*Proc. Natl. Acad. Sci. USA*
Vol. 77, No. 12, pp. 7049–7053, December 1980
Biochemistry

# DNA polymerase α mutants from a *Drosophila melanogaster* cell line

(aphidicolin/drug-resistant cells/DNA synthesis/ethyl methanesulfonate mutagenesis/overproduction)

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*Communicated by I. Robert Lehman, August 18, 1980*

ABSTRACT **Aphidicolin, a tetracyclic diterpenoid antibiotic, is a specific inhibitor of DNA synthesis *in vivo* and DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7) α of eukaryotic organisms. After ethyl methanesulfonate mutagenesis, we have recovered mutants of *Drosophila melanogaster* Schneider cell line no. 2 that grow at concentrations of aphidicolin that completely inhibit wild-type cells. The DNA polymerase α from one of these mutants, *aph-10*, is much more resistant to inhibition by the drug; the apparent $K_i$ of the wild-type enzyme is 12 nM aphidicolin, whereas the apparent $K_i$ of the *aph-10* polymerase is more than 100 nM. (The apparent $K_m$ for dCTP is the same for both enzymes.) Another mutant, *aph-13*, overproduces DNA polymerase α at least 8-fold. The DNA polymerase of this mutant has the same apparent $K_m$ and $K_i$ for dNTPs and aphidicolin as does wild-type polymerase.**

Higher eukaryotic cells contain at least three structurally and probably functionally distinct classes of DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7). On the basis of size, subunit structure, chromatographic properties, sensitivity to inhibitors, and template-primer specificity, these three enzymes have been classified as DNA polymerase α, β, and γ (1–3). DNA polymerase α is a large nuclear enzyme and the most abundant DNA polymerase found in growing cells; it might be a true replicase. Polymerase β is a smaller, basic enzyme associated with the nucleus and may act during DNA repair. Polymerase γ is a large molecular weight enzyme located in the mitochondria and nucleus and has been implicated in the replication of some viruses, but not host DNA (4). To date, however, these polymerases' precise functional roles in DNA replication and repair have not been convincingly established.

Mutants having altered DNA polymerases would be valuable in further understanding the functional roles of these enzymes. In addition, such mutants might prove extremely useful in the biochemical analysis of the enzymes and the reactions they catalyze. Specific inhibitors of DNA replication have been valuable tools in the study of replication in prokaryotes and particularly in eukaryotes, for which genetic analysis is less well developed (3, 5, 6).

Aphidicolin is a specific and potent inhibitor of DNA replication *in vivo* (7–9) and DNA polymerase α reactions *in vitro* (6, 8–13); it does not inhibit the other eukaryotic DNA polymerases. Using aphidicolin as the selective agent, we have sought mutants of a *Drosophila melanogaster* cell line possessing altered DNA polymerase α activities. This strategy has been pursued with various cell lines by others, but mutants possessing altered DNA polymerase α have not been reported (14, 15, †); instead, the resistant mutants were altered in nucleotide biosynthesis pathways (15, †). In this paper, we describe an aphidicolin-resistant mutant that contains an altered DNA polymerase α. Another resistant mutant appears to overproduce DNA polymerase α about 10-fold. A possible overproducing mutant has been described (14), but overproduction was only 2-fold.

*D. melanogaster* is the most thoroughly studied of the eukaryotes, and the three different DNA polymerases have been identified. It has been shown recently that *D. melanogaster* early embryos are an excellent source for the enzymes of DNA replication, particularly DNA polymerase α (16).

## MATERIALS AND METHODS

**Chemicals.** Aphidicolin was provided by A. H. Todd (Imperial Chemical Industries, Macclesfield, England). 2′,3′-Dideoxythymidine 5′-triphosphate (ddTTP), 1-β-D-arabinofuranosylcytosine 5′-triphosphate (aCTP), and other deoxy- and ribonucleotides were purchased from P-L Biochemicals; ethyl methanesulfonate from Eastman; phenylmethanesulfonyl fluoride ($PhMeSO_2F$) from Sigma; [α-$^{32}$P]dCTP (2000–3000 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) from Amersham; [α-$^{32}$P]dTTP (400 Ci/mmol) from ICN; [$^{32}$P]orthophosphate (carrier free), [*methyl*-$^3$H]thymidine, [5-$^3$H]uridine, and L-[4,5-$^3$H]leucine from New England Nuclear; uniformly $^{14}$C-labeled amino acids mixture from Schwarz/Mann; and modified Schneider medium (17) and fetal bovine serum from GIBCO. DNA-agarose was made as published (18).

