Inhibition of the 3′ → 5′ Exonuclease of Human DNA Polymerase ε by Fludarabine-terminated DNA*

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Incorporation of the anticancer drug fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate; F-ara-AMP) into the 3′-end of DNA during replication causes termination of DNA strand elongation and is strongly correlated with loss of clonogenicity. Because the proofreading mechanisms that remove 3′-F-ara-AMP from DNA represent a possible means of resistance to the drug, the present study investigated the excision of incorporated F-ara-AMP from DNA by the 3′ → 5′-exonuclease activity of DNA polymerase ε from human leukemia CEM cells. Using the drug-containing and normal deoxynucleotide oligomers (21-base) annealed to M13mp18(+) DNA as the excision substrates, we demonstrated that DNA polymerase ε was unable to effectively remove F-ara-AMP from the 3′-end of the oligomer. However, 3′-terminal dAMP and subsequently other deoxynucleotides were readily excised from DNA in a distributive fashion. Kinetic evaluation demonstrated that although DNA polymerase ε possesses a higher affinity for F-ara-AMP-terminated DNA (K_m = 7.1 pm) than for dAMP-terminated DNA of otherwise identical sequence (K_m = 265 pm), excision of F-ara-AMP proceeded at a substantially slower rate (V_max = 0.053 pmol/min/mg) than for 3′-terminal dAMP (V_max = 1.96 pmol/min/mg). When the 3′-5′ phosphodiester bond between F-ara-AMP at the 3′-terminus and the adjacent normal deoxynucleotide was cleaved by DNA polymerase ε, the reaction products appeared to remain associated with the enzyme but without the formation of a covalent bond. No further excision of the remaining oligomers was observed after the addition of fresh DNA polymerase ε to the reaction. Furthermore, the addition of DNA polymerase α and deoxynucleoside triphosphates to the excision reaction failed to extend the oligomers. After DNA polymerase ε had been incubated with 3′-F-ara-AMP-21-mer for 10 min, the enzyme was no longer able to excise 3′-terminal dAMP from a freshly added normal 21-mer annealed to M13mp18(+) template. We conclude that the 3′ → 5′ exonuclease of human DNA polymerase ε can remove 3′-terminal F-ara-AMP from DNA with difficulty and that this excision results in a mechanism-mediated formation of “dead end complex.”

Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate; F-ara-AMP)* is a major new drug in the treatment of hematologic malignancies (1–3). The action of fludarabine, mediated by its 5′-triphosphate metabolite F-ara-ATP, is characterized by its inhibitory effect on DNA metabolism (4, 5). Previous studies revealed that several mechanisms may be involved in the drug-induced cytotoxicity. F-ara-ATP competes with dATP for incorporation into DNA by several DNA polymerases (6–10), including DNA polymerase ε (pol ε) (10), which possesses 3′ → 5′ exonuclease activity (11, 12). Because F-ara-ATP also inhibits ribonucleotide reductase (6–8) and decreases dNTP pools (13), the incorporation of F-ara-ATP into DNA may be increased as a result of raising the ratio of F-ara-ATP to dATP in cells. The incorporated F-ara-ATP, found predominantly at the 3′-termini, impairs the function of DNA polymerases by effectively terminating DNA strand elongation (10). Acting at an additional process in DNA synthesis, F-ara-ATP inhibits primer RNA synthesis by DNA primase and thus may affect lagging strand initiation (14, 15). Finally, the 3′-terminal F-ara-AMP in DNA is a poor substrate for human DNA ligase I (16). F-ara-ATP also interacts directly with DNA ligase I to inhibit the enzyme (16). In whole cells, F-ara-ATP incorporation in DNA is associated with the loss of large fragments of DNA from surviving cells (17) and is required for drug-induced DNA fragmentation in cells undergoing apoptosis (18–20). The amount of F-ara-AMP incorporated into cellular DNA is linearly correlated with the loss of clonogenicity (10, 21). It is therefore likely that this is essential for drug-induced lethality.

The 3′ → 5′ exonuclease activities associated with prokaryotic and eukaryotic DNA polymerases that remove terminal mismatched nucleotides increase the fidelity of DNA replication (22–27). Conditions that inhibit exonucleolytic proofreading decrease the fidelity of DNA polymerization (27, 28), and cells lacking this activity exhibit higher mutation rates than those that express the wild type enzymes (29–31). Because the toxicity of many anticancer and antiviral nucleotide analogues is expressed only after their incorporation into nascent DNA chains, such analogues may also be recognized as substrates for 3′ → 5′ exonucleases. Thus, the ability of exonucleases to excise antimetabolites may serve as a drug resistance mechanism. As a corollary, incorporated analogues that resist excision removal are predicted to be highly cytotoxic.

