# Inhibition of DNA Primase by 9-β-D-Arabinofuranosyladenosine Triphosphate†

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ABSTRACT: 9-β-D-Arabinofuranosyladenosine triphosphate (araATP) is a potent inhibitor of DNA primase. Primase readily incorporates araATP into primers, and primers containing araAMP are then elongated by DNA polymerase α (pol α) upon addition of dNTPs. AraATP did not inhibit utilization of primers under conditions where the ability of pol α to elongate primers was independent of the dATP concentration. The fraction of primers elongated by pol α was reduced by araATP only when elongation was dependent upon the dATP concentration. When the $K_i$ for primase was measured in terms of the inhibition of the synthesis of primers that can be utilized by pol α, we obtained $K_i$ = 2.7 μM (37 °C) and 2.0 μM (25 °C). Inhibition was competitive with ATP. Inhibition of pol α activity by araATP was measured under conditions where primase-catalyzed primer synthesis was required for the pol α activity. The decreased pol α activity was due to primase inhibition, and at constant dATP, araATP inhibition was competitive with ATP and gave $K_i$ = 1.2 μM, similar to the $K_i$ for primase alone. Increasing the dATP concentration had no effect on inhibition. In combination with previously reported in vivo data, we conclude that DNA primase is the primary in vivo target of the arabinofuranosyl nucleotides, not pol α.

DNA polymerase α (pol α) is a major replicative DNA polymerase (Kornberg, 1980; Lehman & Kaguni, 1989). It copurifies as a complex with DNA primase, such that on single-stranded DNA the primase will synthesize RNA primers that the pol α then elongates (Wang et al., 1984; Grosse & Krauss, 1985; Yoshida et al., 1983). DNA replication is a prime target of cancer chemotherapeutics; hence, pol α has been well studied as a target for inhibitors. Primase is less well studied, although it would be an ideal target for chemotherapeutics, particularly since as far as is known primase is only involved in DNA replication.

The arabinofuranosyl nucleotides are potent inhibitors of DNA replication in vivo (Dicioccio & Srivastava, 1977). One of them, 1-β-D-arabinofuranosylcytosine (araC)[1] is a potent chemotherapeutic agent used for the treatment of some leukemias (Howard et al., 1968; Weil et al., 1980). 9-β-D-Arabinofuranosyladenosine (araA), while not effective as a cancer treatment, is an antiviral agent (Keeney & Buchanan, 1975). The active form of each of these compounds is thought to be the triphosphate.

In vitro, araATP and araCTP are both potent inhibitors of pol α. They are competitive inhibitors with dATP and dCTP, respectively, and their $K_i$s (ca. 1 μM) are similar to the $K_M$s for dNTPs (Dicioccio & Srivastava, 1977; Parker et al., 1988). AraNTPs could inhibit both via direct competitive inhibition of dNTP polymerization and, if incorporated into the growing DNA chain, by greatly reducing the rate of polymerization of the next nucleotide. In vivo, addition of araA/araC to growing cells results in incorporation of araNMPs into DNA primarily at internucleotide positions, but also at 3′ termini (Major et al., 1981, 1982). AraATP and araCTP are also inhibitors of primase. Measurements using either a filter binding assay or elongation of primers by DNA pol I (*Escherichia coli*) have given $K_i$ values of 20–30 μM (Parker & Cheng, 1987; Yoshida et al., 1985). On the basis of the lower $K_i$ for pol α, it has been proposed that inhibition of primase is only a secondary target of araNTPs in vivo.

On a poly(dT) template, the products of primase (calf thymus) are 2–10 nucleotides long. The dominant products are 2–3 and 8–9 nucleotides long (Kuchta et al., 1990). Importantly, only those primers ≥7 nucleotides are competent to be utilized by the polymerase—shorter primers cannot be elongated even with elevated dNTP concentrations (Kuchta et al., 1990). Thus, primase has only truly synthesized a product once the primer reaches 7 nucleotides. We therefore considered the possibility that previous data may understate the potency of araATP inhibition of primase. Only primers ≥7 nucleotides should be considered when primase activity is being measured since only these primers can be utilized by pol α. It is unclear, however, what length primers are detected by the filter binding assay, and pol I was recently reported to only elongate primers >15 nucleotides long (Suzuki et al., 1989). In this report we explore the mechanism and potency of araATP inhibition of DNA primase and find that, indeed, it is a very potent inhibitor of DNA primase.

## Materials and Methods

Unless noted, all materials and methods were as described previously (Kuchta et al., 1990). AraATP and aphidicolin were from Sigma, and oligo$(A)_{12-18}$ was from Pharmacia. $DNA_G$ was synthesized on an Applied Biosystems DNA synthesizer and then purified on an Applied Biosystems OPC system. DNA duplexes were formed as described previously (Kuchta et al., 1987).

DNA pol α·primase was purified from calf thymus as described previously with the following modifications (Chang et al., 1984). In addition to PMSF as a protease inhibitor, the cell breakage buffer also contained 1 mM EDTA and 5 mM benzamidine. After protamine sulfate precipitation of nucleic acids and clarification with Nonidet P40, the extract was loaded onto a phosphocellulose column (36 cm × 6 cm) equilibrated with 20 mM potassium phosphate, pH 7.5. This

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[1] Abbreviations: araA, 9-β-D-arabinofuranosyladenosine; araC, 1-β-D-arabinofuranosylcytosine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, sodium salt; PMSF, phenylmethanesulfonyl fluoride; pol α, DNA polymerase α; pol δ, DNA polymerase δ; Tris, tris(hydroxymethyl)aminomethane, hydrochloride salt.