**Cell Line and Growth Conditions.** *D. melanogaster* established cell line no. 2 (Schneider no. 2) (19) was provided by I. Schneider of the Walter Reed Army Institute of Research. The cells were grown in modified Schneider medium (17) supplemented with 10% fetal bovine serum at 25–26°C. Plastic petri dishes or bottles (Corning) were used for most manipulations. For large-scale cell growth, roller bottles were used.

**Partial Purification of DNA Polymerase.** Cells were grown in suspension culture to $5 \times 10^7$ per ml in two 1-liter roller bottles each containing 150 ml of medium, collected by centrifugation at $650 \times g$ for 5 min, suspended in 10 ml of 10 mM Tris·HCl, pH 7.5/1 mM EDTA/4 mM $MgCl_2$/6 mM 2-mercaptoethanol/0.025% Triton X-100/0.1 mM $PhMeSO_2F$, and homogenized with a Teflon-pestle glass homogenizer; then 1 M KCl was added. After 60 min at 0°C, the homogenate was centrifuged at 20,000 rpm for 30 min in a Spinco SW 27 rotor. The supernatant was dialyzed against buffer A [20 mM Tris·HCl, pH 8.0/1 mM EDTA/10% (vol/vol) glycerol/0.1 mM $PhMeSO_2F$/10 mM 2-mercaptoethanol] for 4 hr at 0°C (frac-

Abbreviations: ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; aCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate.

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† Chang, C. C., Lie, P. K., Warren, S. T., Sabourin, C. L., Schulty, R. A., Glatzer, L., Boezi, J. A. & Trosko, J. E. (1980) *11th Annual Meeting of the Environmental Mutagen Society*, Nashville, TN, pp. 80–81 (abstr.).

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tion I), and applied to a DNA-agarose column (2 × 10 cm) equilibrated with buffer A. DNA polymerases were eluted with 50 ml each of 0.05, 0.15, 0.3, 0.5 and 1 M KCl/buffer A. DNA polymerase activity eluting at 0.15 and 0.3 M KCl was pooled and dialyzed against buffer B [25 mM $KPO_4$, pH 6.8/0.1 mM $PhMeSO_2F$/10% (vol/vol) glycerol/10 mM 2-mercaptoethanol] (fraction II). It was then applied to a hydroxylapatite column (2 × 5 cm) equilibrated with buffer B. The column was washed with buffer B and the enzyme was eluted with a 150-ml linear gradient of 0.025–0.5 M $KPO_4$ (pH 6.8) containing 10% (vol/vol) glycerol, 0.1 mM $PhMeSO_2F$, and 10 mM 2-mercaptoethanol. DNA polymerase $\alpha$ was eluted at about 0.2 M $KPO_4$, pooled, dialyzed against 50% (vol/vol) glycerol/50 mM Tris·HCl, pH 7.5/1 mM EDTA/10 mM 2-mercaptoethanol/0.1 mM $PhMeSO_2F$ and stored at −20°C (fraction III).

**Assay of DNA Polymerase Activity.** DNA polymerase $\alpha$ was assayed at 30°C for 30 min in 35 mM Na Hepes buffer, pH 7.8/10 mM $MgCl_2$/1 mM dithiothreitol/250 $\mu$g of activated calf thymus DNA (16) per ml/33 $\mu$M dGTP, dATP, dCTP, [$\alpha$-$^{32}$P]- or [$^3$H]dTTP (specific activity 0.5 Ci/mmol)/5 mM ATP/100 $\mu$g of bovine serum albumin per ml. When crude extracts were used, each rNTP was added to the reaction mixture at 100 $\mu$M. (Large amounts of rNTPs prevented degradation of dNTPs in crude extracts and were required to maintain linear DNA synthesis beyond 15 min.) The DNA polymerase $\beta$ assay was essentially the same as the assay for DNA polymerase $\alpha$ except that 2 mM *N*-ethylmaleimide was added. DNA polymerase $\gamma$ was assayed as published (20). One unit of activity is the amount that catalyzes the incorporation of 1 nmol of dNTPs into acid-insoluble material in 60 min at 30°C. A previously defined unit (16) was 1 nmol of dNTPs incorporated in 60 min at 37°C. Our units therefore represent at least 3-fold more enzyme because of the temperature difference.

