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# Mitochondrial DNA Replication Does Not Involve DNA Polymerase α*

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**Aphidicolin, a known inhibitor of DNA polymerase α, is a potent inhibitor of nuclear DNA synthesis in HeLa cells but has no effect on the replication of mitochondrial DNA. Parallel experiments with mitochondria incubated *in vitro* also show no inhibition of DNA synthesis by aphidicolin; however, DNA synthesis in these isolated mitochondria is completely blocked by dideoxycytidine triphosphate, which inhibits DNA polymerase γ but not the α polymerase. The replication of mitochondrial DNA therefore requires only one DNA polymerase of the γ type.**

The circular DNA genome of mitochondria replicates in an asymmetric fashion, in which one of the parental strands of the DNA is copied before replication of the complementary strand is initiated (1). In murine mitochondria, copying of the L strand is two-thirds complete before H strand replication commences (2). At present, strand displacement syntheses of this magnitude are not known to occur in the replication of the host nuclear DNA. It was, therefore, of interest that DNA polymerase γ, identified as the mitochondrial DNA polymerase (3), has been implicated in another case of strand displacement synthesis, namely the replication of adenovirus DNA in the cell nucleus (4). Adenovirus DNA, a linear duplex, is also synthesized asymmetrically by a presumed strand displacement mechanism (5). In addition, however, the replication of adenovirus DNA apparently requires DNA polymerase α (6), and the specific role of the α and γ polymerases in the biosynthetic pathway leading to a mature adenovirus DNA molecule remains unknown.

Recently, it has been reported that DNA polymerase α, in addition to the γ polymerase, is involved in mitochondrial DNA replication (7). If so, this would provide a striking similarity between the replication of adenovirus and mitochondrial DNA. We have analyzed the synthesis of HeLa cell nuclear and mitochondrial DNA *in vivo* and *in vitro* with isolated mitochondria in the presence of specific inhibitors of DNA polymerase activity. By separating and isolating the nuclear and mitochondrial DNA, we have shown that DNA polymerase α is not involved in mitochondrial DNA replication, a process which requires only the γ polymerase.

## MATERIALS AND METHODS

Deoxyribonucleotides were purchased from Sigma; [*methyl*-$^3$H]-thymidine was from New England Nuclear; [$^{32}$P]orthophosphate was from Amersham; DNase I and RNase A were obtained from Worthington; proteinase K was from E. Merck; and *Hin*dIII endonuclease was from Bethesda Research Laboratories. Aphidicolin was a gift from Dr. Hesp (Imperial Chemical Industries, England) and pBR322 DNA was kindly supplied by David Wing, Roche Institute of Molecular Biology. Agarose was from Seakem, CsCl was from E. Merck, and ethidium bromide was from Calbiochem. 2′,3′-Dideoxy-GTP and 2′,3′-dideoxy-CTP were obtained from P-L Biochemicals.

*Cell Culture*—HeLa S3 cells were grown in spinner culture in F-13 culture medium (Gibco) with 5% horse serum, 50 units of penicillin, and 50 μg of streptomycin per ml. Cells were harvested or pulse-labeled during exponential growth (3 to 5 × $10^5$ cells/ml) at 37°C.

*Labeling Conditions for Cells*—In order to be able to correct for differential losses during the isolation of mitochondria or extraction of mitochondrial DNA, cells were prelabeled for 24 h with [$^3$H]-thymidine (77 Ci/mmol, 2 μCi/ml). For pulse labeling of the cells with [$^{32}$P]orthophosphate, 200 ml of cell suspension were centrifuged under sterile conditions at 600 × *g* for 15 min, and the cell pellet was washed twice with 0.1 volume of phosphate-free F-13 medium at room temperature and resuspended in the original volume of phosphate-free F-13 medium. After 90 min at 37°C, aphidicolin dissolved in $Me_2SO$[1] (1 mg/ml) or an equal volume of $Me_2SO$ (without the drug) was added to parallel cultures. Thirty minutes later, cells were pulse-labeled for 2 h with [$^{32}$P]orthophosphate (40 μCi/ml). Under these conditions, control cells showed 80 to 85% of the [$^3$H]thymidine incorporation into DNA when compared to cells cultured in medium with normal amounts of phosphate.

*Isolation of Nuclei and Mitochondria*—The cell suspension was chilled on ice, and cells were collected by centrifugation (850 × *g*, 10 min, 4°C) and washed twice with phosphate-buffered saline (Gibco). After swelling in 3 to 9 volumes of hypotonic buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.9) for 5 min on ice, the cells were disrupted by 7 to 10 strokes in a Dounce homogenizer. The homogenate was brought to 0.25 M sucrose and centrifuged at 850 × *g* for 5 min at 4°C, and the supernatant was carefully removed and recentrifuged. Mitochondria were pelleted from the supernatant by centrifugation at 10,000 × *g* for 20 min at 4°C.

