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Aphidicolin inhibits DNA synthesis by DNA polymerase α and isolated nuclei by a similar mechanism

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ABSTRACT

Aphidicolin is a selective inhibitor of DNA polymerase α. In contrast to earlier reports, the drug was found to inhibit DNA synthesis catalyzed by DNA polymerase α and isolated HeLa cell nuclei by a similar mechanism. For both systems aphidicolin primarily competed with dCTP incorporation. However, the apparent V_{max} for dCTP incorporation was reduced by 50-60% at relatively low concentrations of aphidicolin, thus the mechanism of inhibition is complex. Furthermore, a 2-5 fold increase in apparent K_m for dTTP was observed in the presence of aphidicolin, but the apparent K_m values for dATP and dGTP were essentially unaltered. This, together with additional evidence, suggested that the mechanism of action of aphidicolin involves a strong competition with dCMP incorporation, a weaker competition with dTMP incorporation and very little, if any, competition with dGMP and dAMP incorporation.

INTRODUCTION

The antibiotic aphidicolin is a selective inhibitor of DNA polymerase α (1) and has recently been used extensively to study the function of eukaryotic DNA polymerases (1-7). Its potential as a possible anti-cancer agent is also being explored. The inhibition of the α-polymerase was reported to be due to competition with dCMP incorporation (8). In contrast, inhibition of DNA synthesis in uninfected isolated nuclei was apparently non-competitive with respect to dCTP (6). Furthermore, aphidicolin apparently inhibited adenovirus synthesis in isolated nuclei by competing with dTMP incorporation (9). Recently it was shown that aphidicolin competed with the poly(dA)-directed incorporation of dTMP (10). In this paper we have compared the effect of aphidicolin on DNA synthesis catalyzed by DNA polymerase α and by isolated HeLa cell nuclei. In contrast to earlier reports (6,8), we find that the mechanism of action is qualitatively similar in the two systems.

MATERIALS AND METHODS

Chemicals. All non-radioactive nucleotides were purchased from PL Bio-

chemicals. Radioactively labelled nucleotides and nucleosides were obtained from the Radiochemical Centre, Amersham. Aphidicolin was kindly donated by A.H. Todd, ICI. Other chemicals were obtained as described previously (11). The concentrations of deoxynucleoside 5-triphosphates were measured spectrophotometrically and adjusted if necessary.

Cells and enzymes. HeLa cells were grown in suspension culture (12) and a cell extract (cytosol) prepared as described previously (13). DNA polymerase α was prepared essentially according to a published procedure (14) with minor modifications as specified in figure 1. One unit of DNA polymerase activity is defined as 1 nmol of total deoxynucleoside monophosphate incorporated into DNA in 60 min at 37°C.

Assay for DNA polymerase α. The reaction mixture (100 µl) contained 50 mM Tris-HCl pH 8.5, 7.5 mM $MgCl_2$, 2 mM 2-mercapto-ethanol, 50 µg of activated salmon sperm DNA and the four deoxy-ribonucleoside triphosphates (one of which was ^{3}H-labelled) in concentrations specified in the tables and figure legends. Incubations were carried out at 37°C for 60 min and the incorporated radioactivity assayed as described previously (4).

Assay for DNA polymerase γ. The reaction mixture contained (in 70 µl) 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 0.5 mM poly(rA)·oligo $(dT)_{12-18}$, 2.5 mM dithiothreitol, 5 µM [^{3}H]dTTP (2 µCi/nmoles), 35 µg bovine serum albumin and 100 mM KCl.

DNA synthesis in isolated HeLa cell nuclei. Nuclei were isolated from cells synchronized by the amethopterin/adenosine method (12) and stored at -70°C in a buffer containing 70% glycerol as described previously (11). The cells were prelabelled with ^{14}C-thymidine (0.05 µCi per 600 ml cell suspension) for two generations prior to synchronization to allow an exact estimation of the amount of DNA per assay in subsequent *in vitro* experiments. The incubation mixture has been optimalized for DNA replication (12) and contained (in 100 µl) 65 mM Tris-HCl (final pH 8.1), 65 mM NH_4Cl, 10% glycerol, 50 mM glucose, 1 mM EGTA, 0.05 mM CTP, UTP GTP, 10 mM ATP, 10.7 mM $MgCl_2$, about $2x10^6$ nuclei and the four dNTPs at concentrations and specific radioactivities as specified in figures and tables. Incubations were performed at 37°C for 15 min. The reactions were stopped and the radioactivity incorporated assayed as described previously (4).