**Isolation of Aphidicolin-Resistant Mutants.** *D. melanogaster* cell line no. 2 was grown in 10 10-cm petri dishes to about $3 \times 10^7$ cells per dish at 25°C and 10 mM ethyl methanesulfonate was added to the growth medium. After 5 hr of treatment, yielding 20% survival by the colony-forming assay, the medium was removed, the cells were washed twice with 5 ml of 0.1 M $KPO_4$, pH 7.0/0.15 M NaCl (phosphate/saline buffer), fresh medium was added, and the surviving cells were grown at 22°C for 1 week to allow expression. The cells were suspended, pooled, transferred to 50 24-well plastic dishes, and incubated for 1 week at 22°C in medium containing 25 $\mu$M aphidicolin. Any cells that grew in a well were then further cultured at 22°C in media containing 50 $\mu$M and finally 100 $\mu$M aphidicolin.

**Rates of DNA, RNA, and Protein Synthesis.** About $5 \times 10^6$ cells were grown for 48 hr in a 3.5-cm-diameter plastic petri dish containing 1.5 ml of medium, and they were pulse-labeled with [$^3$H]thymidine, [$^3$H]uridine, or [$^3$H]leucine at 1 $\mu$Ci/ml for 30 min at 25°C to measure DNA, RNA, or protein synthesis, respectively. After pulse-labeling, cells were centrifuged and suspended in phosphate/saline buffer; 5% trichloroacetic acid was added, and acid-insoluble material was collected on a Whatman GF/C filter. The radioactivities were measured by liquid scintillation spectrometry.

**Cellular Nucleotide Pools.** Cells were grown for 24 hr at 25°C in 3.5-cm plastic petri dishes containing 1.5 ml of medium, and 100 $\mu$Ci of carrier-free [$^{32}$P]orthophosphate was added. After another 48-hr incubation the cells were collected by centrifugation, washed twice with 2 ml of phosphate/saline buffer, and suspended in 0.5 ml of 5% trichloroacetic acid. After 30 min at 0°C with occasional shaking, the cell suspension was centrifuged. The supernatant was extracted with ether several times until its pH became neutral and then was lyophilized. The $^{32}$P-labeled nucleotides were dissolved in 20 $\mu$l of water and separated by polyethyleneimine-cellulose thin-layer chromatography as published (21), using unlabeled dNTP and rNTP markers. Radioactive nucleotides were identified by UV monitoring and autoradiography, and the radioactivity of each spot was measured by scintillation spectrometry.

**Nuclease Assay.** Reaction mixtures were identical with those for DNA polymerase assay except that native or heat-denatured *Escherichia coli* [$^3$H]DNA (1 $\mu$g, $5 \times 10^4$ cpm/$\mu$g) was the substrate. The reactions were terminated by addition of 0.2 ml of cold 10% trichloroacetic acid containing 1% potassium pyrophosphate. After 10 min, the tubes were centrifuged at 10,000 rpm for 10 min in a Sorvall SS34 rotor. The radioactivity of 0.1 ml of the supernatant was measured in Ultrafluor scintillation fluid (New England Nuclear).

**Other Methods.** Sodium dodecyl sulfate/7% polyacrylamide gel electrophoresis of $^{14}$C-labeled proteins was carried out as published (22); the gel was dried and autoradiographed for 6 days at room temperature. Protein markers were purchased from Bio-Rad. Protein concentrations were measured by the Lowry method (23).

## RESULTS

**Effect of Aphidicolin on Macromolecule Synthesis in *D. melanogaster* Cells.** The aphidicolin sensitivities of cell growth and of DNA, RNA, and protein synthesis were measured in wild-type *D. melanogaster* cells (Fig. 1). About 0.2 $\mu$M aphidicolin inhibited both cell growth and [$^3$H]thymidine incorporation by 50%, whereas neither RNA nor protein synthesis was significantly inhibited by even 20 $\mu$M drug. The total DNA polymerase activity in crude extracts was also measured; it was about 10 times more sensitive than *in vivo* DNA synthesis and cell growth. The small residual DNA polymerase activity (5%) in the presence of 25 $\mu$M aphidicolin might be due to DNA polymerase $\beta$, $\gamma$, or both, because this residual synthesis was completely inhibited by 40 $\mu$M ddTTP in the presence of 20

