

Termination of DNA Synthesis by 9- β -D-Arabinofuranosyl-2-fluoroadenine

A MECHANISM FOR CYTOTOXICITY*

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The action of 9- β -D-arabinofuranosyl-2-fluoroadenine (F-ara-A) on DNA synthesis was evaluated both in whole cells and *in vitro*. 9- β -D-Arabinofuranosyl-2-fluoroadenine was converted to its 5'-triphosphate 9- β -D-arabinofuranosyl-2-fluoroadenine 5'-triphosphate (F-ara-ATP) in cells and then incorporated into DNA in a self-limiting manner. More than 94% of the analogue was incorporated into DNA at the 3' termini, indicating a chain termination action. *In vitro* DNA primer extension experiments further revealed that F-ara-ATP competed with dATP for incorporation into the A site of the extending DNA strand. The incorporation of F-ara-AMP into DNA resulted in termination of DNA strand elongation. Human DNA polymerase α incorporated more F-ara-AMP into DNA than polymerase ϵ (proliferating cell nuclear antigen-independent DNA polymerase δ) and was more sensitive to inhibition by F-ara-ATP. On the other hand, DNA polymerase ϵ was able to excise the incorporated F-ara-AMP from DNA *in vitro*. The incorporation of F-ara-AMP into DNA was linearly correlated both with inhibition of DNA synthesis and with loss of clonogenicity; thus it may be the mechanism of cytotoxicity.

9- β -D-Arabinofuranosyl-2-fluoroadenine (F-ara-A)¹ is an analogue of adenosine and deoxyadenosine that shows potent antitumor activity in experimental systems (1–4). The fluorine at the 2-carbon of the purine ring makes F-ara-A resistant to adenosine deaminase (1, 2), thus overcoming the drawback of 9- β -D-arabinofuranosyladenine, which has limited therapeutic activity because of its rapid deamination by this enzyme (5, 6). Clinical trials have shown that F-ara-A 5'-phosphate, the more soluble formulation of F-ara-A for clinical use, is effective in the treatment of indolent lymphocytic neoplasms (7, 8).

Previous studies have demonstrated that F-ara-A is trans-

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¹ The abbreviations used are: F-ara-A, 9- β -D-arabinofuranosyl-2-fluoroadenine; F-ara-ATP, 9- β -D-arabinofuranosyl-2-fluoroadenine 5'-triphosphate; pol α , DNA polymerase α ; pol ϵ , DNA polymerase ϵ ; PCNA, proliferating cell nuclear antigen; HPLC, high-performance liquid chromatography.

ported into the cells where it is phosphorylated to F-ara-ATP (1, 2). Incorporation of F-ara-ATP into both DNA and RNA is associated with inhibition of nucleic acid synthesis (9–11). Enzyme assays indicate that F-ara-ATP inhibits DNA polymerases and ribonucleotide reductase (12–14). Recent studies also demonstrated that this compound induces genetic damage by causing deletions of DNA fragments from the cellular genome (15). However, the molecular mechanisms by which F-ara-A inhibits DNA synthesis and induces cytotoxicity remain to be elucidated.

Our studies were conducted to further investigate the mechanisms of action of F-ara-A both in whole cells and *in vitro* systems. Here we describe the incorporation of F-ara-A into DNA and its chain termination activity on DNA synthesis in human leukemia cells. The differential actions of F-ara-ATP on human DNA polymerase α (pol α) and DNA polymerase ϵ (pol ϵ) were demonstrated, and the molecular mechanisms responsible for cytotoxicity were discussed in the context of present knowledge.

EXPERIMENTAL PROCEDURES

Materials—F-ara-A was supplied by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. F-ara-ATP was chemically synthesized in our laboratory by Dr. Alina Sen, using a described procedure (16). HPLC analysis (at $A_{262\text{ nm}}$, full scale: 0.005) of the F-ara-ATP product detected no contamination with dATP. [8-³H]F-ara-A (specific activity 11 Ci/mmol) and [2-¹⁴C]thymidine (specific activity 56 mCi/mmol) were prepared by Moravek Biochemicals, Inc., Brea, CA. [*methyl*-³H]Thymidine (specific activity 52 Ci/mmol), [*methyl*-³H]dTTP (specific activity 58 Ci/mmol), and [γ -³²P]ATP (specific activity 3000 Ci/mmol) were purchased from ICN Radiochemicals, Irvine, CA. Cellulose phosphate (P 11) and diethylaminoethyl cellulose (DE52) were the products of Whatman BioSystems Ltd. Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad. Single-stranded DNA-cellulose and spleen phosphodiesterase were purchased from Sigma. Micrococcal nuclease was the product of Worthington Biochemical Corp., Freehold, NJ. The M13 dideoxy sequencing kit was from Bethesda Research Laboratories. The M13mp18(+) strand DNA, the 17-base M13 sequencing primer (5'-d(GTAAACGACGGCCAGT)-3'), activated calf thymus DNA, and ultrapure dATP, dCTP, dGTP, and dTTP were obtained from Pharmacia LKB Biotechnology Inc.

Cell Culture—Human T lymphoblastoid cells, CCRF-CEM, were maintained in exponential growth in RPMI 1640 suspension culture medium supplemented with 5% fetal bovine serum. The cell number and volume were determined as described (17).

Effect of F-ara-A and Aphidicolin on Cell Clonogenicity—Cells were incubated with various concentrations of the drugs for 5 h and washed twice with drug-free warm medium. Depending on the drug concentrations, 200–800 cells were mixed with 1.3 ml of 0.25% soft agar in Dulbecco's medium supplemented with 20% fetal bovine serum (pre-warmed to 37 °C) and incubated in a 35 × 10-mm tissue culture dish for 10 days (humidified 5% CO₂, 37 °C). At the end of the incubation period, colonies of more than 40 cells were scored under a microscope.

The cytotoxic effect of the drugs was expressed as a percentage of survival relative to that of untreated control cells.

Quantitation of Intracellular F-ara-ATP and Detection of Its Incorporation into DNA—Cells were incubated with various concentrations of F-ara-A for 5 h, washed with cold phosphate-buffered saline (8.1 g of NaCl, 0.22 g of KCl, 1.14 g of Na₂HPO₄, and 0.27 g of KH₂PO₄/liter of H₂O, pH 7.4), and nucleotides were extracted with 0.4 N HClO₄ (2). The intracellular F-ara-ATP was quantitated by HPLC analysis (17). To determine the incorporation of F-ara-A into DNA, cells were incubated with various concentrations of [³H]F-ara-A for 5 h. The [³H]F-ara-A was purified by reverse phase HPLC to more than 99.9% purity and diluted with unlabeled F-ara-A to a specific activity of 0.45 Ci/mmol before each experiment. At the end of the incubation period, the cells were washed twice with cold phosphate-buffered saline, lysed in 4 M guanidinium isothiocyanate, and DNA was separated from RNA by CsCl gradient centrifugation (18). The DNA fraction was treated with RNase A (50 µg/ml) at 37 °C for 45 min, with proteinase K (50 µg/ml) for 45 min, and then extracted with phenol and chloroform. DNA was precipitated twice with 3 volumes of ethanol, dissolved in H₂O, and quantitated by UV absorbance at 260 nm. The [³H]F-ara-AMP in DNA was measured by liquid scintillation counting.

