0026-895X/87/030330-10$02.00/0

MOLECULAR PHARMACOLOGY, 32:330-339

Sequence-Specific Effects of Ara-5-aza-CTP and Ara-CTP on DNA Synthesis by Purified Human DNA Polymerases *In Vitro*: Visualization of Chain Elongation on a Defined Template

ALAN J. TOWNSEND and YUNG-CHI CHENG
Pharmacology Department, University of North Carolina, Chapel Hill, NC 27514
Received December 2, 1986; Accepted June 12, 1987

SUMMARY

1-β-D-arabinofuranosyl-5-aza-cytosine (ara-5-aza-Cyd) is an analog of 1-β-D-arabinofuranosylcytosine (ara-C), which resembles ara-C in anabolic metabolism, incorporation into DNA, and inhibition of DNA replication. Human T-lymphoblastic cells (Molt-4) incorporate three- to fivefold more ara-5-aza-Cyd than ara-C into DNA during 5–8 hr exposure. Although ara-5-aza-Cyd and its triphosphate metabolite are unstable in aqueous solution, the aza-analog was much more stable in solution when incorporated into native DNA isolated from Molt-4 cells. By using gapped duplex DNA as a substrate for purified human DNA polymerases alpha and beta, inhibition of [^{3}H]-dCTP incorporation by ara-5-aza-CTP and ara-CTP was competitive, with K_i values for alpha of 11 and 1.5 μM, respectively. K_i values for polymerase beta were 39 and 7.6 μM, respectively. A DNA elongation assay was adapted from DNA sequencing technology, using singly primed bacteriophage M13mp19 or M13mp9 (+)-DNA. Elongation of 5′-[^{32}P]-labeled primer by polymerase alpha is slowed considerably by incorporation of one ara-CMP and to a lesser extent after incorporation of one ara-5-aza-CMP. Neither analog significantly affected elongation by polymerase beta after a single incorporation. However, neither polymerase alone could appreciably extend the growing chain if two consecutive ara-5-aza-CMP or ara-CMP analogs were incorporated. Thus, if similar mechanisms are operant in intact cells, the greater incorporation of ara-5-aza-Cyd than ara-C into DNA may be due to a more facile elongation of the nascent DNA strand by polymerase alpha after incorporation of a single analog. The effect *in vitro* of incorporation of either analog on DNA chain elongation is widely variable, depending on the identity of the polymerase involved and the sequence of the DNA template being copied.

Ara-5-aza-Cyd is a relatively new antitumor agent (1) that incorporates both the 2′-β ("up") pentose configuration found in cytosine arabinoside (ara-C) and the nitrogen substitution at position 5 of the cytosine ring that is found in 5-aza-Cyd and 5-aza-dCyd. Each of these modifications alone has resulted in compounds with potent antileukemic activity (2, 3). The new hybrid analog has been reported to have better antileukemic activity in the mouse L1210 model (4) and much greater activity against human colon, lung, and mammary tumor xenografts in nude mice (5) than ara-C or the 5-aza-derivatives, none of which has previously proven effective against nonhematologic tumors (6). We have previously investigated the metabolism and mode of action of this pyrimidine analog in human T-lymphoblastic cells (Molt-4) (7). We reported that ara-5-aza-Cyd and ara-C shared anabolic pathways, inhibited DNA, but not RNA or protein synthesis, and had similar cytotoxicity against these cells as measured by clonogenic survival. The enzyme deoxycytidine kinase is required for the initial phosphorylation step, and deletion of the enzyme results in a high degree of resistance to ara-C, 5-aza-dCyd, and ara-5-aza-Cyd (7, 8). Unlike ara-C, 5-aza-Cyd, and 5-aza-dCyd, however, ara-5-aza-Cyd was not significantly catabolized by Cyd-dCyd deaminase. This feature could prove advantageous, since deamination is the primary catabolic route of inactivation of ara-C (9) and results in a short half-life of the nucleoside *in vivo*. Also unlike the other nucleosides, ara-5-aza-Cyd does not competitively inhibit deamination of dCyd (7). Thus, although a high tumor deaminase level would tend to lower the potency of ara-C, 5-aza-Cyd, and 5-aza-dCyd, it might actually confer a greater sensitivity to ara-5-aza-Cyd in these cells, since they would be expected to have lower competing pools of dCyd due to selective removal by deamination. This phenomenon, which has been termed "collateral sensitivity" (10), could result in

This work was supported by Grant CA-27448 from the National Cancer Institute, National Institutes of Health, Bethesda, MD.

ABBREVIATIONS: ara-5-aza-Cyd, 1-β-D-arabinofuranosyl-5-aza-cytosine; ara-C, 1-β-D-arabinofuranosylcytosine; 5-aza-Cyd, 5-aza-cytidine; 5-aza-dCyd, 5-aza-2′-deoxycytidine; ara-5-aza-CTP, 1-β-D-arabinofuranosyl-5-aza-cytosine-5′-triphosphate; ara-CTP, 1-β-D-arabinofuranosylcytosine-5′-triphosphate; 5-aza-dCTP, 5-aza-2′-deoxycytidine-5′-triphosphate; ssDNA, single-stranded DNA; ACS, aqueous counting scintillant; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; DTT, dithiothreitol; pol alpha, human DNA polymerase alpha; pol beta, human DNA polymerase beta; AMP, adenosine-5′-monophosphoric acid; "C" sites, positions on the nascent DNA strand where a dCyd incorporation is specified by the template; PBS, phosphate-buffered saline (0.14 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.15 mM KH_2PO_4 (pH 7.2); HPLC, high pressure liquid chromatography; BSA, bovine serum albumin.

enhanced sensitivity of solid tumors to ara-5-aza-Cyd, since these frequently exhibit high levels of this enzyme and are generally unresponsive to ara-C (11). Ara-5-aza-Cyd also exhibited another potentially important difference from ara-C in its disposition in Molt-4 cells. It was found that despite the fact that ara-5-aza-CTP accumulation was less than half that of ara-CTP, Molt-4 cells incorporated more than three times as much ara-5-aza-Cyd as ara-C into DNA during the first 8 hr of exposure (7). The degree of incorporation of ara-C into DNA has been closely correlated with cytotoxicity (12). Hence, the new analog may also have an advantage in this important determinant of ara-C lethality, if its mechanism of action is similar.

In an effort to better understand the differences in disposition and mode of action of ara-5-aza-Cyd and ara-C, we have studied the behavior of purified human DNA polymerases alpha and beta in the presence of ara-5-aza-CTP and ara-CTP. The first approach utilized was the classic kinetic assay for inhibition of [^{3}H]-dCTP incorporation, with gapped duplex DNA as substrate, in the absence or presence of the competing triphosphates, ara-5-aza-CTP and ara-CTP. The second approach, a DNA elongation assay, was developed to study the site-specific effects of the local DNA template sequence on DNA primer extension when ara-5-aza-CTP, ara-CTP, or 5-aza-dCTP are substituted for dCTP. This assay employs as substrate a prelabeled 17-base primer annealed to M13 circular (+)-DNA, followed by electrophoresis and autoradiographic visualization of the elongated products. The substrate efficacy and relative chain-terminating ability of the two triphosphates have been directly compared for both singular and consecutive sites of incorporation along the known sequences specified by different M13 templates. In addition, we have investigated the stability of the new analog after incorporation into DNA, since the nucleoside and triphosphate forms are known to be unstable in aqueous solution (1, 7, 17).