Inasmuch as most of the incorporated F-ara-AMP residues are located at the 3′-termini of the DNA strands (10), 3′-terminal F-ara-AMP might well be excised by the 3′ → 5′ exonuclease activities associated with DNA polymerases. Indeed, preliminary studies indicated limited excision of 3′-terminal F-ara-AMP by pol ε (10). In the present study, we used an in vitro DNA excision assay to quantitatively investigate the

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1 The abbreviations used are F-ara-AMP, 9-β-D-arabinofuranosyl-2-fluoro adenine 5′-monophosphate (fludarabine); F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoro adenine 5′-triphosphate; pol ε and pol α, DNA polymerase ε and α, respectively.
ability of $3' \rightarrow 5'$ exonuclease activity of human DNA pol $\epsilon$ to remove F-ara-AMP from the $3'$-end of DNA. For comparison, excision of normal nucleotides was also evaluated. Our results demonstrated that DNA pol $\epsilon$ bound to F-ara-AMP-terminated DNA with high affinity but excised the analogue from DNA at a low velocity. Once the phosphodiester bond between the $3'$- F-ara-AMP and its adjacent nucleotide was cleaved by pol $\epsilon$, the excision products appeared to remain associated with the enzyme, inactivating the exonuclease and preventing further exonucleolytic degradation or polymerization of the DNA products.

**EXPERIMENTAL PROCEDURES**

**Materials—** F-ara-A was kindly provided by Dr. V. L. Narayanan (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, National Institutes of Health). F-ara-ATP was synthesized as described previously (32). The M13mp18 (+) single-stranded DNA and the 17-mer M13 universal sequencing primer (5'-GTAAAGCACGGCCAGTGACAAAGTCAGGC-3'), complementary to sites 6290–6306 of M13mp18 (+) DNA, were obtained from Pharmacia Biotech Inc. Synthetic oligonucleotide CAGT-3', described previously (32). The M13mp18 templateDNA strands, a ratio of 1 oligomer to 10 copies of M13 DNA was used (see below).

**Preparation of Normal DNA Substrate for Excision by pol $\epsilon$—** The 17-mer M13 sequencing primer was labeled at its 5'-terminus with [$\gamma$-32P]ATP by T4 polynucleotide kinase and annealed to the complementary site of the M13mp18 (+) single-stranded DNA template as described previously (10). The labeled 17-mer primer/M13mp18 template was incubated with pol $\epsilon$ and 100 $\mu$M each of dCTP, dGTP, and 10 $\mu$M F-ara-ATP at 37°C for 30 min in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 8 mM MgCl$_2$, 0.5 mM dithiothreitol, and 10 mM NaCl. F-ara-ATP was incorporated into the primer at site 21 opposite the T site of the M13mp18 template. The reaction products were separated by electrophoresis through a 15% polyacrylamide sequencing gel. The 21-mer excision products appeared to remain associated with the enzyme, inactivating the exonuclease and preventing further exonucleolytic degradation or polymerization of the DNA products. The 21-mer band containing F-ara-AMP-terminated DNA by pol $\epsilon$ was excised from the gel and recovered from the gel slice as described previously (10). The purified F-ara-AMP-21-mer was annealed to M13mp18 (+) DNA to generate the following DNA hybrid,

$5'-$GTAAAGCACGGCCAGTGACAAAGTCAGGC$3' (Sequence 1)

$3'-$CATTCTTGTGGCCGTACAGGTTCCGAACGTCGAA$5'$ (Sequence 2)

where F indicates the incorporated F-ara-AMP. The 21-mer M13 sequencing primer was labeled at its 5'-terminus with [$\gamma$-32P]ATP by T4 polynucleotide kinase and annealed to the complementary site of the M13mp18 (+) DNA strands, a ratio of 1 oligomer to 10 copies of M13 DNA was used (see below).

