The incorporation of cytosine arabinoside monophosphate (araCMP) into DNA at internucleotide linkages by DNA polymerase $\alpha$ (DNA pol $\alpha$) has been investigated by using oligonucleotide primed DNA templates. The products of reactions catalyzed by DNA pol $\alpha$ in vitro were analyzed on polyacrylamide gels to measure insertion of araCMP, extension from an araCMP 3' terminus, and binding of the enzyme to an araCMP 3' terminus. The results show that insertion of araCMP opposite dGMP in the DNA template is about 3-fold less efficient than insertion of dCMP. Extension from an araCMP 3' terminus by addition of the next complementary nucleotide is approximately 2000-fold less efficient than extension from a correctly base-paired 3' terminus. In the absence of the second substrate, dNTP, DNA pol $\alpha$ binds with approximately equal affinities to DNA templates that contain oligonucleotide primers with araCMP or dCMP positioned at the 3' terminus. In the presence of dNTP, the enzyme extends the araCMP 3' terminus or dissociates, but it is not trapped at the araCMP 3' terminus in a nonproductive ternary complex as is observed at the dCMP 3' terminus. To determine if slow phosphodiester bond formation contributes to the observed extension rate from the araCMP 3' terminus by DNA pol $\alpha$, oligonucleotide primers with araCMP positioned at the 3' terminus were elongated by addition of the $\alpha$-phosphorothioate analogue of the next complementary nucleotide. The rate of extension from araCMP by addition of 2' deoxyadenosine 5'-O-phosphorothioate (dAMP$\alpha$S) was 6-fold slower than by addition of dAMP, indicating that bond formation is partially rate limiting in the extension reaction. Thus, inefficient extension from the araCMP 3' terminus is the major determinant contributing to the low incorporation frequency of araCMP into DNA by DNA pol $\alpha$, and this inefficiency can be attributed, in part, to slower phosphodiester bond formation at the araCMP 3' terminus.

DNA polymerase $\alpha$ (DNA pol $\alpha$)\(^1\) is one of the principal enzymes that replicates genomic DNA in human cells (Wang, 1991; Kornberg and Baker, 1992). The essential role for this enzyme in DNA replication makes it a potential target for chemotherapeutic agents like 1-beta-D-arabinofuranosylcytosine (araC) that inhibit DNA synthesis in leukemia cells. The active metabolite araCTP is believed to be responsible for inhibition of DNA synthesis in cells. Incorporation of araCMP into cellular genomic DNA has been detected at internucleotide positions and at 3' termini (Major et al., 1982; Kufe et al., 1984). The amount of araCMP incorporated into DNA at internucleotide positions has been correlated with cell lethality, suggesting that the position of araCMP in DNA is an important parameter in the mechanism(s) of araC-induced cell death (Kufe et al., 1980; Major et al., 1981). Although the mechanism(s) of internucleotide incorporation of araCMP is not clearly established, addition of araCMP to a growing DNA chain and subsequent elongation from the araCMP 3' terminus by DNA pol $\alpha$ could lead to incorporation at internucleotide positions in DNA.

Polymerization of araCMP into DNA by DNA pol $\alpha$ requires insertion and subsequent extension from the araCMP 3' terminus. Insertion of araCMP opposite dGMP in the DNA template during in vitro synthesis by DNA pol $\alpha$ has been demonstrated using homopolymers (Cozzarelli, 1977) and natural DNA templates (Townsend and Cheng, 1987). Extension from an araCMP 3' terminus has been more difficult to demonstrate due to exonuclease activity in some DNA pol $\alpha$ preparations. Townsend and Cheng (1987) showed that elongation by DNA pol $\alpha$ was slowed by addition of a single araCMP to the 3' terminus of a growing DNA chain, and no appreciable elongation was detected after two consecutive araCMP additions. Synthesis past sites of single araCMP insertion indicated that DNA pol $\alpha$ extended from an araCMP 3' terminus. Using an oligonucleotide template-primer with araCMP positioned at the 3' terminus, Mikita and Beardsley (1988) showed that araCMP was preferentially excised by an exonuclease activity and no extension from araCMP by HeLa cell DNA pol $\alpha\_2$ (Skarnes et al., 1986) could be detected. The human DNA pol $\alpha$ has no detectable proofreading exonuclease as an integral part of the polymerase catalytic polypeptide (Copeland and Wang, 1991), and this DNA polymerase has been purified devoid of exonuclease activity (Chang et al., 1984; Wahl et al., 1984; Wang et al., 1984; Reyland and Loeb, 1987; Nasheuer and Groese, 1987). The role of exonuclease as proofreading enzymes for DNA pol $\alpha$ in some preparations has not been clearly established (Chen et al., 1978; Skarnes et al., 1986; Ottiger et al., 1987; Cotterill et al., 1987; Bialek et al., 1989).