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was then washed with 1 L of the same buffer, and protein was eluted with 10% glycerol, 0.5 M NaCl, and 0.1 M potassium phosphate, pH 7.5. The eluate containing pol $\alpha$ (ca. 300 mL) was twice dialyzed against 2 L of 20 mM potassium phosphate, pH 7.5, and then loaded onto the monoclonal antibody column. The column was washed with 50 mL of 0.5 M NaCl and 50 mM potassium phosphate, pH 7.5, followed by 250 mL of 50 mM Tris, pH 7.4. Pol $\alpha$·primase was eluted with 3.2 M $MgCl_2$, and the fractions containing protein were immediately diluted 1:2 with 10% glycerol, 1 mM DTT, and 50 mM Tris, pH 7.4. Pol $\alpha$·primase was then dialyzed against 50% glycerol, 1 mM DTT, and 50 mM potassium phosphate, pH 7.5, and stored at −20 °C.

*$K_i$ Measurements (Primase).* Assays (10 $\mu$L) contained pol $\alpha$·primase (1–2 units of primase), 0.1 mM poly(dT), [$\alpha$-$^{32}$P]ATP (ca. 15000 cpm pmol$^{-1}$), araATP, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.4. Assays were quenched into gel loading buffer (90% formamide) after 30 min at 37 °C and subjected to denaturing polyacrylamide gel electrophoresis (18% acrylamide, 8 M urea). The products corresponding to primers ≥7 nucleotides long were excised from the gels and quantified by scintillation counting. Controls demonstrated that the rate of primer synthesis was linear for at least 60 min. Assays at 25 °C were performed similarly, except the incubation time was increased.

*Elongation of Primers by Pol I.* Primers were synthesized in 10-$\mu$L assays as described above. After 60 min at 37 °C, the reactions were heated to 75 °C for 5 min to inactivate the pol $\alpha$·primase. dATP was added to a final concentration of 50 $\mu$M. The Klenow fragment of pol I (4 units) was added, and the reactions were incubated at 20 °C. At various times, aliquots were quenched into gel loading buffer and then analyzed by gel electrophoresis followed by autoradiography.

*DNase I Treatment of Pol $\alpha$ Elongated Primers.* Pol $\alpha$ elongated primers were synthesized in assays (10 $\mu$L) containing 100 $\mu$M [$\alpha$-$^{32}$P]ATP and 25 $\mu$M dATP. They were incubated at 25 °C for 60 min and then heated to 75 °C for 5 min to inactivate the pol $\alpha$·primase. The polymerase elongated primers were generated at 25 °C to ensure maximal utilization of primers. DNase I was added to a final concentration of 0.32 $\mu$g $\mu$L$^{-1}$, and the reactions were incubated at 37 °C. At various times aliquots were quenched with gel loading buffer.

*Primase-Coupled Pol $\alpha$ Activity.* Assays to measure the rate of polymerase activity when coupled to primer synthesis typically contained 0.2 $\mu$g of pol $\alpha$·primase, 5 mM $MgCl_2$, 50 mM Tris, pH 7.4, and various concentrations of ATP, [$\alpha$-$^{32}$P]dATP (ca. 4000 cpm pmol$^{-1}$), and araATP. Reactions were stopped by the addition of EDTA to a final concentration of 20 mM, and the amount of [$^{32}$P]dATP incorporated into oligonucleotides was determined with a filter binding assay. Only one time point was taken for each assay, and then several portions of each assay were spotted onto DE81 filters to increase accuracy (Bryant et al., 1983). To determine the appropriate length of time, control experiments with multiple time points gave the linear time range of the assay.

## Results

*Inhibition of Primase-Coupled Polymerase Activity.* Treatment of cells with araA/araC ultimately results in decreased DNA synthesis (Dicioccio & Srivastava, 1977). This could reflect primase or polymerase inhibition since a large fraction of DNA synthesis by pol $\alpha$ during replication is likely directly coupled to primer synthesis. Perhaps the most important aspect of araATP inhibition is therefore inhibition of primase-coupled DNA synthesis. To test the coupled reaction, we used poly(dT) as template. In order for pol $\alpha$ to polymerize [$\alpha$-$^{32}$P]dATP, primase must first synthesize a primer. Inhibition of coupled dATP polymerization was measured at 25 °C, conditions where increasing dATP concentration does not affect the fraction of primers elongated (Kuchta et al., 1990). With 5 $\mu$M araATP, 25 $\mu$M [$\alpha$-$^{32}$P]dATP, and 100 $\mu$M ATP, dATP polymerization was inhibited 64%. This is consistent with primase inhibition, not pol $\alpha$ inhibition, since under these conditions pol $\alpha$ should have been inhibited only 35% [assuming $K_i$(araATP) = 1 $\mu$M and $K_M$(dATP) = 3 $\mu$M (see below)].

Table I: Effect of Increasing dATP Concentrations on Primase-Coupled Pol $\alpha$ Activity

| | fractional activity[a] | | |
|---|---|---|---|
| | 3 $\mu$M araATP | | 10 $\mu$M araATP |
| [dATP] ($\mu$M) | 25 °C | 37 °C | 37 °C |
| 10 | 0.40 | 0.46 | 0.21 |
| 20 | 0.44 | 0.56 | 0.31 |
| 30 | 0.49 | 0.52 | 0.34 |
| 40 | 0.42 | 0.50 | 0.36 |
| 50 | 0.42 | 0.51 | 0.40 |

[a] Assays were performed as described under Materials and Methods. Fractional activity was calculated as activity in the presence of araATP divided by the activity in the absence of araATP. The araATP concentration is noted above the temperatures at which the assay was performed.