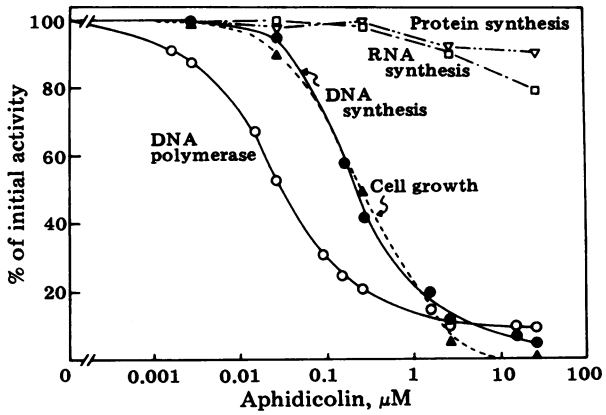


FIG. 1. Aphidicolin sensitivity of macromolecule synthesis in a *D. melanogaster* cell line. Wild-type cells (Schneider no. 2) were grown in 3.5-cm petri dishes containing 2.0 ml of medium for 48 hr, whereupon various amounts of aphidicolin were added. After 5 min, [$^3$H]thymidine, [$^3$H]uridine, or [$^3$H]leucine was added to measure DNA, RNA, or protein synthesis, respectively. After 30 min the cells were suspended, centrifuged, and resuspended in 1 ml of phosphate/saline buffer, and acid-insoluble radioactivities were measured. The 100% values for the DNA, RNA, and protein synthesis were 10,164, 5480, and 1955 cpm, respectively. For cell growth, predetermined numbers of cells were grown for 4 days in the presence or absence of aphidicolin and total cell number was measured with a hemocytometer. For DNA polymerase assays, about $10^7$ cells were lysed and the lysate was dialyzed against 20 mM Tris·HCl, pH 7.5/1 mM EDTA/10% glycerol/0.1 mM $PhMeSO_2F$/10 mM 2-mercaptoethanol and used for the assays of DNA polymerases. DNA polymerase activities were measured as in *Materials and Methods* except that 0.25 $\mu$M dCTP and 100 $\mu$M of each rNTP were added. The 100% value was 41 pmol of [$^{32}$P]dTMP incorporation per assay.

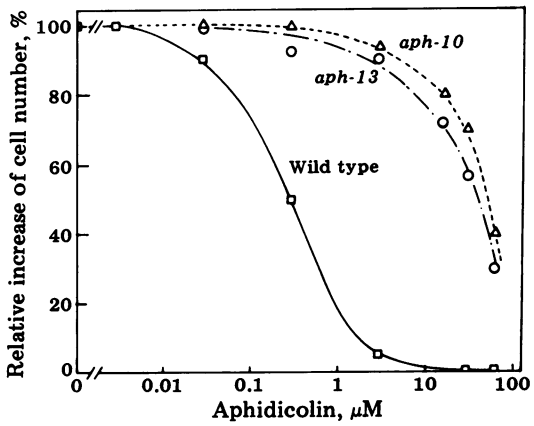


FIG. 2. Aphidicolin sensitivity of wild-type and aphidicolin-resistant cells. Relative increase of cell number was measured as in Fig. 1.

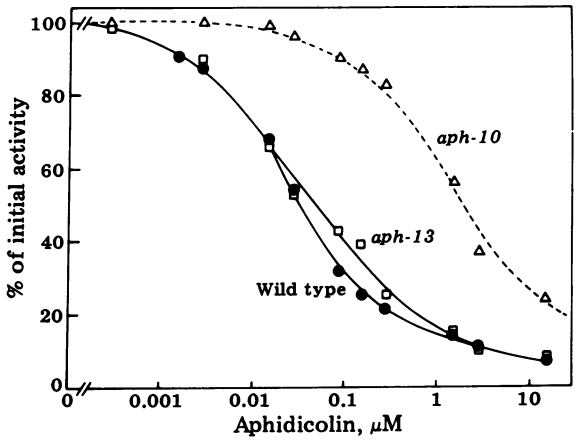


FIG. 3. Aphidicolin sensitivity of DNA polymerase α from *aph-10*, *aph-13*, and wild-type cells. An inoculum of $10^6$ cells was grown at 25°C for 4 days, collected, and lysed, and aphidicolin sensitivity of DNA polymerase α in the crude extracts was measured as in Fig. 1.