Materials and Methods

Cell culture. Human T-lymphoblastic cells (Molt-4) were maintained as previously described (7) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). Log-phase cells were resuspended in fresh medium before all experiments.

Materials. Ara-5-aza-Cyd was synthesized by the Drug Synthesis and Chemistry Branch of the National Cancer Institute and provided by Dr. David Cooney. 5-aza-dCyd was provided by Mack, Inc. (Bavaria, FRG). All other nucleotides were purchased from Pharmacia, Inc. (Piscataway, NJ), unless otherwise indicated. Commercial dATP, dCTP, dTTP, and dGTP were ultrapurified by anion-exchange HPLC as previously described (7), followed by chromatography on a 2.5 × 30 cm column of DEAE-Sephadex A-25 formate (Pharmacia) with a 0–2 M ammonium formate elution gradient. The ultrapure triphosphates were lyophilized to dryness several times, then desalted on a column of Sephadex G-10 equilibrated with 10 mM Tris/HCl (pH 7.5) and 0.2 mM EDTA. Ara-CTP was synthesized from ara-C (Sigma) by the chemical method previously described (13) and purified as above. The final ultrapurified nucleotide preparations were checked for purity by anion-exchange HPLC. DEAE-cellulose (DE-52), phosphocellulose (P-11), DEAE-coated discs (DE-81), and glass fiber discs (GF/A) were from Whatman, Inc. (Clifton, NJ). Calf thymus DNA was obtained from Sigma (St. Louis, MO) and gapped using DNase I as previously described (14). Single-stranded DNA-cellulose was also from Sigma. Proteinase K, DNase I, and DTT were from Boehringer Mannheim, Inc. (Indianapolis, IN). 5-[^{3}H]-dCTP (28 Ci/mmol) and 5-methyl-[^{3}H]-dTTP (23 Ci/mmol) were from ICN, Inc. (Irvine, CA), and γ-[^{32}P]-ATP and ACS scintillant were from Amersham, Inc. (Arlington Heights, IL). 5-[^{3}H]-dCyd (18 Ci/mmol), 5,6-[^{3}H]-ara-C (18 Ci/mmol), 6-[^{3}H]-5-aza-dCyd (8 Ci/mmol), and 6-[^{3}H]-ara-5-aza-Cyd (2.8 Ci/mmol) were purchased from Moravek, Inc. (Brea, CA). Phages M13mp19 and M13mp9 were the generous gift of Dr. Clyde Hutchison III of the University of North Carolina Microbiology Department. Phages were grown in *Escherichia coli* strain K12-JM-103 (Pharmacia) and single-stranded circular (+)-DNA recovered as described (15). The 17-base oligodeoxynucleotide primer (5′-GTAAAACGACGGCCAGT-3′) was synthesized with an American Bionuclear (Emeryville, CA) automated DNA synthesizer by Dr. Dana Fowlkes, Pathology Department, University of North Carolina School of Medicine, according to the manufacturers specifications.

Enzymatic synthesis of aza-triphosphates. Ara-5-aza-CTP and 5-aza-dCTP were too labile to be prepared by the chemical method and were therefore synthesized enzymatically as follows. Proteins were extracted from 15 g leukopheresed human chronic lymphocytic leukemia cells and carried through the 1% streptomycin sulfate precipitation of nucleic acids as previously described (16). The resulting supernatant was dialyzed against 25 mM Tris/HCl (pH 7.5), 2 mM DTT, 1 mM EDTA, and 10% glycerol (buffer A) and applied to a 1.5 × 15 cm column of Blue Sepharose (Pharmacia) equilibrated with buffer A. Cytidylate kinase (E.C. 2.7.4.14) and nucleosidediphosphate kinases (E.C. 2.7.4.6) were retained on the column and were step-eluted with buffer A containing 1.5 M KCl. The unadsorbed fraction contained deoxycytidine kinase (E.C. 2.7.1.74), which was concentrated fivefold by ammonium sulfate precipitation (65%) followed by dissolution of the pellet in 1/5 volume buffer A. Both enzyme fractions were dialyzed against buffer A and stored at −70°C. The enzymatic synthesis was conducted in two steps. The monophosphorylation reaction was performed at 23°C for 1.5 hr in 12.5 ml buffer containing 1,875 μmol Tris/HCl (pH 7.5), 125 μmol ATP, 125 μmol $MgCl_2$, 125 μmol NaF, 25 μmol DTT, 2.5 mg BSA, 1.25 ml glycerol, 18.7 μmol nucleoside, and approximately 200 units (5 ml) of dialyzed deoxycytidine kinase (1 unit = 1 nmol dCMP formed/min at 37°C). An additional 62.5 μmol ATP plus the mono/diphosphate mixture (>1,000 units each) was added in a total volume of 2.5 ml, and the reaction mixture (15 ml) was incubated at 37°C for 30 min. The reaction was stopped by addition of EDTA (50 mM final), and the analog triphosphates were purified by DEAE-Sephadex chromatography at 4°C as described above. The yield of the enzymatic synthesis was 60–65%, and the final products each chromatographed as a single peak on anion-exchange HPLC.

Stability in native DNA. Log-phase human T-lymphoblastic cells (Molt-4, American Type Culture Collection, Rockville, MD) were incubated at 10^6 cells/ml as previously described (7) for 5 hr with various [^{3}H]-nucleosides (0.2 μM, 1.0 μCi/ml), and the DNA was purified as previously described (7). The DNA from 2–3 × 10^7 cells was redissolved in 1.0 ml PBS containing 1 mM EDTA, sheared 50 times with a 22-gauge needle (to reduce the viscosity), and incubated at 37°C. At 0, 2, 8, and 24 hr, duplicate 100 μl aliquots were removed and spotted onto DE-81 discs and stored at −20°C. Alternatively, a portion of the DNA was dissolved in 5 mM Tris/HCl (pH 7.2) and denatured by sonication with a Branson microtip sonifer set to 40% of maximum power for 3 × 20 sec bursts. Partial denaturation was verified by a low A_{260} hyperchromic shift at 80°C of only 14%, compared with 40% for the gapped duplex DNA. The solution of denatured DNA was adjusted to 140 mM NaCl, 0.65 mM KPO_4 (pH 7.2, i.e., same as PBS), and 1 mM EDTA. This DNA solution was incubated at 37°C for 1, 2, 4, or 8 hr, and aliquots were spotted onto DE-81 discs as described above. The discs were kept at −20°C and washed together three times for 5 min each in 0.3 M ammonium formate (pH 7.5) to remove soluble radioactivity and counted in 10 ml ACS scintillant. For comparison, [^{3}H]-ara-5-aza-CTP was also incubated in PBS at 37°C and spotted onto discs and washed three times with 1 mM ammonium formate. Under these conditions, the irreversible loss of the 6-carbon of the triazine ring as [^{3}H]-formate is readily quantitated by measuring the decrease in radioactivity retained on the anion-exchange disc after selective washing to remove labeled formate.