Further evidence that primase is the inhibited enzyme in these primase-coupled dATP polymerization assays comes from the small effects of increasing dATP concentration. With constant ATP (100 $\mu$M) and araATP (3 $\mu$M), increasing the dATP concentration from 10 to 50 $\mu$M did not overcome inhibition due to araATP at either 25 or 37 °C (Table I). Data are given as the rate of dATP polymerization in the presence of araATP divided by the rate of dATP polymerization without araATP in order to compensate for (i) inhibition of primase by dATP and (ii) increased utilization of primers due to increasing dATP concentrations [37 °C only (Kuchta et al., 1990)]. With a higher araATP concentration (10 $\mu$M), conditions where pol $\alpha$ inhibition could become significant, increasing dATP from 10 to 50 $\mu$M reduced the amount of inhibition by only 24% (Table I).

These small effects with dATP contrast with the effect of varying the ATP concentration on the coupled rate of [$\alpha$-$^{32}$P]dATP polymerization. With 25 $\mu$M dATP and at 25 °C, a plot of [ATP]$^{-1}$ versus (dATP polymerization rate)$^{-1}$ was linear, with an apparent $K_M$ of 180 $\mu$M. AraATP inhibited dATP polymerization competitively with respect to ATP and gave a $K_i$ of 1.2 $\mu$M (data not shown). These data were obtained at 25 °C since the same fraction of primers was utilized by the polymerase regardless of araATP concentration. At 37 °C the data were more complicated due to inhibition of the primase to polymerase activity switch (not shown). Together, these data indicate that inhibition of the coupled assay is primarily due to primase inhibition; pol $\alpha$ inhibition contributes only secondarily.

*Incorporation of AraATP into Primers.* The data on the coupled assay indicated that primase inhibition was a significant mechanism by which araATP could inhibit DNA replication; hence, we next examined the effects of araATP on primase alone. Previous work was inconclusive as regards incorporation of araNTPs into primers (Parker & Cheng, 1987; Yoshida et al., 1985). We thus examined primer synthesis on a poly(dT) template with 100 $\mu$M [$\alpha$-$^{32}$P]ATP and various concentrations of araATP (Figure 1a). When compared to "normal" primers (lane a), those synthesized in the

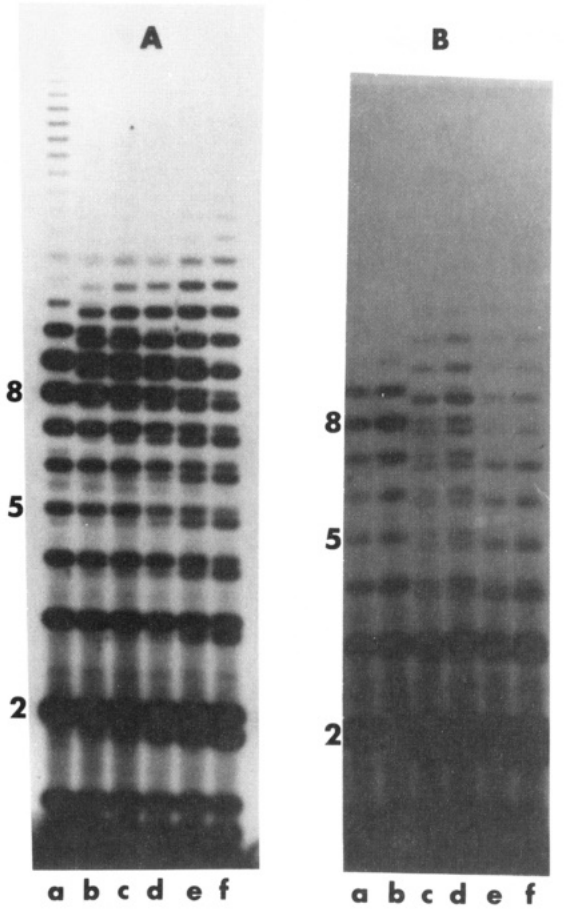



FIGURE 1: Effect of araATP on primers. (A) Primers were synthesized with 100 $\mu$M [$\alpha$-$^{32}$P]ATP and 0 $\mu$M araATP (lane a), 1 $\mu$M araATP (lane b), 2 $\mu$M araATP (lane c), 4 $\mu$M araATP (lane d), 10 $\mu$M araATP (lane e), and 20 $\mu$M araATP (lane f). In lane a, the longer products (ca. 16–19 nucleotides long) are primer-dimers. (B) Primers were synthesized with [$\gamma$-$^{32}$P]ATP and 0 (lanes a and b), 5 (lanes c and d), or 20 $\mu$M araATP (lanes e and f). Lanes a, c, and e are the products after 30 min, and lanes b, d, and f are after 60 min.

