Inhibition of DNA Primase and Polymerase α by Arabinofuranosylnucleoside Triphosphates and Related Compounds†

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ABSTRACT: Inhibition of DNA primase and polymerase α from calf thymus was examined. DNA primase requires a 3′-hydroxyl on the incoming NTP in order to polymerize it, while the 2′-hydroxyl is advantageous, but not essential. Amazingly, primase prefers to polymerize araATP rather than ATP by 4-fold (k_{cat}/K_M). However, after incorporation of an araNMP into the growing primer, further synthesis is abolished. The 2′- and 3′-hydroxyls of the incoming nucleotide appear relatively unimportant for nucleotide binding to primase. Polymerization of nucleoside triphosphates by DNA polymerase α onto a DNA primer was similarly analyzed. Removing the 3′-hydroxyl of the incoming triphosphate decreases the polymerization rate >1000-fold (k_{cat}/K_M), while a 2′-hydroxyl in the ribo configuration abolishes polymerization. If the 2′-hydroxyl is in the ara configuration, there is almost no effect on polymerization. An araCMP or ddCMP at the 3′-terminus of a DNA primer slightly decreased DNA binding as well as binding of the next correct 2′-dNTP. Changing the primer from DNA to RNA dramatically and unpredictably altered the interactions of pol α with araNTPs and ddNTPs. Compared to the identical DNA primer, pol α discriminated 4-fold better against araCTP polymerization when the primer was RNA, but 85-fold *worse* against ddCTP polymerization. Additionally, pol α elongated RNA primers containing 3′-terminal araNMPs more efficiently than the identical DNA substrate.

A variety of nucleotide analogues inhibit DNA synthesis and are clinically useful. These include the arabinofuranosyl nucleosides arabinofuranosylcytosine (araC),[1] which is used to treat certain leukemias, and araA, which exhibits antiviral properties (Weil et al., 1980; Keeney & Buchanan, 1975). In each case, the active form of the compound is thought to be the triphosphate. Both compounds inhibit DNA replication in vivo, and this is a likely source of their cytotoxicity. AraA and araC are readily incorporated into DNA during replication, primarily at internucleotide linkages, but also at the 3′-terminus (Major et al., 1981, 1982). As the triphosphate, they potently inhibit several enzymes involved in DNA replication, including the DNA polymerases and DNA primase (Dicioccio & Srivastava, 1977; Lee et al., 1980; Kuchta & Willhelm, 1991). Thus, the araNTPs could potentially disrupt DNA replication by inhibiting initiation of new replicons (primase inhibition), by simple competitive inhibition with respect to a 2′-dNTP (polymerase inhibition), or via incorporation into the DNA and a large reduction in the rate of polymerization of the next 2′-dNTP (polymerase inhibition).

DNA primase and pol α are essential for DNA replication. The two activities copurify as a four-peptide complex (Kaguni et al., 1983; Brooks & Dumas, 1989). On single-stranded DNA, primase synthesizes short RNA oligomers that pol α then elongates via 2′-dNTP polymerization. While primase synthesizes products 2–10 nucleotides long, pol α only utilizes those primers at least 7 nucleotides long, both in vivo and in vitro (Kuchta et al., 1990; Kitani et al., 1984; Hay et al., 1984). Previously, we found that araATP is a more potent inhibitor of primase than of pol α. AraATP is readily polymerized into primers and results in increased synthesis of short primers that pol α cannot elongate. Pol α polymerizes dNTPs onto DNA primers containing an araNMP at the 3′-terminus much slower than if the primer contains a 2′-dNMP at the 3′-terminus (Mikita & Beardsley, 1988). Curiously, however, we observed that pol α polymerizes multiple araAMPs onto primase-synthesized RNA primers. These data raise the possibility that inhibitors may interact differently with pol α when the primer is RNA.

In addition to the araNTPs, several clinically useful nucleotide analogues contain modifications in the 2′- and 3′-hydroxyls. Included in this group are 3′-azido-3′-deoxythymidine and the dideoxy nucleotides, which exhibit potent anti-HIV activity (Larder et al., 1989). In these experiments, we further examine primase inhibition by araATP and related nucleotides, and also examine pol α inhibition. They provide insights into the role of the 2′- and 3′-hydroxyls during nucleotide binding and polymerization by each enzyme and show that RNA primers greatly modulate pol α inhibition by nucleotide analogues relative to DNA primers.

Experimental Procedures

Materials

Pol α was purified from calf thymus using immunoaffinity chromatography as described previously (Kuchta & Willhelm, 1991). One unit of pol α polymerizes 1 nmol of 2′-dNTP h^{-1} on activated calf thymus DNA at 37 °C, and 1 unit of primase polymerizes 1 nmol of ATP h^{-1} on a poly(dT) template. [^{3}H]AraAMP and [^{3}H]dATP were from Moravek Biochemicals, while all other radiolabeled compounds were from ICN. 3′-AraAMP was generously provided by Wayne Miller at Burroughs–Wellcome Corp. All chemicals were reagent grade

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[1] Abbreviations: araA, 9-β-D-arabinofuranosyladenosine; araC, arabinofuranosylcytosine; ddNTP, 2′,3′-dideoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid (Na salt); pol α, DNA polymerase α:primase; Tris, tris(hydroxylmethyl)aminomethane (HCl salt).

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Chart I: Synthetic Primer-Templates Used

DNA_G	TCC ATA TCA CAT (3') AGG TAT AGT GTA GAT CTT ATC ATC T
DNA_{42}	GCG CCG AAA (3') TTT CGC GGC TTT GTAAAA GTCCCC GTAAAA GTCCCC GTAAAA
RNA_{42}[1]	GCG CCG AAA (3') TTT CGC GGC TTT GTAAAA GTCCCC GTAAAA GTCCCC GTAAAA

[a] The primer strand is all RNA, while the DNA strand is all DNA.

or better. DNA oligonucleotides of defined sequence were obtained from Oligos, Etc., while the RNA primer was synthesized on an Applied Biosystems DNA synthesizer. Duplexes were formed as described previously (Kuchta et al., 1987), and are listed in Chart I. $(dT)_{25-30}$•$(rA)_{12-18}$ duplex was annealed in a 2:1 base ratio.

DNA_G containing a ddCMP polymerized onto the primer 3'-terminus was synthesized using Klenow fragment [(exo⁻), kindly provided by Dr. C. Joyce] and a 20-fold excess of ddCTP. DNA_G containing a 3'-terminal araCMP was synthesized similarly, except ddCTP was replaced with araCTP. DNA_G containing a 3'-terminal 2'-dCMP was synthesized using pol α and a 2-fold excess of 2'-dCTP. DNA was purified by EtOH precipitation (Maniatis et al., 1982). Each DNA was 5'-^{32}P-labeled and analyzed by gel electrophoresis followed by phosphorimagery to ascertain that all of the primer strand was elongated. Additionally, we added Klenow fragment (exo⁺) and four dNTPs to elongate each of the DNAs and show that all of the primer strand could be elongated (the exonuclease removes the 3'-terminal araCMP or ddCMP, and then the polymerase activity elongates it).