**Preparation of Normal DNA Substrate for Excision by pol $\epsilon$—** A 21-base oligomer with nucleotide sequence identical to the 3'-F-ara-AMP-21-mer except that dAMP was substituted for F-ara-AMP was chemically synthesized by Genosys. The normal 21-mer and the 17-mer M13 sequencing primer were separately labeled at the 5'-ends with [$\gamma$-32P]ATP and purified by polyacrylamide sequencing gels as described above. The 32P-labeled normal oligomers were annealed to the M13mp18 (+) DNA to produce the following DNA hybrid substrates for excision assay by pol $\epsilon$.

$5'-$GTAAAGCACGGCCAGTGACAAAGTCAGGC$3' (Sequence 1)

$3'-$CATTCTTGTGGCCGTACAGGTTCCGAACGTCGAA$5'$ (Sequence 2)

To assure that all 32P-labeled oligomers were hybridized to the complementary M13mp18 (+) DNA strands, a ratio of 1 oligomer to 10 copies of M13 DNA was used. Under these conditions, no single-stranded 32P-labeled oligomer was present in the mixture as evidenced by a complete conversion of all primers to high molecular weight DNA products when the mixtures were incubated with DNA pol $\epsilon$ in the presence of the four DNA nucleotides.

**DNA Primer Excision Assay—** The normal and F-ara-AMP-terminated DNA hybrids described above were used as the substrates for pol $\epsilon$. The reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 8 mM MgCl$_2$, 0.5 mM dithiothreitol, 10 mM NaCl, 0.2 unit of DNA polymerase $\epsilon$, and the indicated concentration of DNA substrates. The reactions were carried out at 37°C for the indicated times up to 40 min. The reaction products were analyzed by ethophoresis through 10 or 15% polyacrylamide sequencing gels. After autoradiography, the radioactivity associated with each DNA band in the gels was quantitated by a Betascope 603 blot analyzer under conditions recommended by the manufacturer (Betascope Corporation, Waltham, MA).

**DNA Polymerization Assay—** The normal and F-ara-AMP-terminated DNA hybrids described above were used as the substrates for polymerization by pol $\epsilon$ or pol $\alpha$. The reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 8 mM MgCl$_2$, 0.5 mM dithiothreitol, 10 mM NaCl, 20 $\mu$g/ml bovine serum albumin, 100 $\mu$M each of dATP, dCTP, and dGTP, the indicated concentration of DNA substrates, pol $\epsilon$, and/or pol $\alpha$. The reactions were carried out at 37°C for the indicated times, and analyzed by ethophoresis through a 15% polyacrylamide sequencing gel. After autoradiography, the radioactivity in the gels was quantitated by a blot analyzer as described above.

**Kinetic Analysis—** To determine the apparent $K_m$ and $V_{max}$ values for the excision of normal and F-ara-AMP-3'-terminated DNA by pol $\epsilon$, various concentrations of the respective DNA constructs were incubated with pol $\epsilon$ in 10-ul excision reaction mixtures (see above) at 37°C for 10 min. Under these conditions, the reaction rate remained linear for at least 15 min. The reaction products were then analyzed and quantitated as described above. The sum of radioactivity associated with all excision products (20-mer and less) in each lane was used to calculate the reaction rate and then plotted against the respective DNA substrate concentrations. The apparent $K_m$ and $V_{max}$ values were calculated by the Michaelis-Menten equation with a computer-assisted program (33). To determine the $K_m$ of 3'-F-ara-AMP-21-mer/M13mp18 DNA in inhibiting the excision of normal oligomer/M13mp18 DNA by pol $\epsilon$, various concentrations of normal 32P-labeled DNA substrates were incubated with pol $\epsilon$ for 15 min in the presence of different fixed concentrations of nonradioactive 3'-F-ara-AMP-21-mer/M13mp18 DNA. Nonradioactive normal 21-mer/M13mp18 DNA were used in parallel as controls. The reaction products were analyzed and quantitated as described above, and the $K_m$ value was calculated by the Michaelis-Menten equation with a computer-assisted program (33).

**Gel Retardation Assay—** The following normal or F-ara-AMP-containing oligomers were used as the substrates for binding to pol $\epsilon$.