Enzyme purification. DNA polymerases were purified from ex-

tracts of human acute myeloblastic leukemia cells obtained by leukopheresis. The cells were first resuspended for 10 sec in ice-cold 1 mM phosphate buffer to lyse contaminating erythrocytes, then adjusted to normality with PBS (×10), washed twice in cold PBS and resuspended in 4 vol buffer B (50 mM KPO_4 (pH 7.5), 2 mM DTT, 2 mM EDTA, 1 mM PMSF, and 10% glycerol). The cells were frozen and thawed twice, then homogenized by 40 strokes in a Dounce homogenizer. The KPO_4 concentration was adjusted to 300 mM, and the suspension was centrifuged at 27,000 × *g* for 30 min. The resulting supernatant was passed through a 50-ml column of DEAE-cellulose equilibrated with buffer B plus 300 mM KPO_4, (pH 7.5) and the unadsorbed fractions pooled and dialyzed overnight against buffer B. The dialysate (180 ml) was recentrifuged and applied to a 150-ml DEAE-cellulose column equilibrated with buffer B, then washed with 300 ml buffer B, and eluted with a 600-ml linear gradient of 50–500 mM KPO_4 buffer B. The unadsorbed fractions, which contained pol beta, and the gradient fractions containing pol alpha were pooled separately and dialyzed against buffer B. These fractions were applied to separate 120-ml columns of phosphocellulose that were pre-equilibrated with buffer B. The columns were washed with 250 ml buffer B and eluted with 600 ml gradients of 50–500 mM KPO_4 for pol alpha or 0–1,200 mM KCl for pol beta. Fractions containing peak activity were pooled and dialyzed against 25 mM Tris/HCl (pH 7.5), 2 mM DTT, 0.2 mM EDTA, and 20% glycerol and applied to separate 10 ml ssDNA-cellulose columns equilibrated with the same buffer. After washing with 20 ml of this buffer, the columns were eluted with 60 ml gradients of 0–1,200 mM KCl in equilibration buffer. Fractions containing peak activities were pooled and dialyzed against equilibration buffer containing 30% glycerol and stored at −70°C until use. This procedure generally resulted in a 200–500-fold purification of pol alpha (500–1000 units/mg). The specific activity of purified pol beta was about 1,000–2,000 units/mg (1 unit polymerase activity is defined as the amount that catalyzes the incorporation of 1 nmol dTMP into activated DNA per hour at 37°C).

Enzyme assay. The reaction mixture for pol alpha contained 50 mM Tris/HCl (pH 7.5), 8 mM $MgCl_2$, 2 mM DTT, 100 μg/ml gapped calf thymus DNA, 100 μM each dATP, dTTP, and dGTP, 100 μg/ml BSA, variable concentrations of [^{3}H]-dCTP (1.0 Ci/mmol) and competing triphosphate, and 1–5 μl enzyme (0.05–0.25 units) in a total volume of 50 μl. The reaction mixture for pol beta was identical except 60 mM KCl was added. In the substitution assay, (Fig. 2), dCTP was omitted, and [^{3}H]-dTTP (10 μM, 10 μCi/ml) was used to monitor polymerization. All assays were 30–60 min at 37°C, after which 40 μl of the mixture were spotted onto GF/A discs and washed three times in cold 5% TCA/10 mM sodium pyrophosphate and once in 95% ethanol and counted in 8 ml ACS scintillant.

DNA elongation assay. Primer was prelabeled by incubation of 0.06 A_{260} U with 200 μCi γ[^{32}P]-ATP (3,000 Ci/mmol) and 5–10 U T4 polynucleotide kinase (Boehringer) in 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 5 mM DTT in a total volume of 0.2 ml for 30 min at 37°C, followed by boiling for 3 min. The annealing was done by combining 1.0 μg M13 ssDNA, 1.0 μl 50 mM Tris/2 mM EDTA, and 8 μl H_2O/μl labeled primer and heating the mixture to 65°C followed by slow cooling to room temperature over 60 min.

The DNA elongation mixture contained, in a total volume of 7.5 μl, 30 mM Tris/HCl (pH 7.5), 8 mM $MgCl_2$, 2 mM DTT, 50 μM each dATP, dTTP, and dGTP, 25 μg/ml labeled M13 template/primer, 100 μg/ml BSA, DNA polymerase alpha (0.15 units) or beta (0.03 units), and the indicated concentration of dCTP or analog. After incubation at 37°C for the indicated times, the reaction was stopped by addition of 3.5 μl of 50 mM EDTA and 0.3% bromphenol blue in formamide, boiled for 3 min, then placed on ice. The samples (in 1.5 ml microfuge tubes) were centrifuged briefly and loaded onto a 10% polyacrylamide DNA sequencing gel (apparatus was from BRL, Bethesda, MD) and electrophoresed for 2.5 hr at 30 W constant power. The gel plates were separated, and the wet gel, still adhered to one plate, was wrapped with polyethylene film and apposed to a 14″ × 17″ sheet of Fuji RX type x-ray film (Chesapeake X-ray, Farmville, NC), and exposed for 16 hr and developed.

Results

Stability in DNA. The 5-aza-substitution results in a triazine ring that is inherently unstable in aqueous solution, decomposing by hydyolytic ring-opening followed by loss of the [^{3}H]-labeled six-carbon atom as [^{3}H]-formic acid (1, 4, 17). The hydrolysis is pH and temperature dependent, occurring much faster at extremes of pH or at 37°C than at neutral pH or at 4°C (7, 17). Thus ara-5-aza-Cyd spontaneously breaks down at 37°C in PBS with a $t_{½}$ = 12 hr, whereas the triphosphate is even more unstable, with a $t_{½}$ = 4–5 hr (7) (Fig. 1). It was observed previously that ara-5-aza-Cyd was incorporated into DNA to a three- to fivefold greater extent than ara-C under identical conditions despite the instability and twofold lower accumulation of the triphosphate in Molt-4 cells at the time of harvest (7).

Since the nucleoside and triphosphate were unstable, it was of interest to determine the stability of ara-5-aza-Cyd in DNA, since decomposition after incorporation would lead to the formation of potentially genotoxic apyrimidinic sites in the DNA. After exposure of Molt-4 cells to [^{3}H]-ara-5-aza-Cyd for 5 hr, nucleic acids were extracted and purified and then incubated at 37°C in PBS. At the times indicated in Fig. 1, aliquots were removed and spotted onto DE-81 discs, washed free of soluble radioactivity, and counted in a scintillation counter to determine the remaining DNA-associated radioactivity. Surprisingly, the new analog appeared to be quite stable in native DNA at 37°C (Fig. 1), as was another 5-aza-substituted pyrimidine, 5-aza-dCyd (not shown). Some stabilization was lost on denaturation of the DNA by sonication, resulting in about 15% loss of label after 8 hr at 37°C. At the end of the incubation period (24 hr), the radioactivity in native DNA was >90% solubilized by exposure to 50 μg/ml DNase I for 5 min. This is consistent with our previous finding that the analog is incorporated exclusively into DNA (7). An aliquot of the DNase 1 digest was treated with spleen phosphodiesterase and alkaline