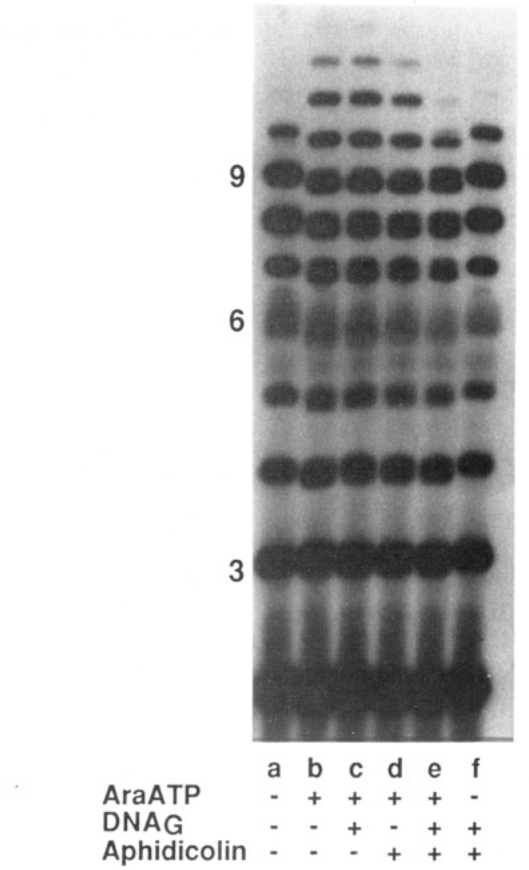



FIGURE 2: Effect of inhibiting pol $\alpha$ on araATP incorporation into primers. Assays were performed as described under Materials and Methods. Each assay contained 100 $\mu$M [$\alpha$-$^{32}$P]ATP. The presence of araATP (5 $\mu$M), aphidicolin (100 $\mu$M), and $DNA_G$ (15 $\mu$M) was as noted.

presence of araATP were greatly altered. First, addition of araATP resulted in new products >9 nucleotides long. These were not the usual double-length primers (primer-dimers), which are typically 16–20 nucleotides. Second, a smaller fraction of products were ≥7 nucleotides long. With 100 $\mu$M ATP and no araATP, 15% of the products were at least 7 nucleotides long, while only 9% of the products were this long in the presence of 6 $\mu$M araATP.

Most important, however, was the appearance of bands of altered mobility that migrated slightly faster than the normal primer. These new products first appear for the 9- and 10-mer (lanes b and c) but eventually appear even for the 2-mer (lane f) as the araATP concentration increased. The fraction of each length primer that is present as the new, higher mobility species increased as the araATP concentration increased.[2] Thus, primase incorporated araATP into the primers. We considered the possibility that the new species were due to a large decrease in mobility of the normal length product. However, observation of a new product that migrated faster than the 2-mer requires that these new products are of increased mobility compared to the normal product.

We examined the unlikely possibility that the pol $\alpha$ was actually responsible for incorporation of araATP. Two lines of evidence ruled this out. First, products containing araAMP were observed for primers as short as the 2-mer, while we previously demonstrated that pol $\alpha$ will not elongate primers less than 7 nucleotides long (Kuchta et al., 1990). Second, we inhibited the polymerase and found that the araAMP-containing products were still formed (Figure 2). Addition of aphidicolin (100 $\mu$M) and a synthetic template such as $DNA_G$ (15 $\mu$M) resulted in >95% inhibition of pol $\alpha$ but did not decrease primase activity (lanes a and f and unpublished results). Inhibition of the polymerase in this manner did not inhibit the formation of the new products 2–10 nucleotides long (lane e). Therefore, incorporation of araATP into these primers is due to the primase, not the polymerase. However, inhibition of pol $\alpha$ prevented formation of new products 11–13 nucleotides long. The implications of this will be discussed later.

TCCATATCACAT$^{3'}$
AGGTATAGTGTAGATCT TATCTCT
$DNA_G$

AraATP was not incorporated solely into the 5′ terminus of the primers. Primers were synthesized with [$\gamma$-$^{32}$P]ATP and araATP (Figure 1B), and the araAMP-containing products were still observed. Only the 5′-terminal nucleotide retains the $\gamma$-phosphate; thus, araATP must be incorporated into internucleotide linkages and/or at the 3′ terminus. These data do not, however, rule out the possibility that some of the primers contain an araATP at the 5′ terminus.

DNA pol I (*E. coli*) has been reported not to elongate primers containing a 3′-terminal araNMP (Suzuki et al., 1989). The ability of pol I (Klenow fragment) to elongate

[2] The resolution of the araAMP-containing primers and the normal primers was not sufficient to accurately quantify the relative amounts of each.

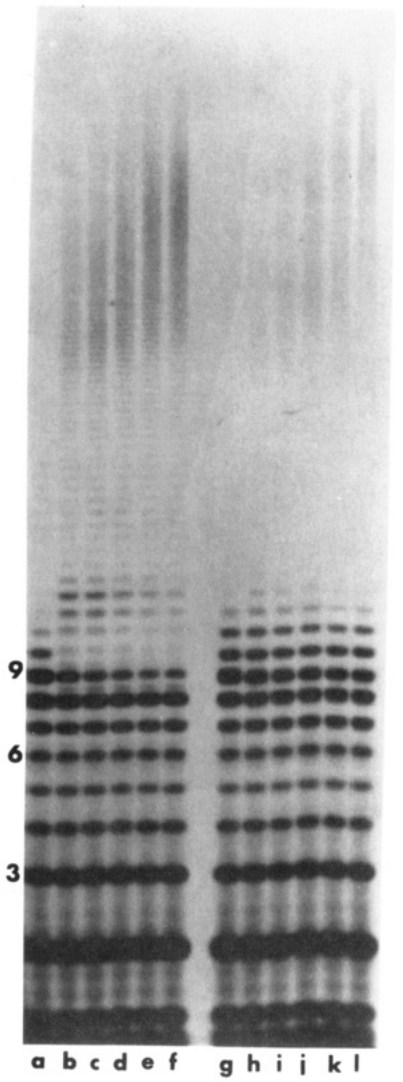



FIGURE 3: Klenow fragment is unable to elongate primers containing araAMP. Assays were performed as described under Materials and Methods and contained 0 $\mu$M araATP (lanes a–f) or 2 $\mu$M araATP (lanes g–l). Products were analyzed prior to addition of Klenow (lanes a and g) and then 1 min (lanes b and h), 2 min (lanes c and i), 5 min (lanes d and j), 10 min (lanes e and k), and 15 min (lanes f and l) after addition of Klenow Fragment.

