitochondria to synthesize DNA has been demonstrated both by studies *in vivo* (Nass, 1967; Parsons and Rustad, 1968; Gross and Rabinowitz, 1969) and *in vitro* (Parsons and Simpson, 1967; Brewer *et al.*, 1967; Karol and Simpson, 1968). A distinct mitochondrial DNA polymerase has been found in yeasts (Wintersberger, 1966) and in two separate studies a DNA polymerase has been partially purified from rat liver mitochondria (Kalf and Chih, 1968; Meyer and Simpson, 1968, 1970). Unlike other cellular DNA polymerases, the mitochondrial enzyme was reported to be stimulated by high ionic strength and to show a preference for a mitochondrial DNA template, and in fact to synthesize a double-stranded replica of the mitochondrial DNA template (Kalf and Chih, 1968).

These unique properties have prompted us to examine the DNA polymerases found in the mitochondria of cultured human cells. At least three other DNA polymerases have been partially purified from HeLa cells and characterized. Nuclei of cultured human cells contain two distinct DNA-dependent DNA polymerases designated nuclear D-DNA polymerases I (mol wt ~30,000–40,000) and II (mol wt ~250,000) (Weissbach *et al.*, 1971; Bolden *et al.*, 1972). However, the cytoplasm of these cells contains the bulk of the cellular DNA-dependent DNA polymerase (cytoplasmic D-DNA polymerase). The major cytoplasmic D-DNA polymerase is very similar to and possibly identical with the nuclear D-DNA polymerase II (Weissbach *et al.*, 1971). Another type of DNA polymerase, designated R-DNA polymerase, has been found in cultured human cells and in a wide variety of other eukaryotic cell types (Fridlender *et al.*, 1972; Bolden *et al.*, 1972; Fry and Weissbach, 1973). This enzyme is able to copy polyriboadenylic acid as well as activated DNA and is thus distinguished from the D-DNA polymerases. Although it is highly probable that human cells contain another unique mitochondrial DNA polymerase (in addition to the DNA polymerases described above), such an enzyme has not yet been described.

In this article we report the partial purification of a new and distinct DNA-dependent DNA polymerase from HeLa cell mitochondria. This enzyme, designated HeLa cell mitochondrial D-DNA polymerase, has been characterized and shown to be different from the other DNA polymerases found in either the nucleus or cytoplasm.

Materials and Methods

Materials. [^{3}H]Deoxyribonucleoside triphosphates were purchased from Schwarz-Mann, Orangeburg, N. Y. The oligonucleotides $(dT)_{12-18}$, $(dA)_{12-18}$, $(dG)_{12-18}$, and $(dC)_{12-18}$ were obtained from Collaborative Research Inc., Waltham, Mass. Poly(A), poly(C), poly(U), poly(dT), poly(I), and poly[d(A-T)] were obtained from Miles Laboratories, Kankakee, Ill. Preparation of oligomer–homopolymer hybrids was described in a previous article (Fridlender *et al.*, 1972). Activated DNA was prepared by treatment with DNase I as previously described (Schlabach *et al.*, 1971) except that the DNA was extracted with phenol–chloroform (1:1, v/v), after the DNase I digestion. RNA from Qβ phage was prepared by Dr. A. Ramel of the Research Division of Hoffmann-La-Roche, Inc. Rat liver mitochondrial DNA was a gift of Dr. De-Maw Chuang of the Roche Institute of Molecular Biology. HeLa nuclear RNA was prepared according to the procedure of Girard (1967) and was further purified by two subsequent digestions with DNase followed by extractions with phenol. ^{32}P-Labeled circular λ DNA and ^{3}H-labeled supercoiled λ DNA were the generous gifts of Dr. L. Enquist of the Roche Institute of Molecular Biology. Diethylaminoethylcellulose (DE-52) and phosphocellulose (P-11) were supplied by Whatman, Co., England. Sephadex G-200 was a product of Pharmacia Inc., Uppsala, Sweden.

Growth of Cells. HeLa S-3 cells were grown in suspension cultures at 37° in F-13 medium (Gibco) supplemented with 5% fetal calf serum (Gibco). Cells were harvested at about 5×10^5 cells/ml and washed once with 0.001 M potassium phosphate (pH 7.3) containing 0.32 M sucrose and 0.002 M $MgCl_2$. The cells were either used immediately or stored under liquid nitrogen until used.

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Preparation of Subcellular Fractions. HeLa S-3 cells (20 g) were suspended in 20 ml of 1 mM potassium phosphate (pH 7.0)–10 mM NaCl per gram of cells. After 30 min at 0° the cells were broken in this buffer with a Dounce homogenizer. The nuclei were separated from the cytoplasm by centrifugation at 1000*g* for 10 min. The mitochondria and membranes were subsequently separated from the cytoplasm by centrifugation at 20,000*g* for 20 min. The mitochondria were purified from the resulting particulate fraction by six washes with 0.001 M EDTA (pH 7.0), 0.25 M sucrose, and 0.5 mM dithiothreitol as described by Meyer and Simpson (1970). In a typical preparation, 1.0 g of HeLa cells (wet weight) yielded 1.0–1.5 mg of mitochondrial protein.

Purification of DNA Polymerases from HeLa Cell Nuclei, Cytoplasm, and Mitochondria. DNA-dependent DNA polymerases from HeLa cell nuclei and cytoplasm were purified by DEAE column chromatography and phosphocellulose chromatography as described in a previous report (Weissbach *et al.*, 1971).

