Arabinofuranosyl Nucleotides Are Not Chain-Terminators during Initiation of New Strands of DNA by DNA Polymerase α-Primase†

Harry C. Thompson and Robert D. Kuchta*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received April 7, 1995; Revised Manuscript Received June 28, 1995®

ABSTRACT: Polymerization of NTPs and arabinofuranosyladenosine triphosphate (araATP) during DNA polymerase α catalyzed elongation of primase-synthesized primers was examined. After primase synthesizes a primer, pol α normally polymerizes multiple dNTPs onto this primer. In the absence of a required dNTP, however, primers were still elongated by up to 35 nucleotides via polymerization of the corresponding NTP in place of the missing dNTP. During the elongation of exogenously added primer/templates, however, NTPs were not readily polymerized. AraATP was readily incorporated into products during elongation of primase-synthesized primers. Importantly, polymerization of araATP *did not* result in chain termination; rather, the next correct nucleotide was added such that araATP was simply an alternate substrate. In contrast, polymerization of araATP during elongation of exogenously added primer/templates resulted in strong chain termination. Thus, elongation of primase-synthesized primers by pol α-primase is fundamentally different than elongation of exogenously added primer/templates with respect to interactions with dNTP analogs. Furthermore, these data provide a rationale for how araNMPs are efficiently incorporated into internucleotide linkages of DNA in whole cells and suggest that the initiation of new strands of DNA by pol α-primase may be a unique target for inhibiting replication.

9-β-D-Arabinofuranosyladenosine (araA)[1] and 1-β-D-arabinofuranosylcytosine (araC) are important chemotherapeutic agents (Weil et al., 1980; Keeney & Buchanan, 1975; Howard et al., 1968). Both compounds are cytotoxic, likely due to inhibition of DNA replication by the triphosphates, araATP and araCTP. A curious feature of araA and araC, however, is that when cells are treated with low levels of these compounds, araA and araC are incorporated into DNA primarily at internucleotide linkages (Major et al., 1981; Kufe et al., 1983). Thus, they are polymerized into the DNA, and subsequent nucleotides are then added. However, studies with purified eucaryotic DNA polymerases have found that araATP and araCTP are strong chain-terminators (Huang et al., 1991; Lee et al., 1980; Mikita & Beardsley, 1988; Ohno et al., 1989; Perrino & Mekosh, 1992; Reid et al., 1988). After polymerization of an araNTP, DNA polymerases incorporate the next correct dNTP at very slow rates. Thus, it is unclear as to how araA and araC are so easily incorporated into internucleotide linkages in cells.

DNA pol α is a key replicative DNA polymerase that copurifies as a complex with a second enzyme, DNA primase. On single-stranded DNA, primase synthesizes an RNA primer that is then transferred intramolecularly to the pol α active site and dNTPs are polymerized (Copeland & Wang, 1993; Sheaff et al., 1994). Interestingly, Hu et al. (1984) observed that during elongation of primase-synthesized primers (i.e., primase-coupled pol α activity), both NTPs and dNTPs could be incorporated under conditions of very low dNTP concentrations, a reaction that is not normally observed with exogenously added primer/templates. Due to the nature of the template used for these studies (ssM13 DNA), however, the identity and frequency of NTP incorporation could not be ascertained. More recently, we found that pol α interacts with acyclic nucleotide analogs very differently during primase-coupled pol α activity compared to when pol α elongates an exogenously added DNA primer/template (Ilsley et al., 1995). Together, these results suggested that araNTPs might affect pol α very differently during elongation of a primase-synthesized primer than during elongation of an exogenously added primer/template.

Using single-stranded templates of defined sequence, we examined in detail the ability of pol α-primase to polymerize NTPs and araNTPs during elongation of a primase-synthesized primer. In the absence of a required dNTP, the cognate NTP was frequently polymerized instead. Additionally, araATP was readily incorporated into internucleotide linkages during primase-coupled pol α activity, even if dATP was present in the reaction. Thus, during initiation of a new strand of DNA, araATP is *not* a chain-terminator, but is instead an alternate substrate.

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials were as described previously and were of the highest quality commercially available (Sheaff et al., 1991, 1994). Calf thymus pol α-primase was purified using immunoaffinity chromatography (Kuchta et al., 1990). [^{3}H]AraATP was synthesized as previously described (Kuchta et al., 1992). RNA primers containing a 5′-triphosphate were synthesized using T7 RNA polymerase

† This work was supported by a grant from the National Science Foundation (DMB-9105808).