Methods

Unless noted, all assays contained 5 mM $MgCl_2$ and 50 mM Tris, pH 7.5, and were performed at 37 °C. Rates were measured under initial velocity conditions. Radioactivity incorporated into products was measured either by using a DE81 filter binding assay (Sheaff et al., 1991) or by polyacrylamide gel electrophoresis [18% acrylamide with the Tris/borate/EDTA buffer increased 1.5-fold in the gel, but standard concentration in the running buffer (Sheaff et al., 1991)] followed by phosphorimagery on a Molecular Dynamics PhosphorImager.

Pol α Activity. Pol α activity was typically measured in assays containing 50 μM poly(dT)•oligo(rA) (20:1 base ratio) and [α-^{32}P]dATP, and the amount of ^{32}P incorporated into DNA was measured. To obtain K_is, the data were analyzed using Dixon plots. To determine the relative k_{cat}/K_M for araCTP versus 2'-dCTP, assays contained 0.4 μM DNA_G (5'-^{32}P-labeled in the primer strand), 10 μM each of 2'-dNTP, and various amounts of araCTP. Assays were allowed to proceed until all starting material was elongated and then quenched by the addition of 3 parts gel loading buffer. Products were separated by gel electrophoresis, and the amount of primer elongated by 1 nucleotide and by >1 nucleotide was quantified by phosphorimagery. Incorporation of araCTP into the DNA resulted in a product that is not readily elongated by pol α. Thus, the primer elongated by one nucleotide represents polymerization of araCTP, and longer products reflect polymerization of 2'-dCTP opposite the template G. The relative k_{cat}/K_M can then be obtained as the x intercept from a plot of (fraction of primers containing 2'-dCMP)$^{-1}$ versus [araCTP]/[dCTP] (Fersht, 1985). Background levels for quantitation of the primer elongated by one nucleotide were obtained from reactions containing all four dNTPs and no araCTP, and the background for longer polymerization products was obtained from reactions where no dNTPs were present. Analogous procedures were used for determining the relative k_{cat}/K_M values for ddCTP versus 2'-dCTP, and araATP or ddATP versus 2'-dATP. In measurements of araNTP polymerization, control experiments showed that <3% of the araNMP-terminated primers were elongated under the experimental conditions. For rNTPs, the initial reactions were similar. However, unlike with the araNTPs and ddNTPs, polymerization of a rNTP by pol α would give a product that could be further elongated via 2'-dNTP polymerization. Thus, to discriminate between products that had an rNMP rather than a 2'-dNMP incorporated, we treated the reactions with 0.2 M Na_2CO_3 for 30 min at 95 °C to hydrolyze those molecules containing an rNMP prior to electrophoresis. Control reactions confirmed that these conditions were sufficient to completely hydrolyze oligo(rA).

Relative Polymerization Rates onto Primers Containing an AraNMP or 2'-dNMP at the Primer 3'-Terminus. The primer strand of RNA_{42} was 5'-^{32}P-labeled using polynucleotide kinase and [γ-^{32}P]ATP. The reaction mix was heated to 65 °C and the template strand added. After the reaction was allowed to slowly cool to room temperature, labeled RNA_{42} was purified by chromatography on Sephadex G-25 (30% EtOH in H_2O). RNA_{42} was lyophilized to dryness and resuspended in 50 mM Tris, pH 7.5.

An araCMP or 2'-dCMP was added to the 3'-terminus of the primer by incubating ca. 20 units of pol α, ca. 4 μM RNA_{42}, and 22 μM araCTP or 2'-dCTP in a final volume of 10 μL for 15 min at 37 °C. The reaction was heated to 65 °C for 5 min and allowed to slowly cool to room temperature. dATP (25 μM) and 2'-dTTP (25 μM) were added, and then polymerization was initiated via addition of pol α (final volume 15 μL). After various times at 37 °C, 1.5-μL aliquots were quenched with 10 μL of gel loading buffer. Products were separated by gel electrophoresis, and the amounts of starting material and products were quantified using phosphorimagery. Polymerization onto 2'-dCMP or araCMP-terminated DNA_{42} or DNA_G was measured similarly. For measurement of polymerization onto araAMP or 2'-dAMP-terminated primers, a similar strategy was employed except the dNTPs used were varied as required.

Primase Activity. Primase assays (10 μL) typically contained 50 μM poly(dT) and 100 μM [α-^{32}P]ATP (ca. 2000 cpm pmol^{-1}). Assays were quenched by the addition of 3 volumes of gel loading buffer (90% formamide). Products were separated by gel electrophoresis and visualized using phosphorimagery. The relative molar amount of each length product was calculated by correcting the amount of [^{32}P]AMP present in each primer for their different lengths. To calculate K_is, the amount of ^{32}P incorporated into primers ≥7 nucleotides long was measured. Data were analyzed via Dixon plots (v^{-1} versus [inhibitor]). The relative k_{cat}/K_M for araATP polymerization versus ATP polymerization was determined similarly to the k_{cat}/K_M measurements for pol α. Products from primase assays containing various ratios of [araATP]/[ATP] were separated by gel electrophoresis (20% acrylamide), and the amount of each product was determined by phosphorimagery. Assays contained 5 μM DNA_G and 200 μM aphidicolin to inhibit pol α. Since incorporation of an araATP into a primer results in termination, the amount of araAMP containing primers of length n+1 nucleotides represents the total amount of araATP polymerization onto the primer of length n. The total amount of polymerization of ATP onto the primer of length n is the sum of all other products ≥n+1 nucleotides long. The fraction of primers of length n where ATP was polym-

erized is given by (primers of length $\geq n+1$ nucleotides long where ATP was polymerized)/(total primers of length $\geq n+1$ nucleotides long). Then, $(k_{cat}/K_M)_{ATP}/(k_{cat}/K_M)_{araATP}$ is obtained from a plot of [fraction of primers where ATP was polymerized]$^{-1}$ versus [araATP]/[ATP].

Synthesis of [γ-^{32}P]dATP and [γ-^{32}P]AraATP. Unlabeled araATP (119 nmol) was incubated with 150 nmol of glucose and 0.6 unit of hexokinase in 50 μL of 5 mM $MgCl_2$ and 50 mM Tris, pH 7.4, for 60 min at 37 °C. TLC on PEI-cellulose (0.3 M NaP_i, pH 7.0) showed that all of the araATP was converted to araADP. The reaction was heated to 70 °C for 5 min to inactivate the hexokinase. The araADP was converted to [γ-^{32}P]araATP using [^{32}P]P_i and the method of Johnson and Walseth (1979). [γ-^{32}P]dATP was synthesized similarly, except we started with commercially available dADP.