To determine if DNA pol $\alpha$ might polymerize araCMP into DNA at internucleotide positions during DNA synthesis in vitro, we purified the DNA pol $\alpha$-primase complex devoid of exonuclease activity from human leukemia cells. Our results

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\(^{3}\) The abbreviations used are: DNA pol $\alpha$, DNA polymerase $\alpha$; araCMP, cytosine arabinoside monophosphate; araC, 1-beta-D-arabinofuranosylcytosine; dAMP$\alpha$S, 2'-deoxyadenosine 5'-O-phosphorothioate; (S)-dATP$\alpha$S, (S, isomer) deoxycytidine 5'-O-(1-thiotriphosphate); dNTP, deoxynucleoside triphosphate; ddCTP, deoxy- cytidine triphosphate.
Incorporation of araCMP by DNA Pol α

Prepared from thawed cells by Dounce homogenization, and DNA pol α, we investigate the contributions of enzyme binding and phosphodiester bond formation to elongation from the araCMP 3’ terminus. Using two different binding assays (Creighton et al., 1992; Fisher and Korn, 1981b), we show that DNA pol α binds with approximately equal affinities to DNA templates containing oligonucleotide primers with araCMP or dCMP at the 3’ termini and that in the presence of dNTPs, the enzyme is not trapped in a nonproductive ternary complex at the araCMP 3’ terminus. Extension from an araCMP 3’ terminus has been measured in the presence of dATP or dATP to determine the elemental effect. This result indicates that phosphodiester bond formation is partially rate-limiting in the araCMP extension reaction during in vitro DNA synthesis.

EXPERIMENTAL PROCEDURES

MATERIALS—Radiolabeled ATP and unlabeled (S)-dATP were purchased from Amersham Corp. Unlabeled dNTPs, araCTP, dCTP, and bovine serum albumin (radioimmunoassay grade) were from Sigma. The bacteriophage T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, and E. coli DNA polymerase I (large fragment) were purchased from Promega. Oligonucleotides were synthesized complementary to the single-stranded phagemid DNA. Phagemid DNA (pBluescript I KS–) was purchased from Stratagene. DNA pol α-primase complex was purified by immunoadfinity chromatography from extracts of cells obtained by leukopheresis of a patient diagnosed with acute myeloblastic leukemia. Cells (greater than 99% blasts) were first washed in ice-cold 1 mM potassium phosphate, pH 7.5, for 3 min to lyse red blood cells. An equal volume of 2× phosphate buffered saline (3 mM KH2PO4, 16 mM Na2HPO4, 270 mM NaCl, 5 mM KCl) was added, and blasts walls were collected by centrifugation and stored as pellets at −80°C. Extracts were prepared from thawed cells by Donnec homogenization, and DNA pol α was purified as described (Permo and Loeb, 1989). One unit of DNA pol α catalyzes the incorporation of 1 pmol of (S)-[γ-32P]dTMP into 0.022 pmol of 15-mer-primed eX174 am3 DNA in 30 min at 30°C.

DNA Templates and Primers—Single-stranded DNA templates were prepared from phagemid-containing strains as described (Vieira and Messing, 1987). Phagemids were digested originally from pBluescript II KS– DNA. The sequences of the single-stranded DNA templates 1 and 2 (referred to as templ and templ, respectively) are indicated in Fig. 1A and 2A except for position 2949 (Stratagene map). The C at position 2949 in aC template was protected after incubation for times up to 1 min (not shown). The effectiveness of the polymerase trap in a 15-sec extension reaction is shown in Fig. 5D. After the 15-sec extension incubation at 37°C, reactions were stopped by addition of 45 μl of 95% formamide, 10 μM EDTA. Samples (5 μl) were analyzed by electrophoresis through a 15% urea-polyacrylamide gel, and bands were quantified. The binding affinity of DNA pol α for the araCMP 3’ terminus relative to the dCMP 3’ terminus KDpol was calculated using the following equation as described by Creighton et al. (1992).

Kpol = KDpol + Kpol EMP × EXpol EMP − EXpol EMP (Eq. 1)

EXpol EMP is equal to the amount of radioactivity detected in the 28-mer + 29-mer bands in the absence of unlabeled dC-primed DNA. The extended percentage, EXpol EMP, is equal to the amount of radioactivity detected in the 28-mer + 29-mer bands in the presence of unlabeled araCMP-primed DNA divided by the amount of radioactivity detected in the 28-mer + 29-mer bands in the absence of unlabeled araCMP-primed DNA.

The amount of radioactivity detected in the 28-mer + 29-mer bands in the absence of unlabeled DNA was defined as 100%. Binding reactions were done using unlabeled competitor araCMP- and dCMP-primed DNA, and the amount of radioactivity present in the 28-mer + 29-mer bands was determined. The extension percentage, EXpol EMP, was calculated using the following equation as described by Creighton et al. (1992).

RESULTS

Insertion of araCMP by DNA Pol α—The incorporation of araCMP into DNA at internucleotide linkages requires both insertion and subsequent extension from the araCMP 3’ terminus. We studied insertion of araCMP and extension from the araCMP 3’ terminus by DNA pol α by analyzing the products of a time course reaction on polyclarlamide gels (Fig. 1). A 2’-5’-end-labeled oligonucleotide primer (20-mer1) was hybridized to a single-stranded DNA template (templ) so that the dGMP target site for araCMP insertion was positioned five nucleotides away from the 3’ primer terminus (Fig. 1A). To reach the target site DNA pol α incorporates dTMP opposite dAMP at the first four template positions elongating the 20-mer primer
Incorporation of araCMP by DNA Pol α. The 20-mer oligonucleotide (20-mer) was hybridized to DNA template 1 (temp 1) to produce a primed-template (A). Two time course reactions (150 µl) were prepared, as described under “Experimental Procedures,” each containing template-primer (30 pmol), DNA pol α (26 units), and 10 µM dTTP and araCTP or 10 µM dTTP and dCTP. Samples (20 µl) were removed from each reaction at the indicated times after incubation at 37 °C (B, lanes 2–4 and 8–10). At 30 min, 10 µM dGTP was added to each reaction, incubation was continued, and samples were removed at the indicated times (B, lanes 5–7 and 11–13). Lane 1 was a sample before nucleotide addition. Additional reactions (20 µl) were prepared (C) containing template-primer (4 pmol), DNA pol α (3.5 units), and 10 µM dTTP (lane 2), 10 µM dTTP and dATP (lane 3), 10 µM dTTP and dGTP (lane 4), 10 µM dTTP and araCTP (lane 5), or 10 µM dTTP and dCTP (lane 6). Lanes 1 and 7 were reactions without nucleotides. Incubations were 30 min at 37 °C. Positions of the 20-mer primer and oligonucleotide products are indicated by arrows.