Normal 5'-$GTAAAGCACGGCCAGTGACAAAGTCAGGC$3' (Sequence 1)

$3'-$CATTCTTGTGGCCGTACAGGTTCCGAACGTCGAA$5'$ (Sequence 2)

F-ara-A 5'-$GTAAAGCACGGCCAGTGACAAAGTCAGGC$3' (Sequence 1)

$3'-$CATTCTTGTGGCCGTACAGGTTCCGAACGTCGAA$5'$ (Sequence 2)

**RESULTS**

An in vitro DNA excision assay was used to investigate the ability of $3' \rightarrow 5'$ exonuclease activity associated with DNA polymerase $\epsilon$ to remove the incorporated F-ara-AMP residues from the 3'-ends of DNA. A 21-base oligomer with F-ara-AMP at its 3'-end annealed to the complementary region of M13mp18 (+) DNA was used as the substrate for excision by pol $\epsilon$. The same amount of normal 21-mer/M13mp18 DNA hybrid of identical sequence with dAMP at the 3'-end of the oligomer was present in the mixture as evidenced by a complete conversion of all primers to high molecular weight DNA products when the mixtures were incubated with DNA pol $\epsilon$ in the presence of the four DNA nucleotides.
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Various concentrations of the 32P-labeled 3′-F-ara-AMP-21-mer/M13mp18 DNA or 3′-dAMP-21-mer/M13mp18 DNA were incubated with DNA polymerase ε at 37 °C for 10 min, during which time the reaction rate remained linear. The total DNA excision products and the remaining substrates were quantitated by a blot analyzer, and the apparent $K_m$ and $V_{max}$ values were calculated as described under “Experimental Procedures.” Each value represents the mean ± S.E. of four experiments, each comprised of three to four separate gel determinations.

| DNA substrate         | $K_m$  | $V_{max}$ |
|-----------------------|--------|-----------|
| F-ara-AMP-21-mer      | 7 ± 2  | 0.053 ± 0.01 |
| Normal 21-mer         | 265 ± 32 | 1.96 ± 0.16 |
| Normal 17-mer         | 242 ± 27 | 0.79 ± 0.09 |

**Fig. 1. Excision of nucleotides from normal and F-ara-AMP-terminated DNA by 3′ → 5′ exonuclease of pol ε.** The 32P-S-end-labeled 3′-F-ara-AMP-21-mer and the normal 21-mer (162 pmol each) separately annealed to M13mp18 templates were incubated with 0.2 unit pol ε for the indicated times. The reaction products were analyzed by a 15% polyacrylamide sequencing gel and visualized by autoradiography as described under “Experimental Procedures.” A, autoradiograph of the sequencing gel. Lane 1, 3′-F-ara-AMP-21-mer/M13mp18 DNA incubated without pol ε and without dNTPs; lanes 2–7, 3′-F-ara-AMP-21-mer/M13mp18 DNA incubated with pol ε and without dNTPs for 3, 5, 10, 15, 25, and 40 min, respectively; lane 8, normal 21-mer/M13mp18 DNA incubated without pol ε and without dNTPs; lanes 9–14, normal 21-mer/M13mp18 DNA incubated with pol ε and without dNTPs for 3, 5, 10, 15, 25, and 40 min, respectively. B, the radioactivity associated with the excision products in each lane was quantitated by a Betascope blot analyzer and expressed as percentage of total radioactivity input. ●, normal 21-mer/M13mp18 DNA; ○, F-ara-AMP-21-mer/M13mp18 DNA. Each point represents the mean ± S.E. from four experiments.
3'-end of the oligomer, the enzyme was not able to further excise normal nucleotides from the 20-base oligomer and, perhaps, protected the 20-mer product from further exonucleolytic degradation. As the incubation time was prolonged, only a barely visible band at the 19-mer position was revealed (Fig. 1, lanes 6 and 7). In contrast, pol ε removed nucleotides consecutively from the normal oligomer (Fig. 1A, lanes 9–14). These observations were further confirmed by scanning the radioactivity profiles associated with each DNA band by a Betascope blot analyzer. In the 25-min reaction, at least 12 excision profiles were revealed from the normal 21-mer/M13mp18 substrate (data not shown). When 3'-F-ara-AMP-21-mer/M13mp18DNA was incubated with pol ε for the same time, only a relatively small excision product peak corresponding to 20-mer was detected, which reflected the slow cleavage of the phosphodiester bond between 3'-F-ara-AMP and the adjacent nucleotide at position 20. A much smaller but identifiable 19-mer peak was also revealed. Thereafter, no peaks of smaller sizes were detected. Thus, it appears that after F-ara-AMP was excised, the 3' → 5' exonuclease activity of pol ε was greatly decreased relative to its utilization of the 20-mer/M13mp18 oligomer.