Synthesis of [^{3}H]AraATP. [^{3}H]AraAMP (7.7 nmol, 13 Ci mmol^{-1}) was incubated with ca. 5 units of adenylate kinase (chicken muscle, Sigma), ca. 5 units of pyruvate kinase, 10 nmol of ATP, and 600 nmol of phosphoenolpyruvate in 100 μL of 5 mM $MgCl_2$ and 50 mM Tris, pH 7.5. After 90 min at 37 °C, activated charcoal was added. The charcoal was extensively washed with H_2O, and then [^{3}H]-araATP eluted with 60:2:38 EtOH:NH_3:H_2O (15% final yield) (Dr. D. Shewach, personal communication). TLC on PEI-cellulose showed that >95% of the [^{3}H] comigrated with authentic araATP.

Ratio of AraAMP to AMP in Primers. Primers were synthesized in assays (20 μL) containing 100 μM [α-^{32}P]ATP, 1.3 or 2.6 μM [^{3}H]araATP, 50 μM poly(dT), 10 mM dithiothreitol, 50 μM aphidicolin, and 4.5 μM DNA_G. Reactions were quenched by the addition of 10 μL of 50 mM EDTA and 20 μL of H_2O. Primers were separated from remaining ATP and araATP by chromatography on a 1-mL Sephadex G-25 column with 30% EtOH in H_2O as solvent. Primer-containing fractions were pooled and lyophilized to dryness. The residue was resuspended in 10 μL of H_2O and 30 μL of gel loading buffer. Primers were separated by gel electrophoresis (22% acrylamide) and located by autoradiography. Bands (0.5–1.0 cm^2) containing each primer were excised from the gel, and 1 mL of H_2O added. To speed elution of the primers from the gel, samples were subjected to two freeze–thaw cycles. Scintillation fluid (10 mL, National Diagnostics Econoscint) was added, and the amount of ^{3}H and ^{32}P in each sample was determined by scintillation counting (Packard 1600TR scintillation counter).

Location of AraAMP and 2′-dAMP in Primers. Primers were synthesized and purified from reactions identical to those described above for determining the ratio of araAMP/AMP in primers, except that the ^{32}P was 80 cpm pmol^{-1}. Primers were hydrolyzed to 3′-NMPs and nucleosides by treatment with 0.5 unit of spleen phosphodiesterase and 4 units of RNase T_2 (Sigma) for 30 or 60 min at 37 °C. Products were separated by TLC on silica gel with a solvent of iPrOH/NH_4OH/H_2O (63:30:7), and the amount of radioactivity was determined by cutting the TLC into small pieces and adding 0.5 mL of H_2O followed by 5 mL of scintillation fluid. Similar procedures were used to examine 2′-dAMP incorporation into primers, except reactions contained [^{3}H]2′-dATP instead of [^{3}H]araATP.

Binding of DNA and Nucleotides to Pol α. The K_D value of DNA was measured as a K_i as described previously (Sheaff et al., 1991). The K_d values for nucleotide binding to pol α·DNA complexes were measured using the equilibrium inhibition assay and associated kinetics (Sheaff et al., 1991).

Table I: Inhibition of Primase by NTPs

	K_i[a] (μM)	polymerization by primase
araATP[b]	2	yes
2′-dATP	15	yes
3′-dATP	45	no
ddATP	85	no
CTP	230	no
araCTP	45	yes (!)
2′-dCTP	500	no
3′-dCTP	300	no
ddCTP	580	no
ATP[b]	100 (K_M)	yes

[a] In each case, primase was assayed using a poly(dT) template (see Experimental Procedures). [b] Values for araATP and ATP are from Kuchta and Willhelm (1991).

The substrate DNA was $(dT)_{25-30}$·$(rA)_{12-18}$ (60 μM nucleotide), and polymerization of [α-^{32}P]dATP (20 μM) was measured. Assays contained 0–1.5 μM inhibitor DNA (e.g., DNA_G containing araCMP at the primer 3′-terminus) and various concentrations of the next correct nucleotide (dTTP or ddTTP). Control experiments demonstrated that the dATP did not inhibit polymerization of dCTP onto DNA_G (i.e., dATP binds very weakly to the pol α·DNA_G complex).

Results

Primase Inhibition by Nucleotides. We examined ATP analogues as primase inhibitors in order to understand the role of the 2′- and 3′-hydroxyls. For rate measurements, we only considered those primers at least seven nucleotides long to be products since pol α can only elongate these primers (Kuchta et al., 1990), and the function of primase is to synthesize primers that pol α can use. For each compound, competitive inhibition with ATP was observed (Table I). Inhibition was due both to a decreased total number of primers and to a decreased fraction of the primers reaching seven nucleotides in length (not shown). Interestingly, even though primase activity was always measured on a poly(dT) template, replacing the adenosine compound with the corresponding cytosine analogue had relatively little effect on the potency of inhibition, particularly if the analogue was not polymerized (3′-dATP versus 3′-dCTP, and ddATP versus ddCTP). Removal of either the 2′- or the 3′-hydroxyl decreased inhibition; hence, both the 2′- and 3′-hydroxyls are important for interacting with primase.

For those nucleotides that pol α also utilized,[2] we needed to rule out the possibility that observed inhibition of primase was due to interactions at the polymerase active site. We previously showed that addition of DNA_G and aphidicolin to pol α inhibits pol α activity via formation of a tightly bound pol α·DNA_G·aphidicolin ternary complex (Sheaff et al., 1991). Primase inhibition by araATP and ddATP, which are polymerized onto primers by pol α, was not altered when pol α was inhibited with aphidicolin and DNA_G. Since pol α rapidly elongated primers with 2′-dATP, the K_i for 2′-dATP in Table I was only measured with pol α inhibited.

Polymerization of Nucleotides by Primase. In addition to ATP, both araATP and 2′-dATP were incorporated into primers (Table I and Figure 1). Incorporation of each analogue resulted in products of altered electrophoretic mobility. Inhibiting pol α with DNA_G and aphidicolin did not prevent incorporation of the analogues into primers, indicating that primase was in fact responsible for their polymerization. 3′-dATP and ddATP were not polymerized into primers

[2] Pol α activity specifically refers to the DNA polymerase activity.