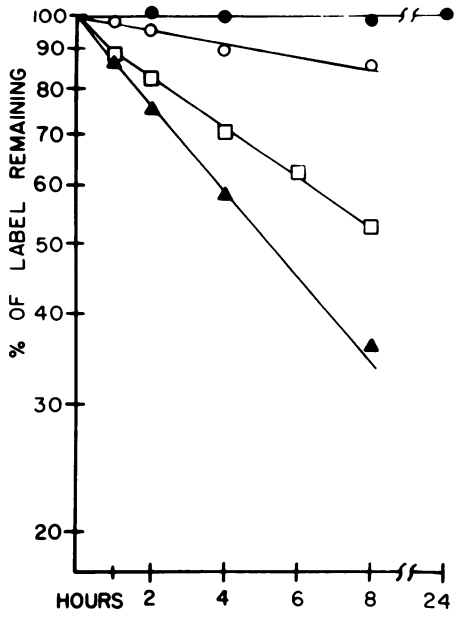

Fig. 1. Chemical stability in PBS at 37°C of ara-5-aza-Cyd (□), ara-5-aza-CTP (▲), [^{3}H]-ara-5-aza-Cyd (●) in native DNA isolated from Molt-4 cells exposed to 0.2 μM, 1.0 μCi/ml [^{3}H]-ara-5-aza-Cyd for 5 hr and stability (○) in denatured DNA under the same conditions.

phosphatase, and the identity of the labeled nucleoside was verified by cation-exchange HPLC as previously described (7). Thus, it may be concluded that the aza-analogs are considerably more stable when incorporated into native DNA; however, this may not be the case in DNA that is single-stranded or maintained under denaturing conditions.

Kinetic studies. The interaction of the triphosphates of ara-5-aza-Cyd and ara-C with purified human DNA polymerases alpha and beta was examined, using gapped double-stranded ("activated") DNA as substrate. Linear double-reciprocal plots were obtained in each case, indicating the expected kinetics of inhibition which were competitive with respect to dCTP (Table 1). The K_i values for pol alpha were 1.5 and 11.3 μM for ara-CTP and ara-5-aza-CTP, respectively. The K_m value for dCTP and the K_i value for ara-CTP are in agreement with those previously published by other laboratories for pol alpha (18, 19). With pol beta, the K_m value for dCTP was similar, but the K_i value was about twofold lower than our previously obtained result (11), possibly due to differences in the source of the enzyme or the purification protocol. The K_i values for pol beta were approximately four- to fivefold higher than for pol alpha for both analogs, compared with a twofold higher K_m for dCTP, indicating a slightly weaker competitive inhibition of this enzyme (Table 1). The K_i values indicate a weaker affinity of the polymerases for ara-5-aza-CTP than for ara-CTP in competition with dCTP, even though the aza-analog is incorporated into cellular DNA to a greater extent. It was therefore of interest to examine the relative rates of elongation when the two polymerases were forced to utilize only the analog triphosphates in the absence of dCTP.

Substitution assay. The first approach employed was a simple substitution assay, using an assay mixture containing gapped duplex DNA and dATP, dTTP, dGTP, [^{3}H]-dTTP, and analog triphosphate, without dCTP. The linear slope of the "background" incorporation of label by pol alpha in the absence of dCTP is about 29% of the rate with the complete mixture containing 10 μM dCTP, after the initial 20 min incubation (Fig. 2). When 5-aza-dCTP was substituted for dCTP, the rate was almost as great as that with dCTP, consistent with a previous report that this analog is a very good substrate for pol alpha (20). Substitution of ara-CTP for dCTP resulted in only a slightly greater amount of thymidylate incorporation at 20 min than in the control reaction without dCTP. Similarly, ara-5-aza-CTP could support a slightly greater rate of incorporation over the initial 20 min than ara-CTP or the control reaction; however, beyond 20 min neither arabinosyl triphosphate appeared to support rates of DNA synthesis measurably different from those of the control reaction, as judged by the slopes of their plots in Fig. 2A.

When the experiment was repeated using pol beta, a similar pattern was observed for all substrates except 5-aza-dCTP, which was only slightly better than the arabinosides during the initial phase (Fig. 2B). Thus, neither arabinosyl triphosphate could support a rate of [^{3}H]-TTP incorporation that was measurably greater with either polymerase than the background, after the initial 20 min of reaction. However, previous experiments in this laboratory[1] and others (21) have shown that labeled ara-CTP can be incorporated by pol alpha into gapped duplex DNA, albeit at low levels, primarily at the 3′-termini of the growing chain. The information obtainable by this approach is limited by the heterogeneous nature of the gapped duplex DNA substrate and by the low sensitivity at the reaction rates being observed. The heterogeneity in the template also complicates comparison of the two polymerases, since pol alpha is a much more processive enzyme than pol beta, which incorporates only one or a few nucleotides per DNA binding event. Thus chain elongation by pol alpha might be affected more by the local sequence of the template being copied than pol beta, when one of the normal four nucleotides is absent or replaced by an analog that is a relatively poor substrate. Indeed, the background slope without dCTP is relatively higher for pol beta (48%) than pol alpha (29%), compared with the slope with all four nucleotides present (Fig. 2). The need for a sensitive

TABLE 1

Analog substitution for dCTP

Michaelis-Menten constants for ara-5-aza-CTP, ara-CTP, and dCTP with human DNA polymerases alpha and beta, determined by double-reciprocal analysis, using a gapped duplex ("activated") DNA substrate. All values expresed as micromolar concentration.

	Polymerase alpha		Polymerase beta	
	K_m	K_i	K_m	K_i
dCTP	3.3 ± 0.3[a]		8.0 ± 1.2	
ara-CTP		1.5 ± 0.6		7.6 ± 1.9
ara-5-aza-CTP		11.3 ± 1.5		38.8 ± 2.5

[a] Values given are means ± standard deviation of at least four separate determinations.

[1] Townsend, A. J., and Y-C. Cheng, unpublished results.

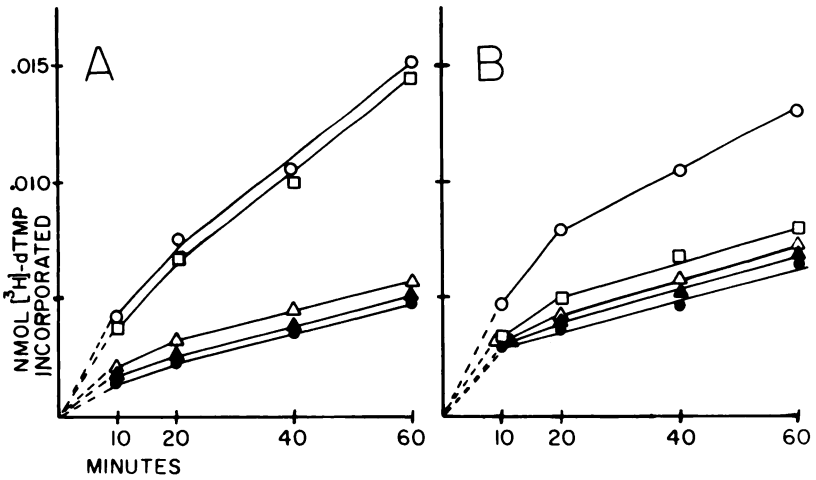

Fig. 2. A, Incorporation of [^{3}H]-dTMP into gapped duplex DNA by pol alpha in the presence (○) or absence (●) of 10 μM dCTP or with 10 μM 5-aza-dCTP (□), ara-CTP (▲), or ara-5-aza-CTP (△) substituted for dCTP. B, same, with pol beta instead of pol alpha. Assay conditions are described in text.

and well-defined system for examination of polymerization events at the molecular level prompted us to develop the assay described below, using modifications of existing technology.