μM dTTP (data not shown). Under these conditions, DNA polymerase α is not inhibited by ddTTP (unpublished result). Moreover, about 90% of the initial activity was also inhibited by 2 mM *N*-ethylmaleimide, suggesting it to be due to DNA polymerase α. From these considerations, 25 μM aphidicolin was adopted for selection of aphidicolin-resistant mutants.

**Isolation of Aphidicolin-Resistant Cells.** Ninety-one isolates resistant to 25 μM aphidicolin were selected from *D. melanogaster* cell line no. 2. The frequency of resistant cells recovered was thus about $10^{-7}$. All resistant cells were able to grow in the presence of 25 μM aphidicolin, but the growth rate in the presence of the drug was about half that in the absence of drug. In order to reject permeability mutants, mutants that were able to grow in the presence of 100 μM aphidicolin were further selected from these 91 resistant cell lines. In this paper, these five high-resistant mutants are described. Fig. 2 shows the growth of two representative high-resistance cell lines (*aph-10* and *aph-13*) in the presence of various amounts of the drug. At 60 μM the rate of cell growth of *aph-10* and *aph-13* was about 40% (generation time about 40 hr) of that in the absence of drug (generation time 16–18 hr). These cells were about two orders of magnitude more resistant to aphidicolin than were wild-type cells; even in the presence of 350 μM aphidicolin they grew at about 10–20% of their growth rate in the absence of drug. This drug resistance could be maintained for at least 3 months (over 80 generations) in the absence of drug. These resistant cells are neither temperature sensitive nor cold sensitive, nor do they exhibit increased sensitivity to UV inactivation (data not shown).

**Aphidicolin Sensitivity of Mutant DNA Polymerase α.** We tested whether *aph-10* and *aph-13* have an altered DNA polymerase α resistant to aphidicolin. As shown in Fig. 3, DNA polymerase α activity from *aph-10* was significantly more resistant to the drug than was wild-type enzyme. However, the enzyme from *aph-13* showed the same sensitivity as that from wild-type cells.

Aphidicolin is a true competitive inhibitor with respect to the concentration of dCTP in the DNA polymerase α reaction (11). DNA polymerase α from wild-type, *aph-10*, and *aph-13* cells was partially purified as summarized in Table 1. The aphidicolin sensitivity of each polymerase was then measured in the presence of several concentrations of dCTP and aphidicolin (Fig. 4). The apparent inhibitor constant ($K_i^{app}$) for aphidicolin of the polymerase from *aph-10* is 10 times higher than that of wild-type enzyme, whereas the apparent Michaelis constant ($K_m^{app}$) for dCTP is the same as that of wild-type enzyme (Table 2). On the other hand, both enzymes were nearly equally inhibited by aCTP (Table 2), *N*-ethylmaleimide, and ddTTP (data not shown). The possibility still existed that a contaminant copurified with the mutant DNA polymerase α was actually causing its apparent aphidicolin resistance by modifying the effects of the drug on the normal polymerase (24). To eliminate this possibility, a mixing experiment was carried out with wild-type and *aph-10* DNA polymerases. As shown in Fig. 5, the relative aphidicolin-resistant activity in the mixtures paralleled the relative mutant polymerase present. Furthermore, the resistant activity in the mixture displayed the same sensitivity to aphidicolin as did the mutant enzyme alone (data not shown). These results support our conclusion that the mutant polymerase itself is resistant to aphidicolin.

***aph-13* Cells Overproduce DNA Polymerase α.** Although the growth of *aph-13* cells was as resistant to aphidicolin as was that of *aph-10* cells, DNA polymerase α activity from *aph-13* cells had the same sensitivity to the drug as did the activity of wild-type enzyme (Figs. 2 and 3). Also, each dNTP and rNTP pool size of this mutant cell (in either the presence or absence of aphidicolin) was the same as that of wild type cells (data not shown). However, the specific activity of DNA polymerase α in crude extracts from *aph-13* cells was about 8 times higher than from wild-type cells (Table 3). Another resistant mutant, *aph-16*, was slightly less resistant to aphidicolin than *aph-13*, and also exhibited an increased DNA polymerase α specific activity. In neither *aph-13* nor *aph-16* was the increased level