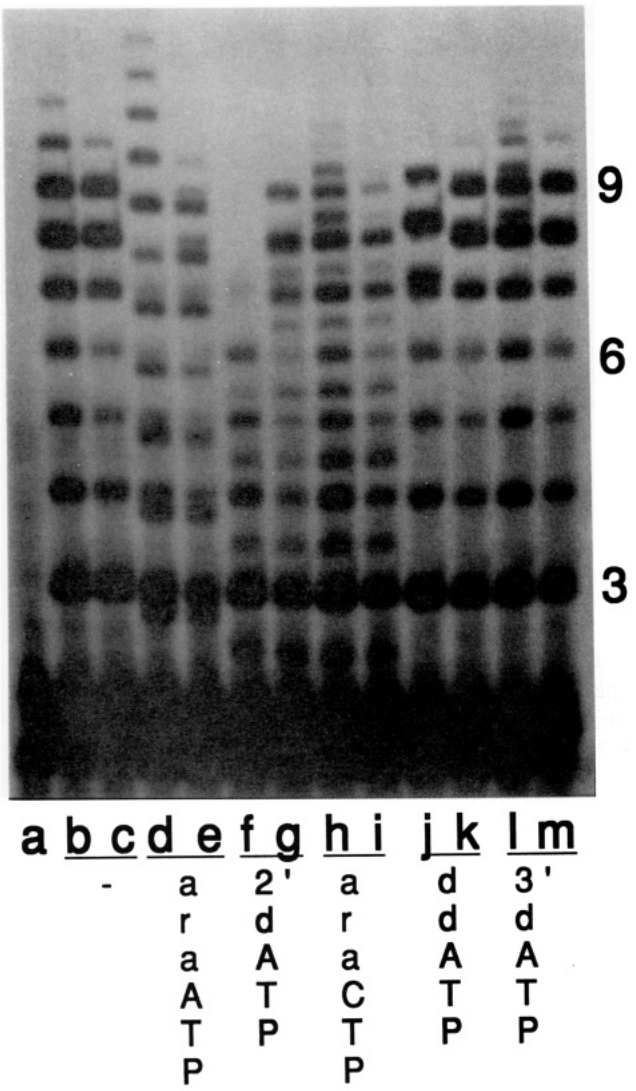


FIGURE 1: Polymerization of NTPs by primase. The length of primers is noted next to lane m. Assays contained pol α-primase, 50 μM poly(dT), 100 μM [α-^{32}P]ATP, and nothing (lanes b and c), 5 μM araATP (lanes d and e), 20 μM 2′-dATP (lanes f and g), 50 μM araCTP (lanes h and i), 100 μM ddATP (lanes j and k), or 50 μM 3′-dATP (lanes l and m). The assay in lane a lacked primase. Reactions for lanes c, e, g, i, k, and m also contained 100 μM aphidicolin and 7 μM DNA_G to inhibit pol α. In lane f, the absence of primers 7–10 nucleotides long is due to their elongation by pol α.

(Figure 1). When 3′-dATP was added to primase assays, new products of slightly lower mobility and corresponding to each of the 7–10-nucleotide-long primers were observed, indicating that 3′-dAMP was incorporated into products (lane l). However, there were no corresponding products for the 2–6-nucleotide-long primers, suggesting that pol α polymerized the 3′-dAMP onto the primers. This was confirmed by including DNA_G and aphidicolin to inhibit pol α (lane m), which eliminated these new products. Similarly, pol α polymerized ddATP onto primers; however, there was no detectable polymerization of ddATP by primase (lanes j and k).

With a poly(dT) template, there was no detectable polymerization of CTP, 2′-dCTP, 3′-dCTP, or ddCTP. This was shown by (i) no observed products of altered electrophoretic mobility when these analogues were included in assays, (ii) no detectable incorporation into primers of ^{32}P from [α-^{32}P]CTP or [α-^{32}P]2′-dCTP and, (iii) in assays containing [α-^{32}P]ATP and either 3′-dCTP or ddCTP, elongation of all primers 7–10 nucleotides long by pol α upon addition of 2′-dATP. Incorporation of 3′-dCTP or ddCTP would have resulted in a species that could not be elongated. Surprisingly, however, primase incorporated araCTP into primers as demonstrated by the appearance of products of altered electrophoretic mobility (Figure 1, lanes h and i). In order to test for pol α catalyzed elongation of araC-containing primers, primers were first synthesized in assays containing [α-^{32}P]ATP and araCTP, and then 2′-dATP was added. While primers of normal electrophoretic mobility were rapidly elongated, pol α was unable to elongate the araC-containing primers (not shown).

Table II: Relative k_{cat}/K_M for Polymerization of AraATP versus ATP by Primase

primer length[a]	$(k_{cat}/K_M)_{araATP}/(k_{cat}/K_M)_{ATP}$	primer length[a]	$(k_{cat}/K_M)_{araATP}/(k_{cat}/K_M)_{ATP}$
8	24	4	2.3
7	15	3	4.3
6	7.1	2	4.2
5	3.8		

[a] Primer length refers to the length of the primer to which either araATP or ATP is being polymerized.

Primase Prefers To Polymerize AraATP Rather than ATP. The selectivity for polymerization of araATP vs ATP by primase was measured. Incorporation of an araAMP into primers alters their electrophoretic mobility and, as will be shown below, results in chain termination. Thus, we can determine how frequently primase polymerizes araATP or ATP onto a given length primer as a function of [araATP]/[ATP], and hence selectivity. The relative $(k_{cat}/K_M)_{araATP}/(k_{cat}/K_M)_{ATP}$ for polymerization onto primers 2–8 nucleotides long is shown in Table II. Pol α can polymerize nucleotides onto primers ≥7 nucleotides long; thus, the significantly higher k_{cat}/K_M ratios for the longer primers may reflect small amounts of araATP polymerization by pol α, even though assays contained 200 μM aphidicolin and 5 μM DNA_G. From the shorter primers, however, primase clearly prefers to polymerize araATP rather than ATP.

Location of AraATP and 2′-dATP in Primers. Primase readily polymerized araATP; hence, we determined where in primers the araAMP was located. The number of araAMPs incorporated into each length primer was quantified by synthesizing primers from [α-^{32}P]ATP and [^{3}H]araATP. Primers were separated by gel electrophoresis, and the amount of ^{3}H and ^{32}P in each length product was determined. Importantly, 50 μM aphidicolin and 4.5 μM DNA_G were included to inhibit pol α. Primers 6–9 nucleotides long were analyzed. With 2.6 μM araATP, those primers that migrated identically to primers formed in the absence of araATP contained 0.08 ± 0.04 araAMP per primer, while primers of altered electrophoretic mobility contained 0.89 ± 0.1 araAMP per primer. Thus, incorporation of araAMP into a primer alters the electrophoretic mobility, and primase incorporates only one araAMP per primer. Similar data were obtained with 1.3 μM araATP—primers of "normal" electrophoretic mobility contained 0.03 ± 0.01 araAMP per primer, while those of altered electrophoretic mobility contained 0.7 ± 0.1 araAMP per primer.

