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An autoradiographic demonstration of nuclear DNA replication by DNA polymerase α and of mitochondrial DNA synthesis by DNA polymerase γ

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ABSTRACT

The incorporation of thymidine into the DNA of eukaryotic cells is markedly depressed, but not completely inhibited, by aphidicolin, a highly specific inhibitor of DNA polymerase α. An electron microscope autoradiographic analysis of the synthesis of nuclear and mitochondrial DNA in vivo in Concanavalin A stimulated rabbit spleen lymphocytes and in Hamster cell cultures, in the absence and in the presence of aphidicolin, revealed that aphidicolin inhibits the nuclear but not the mitochondrial DNA replication. We therefore conclude that DNA polymerase α performs the synchronous bidirectional replication of nuclear DNA and that DNA polymerase γ, the only DNA polymerase present in the mitochondria, performs the " strand displacement " DNA synthesis of these organelles.

INTRODUCTION

Nuclear DNA is replicated through the advancement of many oppositely growing forks on which synchronous and semicontinous synthesis occurs on both strands . Replication of the circular DNA of animal mitochondria occurs instead by asynchronous, continous and unidirectional (strand displacement) synthesis (1).At present, strand displacement synthesis, at least to the extent found in mt-DNA replication, is not believed to occur in the replication of nuclear DNA. An additional difference between nuclear and mitochondrial DNA replication is the unwinding of nucleosomal DNA in front of the fork and subsequent reassembly of nucleosomes behind it (1).

Indirect evidence has involved DNA polymerase α in the synthesis of DNA in the nucleus (2-10) and γ-polymerase has been identified as the mitochondrial DNA polymerase (11-13). However some studies have suggested a role of α-polymerase in mt-DNA synthesis (14) and the detection of γ-polymerase in the nuclei (9) has suggested a possible role of this enzyme in nuclear DNA replication. In order to investigate these possibilities we have used aphidicolin (15), a specific inhibitor of DNA polymerase α

(6,16). Aphidicolin drastically reduces the incorporation of thymidine into DNA in a number of animal (6,16,17) and plant cells (18). However it is not certain that aphidicolin inhibits only nuclear DNA synthesis nor that nuclear DNA synthesis is fully inhibited by the drug, since a residual aphidicolin-resistant incorporation of thymidine (2-3 %) is observed in animal cells. We have therefore analyzed, by electron microscope autoradiography, the synthesis of nuclear and mitochondrial DNA in vivo in Concanavalin A stimulated spleen lymphocytes and in Hamster cell cultures in the absence and in the presence of aphidicolin. Rabbit spleen lymphocytes in culture are an appropriate system to this purpose since we have recently shown (19) that they maintain the integrity of their cytoplasm in parallel with increasing mitochondrial activity during the first 24 hr in culture. Our results show that α-polymerase is used in nuclear DNA replication and that DNA polymerase γ performs mitochondrial DNA synthesis.

MATERIALS AND METHODS

Chemicals : All chemicals were those used in previous works (10,16,19-21).

Cell culture : Rabbit spleen lymphocytes were isolated and cultured as previously described (22). Chinese Hamster cells (V 79/589 FR, male) were grown in minimum Eagle's medium with 10 % faetal calf serum. The cells, removed from confluence, were resuspended in fresh medium and used 24 hr later for pulsing with (Me-^{3}H)-thymidine (19,22).

Measurement of rate of DNA synthesis : as described by Pedrali-Noy and Spadari (16).

Preparation of cell extracts and enzymatic assays : Cell extracts and assays for DNA polymerases α, β, γ and cytochrome C oxidase have been described elsewhere (19).

Determination of the ATP pool : was as described by Hardt et al. (19).

Light autoradiography : Samples for light autoradiography were prepared as in previous works (19,22).

