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The effect of aphidicolin on DNA synthesis in isolated HeLa cell nuclei

Erik Wist and Hans Prydz

Institute of Medical Biology, University of Tromsø, and Department of Microbiology, Dental Faculty, University of Oslo, Norway

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SUMMARY

The effect of the inhibitor aphidicolin on DNA synthesis in isolated nuclei from HeLa cells and on the activities of partially purified DNA polymerases has been tested. Aphidicolin inhibited DNA synthesis and DNA polymerase α very efficiently whereas DNA polymerases β and γ were insensitive to the drug. The results indicate that DNA polymerase α is the polymerase active during elongation as well as in the gapfilling process of discontinuous DNA synthesis.

INTRODUCTION

The antibiotic aphidicolin produced by the mold Cephalosporium aphidicola Petch, was isolated and characterized chemically by Brundret et al. in 1972 (1) and shown to be a potent inhibitor of DNA synthesis by Bucknall et al. (2). Aphidicolin inhibited the growth of herpes simplex virus in tissue culture as well as in rabbit eye (2). Ohashi et al. (3) found a differential effect of the inhibitor on various DNA polymerases isolated from regenerating rat liver. Recently, Ikegami et al. (4) showed that aphidicolin inhibited DNA synthesis in sea urchin embryos. DNA polymerase α isolated from the nuclei of sea urchin blastulae was strongly inhibited, whereas DNA polymerases β and γ were not (4).

As part of our efforts to characterize DNA polymerases active in DNA synthesis in isolated HeLa cell nuclei (5,6) the effects of aphidicolin on this process and on partially purified DNA polymerase preparations have been investigated. Our results are in accordance with the observations of Ikegami et al. (4). In addition, nascent DNA synthesized in the presence of aphidicolin has

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been analyzed to see the effect of this specific polymerase α inhibitor on the elongation and gapfilling steps in discontinuous DNA synthesis.

MATERIALS AND METHODS

Aphidicolin was kindly provided by Imperial Chemical Industries and was dissolved in dimethyl sulfoxide (DMSO, Sigma). All other chemicals were obtained as described previously (6). Methods for suspension culture, synchronization and harvesting of HeLa S_3 cells (7), isolation and storage of nuclei (8), incubation of nuclei (6), isolation and assay of the DNA products (9), preparation and assays for the various DNA polymerases (6) have been described previously.

DNA was estimated by the method of Burton (10).

RESULTS

Effect of aphidicolin on ^{3}H-thymidine incorporation in whole HeLa cells.

Incorporation of ^{3}H-thymidine into DNA of HeLa cells in suspension culture was tested at different concentrations of aphidicolin (table I). Aphidicolin inhibited DNA synthesis very strongly; 0.2 µg/ml gave 86% inhibition. At a concentration of 2 µg/ml DNA synthesis was completely blocked. DMSO alone at the concentrations used had no effect on DNA synthesis.

Table I

Effect of aphidicolin on DNA synthesis in whole HeLa S_3 cells.

HeLa S_3 cells in suspension culture (2×10^5 cells/ml) were labelled with ^{3}H-thymidine (0.1 Ci/ml) for 10min at 37°C. The reactions were stopped by adding ice cold culture medium with excess thymidine (100 µg/ml).

Aphidicolin µg/ml	^{3}H-cpm incorporated	% of control
0	1248	100
0.2	175	14
2.0	0	0

Effect of aphidicolin on DNA synthesis in isolated HeLa S_3 nuclei and on DNA polymerases α, ß and γ.

Aphidicolin very efficiently inhibited (non-competitively, data not shown) incorporation of ^{3}H-dTTP into DNA of isolated nuclei. At a concentration of 2 µg/ml aphidicolin 75% inhibition was observed. The DNA polymerase α was inhibited to the same extent as nuclear DNA synthesis. The activities of DNA polymerases ß and γ were not affected by aphidicolin at the concentrations tested (figure 1). DMSO at the concentrations used in these experiments had no effect on DNA synthesis nor on the activities of the DNA polymerases.