Table 1. Purification of DNA polymerase α from *D. melanogaster* cell lines

| | | Wild type | | | *aph-13* | | |
|---|---|---|---|---|---|---|---|
| Fraction | Purification step | Activity, units | Specific activity, units/mg | Purification, fold | Activity, units | Specific activity, units/mg | Purification, fold |
| I | Crude extract | 449 | 4.1 | 1 | 398 | 2.1 | 1 |
| II | DNA-agarose | 351 | 61.5 | 15.0 | 311 | 26.5 | 12.6 |
| III | Hydroxylapatite | 219 | 183.2 | 44.7 | 156 | 80.5 | 38.3 |

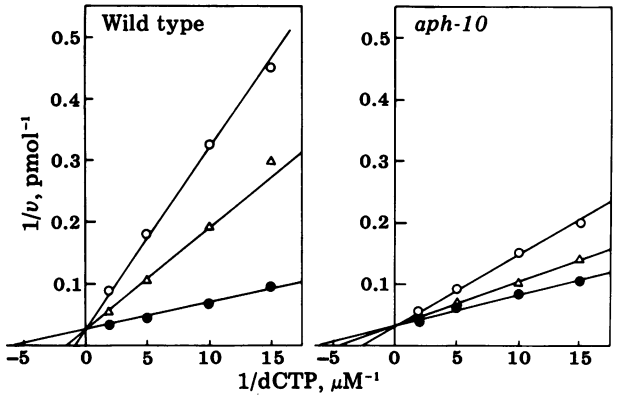


FIG. 4. Inhibition of DNA polymerase α from wild-type and *aph-10* cells by aphidicolin as a function of dCTP concentration. The reaction mixtures contained 0.03 unit of partially purified DNA polymerase α from either wild-type or *aph-10* cells, the indicated amount of dCTP, and no (●), 28.9 nM (△), or 86.7 nM (○) aphidicolin.

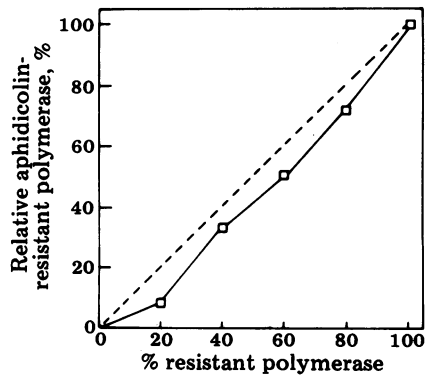


FIG. 5. Aphidicolin sensitivity of wild-type and *aph-10* DNA polymerase α mixtures. Samples of the partially purified wild-type or *aph-10* DNA polymerase α were mixed in various portions, keeping the total activity constant. Each mixture was then assayed in the presence of either 0 or 0.3 μM aphidicolin as described in Fig. 3, and the ratio of activity at 0.3 μM to that at 0 μM aphidicolin was determined.

of polymerase activity due to changes in cell size or nuclease activity: wild-type and mutant cells were of the same size, and their levels of both endonuclease and exonuclease were similar. Moreover, a mixture of wild-type and mutant crude extracts exhibited the level of polymerase α activity expected from a simple average (data not shown). An increase in specific activity by overproduction of DNA polymerase α in crude extracts from *aph-13* and *aph-16* was also suggested by the result of sodium dodecyl sulfate/polyacrylamide gel electrophoresis after labeling the cells with a $^{14}C$-labeled amino acid mixture (Fig. 6). At least one 150,000-dalton polypeptide with mobility similar to that of the largest subunit of DNA polymerase α from *D. melanogaster* eggs (16) was overproduced. (No such overproduction of polypeptides similar to other subunits of DNA polymerase α could have been detected in *aph-13* and *aph-16* under these conditions, because the regions where they migrate were crowded with other polypeptides.) This result is consistent with the altered specific activities of DNA polymerase α in crude extracts from these mutants, assuming that the *ca.* 150,000-dalton polypeptide overproduced in *aph-13* and *aph-16* is the largest subunit of DNA polymerase α (16).

Two other aphidicolin-resistant mutants, *aph-14* and *aph-22*, had neither an altered $K_i^{app}$ for aphidicolin nor an increased specific activity of DNA polymerase α. However, dTTP pool sizes of these mutants were increased severalfold.