DNA elongation assay. The dideoxynucleotide sequencing methodology first described by Sanger *et al.* (22) was combined with the M13 sequencing vectors developed by J. Messing (15) and adapted for use as a DNA elongation assay for mammalian polymerases. This system allows indirect visualization of site-specific events along a circular ssDNA template of known sequence, primed at a single site with a unique complementary 17-base oligodeoxynucleotide that is labeled at the 5′-end with [^{32}P]. Since this primer anneals to the 3′-end of the distinct multiple cloning sites of each of the individual M13 sequencing vectors, the initial template sequence copied by the polymerase may be varied by using ssDNA of different M13 strains annealed to the common primer. In the template sequence specified by the phage vector M13mp19, the first "C" residue specified is flanked by a "T" and a "G" on the 5′ and 3′ sides, respectively. In the vector M13mp9, the first "C" site specified has the sequence "CCC." Thus, it is possible to assess the effect of base stacking and ultimately the effect of flanking sequences on the incorporation of normal and abnormal nucleotides by human polymerases. After incubation of this substrate with purified polymerase and various reaction mixtures, the product is denatured and the elongated [^{32}P]-primer electrophoresed on the sequencing gel and visualized by autoradiography.

In the absence of dCTP, it may be seen that the patterns of incorporation of ara-CTP, ara-5-aza-CTP, and 5-aza-dCTP by pol alpha into the nascent elongating DNA strand are different for each analog triphosphate. Incorporation of 5-aza-dCTP is relatively efficient, as predicted from the data of Fig. 2, although significantly less than with dCTP (Fig. 3A, lanes J–L and Fig. 3B, lanes K–L). Incorporation of either arabinoside analog at the single "C" site first specified by M13mp19 slows but does not stop chain elongation by pol alpha (Fig. 3A, lanes D–I). Incorporation of ara-5-aza-CTP at this site appears to cause less of a slowdown or "pause" by pol alpha at this site than does ara-CTP, consistent with the greater observed incorporation of ara-5-aza-Cyd into Molt-4 DNA. Interestingly, when either arabinosyl analog is substituted by pol alpha into the consecutive "C" sites first specified in the M13mp9 template, nearly complete chain termination occurs (Fig. 3B, lanes E–I).

Since the analog triphosphates themselves are not labeled, the possibility must be considered that strand elongation at "C" sites in the absence of dCTP could occur by misincorporation of dATP, dGTP, or dTTP, rather than site-specific incorporation of the analogs. However, several observations with this assay system support the conclusion that the analogs themselves are incorporated at sites where insertion of a "C" residue is specified by the template. 1) In the absence of dCTP or analog, most of the primer is elongated only five bases and accumulates at the "T" site immediately preceding the first "C"

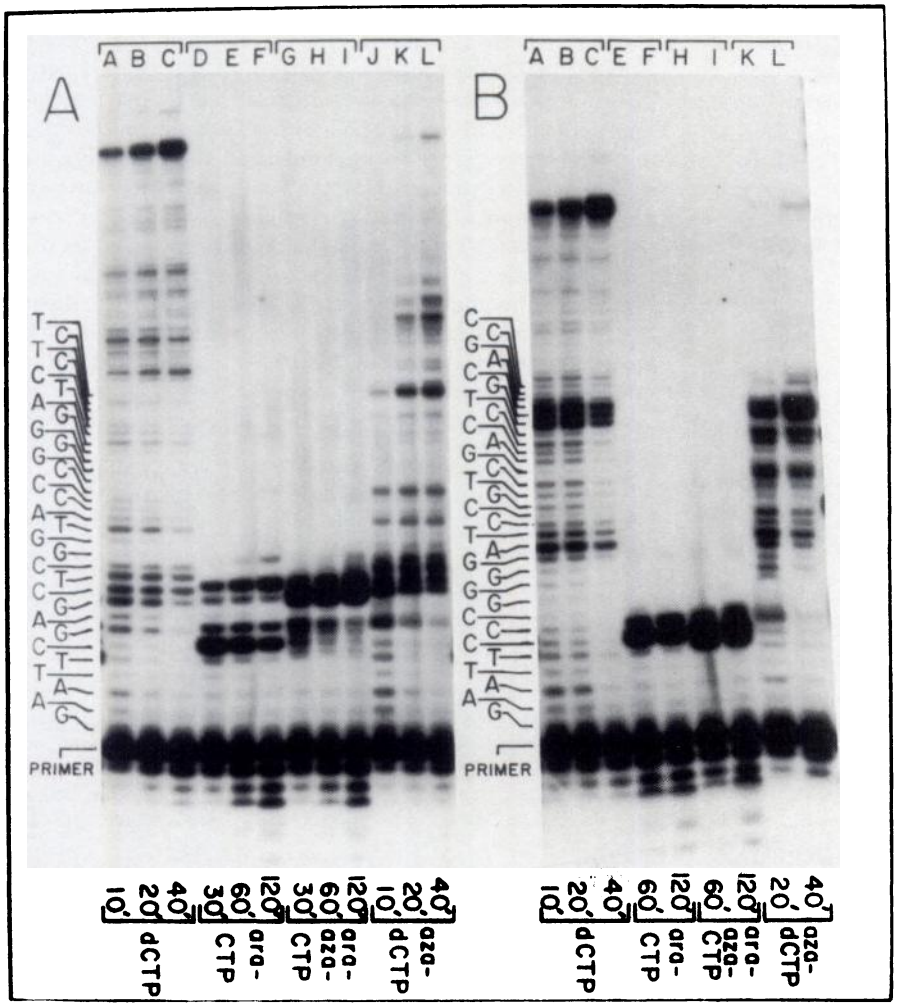

Fig. 3. A, elongation by pol alpha (0.15 units) of 5′-[^{32}P]-labeled primer annealed to M13mp19 (+)-DNA template and incubated for the indicated times in the presence of 100 μM dCTP, ara-CTP, ara-5-aza-CTP, or 5-aza-dCTP and 50 μM each of dATP, dGTP, and dTTP. B, same, except M13mp9 template is annealed to the primer. Note that the polymerase first encounters a single "C" incorporation site with the M13mp19 template (Fig. 3A), whereas with the M13mp9 template, a triple "CCC" site is first encountered. Assay conditions are described in the text.

site (not shown). 2) In the presence of the arabinosyl analogs, the polymerase readily fills this first "C" site, and the primer accumulates at the first (and subsequent) "C" sites (Fig. 3). 3) When dCTP (5 μM) and arabinosyl analog (50 μM) are both present, primer accumulates at multiple "C" sites, rather than at the sites preceding them (not shown). This would be expected if inhibition of elongation occurred after incorporation of a fraudulent base at these sites. These observations, together with those of other workers using *in vitro* assay systems (21), indicate that the arabinosyl analogs are inserted at the sites where a "C" residue is specified, causing a slowdown in subsequent elongation of the nascent strand.