Degradation of DNA to Internal 3'-Nucleotides and Terminal Nucleosides—DNA (100 µg) isolated from cells labeled with [³H]F-ara-A and [¹⁴C]thymidine was dissolved in 200 µl of H₂O and denatured by boiling (2 min) and rapid cooling in an ice bath. The DNA was then degraded to its internal 3'-nucleotides and terminal nucleosides by the sequential action of micrococcal nuclease and spleen phosphodiesterase (19). The digests were separated by reverse-phase HPLC, and the radioactivity associated with the respective 3'-monophosphates and nucleosides was quantitated by liquid scintillation counting.

Determination of DNA Synthesis in Whole Cells—Cells in exponential growth phase were treated with various concentrations of drugs for a desired period and incubated with [³H]thymidine for 30 min. The cells were collected on a 25-mm glass fiber disc (prewetted with 1% sodium pyrophosphate) by filtration and then washed three times with 4 ml of cold 0.4 N HClO₄ and twice with 2 ml of ethanol. Radioactivity retained on the filter disc was determined by liquid scintillation counting.

Determination of DNA Synthesis in Isolated Nuclei—Cells (2.5 × 10⁷) treated with F-ara-A or aphidicolin were washed in cold phosphate-buffered saline, and the nuclei were prepared according to the method previously described (20). The nuclei were suspended in 100 µl of buffer containing 50 mM Tris-HCl (pH 8.0), 25% glycerol, 5 mM magnesium acetate, 0.1 mM EDTA, and 5 mM dithiothreitol. Two-hundred microliters of the reaction mixture (40 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 50 mM NaCl, and 100 µM of dATP, dCTP, dGTP, and [³H]dTTP, specific activity 100 mCi/mmol) were then added to the nuclei suspension and incubated at 37 °C for 15 min. The nuclei were collected on a glass fiber filter disc, washed with 0.4 N HClO₄ and ethanol, and the radioactivity retained on the filter was determined as described above.

Purification of DNA Pol α and Pol ϵ —DNA polymerases α and ϵ were purified from CCRF-CEM cells and characterized with respect to template preference, associated 3' → 5' exonuclease activity, and differential inhibition by N²-(*p*-n-butylphenyl)dGTP as described previously (21, 22). The dependence of the purified pol ϵ on PCNA was examined as described by Tsurimoto and Stillman (23), using poly(dA)_{8,100}/(dT)₁₆ as primed template. This enzyme was highly processive in the absence of PCNA, producing poly(dT) chains of greater than 1000 nucleotides; addition of PCNA did not stimulate the enzyme activity. Under the same reaction conditions, PCNA-dependent pol δ from calf thymus produced poly(dT) chains of similar size in the presence of PCNA. In the absence of PCNA calf thymus pol δ generated short chains of less than 30 nucleotides. Thus, the pol ϵ purified from CEM cells is a PCNA-independent enzyme (24), which had been designated previously as PCNA-independent DNA polymerase δ (21, 25).

DNA Primer Extension Assay—The 17-base oligonucleotide was labeled with ³²P at the 5'-end and annealed to its complementary site on a single-stranded M13mp18(+) DNA template as described previously (21). The primer extension reaction mixture (10 µl) contained 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 0.5 mM dithiothreitol, 10 mM NaCl, 20 µg bovine serum albumin/ml, 40 ng of labeled primer/template, 0.15 units of DNA pol α or pol ϵ , 30 µM each of dCTP, dGTP, and dTTP, and various concentrations of dATP and F-ara-ATP. The reactions were carried out at 37 °C for 30 min and analyzed

by electrophoresis through a 10% polyacrylamide sequencing gel. The dideoxy sequencing of the M13mp18(+) DNA template was done as described by Sanger (26). After autoradiography, the sequencing gels were further analyzed using a Betascope 603 blot analyzer (Betagen Corporation, Waltham, MA). The radioactivity in each DNA band was quantitated under conditions recommended by the manufacturer.

Preparation of a 21-Base Oligomer with F-ara-AMP Incorporated at the 3'-End—The ³²P-labeled 17-base primer/M13mp18 DNA hybrid



was incubated with 30 µM each of dCTP and dGTP, 20 µM F-ara-ATP, and DNA polymerase α at 37 °C for 30 min. The reaction products were separated by electrophoresis through 10% polyacrylamide sequencing gel. The band containing the 21-base DNA fragment with F-ara-AMP at its 3'-end located at the first A site was cut out, and the oligomer was recovered from the gel by incubating the gel slice with 0.5 M NH₄Ac and 1 mM EDTA at 45 °C for 3 h with occasional vortexing. The 21-base oligomer-3'-F-ara-AMP was precipitated with ethanol and dissolved in H₂O.

Preparation of ³H F-ara-ATP—[³H]F-ara-A was purified to greater than 99.9% purity by reverse phase HPLC and then incubated with CCRF-CEM cells for 5 h to allow accumulation of cellular [³H]F-ara-ATP. The cells were extracted with 0.4 N perchloric acid and neutralized with KOH. [³H]F-ara-ATP was purified by HPLC using a Partisil-10 SAX ion-exchange column (Whatman, Inc.). The labeled compound was further purified and desaltsed by chromatography through a column of Sephadex A-25, using 1.0 M ammonium bicarbonate as eluting buffer. The [³H]F-ara-ATP peak fraction was lyophilized and dissolved in H₂O. HPLC analysis of the purified [³H]F-ara-ATP detected no radioactivity coeluting with authentic dATP, dCTP, dGTP, or dTTP.

DNA-dependent Nucleotide Turnover Assay—The unlabeled 17-base primer was annealed to M13mp18(+) DNA and used as the template in the assay system. The reaction mixture (50 µl) contained 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 0.5 mM dithiothreitol, 10 mM NaCl, 20 µg bovine serum albumin/ml, 30 µM each of dGTP and dCTP, 8 × 10⁶ dpm of [³H]F-ara-ATP, and 0.8 units of pol ϵ without or with 3 µg of primed M13mp18 DNA template. The reaction was incubated at 37 °C for 1 h, and the products were analyzed for free [³H]F-ara-AMP by HPLC using a Partisil-10 SAX ion-exchange column and a Flo-One/Beta radioactive flow detector (Packard Instrument Company, Laguna Hills, CA). A linear gradient from 0.005 M NH₄H₂PO₄ (pH 2.8) to 0.75 M NH₄H₂PO₄ (pH 3.7) over 50 min, and then isocratic at 0.75 M NH₄H₂PO₄ (pH 3.7) for an additional 10 min, 1.2 ml/min, was used.