For studying *in vitro* mitochondrial DNA synthesis, the mitochondria were resuspended in 300 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA in a volume equal to the starting cell pellet and used directly or after storage at −70°C. The concentration of mitochondrial protein was determined by the method of Lowry *et al.* (8).

To prepare nuclei, the first 850 × *g* pellet obtained from the homogenate was washed with 25 volumes of 0.25 M sucrose, 1.5 mM $MgCl_2$, 50 mM NaCl, 5 mM Tris-HCl, pH 7.2, and stored at −70°C.

*DNA Synthesis in Isolated Mitochondria*—The *in vitro* incubation of mitochondria with DNA precursors was performed essentially as described by Koike *et al.* (9). The incubation was carried out in a total volume of 100 μl with 100 μCi/ml of [$^3$H]thymidine (82 Ci/mmol) as a radioactive DNA precursor. When necessary, large scale incubations of 25 ml were carried out. To measure the incorporation of [$^3$H]thymidine into acid-insoluble material, the incubation mixture was chilled on ice and 0.1 ml of bovine serum albumin (5 mg/ml) in 100 mM EDTA and 2 ml of 5% trichloroacetic acid, 1% sodium pyrophosphate were added. After five washes of the resultant precipitate with 5% trichloroacetic acid, 1% sodium pyrophosphate, the pellet was dissolved in 0.3 ml of NCS (Amersham) and the radioactivity was determined in a Beckman LS-250 liquid scintillation counter after addition of 3 ml of ACS (Amersham) containing 2 ml of glacial acetic acid/liter.

*Extraction and Analysis of DNA*—DNA from *in vivo*-labeled mitochondria was extracted and analyzed according to Storrie and Attardi (10) with the following modifications: the DNase- and RNase-treated mitochondria, prepared from 0.5 ml of packed cells, were digested at 37°C for 30 min with 100 μg of proteinase K/ml containing 1% sodium dodecyl sulfate and 10 mM EDTA, in 2.5 ml of 10 mM

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[1] The abbreviation used is: $Me_2SO$, dimethyl sulfoxide.

Tris-HCl, pH 7.4. Omitting the high speed pelleting step, and after addition of 0.1 volume of 3 M sodium acetate to the solution, the DNA was precipitated by 2 volumes of ethanol at −20°C. The DNA precipitate was dissolved in 1 ml of 10 mM Tris, 10 mM EDTA, pH 7.4, and sedimented through a two-step CsCl/ethidium bromide gradient consisting of 3 ml of CsCl ($\rho$ = 1.4 g/ml) layered over 1 ml of CsCl ($\rho$ = 1.7 g/ml). The CsCl solutions contained 10 mM Tris, pH 7.4, 10 mM EDTA, and 200 μg/ml of ethidium bromide. Centrifugation was carried out at 38,000 rpm for 5 h at 20°C in an SW 65 rotor. The gradients with $^{3}H/^{32}P$-labeled DNA were fractionated into 80-μl aliquots, each of which was treated with 0.3 M KOH overnight at 37°C to render any contaminating RNA acid-soluble. The DNA in each fraction was precipitated, after adding 30 μg of heat-denatured salmon sperm DNA as carrier, with 2 ml of 10% trichloroacetic acid and the precipitate was collected on GF/C glass fiber filters (Whatman). The filters were subsequently washed with 12.5 ml of 10% trichloroacetic acid containing 1% sodium pyrophosphate, then 5% trichloroacetic acid, and finally with ethanol. Radioactivity was determined after addition of LCS (Amersham) and the $^{3}H$ counts per min were corrected for spillover from $^{32}P$.

The *in vitro*-labeled mitochondria, after being pelleted from the incubation mix at 10,000 × *g* for 20 min at 4°C, were lysed in 5 ml of a solution containing 200 μg/ml of proteinase K, 1% sodium dodecyl sulfate, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, for 30 min at 37°C. Following ethanol precipitation, the proteinase K treatment was repeated (in 1 ml) and the nucleic acid solution was extracted with an equal volume of phenol/chloroform (1:1) saturated with 10 mM Tris-HCl, pH 7.9, containing 1 mM EDTA. The nucleic acids were reprecipitated by ethanol as above, and the pellet was dissolved in 1 ml of 10 mM Tris, 10 mM EDTA, pH 7.4, and sedimented through a two-step CsCl/ethidium bromide gradient. To increase the resolution, the mitochondrial DNA fractions from the first run were centrifuged again through CsCl/ethidium bromide as follows. The fractions from the gradient were pooled and ethidium bromide was removed by two extractions with isobutyl alcohol previously equilibrated with saturated CsCl solution. After dilution of the CsCl solution with 3 volumes of water, DNA was precipitated with 2 volumes of ethanol at −20°C overnight, and 25% of this DNA was rerun on a second two-step CsCl/ethidium bromide gradient. Fractions were collected from the bottom of the gradient, and 10-μl aliquots were spotted onto GF/C glass fiber filters which were washed twice in 5% trichloroacetic acid and ethanol. The filters were rinsed with ether, dried, and immersed in LSC, and radioactivity was determined.