## DISCUSSION

We have isolated a mutant that has an altered DNA polymerase α from *D. melanogaster* established cell line no. 2, using aphidicolin as a selective agent. DNA polymerase α partially purified from the aphidicolin-resistant mutant *aph-10* showed at least an order of magnitude higher resistance to the drug than did wild-type enzyme (Table 2 and Figs. 3 and 4) but the same $K_m^{app}$ for dCTP as wild-type enzyme (Fig. 4 and Table 2). However, aCTP, another competitive inhibitor for dCTP of DNA polymerase α (25), equally inhibited both wild-type and *aph-10* DNA polymerase α (Table 2). This suggests that the aphidicolin binding site might be located near but not at the dCTP binding site on the DNA polymerase α molecule; alternatively, the aphidicolin binding site might be apart from the binding site of dCTP, but be functionally closely related to the dCTP binding site, so that drug binding effectively interferes with dCTP binding.

Aphidicolin generally inhibits DNA polymerase α in higher eukaryotes (8–13). However, the target site is not known. *D. melanogaster* DNA polymerase α might be composed of several subunits (16), although the details of subunit structure have not been rigorously established. Moreover, proteolysis introduces additional confusion concerning the subunit structure of DNA polymerase α (26). It is well known that *E. coli* DNA polymerase III holoenzyme is composed of several protein subunits and that the largest, the *dnaE* gene product, exhibits the fundamental polymerization activity (27). By analogy to this prokaryotic system, it is possible that the dCTP binding site might be located in the main polymerizing subunit of DNA polymerase α, presumably the largest subunit; it is also likely that the aphidicolin binding site is located in the same subunit. A final conclusion, however, awaits analysis using homogeneous (16) DNA polymerase α.

It is clear that a better understanding of the role of DNA polymerase α in both DNA replication and repair processes will require the isolation of conditionally lethal temperature-sensitive mutants. Unfortunately, no temperature-sensitive mutants

Table 2. Properties of DNA polymerase α from *D. melanogaster* cell lines

| Cell line | $K_m^{app}$ for dCTP, nM | $K_i^{app}$ for aphidicolin, nM | $K_i^{app}$ for aCTP, μM |
|---|---|---|---|
| Wild type | 160 ± 10 | 12.6 ± 2.5 | 7.5 ± 1.0 |
| *aph-10* | 167 ± 12 | 120.0 ± 10.5 | 5.3 ± 1.3 |
| *aph-13* | 170 ± 10 | 10.0 ± 3.8 | 8.0 ± 1.0 |

The $K_m^{app}$ for dCTP and $K_i^{app}$ for aphidicolin of DNA polymerase α from wild-type and *aph-10* cells were calculated from Fig. 4. Those of *aph-13* DNA polymerase α were estimated as in Fig. 4 from a different experiment. The $K_i^{app}$ for aCTP of DNA polymerase α was estimated in the same manner as in Fig. 4 in the presence of 0, 1, and 10 μM aCTP and various concentrations of dCTP. The data include the standard errors of each estimate.

Table 3. Specific activity of DNA polymerase α in crude extracts from *D. melanogaster* cell lines

| Cell line | Specific activity, units/mg protein |
|---|---|
| Wild type | 4.01 ± 0.60 |
| *aph-10* | 2.28 ± 0.45 |
| *aph-13* | 26.8 ± 0.7 |
| *aph-16* | 8.40 ± 0.48 |

The crude extracts were prepared as described in *Materials and Methods*, except that the cultures of the aphidicolin-resistant cell lines contained 20 μM aphidicolin. DNA polymerase α activity was measured in the presence of 100 μM rNTPs. The data are means ± standard errors from three independent extracts.