primers synthesized in the presence and absence of araATP was measured (Figure 3). The elongation reaction by Klenow fragment was measured at 20 °C in order to enhance the stability of the RNA·DNA duplexes. While it readily elongated primers that comigrated with "normal" primers, consistent with these primers not containing an araAMP at the 3′ terminus, it was unable to elongate araAMP-containing primers, suggesting that these products contain an araAMP at or near the primer 3′ terminus. It is also of interest that Klenow was only able to extend those primers at least 9 nucleotides long. This is further support for the conclusion that elongation of primers by pol I is a poor method for measuring primase activity (Kuchta et al., 1990; Suzuki et al., 1989). The difference between our data and previous data reporting that primers must be ca. 15 nucleotides to be extended by pol I at 37 °C is likely due to our extension assays being performed at 20 °C (Suzuki et al., 1989).

*Primer Elongation by Pol $\alpha$.* In the presence of ATP and dATP, pol $\alpha$ is only capable of utilizing primers $\geq$7 nucleotides long, even at elevated dATP concentrations (Kuchta et al., 1990). Additionally, at 37 °C the polymerase elongates only some of these primers, while at 25 °C it utilizes virtually all

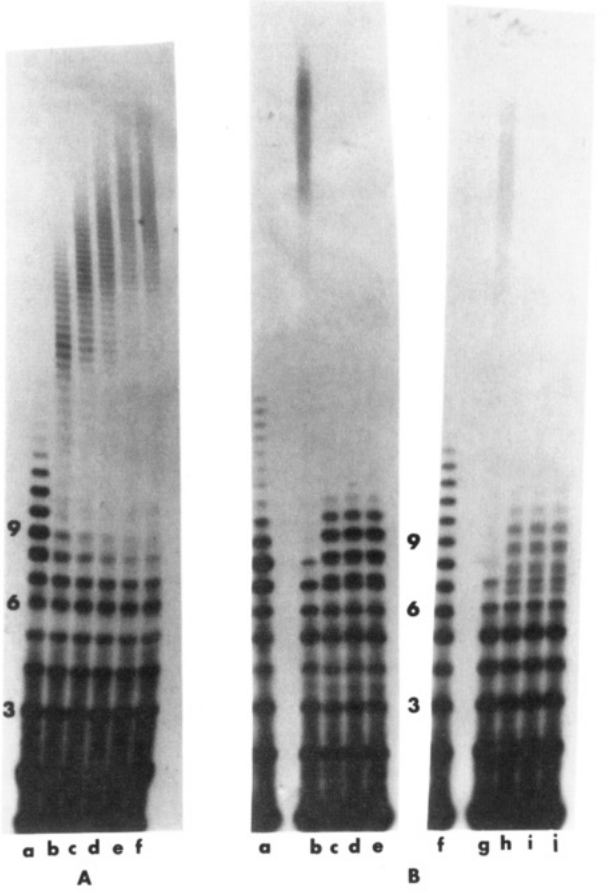



FIGURE 4: Utilization of primers by pol $\alpha$. (A) Assays were performed at 37 °C as described under Materials and Methods. They contained 100 $\mu$M [$\alpha$-$^{32}$P]ATP, 5 $\mu$M araATP, and 0 $\mu$M dATP (lane a), 5 $\mu$M dATP (lane b), 10 $\mu$M dATP (lane c), 15 $\mu$M dATP (lane d), 20 $\mu$M dATP (lane e), or 25 $\mu$M dATP (lane f). (B) Pol $\alpha$ elongated primers were synthesized in assays containing 100 $\mu$M [$\alpha$-$^{32}$P]ATP and 25 $\mu$M dATP and then treated with DNase I as described under Materials and Methods. Lane a shows the products in the absence of dATP. Lane b shows the products prior to DNase treatment, and lanes c–e are 15, 30, and 45 min after addition of DNase. Lanes f–j correspond to lanes a–e, except the reactions also contained 10 $\mu$M araATP.

of those primers of sufficient length (Kuchta et al., 1990). Figure 4A shows utilization of primers at 37 °C with increasing dATP concentration. As expected, increasing the dATP concentration increased the fraction of primers elongated. Impressively, the polymerase utilized both the normal and araAMP-containing primers. Inclusion of araATP did not, however, allow the polymerase to utilize primers less than 7 nucleotides long. Thus, araATP does not affect which primers pol $\alpha$ can elongate.

AraATP can, however, affect the fraction of primers elongated by pol $\alpha$. The fraction of primers elongated by the polymerase at 37 °C was measured at constant dATP and increasing araATP (Table II). Increasing araATP decreased the fraction of primers utilized such that with 25 $\mu$M araATP and 25 $\mu$M dATP the fraction of primers elongated decreased 40%. This is also consistent with the ability of pol $\alpha$ to elongate primers being dependent upon the rate of polymerization, as was previously suggested by increased primer utilization with increasing dATP concentrations (Kuchta et al., 1990). In contrast, at 25 °C increasing araATP had almost no effect on the fraction of primers utilized—ca. 90% were elongated with 25 $\mu$M araATP and 25 $\mu$M dATP (Table II). Similarly, with 25 $\mu$M araATP and 10 $\mu$M dATP, conditions where the pol $\alpha$ should retain only 15% activity, the fraction of primers elongated remained near 90%.