For isolation of $^{3}H$- and $^{32}P$-labeled nuclear DNA, a 30-μl suspension containing 5 × $10^{6}$ nuclei was lysed in 2 ml containing 200 μg/ml of proteinase K, 1% sodium dodecyl sulfate, 10 mM EDTA, and 10 mM Tris-HCl, pH 7.4. After 30 min of incubation at 37°C, the lysate was extracted with an equal volume of phenol/chloroform. The nucleic acids were precipitated from the aqueous phase after addition of 0.1 volume of 3 M sodium acetate and 3 volumes of ethanol at −70°C for 30 min. The pellet was dissolved in 1 ml of 0.3 M KOH and kept at 37°C overnight. Aliquots were spotted onto GF/C filters, washed, and counted as described above.

*Analysis of Mitochondrial DNA on Agarose Gel*—For further analysis of mitochondrial DNA, cells were labeled for 4 h with [$^{3}H$]thymidine (15 Ci/mmol; 10 μCi/ml) in F-13 medium. The mitochondrial DNA was sedimented through a two-step CsCl/ethidium bromide gradient, and fractions of Peaks Ia, Ib, and II (Ref. 10; Fig. 1) were pooled, freed of ethidium bromide, diluted with water, and precipitated with ethanol as described above. Five micrograms of pBR322 DNA and 12.5 μg of adenovirus 5 DNA were added to serve as both carrier and marker for gel electrophoresis. The DNA pellets were dissolved in 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, containing 20% sucrose and 0.005% bromphenol blue. Aliquots (50 μl) were subjected to electrophoresis on cylindrical 0.6% agarose gels (0.5 × 11 cm) in electrophoresis buffer (40 mM Tris-HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA) at 20 V and 1.7 mA/tube for 5.5 h.

For digestion of mitochondrial DNA with restriction endonuclease *Hin*dIII, the DNA was incubated in 20 mM Tris-HCl, pH 7.4, containing 7 mM $MgCl_2$, 60 mM NaCl, and 2 units/μg of DNA of *Hin*dIII were added. After 30 min at 37°C, the same amount of endonuclease was added and incubation was continued for another 60 min. The reaction mixture was extracted with phenol/chloroform and the DNA was precipitated with ethanol. The pellet was dissolved in 20 μl of electrophoresis buffer, containing 0.01% bromphenol blue and 25% glycerol, and the DNA fragments were separated by electrophoresis on a 1% horizontal agarose gel for 15 h.

Marker DNAs were visualized by soaking the gels in 1 μg/ml of ethidium bromide in water for 15 min and viewing under UV light. For determination of radioactivity, the cylindrical and slab gels were sliced into 2- and 1-mm slices, respectively. The slices were dissolved in 50 μl of 5 M sodium perchlorate at 60°C for 20 min. After addition of 100 μl of water and 3.5 ml of ACS (Amersham), the radioactivity in each slice was counted in a liquid scintillation spectrometer. The molecular weights of the radioactive DNA fragments were calculated according to Southern (11).

*DNase I Digestion of Nuclear DNA*—To determine the purity of the isolated nuclear DNA, the KOH-treated DNA was neutralized and dialyzed against 10 mM Tris-HCl, pH 7.5, 10 mM NaCl. The DNA solutions were made up to 6 mM $MgCl_2$, and 100 μg/ml of DNase I were added. After incubation at 37°C for 30 min, aliquots were removed and the acid-insoluble radioactivity was determined as above.

*DNA Polymerase Assays*—HeLa cell DNA polymerase α, β, and γ were prepared as described previously (12, 13). The assay conditions used were those described by Pedrali Noy and Weissbach (14). The vaccinia virus-induced DNA polymerase and the herpes simplex virus-induced DNA polymerase preparations have been described previously (15, 16).

## RESULTS

*Mitochondrial DNA Synthesis in HeLa Cells in the Presence of Aphidicolin—In vivo* studies were performed by labeling the DNA of HeLa cells with [$^{3}H$]thymidine for 24 h.