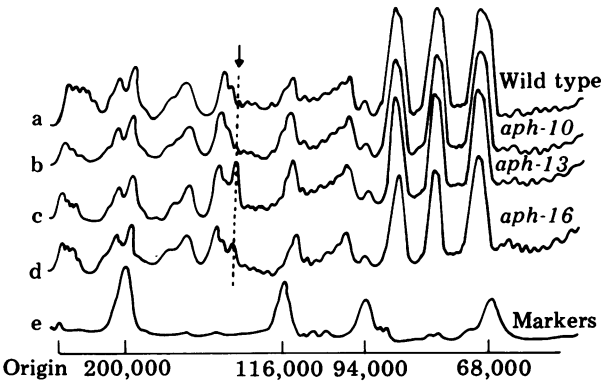


FIG. 6. Analysis of cellular proteins from wild-type and various aphidicolin-resistant mutants. About $5 \times 10^6$ cells were grown for 24 hr in 1.5 ml of medium containing either 0 (wild-type cells) or 40 (aphidicolin-resistant) $\mu$M aphidicolin; 10 $\mu$Ci of $^{14}$C-labeled amino acids mixture was added, and the cells were grown for another 48 hr. The cells were collected, washed twice with phosphate/saline buffer, suspended in 0.1 ml of 0.5% sodium dodecyl sulfate/0.5 mM PhMe-$SO_2F$, and sonicated for 20 sec with a Branson Sonifier. Equal amounts of acid-insoluble radioactivity from the supernatants were applied to 7% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Autoradiographs of the dried gels were traced with a Joyce–Loebl microdensitometer. The bottom line shows the trace of the marker proteins stained with Coomassie blue. The marker proteins were myosin (200,000 daltons), $\beta$-galactosidase (116,500 daltons), phosphorylase b (94,000 daltons), and bovine serum albumin (68,000 daltons). The arrow marks a protein with a mobility corresponding to about 150,000 daltons that was overproduced.

were detected among our isolates. *aph-13* is a more useful mutant than *aph-10* for the study of the enzymology of DNA polymerase $\alpha$ because its specific activity may be at least eight-fold higher than that of the wild-type enzyme (Table 3). Polyacrylamide gel electrophoresis analysis of $^{14}$C-labeled proteins in wild-type and mutant cells (Fig. 6) suggests this conclusion. Nevertheless, it is not completely eliminated that the $\approx$150,000-dalton polypeptide detected in *aph-13* and *aph-16* is not related to the subunit of DNA polymerase $\alpha$. A preliminary experiment, however, showed that the $\approx$150,000-dalton polypeptide overproduced in *aph-13* and *aph-16* (Fig. 6) was copurified with the activity of DNA polymerase $\alpha$ through DNA-agarose and hydroxylapatite column chromatographies. It is obvious that overproduction of DNA polymerase $\alpha$ in *aph-13* and *aph-16* should be more rigorously established. A possibly similar type of mutant has been isolated from FM3A mouse mammary carcinoma cells (14); however, its increase of specific activity of DNA polymerase $\alpha$ was much less, only 2-fold. In neither case is the mechanism of increase of specific activity known. There are at least two possibilities. One is a regulatory mutation controlling the DNA polymerase $\alpha$ structural gene, the corresponding mRNA being overproduced without any increase in DNA polymerase $\alpha$ gene number. Another possibility is a gene duplication (or multiplication) mutation in which the copy number of the DNA polymerase $\alpha$ gene is increased. There are several examples of such gene amplification strategies in eukaryotic systems. Mutants that are resistant to certain inhibitors of nucleotide biosynthesis often have many copies of the gene (28, 29). If the concentration of inhibitor is increased, higher gene amplification mutants are spontaneously generated without any mutagen treatment (28, 29). In our case, although overproduction mutants of DNA polymerase $\alpha$ could be isolated without ethyl methanesulfonate treatment, their frequency was less than $10^{-8}$ (unpublished results). Thus far, using multiple-step selection with increasing concentrations of aphidicolin, we have not isolated a mutant that overproduces DNA polymerase $\alpha$ to a greater extent than *aph-13*. This may be due to lethality of large amounts of DNA polymerase $\alpha$.

Our success in isolating an aphidicolin-resistant DNA polymerase $\alpha$ mutant from an established cell line encourages attempts at isolation of a drug-resistant fly with an altered DNA polymerase $\alpha$. Preliminary results, however, suggest that an aphidicolin concentration at least two orders of magnitude higher than that inhibiting the established cell line is required to inhibit any stage of *Drosophila* development. Either an inhibitor that reduces the effect of aphidicolin or drug detoxification might occur in the intact organism (24) but not in the established cell line. Alternatively, a permeability barrier may protect the intact organism.

We are indebted to Dr. I. Schneider for *D. melanogaster* cell line no. 2, to Dr. A. H. Todd for aphidicolin, to Drs. N. R. Cozzarelli and M. A. Conkling for critical reading of the manuscript, to Dr. J. W. Drake for his constant interest and support and critical reading of the manuscript, and to Dr. R. Wu for his cell culture facility. K.N. was a Visiting Fellow from Japan under the National Institutes of Health Visiting Program.

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