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EFFECT OF APHIDICOLIN AND 2',3'-DIDEOXYTHYMIDINE ON MITOCHONDRIAL DNA REPLICATION

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SUMMARY

Mitochondrial DNA replication in Chinese hamster cells has been found to be inhibited by aphidicolin and 2',3'-dideoxythymidine in vivo. 2',3'-dideoxythymidine 5'-triphosphate efficiently inhibits DNA synthesis in isolated mitochondria but the inhibition by aphidicolin is reduced. This suggests either that DNA polymerases α and γ are both involved in mitochondrial DNA replication or that aphidicolin has a secondary site of action other than DNA polymerase α.

INTRODUCTION

It has been known for many years that mitochondria possess their own DNA polymerase and recently this enzyme has been equated with DNA polymerase γ (1,2). A phylogenetic survey has suggested that γ-polymerase is the only DNA polymerase found in digitonin-washed mitochondria and is therefore solely responsible for mt DNA* replication (3). A similar conclusion has been reached by Hübscher et al. who noted the sensitivity of DNA synthesis in rat brain synaptosomes in vitro to ddTTP*, a known inhibitor of DNA polymerase γ (4).

DNA polymerase γ has also been implicated in the replication of human adenoviruses 2 and 5 through their extreme sensitivity to ddTTP in vitro (5,6) and to ddT* in vivo (7). However, an additional role for DNA polymerase α in adenovirus DNA synthesis is evidenced by its sensitivity to aphidicolin, a highly specific inhibitor of α-polymerases (6,8). Both enzymes are found in adenovirus replication complexes. Since both adenovirus and mtDNA* replication occur via a strand displacement mechanism (9,10) it would

*Abbreviations: dT-thymidine; ddT-2',3'-dideoxythymidine; ddTTP-2',3'-dideoxythymidine 5'-triphosphate; TK-thymidine kinase; HPRT-hypoxanthine phosphoribosyltransferase; mtDNA-mitochondrial DNA

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be of interest to examine the effect of aphidicolin on the synthesis of mtDNA to see if DNA polymerase α is involved in this process. Although this enzyme is not generally detected in highly purified mitochondria it may be present in vivo.

This report described some preliminary experiments using a cytoplasmic TK^- mutant* of a Chinese hamster cell line. Thymidine is incorporated by this mutant exclusively into mtDNA during short labelling periods as shown by analysis of labelled product on CsCl/ethidium bromide gradients (A. G. McLennan, unpublished data). The control cell line (TK^+) used in these experiments was $HPRT^-$*.

MATERIALS AND METHODS

Aphidicolin was the generous gift of I.C.I. Pharmaceuticals, U.K. and was dissolved at 5 mg/ml in DMSO. ddT was from PL-Biochemicals and ddTTP from Boehringer.

Cells: Stocks of a23 (TK^-) and Wg-3h ($HPRT^-$) cells derived from the DON cell line (11) were provided by Dr. J. M. Boyle and were maintained in Dulbecco's modified Eagle's medium (Gibco-Biocult) containing 10% newborn calf serum (Flow) in a humidified atmosphere of 5% CO_2. Cultures were routinely checked for mycoplasma (12).

DNA polymerases: DNA polymerases α, β and γ were separated and partially purified from log phase Wg-3h cells according to published procedures (13). Assay conditions for DNA polymerases α and γ were as described below for isolated nuclei and mitochondria respectively with the addition of activated salmon sperm DNA as primer-template in each case. DNA polymerase β was assayed as for nuclei with activated DNA and 40mM ammediol pH 8.6 as buffer. Assays were processed as previously described (14).

DNA synthesis in vivo: 24-well Linbro plates (Flow) were seeded with 10^5 cells/$2cm^2$ well. When close to confluence, cells were incubated for 3 h with 0.3 ml medium containing 0.1mM hypoxanthine, 0.4 μM aminopterin (Sigma) and 10% dialysed calf serum. Cells were then labelled for 1h (Wg-3h) or 2h (a23) with 0.2 ml of the same medium containing 1 μCi [^{3}H] dT* (65 Ci/mmol, Amersham, U.K.) and inhibitor. Incorporation was terminated with 1ml cold 5% TCA. Monolayers were washed with 3 x 10 min x 2 ml of 5% TCA, then incubated for 1 h at 60° with 0.5 ml 0.1M NaOH. After neutralisation with 0.05 ml 1M HCl, 0.5 ml was added to 3 ml toluene/triton X-100 scintillant and the radioactivity determined.