Mitochondrial DNA-dependent DNA polymerase was purified according to the following procedure. Washed mitochondria were suspended in 1 M potassium phosphate (pH 7.5) containing 0.5 mM dithiothreitol (0.15 ml/mg of mitochondrial protein) and sonicated for 20 sec at a low No. 4 amplitude in the MSE ultrasonic disintegrator. The broken mitochondrial extract was centrifuged at 150,000*g* for 30 min in a Spinco SW 65 rotor and the supernatant fluid was dialyzed for 5 hr against 1000 vol of 0.22 M potassium phosphate (pH 7.5) containing 0.5 mM dithiothreitol. The dialyzed extract was brought to 20% in respect to glycerol and 0.2% for NP-40 (Shell Co., England). The extract was then adsorbed onto a DEAE-cellulose column at a proportion of 1 mg of protein/g of DE-52. The DEAE-cellulose had been equilibrated with 0.02 M potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, 20% glycerol, and 0.2% NP-40. The column was washed with one column volume of this buffer and then eluted with 12 column volumes of a linear gradient from 0.02 to 0.6 M potassium phosphate (pH 8.0) containing 0.5 mM dithiothreitol, 20% glycerol, and 0.2% NP-40.

The pooled peak fractions of D-DNA polymerase activity obtained from DEAE-cellulose (*cf.* Results) were adsorbed directly onto a phosphocellulose column at a ratio of 0.2 mg of protein/g of P-11. The column, which was equilibrated in advance with 0.02 M potassium phosphate (pH 8.0), 0.5 mM dithiothreitol, 20% glycerol, and 0.2% NP-40, was washed with one column volume of the same buffer and eluted with 10 column volumes of a linear gradient from 0.02 to 0.6 M potassium phosphate (pH 8.0) containing 0.5 mM dithiothreitol, 20% glycerol, and 0.2% NP-40.

Gel Filtration of HeLa Mitochondrial D-DNA Polymerase or Sephadex G-200. An aliquot (50 μl) of the pooled peak tubes of mitochondrial D-DNA polymerase activity obtained from phosphocellulose chromatography (Figure 1B) was diluted to 0.5 ml in 0.10 M potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, 20% glycerol, and 0.2% NP-40, and layered on a Sephadex G-200 column (1.5 × 55 cm) equilibrated with the same buffer. The exclusion volume was determined by blue dextran marker and three proteins were used as markers for molecular weight: lactate dehydrogenase (EC 1.1.1.27), alcohol dehydrogenase (EC 1.1.1.1), and ovalbumin.

Assay Conditions for HeLa Nuclear I and II and Cytoplasmic D-DNA Polymerase Activities. The complete reaction mixture contained, in a final volume of 200 μl, 90 μg of bovine serum albumin fraction V, 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 100 μg of activated salmon sperm DNA,

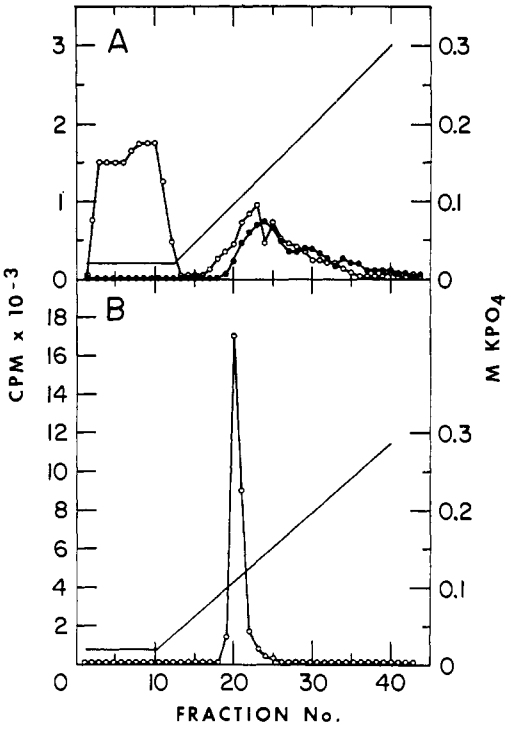

FIGURE 1: DEAE-cellulose and phosphocellulose chromatograms of the HeLa cell mitochondrial extract. (A) DEAE-cellulose chromatogram of the mitochondrial extract. A 1 M potassium phosphate extract of sonicated HeLa cell mitochondria was prepared as described in the text. The dialyzed extract, containing 4.1 mg of protein, was chromatographed on a 4-ml DE-52 column. Elution was carried out as described under Materials and Methods and 30-μl samples of each fraction (0.8 ml) were tested for D-DNA polymerase activity using activated salmon sperm DNA as template. R-DNA polymerase activity was assayed with $(dT)_{12}\cdot$poly(A) as a template. The incubation time was 30 min. The specific activities for all four deoxyribouncleoside triphosphates in the D-DNA polymerase assay and that of the [^{3}H]dTTP in the R-DNA polymerase assay were 12.5 cpm/pmol; (○) D-DNA polymerase; (●) R-DNA polymerase. (B) Phosphocellulose chromatogram of the mitochondrial D-DNA polymerase. The fractions containing the D-DNA polymerase peak (tubes 2–14, Figure 1A), obtained by DEAE-52 column chromatography and containing a total of 1.6 mg of protein, were pooled and adsorbed onto a 2-ml phosphocellulose column. The elution procedure is described under Materials and Methods. Thirty microliter samples of each fraction (0.3 ml) were assayed for D-DNA polymerase activity as in Figure 1A.

and all four deoxyribonucleoside triphosphates at 0.1 mM each containing [^{3}H]thymidine triphosphate (50 cpm/pmol, or as specified). Incubations were carried out at 37°.