to an oligomer of 24 nucleotides. Insertion of araCMP at the target site is indicated by the 25-mer produced upon incubation of the template-primer with DNA pol α and 10 µM dTTP and araCTP (Fig. 1B, lanes 2–4). The amount of araCMP insertion increases with time of incubation. After 30 min 85% of the oligonucleotide is detected at the 25-mer position. In a parallel reaction, the template-primer was incubated with DNA pol α and 10 µM dTTP and dCTP. With the two correct dNTPs present, 90% of the 20-mer primer is elongated to an oligonucleotide product of 25 nucleotides after 30 min of incubation (Fig. 1B, lanes 8–10). These data show that DNA pol α inserts araCMP at the target site almost as efficiently as dCMP, producing 25-mer oligonucleotides with araCMP or dCMP residues positioned at the 3' terminus.

Incorporation of dGMP opposite the four dCMP residues in the DNA template positioned 5' to the target site could result in elongation of the primer from 25 to 29 nucleotides (Fig. 1A). Extension from the araCMP 3' terminus of the 25-mer oligonucleotide is indicated by the appearance of a 29-mer oligonucleotide when 10 µM dGTP is added to the reaction mixture (Fig. 1B, lanes 5–7). However, less than 5% of the 25-mer oligonucleotide is extended to the 29-mer position after 30 min of incubation. In contrast, extension from the dCMP 3' terminus results in elongation of greater than 99% of the 25-mer oligonucleotide to the 29-mer position (Fig. 1B, lanes 11–13). These results demonstrate that insertion of a single araCMP residue dramatically impairs, but does not terminate, subsequent elongation by DNA pol α.

The slow rate of extension from the 25-mer oligonucleotide results from insertion of araCMP at the target site by DNA pol α and not from misinsertion of dTMP or contaminant nucleotides. Misinsertion of dTMP, dAMP, or dGMP at the target site could produce an oligonucleotide migrating to the 25-mer position. To test for possible nucleotide misinsertion, DNA pol α was incubated with the template-primer and nucleotides that were noncomplementary to the target site (Fig. 1C). With 10 µM dTTP only (lane 2), 10 µM dTTP and dATP (lane 3), or 10 µM dTTP and dGTP (lane 4), greater than 95% of the 20-mer primer is extended by just four nucleotides to the 24-mer position corresponding to incorporation of dTMP opposite dAMP in the DNA template. Thus, when araCTP and dCTP are omitted from reactions, misinsertion of dTMP, dAMP, or dGMP at the target site accounts for less than 1% of the primer extended to the 25-mer position. When araCTP or dCTP is added with dTTP to the reactions, 95% of the 20-mer primer is elongated to the 25-mer position, corresponding to araCMP or dCMP insertion at the target site (lanes 5 and 6). The 25-mer oligonucleotide produced upon incubation with araCTP migrates more slowly in the polyacrylamide gel than the 25-mer produced upon incubation with dCTP. The difference in migration between these two 25-mers is most likely a consequence of the different structures of the arabinose and deoxyribose sugars inserted into the oligonucleotide primers at the 3' terminus and is further evidence for insertion of araCMP at the target site by DNA pol α.

To confirm that the 29-mer oligonucleotide is a product of extension from the araCMP 3' terminus by DNA pol α (Fig. 1B, lanes 5–7), we purified the 25-mer-araC oligonucleotide and hybridized it to DNA temp 1 to prepare homogeneous template-primer with 3' terminal araCMP positioned opposite dGMP in the DNA template (Fig. 2). The products of elongation from the 25-mer-araC template-primer are compared with those from the 25-mer-dC template-primer that

FIG. 1. Incorporation of araCMP by DNA Pol α. Two 25-mer oligonucleotides were prepared, as described under “Experimental Procedures,” with araCMP (25 mer-araC) or dCMP (25 mer-dC) positioned at the 3' terminus and hybridized separately to DNA template 1 (temp 1). Reactions (20 µl) contained 0.04 pmol of 25-mer-araC template-primer (lanes 1 and 3–8) or 25-mer-dC template-primer (lanes 2 and 9), 10 µM dGTP, and the indicated amount of DNA pol α. Incubations were 30 min at 37 °C. Positions of the 25-mer primers and oligonucleotide products are indicated.