Other methods. Protein (16) and DNA (17) were measured according to standard procedures.

RESULTS

Purification of DNA polymerase α. DNA polymerase α was partially purified from cytosol as specified in the legend to figure 1. In the final step, contamination of DNA polymerase α by DNA polymerase γ was very small (fig. 1). The final specific activity of DNA polymerase α was 19754 units/mg protein.

Effect of aphidicolin on DNA polymerase α activity and DNA synthesis in isolated nuclei. A reexamination of the mechanism of action of aphidicolin seemed desirable because conflicts among earlier reports render the picture rather confusing (6,8-10).

We examined the rate of [^{3}H]dCMP incorporation as a function of the [^{3}H]-dCTP concentration in the presence of a set of fixed aphidicolin concentrations. Figure 2A shows plots of S/V against S of the data concerning DNA polymerase α. In this type of plot, in the absence of inhibitor, the slope

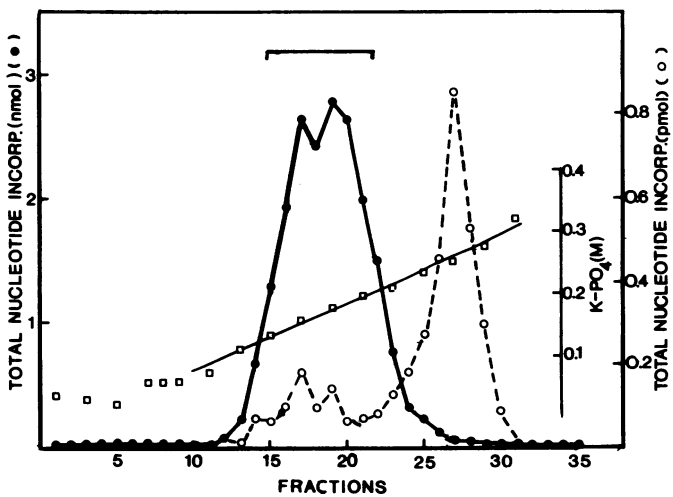

Figure 1. Purification of DNA polymerase α by chromatography on hydroxyl apatite. DNA polymerase α was purified by chromatography on DEAE-cellulose (DE-52, Whatman) and phosphocellulose (P11, Whatman) according to ref. 14, except that all the buffers also contained 1 mM phenylmethylsulfonyl fluoride (PMSF). The active fractions from the phosphocellulose column were pooled, dialyzed against 0.02 M potassium phosphate (pH 7.5), 20% glycerol, 2 mM mercapto-ethanol and 1 mM PMSF. The dialyzed material was loaded onto a column of hydroxylapatite (15 ml) (Bio-Gel HT, Bio-Rad) equilibrated with the same buffer. The column was washed with two column volumes of the same buffer and eluted with 10 column volumes of a linear salt gradient (0.02-0.35 M potassium-phosphate, pH 7.5). Fractions of 4 ml were collected and the activity of DNA polymerase α (—●—) and DNA polymerase γ (--o--) determined in aliquots of 10 µl and 15 µl, respectively.