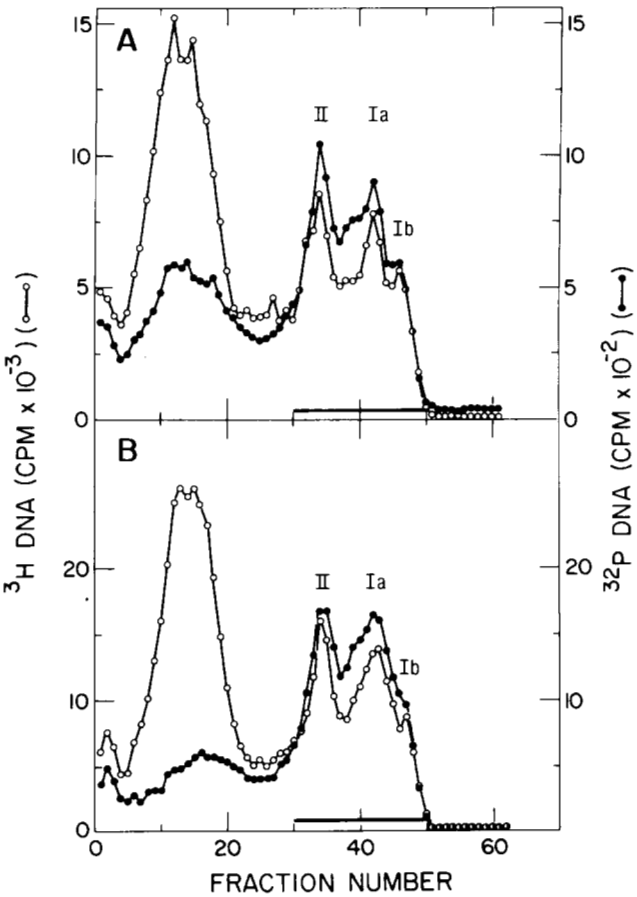


FIG. 1. **Sedimentation patterns in a two-step CsCl/ethidium bromide gradient of DNA from mitochondria of HeLa cells grown in the absence (*A*) or presence (*B*) of aphidicolin.** HeLa cells (7 × $10^{7}$), prelabeled with [$^{3}H$]thymidine, were incubated for 2.5 h in phosphate-free medium with 2 μg/ml of aphidicolin in $Me_2SO$ or with an equivalent volume (0.2%) of $Me_2SO$. During the last 2 h of incubation, cells were pulse-labeled with [$^{32}P$]orthophosphate. Mitochondrial DNA was isolated as described under "Materials and Methods." Sedimentation was performed for 5 h at 38,000 rpm in an SW 65 rotor at 20°C. ○, [$^{3}H$]DNA; ●, [$^{32}P$]DNA. Sedimentation is from *left* to *right*.

These prelabeled cells were subsequently pulse-labeled with [$^{32}$P]phosphate for 2 h in the presence or absence of aphidicolin and the mitochondrial DNA was isolated and analyzed by discontinuous CsCl/ethidium bromide gradient centrifugation as described by Storrie and Attardi (10) (all under "Materials and Methods"). These discontinuous gradients permit separation of the circular mitochondrial DNA from any contaminating linear DNA of the nucleus and enabled us to determine the ratio of newly synthesized $^{32}$P-labeled mitochondrial DNA to the pre-existing $^{3}$H-labeled DNA. By comparing the ratio of pre-existing $^{3}$H-labeled mitochondrial DNA to the newly formed $^{32}$P-labeled mitochondrial DNA, it is possible to avoid significant errors which might be introduced during the isolation of the mitochondrial DNA.

Fig. 1*A* shows the sedimentation pattern in such a two-step CsCl/ethidium bromide gradient of 2 h [$^{32}$P]$PO_4$ pulse-labeled mitochondrial DNA obtained from normally growing HeLa cells. This CsCl pattern shows two main bands in the lower portion of the gradient, corresponding to closed-circular (*Ia*) and open-circular (*II*) mitochondrial DNA (10). In order to prove the identity of these DNA species, parallel experiments were carried out in which HeLa cells were pulse-labeled with [$^{3}$H]thymidine for 4 h prior to separation of the mitochondrial DNA in CsCl/ethidium bromide gradients as described under "Materials and Methods." The $^{3}$H-labeled mitochondrial DNA was isolated in a CsCl/ethidium bromide gradient similar to that shown in Fig. 1. The DNA in Peaks Ia and II were subjected to electrophoresis in agarose gels. Peak II shows one major band whose mobility (Fig. 2*A*) corresponds to open-circular mitochondrial DNA (17), whereas Peak Ia contains, in addition, the faster migrating closed-circular form (Fig. 2*B*, Fractions 25 to 28). The DNA of Peak Ib also consists of mitochondrial DNA, as demonstrated by analysis of the DNA fragments produced from this DNA by the action of restriction endonuclease *Hin*dIII, as shown in Fig. 2*C*. The molecular weights of the major DNA fragments, as determined by agarose gel electrophoresis, were 10.4, 5.2, and 0.87 kilobase pairs (fragments 1, 2, and 3 in Fig. 2*C*), which is close to the values reported by Ojala and Attardi (10.3, 5.4, and 0.81 kilobase pairs) (18). The gel analysis also showed that Peaks Ia and II were essentially mitochondrial DNA, since only minor contaminants were detected. The band of lowest density close to the top of the CsCl/ethidium bromide gradient (tubes 1 to 20 of Fig. 1) contains degraded nuclear DNA (10), which migrates slower than do adenovirus DNA markers in gel electrophoresis, and a small amount of linear full length mitochondrial DNA (data not shown).