* To whom correspondence should be addressed. Telephone: (303) 492-7027. FAX: (303) 492-5894. EMail: kuchta@spot.colorado.edu.

® Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: araA, 9-β-D-arabinofuranosyladenosine; araC, 1-β-D-arabinofuranosylcytosine; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid (Na salt); pol α, DNA polymerase α; Tris, tris(hydroxymethyl)aminomethane (HCl salt).

0006-2960/95/0434-11198$09.00/0

(Milligan et al., 1987). Primer/templates were annealed as previously described (Sheaff & Kuchta, 1993), and the concentrations of DNA are given in terms of 3′-terminii.

Methods

Unless noted, all assays were performed under initial velocity conditions at 37 °C and contained 50 mM Tris, pH 7.5, and 5 mM $MgCl_2$.

Pol α Assays. Pol α activity was measured as described previously (Kuchta et al., 1992). Assays on exogenously added primer/templates typically contained 1 μM DNA, 2−10 μM [α-^{32}P]dNTPs, and 0.66−6.6 nM pol α, and were quenched by the addition of 1−2 volumes of gel loading buffer (90% formamide). The products were separated by denaturing gel electrophoresis (18% acrylamide, 8 M urea) and analyzed by phosphorimagery (Molecular Dynamics).

Primase-Coupled Pol α Assays. Assays were performed as described previously (Sheaff et al., 1991), and typically contained 1 μM DNA template, 50−100 μM NTPs, 2−10 μM [α-^{32}P]dNTPs, 0.1 mg/mL BSA, and 0.66−6.6 nM pol α. Assays were stopped by the addition of 1−2 volumes of gel loading buffer, and the products were analyzed using gel electrophoresis and phosphorimagery.

Relative Amounts of AraAMP and dAMP Incorporation during Primase-Coupled Pol α Activity. The relative amounts of araAMP and dAMP incorporated into products during primase-coupled pol α activity were determined using double-label assays similar to those described by Kuchta et al. (1992). Reactions (5 μL) contained 1 μM $d(TC)_{30}$, 0−15 μM [^{3}H]araATP (29 000 dpm/pmol), 2 μM [α-^{32}P]dATP (29 000 dpm/pmol), 250 μM ATP and GTP, 2 μM dGTP, 0.1 mg/mL BSA, and 3.3 nM pol α. Products were gel-purified and resuspended in H_2O, and the relative amounts of [^{3}H]araAMP and [α-^{32}P]dAMP incorporated into products were determined by double-label scintillation counting. The relative $[V_{max}/K_{M(dATP)}]/[V_{max}/K_{M(araATP)}]$ was calculated from these data as previously described (Kuchta et al., 1992).

Relative Amounts of AraAMP and dGMP Incorporated during Primase-Coupled Pol α Activity on $d(TC)_{30}$. Double-label assays were performed on the $d(TC)_{30}$ as described above except the assays now contained $d(TC)_{30}$, ATP, GTP, [^{3}H]araATP and [α-^{32}P]dGTP.

Purification of Individual Bypass Products. Reactions (40 μL) contained 0.5 mg/mL BSA, 1 μM $d(TC)_{30}$, and 100 μM each ATP, GTP, and [α-^{32}P]dNTP(s) (8 μCi). After separation of the products using denaturing gel electrophoresis, individual product bands were excised from the gel and the ^{32}P-labeled products extracted into H_2O (Kuchta et al., 1992). Products were recovered by *i*PrOH precipitation (Maniatis et al., 1982), dried, and resuspended in 10 mM Tris, pH 7.5.

Base Hydrolysis of Products. Products were treated with 400 mM K_2CO_3 for 10 min at 90 °C. Gel loading buffer was added, and products were analyzed using gel electrophoresis and phosphorimagery.

DNase I Cleavage of Products. Reactions (8 μL) contained ^{32}P-labeled product, 0.5 mg of DNase I, and 6.25 mM $MgCl_2$. After incubation at 37 °C for 60 min, gel loading buffer was added, and products were analyzed by gel electrophoresis and phosphorimagery.

Nuclease Digestion of Products. Reactions (15 μL) were incubated at 37 °C for 10 min with 3 units of micrococcal nuclease, purified $d(TC)_{30}$ bypass product, 20 mM Tris, pH 7.6, and 5 mM $CaCl_2$. Phosphodiesterase II (0.04 unit) was then added to the reaction mixture. At intervals between 2 and 30 min, 2.5 μL aliquots were loaded onto a silica gel-60 TLC plate. The TLC plate had been prespotted with 2 μL of 50 mM EDTA as a quench solution. Products were resolved using a solvent of 6:3:1.2 *i*PrOH/NH_4OH/H_2O.

