The Effects of Cytosine Arabinoside on RNA-primed DNA Synthesis by DNA Polymerase α-Primase*

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Oligonucleotides containing a specific initiation site for polymerase α-primase (pol α-primase) were used to measure the effects of cytosine arabinoside triphosphate and cytosine arabinoside monophosphate (araCMP) in DNA on RNA-primed DNA synthesis. Primase inserts araCMP at the 3' terminus of a full-length RNA primer with a 400-fold preference over CMP. The araCMP is elongated efficiently by pol α in the primase-coupled reaction. Extension from RNA 3'-araCMP is 50-fold less efficient than from CMP, and extension from dCMP. Using araCMP-containing templates, primer synthesis is reduced 2-3-fold, and RNA-primed DNA synthesis is reduced 2-8-fold. The efficiency of polymerization past a template araCMP by pol α is reduced 180-fold during insertion of dGMP opposite araCMP and 35-fold during extension from the araCMP:dGMP 3' terminus. These results show that the pol α-primase efficiently incorporates araCMP as the border nucleotide between RNA and DNA and suggest that the inhibitory effects of araC most likely result from slowed elongation of pol α and less so from inhibition of primer synthesis by primase.

In cells, RNA-primed DNA fragments are synthesized at origins of replication and on lagging strands at replication forks (1, 2). Initiation of DNA chains requires oligonucleotide synthesis to provide 3'-hydroxyl termini for DNA polymerase elongation. The tight association between DNA polymerase α (pol α) and primase indicate that pol α-primase complex is responsible for RNA-primed DNA synthesis. Primase synthesizes RNA primers that are about 10 Nts in length de novo, and pol α elongates the primers.

The aranucleotides inhibit DNA replication in animal cells (3). Inhibition by the triphosphate forms of these analogues might result during RNA-primed DNA synthesis by pol α-primase (4). Incorporation into RNA primers by primase and into DNA by pol α is at positions opposite complementary nucleotides (3-10). Primase inserts araATP and FaraATP more efficiently than ATP (9, 11), and pol α inserts these analogues as efficiently as deoxynucleotides (12). Insertion of aranucleotides terminates primer chain elongation by primase (9, 11), and 3'-aranucleotides in DNA decrease elongation by pol α 2000-fold (12). In contrast, aranucleotides at RNA 3' termini only moderately decrease elongation by pol α (9, 11). The synthesis of nonfunctional primers less than 7 Nts in length using homopolymer templates has made it difficult to quantitate effects of aranucleotides during RNA-primed DNA synthesis and to distinguish effects on primase and pol α.

Oligonucleotide templates permit analysis of sequence-specific effects of aranucleotides on RNA-primed DNA synthesis. The sequences of some primer initiation sites have been identified (13-16), and these sequences support initiation in oligomer templates (15, 16). Using an oligonucleotide containing an initiation site for pol α-primase, primers with the sequence 5'--GGAAGAAGC-3' are generated (16). Incorporation of araCMP at the 3' terminus of this full-length RNA primer and its effect on RNA-primed DNA synthesis were measured. In addition, cytosine residues within the initiation sequence and one nucleotide downstream were replaced with arabinocytosines. With these araCMP-containing templates, primer RNA synthesis and DNA synthesis were measured independently and during processive synthesis of RNA-primed DNA fragments.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled nucleotides were from Amersham. Unlabeled dNTPs, araCTP, and bovine serum albumin were from Sigma. The NTPs were from Pharmacia Biotech Inc. Enzymes were from Promega or U. S. Biochemical Corp. The 40-mer DNA was from Operon Technologies. The 9-mer, 10-mer, and 11-mer DNA primers and araCMP-containing templates (araCMP21, araCMP23, and araCMP31) were synthesized in the Cancer Center of Wake Forest University. The 10-mer RNA was from Oligo's etc. pol α-primase was purified (17), and unit definitions were as described (17, 18). Recombinant primase was a generous gift of Dr. William Copeland (NIHs, National Institutes of Health).

