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Inhibition of DNA Primase by Nucleoside Triphosphates and Their Arabinofuranosyl Analogs

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SUMMARY

DNA primase (EC 2.7.7.6) produces an RNA oligomer of approximately 10 bases, which is required by DNA polymerase α (EC 2.7.7.7) for the initiation of DNA synthesis. We partially purified DNA primase from acute lymphocytic leukemia cells from patients using several chromatography columns. Poly(dT) and poly(dC), but not poly(dA) or poly(dG), were good templates for ribonucleoside triphosphate (rNTP)-dependent DNA synthesis (i.e., DNA primase activity), and they were used in the study of the effect of natural and arabinofuranosyl nucleoside triphosphates on DNA primase activity. The K_m for GTP in the poly(dC) primase assay was approximately 175 μM. All noncomplementary natural rNTPs and deoxyribonucleoside triphosphates (dNTPs) inhibited poly(dC) primase activity to a similar extent (K_i values of ATP and CTP were 610 and 517 μM, respectively). 1-β-D-Arabinofuranosylcytosine 5′-triphosphate (araCTP) and 9-β-D-arabinofuranosyladenine 5′-triphosphate (araATP) were more potent inhibitors of poly(dC) primase activity than were CTP and ATP (K_i values were approximately 125 μM). araCTP, araATP, CTP, and ATP inhibited DNA primase activity in a manner competitive with GTP. The concentration required to inhibit poly(dC) DNA primase activity by 50% was determined for a number of arabinofuranosyl nucleoside triphosphate analogs, and the relative potency of inhibition of DNA primase activity was as follows: rNTP = dNTP = 5-aza-dCTP < ara-5-azaCTP = araTTP = araATP = araCTP < 2-fluoro-araATP = 2′-azido-2′-deoxy-araCTP < 2′-fluoro-araTTP = 2′-fluoro-5-iodo-araCTP = 2′-fluoro-5-methyl-araCTP. In the poly(dT) primase assay ATP did not follow classic Michaelis-Menten kinetics (ATP exhibited positive cooperativity with a Hill coefficient of 2.0). However, this assay was very sensitive to araCTP (apparent K_i of 25 μM). In summary, these experiments suggested that DNA primase is controlled by the levels of ribonucleoside triphosphates, and that the perturbation of these pools by any agent could lead to the inhibition of DNA primase and thereby inhibit DNA synthesis. Furthermore, aranucleoside triphosphate analogs directly inhibited DNA primase, and it is possible that this effect may contribute to the cytotoxicity of these compounds.

Inhibition of DNA replication is believed to play an important role in the cytotoxicity of some clinically useful antitumor agents, such as araC, methotrexate, and 5-fluorouracil (1). The enzymes DNA polymerase α, dihydrofolate reductase, and thymidylate synthetase have been shown to be important intracellular targets of these compounds. In this report we have studied the effect of arabinofuranosyl nucleoside triphosphate analogs on another enzyme important to DNA replication, DNA primase.

DNA polymerase α cannot start DNA synthesis on an unprimed single strand DNA template. It can only add dNTPs to a 3′-hydroxyl of an existing DNA or RNA chain which is annealed to the template DNA. Initiation of DNA synthesis on single-stranded DNA requires the production by DNA primase of a ribonucleotide oligomer of about 10 bases. The 3′-hydroxyl of this primer is then used by DNA polymerase α to begin DNA synthesis. DNA primase has recently been purified from a number of eukaryotic sources (2–11).

araC is an important agent used in the treatment of acute myelocytic leukemia (12, 13). The anabolism of araC to araCTP is required for the cell-killing action of this compound. araCTP is a substrate for DNA polymerase α and β, and this interaction is believed to play a major role in the cytotoxicity of this agent. Recent studies have shown that araCTP and araATP could also inhibit DNA primase purified from several sources (4, 9, 14). This suggested that other nucleoside triphosphate analogs may also inhibit DNA primase and may contribute to their cytotoxicity. In this study we have partially purified DNA