Table 1: Synthetic DNAs of Defined Sequence Used

DNA_G	TCCATATCACAT(3′) AGGTATAGTGTAGATCTTATCATCATCT
10/25mer	GCGCCGAAAC(3′) GTACGCGGCTTTGTGTAATAGTAAG
$d(ACT)_{20}$	(5′)ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT
$d(TCC)_{20}$	(5′)TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC
$d(TTC)_{13}$	(5′)TTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTC
$d(TC)_{30}$	(5′)TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$dA(GA)_4$	(5′)AGAGAGAGA
$d(GGA)_3$	(5′)GGAGGAGGA

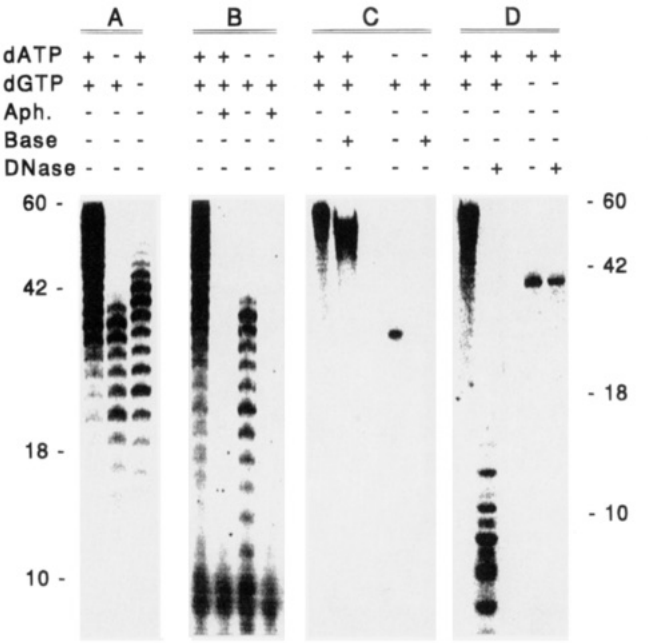

FIGURE 1: Incorporation of NTPs during primase-coupled pol α activity. Panel A shows the products synthesized during primase-coupled pol α activity in assays containing $d(TC)_{30}$, ATP, GTP, and either [α-^{32}P]dATP plus dGTP, [α-^{32}P]dATP alone, or [α-^{32}P]-dGTP alone, as noted in the figure. Panel B shows the effect of inhibiting pol α activity by adding 10 μM DNA_G and 100 μM aphidicolin to primase-coupled pol α activity assays containing either [α-^{32}P]dATP plus dGTP or [α-^{32}P]dATP alone. Individual products from assays were gel-purified and treated with base (panel C) or DNase (panel D). The mobilities of DNA standards of known length shown to the left of panel A are for panels A and B, while those to the right of panel D are for panels C and D.

RESULTS

In the presence of a single-stranded template [($d(TC)_{30}$, Table 1], ATP, GTP, dATP, and dGTP, primase synthesized RNA primers that were then elongated by pol α (Figure 1). Since the primase-synthesized primer is ca. 10 nucleotides long, pol α polymerized 40−50 dNTPs onto each primer. Curiously, however, primase-synthesized primers were still elongated when either dATP or dGTP was omitted from the reactions (Figure 1).

Formation of these unusual products absolutely required pol α activity. First, these elongated products contained

dNMPs since the only ^{32}P-label in the assays was an [α-^{32}P]-dNTP (Figure 1). Second, inhibiting pol α activity eliminated the synthesis of these elongated products. Pol α activity can be specifically and potently inhibited by including a synthetic primer/template (DNA_G, Table 1) and aphidicolin in the assays. This results in the formation of a pol α·DNA_G·aphidicolin ternary complex that cannot bind other primer/templates, but where primase remains active (Sheaff et al., 1991). Inhibiting pol α with DNA_G and aphidicolin potently inhibited the synthesis of products in assays containing $d(TC)_{30}$, ATP, GTP, and either [α-^{32}P]-dATP plus dGTP or only [α-^{32}P]dGTP (Figure 1). Thus, pol α activity is required for elongation of primase-synthesized primers in the absence of a cognate dNTP.

On a M13 DNA template at low dNTP concentrations, pol α-primase can elongate primase-synthesized primers via mixed dNTP and NTP polymerization (Hu et al., 1984). To demonstrate that this was also occurring on these synthetic templates of defined sequence, three approaches were used.