FIG. 2. Extension from an araCMP 3' terminus by DNA Pol α. Two 25-mer oligonucleotides were prepared, as described under “Experimental Procedures,” with araCMP (25 mer-araC) or dCMP (25 mer-dC) positioned at the 3' terminus and hybridized separately to DNA template 1 (temp 1). Reactions (20 µl) contained 0.04 pmol of 25-mer-araC template-primer (lanes 1 and 3–8) or 25-mer-dC template-primer (lanes 2 and 9), 10 µM dGTP, and the indicated amount of DNA pol α. Incubations were 30 min at 37 °C. Positions of the 25-mer primers and oligonucleotide products are indicated.

### Notes
- The incorporation of araCMP by DNA Pol α results in the formation of an oligomer of 24 nucleotides. Insertion of araCMP at the 25-mer position is indicated by the 29-mer product. However, the slow rate of extension from the 25-mer oligonucleotide is due to misinsertion of dTMP or dAMP, which account for less than 1% of the primer extended to the 25-mer position.
- The 29-mer oligonucleotide produced by araCTP migrates more slowly than the 25-mer produced by dCTP, indicating differences in the structures of the arabinose and deoxyribose sugars.
- Incorporation of dGMP opposite the four dCMP residues results in elongation of the primer to 29 nucleotides, but this is less than 5% of the 25-mer primer.
- Extension from the araCMP 3' terminus is more efficient than from the dCMP 3' terminus, indicating a dramatic impairment in extension when one araCMP residue is present.
contains a paired 3' terminus. The presence of araCMP at the 3' terminus of 25 mer-araC is apparent by its slower migration relative to 25-mer-dC in the polyacrylamide gel (Fig. 2, lanes 1 and 2). Extension from the araCMP 3' terminus by DNA pol α upon incubation with the next complementary nucleotide is indicated by the appearance of 29-mer and 30-mer oligonucleotide bands. With addition of increasing amounts of DNA pol α elongation of up to 67% of the 25-mer-araC oligomer is detected after 30 min of incubation (Fig. 2, lanes 3–8). The 29-mer results from extension of four dGMP residues opposite dCMPs in the template. The 30-mer is likely produced by misinsertion of dGMP opposite dTMP. The amount of DNA pol α activity required for elongation of 67% of the 25-mer-araC oligomer contrasts sharply with the 30-fold less DNA pol α required to extend an equal amount of 25-mer-dC oligomer to the 29-mer position (Fig. 2, lanes 8 and 9). This difference illustrates the inefficient extension from an araCMP 3' terminus relative to a correctly paired 3' terminus. The 29-mer produced by extension from 25-mer-araC migrates more slowly in the polyacrylamide gel than the 29-mer produced by extension from 25-mer-dC (compare lanes 8 and 9). This slower mobility is most probably due to araCMP at an internucleotide position in the 29-mer oligonucleotide. Thus, DNA pol α extends the araCMP 3' terminus, incorporating the arabinoside into DNA at internucleotide linkages during in vitro DNA synthesis.

**Steady State Kinetic Analysis of araCMP Incorporation**—We used a steady state kinetic assay (Boosalis et al., 1987; Petruska et al., 1988) to precisely quantify the insertion and extension steps that determine the efficiency of araCMP incorporation into DNA by human DNA pol α. The 24-mer oligonucleotide was hybridized to DNA temp2 so that the 3' terminus was positioned 1 base away from the template dGMP at the target site (Fig. 3). In separate reactions, incorporation of araCMP or dCMP at the target site is detected as an increase in the length of the oligonucleotide primer from 24 to 25 nucleotides. The amount of 25-mer detected upon incubation of the template-primer with DNA pol α is dependent on the concentration of araCTP or dCTP in the reaction. Quantitation of the oligonucleotide products shows saturation kinetics for insertion of araCMP and dCMP with apparent $K_m$ values of 8.6 and 3.8 μM, respectively (Table I). The apparent $V_{max}$ values are 3.0% min$^{-1}$ and 4.1% min$^{-1}$ for insertion of araCMP and dCMP. The efficiency of nucleotide insertion by DNA pol α can be determined from the value of $V_{max}/K_m$ (Boosalis et al., 1987). The $V_{max}/K_m$ values of 0.35% min$^{-1}$/μM for araCMP insertion and 1.1% min$^{-1}$/μM for dCMP insertion differ by only 3-fold, indicating that DNA pol α inserts araCMP into DNA efficiently.

The major determinant of araCMP incorporation into DNA at internucleotide linkages by DNA pol α is extension from the araCMP 3' terminus. To determine the extension frequency from an araCMP 3' terminus relative to extension from a dCMP 3' terminus, the 25-mer-araC and 25-mer-dC oligonucleotides were hybridized to DNA temp2, and the ability of DNA pol α to extend these primers was measured. Extension from the 3' terminus by incorporation of the next complementary nucleotide is detected as an increase in the length of the oligonucleotide primer from 25 to 26 nucleotides. The kinetics of extension from the araCMP and dCMP 3' termini are illustrated in Fig. 4. Extension from both araCMP and dCMP showed typical saturation kinetics. However, the apparent $K_m$ value for next nucleotide addition onto the araCMP 3' terminus was 1000 μM, in contrast to the apparent $K_m$ value of 1.0 μM for extension from the dCMP 3' terminus (Table I). The apparent $V_{max}$ values for araCMP and dCMP extension were 3.3% and 7.8% min$^{-1}$, respectively. Using $V_{max}/K_m$ values as a measure of the ability of DNA pol α to extend the araCMP 3' terminus relative to the dCMP 3' terminus indicates that extension from an araCMP terminus is 2000-fold less efficient than extension from a correctly paired 3' terminus.