DNA synthesis in isolated nuclei: Nuclei were isolated from log phase a23 cells (15), washed 3x with 10mM tris-HCl pH 7.5, 0.32M sucrose, 5mM 2-mercaptoethanol, 1mM EDTA and resuspended at 2 x 10^7 nuclei/ml in this buffer. DNA synthesis was measured in an assay volume of 100μl containing 40mM Hepes-NaOH pH 7.5, 5mM tris-HCl pH 7.5, 0.32M sucrose, 40mM KCl, 10mM $MgCl_2$, 4mM ATP, 2.5 mM 2-mercaptoethanol, 0.5mM EDTA, 0.1 mM each of dATP, dGTP, dCTP, 0.33μM [^{3}H] dTTP (30 Ci/mmol, Amersham) and

10^6 nuclei. (Ethanol was removed from the [^{3}H]dTTP under a stream of N_2 gas). Assays were incubated for 20 min at 37°, stopped with 100µl cold 10% TCA, 10mM $Na_4P_2O_7$, 1mM dT and then processed for counting as above.

DNA synthesis in isolated mitochondria: The 'low speed cytoplasm' of the nuclear preparation from a23 cells was spun repeatedly at 800g for 10 min each time at 4° until no further pellet was produced. Mitochondria were sedimented at 15,000g for 10 min, washed once and resuspended in 25mM tris-HCl pH 8.0, 0.25M sucrose, 10mM NaCl, 1mM EDTA at 10mg mitochondrial protein/ml. DNA synthesis was measured in an assay volume of 100µl containing 25mM tris-HCl pH 8.0, 15mM KPO_4 pH 7.9, 50mM sucrose, 10mM NaCl, 7mM $MgCl_2$, 4mM Na pyruvate, 4mM Na succinate, 1mM EDTA, 50µM each dATP, dGTP, dCTP, 0.33µM [^{3}H]dTTP (30 Ci/mmol) and 2 mg mitochondrial protein. Assays were incubated for 60 min at 37° and processed further as described for nuclei.

RESULTS AND DISCUSSION

It is important to establish that the DNA polymerases of Chinese hamster cells respond to ddTTP and aphidicolin in the manner typical of other mammalian cell polymerases. In order to compare as accurately as possible the behaviour of the enzymes in isolation and in nuclear and mitochondrial preparations, polymerase α was assayed under 'whole nuclei' conditions and polymerase Υ under 'whole mitochondria' conditions. Polymerase-α was not assayed under mitochondrial conditions. The results in Figures 1A and 1B show that the Chinese hamster DNA polymerases behave as expected, polymerase α being sensitive to aphidicolin but not ddTTP up to a ddTTP/dTTP ratio of at least 1, while polymerases β and Υ are sensitive to ddTTP but not aphidicolin. DMSO had no effect on the activity of the polymerases at concentrations up to the maximum added with the aphidicolin. DMSO controls were included in all other experiments.

The use of ddT as an inhibitor of DNA synthesis *in vivo* requires that it is taken up and phosphorylated efficiently by the host thymidine kinases. Although we have not directly examined the uptake and phosphorylation of ddT by Don cells, it is known that ddT is phosphorylated efficiently by rodent cells (17), and examination of the Ki values of Wg-3h cytoplasmic thymidine kinase and a23 mitochondrial thymidine kinase for ddT suggest that it is phosphorylated at a rate similar to dT and that the kinases