Base Hydrolysis. Individual products were gel-purified from assays containing $d(TC)_{30}$, ATP, GTP, and either dATP plus dGTP, dATP alone, or dGTP alone. Treating the products from assays containing both dATP and dGTP with base only decreased the product length by ca. 10 nucleotides due to hydrolysis of the primase-synthesized primers (Figure 1). However, if either dATP (Figure 1) or dGTP (data not shown) was omitted from the initial reaction, treatment with base reduced the products from 30−40 nucleotides long to <5 nucleotides long, consistent with polymerization of NTPs throughout the products.

DNase Digestion. DNase I treatment of the products from assays containing $d(TC)_{30}$, ATP, GTP, dATP, and dGTP reduced them to a length of 6−12 nucleotides (Figure 1). Since DNase will only hydrolyze oligonucleotides to within one or two bases of a NMP (Hu et al., 1984; Kuchta et al., 1990), these remaining products consisted of the primase-synthesized primer and one or two dNMPs. In contrast, DNase did not affect the products from assays containing $d(TC)_{30}$, NTPs, and only [α-^{32}P]dATP (Figure 1) or [α-^{32}P]-dGTP (data not shown), suggesting that in the absence of a required dNTP, an NTP was polymerized.

Nuclease Treatment. Products purified from assays containing $d(TC)_{30}$, ATP, GTP, and either dGTP plus [α-^{32}P]-dATP or [α-^{32}P]dATP alone were treated with micrococcal nuclease and phosphodiesterase II to generate 3′-NMPs. Importantly, this allows one to identify the 5′ neighbor of a [^{32}P]dNMP that was incorporated in a product. For example, >99% of the ^{32}P comigrated with 3′-dGMP after nuclease treatment of assays containing dGTP and [α-^{32}P]dATP, indicating that the 5′ neighbor of [^{32}P]dAMP was dGMP. When dGTP was omitted, the product should be 3′-[^{32}P]GMP if GTP was polymerized in place of dGTP. Nuclease treatment of the products from assays containing just [α-^{32}P]-dATP revealed that, indeed, 93% of the ^{32}P comigrated with 3′-GMP. Thus, if a required dNTP is missing during elongation of a primase-synthesized primer, the cognate NTP can be incorporated in its place.

Hu et al. (1984) demonstrated that low concentrations of dNTPs could eliminate the polymerization of NTPs during elongation of primase-synthesized primers on an M13 template. Similarly, we found that polymerization of NTPs during elongation of primase-synthesized primers on $d(TC)_{30}$ was abolished when 500 nM dATP was added to assays that

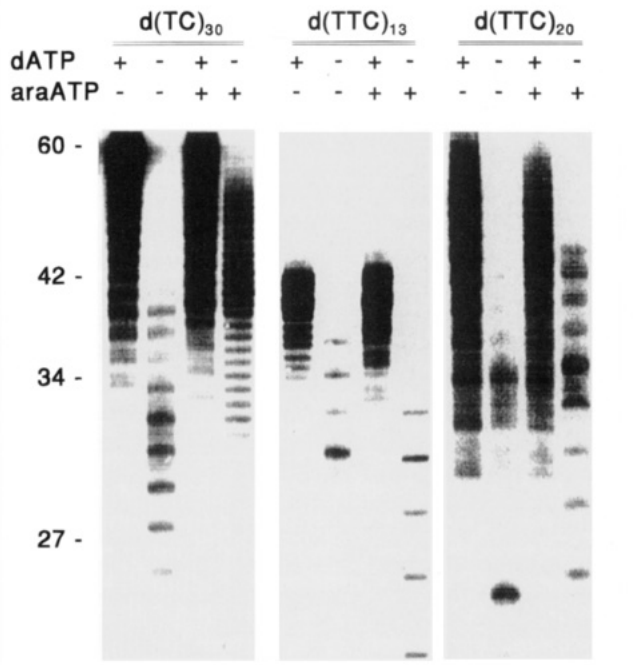

FIGURE 2: Primase-synthesized primers are elongated in the absence of a required dNTP on multiple templates. Primase-coupled pol α assays contained ATP, GTP, [α-^{32}P]dGTP, and dATP and/or araATP as noted.