Further experiments were designed to investigate the mechanism by which the 3' → 5' exonuclease activity of pol ε was inactivated after removal of F-ara-AMP from the 3'-end of the DNA. In sequential reaction experiments, pol ε was first incubated with nonradioactive F-ara-AMP-21-mer/M13mp18 DNA for 10 min, and then the 32P-labeled normal 21-mer/M13mp18 DNA was added to the reaction for an additional 20 min. Preincubation of pol ε with 81 pm or 162 pm of the nonradioactive 3'-F-ara-AMP-21-mer/M13mp18 DNA resulted in a decrease in exonuclease activity by 69 and 78%, respectively, against the 32P-labeled normal substrate (Fig. 2, lanes 3 and 4). In contrast, preincubation of pol ε with the same concentrations of nonradioactive normal 21-mer/M13mp18 DNA did not significantly diminish the exonuclease activity against labeled normal substrate (Fig. 2, lanes 5 and 6).

To determine the kinetics of inhibition by 3'-F-ara-AMP-21-mer/M13mp18 DNA, various concentrations of 32P-labeled normal 21-mer/M13mp18 DNA substrates were incubated with pol ε in the presence of different fixed concentrations of nonradioactive analogue-terminated or normal DNA hybrids. The dose effect of the two DNA hybrids on the 3' → 5' exonuclease activity of pol ε is illustrated in Fig. 3A. Only the F-ara-AMP-containing DNA showed inhibited activity. When the velocity of the reaction was plotted as the function of substrate (32P-labeled normal 21-mer/M13mp18 DNA) concentrations in a double reciprocal plot (Fig. 3B), the lines representing reactions without and with different fixed concentrations of the inhibitor (nonradioactive 3'-F-ara-AMP-21-mer/M13mp18) converged at the ordinate, indicating that the nature of this inhibition was most likely competitive. In fact, computer analysis (33) of the plots revealed a competitive inhibition with a Ki value of 7.5 pm. This is consistent with the high affinity of pol ε for F-ara-AMP-21-mer/M13mp18 DNA as evidenced by its low Km for excision (7 pm, Table I).

Because the nonradioactive normal DNA (3-162 pm) did not inhibit the enzyme activity, the loss of excision activity observed in samples preincubated with F-ara-AMP-terminated DNA was not simply due to competition between the nonradioactive DNA (3-162 pm) and the 32P-labeled DNA substrate (1400 pm). Rather, we postulated that when pol ε was preincubated with 3'-F-ara-AMP-21-mer/M13mp18 DNA, the enzyme remained associated with the F-ara-AMP-terminated DNA with high affinity and was effectively trapped by the reaction products after the phosphodiester bond between 3'-F-ara-AMP and the adjacent nucleotide (position 20) was cleaved. This hypothesis predicts that the 3'-end of the oligomer (20-mer) would be blocked by pol ε after cleavage of F-ara-AMP. Two different approaches were taken to test this postulate.

In the first experiment, pol ε was incubated with 32P-labeled F-ara-AMP-21-mer/M13mp18DNA for 15 min to generate the 20-base excision product. Our hypothesis predicts that if the enzyme remained bound to DNA, the 3'-end of the 20-mer would not be accessible for further excision by freshly added pol ε. Indeed, the further addition of pol ε to reactions containing F-ara-AMP-terminated DNA preincubated with pol ε led to the accumulation of more 20-base excision product but did not result in significant removal of nucleotide from the 20-mer (Fig. 4, lanes 3–6). This is consistent with the conclusion that the 3'-end of the oligomer was protected or blocked by the enzyme. When the amount of pol ε was increased to a total of 5 μl (0.1 unit/μl), a light band was visible at the 19-mer position (lane 6), suggesting that the binding of the preincubated pol ε to the 20-mer/M13mp18 DNA was tight but still reversible. No mobility shift of the 20-mer was observed on denaturing polyacrylamide gels, indicating that pol ε dissociated from the DNA under denaturation conditions (95°C, 50% formamide, and 8 M urea). Therefore, the binding between pol ε and DNA did not appear to involve the formation of a covalent bond. In contrast, the addition of pol ε to reactions preincubated with pol ε and normal DNA led to almost complete digestion of the normal 21-mer (Fig. 4, lanes 9 and 10).