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ABBREVIATIONS: araC, 1-β-D-arabinofuranosylcytosine; araCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; araATP, 9-β-D-arabinofuranosyladenine 5′-triphosphate; 2-F-araATP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate; 2-F-araATP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate; ara-5-azaCTP, 1-β-D-arabinofuranosyl-5-azacytosine 5′-triphosphate; 5-aza-dCTP, 2′-deoxy-5-azacytidine 5′-triphosphate; araTTP, 1-β-D-arabinofuranosylthymine 5′-triphosphate; 2′-F-araTTP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)thymine 5′-triphosphate; cytarazid-TP, 2′-azido-2′-deoxyarabinofuranosylcytosine 5′-triphosphate; FIAC-TP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine 5′-triphosphate; FMAC-TP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine 5′-triphosphate; rNTP, ribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

primase from human leukemia cells and studied the effect of various arabinofuranosyl nucleoside triphosphate analogs on its activity. The results indicated that human DNA primase was inhibited by arabinofuranosyl nucleoside triphosphate analogs and suggested that inhibition of this enzyme could play a role in their cytotoxicity.

Materials and Methods

Chemicals and supplies. The nucleoside triphosphates used in this study (dATP, dCTP, dGTP, TTP, ATP, CTP, GTP, UTP, and dUTP), DNase I from bovine pancreas (type II), and single-stranded DNA-cellulose were obtained from Sigma Chemical Co. (St. Louis, MO). DEAE-cellulose (DE52) and cellulose phosphate (P11) were obtained from Whatman Chemical Separation Inc. (Clifton, NJ). [*methyl*-^{3}H]TTP (20 Ci/mmol), [8-^{3}H]dGTP (20 Ci/mmol), [8-^{3}H]dATP (20 Ci/mmol), and [α-^{32}P]GTP (3000 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). Poly(dC), poly(dT), and oligo(dG)$_{10}$-primed poly(dC) were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). *Escherichia coli* DNA-polymerase I (endonuclease-free) and proteinase K were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). araATP and araCTP were obtained from Sigma. The nucleoside triphosphate analogs, 2-F-araATP, araTTP, 2′-F-araTTP, cytarazid-TP, FIAC-TP, and FMAC-TP, were synthesized from the nucleoside analog and purified as described previously (15). ara-5-azaCTP and 5-aza-dCTP were synthesized in two steps from the nucleoside analog using deoxycytidine kinase followed by a mixture of pyrimidine monophosphate kinase and nucleoside diphosphate kinase. The triphosphates were then purified by DEAE-Sephadex A-25 column chromatography (15), and were given to us by Alan Townsend of this laboratory. In the experiments reported here the concentration of salt in each nucleoside triphosphate was below the concentration required to inhibit DNA primase activity. All other chemicals were of standard analytical grade.

DNA primase and DNA polymerase assay. DNA primase activity was defined as rNTP-dependent DNA synthesis which occurs on single-stranded DNA templates. Thus, the assay involves two enzymes, DNA primase and DNA polymerase. DNA primase was routinely assayed during purification in 50-μl reactions containing 25 mM Tris, pH 8.0, 0.2 mg/ml of BSA, 10 mM $MgCl_2$, 0.125 unit/ml of poly(dC), 1.0 mM GTP, 10 μM [^{3}H]dGTP (1 Ci/mmol), 6 unit/ml of *E. coli* polymerase I, and 5 μl of enzyme preparation. After 20 min at 37°, the DNA in 40 μl of each assay was precipitated onto glass fiber filters with a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. The filters were washed twice with this trichloroacetic acid solution, washed twice with 95% ethanol, dried, and then counted for radioactivity. The inhibition studies were done as described above except that *E. coli* polymerase I was not added to the reaction mixtures. These reactions relied on the endogenous DNA polymerase α to lengthen the RNA primer with [^{3}H]dGTP. The addition of excess purified DNA polymerase α, freed of DNA primase activity, did not affect the DNA primase reaction, indicating that this primase assay was dependent on the amount of DNA primase in the reaction. Inhibition of DNA primase was also assayed in 50-μl reactions containing 25 mM Tris, pH 8.0, 0.2 mg/ml BSA, 10 mM $MgCl_2$, 0.125 unit/ml of poly(dT), 4 mM ATP, 10 μM [^{3}H]dATP (1 Ci/mmol), and 5 μl of enzyme preparation. [^{3}H]dATP incorporated into acid-insoluble product was determined as described above. A unit of DNA primase activity was defined as that amount which allowed incorporation of 1 nmol of dNTP into acid-insoluble material per hr.