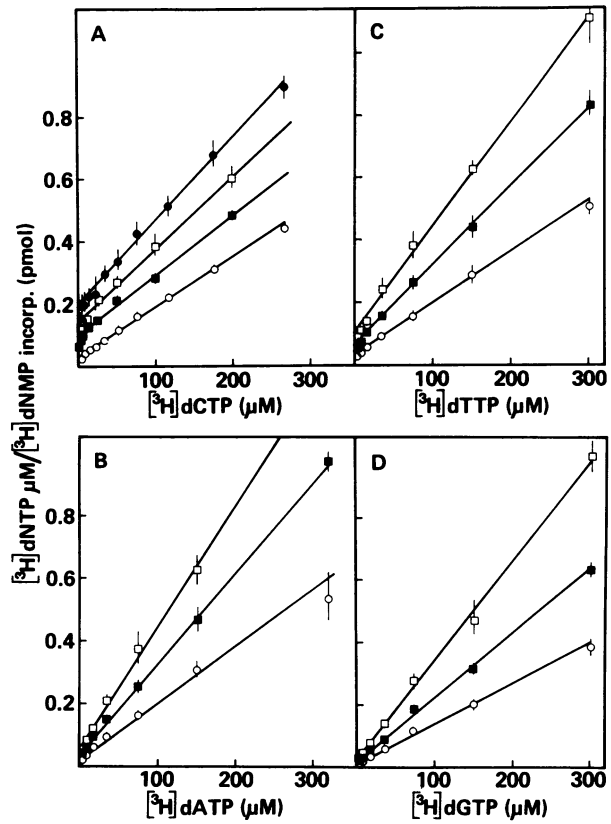

Figure 2. Effect of aphidicolin on DNA polymerase α-catalyzed incorporation of [^{3}H]dCMP, [^{3}H]dGMP, [^{3}H]dTMP or [^{3}H]dAMP. All incubations were performed with about 6 units of DNA polymerase α at 37°C for 30 min. The concentration of free Mg^{2+} was kept constant. All the experiments were carried out three times with duplicates for each concentration. Standard deviations are indicated. Panel A: DNA polymerase α was incubated in an assay mixture containing varying concentrations of [^{3}H]dCTP and 100 μM of the three unlabelled dNTPs. Aphidicolin was present at 0 μM (—o—), 1.2 μM (—■—), 2.4 μM (—□—) or 4.8 μM (—●—). In panels B, C and D the unlabelled dNTPs were present at 600 μM and the concentrations of aphidicolin were 0 μM (—o—), 6 μM (—■—) or 12 μM (—□—). Incorporated radioactivity was assayed as described previously (4).

is $1/V_{max}$ and the abscissa intercept is $-K_m$. Competitive inhibition is characterized by parallel lines.

The data demonstrate that at low aphidicolin concentrations, the mechanism of action approaches simple competitive inhibition, but as the aphidicolin increases, the pattern deviates more and more from pure competitive inhi-

bition as a drop in the apparent V_{max} by about 55% was observed. Table 1, which also includes additional kinetic data, summarizes these results. In the experiments with [^{3}H]dCTP, the unlabelled triphosphates (dATP, dGTP and dTTP) were present at a concentration of 100 μM. As demonstrated later, the concentration of the other triphosphates will influence the kinetics of dCMP incorporation considerably. In figure 2B-D we have investigated the effect of aphidicolin on the incorporation of [^{3}H]dAMP, [^{3}H]dTMP and [^{3}H]dGMP, respectively. In these experiments the non-labelled triphosphates were present at very high (600 μM) concentrations. Aphidicolin inhibited the incorporation of [^{3}H]dAMP and [^{3}H]dGMP non-competitively. The results concerning [^{3}H]dTMP incorporation indicated a mixed type of inhibition, a 2-3 fold increase in the apparent K_m for [^{3}H]dTTP was observed in the presence of aphidicolin, indicating that the mechanism of inhibition may in part be competitive. Incorporation of [^{3}H]dUMP also showed a mixed type of inhibition (data not shown).

Figure 3 illustrates the same type of experiments, but here we have examined the effect of aphidicolin on DNA synthesis in isolated cell nuclei. Nuclear DNA synthesis appears to be catalyzed by DNA polymerase α, although a minor role for the β- or γ-polymerase has not been ruled out (reviewed in ref.

Table I. Effect of aphidicolin on the activity of DNA polymerase α.*

Aphidicolin (μM)	Radiolabelled substrate	Apparent K_m (μM)	Apparent V_{max} (% of control)
0	[^{3}H]dCTP	14.6	100
0.6	"	31.3 (223%)	81.8
1.2	"	41.0	75.0
2.4	"	65.8	61.1
4.8	"	75.3	45.0
0	[^{3}H]dTTP	9.8	100
6	"	15.0	59.6
12	"	23.1	50.7
0	[^{3}H]dATP	5.6	100
6	"	6.1	62.2
12	"	6.9	54.1
0	[^{3}H]dGTP	3.3	100
6	"	3.2	62
12	"	3.5	33

*Incubation conditions were as in fig. 2, but with some additional data included for [^{3}H]dCMP incorporation. Values in paranthesis represent results from single experiments, and are given as the % of the control (without aphidicolin) in that experiment. The other results are the mean values from three experiments.