RESULTS

F-ara-A Is Phosphorylated to F-ara-ATP and Incorporated into DNA at Terminal Positions in Whole Cells—When CCRF-CEM cells were incubated with 0.3–100 µM F-ara-A for 5 h, the accumulation of intracellular F-ara-ATP increased in proportion to the concentration of F-ara-A in the culture medium (Fig. 1). At 100 µM F-ara-A, cells were able to phosphorylate the drug efficiently and accumulated an intracellular concentration of 1270 µM F-ara-ATP. The accumulation of F-ara-ATP did not reach a plateau at this drug concentration, suggesting that neither the transport mechanism nor the enzymes responsible for the phosphorylation of F-ara-A to its triphosphate had been saturated under these conditions. In contrast, the incorporation of F-ara-ATP into DNA was self-limited. At 0.3–10 µM F-ara-A, the amount of the F-ara-AMP incorporated into DNA increased with the drug concentrations. However, no further incorporation was detected at F-ara-A concentrations greater than 10 µM. The plateau value of incorporation ranged between 55 and 58 pmol F-ara-AMP/mg DNA.

The effect of such an incubation on DNA synthesis is shown in Fig. 2A. [³H]Thymidine incorporation was inhibited in a concentration-dependent manner. The extent of DNA synthesis inhibition was linearly related to the amount of incorporated F-ara-AMP in DNA ($r = -0.996$, Fig. 2B), a finding

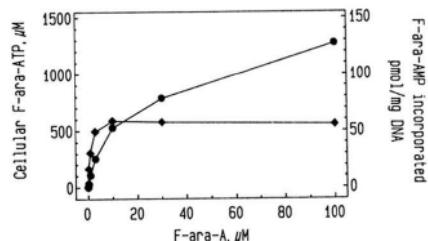


FIG. 1. Accumulation of cellular F-ara-ATP and incorporation of F-ara-AMP into DNA. CCRF-CEM cells were incubated with indicated concentrations of either nonradioactive F-ara-A or [³H]F-ara-A for 5 h. The cells treated with nonradioactive F-ara-A were extracted with 0.4 N perchloric acid and then analyzed by HPLC for F-ara-ATP. DNA was isolated from the cells labeled with [³H]F-ara-A, and incorporation of F-ara-AMP into DNA was measured as described under "Experimental Procedures." ●, cellular F-ara-ATP, micromolar; ♦, F-ara-AMP incorporated into DNA, picomoles/mg DNA. Each point represents average of two determinations.

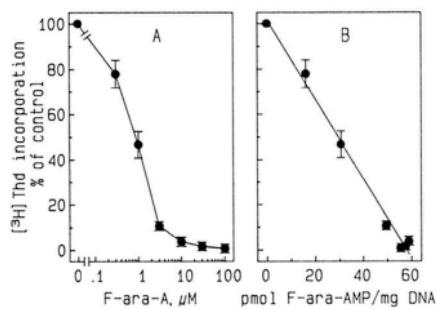


FIG. 2. Inhibition of DNA synthesis by F-ara-A (A) and its correlation with the incorporation of F-ara-AMP into DNA (B). CCRF-CEM cells were incubated with the indicated concentrations of F-ara-A for 4.5 h and then labeled with [³H]thymidine (0.3 μCi/ml) for 30 min. The incorporation of [³H]dTMP into DNA was determined as described under "Experimental Procedures" and expressed as the percentage of the control value (1.77×10^6 dpm/10⁶ cells). Incorporation of F-ara-AMP into DNA was determined as described in Fig. 1. Each point represents pooled data of three to four separate experiments.

that may provide a basis for understanding the self-limiting action of F-ara-A incorporation into DNA. Because most DNA synthesis was inhibited, no additional F-ara-ATP could be incorporated.

To determine whether F-ara-AMP was incorporated into DNA internally or terminally, DNA isolated from cells labeled with [³H]F-ara-A and [¹⁴C]dThd was degraded to internal 3'-nucleotides and terminal nucleosides by the sequential action of micrococcal nuclease and spleen phosphodiesterase. At F-ara-A concentrations of 3–100 μM, greater than 94% of the incorporated F-ara-AMP residues were detected as terminal nucleosides after the enzymatic degradation. In contrast, only about 15% of the incorporated dTMP appeared at the terminal positions in control and in F-ara-A-treated cells. The internal/terminal distributions of the incorporated F-ara-AMP or dTMP were independent of drug concentrations (data not shown). The finding that more than 94% of the incorporated F-ara-AMP residues were located at the 3' termini of the DNA strands strongly suggested that this compound acts as a DNA chain terminator. Control incubations of authentic F-ara-AMP with the enzymes demonstrated less than 0.2% dephosphorylation to F-ara-A.

F-ara-ATP Was Incorporated into the A Sites and Caused DNA Chain Termination in Vitro—A DNA primer extension assay (21, 26) was used to evaluate the molecular action of F-ara-ATP on DNA strand elongation *in vitro*. The ability of DNA polymerases α and ϵ to extend the primer was evaluated

in the absence and presence of F-ara-ATP. Fig. 3 shows the action of F-ara-ATP on DNA primer extension in the absence of dATP. The dark band at the bottom of lane P indicates the position of the 17-base primer. Lanes T, G, C, and A are sequencing ladders of the growing DNA strand starting at the 3'-end of the 17-base primer.

In a complete reaction that contained 30 μM each of dATP, dCTP, dGTP, and dTTP, both pol α and pol ϵ extended the primer to a high molecular weight DNA strand (Fig. 3, *pol* α and *pol* ϵ , lane 6). The dense bands at 47 and 86 nucleotides from the 3'-end of the primer in each lane reflect a pause site for pol α and pol ϵ , probably due to the secondary structure of the M13mp18(+) DNA template (27). When dATP was omitted from the mixture (A⁻ reaction), the primer extension stopped before the first A site, visualized as a *dense band* at the site one nucleotide before the first A site (Fig. 3, *pol* α and *pol* ϵ , lane 1). The *light band* at the first A site in lane 1 represents a small amount of noncomplementary nucleotide misincorporation into the A site by the enzymes, suggesting their error-prone property (28). Pol ϵ , however, was relatively more accurate in replicating the DNA template because it misincorporated less noncomplementary nucleotides than pol α (compare lane 1 in *pol* α and *pol* ϵ). Quantitation of the radioactivity associated with the misincorporation band (the first A site of lane 1, *pol* α or *pol* ϵ) by a Betascope 603 blot analyzer detected 752 counts for the *pol* α reaction and 334 counts for the *pol* ϵ reaction (15-min counting period). In this and two other gels analyzed for radioactivity in the first A band, the ratio of counts incorporated by *pol* α to that by *pol* ϵ was 2.1 ± 0.27 .