A small but significant exonuclease activity was present in the purified pol alpha preparation that could hydrolyze single-stranded (unhybridized) primer in the 3′–5′ direction (Fig. 3, bottom); the activity was only marginally detectable with fully annealed primer/M13 DNA. This activity was almost completely inhibited by 2.0 mM 5′-AMP, a concentration that had no significant effect on the rates of DNA synthesis with either polymerase (data not shown). The DNA elongation experiments were repeated in the presence of 2.0 mM 5′-AMP, to assess whether the exonuclease could be affecting the rate of elongation with either analog. Under this condition, slowdown due to ara-CTP incorporation at the first isolated "C" site specified by M13mp19 is drastically reduced and even more so for slowdown due to ara-5-aza-CTP incorporation at this site (Fig. 4A, lanes D–I). However, no significant "read-through" by pol alpha of the consecutive "C" sites specified by M13mp9 is observed with either arabinoside even in the presence of 5′-AMP (Fig. 4B, lanes E–I).

Pol beta elongated the nascent strand through the single "C" site of M13mp19 with either arabinosyl analog triphosphate, even more efficiently than pol alpha, even though it is present at only 20% of the activity of pol alpha (Fig. 5A, lanes D–I). Pol beta, like pol alpha, increments one base to fill the "C" site when ara-CTP or ara-5-aza-CTP analog is present, again supporting the conclusion that the "C" sites are being filled by insertion of the analogs. However, also like pol alpha, pol beta is nearly completely stopped by incorporation of two consecutive arabinosides into M13mp9 (Fig. 5B, lanes E–I). The presence of 5′-AMP had no effect on elongation by pol beta, which did not contain significant exonuclease activity (data not shown).

When pol alpha and pol beta were both present in the reaction mixture, elongation was better with either arabinosyl analog at the single "C" site of M13mp19 than when the same activity of pol alpha alone was used (compare Fig. 3A, lanes D–I and Fig. 6A, lanes D–I). In addition, the two enzymes together

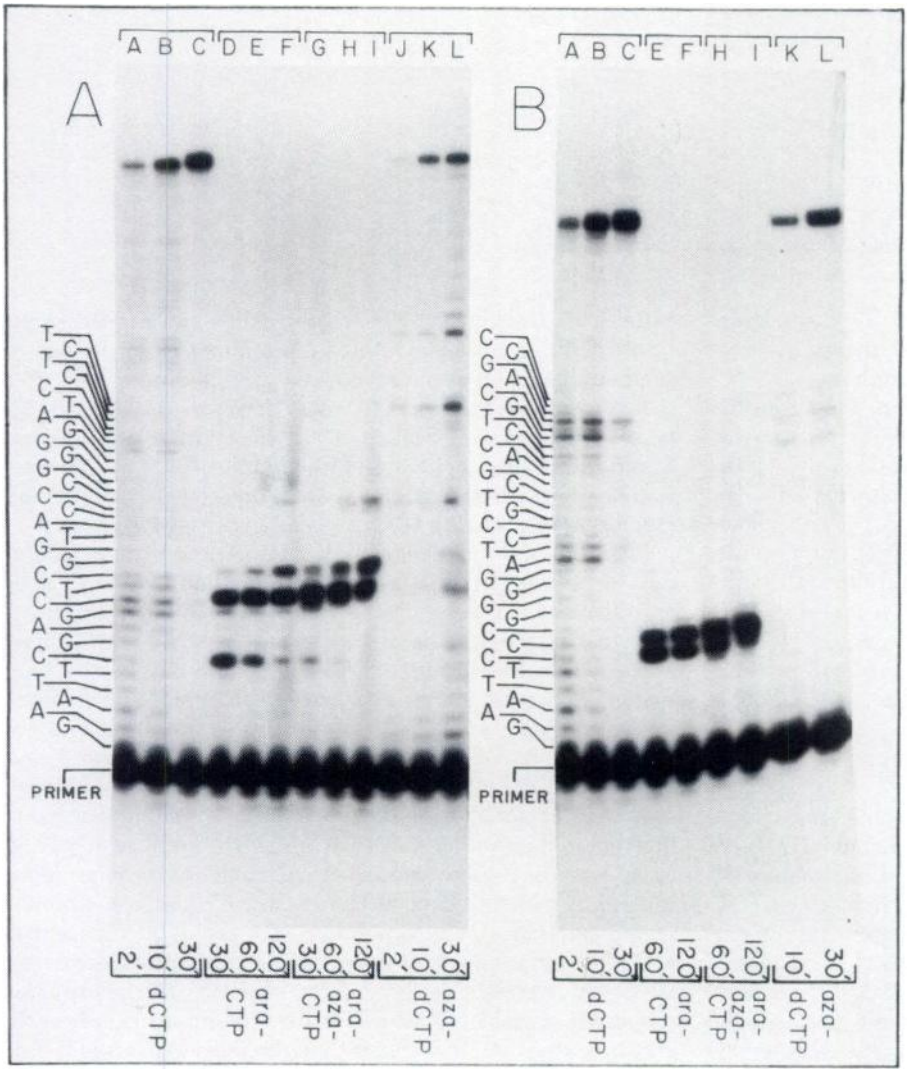

Fig. 4. Elongation by pol alpha under the same conditions as in Fig. 3, except for the inclusion of 2.0 mM 5′-AMP in the reaction mixtures.

