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THE MODE OF ACTION OF APHIDICOLIN ON DNA SYNTHESIS IN ISOLATED NUCLEI

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SUMMARY

Aphidicolin (a specific inhibitor of DNA polymerase-α) inhibited DNA synthesis in isolated nuclei from sea urchin embryos but ddTTP (an inhibitor of DNA polymerases-β and -γ) did not, indicating that DNA polymerase-α was responsible for DNA synthesis in isolated nuclei. DNA synthesis in isolated nuclei was inhibited by aphidicolin noncompetitively with respect to each of dNTPs indicating that properties of in situ DNA polymerase activity in isolated nuclei are different from those of the purified DNA polymerase-α which was inhibited by aphidicolin competitively with respect to dCTP and noncompetitively with respect to the other 3 dNTPs. Similar results were obtained using HeLa cell nuclei.

INTRODUCTION

Previously we demonstrated that aphidicolin prevented mitosis of sea urchin embryos by interfering DNA synthesis and DNA polymerase-α activity (1) and that the mode of inhibition by aphidicolin was competitive with only dCTP but not with the other 3 dNTPs (2). DNA polymerase-α from adenovirus-infected KB cells is also inhibited by the drug competitively with respect to only dCTP (3). However, the endogenous DNA polymerase activity of the adenovirus DNA replication complex which is isolated from adenovirus-infected KB cell nuclei is inhibited competitively with respect to only dTTP (3). DNA polymerases-α and -γ are identified in the extract of the replication complex (4). These results indicated that DNA polymerases-α and -γ are organized to perform viral DNA replication in the replication complex.

In this report, we have studied the inhibitory mode of action by aphidicolin of DNA synthesis in isolated nuclei from sea urchin embryos and

Abbreviations used: dNTP(s), deoxynucleoside triphosphate(s); ddTTP, 2',3'-dideoxythymidine triphosphate.

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HeLa cells and obtained the results showing that properties of DNA polymerase-α integrated in the nucleus is different from those of the isolated enzyme.

MATERIALS AND METHODS

Tritiated dTTP (43 Ci/mmole), [^{3}H]dGTP (12 Ci/mmole), [^{3}H]dATP (24 Ci/mmole) and [^{3}H]dCTP (19 Ci/mmole) were obtained from Radiochemical Centre, Amersham. dATP, dCTP, dTTP, dGTP and ATP were the products of Yamasa, Chiba and Boehringer Mannheim. ddTTP was purchased from P-L Laboratory. Aphidicolin was gifts from Drs. S. Ikegami and M. Ohashi. Activated DNA was prepared according to the method of Fansler and Loeb (5) using calf thymus DNA (Sigma). DNA content was determined by the method of Burton (6).

Isolation of nuclei from sea urchin embryos and determination of DNA synthesis

Embryos at the hatching blastula stage of the sea urchin, Anthocidaris crassispina, were washed with 10 volumes of 1M glucose three times, suspended in buffer A (0.25 M sucrose, 20 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and 1 % Triton X-100) and homogenized 2 strokes with a Teflon-glass homogenizer. The homogenate was centrifuged at 4,000 rpm for 10 min and the crude nuclear pellet was resuspended in buffer A followed by centrifugation at 2,000 rpm for 10 min. The nuclear fraction which was homogenous microscopically, was then suspended in 80 % glycerol containing 5 mM 2-mercaptoethanol and 5 mM $MgCl_2$ and stored at -20 °C. Under the above conditions, the nuclear fraction did not lose activity of DNA synthesis for a week.

Determination of DNA synthesis in sea urchin blastula nuclei was carried out by a modification of the method of Shimada (unpublished).
Five µl of the isolated nuclear suspension containing 12 µg of DNA were incubated in the reaction mixture containing 50 mM Tris-HCl, pH 8.0, 2 mM 2-mercaptoethanol, 2 mM ATP, 2 mM $MgCl_2$, 0.4 M sucrose, 50 µM each of dATP, dCTP and dGTP and 1 µCi [^{3}H]dTTP in a final volume of 0.1 ml. After incubation at 20 °C for 30 min, a 90-µl aliquot was applied on a paper disc and the acid-insoluble radioactivity was counted according to the method of Bollum (7).