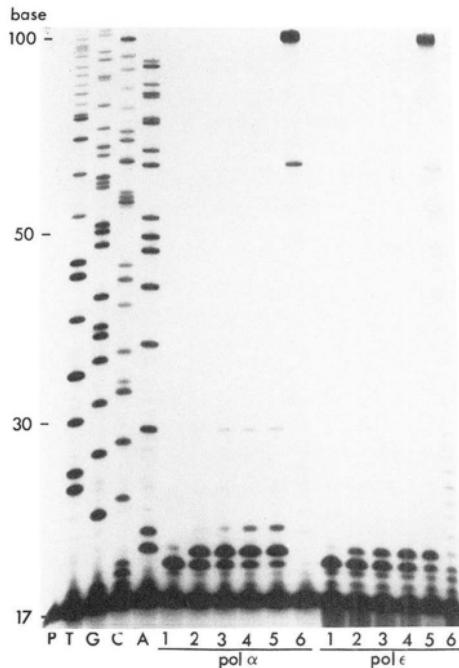


FIG. 3. Incorporation of F-ara-AMP into DNA and its chain termination effect on DNA synthesis by DNA polymerases α and ϵ . The ability of human DNA pol α and pol ϵ to extend the ³²P-labeled primer/template was evaluated in the presence of indicated concentrations of F-ara-ATP and deoxynucleotides as described under "Experimental Procedures." Lane P, the labeled 17-base primer; lanes T, G, C, and A, dideoxynucleotide sequencing of the M13mp18(+) template with ddTTP, ddGTP, ddCTP, and ddATP, respectively, indicating the respective T, G, C, and A sites from the 3'-end of the ³²P-labeled primer; lanes 1–5, A⁻ reactions (contained 30 μM each of dCTP, dGTP, and dTTP) with 0, 1, 3, 10, and 30 μM F-ara-ATP, respectively; lane 6, complete reaction (contained 30 μM each of dATP, dCTP, dGTP, and dTTP) without F-ara-ATP.

When various concentrations of F-ara-ATP were included in the A⁻ reaction, both pol α and pol ϵ were able to use the nucleotide analogue as an alternate substrate for incorporation into the A site (Fig. 3, pol α and pol ϵ , lanes 2–5). Incorporation of F-ara-ATP into the extending primer terminated DNA synthesis, as was evident from the dense band at the first A site. Pol α , however, incorporated more F-ara-AMP than pol ϵ . Furthermore, pol α incorporated more F-ara-AMP with increasing concentrations of F-ara-ATP, which resulted in a gradual decrease of the band's density before the first A site (Fig. 3, pol α , lanes 2–5). This was less obvious in the case of the pol ϵ reaction (Fig. 3, pol ϵ , lanes 2–5). In the presence of 30 μM F-ara-ATP, for example, 6445 counts were detected at the first A site in the pol α reaction (Fig. 3, pol α , lane 5), while pol ϵ incorporated only 3427 counts under the same conditions (pol ϵ , lane 5). Furthermore, pol α was able to extend a small portion of the primer beyond the first A site in the absence of dATP and in the presence of F-ara-ATP. The bands at the second and third A sites in Fig. 3 represent the incorporation of F-ara-ATP into the respective sites and termination of DNA synthesis as a consequence (pol α , lanes 3–5). No primer was extended beyond the first A site by pol ϵ , even with up to 30 μM F-ara-ATP (pol ϵ , lanes 2–5). The radioactivity detected below the 17-base primer band represents degraded products resulting from the 3' \rightarrow 5' exonuclease activity associated with pol ϵ (Fig. 3, pol ϵ , lanes 1–6). When analyzed in a 20% polyacrylamide sequencing gel, the degraded products appeared as smaller bands below the primer (see Fig. 6).

Competition Between F-ara-ATP and dATP for Incorporation—The action of F-ara-ATP on DNA pol α and pol ϵ was then examined in the presence of dATP. As illustrated in Fig. 4, F-ara-ATP competed with dATP for incorporation into the A site of the elongating DNA strand. With increasing concentrations of dATP, the amount of F-ara-AMP incorporated into DNA decreased, and the chain termination effect at the A sites was gradually reversed (Fig. 4, pol α and pol ϵ , lanes 3–7). Detailed comparisons of pol α and pol ϵ further revealed

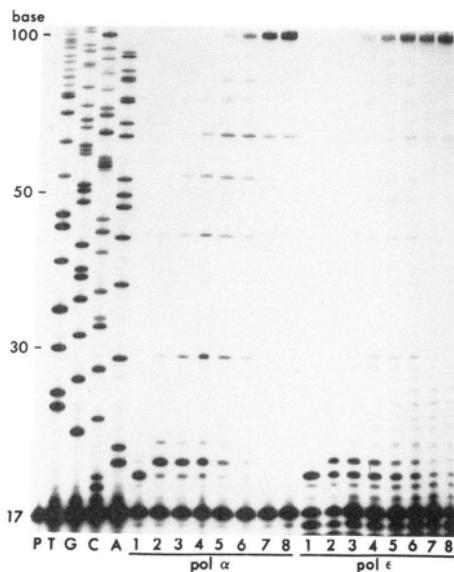


FIG. 4. Competition between F-ara-ATP and dATP for incorporation into DNA catalyzed by DNA polymerases α and ϵ . DNA primer extension assay is described in the legend to Fig. 3. Lanes P, T, G, C, and A, same as described for Fig. 3; lane 1, A⁻ reaction (30 μM each of dCTP, dGTP, and dTTP); lanes 2–7, A⁻ reaction plus 10 μM F-ara-ATP and 0, 0.3, 1, 3, 10, and 30 μM dATP, respectively; lane 8, complete reaction (30 μM each of dATP, dCTP, dGTP, and dTTP) without F-ara-ATP.

differences in their substrate specificity. For example, in the reaction catalyzed by pol ϵ , 1 μM dATP competed efficiently with 30 μM F-ara-ATP and reversed the termination effect, permitting the elongation of the primer to a higher molecular weight DNA strand (Fig. 4, pol ϵ , lane 4). The Betascope detected 676 counts at the uppermost band (high molecular weight DNA). In contrast, in the case of pol α , 1 μM dATP was unable to achieve a similar degree of competition with 30 μM F-ara-ATP; only 136 counts were associated with the high molecular weight DNA band (Fig. 4, pol α , lane 4). Thus pol ϵ was more substrate-specific than pol α and showed a preference for dATP over F-ara-ATP as a substrate for incorporation. These results were consistent with the findings that pol ϵ used F-ara-ATP less efficiently than pol α (Fig. 3).