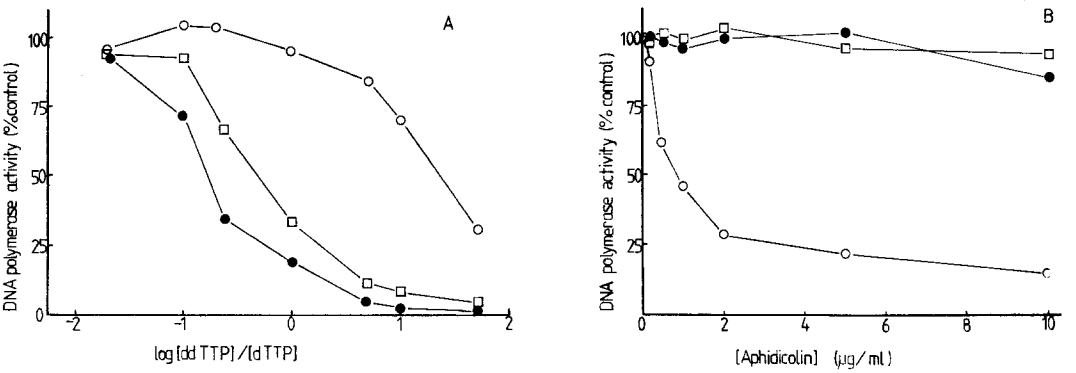

Fig. 1: Effect of (A) ddTTP and (B) aphidicolin on the isolated DNA polymerases of Wg-3h cells. (○) DNA polymerase α (100% = 8,436 cpm); (□) DNA polymerase β (100% = 1,230 cpm); (●) DNA polymerase γ (100% = 325 cpm). Each point is an average of three determinations.

are not the major site of inhibition by this compound (A.G. McLennan, unpublished data).

In order to reduce differences between the applied ratio of ddT/dT and the resultant intracellular ratio of ddT and dT nucleotides, the dT nucleotide pools were reduced before labelling by pre-incubation of the cells with aminopterin. This also had the desired effect of increasing the specific activity of the labelled mtDNA in a23 cells. Labelling of a23 cells for longer than 2h resulted in the appearance of label in material characteristic of nuclear DNA in CsCl/ethidium bromide gradients. This suggests that either the mutant is leaky or that nuclei can utilise mitochondrially phosphorylated precursors. Up to 2h. label was all in mtDNA.

Figure 2A shows that nuclear DNA synthesis in Wg-3h cells as measured by [^{3}H]dT incorporation exhibits a sensitivity to ddT comparable to that of DNA polymerease α to ddTTP whereas dT incorporation into a23 mitochondria *in vivo* is much more sensitive, like DNA polymerase γ. (Since DNA polymerase β has not been found in association with mitochondria, its involvement can be discounted). The reasons for the stimulation of mtDNA synthesis by low concentrations of ddT are unknown, but this seems to occur only *in vivo* (see Figure 3A). When examined for

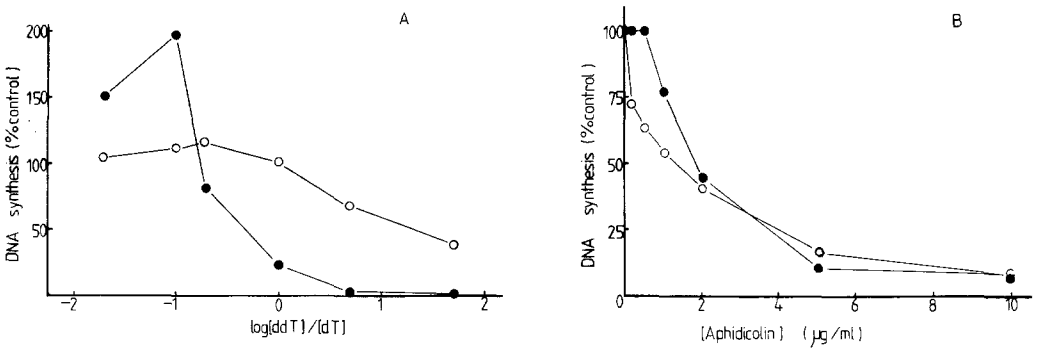

Fig. 2: Effect of (A) ddT and (B) aphidicolin on nuclear DNA synthesis in Wg-3h cells (O, 100% = 106,862 cpm) and on mtDNA synthesis in a23 cells (●, 100% = 3,740 cpm). Each point is an average of three determinations.

sensitivity to aphidicolin, mtDNA synthesis in vivo is inhibited to the same extent as nuclear DNA synthesis (Figure 2B), except at low concentrations of the drug. Interestingly, adenovirus 2 DNA synthesis is also unaffected by low concentrations of aphidicolin, although the lag extends up to 2μg/ml for adenovirus (6). The most attractive explanation of these results is that mtDNA replication, like adenovirus, requires both DNA polymerases α and γ.