In the second approach to investigating the consequences of
3'-terminal F-ara-AMP excision, we reasoned that if pol ε was not tightly associated to the initial excision products, the 3'-end of the 20-mer should be available for polymerization by DNA pol α in the presence of normal dNTPs. Fig. 5 demonstrates that when F-ara-AMP-21-mer was incubated with pol α and normal dNTPs after preincubation with pol ε for 15 min, no polymerization products appeared (lane 5). Quantitation of the radioactivity by Betascope revealed 6.6 ± 1.0%, and 6.9 ± 1.5% (mean ± S.D., n = 12) of the total input radioactivity associated with the excision product band (20-mer) in the reaction containing pol ε alone (lane 3) and the reaction containing pol ε and pol α in the presence of normal dNTPs (lane 5), respectively. The same amount of excision product (20-mer) in lane 3 (no pol α) and lane 5 (with pol α) indicates that after F-ara-AMP was excised from the 3'-end of the 21-mer by pol ε, the excision product (20-mer) was not extended by pol α. In contrast, when the normal 21-mer/M13mp18 DNA was preincubated with pol ε for 15 min and then with pol α in the presence of dATP, dCTP, and dGTP, both polymerization and excision products were generated (lane 6). The polymerization products appeared as a single band at the 24-mer position, due to the absence of dTTP on the reaction mixture (the M13mp18(+)) template directs that dTTP be incorporated at sites 25 and 26; see the DNA sequence under “Experimental Procedures”). The intensity of the DNA band at site 24 is visible but weak, probably due to the presence of exonuclease activity of pol ε. Quantitation of the radioactivity by Betascope analysis revealed 4320 counts asso-
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Results are consistent with a mechanism-based inactivation of pol ε by the analogue-containing DNA.

GMP, an inhibitor of the 3' → 5' exonuclease activities (34–36), was used to evaluate the excision of F-ara-AMP from the 3'-end of DNA by pol ε in comparison with that of normal nucleotides. The addition of GMP to reaction mixtures inhibited the 3' → 5' exonuclease activity of pol ε to the same extent when either F-ara-AMP-terminated DNA or normal DNA was used as substrate (data not shown). This inhibition was concentration-dependent between 30 and 1000 μM GMP. For example, excision of terminal F-ara-AMP and normal deoxynucleotides was inhibited by 51 and 55%, respectively, by 300 μM GMP. These results indicate that the presence of F-ara-AMP at the 3'-end of DNA did not affect the inhibitory activity of GMP on pol ε, suggesting that the site in pol ε for GMP action may be separate from the exonuclease catalytic site. Earlier studies (34) demonstrated that the 5'-monophosphate of arabinosyladenine also failed to inhibit the 3' → 5' exonuclease of what is now recognized as pol ε from rabbit bone marrow (35). Taken together, these results suggest that inhibition of pol ε 3' → 5' exonuclease by F-ara-AMP is likely to be a mechanism-based process. On the other hand, reduction of pol ε 3' → 5' exonuclease activity by relative high concentrations (1 μM) of free F-ara-AMP may be mediated by a separate mechanism, perhaps similar to that of inhibition by 5'-GMP.

**DISCUSSION**

The present study demonstrated that DNA polymerase ε recognized and bound to F-ara-AMP-terminated DNA in preference to normal DNA. Excision of the analogue from the 3'-terminus of DNA, however, proceeded at a much slower rate than did removal of 3'-dAMP. Previous studies demonstrated that mismatched deoxynucleotides at the 3'-terminus of DNA caused an induced fit conformational change of T7 DNA polymerase (36, 37). This change slowed the polymerization step and allowed sufficient time for the intramolecular transfer of the 3'-mismatched nucleotide from the polymerase site to the exonuclease site. The kinetic partitioning between exonuclease and polymerase sites favored error correction during DNA replication when a DNA polymerase encountered the 3'-terminal mismatched nucleotide. This was associated with a reduced rate of polymerization (37, 38). A similar interpretation may be

**Fig. 6. Mobility retardation assay of pol ε/DNA complex.** The 32P-5'-end-labeled normal or F-ara-AMP-DNA oligomers (50 pg/20 μl reaction mixture) were separately incubated with pol ε for 10 min at 37 °C, and then 250 pg/20 μl of competing DNA (nonradioactive normal oligomer) was added to the indicated reactions and incubated for an additional 10 min at room temperature. The reaction products were separated in a nondenaturing polyacrylamide gel, and an autoradiograph was made as described under "Experimental Procedures." Lanes 1, 2, and 3, F-ara-AMP-oligomer incubated without pol ε, with pol ε, and with pol ε plus competing DNA, respectively; lanes 4, 5, and 6, normal oligomer incubated without pol ε, with pol ε, and with pol ε plus competing DNA, respectively. CO, enzyme-DNA oligomer complex; FD, free DNA oligomer; EX, excision products.