Electron Microscope autoradiography : Concanavalin A stimulated lymphocytes, cultivated either in the absence (control cells) or in the presence of 15 μM aphidicolin (treated cells), were withdrawn at 15 or 23 hr and incubated with (Me-^{3}H)-thymidine (20 μCi/ml, 5 Ci/mmol, Amersham) for 2 hr. Incubation was stopped by washing the cells several times with PBS. Cell samples were then fixed for 4 hr at 4°C in 0.1 M cacodylate buffer, pH 7.4, 1.6 % glutaraldehyde (buffer A), washed with the same

buffer and postfixed for 1 hr at room temperature in 1 % osmium tetroxide in buffer A They were then contrasted overnight in 2% aqueous uranyl acetate, washed several times with distilled water, dehydrated in alcohol and finally embedded in Epon-Spurr mixture (1:1). Chinese Hamster cells in culture were pulse-labelled for 1 hr with 20 µCi/ml of (^{3}H)-thymidine in the absence (control cells) or in the presence of aphidicolin which was added 30 min before thymidine. After several washes in PBS containing 1 mg/ml of cold thymidine, the cells were fixed for 30 min at 4°C in buffer A and washed overnight with the same buffer. The cells were then detached from the culture boxes, pelleted, fixed for 1 hr at room temperature in 1 % OsO_4 in buffer A, dehydrated with alcohol and embedded in Epon-Spurr mixture. Ultrathin sections, harvested on grids, were covered with a monogranular layer of Ilford L4 emulsion, using a loop. Radioautographs were developed after 2 to 8 months exposure in D 19 developer (Kodak) or a phenidon-containing developer (23) freshly prepared. The sections were contrast-stained for 20 min with uranyl acetate and then with lead citrate (24) for 10 min. They were finally observed in an AEI EM6B electron microscope at 60 KV.

RESULTS

In vivo aphidicolin inhibits the synthesis of nuclear, but not mitochondrial, DNA in rabbit spleen lymphocytes stimulated by Concanavalin A.

Figure 1 shows the results of a typical experiment in which spleen lymphocytes, stimulated with 5 µg/ml of Concanavalin A, were cultured in the absence or in the presence of 15 µM aphidicolin (a concentration that completely inhibits the α-polymerase *in vitro* - 16,21,25 -) and analyzed for the incorporation of (^{3}H)-thymidine. As in previous studies using other animal cells (16), (^{3}H)-thymidine incorporation is drastically affected by the presence of aphidicolin. This inhibition is probably mediated by the inhibitory effect of this drug on the sensitive DNA polymerase α since DNA polymerases β and γ, partially purified from the stimulated lymphocytes, were fully resistant to the drug (data not shown).

We wondered whether the interaction of aphidicolin with DNA polymerase α might affect its de novo synthesis and whether the reduced thymidine incorporation into DNA could be correlated with a lack of induction of the α-polymerase in the lymphocytes (19,22) exposed to the drug. We therefore analyzed the levels of DNA polymerase

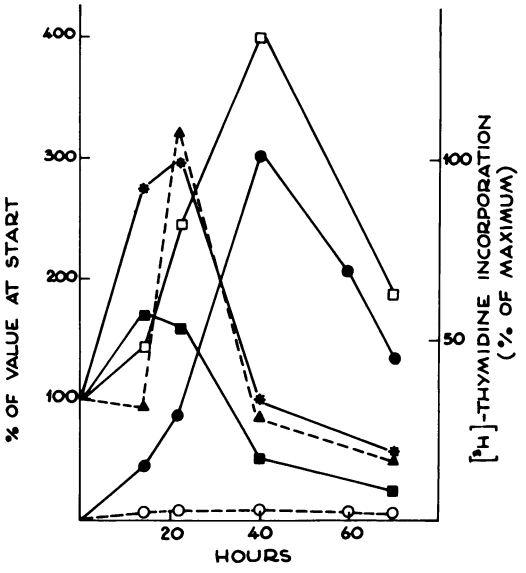


Figure 1. Levels of DNA polymerase α , γ , cytochrome C oxidase, ATP and (^{3}H)-thymidine incorporation in Concanavalin A stimulated lymphocytes cultured in the presence of 15 µM aphidicolin. (^{3}H)-thymidine incorporation (●——●) in cultures grown in the absence of aphidicolin is reported for comparison.
□——□ : DNA polymerase α ; ■——■ DNA polymerase γ ; ✱——✱ Cytochrome C oxidase ; ▲-----▲ ATP ; o-----o (^{3}H)-thymidine incorporation. Values at start : DNA polymerase α : 1.3 U / mg proteins ; DNA polymerase γ : .8 U /mg proteins ; Cytochrome C oxidase : 8.8 mEq / mg proteins.