The presence of one araA per primer suggested that it would be located at either the 5′- or the 3′-terminus. Only the 5′-terminal nucleotide of the primers retains the γ-phosphate of the NTP; hence, we used [γ-^{32}P]araATP to demonstrate that araATP was not incorporated at this position. In assays containing 100 μM ATP and 6.2 μM [γ-^{32}P]araATP, <1% of the primers containing araA had araATP as the 5′-terminal

Table III: Relative k_{cat}/K_M Values for Polymerization of Nucleotides by Pol α

	relative k_{cat}/K_M for template		
	DNA_G	DNA_{42}	RNA_{42}
2′-dATP vs araATP[a]	4		
2′-dATP vs 2′,3′-ddATP[a]	16000	15000	1200
2′-dATP vs ATP[a]	>20000		
2′-dCTP vs araCTP[a]	1.0	2.0	9.2
2′-dCTP vs ddCTP[a]	5000	1300	15
2′-dCTP vs CTP[a]	>20000		
2′-dCTP vs araCTP[b]		3.1	3.1
2′-dCTP vs ddCTP[b]		4900	>3000

[a] This measurement is for the first requirement for 2′-dATP or 2′-dCTP polymerization (noted with an asterisk in the sequence). DNA_{42} = TTT GCG CCG AAA G*T*AAAA GTCCCC GTAAAA GTC-CGC GGC TTT CCC GTAAAA. [b] This measurement is for the second requirement for 2′-dCTP polymerization in DNA_{42} or RNA_{42} (underlined in the sequence).

nucleotide. Control experiments demonstrated that the absence of [γ-^{32}P]araATP incorporation into primers was not due to a lack of primase activity.

To ascertain whether araAMP was located at the primer 3′-terminus, primers were again synthesized from [^{32}P]ATP and [^{3}H]araATP under conditions where pol α was inhibited with aphidicolin and DNA_G. After purification, primers were treated with RNase T2 and phosphodiesterase. Control experiments showed that these conditions were sufficient to completely degrade primers and that 3′-NMPs were not hydrolyzed to nucleosides. Cleavage of a phosphodiester bond by either enzyme generates a 3′-nucleotide and a compound with a free 5′-hydroxyl. Hence, if the araAMP is at the primer 3′-terminus, the hydrolytic product will be araA, while if it is at an internucleotide position, the product will be 3′-araAMP. Prior to enzyme treatment, the primers remained near the origin on silica gel TLC (R_f = 0–0.15). After treatment, <1% of the ^{3}H cochromatographed with 3′-araAMP (R_f = 0.56), while 92 ± 1% cochromatographed with araA (R_f = 0.82). The remaining ^{3}H chromatographed with ATP (3%, R_f = 0.13) or was not readily identifiable (3%). Together, these data indicate that primase incorporates one araAMP per primer and it is located solely at the primer 3′-terminus.

Similar to previous data with yeast primase (Brooks & Dumas, 1989), we observed 2′-dATP polymerization into primers, and incorporation results in products of altered electrohoretic mobility (Figure 1). When primers were synthesized with unlabeled ATP and [α-^{32}P]2′-dATP, only products of altered mobility contained ^{32}P (data not shown). Primers were synthesized using [γ-^{32}P]dATP and unlabeled ATP to examine the 5′-terminal nucleotide of primers. Similar to araATP, less than 1% of the 2′-dATP-containing primers contained 2′-dATP at the 5′-terminus. To determine if 2′-dATP polymerization results in primer termination, primers were synthesized with [^{3}H]2′-dATP, and treated with RNase T2 and phosphodiesterase. Assays contained 200 μM aphidicolin to inhibit pol α. After hydrolysis, 10% of the ^{3}H cochromatographed with 2′-deoxy-3′-AMP, suggesting that 2′-dATP polymerization by primase does not always result in chain termination.

Pol α Polymerization of Nucleotides. Pol α catalyzed polymerization of nucleotides was likewise examined. We compared the relative k_{cat}/K_M values for polymerization of 2′-dATP or 2′-dCTP versus several analogues (Table III). Removal of the 3′-hydroxyl or addition of a 2′-hydroxyl in the ribo configuration greatly decreased the ability of pol α to polymerize the nucleotide. The ability to discriminate against dNTPs lacking a 3′-hydroxyl is also apparent in inhibition studies, where ddATP is a weak competitive inhibitor with respect to 2′-dATP (K_i = 71 μM; data not shown). Surprisingly, pol α did not greatly differentiate between the 2′-dNTP and the araNTP. Parker et al. (1991) similarly observed that pol α did not discriminate between 2′-dATP and 2-fluoro-araATP.

In vivo, pol α must utilize both RNA and DNA primers. We therefore examined the possibility that the nature of the primer alters the ability of pol α to discriminate between araCTP and 2′-dCTP. Two identical primer-templates (RNA_{42} and DNA_{42}, Chart I) were synthesized, and $(k_{cat}/K_M)_{dCTP}/(k_{cat}/K_M)_{araCTP}$ was measured. Pol α discriminated against araCTP 4.5-fold better when the primer was RNA rather than DNA (Table III). However, after polymerization of six dNMPs onto the 3′-terminus of each primer, there was no detectable difference in the discrimination against araCTP.

Similarly, we measured discrimination of pol α against ddATP or ddCTP polymerization onto RNA_{42} or DNA_{42}. Surprisingly, pol α loses much of its ability to discriminate against ddCTP polymerization when the primer is RNA (Table III). Whereas with DNA_{42} the ratio of k_{cat}/K_M for ddCTP versus 2′-dCTP polymerization was 1300, with RNA_{42} the ratio decreased to 15! However, after six dNMPs were polymerized onto the RNA primer, pol α preferred 2′-dCTP >3000-fold, and with the identical all-DNA primer, pol α preferred 2′-dCTP by 4900-fold. Discrimination of pol α against ddATP was also measured on DNA_{42} and RNA_{42}. In this case, however, a 2′-dCMP needed to be polymerized onto both primers for these measurements. Again, there was less discrimination against ddATP polymerization when the primer was RNA (plus one 2′-dCMP) rather than all-DNA (Table III). However, whereas pol α discriminated 87-fold less effectively against ddCTP when the primer was all-RNA, pol α only discriminated 13-fold less effectively against the ddATP with the RNA (plus one 2′-dCMP) primer.