The competition between F-ara-ATP and dATP for incorporation into DNA was further characterized by kinetic studies. The unlabeled 17-base primer was annealed to M13mp18(+) DNA, and the incorporation of various concentrations of [³H]dATP into the primer/template by pol α and pol ϵ was quantitated in the presence and absence of F-ara-ATP. As shown in Fig. 5, when given fixed concentrations of dCTP, dGTP, dTTP, and an excess amount of primer/template, the incorporation of dATP by both enzymes followed Michaelis-Menten kinetics, with K_m values of 2.7 and 1.6 μM for pol α and pol ϵ , respectively. This reaction was inhibited by F-ara-ATP in a competitive manner. Using a computer analytic method (29), K_i values of 1.1 and 1.3 μM were determined for pol α and pol ϵ , respectively. A K_i/K_m value of 0.40 was calculated for pol α , whereas this value was two times greater (0.81) for pol ϵ , indicating that F-ara-ATP was more inhibitory to pol α .

Extension of a F-ara-AMP-terminated Primer—The ability of pol α and pol ϵ to extend a DNA primer with a F-ara-AMP molecule incorporated at its 3' terminus was evaluated in the presence of dATP, dCTP, dGTP, and dTTP, using a 21-base oligomer-3'-F-ara-AMP as the primer. The 21-base primer was prepared as described under "Experimental Procedures" and used as the substrate for extension by pol α and pol ϵ . The reaction products were analyzed by electrophoresis through a 10% polyacrylamide sequencing gel and the relative ability of pol α and pol ϵ to extend the normal 17-base primer

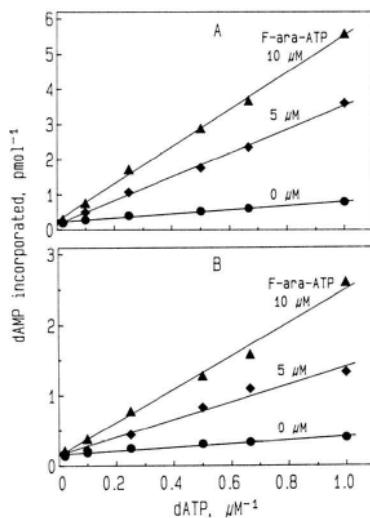


FIG. 5. Kinetic analysis of DNA primer elongation by pol α (A) and pol ϵ (B). Reaction mixture contained 10 $\mu\text{g}/\text{ml}$ of unlabeled primed M13mp18(+) DNA, 50 μM each of dCTP, dGTP, and dTTP, indicated concentrations of [³H]dATP, and the respective polymerases. The reaction was incubated at 37 °C for 30 min, spotted on GF/A filter, washed three times with 5% trichloroacetic acid, and counted by liquid scintillation counting.

and the 21-base primer-3'-F-ara-AMP was quantitated by determining the radioactivity in the respective primer bands and the high molecular weight product band. Table I shows such a quantitation of three separate sequencing gels. Both pol α and pol ϵ extended 35–45% of the normal 17-base primer to high molecular weight DNA. Addition of 300 μ M GMP, an inhibitor of 3' \rightarrow 5' exonuclease (30), did not significantly affect the polymerization of the normal primer ($p > 0.2$). In contrast, pol α and pol ϵ extended only 3.6 and 2.3% of the F-ara-AMP-3'-primer to high molecular weight DNA, respectively. The presence of GMP in the reaction did not affect the activity of pol α ($p > 0.2$), whereas the production of high molecular weight DNA by pol ϵ was significantly reduced ($p < 0.01$). Thus, inhibition of the 3' \rightarrow 5' exonuclease activity resulted in a decreased elongation. These results suggested that pol ϵ might have to remove F-ara-AMP from the 3'-end of the primer and insert dAMP at this site before extending it. Pol α , on the other hand, might directly extend the F-ara-AMP at 3'-end, because the enzyme is relatively less substrate-specific.

Excision of F-ara-AMP from the 3'-End of DNA by Pol ϵ . First, a DNA-dependent nucleotide turnover assay was used to evaluate the possibility that the PCNA-independent pol ϵ may incorporate F-ara-AMP into DNA and then remove it with the intrinsic 3' \rightarrow 5' exonuclease activity. [3 H]F-ara-ATP was incubated with pol ϵ in the presence and absence of primed M13mp18(+) DNA template as described under "Experimental Procedures." The reaction products were analyzed for free [3 H]F-ara-AMP by HPLC using a Flo-One/Beta radioactive flow detector. In the absence of primed M13 DNA, incubation of 10⁶ dpm of [3 H]F-ara-ATP with pol ϵ , dCTP and dGTP (30 μ M each) at 37 °C for 60 min produced 2,492 dpm of free [3 H]F-ara-AMP, which represented the degradation of [3 H]F-ara-ATP to [3 H]F-ara-AMP during the incubation period. When the primed M13mp DNA was included in the reaction, 56,570 dpm of free [3 H]F-ara-AMP were generated from 10⁶ dpm of input [3 H]F-ara-ATP. The DNA template-dependent nucleotide turnover indicated that F-ara-ATP was incorporated into DNA as F-ara-AMP residues, some of which were removed by 3' \rightarrow 5' exonuclease of pol ϵ .

Polyacrylamide gel electrophoresis was used to further evaluate the F-ara-AMP excision by pol ϵ . The 32 P-labeled 21-

base primer-3'-F-ara-AMP and the normal 17-base primers were annealed to M13mp18(+) DNA and used as substrates for excision by pol ϵ in the presence and absence of four dNTPs. The reaction products were analyzed by electrophoresis on a 20% polyacrylamide sequencing gel. Fig. 6 shows the excision of nucleotides from the 3'-ends of both the F-ara-AMP-3'-primer and the normal dTMP-3'-primer. The bands below the primer represent the shorter oligomers that resulted from excision by pol ϵ . The overall amount of removed nucleotides from both primers increased with incubation time, while the radioactivity remaining in the primer bands decreased proportionally. Addition of dNTPs substantially inhibited the excision process. For example, quantitation of the radioactivity in the excised bands revealed that when the normal 17-base primer/M13 DNA was used as substrate, 30 μ M each of dNTP reduced the excision by 80% during a 30-min incubation period (1214 and 6183 counts in all excised bands of lanes 5 and 9, respectively). The same percent of inhibition was observed when the 21-base primer-3'-F-ara-AMP/M13 DNA was the substrate (554 and 2801 counts in all excised bands of lanes 14 and 18, respectively).