**Fig. 7. Effect of free F-ara-AMP on pol ε 3' → 5' exonuclease activity.** DNA polymerase ε (0.1 unit) was incubated with 32P-labeled normal 21-mer/M13mp18 DNA (162 pM) in the presence of the indicated concentrations of free F-ara-AMP at 37 °C for 20 min. The reaction products were analyzed by 15% polyacrylamide gel electrophoresis and quantitated by Betascope. The control value of relative excision was 22.6 ± 0.6% (mean ± S.E.). Each point represents data of five separate gels, mean ± S.E.
applied to the case of an incorporated nucleotide analogue such as F-ara-AMP, which effectively inhibits the polymerization activity of pol ε (10). As was seen with the mismatched terminal nucleotide-induced intramolecular transfer of T7 DNA polymerase, it is likely that when pol ε encounters the 3'-terminal F-ara-AMP, which it cannot readily extend, a similar transfer of the 3'-terminal F-ara-AMP from the polymerase site to the exonuclease site might occur.

The present study showed that the 3' → 5' exonuclease of pol ε bound to 3'-F-ara-AMP-DNA with high affinity (K_m = 7 μM). This was in contrast with substrates with 3'-terminal dAMP, for which the 3' → 5' exonuclease of pol ε exhibited 37-fold less affinity. However, the 3'-terminal F-ara-AMP was a poor substrate for the exonucleolytic activity of pol ε; excision proceeded at a substantially slower rate than it did for 3'-terminal dAMP. Furthermore, the 3' → 5' exonuclease of pol ε became inactive after the 3'-terminal F-ara-AMP had been postulated. Because it seems likely that the enzyme activity would be restored if the excision products were released, we postulate that one or both of the products remained associated with the enzyme after the cleavage of the phosphodiester bond. This hypothesis is supported by the unique excision patterns shown in Fig. 1, in which it is seen that after the phosphodiester bond between the 3'-terminal F-ara-AMP and the penultimate nucleotide was cleaved, pol ε failed to excise the subsequent deoxynucleotides. The mobility retardation experiment (Fig. 6) provided evidence that pol ε was physically associated with F-ara-AMP-DNA. We also attempted to test directly the hypothesis that F-ara-AMP remained associated with the enzyme after cleavage by using [2-3H]F-ara-ATP to label the 3'-terminal F-ara-AMP on the primer. Unfortunately, these experiments were not conclusive because the specific activity of the incorporated [3H]F-ara-AMP at the 3'-termini of DNA was not adequate to detect the small amount of F-ara-AMP excised.

Additional evidence indicates that after excision of F-ara-AMP, the 20-mer/M13mp18 DNA product remained in contact with pol ε. This interpretation is supported by the finding that after incubation of 3'-F-ara-AMP-21-mer/M13mp18 with pol ε, the DNA product was not a substrate for further excision upon the addition of fresh pol ε (Fig. 4, lanes 3-6). Furthermore, after removal of 3'-F-ara-AMP, the inability of newly added pol α and dNTPs to polymerize the 20-mer/M13mp18 DNA product (Fig. 5) is also consistent with the likelihood that one or both excision products remained associated with the enzyme. This association may have contributed to the sustained inhibition of both the exonuclease and polymerase functions. Because no mobility shift of the 20-mer product was observed in denaturing polyacrylamide gels, it is unlikely that pol ε bound to the DNA excision product (20-mer/M13mp18 DNA) by forming a covalent bond.

Inactivation of DNA polymerization by formation of a 3'-analogue DNA-enzyme complex was also observed by Reardon and Spector (39, 40). They demonstrated that when acyclovir triphosphate was incorporated into the 3'-end of DNA primer by herpes simplex virus (type 1) DNA polymerase, upon binding of the next nucleotide as directed by the template, the viral enzyme and the analogue-containing DNA formed a "dead end complex." A similar mechanism was postulated for the inhibition of mammalian DNA polymerase α by 2',3'-dideoxycytidine triphosphate (41). In a different mechanism, aphidicolin inhibited both DNA polymerization and the 3' → 5' exonuclease activity of pol ε simultaneously (42-44) by sequestering the enzyme to the mismatched DNA region (43).