DNA Templates and Primers—The araCMP-containing templates were purified on urea-polyacrylamide gels (12), and potential gel contaminants were removed from recovered oligonucleotides using Sep-Pak C18 cartridges (Waters). The RNA 10-mer (5'--GGAAGAAGC-3') and DNA 9-mer, 10-mer, and 11-mer (5'--GGAAGAAGC(G)-3') were gel purified, recovered, and passed through Sephadex G-25 columns. The araCMP-terminated DNA 10-mer (10-araCMP) was generated by incubating RNA 10-mer with T4 DNA polymerase without dNTPs to produce a 9-mer. The 9-mer was recovered, hybridized to the 40-mer, and elongated with Klenow (exo-) in the presence of araCMP. The araCMP-terminated DNA 10-mer was produced by elongating the DNA 9-mer in the presence of araCTP. The RNA 10-mer:dGMP was generated by elongation of the RNA 10-mer in the presence of dGTP. All primers were gel purified.

Primase, pol α, and Primase-coupled pol α Reactions—All reactions (10 μl) contained 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and pol α-primase. Primase reactions contained 100 pmol of 40-mer template, 6.3 units of primase, and 100 μM NTPs with [α-32P]ATP. The pol α reactions contained 0.25 pmol of [32P]-labeled primer hybridized to the 40-base DNA templates
at a 10:1 molar ratio (template/primer), 0.15 units of pol α, and varying concentrations of nucleotide. Primase-coupled pol α reactions contained 100 pmol of 40-mer template, 3.8 units of primase, 1.8 units of pol α, 10 μM NTPs, and 100 μM dCTP, dGTP, TTP with 10 μM [α-32P]dATP. Incubations were at 37 °C for the indicated times, and reactions were stopped by addition of EDTA to 5 mM. Primase and pol α reactions were subjected to electrophoresis through 20% urea-polyacrylamide gels. Dried gels were quantified using AMBIS radioanalytic imaging system (San Diego) or phosphorimagery (Molecular Dynamics). Primase-coupled pol α reactions were processed for scintillation spectroscopy by collecting acid-insoluble products on glass fiber disks.

RESULTS

Insertion of araCMP into Full-length Primer RNA—pol α-primase generates RNA-primed DNA fragments by synthesizing RNA primers greater than seven nucleotides in length and polymerizing DNA onto the primers. An oligonucleotide containing an initiation site for primer synthesis (16) was used to measure araCMP insertion into quasianucleotide at the 3' terminus of a full-length primer (Fig. 1A). In the absence (Fig. 1B, lane 3) and in the presence of increased concentrations of araCTP (Fig. 1B, lanes 4–14), primase generated RNA primers 9–11 Nts in length. This result demonstrates that pol α-primase synthesizes full-length primer RNA in the presence of araCTP using this template. Insertion of araCMP was apparent from the appearance of the 9-araCMP and 10-araCMP oligomers that migrate more rapidly in the gel than those generated in the absence of araCTP. The araCMP-containing primers were detected even in the presence of a 1000-fold excess of the complementary substrate, CTP (Fig. 1B, lane 4).

The efficiency of araCMP insertion into primer RNA was determined by quantifying the 9- and 10-base products containing CMP and those of altered mobility containing araCMP. A linear relationship was demonstrated between the fraction of primers containing CMP at the 3' terminus and the [CTP]/[araCTP] (Fig. 1C). The inverse slope of this line is equal to the relative (Kcat/Km)araCTP/(Kcat/Km)CMP and is an estimate of the relative efficiencies of araCTP and CTP as substrates for primase (19). Quantitation indicates that araCTP is a 400-fold better substrate for primase than is CTP. To confirm that primase, not pol α, inserts araCMP at this site, primase reactions devoid of pol α were performed using recombinant primase (Fig. 1B, lanes 16–18). Generation of the araCMP-terminated 9- and 10-base products demonstrates that primase is responsible for araCMP insertion.