Quantitation of the radioactivity in the excised bands also revealed that the overall excision rates for both primers were similar. Pol ϵ removed 56.5% of nucleotides from the normal 17-base primer and 61.4% from the F-ara-AMP-3'-primer during a 30-min incubation period (Fig. 6, lanes 9 and 18). However, the pattern of excision of the F-ara-AMP-3'-primer was different from that of the normal primer. Pol ϵ was able to excise at least five nucleotides consecutively from the 3'-

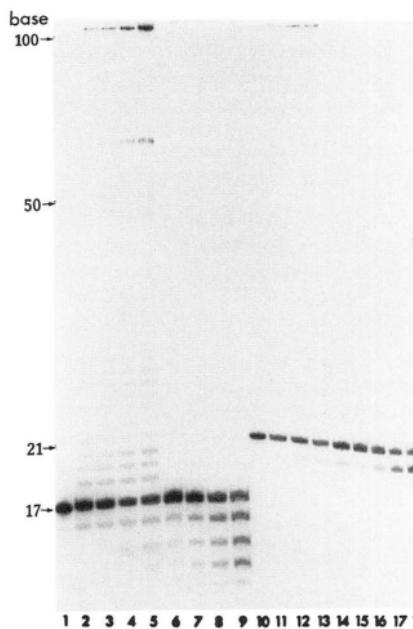


FIG. 6. 3' \rightarrow 5' exonuclease activity of DNA polymerase ϵ . The 5'- 32 P-labeled 17-base primer or 21-base-primer with F-ara-AMP at its 3'-end were separately annealed to M13mp18(+) DNA and used as the substrates for excision by pol ϵ in the presence and absence of dNTP. The reaction products were analyzed by a 20% polyacrylamide sequencing gel and visualized by autoradiography. Lane 1, 17-base primer/M13mp18 DNA incubated without enzyme; lanes 2–5, 17-base primer/M13mp18 DNA incubated with pol ϵ and 30 μ M each of four dNTPs for 2, 5, 15, and 30 min, respectively; lanes 6–9, 17-base primer/M13mp18 DNA incubated with pol ϵ without dNTPs for 2, 5, 15, and 30 min, respectively; lane 10, 21-base primer-3'-F-ara-AMP/M13mp18 DNA incubated without enzyme; lanes 11–14, 21-base primer-3'-F-ara-AMP/M13mp18 DNA incubated with pol ϵ and dNTPs for 2, 5, 15, and 30 min, respectively; lanes 15–18, 21-base primer-3'-F-ara-AMP/M13mp18 DNA incubated with pol ϵ without dNTPs for 2, 5, 15, and 30 min, respectively.

TABLE I
Extension of normal DNA primer and F-ara-AMP-3'-primer by DNA pol α and ϵ

The ability of DNA pol α and pol ϵ to extend the 17-base normal primer and the 21-base primer-3'-F-ara-AMP was examined in the presence and absence of 300 μ M GMP. The reaction products were analyzed in a 10% sequencing gel, and the radioactivity associated with each primer band and high molecular weight product band was quantitated by a Betascope 603 blot analyzer. Values are the mean \pm S.D. of radioactivity quantitated on three separate gels. The extension activity in each reaction was normalized by 10⁴ counts at each primer band. Student's *t* test was used to evaluate the significance of the differences between two means (49).

Sample	High molecular weight product	<i>p</i>
counts		
1. Normal 17-base primer		
Pol α	4541 \pm 907	
Pol α + GMP	5733 \pm 1309	>0.2
Pol ϵ	3685 \pm 763	
Pol ϵ + GMP	3184 \pm 651	>0.5
2. F-ara-AMP-3'-primer		
Pol α	361 \pm 8	
Pol α + GMP	316 \pm 60	>0.2
Pol ϵ	229 \pm 19	
Pol ϵ + GMP	119 \pm 23	<0.01

end of the normal 17-base primer (*lane 9*). The radioactivities in the 16-, 15-, 14-, 13-, and 12-base bands were 2740, 1940, 1674, 334, and 136 counts, respectively. In contrast, F-ara-AMP was removed from the 3'-end of the 21-base primer by pol ϵ , but further excision beyond the 20-base fragment was substantially decreased (*lane 18*). The radioactivities in the 20-, 19-, and 18-base bands were 2365, 477, and 104 counts, respectively. About 80% of the truncated primer remained as 20-base fragment. No significant amount of radioactivity was detected below the 17-base position. It appeared that the 3' \rightarrow 5' exonuclease activity was inhibited once pol ϵ cut F-ara-AMP out from the 3'-end of the primer. It is possible that the excised F-ara-AMP might still bind to the active site of the enzyme and thus impair further excision.

Because the pol ϵ was able to excise the incorporated F-ara-AMP with its intrinsic 3' \rightarrow 5' exonuclease activity, the possibility that this excision may be responsible for the lesser incorporation of F-ara-AMP by this enzyme was evaluated. A small amount of dATP (2 μ M) was added to the A⁻ reaction with 10 μ M F-ara-ATP to support the elongation of the primer and also to allow a detectable amount of F-ara-AMP incorporation at the A sites (Fig. 7, *lane 1*). When 300 μ M GMP was included in the reaction to inhibit the 3' \rightarrow 5' exonuclease activity by 65% (*lane 2*), the incorporation of F-ara-AMP into the A-sites increased and the extension of the DNA primer to high molecular weight DNA decreased. For example, in the presence of GMP, incorporation of F-ara-AMP into the first and second A sites increased to 131 and 129%, respectively. On the other hand, the high molecular weight product band was reduced 3- to 4-fold when the 3' \rightarrow 5' exonuclease activity was inhibited by GMP. This result suggests that pol ϵ was able to excise the nucleotide analogue from the 3'-end of the DNA strand, permitting incorporation of natural deoxynucleotide (dATP) to support further DNA strand elongation.

Incorporation of F-ara-ATP into DNA Is Essential for Cytotoxicity—It is important to understand which mechanism, incorporation into DNA or inhibition of DNA synthesis by competing with dATP, is responsible for cellular lethality. To approach this question, aphidicolin, a compound that inhibits DNA polymerases α , δ , and ϵ but is not incorporated into DNA (31), was used as a control. The effects of F-ara-A and aphidicolin on DNA synthesis and clonogenicity in CCRF-CEM cells were compared in the study summarized in Fig. 8. When cells were treated with F-ara-A for 5 h, both DNA synthesis and cell survival decreased inversely to the drug concentration, IC₅₀ values being 1 and 3 μ M for DNA synthesis and cell survival, respectively. In contrast, aphidicolin had a strong inhibitory effect on DNA synthesis but did not significantly affect cellular clonogenicity under identical incubation conditions. For instance, 0.3 μ M aphidicolin inhibited DNA synthesis by 90% but did not affect cell survival, whereas the same level of DNA synthesis inhibition by 3 μ M F-ara-A resulted in a 50% decrease of clonogenicity. These results suggested that inhibition of DNA synthesis for a relatively short period (5 h) is not critical to cell survival. Figure 9 demonstrates a strong correlation ($r = -0.956$) between the incorporation of F-ara-AMP into DNA and cell survival, suggesting that incorporation of F-ara-AMP into DNA may be responsible for the cytotoxicity. A similar relationship has been reported in studies with HL-60 cells (11).