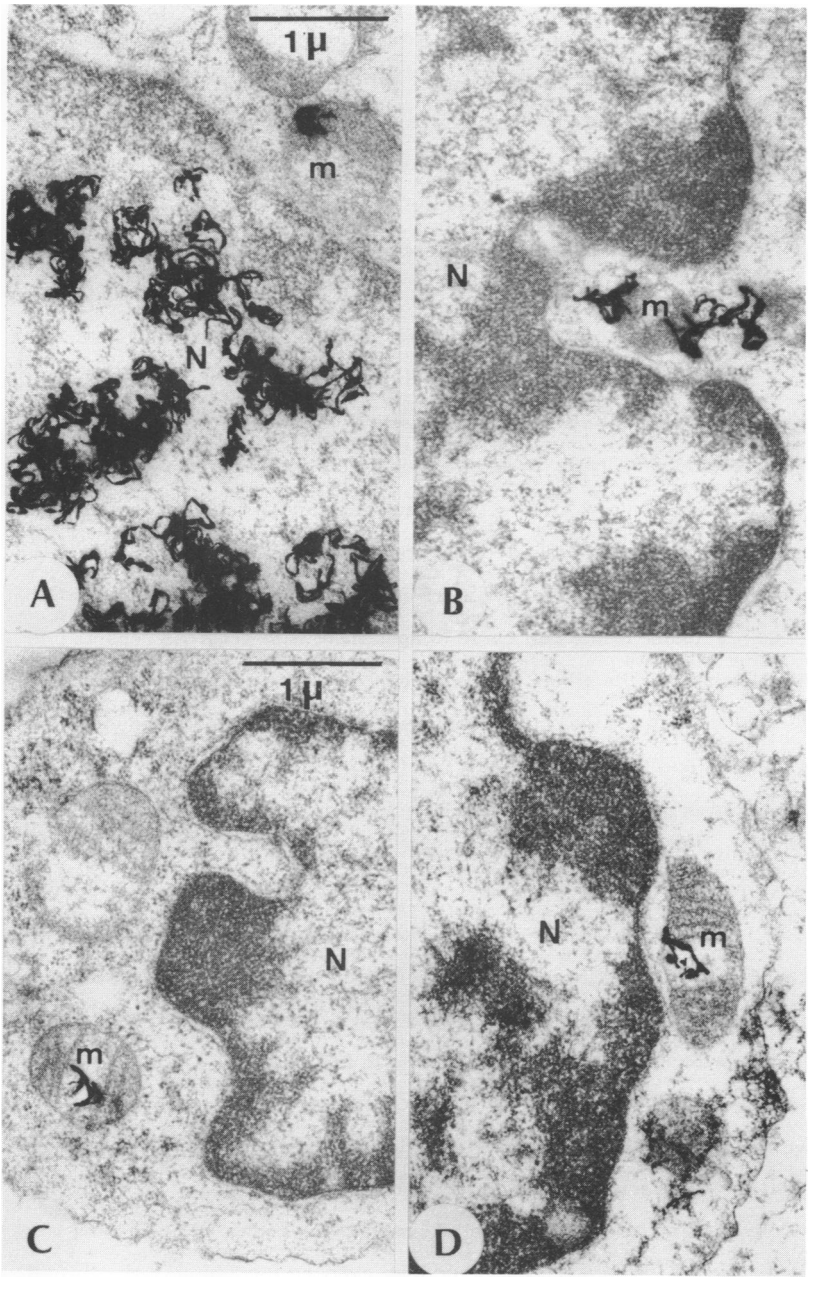
α in Concanavalin A stimulated lymphocytes treated with aphidicolin and found that the levels of the α-polymerase increased during incubation of cell cultures with aphidicolin and paralleled the maximum of DNA synthesis observed in the absence of the drug (Fig.1). DNA polymerase β showed no variations and therefore no correlation with the rate of DNA synthesis in control cells (data not shown). A slight increase in activity of DNA polymerase γ was observed within the first 10-20 hr in culture. Thus the levels of all three DNA polymerases in aphidicolin-treated lymphocytes were similar to those recently reported in stimulated lymphocytes in the absence of aphidicolin (19). In particular the slight increase in activity of γ-polymerase and the increase of cytochrome C oxidase, coupled with the coincident expansion of the ATP pool in the presence of 15 µM aphidicolin, appear consistent with increasing mitochondrial activity

(19) although aphidicolin drastically depressed overall thymidine incorporation.