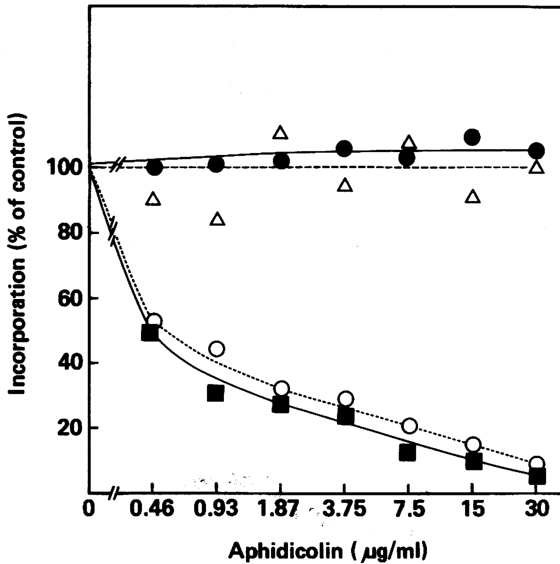

Figure 1. Effect of aphidicolin on DNA synthesis in isolated nuclei and on DNA polymerases α, ß and γ.

Nuclei (20 µg DNA) were incubated at various concentrations of aphidicolin. The incubation mixture contained 50 µM ^{3}H-dTTP of specific activity 100 Ci/mol.
DNA polymerase α, µ and γ were assayed as described (6) in the presence of increasing concentrations of aphidicolin. All reactions were terminated by addition of cold 5% trichloroacetic acid. Acid precipitable radioactivity was assayed as described previously (9).
DNA synthesis in isolated nuclei —■—

DNA polymerase α ----o---- ,ß ——●——, γ .... △ ....

Pulse labelling of nascent DNA in the presence of aphidicolin

DNA synthesis in eukaryotic cells proceeds discontinuously on one or both strands, resulting in the production of primary DNA pieces (Okazaki fragments). The primary DNA pieces are initiated by an RNA primer and elongated by one of the DNA polymerases. The primer is then removed and the resulting gap is filled before the fragments are joined into DNA of larger molecular weight.

If different DNA polymerases with unequal sensitivity towards aphidicolin were involved in elongation of primary DNA pieces and in gapfilling, the presence of aphidicolin would cause a change in the size distribution of DNA product.

Isolated nuclei were pulse labelled with ^{3}H-dTTP in the presence of aphidicolin (1 µg/ml) giving an overall inhibition of DNA synthesis of about 60%. Alkaline sucrose gradient analysis of the product revealed that primary DNA pieces were synthesized under these conditions (figure 2). The distribution of acid precipitable radioactivity in the gradient was similar to that obtained

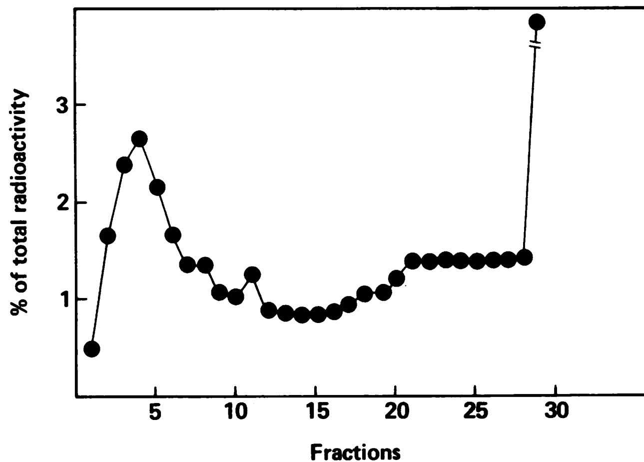

Figure 2. Pulse labelling of nascent DNA in the presence of aphidicolin.

Nuclei (20 µg DNA) were pulse labelled for 60 s with ^{3}H-dTTP (5 µM, specific activity 30 Ci/mmol) in the presence of aphidicolin (1 µg/ml). The reaction was stopped by addition of alkaline lysis solution (8) and the lysate was centrifuged in an alkaline sucrose gradient (36.000 rev/min at 2°C for 20 h in a SW 40 rotor). Fractions were collected from the top.

in control experiments, showing that there was no relative increase of primary DNA pieces, i.e. no preferential inhibition of the gapfilling/ligation process when compared with the elongation of primary pieces.

Effect of aphidicolin on the joining of primary DNA pieces

Further, to study the effect of a complete inhibition of DNA polymerase α on the process of joining of primary DNA pieces the following experiment was performed.