Extension from the araCMP-terminated RNA Primer—Extension from an araCMP-terminated RNA primer could lead to incorporation at internucleotide positions. A steady state kinetic assay (20, 21) was used to measure the efficiency of extension from the araCMP primer RNA by pol α. The RNA 10-araCMP and 10-mer primers were hybridized to the 40-mer template and extended with DNA pol α (Fig. 2). In the presence of increased concentrations of the next complementary nucleotide, increased amounts of 11-mer were detected. Extension from both primers demonstrates saturation kinetics, and the apparent Kcat and Vmax values for the next nucleotide addition were determined (Table I). The Vmax/Km values indicate that pol α extends the araCMP-terminated RNA primer 50-fold less...
efficiently than the CMP-terminated primer. This result contrasts the 2000-fold decrease in extension efficiency that is observed from an araCMP-terminated DNA primer (Table I and Ref. 12). These results show that pol α extends araCMP-terminated RNA primers more efficiently than araCMP-terminated DNA primers.

Extension from the araCMP 3' terminus using the kinetic assay requires that pol α first bind to the template primer then catalyze the addition of deoxynucleoside monophosphate. However, pol α-primase synthesizes an RNA primer and then elongates the primer without dissociating from the DNA template (22). Therefore, it was of interest to determine if insertion of araCMP at the 3' terminus of a full-length RNA primer affects elongation by pol α in a processive reaction. Primase-coupled pol α reactions were performed, and the rate of RNA-primed DNA synthesis in the presence of araCTP (6 fmol of dAMP/min) was equal to the rate in the presence of CTP (7 fmol of dAMP/min). To confirm that araCMP is incorporated as the border nucleotide, primase-coupled pol α reactions were performed, and araCMP in the product was verified by its migration on a polyacrylamide gel (Fig. 3). To limit DNA synthesis by pol α and to eliminate the possibility that dCTP might be inserted as the first deoxynucleotide, only dGTP and ddATP were included for elongation in these reactions. The product containing araCMP (11-araCMP) migrates more slowly in the gel than the 11-mer containing CMP (Fig. 3, compare lanes 4 and 5). In the

![Fig. 2. Extension from 3'-araCMP-terminated RNA primers by pol α.](image)

The extension data from Fig. 2 and from data generated by extension from DNA primers were quantified as described under "Experimental Procedures." The apparent $K_m$ and $V_{max}$ values were determined by non-linear regression to the Michaelis-Menten equation. Rate constants were calculated from $V_{max}/K_m$. Frequencies are relative to extension from 3'-CMP for RNA primers and 3'-dCMP for DNA primers.

| Primer | 3'-NMP | $K_m$ (μM) | $V_{max}$ (%/min) | Rate constant (%/min/μM) | Relative frequency |
|--------|--------|------------|-------------------|--------------------------|------------------|
| RNA    | araCMP | 19 ± 2.6   | 14 ± 0.9          | 0.73                     | 1/53             |
|        | CMP    | 0.11 ± 0.01| 4.3 ± 0.02        | 39                       | 1                |
| DNA    | araCMP | 1000 ± 160 | 8.1 ± 0.5         | 0.0081                   | 1/1600           |
|        | dCMP   | 0.25 ± 0.06| 3.3 ± 0.02        | 13                       | 1                |

![Fig. 3. Incorporation of araCMP during RNA-primed DNA synthesis.](image)
absence of CTP, primase synthesized a 9-mer, and no misincorporation of NTP was detected (Fig. 3, lane 3). These results indicate that araCMP is efficiently incorporated as the border nucleotide between RNA and DNA during RNA-primed DNA synthesis by pol α-primase.