In order to understand the significance of the residual (approximately 2 %) DNA synthesis resistant to aphidicolin, samples of lymphocytes, growing in culture in the presence or absence of the drug, were withdrawn at 15 or 23 hr, incubated with (^{3}H)-thymidine and analyzed by light and electron microscope autoradiography for the intracellular distribution of the incorporated radioactivity. The number of labelled nuclei was evaluated by examining at least 20 samples, each containing approximately 100 cell sections. Forty cell sections were examined at the electron microscope.

The electron microscope results are shown in Figures 2 and 3 and indicate that spleen lymphocytes growing in the absence of aphidicolin, when incubated with (^{3}H)-thymidine carry out both nuclear and mitochondrial DNA synthesis. The addition of 15 µM aphidicolin from the beginning of the culture fully inhibited the incorporation of thymidine into nuclear DNA with no effect on mitochondrial DNA synthesis. In fact quantitative data, obtained by counting the percentage of labelled nuclei in control cells with the light microscope, indicated that approximately 2.5 and 4 % of the lymphocytes incorporated the labelled precursor at 15 and 23 hr respectively, revealing that they were in the S phase of the cell cycle in agreement with previous reports (19,22). In the E.M. analysis the silver grains appear generally dispersed over the whole nucleus (Figures 2A and 3A) but in many cells they are concentrated over the marginated chromatin along the nuclear membrane (Figure 3B). This observation was made with cell samples taken both at 15 and 23 hr. The lymphocytes which have incorporated much (^{3}H)-thymidine into their nucleus, and particularly those with a dispersed label (Figures 2A and 3A) show a considerably less condensed chromatin structure. In the cytoplasm, one or two silver grains are often observed over a few mitochondrial profiles; these labelled mitochondria are observed in the cytoplasm of control lymphocytes whose nuclei are labelled (Figures 2A and 3A) as well as in the cytoplasm of cells which have not incorporated (^{3}H)-thymidine in their nucleus (Figure 2B) revealing that mitochondrial DNA replication occurs independently from nuclear DNA synthesis. Labelled and unlabelled mitochondrial profiles are observed over the same cell sections although some cell sections show no labelled mitochondria.

No label was found in the nuclei of lymphocytes grown in culture for 15 or 23 hr in the presence of 15 µM aphidicolin and then exposed for 2 hr to (^{3}H)-thymidine (Figures 2C , 2D, 3C, 3D) even in the nuclei with a more dispersed chromatin structure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(Figure 3E). Furthermore when 20 samples, each containing approximately 100 cell sections, were examined by light microscope, no labelled nuclei were observed (data not shown).

However the average number of labelled mitochondria per examined cell was 1.5 in aphidicolin-treated cells (Figure 2C, 2D, 3C, 3D, 3E) as well as in control cells.

Similar results were obtained from the analysis of lymphocytes growing in culture for 40 hr except that in the absence of aphidicolin the percentage of cells with labelled nuclei was 12-15 %. This is consistent with earlier observations that maximal DNA synthesis occurs at this time of incubation (19,22).

Selective inhibition by aphidicolin of nuclear DNA synthesis in cultured Hamster cells.

Aphidicolin blocks DNA synthesis in growing Hamster cells (Figure 4). Fifty per cent inhibition is obtained at 0.2 µM aphidicolin, a concentration similar to that causing 50 % inhibition of thymidine incorporation in HeLa cells (16). Since incubation for short time with aphidicolin has no effect upon the pool of dTTP - or on that of the other three deoxyribotriphosphates - (10), the rate of incorporation of (^{3}H)-thymidine truly reflects the rate of cellular DNA synthesis. As in HeLa cells, approximately 2-3 % of DNA synthesis in Hamster cells was found resistant to high doses of aphidicolin. Following incubation for 1 hr with (^{3}H)-thymidine in the absence or in the presence of 15 µM aphidicolin, Hamster cells were analyzed for the intracellular distribution of the incorporated radioactivity by light (data not shown) and by E.M. autoradiography (Figure 5). The examination by light microscope of 20 samples, each containing approximately 100 cell sections, revealed that 60 % of the control cells, incubated with (^{3}H)-thymidine in the absence of aphidicolin, showed highly labelled nuclei. At