Previous work has indicated that pol α polymerization of an araNMP results in termination of the growing DNA strand (Mikita & Beardsley, 1988; Reid et al., 1988). However, we observed that with an *RNA* primer, pol α can first polymerize multiple araATPs and then polymerize 2′-dATP (Kuchta & Willhelm, 1991). This suggested that with an RNA primer, incorporation of an araNMP might not normally result in chain termination. Surprisingly, we found significant amounts of further polymerization after incorporation of araCMP into either DNA_{42} or RNA_{42}, while there was almost no polymerization after an araCMP into DNA_G (Figure 2). We therefore quantified the rate of 2′-dNTP polymerization onto primers containing either an araNMP or a 2′-dNMP at the primer 3′-terminus (Table IV).[3] Rates were measured under a single set of steady-state conditions. In each case, the rate of polymerization onto an araNMP-terminated primer was much less than the corresponding 2′-dNMP-terminated primer. There was a large variation in the rate of polymerization onto araCMP-terminated primers, indicating that the nature of the DNA and perhaps also the 2′-dNTP being polymerized affect the polymerization rate. In both cases examined, polymerization onto an araNMP-terminated RNA primer was significantly faster than polymerization onto the corresponding DNA primer (compare RNA_{42} and DNA_{42}).

How Does the Primer 3′-Terminal Nucleotide Affect Interactions with Pol α? Incorporation of a 2′-dNTP analogue

[3] Polymerization rates onto araAMP/dAMP-terminated primers were only measured with the araAMP/dAMP at the first possible site where it can be incorporated.

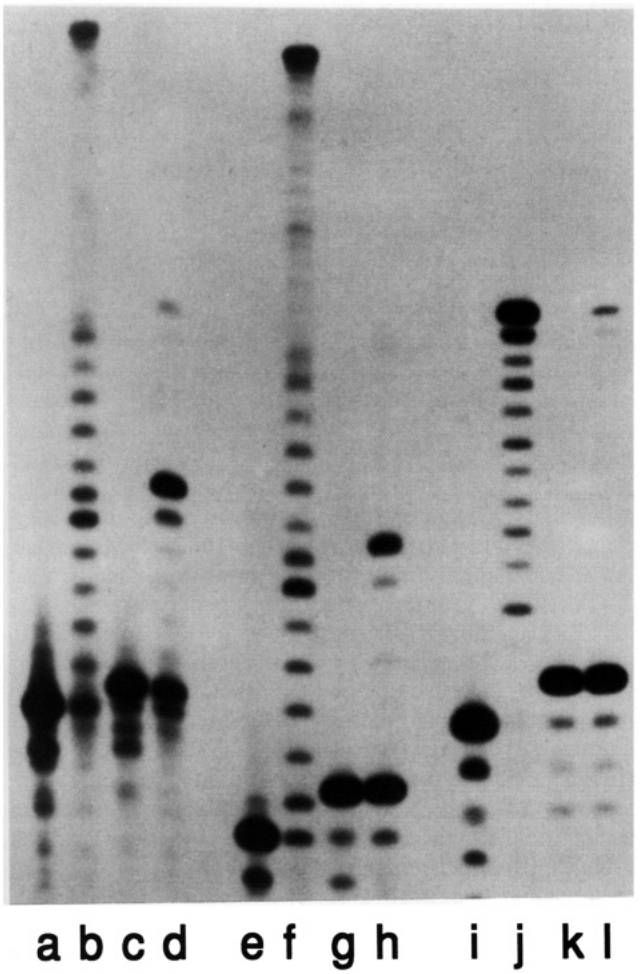


FIGURE 2: Elongation of DNA_G, DNA_{42}, and RNA_{42} containing araCMP at the 3′-terminus. Primer strands were 5′-^{32}P-labeled. Lane a is RNA_{42}, and lane b is RNA_{42} after incubation with pol α and four dNTPs. Lane c shows RNA_{42} after polymerization of an araCMP onto the 3′-terminus, and lane d shows the products after incubation of pol α and dATP, dGTP, and dTTP with the araCMP-elongated RNA_{42}. Lanes e–h and i–l correspond to lanes a–d, except the substrate was DNA_{42} in lanes e–h and DNA_G for lanes i–l. The major stop sites observed in lanes d and h correspond to the second site in each sequence where 2′-dCTP (araCTP) was required.

Table IV: Polymerization onto Template-Primers Containing either a 3′-Terminal AraNMP or 2′-dNMP

template-primer[a]	NMP at 3′-terminus	rate (pmol min^{-1})	rate with 2′-dNMP/ rate with araNMP
DNA_G	dAMP	5.3	41
	araAMP	0.13	
	dCMP	1.5	>250
	araCMP	<0.006	
DNA_{42}	dAMP	2.6	90
	araAMP	0.029	
	dCMP	2.4	114
	araCMP	0.021	
RNA_{42}	dAMP	1.2	17
	araAMP	0.069	
	dCMP	0.74	8.4
	araCMP	0.088	

[a] Primers containing the appropriate 2′-dNMP or araNMP at the 3′-terminus were synthesized by incubating pol α, DNA_G, DNA_{42}, or RNA_{42} and the appropriate dNTPs and araNTPs.

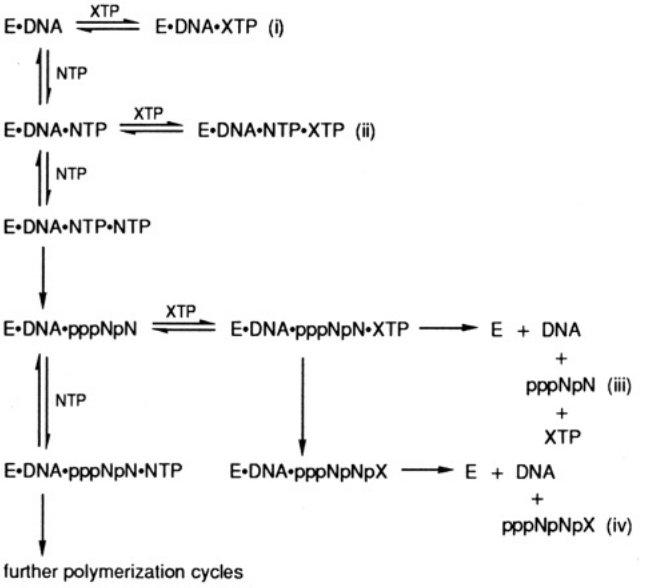
into the growing DNA strand could alter the binding properties of the DNA. We measured the K_D for the pol α·DNA species generated after polymerization of 2′-dCTP, araCTP, or ddCTP onto DNA_G. The K_D for the DNA_G with a 3′-terminal 2′-dCMP = 0.6 μM, similar to the K_D for DNA_G prior to addition of a 2′-dCTP [0.6 μM (Sheaff et al., 1991)]. After polymerization of araCTP, K_D = 1.5 μM, and after polymerization of ddCTP, K_D = 1.3 μM.

Table V: K_D for Nucleotide Binding to Various Pol α·DNA Binary Complexes

nucleotide at primer 3′-terminus[a]	K_D (μM) for dTTP	K_D (μM) for ddTTP
dCMP		250
araCMP	90	1500
ddCMP	35	500

[a] The DNA used was DNA_G elongated by one nucleotide (DNA_{G+C}). AraCMP and ddCMP were incorporated using KF (exo$^-$), and 2′-dCMP was incorporated using pol α. DNA_{G+C} = TCC ATA / AGG TAT TCA CAT C / AGT GTA GAT CTT ATC ATC T.