**Binding of DNA Pol α to the araCMP 3' Terminus**—The extension from a DNA primer by DNA pol α follows an ordered sequential mechanism (Fisher and Korn, 1981a, 1981b). The enzyme first binds DNA at the 3' terminus to form the enzyme-DNA complex (E-DNA), then the enzyme-DNA complex binds dNTP to form the enzyme-DNA-dNTP complex (E-DNA-dNTP). The steady state kinetic analysis shows that the araCMP 3' terminus is a poor substrate for extension by DNA pol α. We used an equilibrium binding assay (Creighton et al., 1992) to determine if inefficient extension from the araCMP 3' terminus might result from a lower
binding affinity of DNA pol α for the araCMP DNA (K_{D(araCMP)}). This assay measures the relative binding affinity (K_{D(araCMP)}) of an enzyme to "mismatched" and matched 3' termini during formation of the enzyme-DNA binary complex. In order to calculate the $K_{D(araCMP)} = K_{D(araCMP)/K_{D(dCMP)}}$, two series of binding reactions were performed (Fig. 5). In one series, the radiolabeled 25-mer-dC template-primer was mixed with increasing amounts of unlabeled competitor 25-mer-araC template-primer and incubated at 37 °C with DNA pol α (Fig. 5A). In a second series, the radiolabeled 25-mer-dC template-primer was mixed with increasing amounts of unlabeled competitor 25-mer-dC template-primer and incubated at 37 °C with DNA pol α (Fig. 5B). In these reactions, the radiolabeled template-primer competes with the unlabeled template-primer for binding to DNA pol α in the absence of the second substrate, dNTP. Since the amount of DNA pol α is less than the amount of competing 25-mer 3' termini, the fraction of enzyme that is bound to each of the two species of DNA reflects the binding affinity of the enzyme for each. To determine the amount of enzyme bound to each of the two competing template-primers, dGTP is added to initiate the elongation reaction, and the products are examined on a polyacrylamide gel. The percentage of radiolabeled 25-mer-dC primer generating oligomers between 26 and 27 nucleotides in length (lane 1 of Fig. 5, A–C). Addition of unlabeled 25-mer-araC template-primer (Fig. 5A, lanes 2–4) or 25-mer-dC template-primer (Fig. 5B, lanes 2–4) to the binding reactions at concentrations equal to 0.33, 1.0, or 2.0 times the molar concentration of radiolabeled 25-mer-dC template-primer results in decreasing band intensities of the extended oligomers. Furthermore, for each of the concentrations of competitor DNA used, the amount of the decrease in extension from the radiolabeled 25-mer-dC is equal to the fraction of unlabeled/total template-primer present in the binding reaction (Fig. 5E). For example, in the presence of equimolar amounts of the unlabeled and radiolabeled template-primers, extension from the radiolabeled 25-mer-dC is decreased by approximately one-half of that observed in the absence of unlabeled template-primer (compare lanes 1 and 3 in Fig. 5, A and B). The comparable results obtained with both the araC-25-mer and DC-25-mer template-primers indi-
cate that there is no apparent difference in binding of DNA pol α to the araCMP and the dCMP 3′ termini.

Competition between the 25-mer-araC and 25-mer-dC template-primers for binding to DNA pol α is a measure of specific binding at the 3′ termini. The 35-mer template was used in order to minimize the effect of nonspecific binding of DNA pol α to single-stranded DNA that was detected when the 2961-nucleotide DNA temp1 was used as template (results not shown). Addition of the unprimed 35-mer template in the binding reactions shows that single-stranded DNA does compete, to a limited extent, with the 25-mer-dC for binding to DNA pol α (Fig. 5C). However, the amounts of unprimed 35-mer template present in the 25-mer-araC and 25-mer-dC binding reactions (Fig. 5, A and B) should be minimal and are not likely to interfere with specific binding to either of the 25-mer-primed templates by DNA pol α.

To assure that DNA pol α elongates only the 25-mer primers that are bound by DNA pol α at the time of dGTP addition, an excess of unlabeled DNA is added along with the dGTP to serve as a polymerase trap (Joyce, 1989). The trapped DNA contains a high concentration of 3′ termini to bind free enzyme and to prevent reinitiation of DNA pol α at the 25-mer 3′ termini during the elongation reaction. The effectiveness of the polymerase trap is demonstrated in a binding reaction containing the trap-DNA and the radiolabeled 25-mer-araC template-primer (Fig. 5D, lane 2). Upon addition of dGTP no extension from the 25-mer-dC is detected, indicating that DNA pol α is bound to the trap-DNA and unable to elongate the radiolabeled 25-mer-dC template-primer. When the trap-DNA is not added along with the dGTP, DNA pol α reinitiates at the 25-mer-dC template-primer as indicated by the 5-fold increase in the amount of extension from the radiolabeled 25-mer-dC primer (compare lane 3 in Fig. 5D with lane 1 in Fig. 5, A–C).