Table II: Fraction of Primers Elongated by the Polymerase as a Function of araATP Concentration

| | fraction elongated[a] | | |
|---|---|---|---|
| | 25 μM dATP | | 10 μM dATP |
| [araATP] (μM) | 37 °C | 25 °C | 25 °C |
| 0 | 0.62 | 0.94 | 0.85 |
| 2.5 | 0.64 | 0.94 | 0.92 |
| 5 | 0.59 | 0.90 | 0.93 |
| 10 | 0.52 | 0.89 | 0.90 |
| 15 | 0.50 | 0.88 | 0.89 |
| 20 | 0.42 | 0.86 | 0.85 |
| 25 | 0.37 | 0.88 | 0.88 |

[a] Assays were at the temperatures noted and contained 100 μM [α-$^{32}$P]ATP. Reactions were quenched with gel loading buffer, and the fraction elongated was determined as described previously (Kuchta et al., 1990).

Inclusion of dATP also obviated accumulation of primers greater than 10 nucleotides long (Figure 4A). This could be due to either that the pol α very efficiently elongates them or that they are no longer produced. To differentiate these possibilities, we synthesized polymerase-elongated primers with [α-$^{32}$P]ATP and unlabeled dATP; hence, the primer moiety was labeled. The products were treated with DNase I to hydrolytically degrade the DNA and subjected to electrophoresis followed by autoradiography. Treatment with DNase I will leave 1–2 dNMPs attached to the primer 3′ terminus, thus giving rise to labeled oligonucleotides 1–2 nucleotides longer than the primers actually utilized by pol α (Hu et al., 1984). Identical products were obtained regardless of araATP inclusion (Figure 4B), indicating that only primers 7–10 nucleotides are being utilized by pol α in the presence of araATP and that dATP prevents formation of the products greater than 10 nucleotides. Since dATP prevented formation of primers greater than 10 nucleotides long, it was unclear if pol α could actually polymerize dNTPs onto them. Pol α·primase was incubated with only araATP and [α-$^{32}$P]ATP to generate these longer primers, and then dATP was added. The primers greater than 10 nucleotides long rapidly disappeared, and longer products appeared (data not shown), indicating that pol α readily polymerizes dATP onto the abnormally long primers.

Inhibition of pol α by $DNA_G$ and aphidicolin obviated synthesis of the "primers" 11–13 nucleotides long (Figure 2). Preventing formation of these longer products also increased the amount of the 8–10 nucleotide long products that correspond to "normal" primers. Pol α does not polymerize NTPs; hence, these long products likely resulted from polymerization of an araATP onto the RNA primer. This also suggests that these primers now contain a 3′-terminal araAMP and thus that pol α will polymerize dATP onto an *RNA* primer containing a 3′-terminal araAMP.

Elongation of RNA primers containing a 3′-terminal araAMP was further examined. The primers formed in a primase assay containing only [α-$^{32}$P]ATP and poly(dT) were purified by EtOH precipitation, and then araATP and pol α·primase were added. This resulted in the simultaneous disappearance of the normal primers and formation of araAMP-containing products (Figure 5, lanes a and b). Addition of only pol α·primase (±100 μM ATP) did not give the new products, and inhibition of pol α with aphidicolin and $DNA_G$ also inhibited formation of these new products (not shown). These data indicate that pol α will polymerize araATP onto RNA primers. The araAMP-containing products 3–7 nucleotides long were due to primase polymerizing araATP onto primers (not shown). Finally, we added dATP to the reactions containing the araAMP-terminated primers

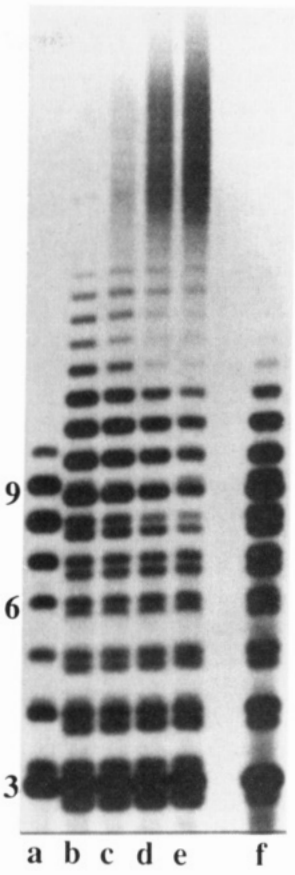



FIGURE 5: Elongation of 3′-araAMP-terminated primers by pol α. The products of a primase assay (100 μM [α-$^{32}$P]ATP) were EtOH precipitated [75% EtOH and 0.3 M NaOAc (Maniatis et al., 1982)] and then resuspended in 10 mM Tris, pH 7.4. These primers were then incubated with 20 μM araATP and pol α·primase for 15 min at 37 °C, at which time dATP was added to a final concentration of 25 μM. Lane a shows the primers after EtOH precipitation. Lane b is the primers after reaction with araATP, and lanes c–e are the products 1, 5, and 10 min after addition of dATP. Lane f shows the primers formed in an assay containing 100 μM [α-$^{32}$P]ATP, 4 μM araATP, and 50 μM poly(dT).

and observed rapid elongation of the primers (Figure 5, lanes c–e). Thus, pol α can elongate araAMP-terminated RNA primers.

*Pol α Inhibition by AraATP.* We measured pol α inhibition by araATP using a poly(dT)·oligo$(A)_{12-18}$ substrate. Inhibition was competitive with dATP, and $K_i$ = 0.9 μM (data not shown). The $K_M$ for dATP was 3.0 μM. These values are similar to previously measured values (Dicioccio & Srivastava, 1977; Parker et al., 1988).