Isolation of HeLa cell nuclei and determination of DNA synthesis

Synchronized HeLa cells were harvested at S-phase and frozen at -80 °C until use. The frozen cells were thawed, resuspended in hypotonic buffer (10 mM Tris-HCl, pH 7.5 and 2 mM $MgCl_2$) at a population of 2.5×10^7 cells/ml and homogenized 10 strokes with a Teflon-glass homogenizer. An equal volume of buffer B (160 mM NaCl, 120 mM Tris-HCl, pH 8.5, 22 mM glucose, 0.1 % Triton X-100, 1 mM EDTA and 2 mM 2-mercaptoethanol) was added to the homogenate and homogenized again as above followed by centrifugation at 1,500 rpm for 5 min. The nuclear pellet was washed once with 10 ml of 1/2 x buffer B containing 15 mg/ml bovine serum albumin and finally resuspended in 1/2 x buffer B containing 15 mg/ml bovine serum albumin at a population of 2.5×10^7 nuclei/ml. Thus obtained nuclear fraction was homogenous microscopically.

For determination of DNA synthesis activity, the standard reaction mixture (0.3 ml) contained 1.25×10^6 nuclei, 33 µM each of dATP, dCTP and dGTP, 3.3 µM [^{3}H]dTTP (0.5 Ci/mmole), 3.3 mM ATP, 50 mM Tris-HCl, pH 8.5, 40 mM NaCl, 5.5 mM glucose, 0.025 % Triton X-100, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 2.5 mg/ml of bovine serum albumin and the indicated amount of aphidicolin where indicated. Incubation was carried out at 37 °C for 10 min. The reaction was terminated with an equal volume of 10 % trichloroacetic acid containing 1 % pyrophosphate and the acid-insoluble radioactivity was counted according to the method of Ono et al. (8).

Preparation and assay of DNA polymerases

DNA polymerases-α and -β from the nuclear fraction of sea urchin blastulae and DNA polymerase-α from HeLa cells were purified and their activities were determined as reported previously (2,8,9).

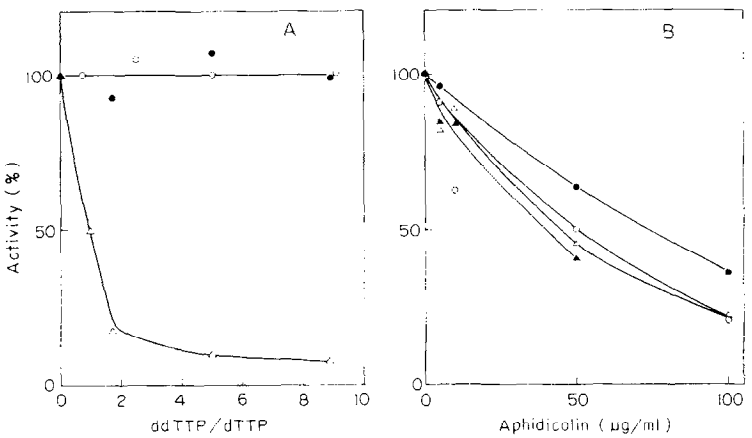

Fig. 1; Effects of ddTTP and aphidicolin on DNA synthesis in isolated nuclei of sea urchin embryos. (A), Effect of ddTTP on DNA synthesis in isolated nuclei (o), DNA polymerases-α (●) and -β (Δ). The reaction mixture for DNA synthesis in isolated nuclei was the same as described in MATERIALS AND METHODS except that [^{3}H]dCTP was used for [^{3}H]dTTP and ddTTP at the indicated concentration and 50 μM dTTP were used. Incorporation of [^{3}H]dCMP in the absence of ddTTP were 4,370 cpm. The reaction mixture for DNA polymerases-α and -β was the same as described in MATERIALS AND METHODS except that [^{3}H]dCTP was used in the place of [^{3}H]dTTP and that ddTTP were changed at 10 μM dTTP. Incorporations of [^{3}H]dCMP in the absence of ddTTP were 2,200 and 2,960 cpm for DNA polymerases-α and -β, respectively. (B), Effect of aphidicolin on DNA synthesis in isolated nuclei. The reaction mixture contained 1 μCi [^{3}H]dNTP without addition of corresponding unlabelled dNTP and 50 μM each of the other 3 dNTP as shown in MATERIALS AND METHODS. Incorporations of [^{3}H]dTMP (o), [^{3}H]dCMP (●), [^{3}H]dAMP (Δ) and [^{3}H]dGMP (▲) in the absence of aphidicolin were 7.2, 8.8, 7.2 and 5.4 pmoles/30 min/12 μg of DNA, respectively.