**Binding of DNA Pol α to the araCMP 3′ Terminus in the Presence of dNTP**—To investigate further the possible mechanism(s) for slow araCMP extension, we assessed binding of DNA pol α to the araCMP 3′ terminus in the presence of the second substrate, dGTP, using a competitive inhibition analysis. This approach was used previously to elucidate the substrate binding mechanism of human KB cell DNA pol α and more recently to study binding of *Drosophila* DNA pol α to template-primers containing abasic sites (Fisher and Korn, 1981b, 1981a; Ng et al., 1989). DNA pol α binds a correctly base-paired ddNMP 3′ terminus when the next complementary nucleotide is present. The absence of a 3′-OH on the terminal residue prohibits nucleophilic attack on the incoming nucleoside triphosphate and subsequent phosphodiester bond formation. Thus, the deoxy-3′ terminus inhibits DNA pol α activity by binding nonproductively to the enzyme and effectively reducing the concentration of free enzyme in the reaction. If DNA pol α binds to an araCMP 3′ terminus and becomes “trapped” in a nonproductive ternary complex, then the 25-mer-araC template-primer should bind DNA pol α and effectively decrease the amount of free enzyme available for elongation of a second correctly base-paired template-primer available in the reaction mixture. Alternatively, if DNA pol α binds to the araCMP 3′ terminus but freely dissociates, the 25-mer-araC template-primer should have little or no effect on elongation from the correctly base-paired template-primer.

We monitored DNA pol α activity using a 20-mer oligonucleotide (20-mer2) hybridized to DNA templ in four separate reactions containing a competitor that was primed with either the 25-mer-araC (Fig. 6A), 25-mer-ddC (Fig. 5B), no 25-mer (Fig. 6 C), or 25-mer-dC (Fig. 6D). The 20-mer and the 25-mer were 32P-labeled in order to detect catalytic activity at each of these primers. The 20- and 25-mer primers hybridize to DNA templ at different positions. DNA pol α was added to each reaction with 50 μM of both dCTP and dGTP, and the products of time course reactions were examined on a polyacrylamide gel. Incorporation of dCMP and dGMP by DNA pol α into the 20-mer2 template-primer is detected as the accumulation of 23-mer oligonucleotide in each of the four reactions (Fig. 6, A–D). Incorporation of dGMP into the 25-mer template-primer is detected as the accumulation of 29-mer oligonucleotide (Fig. 6, A and D). The amount of DNA pol α activity added to the reactions was limiting, so that accumulation of 23-mer oligonucleotide was approximately linear during the first 7 min of the reaction in the absence of competing 25-mer template-primer (Fig. 6C). Quantitation of the oligomer bands (Fig. 7) shows that the rate of 23-mer accumulation in the presence of the 25-mer-ddC template-primer (Fig. 6B) is 8-fold slower than in the presence of the 25-mer-araC or the 25-mer-dC template-primers (Fig. 6, A and D). This slower rate of 23-mer accumulation in the reaction containing the 25-mer-ddC indicates that DNA pol α is inhibited more by the 25-mer-ddC template-primer than by the 25-mer-araC or the 25-mer-dC template-primers. Inhibition by the 25-mer-ddC is presumably due to nonproductive binding of DNA pol α to the deoxy 3′ terminus in the presence of the next complementary nucleotide (Fisher and Korn, 1981a). This differential inhibition is not detected in the absence of dGTP (data not shown). The lack of detectible substrate-induced inhibition by the 25-mer-araC and the 25-mer-dC template-primers indicates that nonproductive ternary complex formation by DNA pol α with these template-primers is minimal. Productive binding to the 25-mer-araC and the 25-mer-dC is evident by the accumulation of 29-mer oligonucleotide (Fig. 6, A and D). However, the rate of extension from the 25-mer-araC is much slower than the rate of extension from the 25-mer-dC as indicated by the much lower amount of 29-mer oligonucleotide synthesized from the 25-mer-araC template-primer. The apparent lack of inhibition by the 25-mer-araC suggests that DNA pol α is not trapped at the 3′ terminus in a nonproductive ternary complex but more likely dissociates from the araCMP DNA.

**Elemental Effect on Extension from an araCMP 3′ Terminus**—The 25-mer-araC primer was elongated using DNA pol α in the presence of the phosphorothioate analogue of the next complementary nucleotide, dATPαS, in order to determine if slow extension from the araCMP 3′ terminus might result from slow phosphodiester bond formation. A rate-limiting step involving formation of a phosphoester bond with a phosphorothioate analogue shows an elemental effect (Benkovic and Schray, 1973; Mizrahi et al., 1985). Thus, if DNA pol α binds to the araCMP 3′ terminus but catalysis of the phosphodiester bond is rate-limiting, then substitution of α-P=S for α-P=O in the incoming nucleotide should reduce the rate of extension from the araCMP 3′ terminus by approximately 100-fold. We determined the rates of elongation from the 25-mer-araC oligonucleotide by DNA pol α upon addition of 50 μM dATPαS or 50 μM dATP (Fig. 8). Elongation from the 25-mer-araC is detected as the appearance of oligonucleotide bands greater than 25 nucleotides in a time course reaction. Quantitation of the products shows that the rate of elongation from the araCMP 3′ terminus upon addition of dATPαS is 6-fold slower than the rate of elongation upon addition of dATP. There is no measurable elemental effect on elongation from the 25-mer-dC paired 3′ terminus (results not shown). These results suggest that phosphodiester bond formation is partially rate-limiting during extension from araCMP by DNA pol α.
Incorporation of araCMP by DNA Pol α