Figure 2. Autoradiographs at the electron microscope of sectioned cells of Concanavalin A stimulated rabbit spleen lymphocytes, withdrawn from culture at 15 hr (see Fig. 1) , incubated for 2 hr in the presence of (^{3}H)-thymidine. Magnification : 18,525
A and B : control cells (grown in the absence of aphidicolin). The Nucleus (N) of the cell in A is highly labelled. One silver grain is localized over a mitochondrial profile (m), in the proximity to the nucleus. The nucleus of the cell in B is not labelled, but a mitochondria, located in the slot of the nucleus, has incorporated tritiated thymidine.
C and D : Aphidicolin (15 µM) treated lymphocytes, with the drug present from the beginning of the culture. No silver grains are observed over the nuclei, but one grain is localized over a mitochondrial profile in each cell.

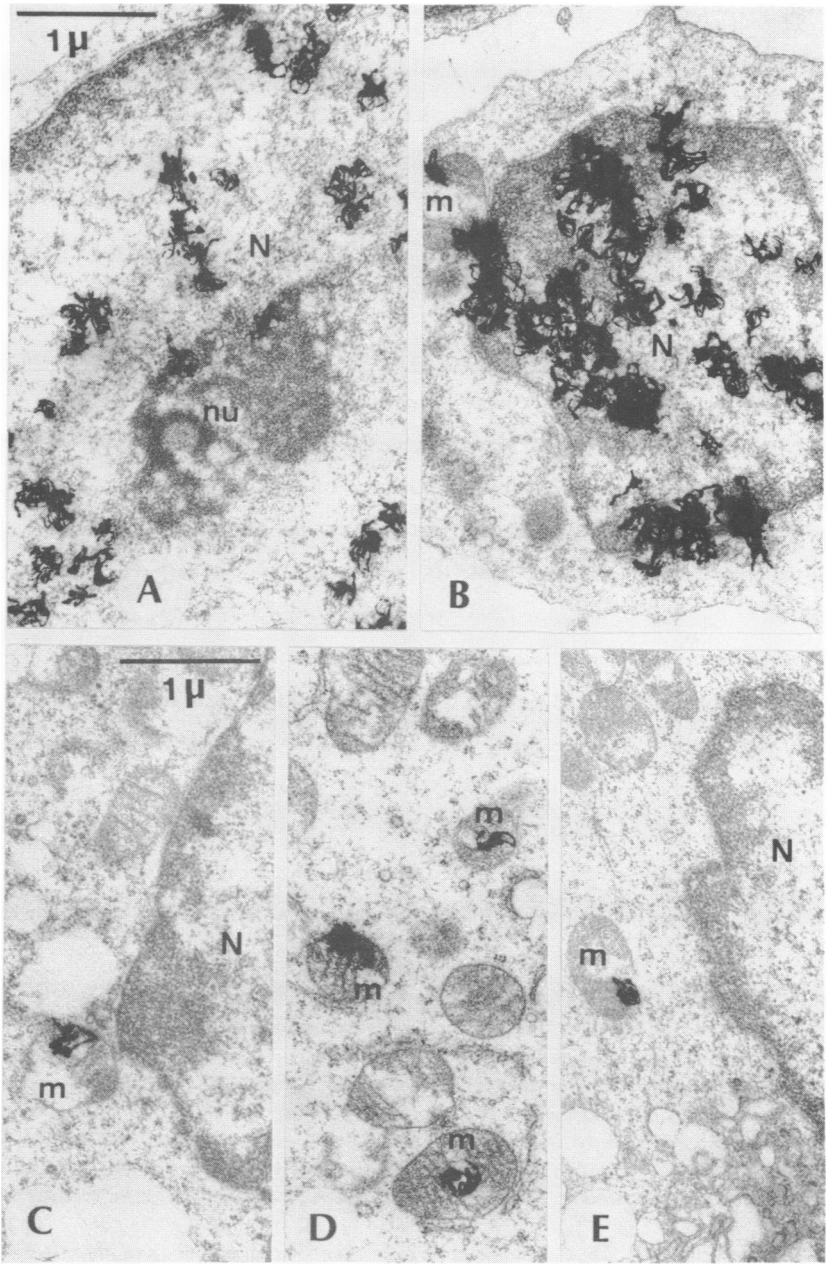
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Figure 3. Autoradiographs at the electron microscope of sectioned cells of Concanavalin A stimulated spleen lymphocytes withdrawn from culture at 23 hr. (see Fig. 1), incubated for 2 hr with (^{3}H)-thymidine. Magnification : 18,525
A and B : control cells (grown in the absence of aphidicolin). Silver grains are dispersed over the nuclear profile (A) or more particularly concentrated over the condensed chromatin (B). nu : nucleolus.
C , D and E : Aphidicolin-treated lymphocytes. The nuclei are not labelled but a silver grain is frequently observed over mitochondrial profiles.