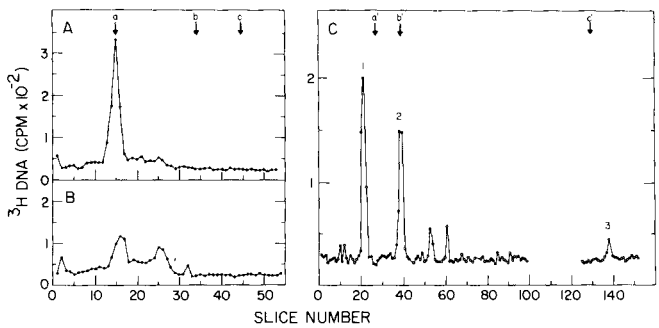


FIG. 2. **Agarose gel electrophoresis of native or *Hin*dIII-digested mitochondrial DNA.** Mitochondrial DNA, isolated from HeLa cells which were pulse-labeled for 4 h with [$^{3}$H]thymidine, was sedimented through a two-step CsCl/ethidium bromide gradient. A similar pattern to that shown in Fig. 1 was obtained. DNAs from Peaks *Ia* and *II* were run on 0.6% cylindrical agarose gels and are shown in *B* and *A*, respectively. DNA from Peak *Ib* was digested with endonuclease *Hin*dIII and the products were analyzed on a 1% agarose slab gel (*C*). The gels were sliced and analyzed for radioactivity. Migration was from *left* to *right*. The positions of marker DNAs are marked by *arrows*. In *A*: *a*, adenovirus DNA; *b*, pBR322 DNA, form II; *c*, pBR322 DNA, form I. In *C*: a′, b′, and c′, *Hin*dIII restriction fragments of adenovirus 5 DNA with sizes of 7.53, 5.29 and 1.05 kilobase pairs, respectively.

TABLE I

*Influence of aphidicolin on nuclear and mitochondrial DNA synthesis in HeLa cells*

HeLa cells ($7 \times 10^7$), prelabeled with [$^{3}$H]thymidine, were incubated for 2.5 h in phosphate-free medium with 2 μg/ml of aphidicolin added in $Me_2SO$ solution or with an equivalent volume of $Me_2SO$ alone. During the last 2 h of incubation, cells were pulse-labeled with [$^{32}$P]orthophosphate. Nuclear and mitochondrial DNAs were isolated as described under "Materials and Methods." Total radioactivity incorporated into mitochondrial DNA was calculated from Fig. 1 (Fractions 30 to 50). The $^{3}$H and $^{32}$P radioactivity incorporated into nuclear DNA was determined by measuring DNase-sensitive radioactivity. In the nuclear DNA fraction from control and aphidicolin-treated cells 90.9% and 60.6% of the $^{32}$P radioactivity, respectively, could be digested by DNase I. Less than 1% of the [$^{3}$H]thymidine-labeled radioactivity was DNase I-resistant in the DNA obtained from both the control and aphidicolin-treated cells.

| Source of DNA | Aphidicolin pretreatment of cells | Total radioactivity incorporated into DNA | | $^{32}$P/$^{3}$H | Inhibition of $^{32}$P incorporation into DNA |
|---|---|---|---|---|---|
| | | $^{32}$P | $^{3}$H | | |
| | | *cpm* | | | % |
| Nuclei | − | $4.93 \times 10^6$ | $200 \times 10^6$ | 0.025 | 89 |
| | + | $0.47 \times 10^6$ | $173 \times 10^6$ | 0.0027 | |
| Mitochondria | − | $1.25 \times 10^4$ | $10.8 \times 10^4$ | 0.12 | 0 |
| | + | $2.41 \times 10^4$ | $20.1 \times 10^4$ | 0.12 | |