FIG. 6. Binding of DNA pol α to the araCMP 3′ terminus in the presence of dNTP. The 25-mer oligonucleotide primers, 25-mer-araC, 25-mer-ddC, and 25-mer-dC, were hybridized to DNA temp1 to produce either a dGMP:araCMP 3′ terminus, a dGMP:ddCMP 3′ terminus, or a dGMP:dC CMP 3′ terminus. An additional hybridization reaction contained DNA temp1 but no 25-mer primer. The 20-mer2 oligonucleotide primer was hybridized to DNA temp1, and four time course reactions (40 µl) were prepared, as described under “Experimental Procedures,” each containing template-primer (0.2 pmol) and DNA pol α (1.4 units). DNA pol α (1.4 units) was added to each reaction, and samples (5 µl) were removed at the indicated times after incubation at 37 °C. Positions of the 20-mer2 and 25-mer primers and the 23- and 29-mer oligonucleotide products are indicated. A small amount of 29-mer was detected in B, presumably from extension of the contaminant 24-mer that was present in the 25-mer-ddC oligonucleotide preparation.

FIG. 7. Quantitation of the competitive binding analysis of DNA pol α to an araCMP 3′ terminus. Reaction products shown in Fig. 6 were quantitated as described under “Experimental Procedures.” Rates of extension from the DNA temp1–20-mer2 primer in the presence of the DNA temp1–25-mer-araC (●), DNA temp1–25-mer-ddC (○), unprimed DNA temp1 (□), or DNA temp1–25-mer-dC (△) were determined from slopes of the linear portions of the curves.

DISCUSSION

DNA pol α contributes to the fidelity of DNA synthesis by its low frequency of extension from mispaired 3′ termini (Petruska et al., 1988; Perrino and Loeb, 1989; Perrino et al., 1989; Mendelman et al., 1990). Similarly, incorporation of araCMP into DNA at internucleotide positions is limited, because DNA pol α extends slowly from araCMP 3′ termini (Townsend and Cheng, 1987; Mikita and Beardsley, 1988). We used oligonucleotide-primed DNA templates to examine the polymerization of araCMP into DNA during in vitro DNA synthesis by DNA pol α purified by immunoaffinity chromatography from human leukemia cells. Using a site-specific kinetic assay (Boosalis et al., 1987), we have measured insertion of araCMP and extension from the araCMP 3′ terminus to evaluate the relative contributions of these two steps to araCMP incorporation into DNA. The results presented here

FIG. 8. Elemental effect on extension from an araCMP 3′ terminus by DNA pol α. The 25-mer-araC oligonucleotide was hybridized to DNA temp2 to produce a dGMP:araCMP 3′ terminus. Two time course reactions (100 µl) were prepared, as described under “Experimental Procedures,” each containing template-primer (0.2 pmol) and DNA pol α (14 units). After incubation for 1 min at 37 °C, a sample (10 µl) was removed, 50 µM dATP (●) or 50 µM dATP (◇) was added, incubation was continued, and samples (10 µl) were removed from each reaction at the indicated times. Oligonucleotide products of dAMP addition (left) and dAMP addition (right) are shown. Quantitation was as described under “Experimental Procedures.” Rates of extension were determined from slopes of the linear portions of the curves.
indicate that araCMP insertion into DNA is only 3-fold less efficient than dCMP insertion. In contrast, extension from an araCMP 3' terminus is 2000-fold less efficient than extension from a correctly base-paired dCMP 3' terminus. Thus, DNA pol α discriminates against araCMP incorporation into DNA almost exclusively at the extension step.

DNA pol α synthesizes DNA by an ordered sequential mechanism in which the enzyme binds to the 3' terminus of a DNA template-primer then to the incoming dNTP (Fisher and Korn, 1981a, 1981b). Extension from the araCMP 3' terminus requires binding of DNA pol α to the araCMP template-primer and dNTP substrates leading to productive ternary complex formation (E-DNA-dNTP) and subsequent phosphodiester bond formation. Inefficient extension from the araCMP 3' terminus could result from poor binding of the enzyme or from slower phosphodiester bond formation or both. Using the equilibrium binding assay (Creighton et al., 1992) we have demonstrated that in the absence of dNTP DNA pol α binds equally well to DNA template-primers with araCMP or dCMP positioned at the 3' terminus of the primer. Thus, we have shown that araCMP positioned at the 3' terminus does not lower the binding affinity of DNA pol α for the DNA during formation of the polymerase-DNA binary complex. However, the equilibrium binding analysis does not measure the precise position of DNA pol α on the template-primer. It is possible that the araCMP residue at the 3' terminus affects the proper positioning of the template-primer within the active site of the enzyme that is required for rapid catalysis. The competitive binding analysis demonstrates that the template-primer containing araCMP at the 3' terminus of the primer does not trap DNA pol α in a nonproductive ternary complex as is observed with the template-primer containing a ddCMP. Thus, in the presence of both substrates, DNA pol α binds to the araCMP 3' terminus and inefficiently catalyzes the extension reaction or dissociates freely from the DNA.