Assay Conditions for Mitochondrial D-DNA Polymerase Activity. The complete reaction mixture contained, in a final volume of 200 μl, 90 μg of bovine serum albumin fraction V, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 100 μg of activated salmon sperm DNA, and all four deoxyribonucleoside triphosphates at 0.1 mM each containing [^{3}H]thymidine triphosphate (50 cpm/pmol, or as specified). Incubations were carried out at 37°.

Assay Conditions for R-DNA Polymerase Activity. R-DNA polymerase activity was monitored with $(dT)_{12}\cdot$poly(A) as primer–template as previously described (Fridlender *et al.*, 1972).

Assay Conditions for Deoxyribonuclease Activity Which Releases Acid-Soluble Products. The mitochondrial and cytoplasmic D-DNA polymerases were incubated under their respective standard polymerase assay conditions with 1.0–2.0 × 10^5 cpm of either ^{3}H-labeled native HeLa DNA (26

TABLE I: Purification of HeLa Cell Mitochondrial D-DNA Polymerase.[a]

Purification Step	Total Units	Total Protein (mg)	% Recovery	Sp Act.
Crude extract	$3.[illegible] \times 10^4$	2250.0		
Crude cytoplasm	$1.[illegible] \times 10^4$	1285.0		
Crude mitochondria	4.72×10^2	257.0		
Washed mitochondria	4.48×10^2	31.0	100	14.5
Mitochondrial extract	3.68×10^2	4.1	82.1	88.7
DEAE-cellulose	3.76×10^2	1.6	102.2	235.0
Phosphocellulose	3.04×10^2	0.09	80.8	3377.7

[a] The purification procedure is described in detail under Materials and Methods. A unit of D-DNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into an acid-insoluble form in 15 min at 37°. Specific activity refers to units per milligram of protein.

cpm/pmol), or ^{3}H-labeled denatured HeLa DNA (122 cpm/pmol). The [^{3}H]DNA replaced both the activated DNA template and [^{3}H]deoxyribonucleoside triphosphates normally used in the assay. The final volume of the reaction mixture was 200 μl and the incubation was carried out for 2 hr at 37°. The reaction was terminated by the addition of 200 μl of denatured salmon sperm carrier DNA (2.5 mg/ml), 1.0 ml of water, and 250 μl of 12% perchloric acid. After centrifugation at 10,000g for 10 min, 1.0 ml of the supernatant fluid was counted for radioactivity in a Triton–toluene scintillation fluid.

Assay Conditions for Endonuclease Activity. The mitochondrial and cytoplasmic polymerases were incubated under their respective standard polymerase assay conditions with 0.3 μg of ^{3}H-labeled supercoiled λ phage DNA (8.8×10^3 cpm/μg) subsituting for the normal template and ^{3}H-labeled substrates. The reaction and all procedures were carried out in sterile tubes. After 10 min at 37°, the reaction was terminated by the addition of 50 μl of 0.1 M EDTA to 100 μl of the mixture. Following the addition of 0.2 μg of ^{32}P-labeled circular λ DNA marker (1.7×10^4 cpm/μg), the mixture was layered on 5.3 ml of 5–20% alkaline sucrose gradient as described by Enquist and Skalka (1973). After centrifugation for 100 min at 45,000 rpm in the SW 50.1 rotor of the Beckman ultracentrifuge, the gradients were separated into 0.16-ml fractions which were counted for radioactivity. In this procedure, the supercoiled ^{3}H-labeled DNA sedimented to the bottom of the gradient remaining on a 0.4-ml 60% sucrose cushion—thus being distinguished from the nicked circular DNA which sedimented with the ^{32}P-labeled marker λ DNA near the top of the gradient (Enquist and Skalka, 1973).

Results

Partial Purification of D-DNA Polymerase from HeLa Cell Mitochondria. Intact HeLa cell mitochondria, isolated and purified as described under Materials and Methods, displayed D-DNA polymerase activity which constituted ~1.5% of the total D-DNA polymerase activity in the crude cell extract. Extraction of the disrupted mitochondria with 1 M potassium phosphate yielded 80% of the polymerase activity found in the intact organelle but only released 13% of its protein. The mitochondrial extract after dialysis was chromatographed on a DEAE-cellulose column and the fractions were assayed for both D-DNA and R-DNA polymerase activities under the respective standard assay conditions for these enzymes. As shown in Figure 1A, the mitochondrial extract contained both D-DNA and R-DNA polymerase activities which are separable on DEAE-cellulose. The bulk of the D-DNA polymerase is not retained by the DE-52 in 0.02 M potassium phosphate under our conditions. In contrast, the R-DNA polymerase activity was adsorbed and then eluted from the column as a broad peak with a maximum at 0.13 M potassium phosphate. The mitochondrial associated R-DNA polymerase was carefully examined for its characteristics and found to resemble in all respects the previously described HeLa cell R-DNA polymerase (Fridlender *et al.*, 1972; Bolden *et al.*, 1972). The R-DNA polymerase isolated from HeLa cell mitochondria could therefore be either an enzyme synthesized and used by the organelle itself, or more probable, a cytoplasmic contaminant of the mitochondrial extract. This article deals exclusively with the mitochondrial DNA-dependent DNA polymerase and no further data concerning the mitochondrial R-DNA polymerase will be presented here.