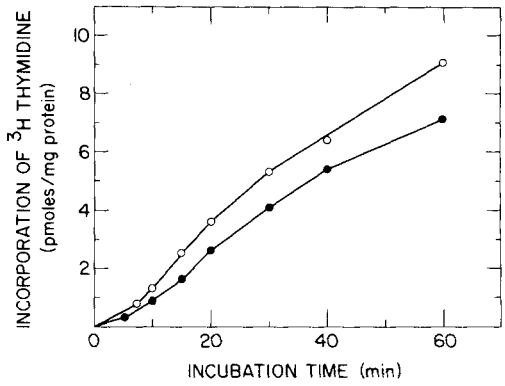


FIG. 3. **Time course of *in vitro* mitochondrial DNA synthesis in the absence and presence of aphidicolin.** Isolated HeLa cell mitochondria were incubated in the reaction medium stated under "Materials and Methods" at 37°C in the absence (○) or presence (●) of 25 μg of aphidicolin/ml. At the indicated times, 0.25-ml aliquots of the reaction mixture were removed and acid-insoluble radioactivity was determined as described under "Materials and Methods."

The sedimentation profile of [$^{32}$P]$PO_4$ pulse-labeled mitochondrial DNA from cells exposed to 2 μg of aphidicolin/ml for 30 min prior to the onset of the [$^{32}$P]phosphate pulse label (Fig. 1*B*) is qualitatively very similar to the control. After normalization for mitochondrial DNA recovery on the basis of the [$^{3}$H]thymidine prelabel, the rate of mitochondrial DNA synthesis is not reduced in the presence of aphidicolin (Table I). In contrast, incorporation of [$^{32}$P]$PO_4$ into nuclear DNA is inhibited by 89% in the same cells under these conditions (Table I). It is worth noting that we usually observe a 98 to 99% inhibition of HeLa nuclear DNA synthesis with 2 μg/ml of aphidicolin with [$^{3}$H]thymidine as a precursor. The slightly lower inhibition observed in this experiment may be due to

normalization of the [$^{32}$P] results to correspond to the DNase sensitivity of the nucleic acid preparation.

*The Influence of Aphidicolin on Mitochondrial DNA Synthesis in Vitro*—In order to provide additional information, the influence of aphidicolin on DNA synthesis in isolated mitochondria was studied. An *in vitro* system described by Koike *et al.* (9), using liver mitochondria from newborn rats, measures mitochondrial DNA synthesis by incorporation of [$^{3}$H]thymidine into trichloroacetic acid-insoluble material in isolated mitochondria. [$^{3}$H]Thymidine is an efficient label in this system, because mitochondria are capable of phosphorylating thymidine to TTP. As shown in Fig. 3, the time course of mitochondrial DNA synthesis using HeLa cell mitochondria is very similar to that of newborn rat liver mitochondria, as reported by Koike *et al.* (9). In the presence of very high levels of aphidicolin (25 μg/ml), the rate of [$^{3}$H]thymidine incorporation into the mitochondrial DNA is depressed by about 20% (Fig. 3). The DNA synthesized either in the presence or absence of 25 μg of aphidicolin/ml in isolated HeLa cell mitochondria during a 1 h-incubation was also analyzed in a two-step CsCl/ethidium bromide gradient. The mitochondrial DNA, newly synthesized in the presence of aphidicolin, showed a sedimentation pattern qualitatively similar to that of the control (Fig. 4). It is worth noting that, in contrast to the sedimentation profile obtained with the mitochondrial DNA isolated from whole cells labeled with [$^{3}$H]thymidine (Fig. 1), the upper band corresponding to contaminating nuclear DNA is missing. In addition, the amount of open-circular mitochondrial DNA exceeds that of the closed-circular form (Fig. 4). Since we did observe a 20% inhibition of mitochondrial DNA synthesis *in vitro* at very high concentrations of aphidicolin, we decided to titrate the level of this drug and measure its effect on mitochondrial DNA synthesis. Fig. 5 shows that lower levels of aphidicolin (1 to 5 μg/ml), which usually inhibit nuclear DNA synthesis by 98 to 99% *in vivo*, exhibit little inhibition of mitochondrial DNA synthesis *in vitro*. We assume that the small inhibition of mitochondrial DNA synthesis by very high levels of aphidicolin could reflect some inhibition of DNA polymerase γ under these conditions.