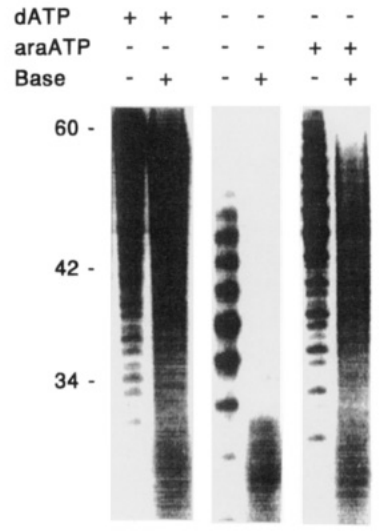

FIGURE 3: Incorporation of araNTPs during primase-coupled pol α activity. Assays contained $d(ACT)_{20}$, NTPs, dTTP, [α-^{32}P]dGTP, and dATP and/or araATP as noted. The effects of base hydrolysis on the products are also shown.

contained 400 μM ATP, 400 μM GTP, and 2 μM [α-^{32}P]-dGTP (data not shown). Thus, dNTPs are very potent inhibitors of this NTP polymerization.

Polymerization of the corresponding NTP in the absence of the required dNTP was not limited to the $d(TC)_{30}$ template (Figures 2 and 3). With several different templates [$d(TTC)_{13}$, $d(TCC)_{20}$, and $d(ACT)_{20}$ (Table 1)], bypass of a template nucleotide in the absence of a required dNTP was observed. Importantly, the fact that pol α-primase synthesized products >34 nucleotides long on $d(TTC)_{13}$ in the absence of dATP indicates that consecutive bases can be bypassed.

Bypass of the template bases during primase-coupled pol α activity was not due to the short length of the templates or their sequence. For example, $d(TC)_{30}$ was annealed to

5′-^{32}P-labeled dA(GA)$_4$ to generate a primer/template. The resulting primer/template was then incubated with pol α-primase, ATP, GTP, and either dATP plus dGTP, dATP alone, or dGTP alone. Whereas the primers were elongated by 10−40 nucleotides when both dATP and dGTP were present, they were elongated by at most 2 nucleotides in the absence of either dATP or dGTP. Similar results were obtained using a primer/template generated by annealing d(GGA)$_3$ to d(TCC)$_{20}$.

Incorporation of AraATP into Internucleotide Linkages during Primase-Coupled Pol α Activity. The unusual incorporation of NTPs during primase-coupled pol α activity described above raised the possibility that interactions of pol α-primase with araNTPs would be greatly altered when pol α elongates a primase-synthesized primer as opposed to an exogenously added primer/template. Figure 3 shows that the products synthesized during primase-coupled pol α activity in assays containing d(ACT)$_{20}$, NTPs, dTTP, dGTP, and araATP were slightly longer than those synthesized when araATP was omitted from the reaction (Figure 3). Furthermore, treating the products synthesized in the presence of araATP with base only reduced their length by ca. 10 nucleotides, consistent with removal of just the primase-synthesized primer. These data suggested that araATP was polymerized when dATP was omitted, but polymerization did not result in chain termination.

Apparent incorporation of araATP followed by polymerization of additional dNTPs was not limited to d(ACT)$_{20}$. On d(TC)$_{30}$, d(TCC)$_{20}$, and d(TTC)$_{13}$, adding araATP to primase-coupled pol α assays that lacked dATP resulted in longer products with altered electrophoretic mobility (Figure 2). Addition of araATP to primase-coupled pol α assays that contained all required dNTPs decreased the amount of product but had only small effects on the length of products. Thus, any araATP that was incorporated during elongation of primase-synthesized primers was not resulting in strong chain termination. Finally, generation of these elongated products absolutely required pol α activity since aphidicolin could completely inhibit their synthesis.

Double-label experiments were used to explicitly show that pol α-primase incorporated araATP into internucleotide linkages during primase-coupled pol α activity. Assays contained d(TC)$_{30}$, ATP, GTP, [α-^{32}P]dGTP, and [^{3}H]-araATP. Products (ca. 38−50 nucleotides long) were separated by gel electrophoresis, and the amounts of [^{32}P]-dGMP and [^{3}H]araAMP in the products were determined. The [^{32}P]dGMP:[^{3}H]araAMP ratio was 1.01 ± 0.18, indicating that equal amounts of dGMP and araAMP were incorporated into products during elongation of the primase-synthesized primers. These data require that after primer synthesis, every other nucleotide polymerized was an araAMP. Since the products are 38−50 nucleotides long and the RNA primer is only 10 nucleotides long, multiple araAMPs were polymerized during elongation of the RNA primer and, most importantly, incorporation of araAMP *did not* result in chain termination.