The pass-through from the DEAE-cellulose column which contained only the D-DNA polymerase activity (tubes 2–15, Figure 1A) was further purified by phosphocellulose column chromatography. As shown in Figure 1B the mitochondrial D-DNA polymerase is eluted from phosphocellulose as a sharp peak at 0.11 M potassium phosphate. All further investigations concerning the requirements and properties of the HeLa cell mitochondrial D-DNA polymerase were carried out using the phosphocellulose fraction (fraction No. 21) of the enzyme. To stabilize this fraction, bovine serum albumin fraction V was added to a final concentration of 500 μg/ml and the polymerase was stored under liquid nitrogen. Table I summarizes the purifications steps of the mitochondrial D-DNA polymerase. It is apparent from our data that in comparison with the activity of the intact organelle, the mitochondrial D-DNA polymerase was purified 234-fold after phosphocellulose chromatography.

Requirements of the HeLa Cells' D-DNA Polymerase. The requirements of the partially purified mitochondrial D-DNA polymerase are summarized and compared to those of the cytoplasmic HeLa cell D-DNA polymerase in Table II. The data in this table show that the mitochondrial D-DNA polymerase differs in its requirements for maximal activity from the cytoplasmic D-DNA polymerase. Although both enzymes require an activated DNA template and Mg^{2+} for maximal activity, the mitochondrial enzyme is stimulated 60% by the addition of 0.05 M KCl, whereas the cytoplasmic polymerase is almost unaffected by salt at this concentration. It should be noted that previous reports demonstrated six- to sevenfold stimulation of rat liver mitochondrial D-DNA polymerase by 0.15 M KCl (Meyer and Simpson, 1968, 1970). In the case of the HeLa cell mitochondrial D-DNA polymerase, a maximal salt stimulation of only 60% was obtained. In the absence of KCl, the optimal Mg^{2+} concentration for the mitochondrial D-DNA polymerase rises twofold. This may be due to the salt effect exerted by the higher $MgCl_2$ concentration. Our results also indicate that, in contrast to the cytoplasmic DNA polymerase, the mitochondrial enzyme does not depend on dithiothreitol for its activity and is only slightly inhibited by *N*-methylmaleimide. Similar observations were reported by Meyer and Simpson (1970) for the rat liver mitochondrial D-DNA polymerase.

TABLE II: Requirements of the HeLa Cell Mitochondrial and Cytoplasmic D-DNA Polymerases.

Mitochondrial D-DNA Polymerase	% Incorpn	Cytoplasmic D-DNA Polymerase	% Incorpn
Complete mixture[a]	100.0	Complete mixture[b]	100.0
− activated DNA	0.0	− activated DNA	0.0
− Mg^{2+}	2.2	− Mg^{2+}	34.6
− KCl	62.5	− KCl	97.9
2.5×10^{-3} mM dithiothreitol[c]	80.7	2.5×10^{-3} mM dithiothreitol[c]	52.7
+ 1.0×10^{-2} mM *N*-methylmaleimide	72.6	+ 1.0×10^{-2} mM *N*-methylmaleimide	7.8

[a] The phosphocellulose fraction of the mitochondrial D-DNA polymerase (0.5 μg) was incubated with activated salmon sperm DNA under the standard assay conditions described under Materials and Methods. The specific activity of the [^{3}H]dNTP was 62.5 cpm/pmol; 100% incorporation was 273 pmol of dNTP incorporated in 60 min. dNTP refers to deoxyribonucleoside triphosphates. [b] Two micrograms of the phosphocellulose fraction of the cytoplasmic D-DNA polymerase was incubated under standard assay conditions described under Materials and Methods except that the complete reaction mixture contained 50 mM KCl. The specific activity of the [^{3}H]dNTP was 62.5 cpm/pmol. 100% incorporation was 3835 pmol incorporated in 60 min. [c] No exogenous dithiothreitol added. This final concentration of dithiothreitol is due to the enzyme solution which contained 5×10^{-4} M dithiothreitol.

TABLE III: Primer Templates' Utilization by the HeLa Cell Mitochondrial and Cytoplasmic D-DNA Polymerases.[a]

Primer Template	^{3}H Substrate	Mitochondrial D-DNA Polymerase (%)	Cytoplasmic D-DNA Polymerase (%)
Native nicked salmon sperm DNA	dNTP	100.0	100.0
Native salmon sperm DNA	dNTP	6.9	0.2
Denatured salmon sperm DNA	dNTP	0.0	1.5
Rat liver mitochondrial DNA	dNTP	12.2	0.0
$(dT)_{12-18}$·poly(dA)	dTTP	0.0	0.1
$(dT)_{12-18}$	dTTP	0.0	0.0
Poly[d(A-T)]	dATP + dTTP	7.5	1.3
$(dG)_{12-18}$·poly(dC)	dGTP	6.6	590.0
Poly(dG)·poly(dC)	dGTP	1.7	0.1
$(dT)_{12-18}$·Qβ-RNA	dNTP	0.0	0.0
$(dT)_{12-18}$·HeLa nuclear RNA	dNTP	0.0	0.0

[a] The activities of the phosphocellulose fractions of the mitochondrial and cytoplasmic D-DNA polymerases at 0.5 and 2.0 μg of protein per incubation, respectively, were assayed under the respective standard assay conditions. Copying of activated salmon sperm DNA by both polymerases was taken as 100% activity. Utilizing this template, the mitochondrial and cytoplasmic enzymes incorporated 159 and 610 pmol of the deoxyribonucleoside triphosphates in 60 min, respectively. The specific activities of the dNTP substrate were 62.5 cpm/pmol all for four deoxyribonucleoside triphosphates, where DNA was used as template. The substrate used with other assays contained 250 cpm/pmol. The concentration of all templates was 40 μg/ml.

The pH optimum of the HeLa cell mitochondrial D-DNA polymerase is 8.5 with 94 and 81% of the activity observed at pH 8.0 and 9.0, respectively. The optimal temperature for the mitochondrial enzyme is 37°.