Nucleic were pulse labelled in the absence of aphidicolin for 60 s and chased with excess cold dTTP in the presence or absence of aphidicolin (figure 3). 71% of the radioactivity in primary DNA pieces synthesized during the pulse was transformed into DNA

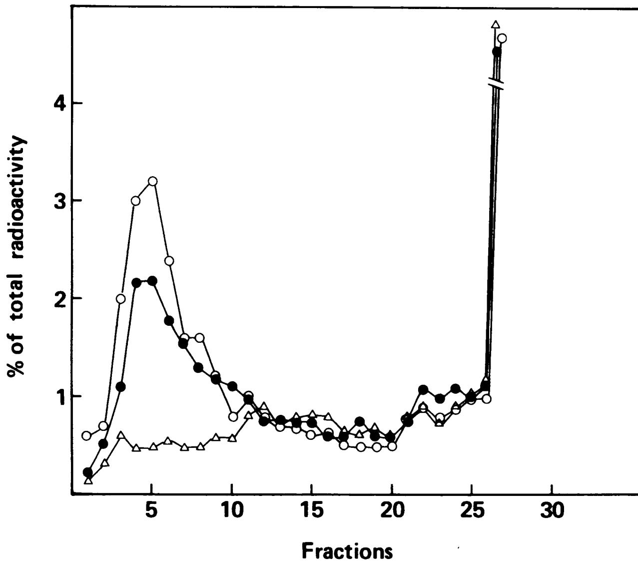

Figure 3. Effect of aphidicolin on the joining of primary DNA pieces.

Nuclei were pulse labelled for 60 s with ^{3}H-dTTP (10 µM, specific activity 30 Ci/mmol). The reaction was either stopped immediately (—— o ——) or followed by a chase with excess dTTP for 180 s in the presence (—— • ——) or absence (——△——) of aphidicolin (100 µg/ml). The reactions were stopped by adding alkaline lysis solution. The lysates were centrifuged in alkaline sucrose gradients (36.000 rev/min at 2°C for 20 h in a SW 40 rotor). Fractions were collected from the top.

of larger molecular weight in a 3 min chase in the absence of aphidicolin. When aphidicolin was present during the chase at a concentration of 100 µg/ml, giving at least 95% inhibition of both nuclear DNA synthesis and DNA polymerase α, only 23% of the radioactivity was transferred to larger molecular weight. These 23% probably represents mainly primary pieces where the primer RNA has been removed and the gapfilling is more or less completed before the actual ligation. A lag in the process of reaching an inhibitory concentration at the sites of gapfilling and ligation may also be involved.

DISCUSSION

The question of which polymerase(s) participate(s) in nuclear DNA synthesis has been the subject of several studies recently (12-14), most of which have utilized virus infected cells as the source of nuclei (12,14).

In an attempt to characterize a DNA synthezising isolated nuclear system (7,8,9,15), we have previously studied the effect of 1-ß-D-arabinofuranosyl cytosine triphosphate (araCTP) and 2',3' dideoxythymidine triphosphate (d_2TTP) (5,6) in this system. These studies provided strong evidence that DNA polymerase α was the main polymerase engaged in nuclear DNA synthesis. Butt et al. (16), however, utilizing extraction procedures, suggested that DNA polymerase ß was the more important enzyme in this process. They suggested that a complex form of DNA polymerase ß took part in the limited extension of Okazaki fragments observed in their system of isolated nuclei (17).

Since both araCTP and d_2TTP are substrates for the polymerases, we have studied the effect of another inhibitor, aphidicolin. This is a tetracyclic diterpenoid and very unlikely to be a substrate for any DNA polymerase. The inhibition is non-competitive in the nuclear system.

Our results confirm previous reports (3,4) on the specificity of the inhibitor with regard to the different DNA polymerases: Aphidicolin has a high degree of specificity towards DNA polymerase α, whereas the ß and γ polymerase activities remain uninhibited.

Utilizing this tool we demonstrate, in accordance with our

previous results with other inhibitors (6), that nuclear DNA synthesis and DNA polymerase α show very similar patterns of inhibition. The polymerase α is apparently responsible in the main for both the elongation of primary DNA pieces and the gap-filling which precedes ligation of such pieces, since during a partial inhibition of polymerase α both elongation and transfer of label from the primary piece region to the high molecular weight region of the alkaline gradient suffered in parallell.

The fact that preformed primary DNA pieces were not transformed into larger molecular weight DNA when aphidicolin was present at concentrations blocking the activity of DNA polymerase α, might be explained by an inhibitory effect on DNA ligase(s). The absence of any accumulation of primary DNA pieces when the inhibitor was present at a concentration giving a 60% inhibition of total DNA synthesis makes this hypothesis less likely.

Some effect of the inhibitor on DNA ligase activity cannot, however, be excluded and the effect of aphidocolin on DNA ligase will be measured directly.

Provided in vitro assays of the polymerases reflect their in vivo responses to inhibitors, it now seems reasonable to conclude that DNA polymerase α is responsible fcr the elongation as well as for the gapfilling in the semiconservative DNA synthesis in isolated HeLa cell nuclei.

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