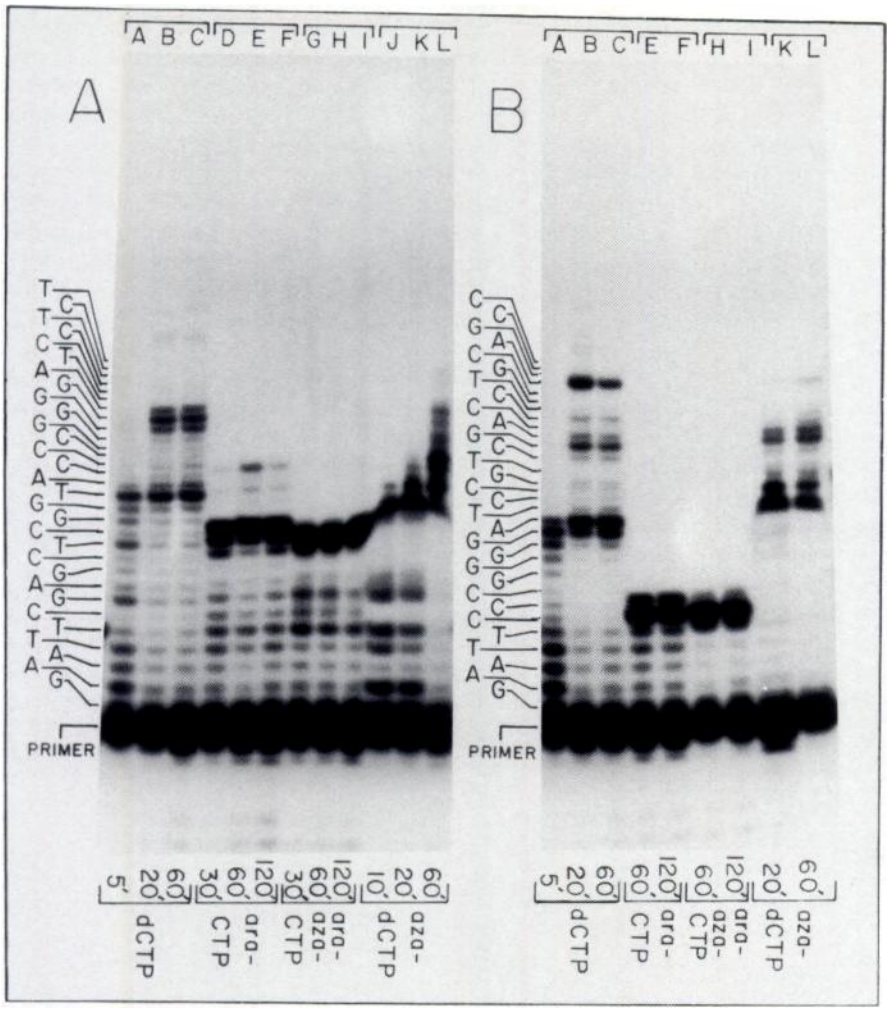

Fig. 5. Elongation of 5′-[^{32}P]-primer by pol beta (0.03 U) under the same conditions as in Fig. 3.

could extend the nascent strand through a "C-T-C" site specified at positions 11–13 of M13mp19, whereas either polymerase alone halts at either the first "C" site (pol alpha) or the "T" site (pol beta). Significantly, the two polymerases together succeed in extending a small amount of primer all the way through the triple "C" site specified at positions 6-9 of the M13mp9 template (Fig. 6B, lanes E–I), indicating that the chain termination produced at this site with either polymerase alone is best considered in the relative, rather than absolute, sense. Also worth mentioning is the observation that in the presence of all four normal dNTPs, the two enzymes in combination can read through a sequence, approximately 75 bases in the 3′-direction, where pol alpha alone is completely arrested (Fig. 6, lanes A–C). The significance of this observation is currently under investigation.

Discussion

Aza-substituted pyrimidine analogs are quite unstable in aqueous solution, and great care must be taken to prevent hydrolytic degradation of these compounds in pharmaceutical preparations, as well as in the laboratory research setting (1, 7, 17, 23). Thus, we were quite intrigued to discover that the new analog, as well as 5-aza-dCyd, are apparently stabilized against hydrolytic degradation by incorporation into DNA. This could be due in part to hydrophobic shielding of the triazine ring from water and other polar nucleophiles within the DNA double helix. This finding tends to argue against the proposal that cytotoxicity of 5-aza-substituted pyrimidines might be due to generation of potentially genotoxic apyrimidinic sites, after their incorporation into DNA (24). However, the renewed lability of the triazine ring in DNA in solution after partial denaturation suggests that DNA containing 5-aza-pyrimidines could be susceptible to such a process during periods of strand separation (e.g., during DNA replication or transcription).

In vitro, ara-5-aza-CTP exibits a weaker competitive inhibition of purified DNA polymerases alpha and beta than does ara-CTP, with K_i values about four- to fivefold higher. When DNA polymerases alpha or beta were incubated with gapped duplex DNA and dATP, dGTP, and [^{3}H]-dTTP, the only sustained increase in rate produced by an analog triphosphate was seen when 5-aza-dCTP was substituted for dCTP with pol alpha. This is consistent with the results of Bouchard and Momparler (20), who also found that 5-aza-dCTP was a good substrate for pol alpha. We have observed that 5-aza-dCyd is incorporated into Molt-4 cell DNA almost as well as dCyd and to at least a 10-fold greater extent than the arabinosides,[1] consistent with the data of Glazer and Knode (23), who observed much greater incorporation of 5-aza-dCyd than either of these arabinosides into human HT-29 colon carcinoma cell DNA. In contrast, neither of the arabinosyl triphosphates supported rates of [^{3}H]-dTTP incorporation significantly greater with either polymerase than the rates without dCTP or

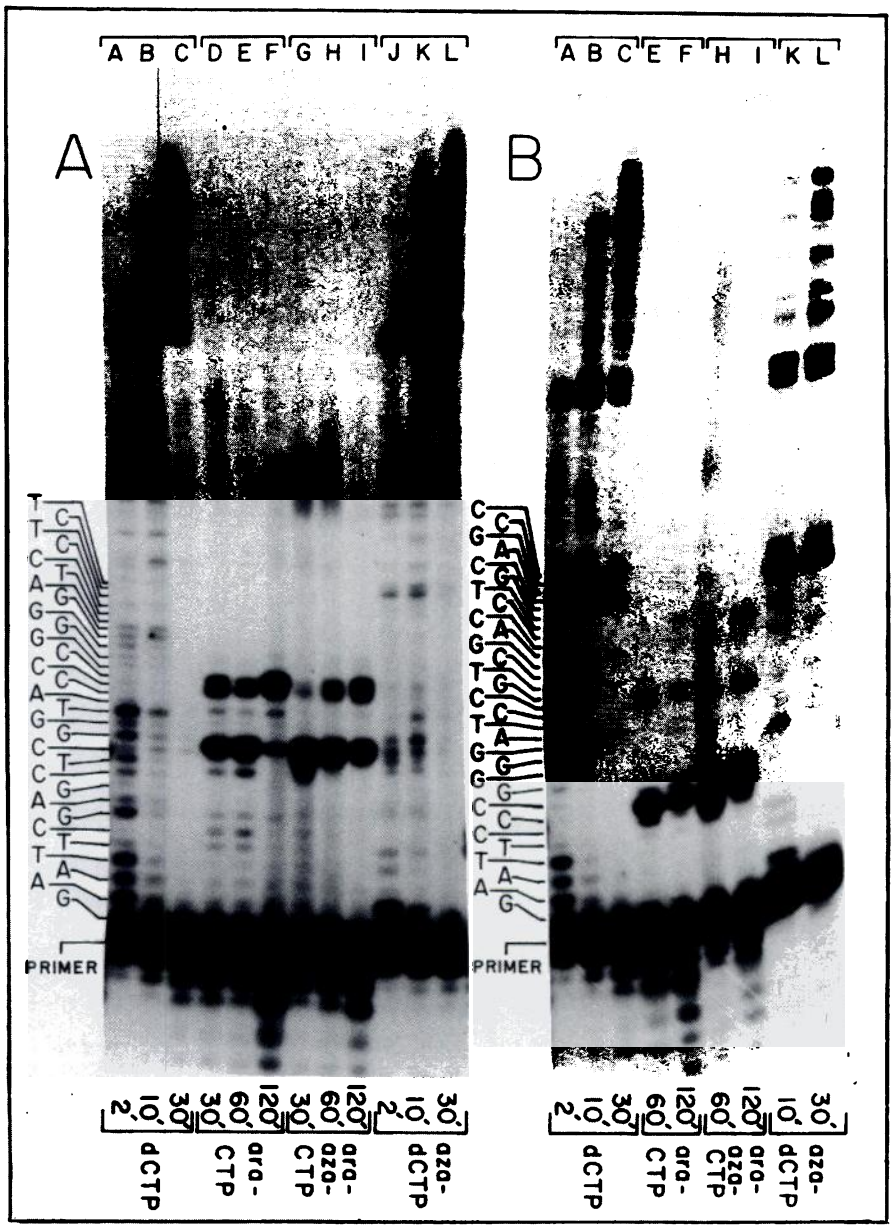

Fig. 6. A, elongation of 5′-[^{32}P]-primer by pol alpha (0.12 units) and pol beta (0.04 units) in combination (0.15 units total) under the same conditions as in Fig. 3.