Template Utilization by the HeLa Cell Mitochondrial and Cytoplasmic D-DNA Polymerases. The relative abilities of the mitochondrial and cytoplasmic D-DNA polymerases to utilize various natural and synthetic templates are shown in Table III. These data indicate that the mitochondrial enzyme behaves as a DNA-dependent DNA polymerase and that it differs somewhat in its template preferences from the cytoplasmic D-DNA polymerase. Both enzymes copy activated DNA at a high rate. The mitochondrial polymerase shows a limited ability to copy native salmon sperm DNA but fails to utilize its denatured form. In contrast, the cytoplasmic D-DNA polymerase does not copy native DNA and shows a very limited ability to utilize denatured DNA. Our findings are different from those of Meyer and Simpson (1968, 1970), who reported a preference for single-stranded DNA by the rat liver mitochondrial D-DNA polymerase and a pronounced ability of that enzyme to copy native DNA. Another interesting feature of the mitochondrial polymerase is its limited ability to copy rat liver mitochondrial DNA, a template which cannot be copied at all by the cytoplasmic enzyme. The ability of both HeLa cell and rat liver D-DNA polymerases to utilize native DNA as a template could be explained by the presence of traces of endonuclease in the polymerase preparations which are able to expose to 3′-OH ends in the native template. As will be shown later in this article, endonucleolytic activity is present in the mitochondrial D-DNA polymerase preparation whereas the cytoplasmic polymerase is devoid of such activity. The ability of the mitochondrial D-DNA polymerase to copy native and mitochondrial DNA templates and the presence of an endonuclease associated with this enzyme could be of considerable significance in view of the circular nature of HeLa cell mitochondrial DNA (Hudson and Vinograd, 1967).

Both cytoplasmic and mitochondrial D-DNA polymerase are free of R-DNA polymerase activity as demonstrated by their complete failure to copy oligomer–homopolymers such as $(dT)_{12-18}$·poly(A), $(dA)_{12-18}$·poly(U), $(dG)_{12-18}$·poly(C) and $(dC)_{12-18}$·poly(I) which can be utilized by various eukaryotic R-DNA polymerases (Bolden *et al.*, 1972; Fry and Weissbach, 1973). These enzymes are also free of terminal deoxyribonucleotidyl transferase since they fail to utilize primers such as $(dT)_{12-18}$ in the absence of an added template strand. The mitochondrial and cytoplasmic D-DNA polymerases fail to copy natural RNA templates primed with oligo(dT) or poly(A)·poly (U) and are obviously different from the RNA-dependent DNA polymerases found in leukoviruses.

The mitochondrial D-DNA polymerase also differs somewhat from the cytoplasmic D-DNA polymerase in showing a higher relative rate of synthesis with the synthetic template poly[d(A-T)]. A more significant difference between the mitochondrial polymerase and the HeLa cell D-DNA polymerases is, however, the exceptional inability with which the

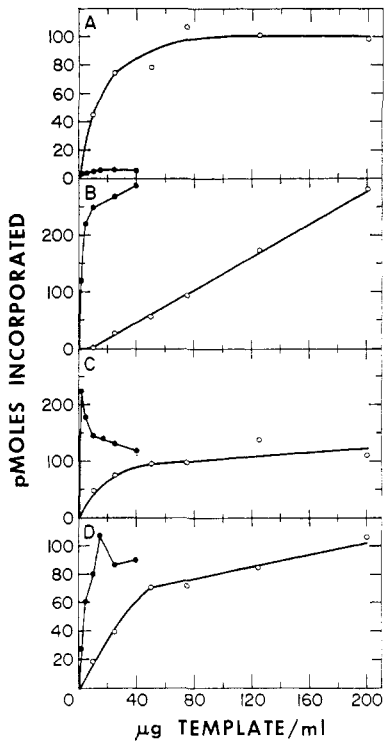

FIGURE 2: (A) Mitochondrial D-DNA polymerase: 0.5 μg of protein of the phosphocellulose fraction was incubated for 15 min under its standard assay conditions with $(dG)_{12}$·poly(dC) or activated DNA templates as indicated in the figure. (B) Cytoplasmic D-DNA polymerase: 2.0 μg of protein of the phosphocellulose fraction was incubated under its standard assay conditions as in A. (c) Nuclear D-DNA polymerase I: 1.0 μg of protein of the phosphocellulose fraction was incubated as in B. (d) Nuclear D-DNA polymerase II: 2.0 μg of protein of the phosphocellulose fraction was incubated as in B; (○) activated DNA; (●) $(dG)_{12}$·poly(dC).

mitochondrial enzyme copies $(dG)_{12}$·poly(dC) when compared to the other DNA polymerases found in HeLa cells. This is further demonstrated in Figure 2 which shows that at 2–10 μg of template/ml, all the HeLa cell D-DNA polymerases copy $(dG)_{12}$·poly(dC) at a rate which is 4- to 25-fold higher than that obtained with an activated DNA template. In contrast, at the same levels of templates, the mitochondrial enzyme copies $(dG)_{12}$·poly(dC) at a rate which is only 10–50% that observed with an activated DNA template. The saturation of all enzymes with $(dG)_{12}$·poly(dC) is attained at levels of 2–10 μg of template/ml and there can be a marked drop in apparent enzyme activity at higher dG_{12}·poly(dC) levels.