By contrast, and as shown in Fig. 5, dideoxycytidine triphosphate, which is a potent inhibitor of DNA polymerases β and γ, inhibits mitochondrial DNA synthesis by 95%. Di-

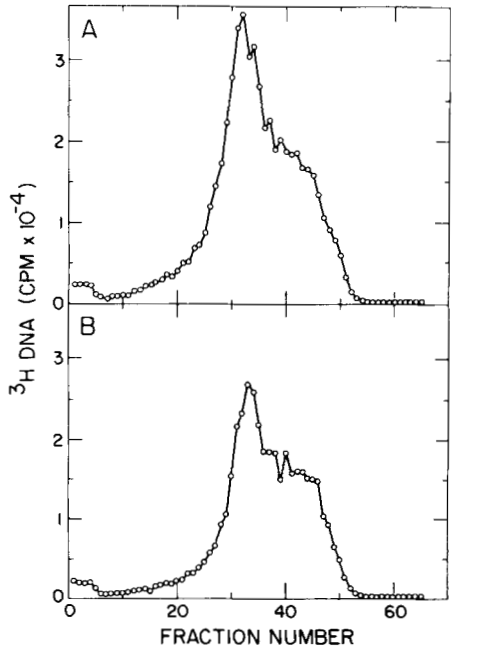


FIG. 4. **Sedimentation patterns in a two-step CsCl/ethidium bromide gradient of mitochondrial DNA, labeled *in vitro* in the absence (*A*) or presence (*B*) of aphidicolin.** Mitochondrial DNA was labeled *in vitro* for 60 min at 37°C in the absence or presence of 25 μg/ml of aphidicolin (see Fig. 3). DNA was extracted and analyzed as described under "Materials and Methods."

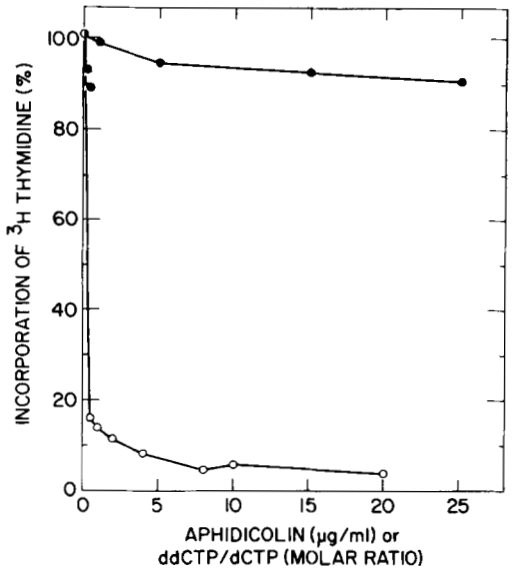


FIG. 5. **Effect of dideoxy-CTP and aphidicolin on *in vitro* mitochondrial DNA synthesis.** Mitochondria were incubated under the same conditions as described in the legend to Fig. 3 in the presence of different concentrations of aphidicolin (●) and dideoxy-CTP (○) for 60 min at 37°C. The concentrations of dATP, dCPT, and dGTP were 30 μM each, and [$^{3}$H]thymidine (100 μCi/ml; 82 Ci/mmol) replaced dTTP in these incubations. The incorporation of [$^{3}$H]thymidine into acid-insoluble material was determined as described under "Materials and Methods."

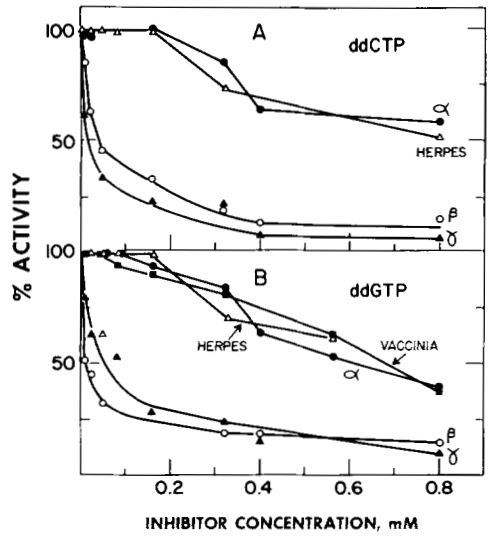


FIG. 6. **Effect of dideoxy-GTP and dideoxy-CTP on purified HeLa cell DNA polymerases α, β, γ, and herpes simplex virus- and vaccinia virus-induced DNA polymerases.** Each enzyme was assayed under optimal conditions in the presence of 250 μg/ml of activated salmon sperm DNA and $MgCl_2$. Assays were performed in the presence of different concentrations of dideoxy-CTP (*A*) and dideoxy-GTP (*B*). The concentration of each of the deoxynucleoside triphosphates was 0.1 mM. Incubation was at 37°C for 30 min. One hundred per cent activity (picomoles of TMP incorporated into acid-precipitable radioactivity) are as follows: DNA polymerase α, 23 pmol; DNA polymerase β, 33 pmol; DNA polymerase γ, 22 pmol; herpes virus-induced DNA polymerase, 27 pmol; vaccinia virus-induced DNA polymerase, 23 pmol.