To investigate this possibility further, the effect of ddTTP and aphidicolin on DNA synthesis in isolated nuclei and once-washed mitochondria was studied (Figure 3). This mitochondrial preparation did contain measurable amounts of DNA polymerase α, mostly (if not all) as

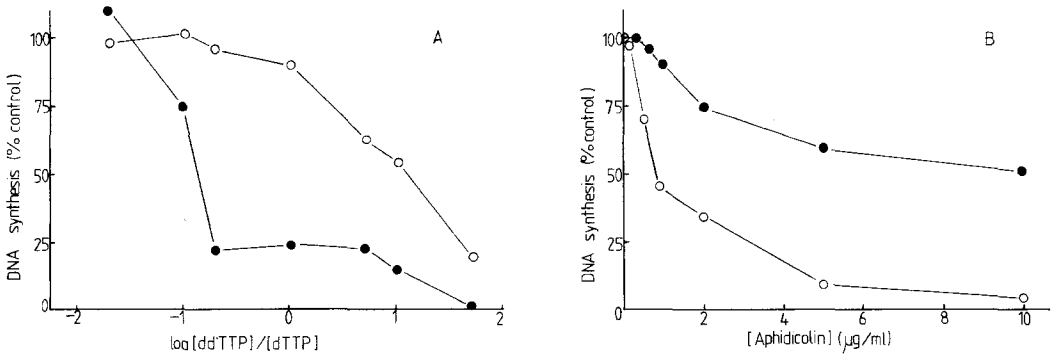

Fig. 3: Effect of (A) ddTTP and (B) aphidicolin on DNA synthesis in isolated a23 nuclei (O, 100% = 4,321 cpm) and isolated a23 mitochondria (●, 100% = 866 cpm). Each point is an average of three determinations.

cytoplasmic contamination. Any contribution of extra-mitochondrial enzymes and DNA to these results was excluded by the finding that treatment with DNase did not destroy any of the incorporated activity. The nuclei and mitochondria retain the expected sensitivities to ddTTP, but the mitochondria *in vitro* appear much less sensitive to aphidicolin than *in vivo*. The degree of inhibition varied between 20 and 50% at 10 μg/ml for different preparations.

These results may indicate that *in vivo*, complete mtDNA replication is dependent on both DNA polymerases α and γ. One possible explanation is that α-polymerase is required for 7S mt DNA synthesis while γ-polymerase catalyses elongation of the 7S mt DNA and L-strand synthesis (10). Such a situation is found during the synthesis of the leading strand of ColE1 plasmid where DNA polymerase I initially extends the RNA primer before polymerase III takes over (18). Alternatively, synthesis of the H and L strands may have different enzymological requirements though this would appear less likely. The possible role of DNA polymerase γ in strand displacement synthesis has recently been discussed (16). Since extensive washing of mitochondria removes all detectable α-polymerase this may result in only the γ-catalysed component of mt DNA synthesis occurring *in vitro*. This may explain the reduced sensitivity of isolated mitochondria to aphidicolin. On the other hand, all detectable DNA polymerase α can be removed from nuclei while still allowing them to perform an ostensibly α-catalysed reaction (19). If catalytically active trace amounts of α-polymerase were retained by mitochondria, the reduced inhibition by aphidicolin may then reflect differences in nucleotide pool sizes *in vivo* and *in vitro* and the recently observed differences in competition of nucleotides with aphidicolin between α-polymerase in isolation and in a replication complex (20).

However, it should be noted that since excision-repair of ultraviolet light-induced damage, which is generally thought to involve DNA

polymerase-β, is also sensitive to aphidicolin (21,22) it now seems possible that aphidicolin has an alternative site of action to DNA polymerase-α *in vivo* which is common to all forms of DNA synthesis. The final interpretation of these results must await the characterisation of the various mt DNA replication intermediates synthesised *in vivo* and *in vitro* in the presence of these inhibitors.

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