Incorporated arabinosyl nucleotide analogues are known to alter the configuration of duplex DNA in a specific manner (45). We speculate that although pol ε readily recognizes and adapts its shape to bind to the 3'-terminal F-ara-AMP, cleavage of the phosphodiester bond is achieved with relative difficulty. It is likely that the conformational changes in pol ε that are required to conduct this reaction are so extensive that the enzyme is unable to release the reaction products. If so, the change in enzyme conformation induced by excision of F-ara-AMP may be a key event responsible for the failure to release the excision products and for the ensuing inactivation of the exonuclease activity. This proposed mechanism of reaction-induced inactivation of pol ε may provide an explanation for the inability of free F-ara-AMP to inhibit the enzyme in the presence of normal DNA (Fig. 7). Because the binding of pol ε to normal DNA did not abnormally change the enzyme conformation, the presence of free F-ara-AMP should not lead to sequestering of the enzyme to its normal DNA substrate. Recent studies using genetic approaches indicated that pol ε, in addition to its polymerization and excision functions, may serve as a sensor involved in S phase checkpoint signaling in yeast (46).

Thus, it will be important to investigate the biological consequences of the sequestering of pol ε by F-ara-AMP-DNA in whole cells.

The data presented in Table I and Fig. 3 suggest that the inhibition of pol ε excision of normal DNA by F-ara-AMP-DNA is probably competitive in nature. This may reflect the initial competitive binding of the two DNA species to the enzyme. It is possible that the fraction of pol ε molecules that bound to F-ara-AMP-DNA might have reacted differentially to produce two possible consequences. A small portion of the enzyme molecules cleaved F-ara-AMP at a slow rate and thereby become sequestered in the dead end complex. Most of the enzyme, however, was unable to cleave the analog and eventually detached from the uncleaved DNA. This portion of pol ε could have been recycled in the reaction, free to enter a second phase of competitive distribution between normal DNA and F-ara-AMP-DNA. This nonproductive binding of pol ε to F-ara-AMP-DNA slowed the excision of normal DNA in a competitive manner. Nevertheless, because a small portion of the enzyme was sequestered in the dead end complex and was unable to recycle, the overall reaction is most likely a mixed-type competitive inhibition.

Although relatively few investigations of the ability of proof-reading exonuclease associates with DNA polymerases to remove nucleotide analogues have been reported, a review of the field suggests that our findings with F-ara-AMP-terminated DNA may not be generalized to other arabinosyl nucleosides or to nucleoside analogues with different nonphysiological carbohydrates that result in either relative or absolute inhibition of DNA elongation. For instance, 2',2'-difluorodeoxycytidine monophosphate, the active form of the new anticancer drug gemcitabine was resistant to excision by DNA pol ε (47) when placed at either the 3'-terminus or in the penultimate position. With respect to antiviral drugs, the incorporated 3'-azidohymidine monophosphate was removed by human pol ε with relative difficulty (48, 49), whereas 2',3'-dideoxy-2',3'-didehydro-2',3'-dideoxyadenosine was not a substrate for the same enzyme (48). The resistance to exonucleolytic action of 3'-terminal nucleotides with phosphorothioate linkages makes them substrates of choice for PCR primers when using thermostable DNA polymerases that possess 3' → 5' exonucleases (50). In contrast, 3'-terminal arabinosylcytosine monophosphate was shown to be a relatively good substrate for excision by the 3' → 5' exonuclease of E. coli K120 fragment (51) and human DNA pol ε (47). Following excision of the analogue, the appearance of excision products of intermediate length indicates that removal of subsequent nucleotides proceeded in a nonprocessive fashion. Comparative studies with DNA terminated by the mono-
phosphates of arabinosyladenine and 2-fluoro-2-deoxyadeno-
nosine will help determine the relative importance of the fluoro
to the 2-carbon and the arabinosyl hydroxyl to the
activity of pol ε against F-ara-AMP.

After incubation of cells with radioactive F-ara-A, more than
94% of the incorporated drug in DNA was located at 3’-termini
(10). This is strongly correlated with loss of viability, suggest-
ing that such terminal incorporation of F-ara-A nucleotide is a
critical mechanism of drug action (10, 19, 21). Further strand
elongation by DNA polymerases is greatly impeded (10), and
ligation of DNA strands by DNA ligase I (16) is inhibited by
elongation by DNA polymerases is greatly impeded (10), and

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