analog. Although the initial rate with ara-5-aza-CTP was slightly greater than with ara-CTP, interpretation of this result is difficult due to the heterogeneous nature of the gapped duplex DNA substrate. An important question is whether the difference lies in the rates of incorporation or removal of these analogs or the rates of subsequent extension from the incorporated 3′-terminal arabinoside. Another important issue is the potential significance of the local sequence specified by the template at the site(s) of incorporation of these analogs. If incorporation of an analog results in a configuration at the 3′-terminal end of the growing chain that is less favorable for further extension, then consecutive incorporation of two or more analogs could conceivably result in an exaggeration of this effect. The DNA primer elongation assay was developed to allow indirect visualization of site-specific polymerization events along a defined, singly-primed M13 phage circular DNA substrate. This system has the desired sensitivity and resolution to permit examination of these issues.

Several conclusions may be drawn from our observations with this system. First, the analog triphosphates could substitute for dCTP at sites where a "C" residue is specified by the template. Although 5-aza-dCTP substituted well for dCTP with both polymerases in this assay, the arabinosyl analogs tended to slow or arrest chain elongation after incorporation at "C" sites. The lack of significant accumulation of primer at the "T" sites at position 5 of both templates, in contrast to the prolonged accumulation at the "C" sites at position 6, strongly suggests that incorporation of the arabinosyl analogs occurred significantly faster than subsequent elongation of the nascent strand. This observation also supports incorporation of the arabinosides, rather than misincorporation of dATP, dGTP, or dTTP at the "C" sites, because in the absence of dCTP or analog, primer accumulates at the "T" site at position 5 (not shown). This one-base increment in the position where the polymerase pauses has also been observed at "A" sites in the absence of dATP and when ara-ATP is substituted (not shown).

When substituted for dCTP, the arabinosyl analogs slowed but did not stop primer elongation by pol alpha at the first isolated "C" site specified by the M13mp19 template. Incorporation of ara-5-aza-CTP appeared to slow subsequent elongation significantly less than ara-CTP incorporation at this single "C" site; this may be the reason it is incorporated into Molt-4 DNA to a greater extent. Pol beta was not measurably affected by incorporation of either arabinosyl nucleotide at this site and may therefore contribute significantly to the incorporation of these analogs into cellular DNA.

The observation that incorporation by either polymerase of either arabinosyl analog at two consecutive sites results in nearly complete chain termination offers a new insight into the mode of action of these two analogs at the molecular level and also poses interesting new questions as well. One critical issue is whether tandem incorporation of these nucleotides also renders the nascent DNA strand inactive as a substrate for elongation in the intact cell, where presumably a multitude of enzymes and modifiers contributes to fidelity of replication and correction of mistakes. Previous work in this laboratory has identified an exonucleolytic enzyme in leukemic cells that copurifies with pol alpha and can remove 3′-terminal ara-C residues from gapped duplex DNA.[2] If this enzyme is present in the nucleus during replication, it could help to circumvent tandem blocks by removing one or more of the incorporated analogs. It would seem that the cell must have some means of coping with the consequences of tandem incorporation, since even at low doses of these analogs the cellular triphosphate concentrations are equal to or higher than the competing dCTP concentration; this would likely lead to accumulation of an insurmountable number of tandem blocks in a relatively short period.

The experiments were repeated in the presence of 2.0 mM AMP, which completely inhibits the exonuclease that is present in our pol alpha preparation, to assess the possibility that slowing of elongation might be due to removal of the incorporated arabinosyl nucleotide. This would lead to redundant incorporation, followed by either elongation or another cycle of removal and reincorporation of another triphosphate. Under this condition and in the absence of dCTP, elongation by pol alpha was enhanced at the first isolated "C" site of M13mp19, suggesting a possible role for the exonuclease in removal of the 3′-terminal incorporated analog. An analogous role has been postulated for the 3′–5′ exonuclease activity that is associated with herpes simplex type 1 DNA polymerase for the removal of 3′-incorporated ara-AMP residues (25): inhibition of the exonuclease by GMP led to a greater incorporation of ara-AMP into gapped duplex DNA. Alternatively, the presence of 5′-AMP may simply exert a permissive influence on pol alpha, allowing more efficient extension after addition of a single analog. Further investigation is required to resolve this question.

The results with pol beta, alone and in combination with pol alpha, indicate a possible role for this enzyme in DNA replication in the presence of ara-5-aza-CTP and ara-CTP. In addition to the observation that pol beta is not measurably slowed by substitution of either arabinoside at the isolated "C" site, it can also, in combination with pol alpha, reverse the slowdown that occurs at these sites when pol alpha alone is used. However neither 5′-AMP nor the combination of polymerases can effectively overcome the block that occurs after tandem incorporation, although the two polymerases together accomplish a detectable "read-through" in some experiments, particularly at the "C-T-C" site previously noted.

Incorporation of ara-C into DNA has been shown to be directly and closely correlated with the lethality of the drug (12). Since this may be a critical determinant in the lethality of other nucleoside analogs as well, it would be helpful to obtain a better understanding of the factors that might possibly influence this parameter in intact cells. Based on these experiments, the greater incorporation of ara-5-aza-Cyd than ara-C into Molt-4 DNA can most readily be correlated with the greater incorporation of the nucleotide by pol alpha at single "C" sites, that are statistically favored over multiple sites in terms of random distribution along the DNA template. However, the dramatic block produced by even a double incorporation suggests that the cell likely has a mechanism for dealing with such an obstacle; otherwise even a short exposure to arabinosyl nucleotides would be disastrous for the DNA synthetic apparatus. The effect of 5′-AMP on DNA extension from a tandem block offers a useful first avenue of approach to this question, namely to determine whether it is the pol alpha itself or an associated polypeptide such as an exonuclease that is modulated by 5′-AMP.

Another interesting question deals with the issue of concerted action by the various DNA polymerases, all of which have been reported to be present in the nucleus (26, 27). The small but measurable extension of the primer by pol alpha and pol beta together after tandem incorporation of ara-5-aza-CTP or ara-CTP suggests a cooperative interaction or complementary functional existence between the two enzymes. Thus, although pol alpha has historically been considered the "target enzyme" in the cytotoxic mode of action of ara-C, our results suggest that a closer look at pol beta may be warranted, if incorporation into DNA is indeed the common denominator of lethal effect for the arabinosides. The facilitation by pol beta of pol alpha "read-through" at the site 75 bases downstream where pol alpha alone is arrested also indicates that both polymerases may have essential roles in the replication of certain sequences in the genome under normal circumstances (i.e., no analogs). These questions are currently under investigation in this laboratory.

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