To ascertain the effect of the nucleotides and their analogs on the DNA polymerase component of the DNA primase assay, DNA polymerase activity was measured as in the routine DNA primase assay except that oligo(dG)-primed poly(dC) (0.044 mg/ml) replaced the poly(dC) as template and the GTP and *E. coli* polymerase I were not added to the reaction. During purification, DNA polymerase activity was measured in 50-μl reactions containing 50 mM Tris, pH 8.0, 0.2 mg/ml of BSA, 10 mM $MgCl_2$, 0.1 mg/ml of activated DNA (16), 10 μM [^{3}H]TTP (1.0 Ci/mmol), a 50 μM concentration each of dATP, dCTP, and dGTP, and 5 μl of enzyme preparation. After the desired time at 37°, the [^{3}H]TTP incorporated into acid-insoluble product was determined as described above. The DNA polymerase associated with DNA primase was identified as DNA polymerase α, because it was inhibited by aphidicolin, araCTP, and *N*-ethylmaleimide, but not by dideoxynucleoside triphosphates.

Purification of DNA primase. DNA primase was partially purified from leukemia cells which were obtained from patients by leukophoresis using routine chromatography procedures. Briefly, the leukemia cells (approximately 60-ml packed cell volume) were sonicated in a 300 mM potassium phosphate buffer at 4° and loaded onto a DEAE-cellulose column equilibrated with 300 mM potassium phosphate buffer, pH 7.0. The unadsorbed fractions were collected and further purified using phosphocellulose, DEAE-cellulose, and single-strand DNA cellulose column chromatography. Because DNA primase activity in the crude extract varied from one experiment to another, and most of the activity detected in the crude extract did not stick to the phosphocellulose column (even though most of the polymerase activity did), we believe that the specific activity for the crude extract was inaccurate. Thus, the first step of the purification shown in Table 1 is after phosphocellulose chromatography. Subsequent chromatography on DEAE-cellulose and single-strand DNA cellulose columns resulted in 10-fold greater purity. The difficulties of measuring DNA primase activity in the crude extract were most likely due to other activities which can prime our template DNA, such as RNA polymerases and terminal transferase. Most DNA polymerase activity was adsorbed onto phosphocellulose. However, only about 33% was associated with DNA primase activity. The identification of two species of polymerase α, one with and one without associated DNA primase activity, has been reported previously (2, 4, 5, 17, 18). The proteins in the single-strand DNA cellulose pool were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19) and stained with silver using the method of Merril *et al.* (20). We found that this preparation still contained 10–15 major protein bands (data not shown), which indicated that we were purifying a replication complex. We are currently investigating the function of some of these proteins.

Analysis of the product of DNA primase. The primer formed by DNA primase was analyzed using 7 M urea, 15% polyacrylamide gel electrophoresis (3). The reaction mixture (75 μl) contained 25 mM Tris, pH 8.0, 0.2 mg/ml of BSA, 10 mM $MgCl_2$, 0.84 unit/ml of poly(dC), 25 or 50 μM [α-^{32}P]GTP (4 Ci/mmol), and 0.15 unit of purified DNA primase. Reactions were done in the presence or absence of 50 μM dGTP. After incubating at 37° for 1 hr, the reactions were stopped by the addition of 150 μl of 10 mM Tris, pH 8.0, 40 mM EDTA, 0.3% sodium dodecyl sulfate, 750 μg/ml of RNA, and 75 μg/ml of proteinase K. These solutions were incubated for 30 min at 37°, extracted with an equal volume of phenol/chloroform (1:1), and the DNA and RNA were precipitated by adding ammonium acetate to 2 M and 3 volumes of 95% ethanol. Ethanol precipitation of the sample was repeated once with a 2 M ammonium acetate/75% ethanol solution and twice with only 95%

TABLE 1

Purification of DNA primase

All columns were equilibrated with 25 mM potassium phosphate buffer pH 7.0, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. DNA primase activity was eluted from a phosphocellulose column (3 × 25 cm) with a 25–500 mM potassium phosphate gradient (600 ml). DNA primase activity was eluted from the DEAE-cellulose (2.5 × 5 cm) and ss-DNA cellulose (1.5 × 3 cm) columns with a 0–1 M potassium chloride gradient in the equilibration buffer (200 ml and 40 ml, respectively).