Primer RNA Synthesis on araCMP-containing Templates—The DNA isolated from araC-treated cells contains araCMP at internucleotide positions (23, 24). To test the effects of araCMP in DNA on RNA-primed DNA synthesis by pol α-primase, three araCMP-containing templates were prepared (Fig. 4). For two of the templates, a dCMP within the priming site (NT 31 or NT 23) was replaced with araCMP. For the third template, the dCMP positioned at the first nucleotide downstream from the priming site (NT 21) was changed to araCMP. Primase initiates synthesis by insertion of the first 9-GTP at nucleotide 31. Nucleotide 23 is seven nucleotides from the initiation start site (16). These two positions were selected to test for differences in primer synthesis that might relate to the position of araCMP within the priming site. Nucleotide 21 was selected to measure the effect of araCMP in the template at the primase to pol α switch position. Synthesis of RNA primers using the araCMP-containing templates was determined in time course reactions (Fig. 4). Products 9–11 Nts in length are detected using all three of the araCMP-containing templates. Quantitation of the oligomer products shows that the rates of primer synthesis are reduced 2-fold using araCMP31 (Fig. 4, lanes 7–11) and araCMP23 (Fig. 4, lanes 12–16) and 3-fold using araCMP21 (Fig. 4, lanes 17–21) relative to the 40-mer template (Fig. 4, lanes 2–6). These results indicate that primase initiates primer synthesis and generates full-length RNA primers at priming sites containing araCMP at a reduced rate.

Primase-coupled pol α Synthesis Using araCMP-containing Templates—The rates of RNA-primed DNA synthesis using the araCMP-containing templates were reduced relative to the 40-mer template (Fig. 5). The levels of inhibition were dependent upon the positions of araCMP within the DNA templates. With the araCMP31 and araCMP23 templates, primase-coupled pol α synthesis was reduced 2- and 3-fold, respectively. The araCMP positioned at the presumed site of primase to pol α switching on the araCMP21 template had the greatest inhibitory effect, reducing primase-coupled pol α synthesis by 8-fold (Fig. 5). These results likely reflect, in part, the reduced rates of primer synthesis on these templates, while the araCMP requires insertion of dGMP opposite araCMP and 40-mer templates, while the araCMP requires insertion of dGMP opposite araCMP and dCMP is 0.11 m. The kne value for addition of dGMP opposite araCMP is 100 m. The Kne value for addition of dGMP opposite dCMP is 0.11 m. The Vmax values are more similar with a 5-fold greater Vmax value obtained using the araCMP21 template. The relative Vmax/Km values for these reactions indicate that pol α inserts dGMP opposite araCMP 180-fold less efficiently than it inserts dGMP opposite dCMP during RNA primer elongation.

Synthesis Past araCMP from an RNA Primer by pol α—To quantitate the effect of araCMP positioned in the DNA template at the RNA-DNA border on elongation by pol α, the kinetic assay was used to measure RNA synthesis independently from RNA primer synthesis (Fig. 6). Synthesis past araCMP requires insertion of dGMP opposite araCMP and extension from the 3′-araCMP:dGMP. In separate reactions, the 10-mer RNA primer was hybridized to the araCMP21 and 40-mer templates and incubated with pol α and increased concentrations of dGTP. Quantitation of oligomer products (Table II) shows that the Kne value for addition of dGMP opposite araCMP is 100 m. The Kne value for addition of dGMP opposite dCMP is 0.11 m. The Vmax values are more similar with a 5-fold greater Vmax value obtained using the araCMP21 template. The relative Vmax/Km values for these reactions indicate that pol α inserts dGMP opposite araCMP 180-fold less efficiently than it inserts dGMP opposite dCMP during RNA primer elongation.

Synthesis past araCMP was measured using the RNA primer containing an additional dGMP at the 3′ terminus. The RNA 10-mer-dGMP primer was hybridized to the araCMP21 and 40-mer templates, and extension from the araCMP:dGMP 3′ terminus by pol α was measured (Fig. 7). The Kne values for next nucleotide addition are 2.4 µM using the araCMP21 and 0.072 µM using the 40-mer templates (Table II). The relative Vmax/Km values for extension from the araCMP:dGMP and dCMP:dGMP termini indicate that extension from araCMP:
dGMP by pol α is 35-fold less efficient than from dCMP:dGMP. These results suggest that araCMP positioned in the template as the border nucleotide between RNA primer and DNA synthesis has a greater inhibitory effect on RNA-primed DNA synthesis than does araCMP positioned in the template within the RNA priming site.