The relative V_{max}/K_M for polymerization of araATP rather than dATP during primase-coupled pol α activity was measured using double-label experiments. Assays contained d(TC)$_{30}$, ATP, GTP, dGTP, [α-^{32}P]dATP, and various concentrations of [^{3}H]araATP. From the relative amounts of [α-^{32}P]dATP and [^{3}H]araATP polymerized into products, one can obtain V_{max}/K_M for polymerization of dATP versus

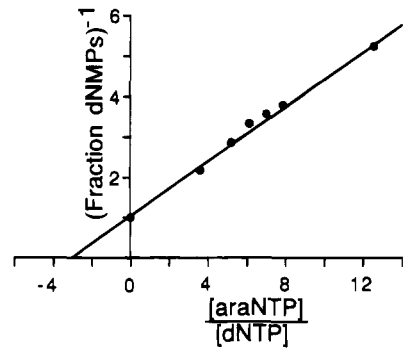

FIGURE 4: Pol α-primase exhibits minimal discrimination against araATP during primase-coupled pol α activity. Assays contained d(TC)$_{30}$, NTPs, and various araATP:dATP ratios as described under Experimental Procedures. The fraction dAMP incorporated into products was calculated as dAMP/(dAMP + araAMP) in products.

araATP (Fersht, 1985; Kuchta et al., 1992). Radiolabeled products were ca. 40−60 nucleotides long (see Figure 2), and the amount of [^{32}P]dAMP and [^{3}H]araAMP present in the products was determined by excising the products from the gel followed by scintillation counting. Increasing the ratio of [^{3}H]araATP to [α-^{32}P]dATP increased the relative amount of [^{3}H]araAMP incorporated into products. For example, when assays contained 2 μM dATP and 2 μM araATP, the products contained 0.45 pmol of dAMP and 0.52 pmol of araAMP, whereas increasing the araAMP concentration to 15 μM resulted in products containing 0.12 pmol of dAMP and 0.51 pmol of araAMP. Quantitative analysis of these data over a range of araATP concentrations revealed that pol α-primase preferred to polymerize dATP rather than araATP by only a factor of 3 (V_{max}/K_M, Figure 4).

Even though previous studies have clearly shown that araATP is a strong chain-terminator during elongation of exogenously added primer/templates, we nonetheless considered the unlikely possibilities that the ability of araATP to act as a chain terminator was affected by the repeating nature of d(TC)$_{30}$ or that primase-synthesized primers are RNA and contain a 5′-triphosphate. 5′-^{32}P-labeled 9mer primers were annealed to d(TC)$_{30}$ and d(TCC)$_{20}$, and elongation of these primers by pol α-primase in assays containing only dGTP and araATP was examined. Similar to previous studies using other primer-templates, incorporation of araATP resulted in strong chain termination (data not shown), indicating that the repeating nature of these templates does not eliminate the chain-termination properties of araATP. To exclude the possibility that the results were due to an RNA primer containing a 5′-triphosphate, a 10/25mer was constructed where the primer was RNA and contained a 5′-triphosphate (Table 1). When all four dNTPs were present, the primer was efficiently elongated (Figure 5). Addition of araATP resulted in pause sites at those positions where araATP could be polymerized, and these products were of slightly altered electrophoretic mobility compared to the normal pause sites in the absence of araATP (Figure 5), consistent with polymerization of araATP resulting in chain termination. Finally, omitting dATP in the presence or absence of araATP resulted in almost no elongation of the primer. Since dATP is the first nucleotide required for elongation of the primer, these data indicate that araATP cannot replace dATP for processive elongation of exogenously added RNA primers containing a 5′-triphosphate. Thus, incorporation of araATP into internucleotide

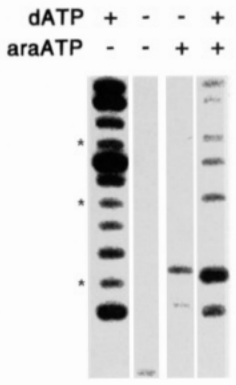

FIGURE 5: Nature of the primer does not affect araATP-induced chain termination. Assays contained 10/25mer, [α-^{32}P]dCTP, dGTP, dTTP, and either dATP, no addition, araATP, or dATP plus araATP as noted in the figure legend. Positions where dATP is the correct nucleotide are marked with an asterisk.

linkages during elongation of primase-synthesized primers is not due to the composition of the primer.