Fractions	Total units	Yield	Specific activity	-Fold purification
Phosphocellulose	248	100	15	1
DEAE-cellulose	188	76	84	6
ss-DNA cellulose[a]	256	103	149	10

[a] ss-DNA cellulose, single-strand DNA cellulose.

ethanol. The samples were lyophilized and resuspended in 20 μl of buffer containing 20 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 2 mM $CaCl_2$ or the same buffer containing 50 μg/ml of DNase I. After 3 hr at 37°, 4 μl of 200 mM EDTA were added to all samples to stop the DNase reaction. The samples were frozen, lyophilized, resuspended in 20 μl of 50% formamide solution, boiled, and loaded onto a 7 M urea, 15% polyacrylamide gel. After electrophoresis the ^{32}P-labeled products of the primase reaction were visualized by autoradiography.

Results

Characterization of DNA primase. The product of DNA primase using poly(dC) as template was labeled with [α-^{32}P]GTP and analyzed by polyacrylamide/urea gel electrophoresis (Fig. 1). The reaction was totally dependent on the addition of template (Fig. 1, lane *A*). The primer formed had a unit length of 12–14 bases (Fig. 1, lanes *B* and *C*) and, in the absence of added dGTP, DNA primase could synthesize another unit or two of primer which was covalently attached to the 3′ end of the previously formed primers. This phenomenon has been observed with DNA primase purified from other sources (2, 3, 21–23) and suggested that DNA primase is a processive enzyme. The high molecular weight products formed in the absence of added dGTP (Fig. 1, lane *B*) are probably due to limited elongation of the primers by DNA polymerase α using dGTP which contaminated the GTP stock (6, 24). If dGTP was included in the reaction, then most of the ^{32}P-primer was attached to polynucleotides of 30–90 bases (Fig. 1, lane *D*) which were degraded by DNase I to products of 12–14 bases (Fig. 1, lane *E*). These results showed that, in the presence of dGTP, DNA polymerase α added dGTP to the 3′ terminus of the primer produced by DNA primase, and that only the unit length primer was formed. Because DNase I treatment of an RNA/DNA hybrid should leave three or four deoxynucleotides at the 3′ end of the primer (6), these results indicated that DNA primase produced a primer of approximately 8–10 bases, which was in agreement with published observations (2–4, 6).

poly(dC) and poly(dT), but not poly(dG) or poly(dA), were efficient templates in the DNA primase reaction (data not shown). The K_m for GTP in the DNA primase reaction utilizing poly(dC) was approximately 175 μM. ATP-dependent incorporation of [^3H]dATP into acid-insoluble product using poly(dT) as template did not follow normal Michaelis-Menten kinetics in that a plot of 1/velocity versus 1/(the concentration of ATP squared) was linear. When these data were plotted according to the method of Hill, as described by Segel (25), ATP exhibited positive cooperativity with a Hill coefficient of 2.0. The concentration of ATP required for half-maximal velocity, calculated from the Hill plot, was 3.4 mM. As noted by other investigators (14), ATP at higher concentrations (greater than 6 mM) inhibited DNA primase activity (data not shown). Because the poly(dT) DNA primase assay did not follow classic Michaelis-Menten kinetics, we chose to use the poly(dC) primase assay to further study the effect of the aranucleoside triphosphates on DNA primase.

Inhibition of DNA primase activity by nucleoside triphosphates and their arabinofuranosyl analogs. poly(dC) DNA primase activity was moderately inhibited by the normal rNTPs (Table 2). AMP and ADP were less potent inhibitors of DNA primase than ATP, suggesting that the β and λ phosphates are important determinants of the inhibitory action of these nucleotides. Because the DNA primase assay was a coupled enzyme assay involving DNA primase plus DNA polymerase α, it was necessary to determine the effect of these inhibitors on DNA polymerase α activity. The ribonucleoside triphosphates had no effect on DNA polymerase activity, indicating that the inhibition of the DNA primase assay by the rNTPs was due to inhibition of the DNA primase component of the assay and not DNA polymerase α. ATP and CTP inhibited poly(dC) primase activity in a manner competitive with GTP (K_i of 610 and 517 μM, respectively). DNA primase was also inhibited to a similar extent by the dNTPs (Table 2). However,