*AraATP Potently Inhibits Primase.* Reported values for the $K_i$ of araATP with primase vary from 20 to 30 μM with either use of a filter binding assay or measurement of incorporation of dNTPs onto primers by DNA pol I (Parker & Cheng, 1987; Yoshida et al., 1985; L. Willhelm and R. Kuchta, unpublished data). However, when we measured primase-coupled pol α activity, we obtained $K_i$ = 1.2 μM, and inhibition was competitive with ATP, consistent with primase inhibition. In the previous measurements it is unclear what length primers were actually measured by the filter binding or pol I extension assays. Since only primers ≥7 nucleotides long are actually competent to be utilized by pol α, only these products should be considered for kinetic measurements. We explicitly measured araATP inhibition of ATP polymerization into primers ≥7 nucleotides long at 25 °C (not shown). Competitive inhibition with respect to ATP was observed, and the $K_i$ was 2.0 μM, substantially less than previous values. This lower $K_i$

likely reflects the fact that inclusion of araATP results in increased production of primers less than 7 nucleotides long (Figure 1). Similar data were obtained at 37 °C, where the $K_i$ was 2.7 $\mu$M. These values for the $K_i$ of araATP on primase alone are very similar to that measured for the coupled assay ($K_i$ = 1.2 $\mu$M) and are consistent with primase as the inhibited enzyme in the coupled assay.

## Discussion

The results presented here show that araATP is a potent inhibitor of DNA primase and that primase incorporates araATP into primers. The $K_i$ for araATP was 2–2.7 $\mu$M, substantially less than the $K_M$ for ATP [100 $\mu$M (Kuchta et al., 1990)]. It is also much less than previously reported $K_i$s for primase (20–30 $\mu$M) and raises the question of the true in vivo target of the arabinofuranosyl nucleotides—primase or pol $\alpha$. Pol $\alpha$ has been considered the target on the basis of inhibition of DNA synthesis and the lower $K_i$ for pol $\alpha$ (Parker & Cheng, 1987; Yoshida et al., 1977). The data presented here show that the $K_i$s for pol $\alpha$ and primase are similar when one considers only those primase products that are competent for elongation by pol $\alpha$.

Ultimately, the most physiologically relevant parameter is the rate of dNTP polymerization coupled to primer synthesis. Two distinct conditions were considered: (i) 37 °C, the physiological temperature but where the polymerase is less efficient at utilizing primers synthesized by primase; (ii) 25 °C, conditions where the polymerase utilizes almost all of the competent primers synthesized even at low (2.5 $\mu$M) dATP concentration (Kuchta et al., 1990). We do not know which conditions more closely represent the situation in vivo. At 25 °C, araATP inhibited the coupled reaction competitively with ATP and gave a $K_i$ (1.2 $\mu$M) that was similar to the $K_i$ for primer synthesis alone (2.0 $\mu$M). With 10 $\mu$M dATP and 3 $\mu$M araATP, increasing the dATP concentration did not decrease inhibition (Table I). Increasing the dATP concentration reduced the extent of inhibition only when the dATP and araATP concentrations were similar (e.g., 10 $\mu$M of each). Importantly, the nucleotide concentrations used for these experiments were very similar to the in vivo concentrations of CTP [ca. 100–300 $\mu$M (Bucher & Swaffield, 1966; Jones, 1980)] and dCTP [ca. 10–30 $\mu$M (Colby & Edlin, 1970; Nicander & Reichard, 1985)][3] —the implications of this are discussed below. These data indicate that under conditions where pol $\alpha$ activity is dependent upon primase activity araATP primarily inhibits primase.

While these in vitro data support primase as the in vivo target enzyme for the arabinofuranosyl nucleotides, it is impossible to know how accurately they reflect in vivo reality since (i) genomic DNA is not poly(dT) and (ii) other proteins likely interact with the pol $\alpha$-primase complex and may change its catalytic properties. Several in vivo results also raise the possibility that primase, not pol $\alpha$, is the actual target. Bell and Fridland found that treatment of growing cells with araC very effectively prevented formation of new short DNA molecules but only secondarily inhibited elongation of preexisting DNA polymers (Bell & Fridland, 1980; Fridland, 1977). This suggests that araC interrupts DNA replication at a very early stage, such as during primer synthesis, with smaller effects upon ongoing DNA synthesis catalyzed by DNA polymerase. More intriguing, however, are the differences between araA and araC cytotoxicity. AraATP and araCTP are equally potent inhibitors of pol $\alpha$ [$K_i$s = 1–3 $\mu$M (Dicioccio & Srivastava, 1977; Parker et al., 1988)] and are competitive with dATP and dCTP, respectively. Similarly, previous data have indicated that araATP and araCTP are equivalent inhibitors of primase ($K_i$s of 20–30 $\mu$M) and are competitive with ATP and CTP, respectively. We have demonstrated that these data for araATP understate the potency of inhibition, and it seems likely that previous araCTP data also understate its potency. Curiously however, araATP is much less effective as a DNA synthesis inhibitor than araCTP in vivo even though araATP and araCTP have equal $K_i$s for both pol $\alpha$ and primase. Equal amounts of DNA synthesis inhibition were obtained only when the intracellular araATP concentration was 16-fold greater than the araCTP concentration (Bell & Fridland, 1980). If DNA synthesis inhibition were due to pol $\alpha$ inhibition, one would have expected araATP and araCTP to have similar properties in vivo since they have equal $K_i$s for pol $\alpha$ and the concentrations of dATP and dCTP are similar (Bucher & Swaffield, 1966; Colby & Edlin, 1970; Jones, 1980; Nicander & Reichard, 1985).[4] However, if the in vivo target is primase, then araCTP should be much more inhibitory than araATP since the ATP concentration is typically 10–20-fold greater than the CTP concentration (Bucher & Swaffield, 1966; Colby & Edlin, 1970; Jones, 1980; Nicander & Reichard, 1985). Ten- to twentyfold less araCTP than araATP should therefore be required to obtain equal amounts of inhibition—the observed result (Bell & Fridland, 1980). Our in vitro experiments were at nucleotide concentrations similar to those found for dCTP and CTP in vivo and clearly showed that primase inhibition was the cause of decreased primase-coupled polymerase activity. On the basis of these in vivo data and the in vitro data described herein, we believe that DNA primase inhibition is the root cause of araA/C-induced cytotoxicity and pol $\alpha$ inhibition contributes only secondarily. Experiments to further test this are in progress.