Scheme I



An additional mechanism by which incorporation of an araNMP or ddNMP could inhibit pol α activity is via formation of a pol α·DNA·dNTP ternary complex. Thus, binding of the next correct 2′-dNTP to pol α·DNA complexes was measured (Table V), where the DNA contained either a 5′-terminal araCMP or ddCMP. It is not possible to directly measure the K_D for 2′-dTTP binding to the pol α·DNA complex where the primer contains a 3′-terminal 2′-dCMP due to the rapid polymerization reaction. Thus, to estimate the effect of changing the 3′-terminal nucleotide from a 2′-dCMP to either araCMP or ddCMP, we determined the K_D for ddTTP binding to each pol α·DNA complex. The ddTTP bound only 2-fold weaker when the 3′-terminus was ddCMP rather than 2′-dCMP, suggesting that the 3′-hydroxyl on the primer is relatively unimportant for binding of the next correct 2′-dNTP. Additionally, as the primer terminus was changed from araCMP to ddCMP, the relative affinity for 2′-dTTP or ddTTP did not change.

Discussion

Primase Inhibition. DNA primase is an essential enzyme for DNA replication and, as far as is known, is only involved in DNA replication. As such, it would be an ideal target for chemotherapeutics aimed at growing cells. Pol α can only elongate primers at least seven nucleotides long; hence, primase inhibitors only need prevent primers from reaching this length.

As shown in Scheme I, NTP analogues (i.e., XTP) could inhibit primase by several mechanisms, including the following: (i) the inhibitor competes with the initial NTP that will form the primer 5′-terminus; (ii) the inhibitor competes with the second, or latter, NTPs that are added to the growing primer, thereby decreasing the rate of primer synthesis by direct competition; (iii) the inhibitor binds to the primase·DNA·primer ternary complex, and allows dissociation of the DNA template and/or the primer; (iv) the inhibitor is incorporated into the growing primer strand, and upon incorporation terminates further polymerization. Within this framework, we examined several NTP analogues.

For each compound tested, inhibition was reflected in a decreased total number of primers synthesized as well as an increased fraction of short primers. For those inhibitors not incorporated into primers, an increased fraction of short primers indicates that inhibition proceeds via mechanism iii while a decreased number of initiations could be due to either mechanism i or mechanism ii—we cannot discriminate between these two possibilities. Incorporation into primers with consequent termination (mechanism iv) is operative with araATP, 2′-dATP, and probably araCTP.

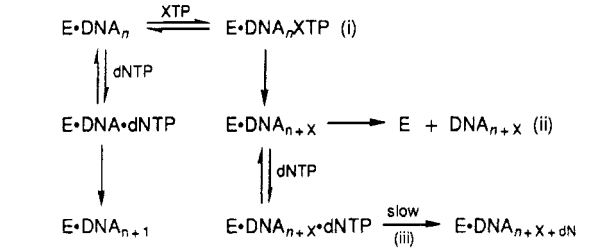
The 2′- and 3′-hydroxyls of the incoming NTP appear relatively unimportant for binding to primase. Inhibition due to just nucleotide binding to primase (mechanisms i–iii in Scheme I) only decreases 2-fold upon removal of the 2′-hydroxyl (compare 3′-dATP and ddATP, and CTP with 2′-dCTP; Table I), while removal of the 3′-hydroxyl had almost no effect (compare CTP with 3′-dCTP, and 2′-dCTP with ddCTP; Table I). Base complementarity also plays a relatively small role in inhibition of primase by NTPs. For those cases where the inhibitor was not incorporated into the primer (3′-dNTPs and ddNTPs), the noncomplementary cytosine analogue inhibited primase activity on poly(dT) only 7-fold less than the complementary adenosine analogue.

The 3′-hydroxyl of the incoming nucleotide is critical for polymerization, since neither 3′-dATP nor ddATP was polymerized. The 2′-hydroxyl is not essential since primase polymerized 2′-dATP. A most surprising feature of primase, however, is its *preference* for nucleotides containing a 2′-hydroxyl in the ara configuration, both for polymerization and for binding. With araATP, primase preferentially polymerized araATP by at least a factor of 4. Additionally, whereas primase did not incorporate CTP into primers on a poly(dT) template, small amounts of araCTP were incorporated.

In addition to preferentially polymerizing araNTPs, primase likely also prefers binding nucleotides with a 2′-hydroxyl in the ara configuration. In primase assays measuring araCTP incorporation, less than 10% of primers contained araCMP under conditions that gave 52% inhibition, indicating that primer termination via araCTP polymerization can only account for 20% of the inhibition. The majority of primase inhibition by araCTP must have been due to simple binding of araCTP at one of the NTP binding sites. However, since araCTP is a 5-fold better inhibitor than CTP, araCTP must bind tighter than CTP. This also suggests that araATP binds better than ATP. Primase must contain two NTP binding sites in order to catalyze the initial polymerization event (2ATP → pppApA), and araNTPs must bind to the site for incoming NTPs to account for their polymerization. However, it remains unknown if araNTPs can likewise bind to the site where the 5′-terminal NTP binds.

While araATP is polymerized by primase, primase will not use araATP as the 5′-terminal nucleotide of the primer. This is consistent with the inability of primase to elongate primers

Scheme II



containing a 3′-terminal araAMP, since for the initial polymerization event, the NTP that will become the primer 5′-terminus is simultaneously the 3′-terminus of the "growing primer". Similarly, 2′-dATP was not utilized as the 5′-terminal nucleotide. Unlike araAMP, however, 2′-dAMP was found both at the primer 3′-terminus and at internucleotide positions. After incorporation of 2′-dAMP into primers, primase appeared to polymerize further ATPs onto the 2′-dAMP residue. An alternative possibility is that after primase incorporated 2′-dAMP into primers, *pol α* then polymerized further 2′-dATPs, even though assays contained 200 μM aphidicolin to inhibit pol α. Using purified yeast primase, Brooks and Dumas (1989) also concluded that primase can incorporate 2′-dAMP into primers and then polymerize further ATPs.

Pol α Inhibition. Potential mechanisms by which a nucleotide analogue (e.g., XTP) could inhibit pol α are summarized in Scheme II and include the following: (i) direct competition with the 2′-dNTP for binding to the pol α·DNA complex; (ii) incorporation of the inhibitor into the DNA and resultant chain termination; (iii) incorporation of the inhibitor into the DNA followed by binding of the next correct 2′-dNTP to generate a stable pol α·DNA$_I$·dNTP complex. Initially, we will only discuss inhibition where the primer is DNA. Pol α discriminated minimally between polymerization of araCTP or araATP versus the corresponding 2′-dNTP [$(k_{cat}/K_M)_{dNTP}/(k_{cat}/K_M)_{araNTP}$]. Similarly, Parker et al. (1991) showed that pol α does not discriminate against 2-fluoro-araATP polymerization. This rapid polymerization of araNTPs indicates that simple competitive binding is relatively unimportant (mechanism i in Scheme II), since the pol α·DNA·araNTP complex will rapidly break down via either araNTP dissociation or polymerization.