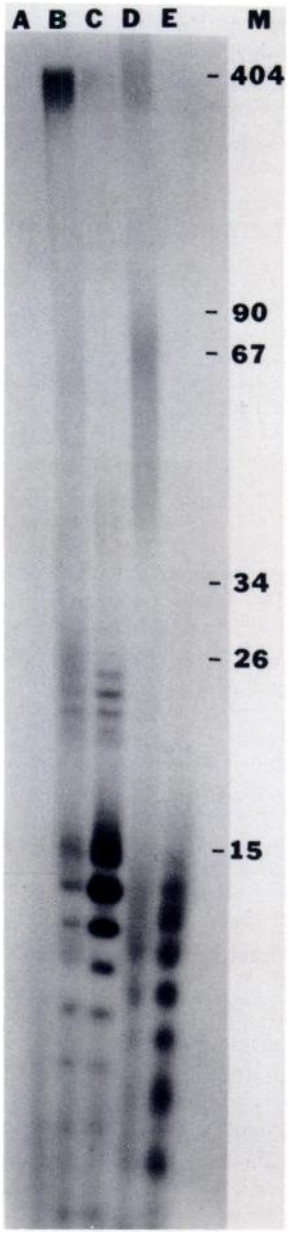

Fig. 1. Production of RNA primer by DNA primase. DNA primase was incubated for 1 hr with poly(dC) and 25 μM [α-^{32}P]GTP in the presence (lanes *D* and *E*) or absence (lanes *B* and *C*) of 50 μM dGTP. The RNA and DNA from each sample were extracted as described in Materials and Methods, and the extracts of lanes *C* and *E* were digested with 50 μg/ml of pancreatic DNase I. Electrophoresis of all samples was done on 7 M urea, 15% polyacrylamide gels. The size of the products was determined by comparison with DNA fragments from an MSP-I digest of PBR 322 DNA which were end labeled with T4 kinase and [λ-^{32}P]ATP (lane *M*). Lane *A* represents an experiment with DNA primase and [α-^{32}P]GTP but no poly(dC).

TABLE 2

Effect of natural nucleotides on DNA primase activity

DNA primase activity was measured as described in Materials and Methods using poly(dC) as template and 100 μM GTP. DNA polymerase α activity was measured using poly(dC) primed with oligo(dG) as template. The concentration of [^{3}H]dGTP in both the primase and polymerase assays was 10 μM. Each result represents the mean ± the standard deviation from three separate experiments.

Compound (1 mM)	Percentage of control primase activity	Percentage of control polymerase activity	K_i primase
			μM
AMP	94 ± 13		
ADP	53 ± 4		
ATP	50 ± 2	96 ± 6	610
CTP	32 ± 1	101 ± 6	517
UTP	29 ± 1	93 ± 8	
dATP	23 ± 4	65 ± 4	
dCTP	17 ± 17	60 ± 11	
dUTP	30 ± 17	67 ± 6	
dTTP	6 ± 3	40 ± 6	

TABLE 3

Effect of aranucleoside triphosphate analogs on DNA primase

DNA primase activity was measured using poly(dC) as template as described in Materials and Methods. DNA polymerase α activity was measured using poly(dC) primed with oligo(dG). The concentration of each compound in the DNA polymerase assay was equal to the highest concentration used to determine the K_i (the concentrations were: araCTP, 400 μM; araATP, 400 μM; and 2-F-araATP, 300 μM).

Compound	K_i primase	Percentage of control polymerase activity
	μM	
araCTP	122	107
araATP	128	76
2-F-araATP	25	116

the dNTPs also inhibited DNA polymerase activity using poly(dC) primed with oligo(dG) as template. The concentration of the dNTPs used in this experiment was 100 times the concentration of the [^{3}H]dGTP used to label the product.

poly(dC) DNA primase activity was more sensitive to araCTP than to CTP or dCTP (K_i of 122 μM, Table 3). araCTP inhibited poly(dC) DNA primase activity in a competitive manner with respect to GTP. Inhibition of the poly(dT) primase assay by araCTP was also competitive with respect to ATP (apparent K_i of 25 μM, data not shown). A comparison of the effect of araCTP, araATP, and 2-F-araATP on poly(dC) primase activity can be seen in Table 3. These three compounds were competitive inhibitors of DNA primase with respect to GTP, and the double reciprocal plots of 1/V versus 1/substrate concentration were linear for all drugs tested. Interestingly, 2-F-araATP was a much more potent inhibitor of DNA primase than was araATP or araCTP. DNA polymerase activity was not inhibited by 400 μM araCTP, 400 μM araATP, or 300 μM 2-F-araATP (Table 3), which was equal to the highest concentration of the analog used in the determination of the K_i, respectively, for DNA primase. Therefore, the effect of these agents on the DNA primase assay can be attributed to the inhibition of DNA primase and not DNA polymerase α.