Synthesis past araCMP from a DNA Primer by pol α—To test the relative effects of araCMP in DNA on pol α polymerization from RNA versus DNA, a kinetic analysis of araCMP bypass using DNA primers was performed (Table II). The position for DNA primer elongation was identical to that chosen for RNA primer elongation. A DNA 10-mer was hybridized to the araCMP21 and 40-mer templates and elongated with pol α in the presence of dGTP. Similarly, a DNA 11-mer was hybridized to the two templates and elongated with pol α. Quantitation indicates that insertion of dGMP opposite araCMP is 50-fold less efficient than insertion opposite dCMP. Extension from the araCMP:dGMP terminus is 160-fold less efficient than from the dCMP:dGMP terminus. These results are similar to those obtained using RNA primers and suggest that pol α synthesis past araCMP is not influenced by the nature of the primer strand. However, the rate of elongation past araCMP in DNA from both RNA and DNA primers is at least 10-fold more efficient than elongation from an araCMP 3′ terminus (Tables I and II), indicating that the position of araCMP in the nascent strand reduces the rate of elongation to a greater extent than does araCMP in the template strand.

**DISCUSSION**

We used a DNA template with a specific primer initiation sequence to study incorporation of araCMP into RNA-primed DNA fragments by pol α-primase. Our results demonstrate that primase incorporates araCMP opposite dGMP as the 3′-terminal nucleotide of a full-length RNA primer very efficiently and that pol α elongates the araCMP-terminated RNA primer. These data suggest that araCMP might be incorporated efficiently into RNA-primed DNA fragments at certain positions during discontinuous DNA synthesis in cells. Previous in vitro studies using homopolymer and bacteriophage templates have shown that araCMP are potent inhibitors of RNA-primed DNA synthesis by pol α-primase (3–9). Using poly(dT) templates, araCMP are detected in truncated RNA primers exclusively at 3′ termini, suggesting that primer chain termination is a likely mechanism of primase inhibition (9, 11). The truncated primers 2–6 Nts are not sufficient in length to support subsequent DNA synthesis by pol α (25). However, primers of at least 7 Nts in length containing a 3′-terminal araCMP are elongated by DNA pol α (Refs. 7, 9, 11, and this study). Since araCMP are incorporated into RNA primers at positions of correct base pairing, it is apparent that the DNA template sequence is an important consideration.

The related nucleotide analogues, FaraATP, araATP, and araCTP, might inhibit DNA replication by different mechanisms that relate to sequence-specific effects. Primer initiation sites are rich in thymidine and cytosine residues (13, 15, 16, 26). The high level of thymidine residues should make it more likely that FaraAMP and araAMP might be incorporated into elongating primers by primase within the first 6 bases, resulting in truncated primers that are not elongated by pol α. In contrast, the less frequent guanosines in initiation sites make it less likely that nonfunctional primers are generated in the presence of araCTP. Previous results using cell lysates indicate that FaraATP inhibits and araCTP stimulates primer RNA synthesis (8). These results might be explained by the generation of a high level of nonfunctional primers in the presence of FaraATP, resulting in the inability to initiate DNA synthesis. In contrast, araCMP might be incorporated more frequently into full-length RNA primers. Our results show that DNA pol α elongates an araCMP-terminated RNA primer more efficiently than an araCMP-terminated DNA primer. Kinetic measurements of extension efficiencies from araCMP termini show a 50-fold reduction for RNA and a 2000-fold reduction for DNA (Table I and Ref. 12). The failure to elongate araCMP-containing DNA 3′ termini is also apparent in the chain-termination effects associated with araCMP (10, 23, 27). Furthermore, extension from araCMP-terminated RNA primers appears to be efficient during primase-coupled pol α synthesis (Fig. 1C).

**Fig. 6.** Insertion of dGMP opposite araCMP from a primer RNA by pol α. The 10-mer RNA primer was hybridized to the araCMP21 (●) and 40-mer (○) templates and extended (60 s, 37 °C) in pol α reactions containing the indicated concentrations of dGTP. Oligonucleotide products were quantified as described under “Experimental Procedures.”