While the $K_i$s of araATP for primase and pol $\alpha$ are similar, araATP is a more potent inhibitor of primase than of pol $\alpha$. The potency of inhibition is dependent upon both the $K_i$ of the inhibitor and the $K_M$ for the normal substrate. Thus, for pol $\alpha$ $K_M/K_i$ = 3, while for primase $K_M/K_i$ = 50 (Kuchta et al., 1990).

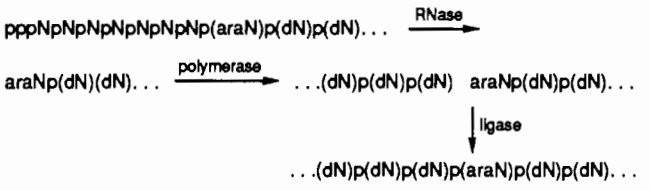
Under conditions where the ability of pol $\alpha$ to elongate primers is dependent upon dATP concentration [37 °C (Kuchta et al., 1990)], inclusion of araATP reduced the ability of the polymerase to elongate primers (Table II). Increasing the dATP concentration would likely increase the rate of polymerization onto the primers at the 3′ terminus, hence at 37 °C the ability of pol $\alpha$ to utilize primers is dependent upon the rate of dATP polymerization. Thus, compounds that inhibit the polymerase would be expected to inhibit the switch, as indeed was observed. At 25 °C the switch is independent of dATP concentration with virtually all primers greater than 6 nucleotides long utilized by pol $\alpha$ (Kuchta et al., 1990). Consistent with the switch now being independent of the dATP polymerization rate, araATP had no effect on the fraction of primers utilized.

It is unclear at what positions the araATP is incorporated into primers. That we were able to label the araA-containing products with [$\gamma$-$^{32}$P]ATP precludes the possibility that all

[3] The exact concentrations of nucleotides in vivo are not known, and there is substantial variation in the reported values. This may be a function of cell type, cell cycle, and experimental methodology. Additionally, there may be compartmentalization of nucleotides within the cell. Thus, the values stated should be considered approximate.

[4] Nicander and Reichard (1985) reported dCTP levels in 3T6 mouse fibroblasts were 7-fold greater than dATP. This would further enhance the cytotoxicity of araATP compared to that of araCTP if pol $\alpha$ were the in vivo target of araCTP.

Scheme I




of the araA is at the primer 5′ terminus (Figure 1B); therefore, araAMP must be located at internucleotide positions and/or the 3′ terminus. Pol I (Klenow fragment) was unable to elongate those primers that contained araAMP, suggestive of araAMP at the 3′ terminus. While pol I (Klenow fragment) is unable to elongate DNA primers containing a 3′-terminal araAMP (Yoshida et al., 1985; Mikita & Beardsley, 1988), it is unknown if pol I can elongate the equivalent RNA primers.

This question is particularly pertinent since treatment of cells with araNTPs results in most of the araNMP located at internucleotide positions, not at 3′ termini (Major et al., 1981, 1982). However, both pol $\alpha$ and pol $\delta$ have been reported to only slowly elongate DNA primers containing a 3′-terminal araNMP (Lee et al., 1980; Mikita & Beardsley, 1988; Reid et al., 1988). Thus, the problem arises of how to incorporate araNMP into internucleotide linkages if neither pol $\alpha$ nor pol $\delta$ will readily elongate DNA primers containing a 3′-terminal araNMP. If pol $\alpha$ is capable of elongating *RNA* primers containing 3′-terminal araNMPs, this would provide a mechanism for incorporation into internucleotide positions (Scheme I). The RNA portion of the primer would be removed by RNase H, the gap filled in by DNA polymerase, and then the 5′-araNMP-terminated DNA strand ligated to the adjoining strand.

While pol $\alpha$ cannot elongate DNA primers containing a 3′-terminal araNMP, our data indicate that pol $\alpha$ readily elongates RNA primers containing a 3′-terminal araAMP (Figure 5). This indicates that the model in Scheme I is feasible. Pol $\alpha$ also polymerizes araATP onto RNA primers (Figures 2 and 5), and pol $\alpha$, not primase, is responsible for the products 11–13 nucleotides long formed in primase assays. We also considered the possibility that the products 11–13 nucleotides long were abortive primer-dimers (i.e., primers 16–20 nucleotides long). This is unlikely since inhibition of pol $\alpha$ with aphidicolin and $DNA_G$ does not inhibit primer-dimers (unpublished results).

DNA primase would be an ideal target for cancer chemotherapeutics. It is absolutely required for DNA replication, and as far as is known, it is only involved in DNA replication. The data presented herein suggest that at least one chemotherapeutic, araC, likely inhibits DNA replication via primase inhibition. Additionally, nucleotide analogues that are designed to inhibit primase should be cytosine analogues since CTP is the least concentrated NTP in vivo (Bucher & Swaffield, 1966; Colby & Edlin, 1970; Jones, 1980; Nicander & Reichard, 1985).

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