The effect of araTTP, 2′-F-araTTP, FIAC-TP, FMAC-TP, and cytarazid-TP on DNA primase activity was compared to the inhibition seen with araCTP, araATP, and 2-F-araATP (Table 4). araTTP inhibited DNA primase to a similar degree as araATP and araCTP. However, 2′-F-araTTP, FIAC-TP, FMAC-TP, and cytarazid-TP were much more potent inhibitors of DNA primase than was either araCTP or araATP. DNA polymerase α was not inhibited by any of these compounds at

TABLE 4

Effect of aranucleoside triphosphate analogs on DNA primase activity

DNA primase activity was measured using poly(dC) as template and 50 μM GTP as described in Materials and Methods. DNA polymerase α was measured using poly(dC) primed with oligo(dG) as template. Each result represents the mean ± the standard deviation from three separate experiments.

Compound	Inhibition of DNA primase (IC_{50})	Pecentage of control polymerase activity[a]
	μM	
araCTP	34 ± 11	98 ± 3
araATP	50 ± 6	94 ± 6
2-F-araATP	13 ± 4	92 ± 9
araTTP	63 ± 8	85 ± 5
2′-F-araTTP	4 ± 1	93 ± 3
cytarazid-TP	13 ± 4	88 ± 2
FIAC-TP	2 ± 1	91 ± 8
FMAC-TP	3 ± 1	99 ± 12

[a] The concentration of each compound used in this experiment was equal to the concentration required to inhibit DNA primase by 90%. The concentration of each compound was: araCTP, 200 μM; araATP, 200 μM; 2-F-araATP, 80 μM; araTTP, 250 μM; cytarazid-TP, 80 μM; 2′-F-araTTP, 20 μM; FIAC-TP, 20 μM; and FMAC-TP, 20 μM.

concentrations which resulted in 90% inhibition of the DNA primase activity (Table 4). The IC_{50} for ara-5-azaCTP and 5-aza-dCTP could not be determined under conditions used in Table 4 because salt in the stock solutions of both ara-5-azaCTP and 5-aza-dCTP inhibited DNA primase at concentrations of drug greater than 50 μM. Because of the interest in ara-5-azaCTP and 5-aza-dCTP as potential antitumor agents, the inhibition of DNA primase by these two agents was compared to that seen with araCTP, dCTP, and CTP using a lower concentration of GTP (5 μM). Under these conditions a 12.5 μM concentration of araCTP, ara-5-azaCTP, and 5-aza-dCTP inhibited DNA primase activity by 65, 53 and 21%, respectively (data not shown). 5-aza-dCTP, dCTP, and CTP inhibited DNA primase activity to a similar extent (approximately 20%). These results indicated that the replacement of the number 5 carbon with nitrogen only minimally affected the ability of the parent compound to inhibit DNA primase activity.

Inhibition of primer synthesis by analogs of nucleoside triphosphates. The effect of the aranucleoside triphosphates on RNA primer formation was studied using polyacrylamide/urea gel electrophoresis. araCTP inhibited primer formation in a dose-dependent manner (Fig. 2). The inhibition of DNA primer synthesis was quantitated with the aid of a densitometer, and, as seen in Fig. 2, the inhibition of primer formation (Fig. 2, row *C*) correlated well with the inhibition of DNA primase activity which was measured by the routine filter disk assay using poly(dC) (Fig. 2, row *B*). RNA primer formation was similarly inhibited by the other aranucleotide analogs, araATP, 2-F-araATP, cytarazid-TP, FIAC-TP, FMAC-TP, araTTP, and 2′-F-araTTP (data not shown). The inhibition of primer formation seen with the aranucleoside triphosphates supported the conclusions drawn from the coupled enzyme assays (i.e., the inhibition of DNA primase was responsible for the inhibition seen in the coupled enzyme assay by these compounds).