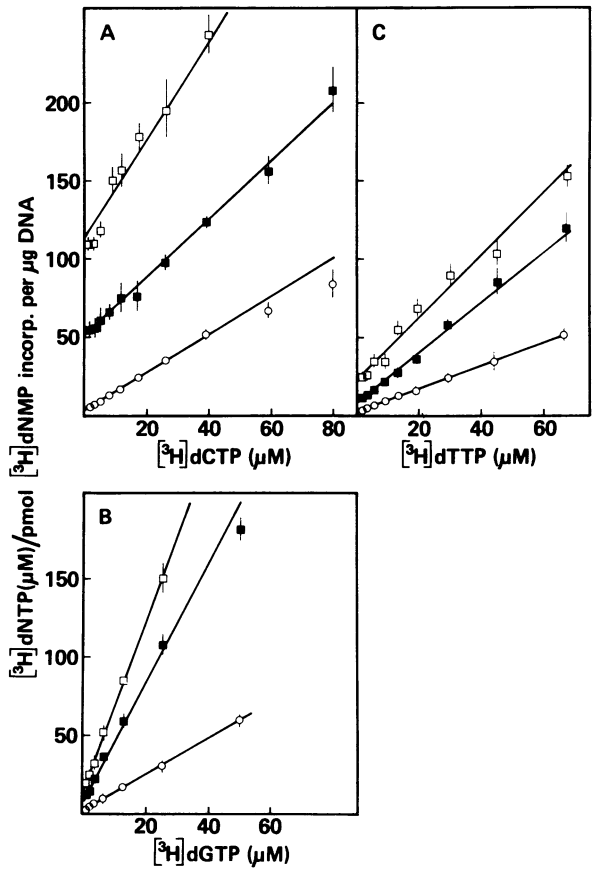

Figure 3. Effect of aphidicolin on nuclear incorporation of [^{3}H]dCMP, [^{3}H]-dGMP or [^{3}H]dTMP. All incubations were performed with isolated HeLa cell nuclei (corresponding to 20-25 μg DNA) at 37°C for 15 min during which incorporation is approaching linearity (12). The concentration of free Mg^{2+} was kept constant. All the experiments were carried out twice in duplicate. Standard deviations are indicated. Panel A: Nuclei were incubated in an assay mixture containing varying concentration of [^{3}H]dCTP and 100 μM of the unlabelled dNTPs. Aphidicolin was present at 0 μM (—o—), 2.4 μM (—■—) or 9.6 μM (—□—). In panel B and C unlabelled dNTPs were present at 400 μM. Aphidicolin was present at 0 μM, (—o—), 6 μM (—■—) or 12 μM (—□—). Incorporated radioactivity was assayed as described previously (4).

18).

Again we find that dCTP can overcome most of the inhibitory effect of aphidicolin, but with increasing inhibitor concentrations, the apparent V_{max} is decreased considerably. In addition, the apparent K_m for dTTP increased 5-fold,

indicating again a mixed type of inhibition. Therefore, although aphidicolin primarily competes with dCTP, a weaker competition with dTMP incorporation seems likely. We also observed a 2-fold increase in the apparent K_m for [^{3}H]-dGTP in the presence of aphidicolin (table II). It was not possible to determine the kinetics of incorporation of [^{3}H]dAMP into DNA of isolated nuclei in a quantitatively satisfactory manner, because the ATP (which is present in the nuclear system at a concentration of 10 mM) seemed to be contaminated by trace amounts of dATP. Thus, the apparent K_m for [^{3}H]dATP was found to be very high (about 50 µM). However, in a similar experiment varying concentrations of added unlabelled dATP (0-600 µM) had only a marginal effect on the rate of [^{3}H]dTMP incorporation, and when absent, the rate was only depressed by about 10%. Taken together these experiments indicate that dATP is supplemented by some other source, probably as a trace contaminant of ATP.

Based on the results described above, we suspected that it would be possible to demonstrate a near pure competitive inhibition of [^{3}H]dCMP incorporation by aphidicolin if the concentrations of dTTP, dATP and dGTP were all increased considerably. This turned out to be correct (fig. 4). When the unlabelled triphosphates were present at 400-600 µM, the pattern of inhibition is essentially competitive at all the concentrations of aphidicolin tested. In the presence of 9.6 µM aphidicolin the apparent K_m for [^{3}H]dCTP increases