Utilization of Single Deoxyribonucleoside Triphosphates with Activated DNA as Template. The ability of the mitochondrial and cytoplasmic D-DNA polymerases to utilize single deoxyribonucleoside triphosphates with activated DNA as template is compared in Table IV. It is apparent from our results that the cytoplasmic enzyme behaves as a typical eukaryotic D-DNA polymerase by incorporating single deoxyribonucleotide triphosphates at a significant rate with an activated DNA template (Schlabach *et al.*, 1971; Sedwick *et al.*, 1972; Fry and Weissbach, 1973). By comparison, the mitochondrial D-DNA polymerase incorporates single deoxyribonucleotide triphosphates at an average rate which is significantly lower than that of the cytoplasmic enzyme. The very low rate of incorporation of single deoxyribonucleotide triphosphates by the mitochondrial D-DNA polymerase resembles the DNA polymerase I of *Escherichia coli* rather than the known eukaryotic D-DNA polymerases (Fry and Weissbach, 1973). Kalf and Chih (1968) have reported that rat liver mitochondrial D-DNA polymerase incorporates dATP at 10% the rate of incorporation of all four deoxyribonucleoside triphosphates. Since these authors used a nonactivated DNA template, it is difficult to make a meaningful comparison between the two enzymes.

TABLE IV: Utilization of All Four *vs.* Single Deoxyribonucleoside Triphosphates by HeLa Cell Mitochondrial and Cytoplasmic D-DNA Polymerases.[a]

Substrate	Enzyme: Mitochondrial D-DNA Polymerase (pmol)	(%)	Enzyme: Cytoplasmic D-DNA Polymerase (pmol)	(%)
[^{3}H]dATP + dNTP[b]	162.5	(100)	916.0	(100)
[^{3}H]dATP	2.5	1.5	29.0	3.2
[^{3}H]dTTP + dNTP	187.7	(100)	680.0	(100)
[^{3}H]dTTP	7.6	4.1	94.0	13.8
[^{3}H]dCTP + dNTP	98.6	(100)	54.7	(100)
[^{3}H]dCTP	1.6	1.6	3.7	6.7
[^{3}H]dGTP + dNTP	51.2	(100)	340.5	(100)
[^{3}H]dGTP	0.0	0.0	8.8	2.6

[a] The mitochondrial and cytoplasmic D-DNA polymerases (0.5 and 2.0 μg of protein of each/assay, respectively) were incubated for 60 min under their respective optimal assay conditions with native nicked salmon sperm DNA as template. The specific activities of the single substrates were 250 cpm/pmol. [b] dNTP refers to a mixture of all four unlabeled deoxyribonucleoside triphosphates.

Properties of the HeLa Cell Mitochondrial D-DNA Polymerase. The partially purified HeLa mitochondrial D-DNA polymerase has the following properties.

(a) MOLECULAR WEIGHT. The molecular weight of the mitochondrial D-DNA polymerase was determined by gel filtration on a Sephadex G-200 column as described under Materials and Methods. The measured molecular weight of the mitochondrial polymerase is 106,000. The molecular weights of both the cytoplasmic and nuclear II D-DNA polymerases as measured by gel filtration are about 250,000 whereas that of the nuclear polymerase I is 30,000–40,000.

(b) HEAT INACTIVATION. Figure 3 shows the inactivation patterns of the mitochondrial and cytoplasmic HeLa D-DNA polymerases at 45° in the presence of 500 μg of bovine serum albumin/ml. Under these conditions, the mitochondrial enzyme is inactivated very rapidly at 45° with a half-life of less than 5 min. The cytoplasmic enzyme, on the other hand, is almost completely resistant to heating under the conditions employed.

(c) INHIBITION OF ATP. ATP is an effective inhibitor of the HeLa mitochondrial D-DNA polymerase. At a concentration of 2.5 mM, ATP inhibits the mitochondrial enzyme 13% and at a concentration of 7.5 mM ATP this enzyme is 76% inhibited. It should be noted, however, that of all the D-DNA polymerases described in HeLa cells, the mitochondrial enzyme is the least sensitive to inhibition by ATP. We have found that the cytoplasmic and the nuclear II D-DNA polymerases are 96% inhibited by 2.5 mM ATP, whereas the nuclear DNA polymerase I is 22% inhibited by ATP at that concentration.

(d) SATURATION BY DNA PRIMERS. Activated and native

TABLE V: Endonucleolytic "Nicking" of Supercoiled λ Phage DNA by the Mitochondrial D-DNA Polymerase.[a]

D-DNA Polymerase	Supercoiled DNA (%)	"Nicked" Circular DNA (%)	Supercoils Nicked (%)
	36.13	63.87	
Cytoplasmic	37.77	62.23	0.0
Mitochondrial	14.05	85.95	61.12

[a] Supercoiled ^{3}H-labeled λ phage DNA was incubated for 10 min at 37° with either mitochondrial (0.2 μg) or cytoplasmic D-DNA polymerases (0.8 μg) under the respective standard polymerase assay conditions. The reaction was terminated and ^{32}P-labeled λ phage circular marker DNA was added as described under Materials and Methods. The reaction mixture was layered on a 5–20% alkaline sucrose gradient and sedimented at 45,000 rpm in the Spinco SW 50.1 rotor for 100 min. When the gradients were fractionated the supercoils were found on the 60% sucrose cushion at the bottom of the tube. The nicked circular or linear DNA was located in the upper third of the gradient. The radioactivity found in each of these peaks was determined and expressed as the per cent of total activity. The control contained the radioactive DNA incubated in a standard reaction mixture in the absence of enzyme.