DISCUSSION

The data presented above show that both NTPs and araNTPs are incorporated into internucleotide linkages during elongation of primase-synthesized primers. In the most extreme case, $d(TC)_{30}$, elongation involves the repeating polymerization of a dNTP followed by either an NTP or an araNTP. These reactions appear to be quite general, since we observed similar results on $d(TCC)_{20}$, $d(TTC)_{13}$, and $d(ACT)_{20}$, and Hu et al. (1984) observed polymerization of NTPs during elongation of primase-synthesized primers on M13 DNA.

Ever since Kufe and co-workers showed that treating cells with low concentrations of araA or araC results in the incorporation of araAMP and araCMP into DNA primarily at internucleotide linkages (Major et al., 1981; Kufe et al., 1983), it has been an open question as to how the araNTPs are incorporated at this position with such high efficiency. Numerous studies have demonstrated that primers containing an araC or araA at the 3′-terminus are not readily elongated by purified polymerases (Huang et al., 1991; Lee et al., 1980; Mikita & Beardsley, 1988; Ohno et al., 1989; Perrino & Mekosh, 1992; Reid et al., 1988). Incorporation during primase-coupled pol α activity provides a likely solution to this enigma, since araATP was readily incorporated into internucleotide linkages during primase-coupled pol α activity, and pol α-primase exhibited minimal discrimination against araATP polymerization [3-fold (V_{max}/K_M)]. Interestingly, treating cells with higher concentrations of araA or araC results in more ara-nucleotides at the 3′-termini of DNA. This change in location might indicate that at higher intracellular araATP and araCTP concentrations, pol δ and/or ε polymerize more araATP and araCTP which then results in chain termination.

Pol α activity is essential for the incorporation of NTPs during elongation of primase-synthesized primers since inhibiting pol α activity using DNA_G and aphidicolin completely blocked this reaction.[2] Two distinct models, however, remain plausible for this reaction: (i) Pol α polymerizes both dNTPs and NTPs during elongation of primase-synthesized primers. Normally, pol α polymerizes NTPs very slowly and prefers dNTPs rather than NTPs as substrates by >20 000-fold [V_{max}/K_M (Kuchta et al., 1992)]. Thus, for pol α to polymerize the NTPs during primase-coupled pol α activity, the rate of NTP polymerization during elongation of primase-synthesized primers must be greatly increased compared to elongation of an exogenously added primer/template. It is unclear if the ability of pol α to discriminate between NTPs and dNTPs is affected, since even low levels of dNTPs abolished NTP incorporation during elongation of primase-synthesized primers. (ii) Pol α polymerizes the dNTPs and primase polymerizes the NTPs during elongation of a primase-synthesized primer. If a required dNTP is not present during pol α-catalyzed elongation of a primase-synthesized primer, the primer/template might translocate into the primase active site and primase then incorporates the cognate NTP. The primer/template now moves back into the pol α active site to allow further dNTP polymerization. Primase readily polymerizes NTPs, consistent with this latter model. However, primase will not easily polymerize NTPs onto primers containing a 3′-terminal dNMP (Kuchta et al., 1992). Furthermore, if this latter model were correct, it raises the question of why primase does not readily polymerize multiple NTPs onto a primase-synthesized primer in the complete absence of dNTPs.[3]

Similarly, either pol α or primase could have incorporated the araATP during primase-coupled pol α activity. Previous studies showed that pol α exhibits minimal discrimination against araATP polymerization when using an exogenously added primer/template as substrate, and primase actually prefers to polymerize araATP rather than ATP (Kuchta et al., 1992). For the reasons cited above, as well as its greater simplicity, our preferred model is that pol α polymerizes both NTPs and araATP during primase-coupled pol α activity, perhaps due to changes in the specificity of pol α for the sugar moiety of nucleotides during primase-coupled pol α activity. Consistent with this idea, we have demonstrated that pol α loses most of its ability to discriminate against polymerization of acyclovir triphosphate during primase-coupled pol α activity (Ilsley et al., 1995).

Why, however, should pol α-primase exhibit altered specificity during primase-coupled pol α activity? Incorporation of araATP at internucleotide linkages and polymerization of NTPs during elongation of primase-synthesized primers are not due to either the composition (RNA) or the presence of a 5′-triphosphate in primase-synthesized primers. Both these and previous studies have shown that pol α polymerizes araNTPs onto DNA and RNA primers (±5′-triphosphate) with similar efficiency, but incorporation results in strong chain termination (Kuchta et al., 1992). Similarly, pol α does not readily polymerize NTPs during elongation of RNA or DNA primer/templates [*vide infra* and Kuchta et al. (1992)]. Altered specificity could be caused by a

[2] Previous studies showed that an anti-pol α antibody reduced, but did not eliminate, the incorporation of NTPs during elongation of primase-synthesized primers on ssM13 DNA (Hu et al., 1984). Since pol α is typically 100-fold more active than primase, even trace amounts of residual pol α activity would have allowed pol α-catalyzed polymerization of both NTPs and dNTPs onto the primase-synthesized primers.