Table II

Effect of aphidicolin on [^{3}H]dNMP incorporation into isolated nuclei.*

Aphidicolin (µM)	Radiolabelled substrate	Apparent K_m (µM)	Apparent V_{max} (pmoles/µg DNA)
0	[^{3}H]dCTP	2.60	0.81
0.6	"	6.8 (251%)	0.80 (94%)
1.2	"	10.4 (385%)	0.62 (81%)
2.4	"	26.0	0.58
9.6	"	37.3	0.30
0	[^{3}H]dTTP	1.55	1.32
6	"	4.40	0.61
12	"	7.70	0.50
0	[^{3}H]dGTP	0.90	0.86
6	"	1.65	0.25
12	"	1.93	0.19

*Incubation conditions were as in fig. 3. The experiments were performed twice and with duplicates for each dNTP concentration. The mean values are presented. Some results are from single experiments and the values (in %) given in parenthesis is the value relative to the control (0 µM aphidicolin) in that experiment.

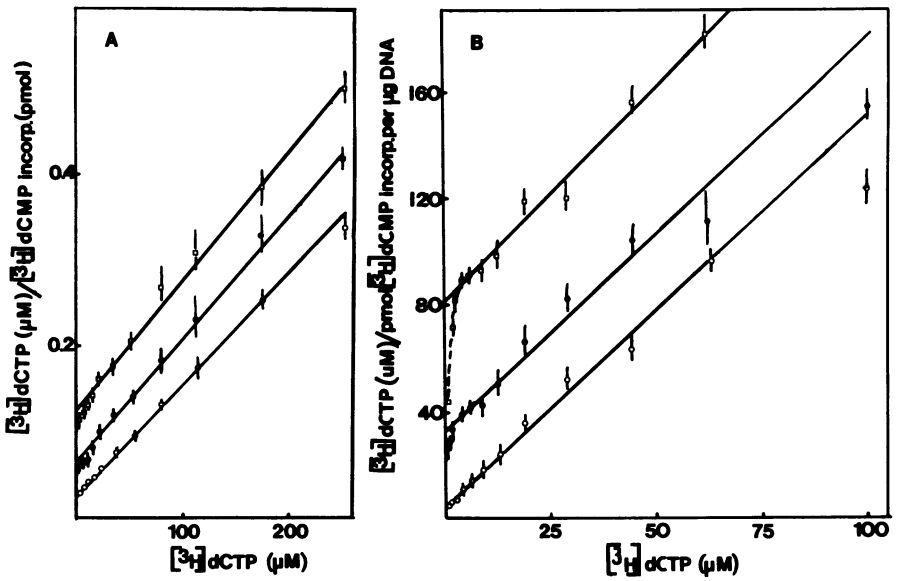

Figure 4. Effect of aphidicolin on [^{3}H]dCMP incorporation in the presence of 400-600 μM of dATP, dGTP and dTTP. Panel A. DNA polymerase α was incubated at 37°C for 30 min in an assay mixture that contained varying concentrations of [^{3}H]dCTP (spes. radioactivity 166 μCi/μmole) and 600 μM of dATP, dGTP and dTTP in the absence (–o–) or presence of aphidicolin at 3.2 μM (–●–) or 9.6 μM (–□–). Panel B. Isolated HeLa cell nuclei (corresponding to 20-25 μg endogenous DNA) were incubated at 37°C for 15 min in an assay mixture that contained varying concentrations of [^{3}H]dCTP (specific radioactivity 250 μCi/μmole) and 400 μM of dATP, dGTP and dTTP, in the absence (–o–) or presence of aphidicolin at 2.4 μM (–●–) or 9.6 μM (–□–). The concentration of free Mg^{2+} was always kept constant. The incorporated radioactivity was assayed as described previously (4).

about 8-fold (from 12 μM to about 84 μM) in the polymerase assays, whereas the apparent V_{max} dropped by only about 9 and 20% for the two aphidicolin concentrations tested (fig. 4A). Analogous results were observed with the nuclear system (fig. 4B). Here the apparent K_m for dCTP increased from 3.1 μM to 51 μM, and the apparent V_{max} was unaltered or dropped by 8% (9.6 μM aphidicolin). It is evident that the apparent K_m for some of the triphosphates is about one order of magnitude lower in the nuclear system than in the isolated polymerase system. This phenomenon has also been observed by others (reviewed in ref. 19), and cannot presently be explained, although it is likely that it is due to interaction of DNA polymerase α with other proteins to form a multienzyme complex. Although not evident from all the figures, the kinetics of dCMP incorporation did not show simple Michaeli-Menten kinetics at

very low and very high dCTP concentrations. Generally the rate of incorporation was higher than expected both at very low (for DNA polymerase α below 10 μM and for nuclei below 3-4 μM) and very high dCTP concentrations (more than 200 μM dCTP). We do not know the reason for this phenomenon, which was not observed for the other triphosphates (with the possible exception of dTTP).