We measured the elemental effect on the rate of extension from an araCMP 3' terminus by DNA pol α to determine if slower phosphodiester bond formation contributes to the inefficient extension from araCMP. Elemental effects have been used to deduce the kinetic mechanism of *E. coli* DNA pol I (Kuchta et al., 1987, 1988) and bacteriophage T7 DNA polymerase (Patel et al., 1991; Wong et al., 1991; Donlin et al., 1991) and provide insights into the mechanism of araCMP incorporation into DNA by the human DNA pol α. The chemistry of DNA polymerization involves nucleophilic attack of the 3'-OH group of the primer nucleotide at the α-phosphate of the incoming triphosphate nucleotide. Substitution of P=S for P=O for the α-phosphate leads to lower reactivity of the thiophosphoryl center relative to the phosphate center (Benkovic and Schray, 1973; Mizrahi et al., 1986). If the bond formation step is limiting in extension from the araCMP 3' terminus by DNA pol α, then elongation with the α-phosphorothioate analogue should be slower than elongation with the normal triphosphate nucleotide. As shown in Fig. 8, the rate of extension from araCMP by addition of dAMP·P=S under steady state conditions is 6-fold slower than extension by addition of dAMP. This moderate elemental effect indicates that phosphodiester bond formation is partially rate-limiting during extension from the araCMP 3' terminus and likely contributes to slow extension from araCMP. Furthermore, the lack of an elemental effect during extension from the correctly base-paired dCMP 3' terminus (not shown) suggests that phosphodiester bond formation is not rate-limiting during correct dNMP incorporation and only becomes limiting after incorporation of araCMP. Therefore, it seems likely that upon insertion of araCMP into a growing DNA strand by DNA pol α, binding of the enzyme to the DNA is not directly affected, but slower bond formation increases the probability of enzyme dissociation and limits incorporation of the arabinoside into DNA at internucleotide linkages.

The kinetics of araCMP incorporation into DNA predict that the geometric requirements believed to be necessary for accurate DNA synthesis (Echols and Goodman, 1991) are preserved in the insertion step but not in the extension step. Efficient insertion of araCMP indicates that the incoming arabinoside triphosphate binds to DNA pol α, allowing base pairing between araCMP and dGMP in the template and subsequent phosphodiester bond formation. Covalent linkage of araCMP to the 3' terminus likely introduces changes in DNA structure that account for inefficient elongation from the araCMP 3' terminus through slower phosphodiester bond formation. Melting studies with oligomers containing araCMP:dGMP pairings indicate that hydrogen bonding is only moderately affected (Beardseley et al., 1988), suggesting that improper base pairing probably cannot account for the observed slow rate of extension from an araCMP 3' terminus. Model building studies and the crystal structure of araCMP in a DNA decamer (Gao et al., 1991) show that the trans-2'-OH group of the arabinose sugar projects into the major groove and could potentially directly affect interactions between DNA pol α and the DNA. Alternatively, the position of the 2'-OH group of araCMP could sterically preclude binding of the next complementary nucleotide. The close contact between the 2'-OH group of araCMP and the CH₂ group of an adjacent dTMP appears to account for some structural distortion in an araCMP decamer (Gao et al., 1991). It seems likely that the duplex DNA binding site of DNA pol α is formed to accommodate absolute Watson-Crick geometry as the polymerase translocates along the double-stranded region during DNA synthesis. Anything less than absolute Watson-Crick geometry, whether imposed from the sugar-phosphate backbone or from base mispairing, might affect phosphodiester bond formation and promote DNA pol α dissociation.

Several mechanisms might exist in cells to account for araCMP incorporation into DNA at internucleotide positions. We have demonstrated here that in the absence of exonucleases, DNA pol α can incorporate araCMP into DNA at internucleotide positions during DNA synthesis in vitro. It remains to be determined if exonucleases function coordinately with DNA pol α in the cell. Nevertheless, the efficient insertion of araCMP by DNA pol α in cells could potentially saturate any editing function, resulting in the kinetically less favorable extension from an araCMP 3' terminus and stable incorporation into DNA. It is also possible that an araCMP-terminated DNA fragment might be covalently linked to the 5' nucleotide of an adjacent DNA fragment by DNA ligase (Mikita and Beardseley, 1988; Ross et al., 1990). In addition, arabinosides are substrates for primase (Yoshida et al., 1985; Parker and Cheng, 1987), and it is possible that incorporation by primase during RNA primer synthesis leads to araCMP incorporation at internucleotide linkages in DNA (Kuchta et al., 1985; Catapano et al., 1991). Although it is possible that ligation and primer RNA synthesis contribute to araCMP incorporation into DNA, we favor DNA polymerization as the most likely mechanism of araCMP incorporation into DNA.

The principal finding in this study is that DNA pol α most likely dissociates from the 3' terminus following insertion of araCMP. This finding has mechanistic implications related to exonucleolytic editing. DNA pol α lacks an inherent editing
exonuclease (Copeland and Wang, 1991), but the exonuclease activity of DNA pol δ can remove arabinosides from the 3′ terminus of DNA (Lee et al., 1980). We have proposed previously a possible mechanism in which the exonuclease of DNA pol δ might excise nucleotides that have been misinserted by DNA pol α at the replication fork (Perrino and Loeb, 1990). A mechanism whereby DNA pol α dissociates from the 3′ terminus upon misinsertion of nucleotides or nucleotide analogues might facilitate repair by the proofreading exonuclease of DNA pol δ or another 3′ → 5′ exonuclease. Regardless of which exonuclease might be responsible, dissociation of DNA pol α would be required to permit access to the 3′ terminus.

REFERENCES


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