the E.M. autoradiography, the silver grains appeared dispersed over the whole surface of the nuclear profiles or, less frequently, concentrated over the marginated chromatin (Figure 5A). The high number of labelled cells reflects the partial synchronization of our initial culture. When 30 cell sections were examined at the E.M., silver grains were also observed over the mitochondrial profiles.

The presence of aphidicolin during incubation of cells with (^{3}H)-thymidine dra-

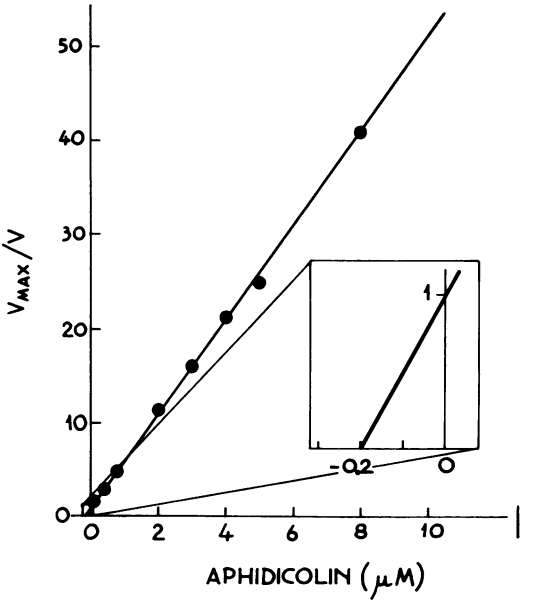
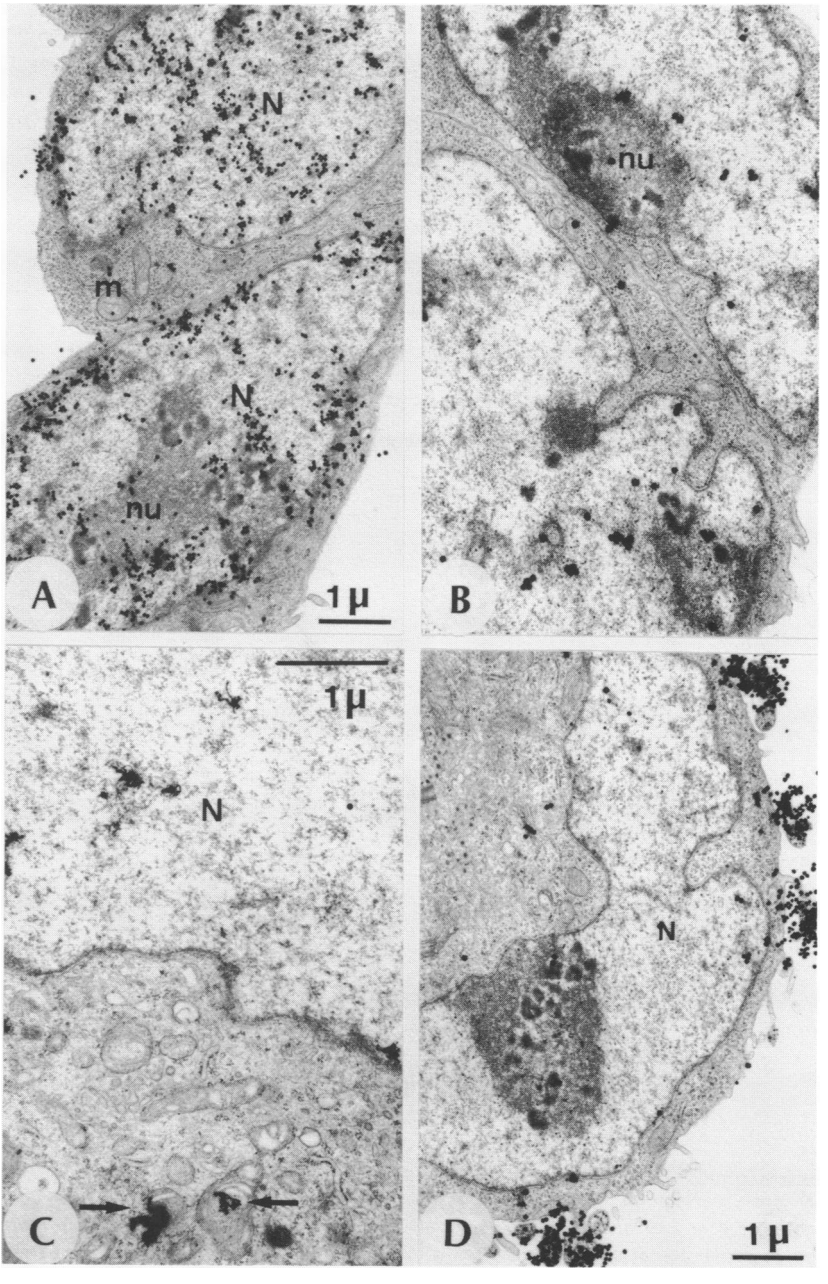