**TABLE II**

| Primer | Template | $K_m$ (mM) | $V_{max}$ (% min) | Rate constant (1/min) | Relative frequency |
|--------|----------|------------|-------------------|-----------------------|-------------------|
| Insertion | RNA 10-mer | araCMP21 | 100 ± 13 | 23 ± 1 | 0.22 | 1/80 |
| DNA 10-mer | dCMP21 | 0.11 ± 0.02 | 4.3 ± 0.2 | 39 | 1 |
| DNA 10-mer | araCMP21 | 100 ± 22 | 27 ± 2 | 0.27 | 1/48 |
| DNA 10-mer | dCMP21 | 0.25 ± 0.06 | 3.3 ± 0.2 | 13 | 1 |
| Extension | RNA 10-mer | araCMP21 | 2.4 ± 0.7 | 11 ± 0.8 | 4.5 | 1/35 |
| DNA 11-mer | dCMP21 | 0.072 ± 0.014 | 11 ± 0.4 | 160 | 1 |

The kinetic effects associated with araCTP (10, 23, 27). Furthermore, extension from araCMP-terminated RNA primers appears to be efficient during primase-coupled pol α synthesis (Fig. 1C).
Thus, a greater level of RNA primer synthesis in the presence of araCTP might be explained by shorter RNA-primed DNA fragments resulting from inhibition of DNA chain elongation and subsequent reinitiation at regions of single-stranded DNA downstream.

During replication, RNA primers are removed, and the resulting DNA fragments are joined by ligase (1, 2). It is not known which enzyme(s) removes RNA primers, nor is it known how the RNA-DNA border is recognized. Our results show that araCMP incorporated at the 3′ terminus of an RNA primer by primase can be elongated by pol α. The rate of RNA primer removal in the presence of araCTP is reduced (28). It is possible that araCMP at the RNA-DNA border is not removed, resulting in 5′-araCMP in DNA fragments. It has been shown that DNA fragments with a 3′-araCMP and 5′-deoxynucleoside monophosphate can be ligated (27). Ligation of 5′-araCMP and 3′-deoxynucleoside monophosphate has not been tested. The inefficient removal of araCMP from the 3′ terminus of an RNA primer and subsequent ligation of DNA fragments might lead to incorporation of araCMP at internucleotide positions.

Since araCMP is detected at internucleotide positions (23, 24), araCMP in the DNA template might have an effect on initiation of RNA-primed DNA synthesis. Our results show that araCMP positioned within the primer site reduces primer synthesis 2–3-fold (Fig. 4). This moderate effect on primer synthesis using araCMP-containing DNA might relate to the low fidelity of primase (29–31). In contrast, araCMP positioned one nucleotide downstream from the priming site significantly reduced primer elongation by pol α in a kinetic analysis and in the primase-coupled pol α reaction (Table II and Fig. 5). These results are similar to those of Mikita and Beardsley (27), who showed that araCMP in the DNA template slowed replication bypass for some polymerases. Thus, the major effect of araCMP in the DNA template appears to be on DNA polymerization. The kinetic analysis reveals large differences in elongation efficiencies of araCMP-containing RNA and DNA primers and small differences in elongation from RNA and DNA primers using araCMP-containing templates. The pol α discriminates against araCMP insertion into DNA 4-fold better using RNA primers than DNA primers (7). Similarly, insertion of dGMP opposite araCMP from an RNA primer is 4-fold less efficient than from a DNA primer (Table II). However, extension from the RNA 10-mer-dGMP positioned opposite araCMP was 5-fold more efficient than from the DNA 11-mer. Overall, the kinetic analysis shows that the efficiency of bypass of araCMP in the template by pol α-primase is very similar for RNA and DNA primers.

The primase and pol α are very likely target enzymes for arabinonucleotides during genomic DNA replication. Using an oligonucleotide template, we have measured the multiple effects that araC has on the synthesis of RNA-primed DNA fragments. Most significantly, we show that araCMP is efficiently incorporated as the border nucleotide between RNA and DNA by the pol α-primase complex.

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