RESULTS

Characterization of DNA polymerase which is responsible for DNA synthesis in isolated nuclei from sea urchin blastulae

DNA polymerase which is responsible for DNA synthesis in isolated nuclei was evaluated using ddTTP and aphidicolin. The effect of ddTTP on DNA polymerases-α and -β and DNA synthesis in isolated nuclei from sea urchin embryos is shown in Fig. 1A. At the ddTTP/dTTP ratio of 10, DNA polymerase-α activity remained unchanged and nearly all activity of DNA polymerase-β was inhibited. On the other hand, endogenous DNA synthesis activity in isolated nuclei was resistant to ddTTP.

As shown in Fig. 1B, approximately half of incorporation of each [^{3}H]dNMP into DNA was inhibited by aphidicolin at a concentration of 50 μg/ml and the incorporation of each [^{3}H]dNMP was completely inhibited at higher concentrations than 200 μg/ml.

Mode of inhibitory action by aphidicolin on DNA synthesis in sea urchin embryo nuclei

The noncompetitive nature of inhibition of dTMP and dCMP incorporations into DNA by aphidicolin is demonstrated by double-reciprocal plots (Fig. 2).

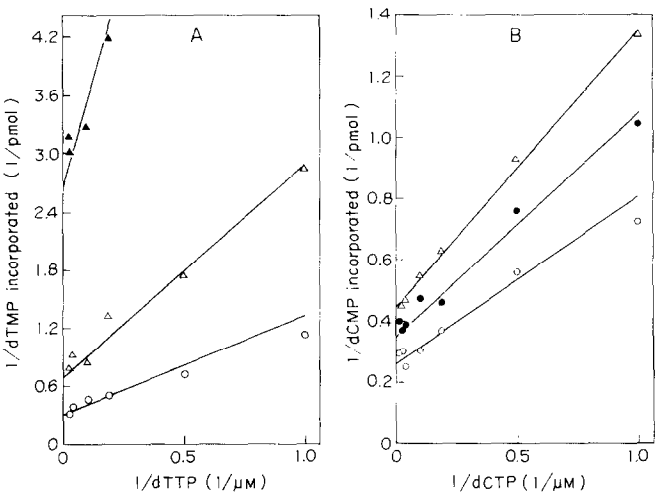

Fig. 2; Double-reciprocal plots of DNA synthesis in sea urchin nuclei as a function of dTTP (A) and dCTP (B). The assay conditions were as described in MATERIALS AND METHODS except that the concentrations of dTTP (A) or dCTP (B) were changed at the fixed concentrations of aphidicolin. ▲, 100 μg/ml of aphidicolin; △, 50 μg/ml of aphidicolin; ●, 10 μg/ml of aphidicolin; o, without aphidicolin.

Approximate Km values for dTTP and dCTP were calculated to be 3.3 and 2 μM, respectively. The Ki value for aphidicolin of 40 μg/ml (118 μM) was almost the same in both cases.

As for dATP and dGTP, the situation was quite the same as dTTP and dCTP. Addition of excess amount of dATP or dGTP to the reaction mixture did not reverse inhibition of DNA synthesis in isolated nuclei by the drug. The extent of inhibition by the drug was not increased when dATP or dGTP was removed from the reaction mixture (data not shown). These data indicated that aphidicolin inhibits noncompetitively with respect to any one of dNTPs in DNA synthesis in isolated nuclei.

Kinetic analysis of inhibition of DNA synthesis in isolated HeLa cell nuclei by aphidicolin

It has been reported that aphidicolin inhibits completely the DNA synthesis in isolated HeLa cell nuclei at a similar concentration to inhibit DNA polymerase-α (10.11). The mode of inhibition of DNA synthesis in isolated HeLa cell nuclei by aphidicolin is shown in Figs. 3A and 3B. Inhibitory mode was similar to that of DNA synthesis in sea urchin nuclei. The Ki value of 0.2 μg/ml (0.6 μM) was determined. Furthermore, the mode of inhibition of HeLa cell DNA polymerase-α by aphidicolin was competitive with dCTP (Fig. 3C) and not competitive with the other 3 dNTPs (data not shown) as demonstrated