[3] Primase will, in some cases, polymerize multiple NTPs onto primase-synthesized primers in the absence of dNTPs (Kuchta et al., 1990). In this reaction, primase polymerizes ca. 8−10 NTPs onto a preexisting primer to generate products twice as long as the original primer. However, this reaction is very slow and results in products of very different sizes compared to polymerization of either dNTPs or dNTPs plus NTPs in the absence of a required dNTP.

"conformational change" of the complex due to primer synthesis, perhaps driven by the favorable ΔG of NTP polymerization. Alternatively, it is possible that primase-synthesized primers and exogenously synthesized primers bind to distinct sites on the enzyme complex. For example, a primase-generated primer/template might interact with both pol α and primase (Sheaff et al., 1994), whereas an exogenously added primer/template might only interact with pol α.

Virtually all previous studies on the effects of nucleotide analogs on pol α activity have used exogenously added primer/templates as substrates. From the studies presented here on the polymerization of araNTPs and NTPs during primase-coupled pol α activity as well as previous work on acyclovir triphosphate (Ilsley et al., 1995), it is apparent that primase-coupled pol α activity has fundamentally different properties than pol α activity on exogenously added primer/templates. Consequently, pol α activity should be examined under both sets of conditions when assessing the potential effects of a nucleotide analog. Furthermore, since primase-coupled pol α activity (i.e., initiation of new strands of DNA) presumably only occurs during DNA replication, it might be possible to take advantage of this altered specificity toward nucleotide analogs to develop novel strategies to inhibit DNA replication without affecting DNA repair.

REFERENCES

Copeland, W. C., & Wang, T. S.-F. (1993) *J. Biol. Chem. 268*, 26179−26189.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman & Co., New York.

Howard, J. P., Albo, B., & Newton, W. A. (1968) *Cancer 21*, 341−345.

Hu, S.-Z., Wang, T. S. F., & Korn, D. (1984) *J. Biol. Chem. 259*, 2602−2609.

Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., & Plunkett, W. (1991) *Cancer Res. 51*, 6110−6117.

Ilsley, D. D., Lee, S.-H., Miller, W. H., & Kuchta, R. D. (1995) *Biochemistry 34*, 2504−2510.

Keeney, R. E., & Buchanan, R. A. (1975) *Ann. N.Y. Acad. Sci. 255*, 185−189.

Kuchta, R. D., Reid, B., & Chang, L. M. S. (1990) *J. Biol. Chem. 265*, 16158−16165.

Kuchta, R. D., Ilsley, D., Kravig, K. D., Schubert, S., & Harris, B. (1992) *Biochemistry 31*, 4720−4728.

Kufe, D. W., Major, P. P., Munroe, D., Egan, M., & Herrick, D. (1983) *Cancer Res. 43*, 2000−2004.

Lee, M. Y. W. T., Byrnes, J. J., Downey, K. M., & So, A. G. (1980) *Biochemistry 19*, 215−219.

Major, P. P., Egan, E. M., Beardsley, G. P., Minden, M. D., & Kufe, D. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3235−3239.

Major, P. P., Egan, E. M., Herrick, D. J., & Kufe, D. W. (1982) *Biochem. Pharmacol. 31*, 2937−2944.

Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783−8798.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Ohno, Y., Spriggs, D., Matsukage, A., Ohno, T., & Kufe, D. (1989) *Cancer Res. 49*, 2077−2081.

Reid, R., Mar, E.-C., Huang, E.-S., & Topal, M. D. (1988) *J. Biol. Chem. 263*, 3898−3904.

Sheaff, R., & Kuchta, R. D. (1993) *Biochemistry 32*, 3027−3037.

Sheaff, R., Ilsley, D., & Kuchta, R. (1991) *Biochemistry 30*, 8590−8597.

Sheaff, R. J., Kuchta, R. D., & Ilsley, D. (1994) *Biochemistry 33*, 2247−2254.

Weil, M., Jacquillat, C., Auclerc, M. F., Achaison, G., Chastung, C., & Bernard, J. (1980) *Recent Res. Cancer Res. 80*, 36−41.

BI950786R