The consequence of incorporated F-ara-AMP in DNA was further studied with respect to DNA synthesis. Cells were first incubated with F-ara-A for 5 h to allow accumulation of F-ara-ATP and its incorporation into DNA. Nuclei were then isolated from the cells and their ability to synthesize DNA in the presence of exogenous dNTP was evaluated by measuring

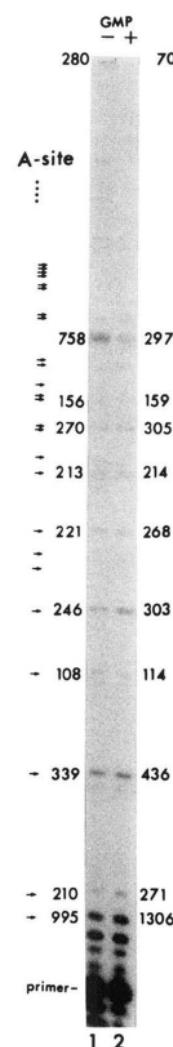


FIG. 7. Effect of 3' \rightarrow 5' exonuclease inhibitor GMP on incorporation of F-ara-ATP into DNA by pol ϵ . The DNA primer extension assay is described in the legend to Fig. 3. *Lane 1*, reaction mixture contained 30 μ M each of dCTP, dGTP, and dTTP, 10 μ M F-ara-ATP, and 2 μ M dATP; *lane 2*, same as *lane 1* plus 300 μ M GMP. Numbers next to bands show radioactivity associated with each band as detected during a 12-min counting time by a Betascope 603 blot analyzer. Arrows indicate the positions of the A sites.

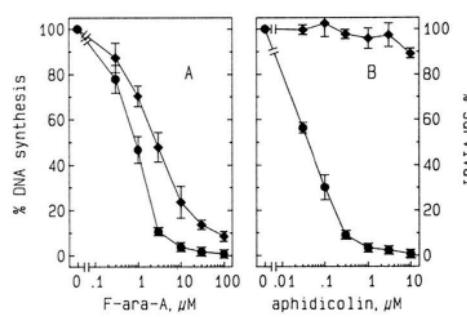


FIG. 8. Effect of F-ara-A or aphidicolin on DNA synthesis and clonogenicity in CCRF-CEM cells. Cells were incubated with indicated concentrations of F-ara-A or aphidicolin for 5 h. The cells were then divided into two portions, one portion labeled with [³H] thymidine to measure DNA synthesis and another portion cultured in soft agar to determine clonogenicity as described under "Experimental Procedures." Each point represents pooled data of three separate experiments. ●, percentage of DNA synthesis; ♦, percentage of cell survival.

the incorporation of [³H]dTTP into the nuclear DNA. Cells incubated with aphidicolin in a parallel experiment were used for comparison. Table II shows that when cells were treated with 1–10 μM F-ara-A, DNA synthesis in the isolated nuclei was significantly reduced. HPLC analysis detected no F-ara-ATP or dNTP in the isolated nuclei (detection limit, 20 pmol/10⁷ nuclei). These results suggested that the incorporated F-ara-AMP residues in the nuclear DNA strands block further DNA replication. The nuclei isolated from cells treated with 10 μM F-ara-A retained only 44% of the control DNA synthesis. This activity may reflect DNA synthesis at the replication forks that were newly formed during the isolation of nuclei and the incubation period and thus had not incorporated F-ara-AMP at the growing DNA strands. In contrast, rather than impairing DNA synthesis, nuclei treated with aphidicolin achieved 141% of the control activity. This suggested that replication sites accumulated as a result of the drug action (32, 33); these replication sites started to function when aphidicolin was removed during isolation of nuclei.

DISCUSSION

The conversion of F-ara-A to F-ara-ATP by cellular enzymes and the inhibition of DNA synthesis by this compound have been known for some time (1, 2, 10, 11). With respect to inhibition of DNA synthesis, two types of enzymes, DNA polymerases and ribonucleotide reductase, were proposed to be the targets of F-ara-ATP (12–14). Our studies demonstrated that this analogue inhibits DNA polymerases by two different mechanisms. First, F-ara-ATP competed with dATP for incorporation into the A sites of the growing DNA strands, acting as a competitive inhibitor of DNA polymerases. Second, once F-ara-ATP was incorporated into DNA, most of

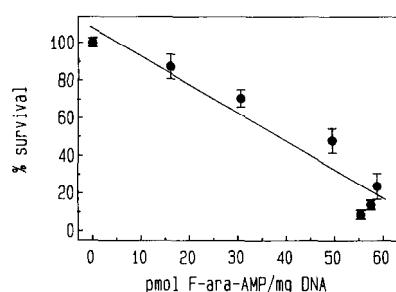


FIG. 9. Relationship between incorporation of F-ara-AMP into DNA and loss of clonogenicity. Quantitation of F-ara-AMP incorporation and determination of cell clonogenicity were described, respectively, in the legends to Figs. 1 and 7. Each point represents pooled data of three to four separate experiments.

TABLE II

DNA synthesis in isolated nuclei from CCRF-CEM cells pretreated with F-ara-A or aphidicolin

Cells were incubated with the indicated concentrations of F-ara-A or aphidicolin for 5 h and then nuclei were isolated from the cells. DNA synthesis in the isolated nuclei was quantitated as described under "Experimental Procedures" and presented as the percentage of the control value (mean ± S.D., 11,768 ± 768 dpm).

Sample	[³ H]dTTP incorporation			
	Exp. 1	Exp. 2	Exp. 3	Mean ± S.D.
% of control				
Control	100	100	100	100
F-ara-A (μM)				
0.3	95	90	103	96 ± 6.5
1	68	76	61	68 ± 7.5
3	66	49	52	56 ± 9.1
10	50	38	43	44 ± 6.0
Aphidicolin, 1 μM	114	169	141	141 ± 28

the DNA strands with F-ara-AMP residues at the 3'-ends could not be extended further. In this case, F-ara-AMP was a DNA chain terminator.

In contrast, parallel studies with arabinosylcytosine in CCRF-CEM cells revealed less than 10% terminal incorporation of the analogue at similarly toxic concentrations. This is consistent with the findings by other laboratories using different cell lines (34, 35) and for results obtained with arabinosyladenine (19, 36). This suggests differences in the mechanisms of action of these two analogues and F-ara-A.