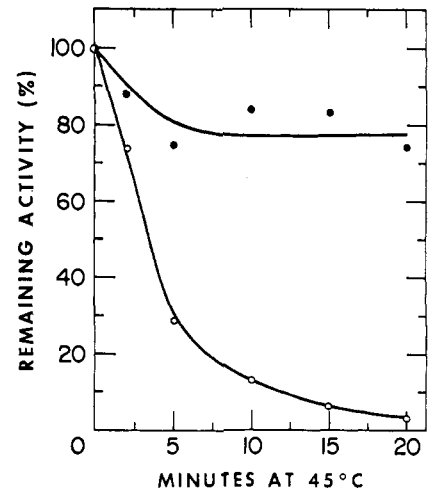

FIGURE 3: Heat inactivation of mitochondrial and cytoplasmic D-DNA polymerases. The phosphocellulose fractions of these enzymes were incubated at 45° for the indicated periods of time in the presence of 500 μg/ml of bovine serum albumin fraction V. At various intervals the enzymatic activities of 0.5 and 2.0 μg of protein of the mitochondrial and cytoplasmic polymerases, respectively, were assayed under their standard assay conditions. The specific activity of the [^{3}H]dNTP was 62.5 cpm/pmol; 100% activity was 271 and 2012 pmol of dNTP incorporated per 60 min for the mitochondrial and cytoplasmic enzymes, respectively; (○) mitochondrial D-DNA polymerase; (●) cytoplasmic D-DNA polymerase.

salmon sperm DNA templates, at 60 μg/ml, saturate 2.5 μg/ml of enzyme protein of the phosphocellulose fraction of the mitochondrial D-DNA polymerase.

(e) ABSENCE OF DNA-DEPENDENT RNA POLYMERASE ACTIVITY IN THE MITOCHONDRIAL D-DNA POLYMERASE. The mitochondrial D-DNA polymerase fails to incorporate ribonucleoside triphosphates with activated DNA as template using either mitochondrial D-DNA polymerase or *E. coli* DNA-dependent RNA polymerase standard assay conditions (Hurwitz, 1963).

(f) PRESENCE OF ENDODEOXYRIBONUCLEASE ACTIVITY IN THE MITOCHONDRIAL D-DNA POLYMERASE PREPARATION. We have not been able to demonstrate any DNase activity in the phosphocellulose fraction of the mitochondrial D-DNA polymerase as measured by the solubilization of highly radioactive (25 cpm/pmol) native or denatured HeLa DNA (see Materials and Methods). On the other hand, using the λ phage supercoiled circular DNA as a probe, we were able to demonstrate low endonuclease activity in the mitochondrial D-DNA polymerase preparation. Mitochondrial and cytoplasmic D-DNA polymerases were incubated with ^{3}H-labeled supercoiled circular λ phage DNA and the λ DNA was consequently analyzed for breaks by sucrose gradient sedimentation as described under Materials and Methods. Table V summarizes the results of this experiment. Our experiment indicates that the mitochondrial D-DNA polymerase preparation contains an endonuclease capable of breaking the λ circular DNA. The cytoplasmic D-DNA polymerase is devoid of such activity. As mentioned earlier, the presence of endonuclease in the mitochondrial D-DNA polymerase preparation can provide an explanation for the unique ability of this enzyme to copy native and mitochondrial DNA templates. It is not clear, however, whether the endonuclease is an integral part of the polymerase or whether it is simply an impurity. It can be calculated from the data in Table V that the endonuclease activity of this preparation hydrolyzes phosphodiester bonds at about 10^{-4} the rate at which the DNA polymerase catalyzes the synthesis of the phosphodiester bond.

Discussion

Three or four DNA polymerase activities have been previously described in HeLa cells. Weissbach *et al.* (1971) have separated two nuclear DNA-dependent DNA polymerases on DEAE-cellulose (designated nuclear D-DNA polymerases I and II), which differ in their molecular weights, their requirements for maximum activity, and their response to various templates (Weissbach *et al.*, 1971; Schlabach *et al.*, 1971). The cytoplasm of HeLa cells contained another DNA polymerase activity which was very similar to and possibly identical with the nuclear polymerase II and which was designated cytoplasmic D-DNA polymerase (Weissbach *et al.*, 1971). Another type of DNA polymerase has been recently found in the HeLa cells as well as in various other eukaryotic cell types (Fridlender *et al.*, 1972; Bolden *et al.*, 1972; Fry and Weissbach, 1973). This polymerase, designated R-DNA polymerase, is able to copy the poly(A) strand of the primer template $(dT)_{12-18}$·poly(A) as well as activated DNA. In this article we described the isolation and partial purification of a new D-DNA polymerase from mitochondria of HeLa cells. This enzyme thus represents the fourth (or fifth) DNA polymerase now known in human cells. This mitochondrial D-DNA polymerase differs from all the previously described HeLa cell DNA polymerases and since it appears to be associated with the organelle, we assume it to be a specific mitochondrial enzyme.

The mitochondrial polymerase has a mol wt of 106,000 whereas that of the cytoplasmic and the nuclear D-DNA polymerase II is 250,000 and that of the nuclear DNA polymerase I is about 40,000. In agreement with previous reports on the requirements of the rat liver mitochondrial D-DNA polymerase (Meyer and Simpson, 1968, 1970) we find that the HeLa cell mitochondrial polymerase requires moderate ionic strength for its maximum activity whereas the cellular D-DNA polymerases are either unaffected or inhibited by salt. In contrast to the cytoplasmic D-DNA polymerase, the HeLa