Discussion

Aranucleoside triphosphates are important agents in the treatment of cancer (1, 12). Most of these agents are known to

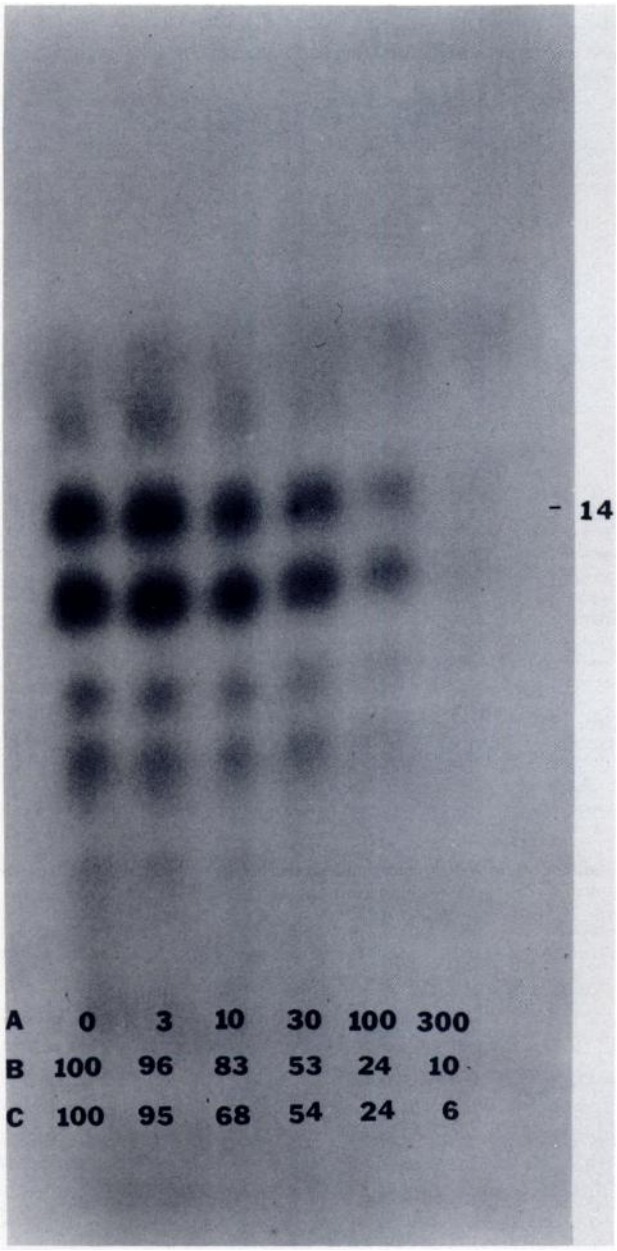

Fig. 2. Inhibition by araCTP of DNA primer formation. DNA primase was incubated with poly(dC), 50 μM dGTP, 50 μM [α-^{32}P]GTP, and 0–300 μM araCTP for 1 hr at 37°. The ^{32}P-labeled products were extracted with phenol/chloroform, precipitated with ethanol, and digested with DNase I as described in Materials and Methods. Below each lane is: row *A*, the concentration of araCTP used in the experiment; row *B*, percentage of control DNA primase activity determined by the routine filter disk assay (Table 4); and row *C*, percentage of control DNA primase activity determined by integration of a densitometric tracing of each lane.

inhibit DNA polymerase α, which is believed to play a role in the mechanism of action of these agents. Considering the importance of DNA primase to the replication of DNA, we studied this enzyme as a potential target in anticancer chemotherapy.

In this report we studied the effect of aranucleoside triphosphates on DNA primase activity using poly(dC) as template. This template offered certain advantages over natural templates such as M13 single-stranded DNA. First, the primase assay using poly(dC) was much more sensitive than the assays using either poly(dT) or M13 single-stranded DNA. Also, DNA primase activity on natural templates is not decreased to a significant degree by the absence of CTP, UTP, or GTP (2, 4, 6, 10, 18). Therefore, the K_m for individual nucleoside triphosphates on natural templates cannot be determined accurately, and the appropriate kinetic experiments cannot be done. Finally, DNA primase is very difficult to measure directly in a quantitative fashion, and measurement of DNA primase activity requires the presence of DNA polymerase to increase the sensitivity of the assay. The use of poly(dC) template to study the effect of noncomplementary aranucleoside triphosphates on DNA primase activity is advantageous, because it allows us to study the inhibition by these compounds of DNA primase activity in the coupled assay with minimal interference from an effect of these compounds on DNA polymerase α activity. One disadvantage to using poly(dC) primase activity assay to study the effect of arabinofuranosyl analogs of cytidine, adenosine, and thymidine is that DNA primase may be more sensitive to these compounds when inhibition is determined against the complementary template. Therefore, the K_i reported in this study represents the highest estimate, and DNA primase may be more sensitive to inhibition by these agents *in vivo*.