Incorporation of araATP or araCTP into DNA resulted in a DNA that was only slowly elongated by pol α (mechanisms ii and iii in Scheme II), consistent with previous work (Mikita & Beardsley, 1988). The actual rate varied considerably as the DNA sequence was varied. That DNA sequence should cause such large changes in polymerization rates is consistent with studies on misincorporation of dNTPs by other DNA polymerases, where varying the DNA sequence can alter the misincorporation rate up to 1000-fold (Fersht et al., 1982; Kuchta et al., 1988; Lai & Beattie, 1988).

The slow rate of elongation was not due to the araNMP-containing DNA not binding to pol α. The K_D for DNA containing a 3′-terminal araCMP (1.5 μM) was similar to the identical DNA containing a 3′-terminal 2′-dCMP (0.6 μM), indicating that the 3′-hydroxyl at the primer terminus is not required for binding. Similarly, a 3′-terminal ddCMP did not greatly alter binding to pol α (K_D = 1.3 μM).

The presence of a ddNMP or araNMP at the primer terminus appears to have only small effects on the binding of the next correct 2′-dNTP to the pol α·DNA complex. The K_Ds for 2′-dTTP or ddTTP binding to DNA$_G$ containing araCMP at the 3′-terminus (DNA$_{G+araCMP}$) increased only 3-fold compared to DNA$_G$ containing ddCMP at the 3′-terminus

($DNA_{G+ddCMP}$). While we cannot directly measure the K_D for 2′-dTTP binding to DNA_G containing 2′-dCMP at the 3′-terminus (DNA_{G+dCMP}), we can estimate it to be ca. 17 μM. This estimate assumes that the ratio of the K_Ds for binding 2′-dTTP to DNA_{G+dCMP}, $DNA_{G+araCMP}$, and $DNA_{G+ddCMP}$ will be the same as for ddTTP binding to these DNAs. The ratio for ddTTP binding to $DNA_{G+araCMP}$ or $DNA_{G+ddCMP}$ was similar to the ratio of 2′-dTTP binding to $DNA_{G+araCMP}$ or $DNA_{G+ddCMP}$, suggesting it will also be true for ddTTP and 2′-dTTP binding to DNA_{G+dCMP} and $DNA_{G+ddCMP}$. Thus, removal of the 3′-hydroxyl from the primer terminus only modestly reduces binding of the next correct 2′-dNTP.

RNA Primers. The pol α·primase complex likely synthesizes at least the initial portion of Okazaki fragments in vivo; hence, pol α must frequently polymerize dNTPs onto RNA primers (Linn, 1991). We previously observed that on a poly(dT) template, pol α will polymerize multiple araAMPs onto a primase-synthesized oligo(A) primer (Kuchta & Willhelm, 1991), suggesting that pol α might interact differently with RNA primers than with DNA primers. Comparing identical RNA and DNA primer-templates (RNA_{42} and DNA_{42}), we found that with the RNA primer, pol α discriminated 4-fold better against araCTP during the polymerization reaction. Conversely, we observed the opposite effect with ddCTP—pol α discriminated 85-fold less well against ddCTP polymerization when the primer was RNA rather than DNA. As dNMPs were polymerized onto the RNA primer, the decreased discrimination against ddNTP polymerization and enhanced discrimination against araCTP polymerization disappeared. Polymerization of just one 2′-dNMP onto RNA_{42} mitigated the effect of the RNA primer, suggesting that the effects of the RNA primer decrease rapidly as the primer 3′-terminus moves away from the last NMP. However, since the nucleotide polymerized directly onto the RNA primer is 2′-dCTP and the next nucleotide is 2′-dATP, we cannot exclude the possibility that the reduced effects also reflect the different nucleotides. For primers containing an araNMP at the 3′-terminus, pol α used RNA primers more effectively than the identical DNA primers. Together, these data indicate that changing the primer from DNA to RNA results in large and unpredictable effects. Most data on purified pol α have been obtained on DNA primers, even though RNA primers are very important in vivo. For understanding how inhibitors function in vivo, particularly compounds such as ddATP or ddCTP that result in chain termination, the data with DNA primers potentially understate their effects on pol α.

General Comments. The araNTPs have been extensively studied, yet the actual mechanism by which they inhibit DNA replication in vivo remains unclear. Kufe and co-workers found that when cells are treated with arabinofuranosylnucleosides, araNMPs are incorporated into the DNA (Major et al., 1981, 1982), although only a small fraction of the araNMPs are found at the 3′-terminii of DNA. In vitro, however, incorporation of araNMPs into the DNA results in a species that pol α and pol δ can only poorly elongate (Lee et al., 1980; Mikita & Beardsley, 1988; Reid et al., 1988). Pol α elongates RNA primers containing a 3′-terminal araNMP much more readily than the corresponding DNA primer; hence, the large amounts of araNMPs found at internucleotide linkages in vivo may reflect their incorporation onto RNA primers and subsequent elongation by 2′-dNTP polymerization. Additionally, in vivo there are likely a large number of other proteins associated with the replicative DNA polymerases which may further modulate their ability to elongate primers containing 3′-terminal araNMPs.

AraATP is a potent inhibitor of both pol α and primase, raising the question of which activity is the target in vivo. Inhibition of either activity could result in reduced rates of DNA synthesis. Recently, Catapano et al. (1991) concluded that inhibition of DNA replication by araCTP is due to DNA polymerase inhibition, while 2-fluoro-araATP inhibits primase. It is curious that two compounds so closely related inhibit DNA synthesis via different pathways in vivo.

What makes RNA primers different? It is not solely due to a 2′-hydroxyl at the 3′-terminus since effects of the RNA primer are detectable after polymerization of a 2′-dNMP or araNMP onto the primer. The different structures of a RNA·DNA duplex (A-helix) and DNA·DNA duplex (B-helix) could be important (Chou et al., 1989; Hall & McLaughlin, 1991; Reid et al., 1983). Alternatively, pol α might specifically interact with 2′-hydroxyls of primer residues distant from the primer 3′-terminus. We are presently examining what makes RNA primers "different". Polymerization of dNTPs onto RNA primers likely only occurs when cells are replicating. Since compounds can differentially inhibit pol α with RNA or DNA primers, polymerization onto RNA primers could be an attractive target for specifically inhibiting DNA replication with lesser effects on DNA repair.

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