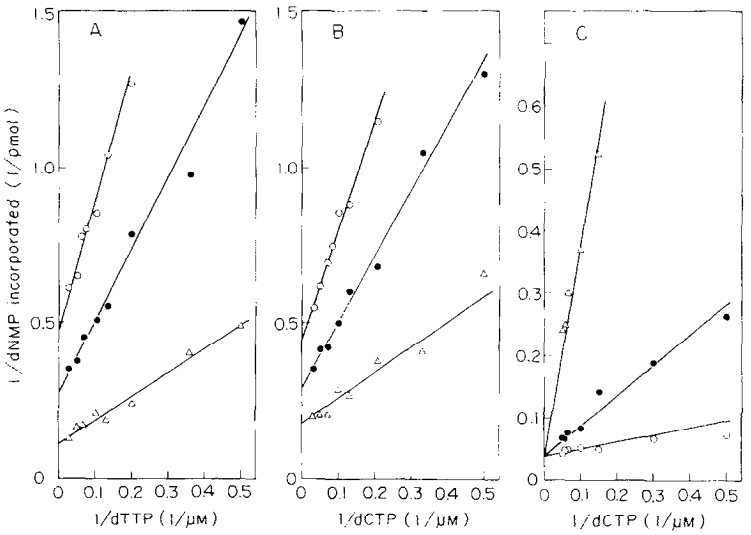

Fig. 3; Inhibition of DNA synthesis in isolated nuclei and activity of DNA polymerase-α from HeLa cells by aphidicolin. (A, B), Double-reciprocal plots of DNA synthesis in HeLa cell nuclei as a function of dTTP (A) and dCTP (B). The assay conditions were as described in MATERIALS AND METHODS except that the concentrations of dTTP (A) or dCTP (B) were changed at the fixed concentrations of aphidicolin. o, 2 μg/ml of aphidicolin; •, 0.2 μg/ml of aphidicolin; Δ, without aphidicolin. (C), Double-reciprocal plots of DNA polymerase-α activity as a function of dCTP. The activity was determined as described in MATERIALS AND METHODS except that the concentrations of dCTP were changed in the presence of aphidicolin. Δ, 6.7 μg/ml of aphidicolin; •, 1.3 μg/ml of aphidicolin; o, without aphidcolin.

with DNA polymerase-α from sea urchin embryos (2), mouse myeloma (2) and KB cells (3). A Ki value was determined to be approximately 0.3 μg/ml (0.89 μM).

DISCUSSION

DNA polymerase responsible for DNA synthesis in isolated nuclei of sea urchin embryos and HeLa cells is thought to be DNA polymerase-α by the followings. ddTTP, which is reported to be an inhibitor of DNA polymerases-β and -γ (12-14), does not inhibit DNA synthesis in isolated nuclei (Fig. 1A, 14). Aphidicolin, a specific inhibitor of eukaryotic DNA polymerase-α (1,11,15,16), inhibits DNA synthesis in isolated nuclei (Fig. 1B, 10,11), although the susceptibility of DNA polymerase-α to the drug was higher than that of DNA synthesis in isolated nuclei of sea urchin blastulae (Fig. 1B). The other lines of evidence are as follows. The major polymerase activity found in the extract of isolated nuclei was DNA polymerase-α in both cases of sea urchin embryos and HeLa cells (unpublished data). The extent of DNA synthesis in isolated HeLa cell nuclei was dependent on the stage of cell cycle from which nuclei were isolated. S-phase nuclei synthesized DNA much more than nuclei at other phases did (unpublished data). DNA polymerase-α activity of isolated nuclei increases when cells are at S-phase (17,18).

Mode of inhibition by aphidicolin on DNA synthesis in isolated nuclei was fairly different from that on DNA polymerase-α activity. The difference seems to be general phenomenon because a similar result was obtained in both sea urchin embryos and HeLa cells. Such a difference may be interpreted as follows. DNA polymerase-α interacts with other components in the nucleus to form a putative replication complex. Such an organized form of DNA polymerase-α is assumed to have different properties from the purified enzyme. We have suggested an interaction between DNA polymerases-α and -γ in the adenovirus DNA replication complex (3).

The susceptibility to aphidicolin was different between DNA polymerase-α activity (Ki, 0.5 μg/ml) (2) and DNA synthesis in isolated nuclei (Ki, 40 μg/ml) (Fig. 2) from sea urchin embryos. However, Ki values for aphidicolin were nearly the same between DNA polymerase-α and nuclei from HeLa cells (Fig. 3,10, 11). The reason for the difference between HeLa cell nuclei and sea urchin nuclei remains to be studied.

Data presented here show that aphidicolin inhibits DNA synthesis in isolated nuclei noncompetitively with respect to each dNTP. This explains our preliminary results which showed in the presence of aphidicolin deoxycytidine had no ability to induce mitotic cell division of the sea urchin embryos and to restore *in vivo* DNA synthesis of mouse JLS-V9 cells. These results are not consistent with the previous data which showed that DNA polymerase-α " a replicative enzyme " is inhibited by aphidicolin competitively with respect to dCTP (2). Data presented here explained this discrepancy because aphidicolin inhibits DNA synthesis in isolated nuclei noncompetitively with respect to each dNTP.

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