Figure 4. In vivo inhibition by aphidicolin of DNA synthesis in exponentially growing Hamster cells. (^{3}H)-thymidine incorporation was measured in the presence of increasing concentrations of aphidicolin. Incubation was at 37°C and 100 µl aliquots were removed in duplicate at 15, 30 and 60 min to follow (^{3}H)-thymidine incorporation. V_{max} is 65,000 cpm incorporated per hr. The values are corrected for incorporation resistant to 150 µM aphidicolin.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stically depressed nuclear DNA synthesis. No silver grains, or very few grains (less than 1 % of the control) were observed over the nuclear profiles of these cells (Figure 5B). However the average number of labelled mitochondria per examined cell was 0.5 in aphidicolin-treated cells (Figure 5C) as well as in control cells. Thus Hamster cells seem a less favorable material than lymphocytes for these studies.

We found that in mycoplasma contaminated cells silver grains were observed around the plasma membrane (Figure 5D) although nuclear DNA replication was fully inhibited by aphidicolin. Thus it appears that DNA synthesis by mycoplasma is resistant to aphidicolin. This may led to an over-estimation of the amount of cellular DNA replication resistant to aphidicolin, when determined by biochemical methods.

DISCUSSION

Our autoradiographic analysis of nuclear and mitochondrial DNA synthesis in Concanavalin A stimulated spleen lymphocytes and in Hamster cell cultures exposed to aphidicolin shows that α-polymerase is used in nuclear DNA replication and is not involved in mitochondrial DNA synthesis. Mitochondrial DNA replication requires therefore only the γ-polymerase which previous studies (11-12) identified as the only DNA polymerase present in mitochondria. Our results are in agreement with the conclusions of Zimmermann et al. (26) who analyzed the synthesis of HeLa cells nuclear and mitochondrial DNA in the presence of aphidicolin by discontinous CsCl / ethidium bromide gradient centrifugation. Our results are also consistent with the observation that in plant cells the synthesis of DNA in chloroplasts, which also occurs by a strand displacement mechanism, and in mitochondria, is mediated (27) by a γ-like polymerase (28) resistant to aphidicolin (18) and does not require the α-like DNA polymerase (29). This

Figure 5. Autoradiographs at the electron microscope of sectioned Hamster cells incubated for 1 hr with (^{3}H)-thymidine . Magnification : 9,300 for A, B, D and 14,625 for C.
A : control (labelled in the absence of aphidicolin) cells showing a highly labelled nucleus (N) and grains over mitochondrial profiles (m). nu : nucleolus.
B and C : Aphidicolin-treated cells showing a very low nuclear labelling and grains over mitochondrial profiles. Aphidicolin was added only 30 min before incubation with (^{3}H)-thymidine.
D : mycoplasma infected cell from an aphidicolin-treated culture. Mycoplasma, associated with the plasma membrane, are highly labelled, whereas nuclear DNA replication was fully inhibited by aphidicolin.

latter enzyme, which is sensitive to aphidicolin (18) is required only for nuclear DNA synthesis (27). However our results differ from those of McLennan (14) who suggested that, in addition to γ-polymera se, DNA polymerase α is a lso involved in the replication of mt-DNA. The mitochondria preparation used in that work contained DNA polymerase α which was not detected in other purified mitochondria preparations (11-1 2) obtained from cells in which mt-DNA synthesis occurred normally. The possibility that in his case the mitochondrial preparation may contain nuclear material should be seriously considered.