TABLE II

*Effect of aphidicolin on detergent-treated mitochondria from HeLa cells*

Mitochondria were pretreated with 0.5% Brij 58 or Nonidet P-40 for 5 min on ice until clearing of the mitochondrial suspension occurred. The cleared mitochondria suspensions were added to an *in vitro* DNA synthesis mixture (final detergent concentration, 0.05%) and [$^3$H]thymidine incorporation into DNA was measured as described under "Materials and Methods."

| Pretreatment of mitochondria | Assay in the presence of aphidicolin (5 μg/ml) | Incorporation of [$^3$H]thymidine | Percentage of control |
|---|---|---|---|
| | | *pmol/mg protein* | |
| None | − | 5.46 | 100 |
| None | + | 6.41 | 114 |
| Brij 58 (0.5%) | − | 5.08 | 100 |
| Brij 58 (0.5%) | + | 5.08 | 100 |
| Nonidet P-40 (0.5%) | − | 3.29 | 100 |
| Nonidet P-40 (0.5%) | + | 2.84 | 86 |

deoxy-TTP is known to be an effective inhibitor of DNA polymerases β and γ, but does not affect the α polymerase (19). We find the same is true of dideoxy-CTP and dideoxy-GTP, and since data showing the specific inhibition of DNA polymerases by these analogs has not been published heretofore, the effect of these analogs is quantitated in Fig. 6. It can be seen that the dideoxynucleotides at 0.1 mM have no effect on the α polymerase from HeLa, the herpes simplex virus-induced DNA polymerase, or the vaccinia-induced DNA polymerase. DNA polymerases β and γ, *in vitro*, are inhibited strongly at this concentration. The inhibition of *in vitro* mitochondrial DNA synthesis by the dideoxynucleoside triphosphates is further evidence for the unique role of DNA polymerase γ in mitochondrial DNA synthesis, since the β polymerase is not detectable in isolated mitochondria (3, 20, 21).

To minimize the possibility that the failure of aphidicolin to inhibit mitochondrial DNA synthesis was caused by its inability to penetrate mitochondria *in vitro*, other experiments were performed using isolated mitochondria which had been pretreated with the detergents Brij 58 or Nonidet P-40 to permeabilize them as measured by clearing of the mitochondrial suspension. The results with the detergent-treated mitochondria are shown in Table II, and it is noted again that aphidicolin does not inhibit mitochondrial DNA synthesis under these conditions.

## DISCUSSION

The results in this study emphasize one of the differences between the replication of the host nuclear DNA and that of the mitochondrial DNA. There is strong evidence which implicates DNA polymerase α in the synthesis of DNA in the nucleus, be it either host (22, 23) or viral, such as Simian virus 40 or adenovirus (6). At present, there are no data to support the role of DNA polymerase γ in host nuclear DNA replication, though it is present in the nucleus (14) and participates in the synthesis of adenovirus DNA (4). The role of the nuclear γ polymerase in uninfected cells can only be a source of speculation at the present time (24).

On the other hand, the participation of DNA polymerase γ in mitochondrial DNA replication is clear. In addition to the studies presented here and the previous identification of the mitochondrial DNA polymerase as a member of the γ class (3, 20, 21), Hübscher *et al.* (25) have used rat brain synaptosomes to demonstrate a role of DNA polymerase γ in mitochondrial DNA replication. The elimination of DNA polymerase α from a role in mitochondrial DNA synthesis as shown herein indicates that the replication of the entire mitochondrial genome is probably catalyzed by DNA polymerase γ, since the β polymerase has never been demonstrated in mitochondria (3, 20, 21, 26). Our results differ from those of McLennan (7), who has reported that DNA polymerase α is involved in mitochondrial DNA replication in chinese hamster cells. Though it is possible that there are different mechanisms for mitochondrial DNA replication in HeLa and chinese hamster cells, it is also plausible that the *in vivo* effects of aphidicolin reported by McLennan reflect contamination of his mitochondrial DNA preparations with nuclear DNA. Thus, he notes in his studies that mitochondria, *in vitro*, are much less sensitive to aphidicolin.

As the only mitochondrial DNA polymerase, the γ polymerase must be capable of catalyzing the synthesis of mitochondrial DNA in at least two separate steps. The first involves priming of the duplex mitochondrial DNA with RNA (27) and subsequent extension of a new DNA chain by the γ polymerase. This leads to strand displacement of the other parental strand in the D loop where it now exists as a single strand (28). The second step requires RNA priming of the displaced parental strand (29) and copying of this single-stranded DNA segment by DNA polymerase γ. The γ polymerase is therefore catalyzing DNA synthesis from an RNA primer formed either on a duplex DNA (first step) or on a single-stranded DNA (second step). It will be of interest to determine which other replication factors are required in each of these steps.

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