DISCUSSION

The selective inhibition of DNA polymerase α by aphidicolin is well established (1-7). In addition to DNA polymerase α, HSV-1 DNA polymerase is highly sensitive to the drug (4,20) with vaccinia virus polymerase being moderately sensitive (20). So far, no non-polymerase enzymes have been found to be inhibited by aphidicolin, although it has been suggested that aphidicolin may possibly have additional targets in intact cells (21).

It is perhaps not surprising that the literature concerning the mode of action of aphidicolin on DNA polymerase α and subcellular systems for DNA synthesis contains some apparent inconsistencies (6,8-10). Considering the fact that DNA polymerase α has five substrates with which aphidicolin might interact, unambiguous experiments are difficult to design. We have tried to overcome some of these problems by keeping the non-radioactive dNTPs at high levels so that any competition between aphidicolin and unlabelled dNTPs would be minimized. Using this approach, we have found clear evidence for competitive inhibition of dCMP incorporation also in a nuclear system, and in addition we have demonstrated competition with dTMP incorporation. In earlier reports, the concentrations of the non-varying triphosphates were generally so low (20-33 μM) that, for instance, the competition of aphidicolin with dCMP incorporation could easily be masked in the HeLa cell nuclei system used (6,8). However, we cannot exclude the possibility that polymerases from different cell types or strains may vary, or that different methods for preparation of enzymes or subcellular systems may influence the mode of action of aphidicolin. Recently it was demonstrated that the incorporation of dTMP into poly(dA)·oligo(dT) was inhibited competitively by aphidicolin (10). We have verified this result and have also found that aphidicolin inhibits incorporation of dTMP into poly d(AT). This effect was reversed by dTTP, but not by dATP or dCTP (data not shown).

We feel that although aphidicolin has no obvious structural resemblance to any of the dNTPs, competition for binding at, or close to, the triphosphate binding site is the mechanism of action that fits the data best although an

allosteric-type of process is also possible. It may be more than a coincidence that the ability of aphidicolin to compete with deoxynucleotide incorporation is correlated to their apparent K_m values. Even though E.coli pol I has only one triphosphate binding site (22), this has not been proven for mammalian DNA polymerases. It is possible then that aphidicolin preferentially competes with a dCTP site. Alternatively, the conformation of the triphosphate binding site may depend on the base to be paired with on the template strand, such that when there is a dGMP residue in the template strand, only dCTP (or aphidicolin) will bind. Such a template-dependent conformation of the triphosphate binding site would also explain the observations that the fidelity of the polymerase reaction is much higher than expected from the relative stability of AT and GC base pairs (23).

Recent genetic evidence also suggests that the main target for aphidicolin in intact cells is indeed DNA polymerase α and that a major mechanism of action in vivo is competition with dCMP incorporation: some aphidicolin resistant mutants are DNA polymerase α hyperproducers (24-26) and were also resistant to cytosine arabinoside. DNA polymerase α from one mutant was aphidicolin resistant (25). Some aphidicolin resistant mutants had elevated concentrations of dATP without a simultaneous increase in dCTP, and incorporation of labelled deoxyribonucleosides into acid-precipitable material was apparently equally depressed by the drug in mutants and wild type (27). The latter results cannot presently be explained, but may suggest additional targets for the drug. Aphidicolin inhibits replication of adenovirus DNA in subcellular systems, but has less effect on intact adenovirus-infected cells (4,9,28). This discrepancy has not yet been explained. Inhibition by aphidicolin of in vitro adenovirus DNA replication is not dependent on the dCTP concentration, but is competitive with dTMP incorporation (9). Different conclusions have therefore been drawn concerning the possible involvement of DNA polymerase α in adenovirus replication (9,28). Our observations and those of Holmes (10) together indicate that aphidicolin also competes with the α-polymerase-catalyzed incorporation of dTMP in complex systems. It is possible that the DNA polymerase α in the replication machinery of adenovirus has an altered structure, such that the relative ability of aphidicolin to compete with each of the four dNTPs is changed.

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