During our study Yoshida *et al.* (14) published their study on the effect of araATP and araCTP on DNA primase activity purified from calf thymus using poly(dT), poly(dI,dT), and M13 single-strand DNA as templates. They showed that araATP competitively inhibited the poly(dT) assay with respect to ATP (K_i equal to 21 μM). We also determined the effect of araATP on human DNA primase activity using the poly(dT) template (data not shown). Our studies could not differentiate whether the inhibitory action of araATP in our poly(dT) assay was due to the inhibition of DNA polymerase α or DNA primase. We found that the noncomplementary arabinofuranosyl nucleoside triphosphate, araCTP, competitively inhibited poly(dT) DNA primase activity with an apparent K_i of 25 μM, and that araCTP and araATP inhibited the poly(dC) DNA primase assay to the same extent (K_i of 125 μM, Table 3). Yoshida *et al.* (14) did not see any inhibition of the poly(dT) primase assay by araCTP. The reason for this discrepancy in our results is not known. Yoshida *et al.* (14) did show that araCTP inhibited the DNA primase assays which utilized M13 single-strand DNA and poly(dI,dT) as templates.

It is not yet known whether araCTP is incorporated into the RNA primer. In our work araCTP inhibited primer formation but did not result in the accumulation of smaller primers in a dose-dependent fashion (Fig. 2). If the inhibition of DNA primer formation were due to the incorporation of araCTP into the DNA primer resulting in the retardation of chain elongation, then we would expect to see the size of the primer decrease with increasing concentrations of araCTP. Because this did not occur, our results suggested that araCTP inhibits DNA primase from the outset, at least for the noncomplementary template. We are currently investigating the ability of DNA primase to incorporate araCTP into the RNA primer. The lack of high radiospecific [α-^{32}P]araCTP is our problem at the moment.

It is interesting that Bell and Fridland (26) have shown that araC and 9-β-D-arabinofuranosyladenine inhibit both the initiation and elongation of DNA synthesis. They speculate that an intracellular target other than DNA polymerase α may be responsible for the inhibition of DNA synthesis initiation seen with this class of compounds. It is possible that the inhibition of DNA primase may account for the inhibition by araATP

and araCTP of the initiation of DNA synthesis that they observed.

We have determined the effect of a number of arabinofuranosyl nucleoside triphosphate analogs on DNA primase activity in an attempt to identify structural features which are important to the inhibitory properties of these compounds. From such studies it may be possible to design new compounds with greater potency against DNA primase or which selectively inhibit DNA primase and not DNA polymerase α. Such compounds may have a different spectrum of antitumor activity and may complement the antitumor drugs which are currently in use. It is evident that the addition of a fluorine atom to either the 2′-carbon of the arabinosyl sugar of araTTP or to the number 2 carbon of the purine ring of araATP markedly increased the potency of these compounds (Table 4). Together these results suggested that 2′,2-difluoro-araATP would be a very potent inhibitor of DNA primase.

The concentrations of ATP, GTP, CTP, and UTP in cells are approximately 2.9, 0.56, 0.34, and 1.0 mM, respectively (27). These values are similar to the K_i for DNA primase of both ATP and CTP (Table 2, Ref. 3), which suggested that the intracellular levels of the rNTPs may play an important role in the regulation of DNA primase. Yamaguchi *et al.* (5) have shown that the alteration of the relative concentrations of the rNTPs in *in vitro* DNA primase assays can affect the composition of the primer which is formed. Together, these results suggested that the perturbation of rNTP pools by any treatment may disrupt DNA synthesis by inhibiting DNA primase.

The results in this paper and those of others (4, 9, 14) showed that DNA primase was inhibited by arabinofuranosyl nucleoside triphosphate analogs and suggested that this may play a role in the cytotoxicity of these agents. However, the K_i for inhibition of DNA primase by araCTP is higher than that for DNA polymerase α (approximately 10 μM, Ref. 12). Therefore, the inhibition of DNA primase may only play a role in the cytotoxicity of araC when high doses of araC are used in the treatment of acute myelogenous leukemia (28) where the concentration of araCTP has been shown to reach more than 70 μM in tumor cells (13, 29, 30). Further studies *in vivo* are necessary to determine the importance of DNA primase as a target for araCTP in the cell-killing action of araC. Certainly the effect of araCTP on DNA polymerase α activity is still a major determinant of this drug's action.

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