Kwant and van der Vliet have shown that the replication of Adenovirus DNA, which occurs by an asymmetrical displacement mechanism, required DNA polymerase γ and is not dependent upon α-polymera se (30). Their more recent evidence supersedes, to our opinion, previous conflicting reports. Thus DNA polymerase α seems to be used for replication involving the synthesis occurring at symmetrical semicontinous forks. Nuclear DNA a nd some viruses, like the simian and papova, are replicated by this mechan ism (1). The replication of DNA by asymmetrical, continous "strand displacement" would seem to require only DNA polymera se γ, in agreement with previous hypotheses (9,30). Mitochondrial DNA, Adenovirus DNA and chloroplasts DNA appea r to be replicated by this mechanism (1). There is a good correlation between the degree of processiveness of DNA polymerases α and γ in vitro with their in vivo functions. DNA polymerase γ is highly processive and once bound to a 3'0H primer synthesizes long DNA chains before being released (31), whereas α-polymerase is less processive (32). DNA polymerase β synthesizes DNA in a highly discontinous fashion (31) and, unlike α and γ-polymerases, preferentially utilizes DNA templates conta ining short gaps (33). This is consistent with the proposed role of β-polymerase in DNA repair synthesis (5,8,25) which requires the filling of short gaps.

An apparent difference in sensitivity of nuclear DNA replication to aphidicolin was observed with spleen lymphocytes and Hamster cells. No silver grains were found in the nuclei of aphidicolin-treated spleen lymphocytes, whereas the nuclei of aphidicolin-trea ted Hamster cells contained approximately 1 % of that found in control cells, except in a minor fraction of cells (less than 5 % of the population) which contained up to 4-5 % of the label observed in control non-treated cells. This difference probably reflects the differing degrees of synchrony of the two cell populations. The Concanavalin A stimulated lymphocytes were cultured in aphidicolin through the experiment,

and were fully synchronized (19), whereas the Hamster cells were only exposed to aphidicolin 30 min prior to pulsing with (^{3}H)-thymidine. Thus the Hamster cells used in the experiment were not fully synchronized and the low level of labelling observed in the nuclei could reflect a limited reaction of the β-polymerase at the level of small gaps. Although it is clear that DNA polymerase β is not the major nuclear DNA replication enzyme, a minor role for DNA replication cannot be excluded. In analogy with the function of bacterial DNA polymerase I , β-polymerase could be necessary for the closing of small gaps such as those originated from the removal of the RNA primers. Finally, if γ-polymerase is also present in the nuclei (9), it seems unable to sustain DNA synthesis in the absence of α-polymerase inhibited by aphidicolin. One cannot however rule out that γ-polymerase might play an accessory role to the α enzyme for DNA replication. Thus aphidicolin resistant thymidine incorporation in non synchronized eukaryotic cells probably arises both from mt-DNA synthesis and from a limited β-polymerase synthesis at small gaps in the nuclear DNA. Aphidicolin resistant thymidine incorporation may be overestimated if mycoplasma contamination is present in the cultures.

The inhibition of nuclear DNA synthesis by aphidicolin allows the normal synthesis and labelling of mt-DNA molecules which may be useful for the reevaluation of the controversial reports on sequence divergence and sequence homology between various mt-DNA's and of the timing and regulation of mitochondrial DNA synthesis in vivo. The specific inhibition by aphidicolin of nuclear replicative DNA synthesis (and therefore cell growth) in eukaryotes, coupled with its activity on neoplastic cells in culture and on animal cancer (34) and with the lack of adverse effects on some important cellular functions such as DNA repair synthesis (25,34) and mt-DNA replication, makes aphidicolin a potentially attractive anti-cancer drug.

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