Both competition and termination mechanisms seem to be directly involved in the inhibition of DNA synthesis, but their roles in inducing cytotoxicity are different. A short-term inhibition of DNA polymerases by aphidicolin did not result in the loss of cell clonogenicity, suggesting that a temporary pause in DNA replication *per se* is not sufficient to generate cytotoxicity. Thus the incorporation of F-ara-AMP into DNA and the subsequent termination of DNA synthesis may be the key mechanism responsible for cell lethality. We have demonstrated that treatment of CHO cells with F-ara-A resulted in deletions of gene fragments (15). Although the competition between F-ara-ATP and dATP may contribute to the inhibition of DNA synthesis, this inhibition as such is not likely to be critical in terms of cytotoxicity. However, F-ara-ATP can be incorporated into DNA only when it competes successfully with dATP for utilization by DNA polymerases. In this regard, during therapy with F-ara-AMP, the dATP concentration in tumor cells must be considered as one of the important factors that determine sensitivity to this drug.

F-ara-ATP has been reported to inhibit ribonucleotide reductase (12–14). The inhibition of ADP reduction may lead to a decrease of cellular dATP, resulting in more incorporation of F-ara-AMP into DNA and thus potentiating the cytotoxic effect. F-ara-ATP is also incorporated into RNA (9–11). Studies with CCRF-CEM cells indicated that neither incorporation of F-ara-AMP into RNA nor inhibition of RNA synthesis was correlated with loss of clonogenicity.²

Two types of mammalian DNA polymerases with 3' → 5' exonuclease activity, namely PCNA-dependent DNA polymerase δ and DNA polymerase ε (PCNA-independent pol δ), were described recently (21–25, 37–40). It has been proposed that pol α and pol δ coordinately catalyze the replication of the lagging and leading DNA strands, respectively (23, 37, 38). *In vitro* experiments suggested that PCNA-dependent pol δ was involved in replication of SV40 DNA (39) and possibly in DNA repair (41, 42), while pol ε showed activity in DNA repair (43, 44). Whether the pol ε can serve as a DNA replication enzyme is not clear at the present time (45). Focher *et al.* (46) consider pol ε (PCNA-independent pol δ), which is a highly processive enzyme, to be a candidate for leading strand DNA synthesis. It would also be interesting to evaluate the action of F-ara-ATP on the PCNA-dependent pol δ in similar experimental systems. However, PCNA failed to stimulate the activity of PCNA-dependent pol δ when primed single-stranded M13 DNA was used as template (23). This problem prevents the direct comparison of the different enzymes under identical experimental conditions, since the use of the primed M13 DNA template is critical in evaluating the site-specific action of F-ara-ATP on the DNA polymerases. Nevertheless, the study of F-ara-ATP action on either the replication enzyme or the repair enzyme is of importance in terms of understanding the mechanism of cytotoxicity of this compound, since the incorporation of F-ara-AMP into DNA and the repair of the F-ara-A-induced DNA lesion are the essential determinants of cell lethality.

² P. Huang and W. Plunkett, unpublished data.

The present study demonstrated that the pol ϵ was able to excise normal nucleotides and the incorporated F-ara-AMP residues from the 3'-ends of DNA *in vitro*. However, the significance of this process for excision of incorporated nucleotide analogue in intact cells is not clear. Under physiological conditions, the presence of dNTPs in intact cells may inhibit the excision of the incorporated fraudulent nucleotide (Fig. 6). The following evidence suggest that in cultured cells pol ϵ was unable to remove the incorporated F-ara-AMP to an extent that overcomes the cytotoxicity of this compound. First, a substantial amount of [³H]F-ara-AMP incorporation was detected in the terminal positions of DNA isolated from cells incubated with [³H]F-ara-A. Second, cells that were treated with F-ara-A for 5 h and then cultured in drug-free medium lost their clonogenicity in linear proportion to the amount of F-ara-AMP incorporation, which was determined at the end of the 5-hour incubation period (Fig. 9). This suggests the cell's inability to remove the incorporated F-ara-AMP and overcome the lethal chain termination action even in the absence of F-ara-A. Third, nuclei isolated from cells treated with F-ara-A had only 44% of control DNA synthesis capacity in the absence of F-ara-ATP (Table II), indicating that pol ϵ was unable to efficiently remove F-ara-AMP from the terminated DNA strands to allow further elongation. However, other experiments showed that incorporated ara-AMP, another analogue of dAMP, were removed from DNA by the 3' \rightarrow 5' exonuclease activity associated with pol ϵ (PCNA-dependent pol δ) or herpes simplex virus DNA polymerase (47, 48). Thus, further studies are required to define the roles of pol ϵ in the excision of the incorporated nucleotide analogue in DNA. Specific inhibitors of the 3' \rightarrow 5' exonuclease activity will be valuable in this type of study.

On the other hand, DNA polymerase α lacks 3' \rightarrow 5' exonuclease activity. This enzyme showed lower substrate specificity than pol ϵ at the A site and incorporated more F-ara-AMP into the DNA primer (Fig. 3). It seems that this enzyme may be a more susceptible target for nucleotide analogues. The finding that F-ara-ATP was more inhibitory to pol α than pol ϵ in the presence of dATP (Figs. 4 and 5) supports this hypothesis.

This study provides evidence that incorporation of F-ara-AMP into DNA renders the polymerases unable to elongate the DNA strand. *In vitro* experiments with pol α showed, however, that this enzyme was able to extend the DNA primer beyond the first several A sites in the presence of F-ara-ATP and the absence of dATP (Fig. 3). This finding suggested that the incorporated F-ara-AMP residue in DNA may not be an absolute chain terminator. Because the F-ara-AMP molecule has a 3'-hydroxyl group, formation of a phosphodiester bond with the subsequent nucleotide is possible. Incorporated F-ara-AMP may create a change in DNA conformation so that pol α may proceed only with great difficulty. The ability of the polymerases to extend a small amount (<5%) of the F-ara-AMP-terminated primer (Table I) supports this hypothesis.

In whole cells, when F-ara-A concentrations in the medium were greater than 10 μ M, the accumulation of cellular F-ara-ATP increased, whereas its incorporation into DNA was saturated (Fig. 1). This self-limited action is likely the result of cessation of DNA synthesis. Thus, to achieve maximum therapeutic effect, clinical administration of this drug might be targeted at the level at which intracellular F-ara-ATP concentration is not much greater than that required to saturate incorporation. Furthermore, prolonged exposure of tumor cells to the drug at an optimal concentration is desirable because cycling cells will continue to enter S phase and

incorporate F-ara-AMP into DNA.

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