mitochondrial polymerase does not require dithiothreitol for its activity. Similar observations were reported for the rat liver mitochondrial D-DNA polymerase (Meyer and Simpson, 1970). Furthermore, with our experimental conditions, the HeLa cell mitochondria D-DNA polymerase has a half-life of ~5 min at 45° whereas the cytoplasmic D-DNA polymerase is almost completely stable at that temperature. Another distinct characteristic of the HeLa cell mitochondrial D-DNA polymerase is its unique template utilization. The mitochondrial enzyme copies the synthetic oligomer–homopolymer $(dG)_{12}\cdot$poly(dC) at a much lower rate than activated DNA. In contrast, all the other HeLa cell D-DNA polymerases copy $(dG)_{12}\cdot$poly(dC) at a much higher rate than the activated DNA template. The magnitude of this difference can be seen if the results are normalized to the values obtained with activated DNA as the template. Relative to this template the other HeLa DNA polymerases utilize $(dG)_{12}\cdot$poly(dC) 25–100 times better than does the mitochondrial DNA polymerase (*cf.* Figure 2). In contrast to the cellular enzymes which fail to copy native salmon sperm DNA and rat liver mitochondrial DNA, the mitochondrial D-DNA polymerase succeeds in copying both these templates to a significant extent. Kalf and Chih (1968) and Meyer and Simpson (1970) have reported that rat liver mitochondrial D-DNA polymerase shows a clear preference for mitochondrial DNA over nuclear DNA and a greater template efficacy of denatured DNA over the native form. We have failed to observe these preferences but nevertheless we could show that the mitochondrial enzyme can copy to some extent both linear double-stranded DNA and circular mitochondrial DNA templates. The finding that our preparation of mitochondrial DNA polymerase contains very low levels of endonuclease activity capable of nicking a covalently closed circular duplex DNA might provide an explanation for the unique ability of this polymerase to copy native and circular templates. The endonuclease could expose free 3′-OH ends in a linear or circular native template for the polymerase to act on. This finding could also have significance in light of the circular nature of the mitochondrial DNA (Hudson and Vinograd, 1967) and the structure of the replicative forms of mitochondrial DNA reported by Kasamatsu *et al.* (1971).

It has been postulated that the replication of the circular mitochondrial DNA requires an endonucleolytic activity to act concomitantly with DNA replicase activity for synthesis of a complete daughter copy of the mitochondrial DNA. As reported by Robberson *et al.* (1972) and Kasamatsu and Vinograd (1973), the first identifiable step of mouse mitochondrial DNA replication is the synthesis of a 450-nucleotide segment by a displacement mechanism to form a D loop. This step is followed by a putative strand nicking system, which allows the continuation of the displacement synthesis. Though we find an enzyme which can nick circular DNA in our mitochondrial DNA polymerase preparations, we do not know whether this endonuclease activity is an integral part of the polymerase or whether it plays a unique role in the replication of the mitochondrial DNA *in vivo*. Further purification of the mitochondrial DNA polymerase may resolve this question.

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References

Bolden, A., Fry, M., Muller, R., Citarella, R., and Weissbach, A. (1972), *Arch. Biochem. Biophys. 153*, 26.
Borst, P. (1970), *Symp. Soc. Exp. Biol. 24*, 201.
Brewer, E. N., Devries, A., and Rusch, H. P. (1967), *Biochim. Biophys. Acta 145*, 686.
Clayton, D. A., and Vinograd, J. (1967), *Nature (London) 216*, 652.
Clayton, D. A., and Vinograd, J. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1077.
Corneo, G., Zardi, L., and Polli, E. (1968), *J. Mol. Biol. 36*, 719.
Enquist, L. W., and Skalka, A. (1973), *J. Mol. Biol. 75*, 185.
Fridlender, B., Fry, M., Bolden, A., and Weissbach, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 452.
Fry, M., and Weissbach, A. (1973), *J. Biol. Chem. 248*, 2678.
Girard, M. (1967), *Methods Enzymol. 12A*, 581.
Gross, N. J., and Rabinowitz, M. (1969), *J. Biol. Chem. 244*, 1563.
Hudson, B., and Vinograd, J. (1967), *Nature (London) 216*, 647.
Hurwitz, J. (1963), *Methods Enzymol. 6*, 23.
Kalf, G. F., and Chih, J. J. (1968), *J. Biol. Chem. 273*, 7907.
Karol, M. H., and Simpson, M. V. (1968), *Science 162*, 770.
Kasamatsu, H., Robberson, D. J., and Vinograd, J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2252.
Kasamatsu, H., and Vinograd, J. (1973), *Nature (London), New Biol. 241*, 103.
Meyer, R. R., and Simpson, M. V. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 130.
Meyer, R. R., and Simpson, M. V. (1970), *J. Biol. Chem. 245*, 3426.
Nass, M. N. K. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1215.
Nass, S. (1967), *Biochim. Biophys. Acta 145*, 60.
Parsons, J. A., and Rustad, R. C. (1968), *J. Cell Biol. 37*, 683.
Parsons, P., and Simpson, M. V. (1967), *Science 155*, 91.
Robberson, D. L., Kasamatsu, H., and Vinograd, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 737.
Sager, R. (1972), Cytoplasmic Genes and Organelles, New York, N. Y., Academic Press, p 12.
Schlabach, A., Fridlender, R., Bolden, A., and Weissbach, A. (1971), *Biochem. Biophys. Res. Commun. 77*, 879.
Sedwick, W. D., Wang, T. S., and Korn, D. (1972), *J. Biol. Chem. 247*, 5026.
Sinclair, J. H., and Stevens, B. J. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 508.
Weissbach, A., Schlabach, A., Fridlender, B., and Bolden, A. (1971), *Nature (London), New Biol. 231*, 167.
Wintersberger, E. (1966), *Biochem. Biophys. Res. Commun. 25*, 1.