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Functional Consequences of the Arabinosylcytosine Structural Lesion in DNA†

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ABSTRACT: Cytosine arabinoside (araC) is a potent antileukemic agent that is misincorporated into DNA in the course of its action. We have developed a chemical synthetic method that allows site-specific introduction of araC into synthetic DNA oligomers. We describe here the utilization of these oligomers as primer/template substrates for in vitro DNA synthesis reactions and as fragments for DNA ligation. These studies were undertaken to investigate the manner in which sites of araC misincorporation constitute sites of DNA dysfunction. AraCMP at the primer terminus dramatically reduced the rate of next nucleotide addition for *Escherichia coli* polymerase I (Klenow fragment) (Pol I), T4 polymerase, HeLa cell polymerase α_2 (Pol α_2), and AMV reverse transcriptase. Polymerases with associated 3′-5′ exonuclease activity preferentially excised araCMP from the primer terminus prior to chain elongation. AraCMP-terminated fragments were ligated more slowly than control fragments by T4 DNA ligase. AraCMP located at an internucleotide site in the template markedly slowed replicative bypass for Pol I, T4 polymerase, and Pol α_2, but not for reverse transcriptase. Synthesis was partially arrested after insertion of the correct nucleotide opposite the lesion site. These results suggest a complex mechanism for the inhibition of DNA replication by araC when it is misincorporated into DNA.

The nucleoside analogue 1-β-D-arabinofuranosylcytosine (araC;[1] Figure 1a) is an important agent in the treatment of various forms of leukemia (Frei et al., 1969; Bodey et al., 1969). Although araC is a formal analogue of the ribonucleoside cytidine (Figure 1b), differing only in configuration about the 2′ carbon, its metabolism more closely resembles that of deoxycytidine (Figure 1c). AraC is converted by a series of kinases to the active metabolite araCTP (Coleman et al., 1975; Hande & Chabner, 1978), which in vitro is an inhibitor of various DNA polymerases, notably DNA polymerase α (Momparler, 1972; Yoshida et al., 1977; Dicioccio & Srivastava, 1977), the putative replicative polymerase in mammalian cells. Kinetically, araCTP behaves as a competitive inhibitor with respect to dCTP (Furth & Cohen, 1968; Dicioccio & Srivastava, 1977). A possible mechanism for its inhibition of DNA synthesis involves misincorporation of the analogue at 3′ termini, followed by a slow or absent rate of addition of the next nucleotide (Momparler, 1972; Fridland, 1977; Cozzarelli, 1977).

At the cellular level, the major biochemical consequence of araC treatment is suppression of replicative (Graham & Whitmore, 1970; Heintz & Hamlin, 1983) and repair (Collins, 1977) DNA synthesis. Treatment of cells with araC results in misincorporation of the analogue into nuclear DNA primarily in internucleotide linkages, with increasing amounts found at chain termini at higher drug concentration (Major et al., 1981, 1982). The total amount of araC misincorporated into cellular DNA correlates very strongly with the cytotoxicity of the drug as measured by clonogenic assays (Kufe et al., 1980), suggesting, but not proving, a causal relationship between araC misincorporation and lethal cellular events.

The sites at which araCMP is misincorporated constitute loci of anomalous structure, or "lesions", in the DNA. Detailed understanding of how the presence of this lesion affects DNA replication processes should further elucidate the mechanism of araC toxicity. Moreover, study of the effects of DNA structural perturbations on the processes of replication may provide basic insights into the structural requirements for normal DNA replication.

Recently, we have developed methodology for chemical synthesis of DNA oligomers containing araCMP at specific sites (Beardsley et al., 1988). In this paper we describe the utilization of these oligomers as substrates for DNA polym-

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[1] Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; DTT, dithiothreitol; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; PDE 2, phosphodiesterase 2; AMV, avian myeloblastosis virus; Pol α_2, HeLa cell polymerase α_2; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; TEAB, triethylammonium bicarbonate; TBE, buffer containing Tris, borate, EDTA, and urea.

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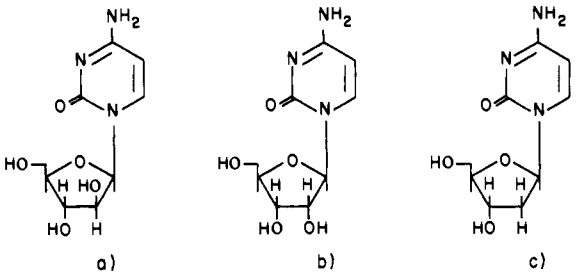

FIGURE 1: Chemical structures of cytosine nucleosides: (a) cytosine arabinoside; (b) cytidine; (c) deoxycytidine.

erase and DNA ligase in in vitro assays in order to study the effects of the araC lesion on DNA replication processes such as primer elongation, exonuclease "editing", and fragment ligation.

Materials and Methods

Chemicals. All chemicals were of reagent grade or better. Unlabeled dNTPs, 5′dNMPs, and 3′dNMPs were obtained from Sigma. 3′-AraCMP was a gift from Dr. E. Wechter of the Upjohn Laboratories. [α-^{32}P]dNTPs (400 Ci/mmol), and [γ-^{32}P]ATP(>3000 Ci/mmol) were obtained from Amersham.

DNA Substrates. DNA oligonucleotides, the sequences of which are given below, were synthesized on an Applied Biosystems automated DNA synthesis machine using phosphoramidite chemistry. Two 16-mer primers were synthesized:

5′ TCTAGAGTCGACCTGaraC 3′ (P16a)

and

5′ TCTAGAGTCGACCTGdeoC 3′ (P16d)

These sequences differ only in the sugar of the nucleotide at the 3′ terminus and are designated P16a (arabinose) and P16d (deoxyribose). The P16a primer required 5′-(dimethoxytrityl)-N^4-benzoyl-2′-acetyl-araC functionalized silica as the solid support for automated synthesis. This was prepared as described (Beardsley et al., 1988).

A 12-mer primer, which was used in the studies with the araC-containing template, and a 10-mer fragment for the ligation assay were also synthesized:

5′ TCTAGAGTCGAC 3′ (P12)

and

5′ AGGCATGCAA 3′ (L10)

Two 20-mer templates, complementary to the 12- and 16-mer primers, were also prepared:

3′ AGATCTCAGCTGGAaraCGATGC 5′ (T20a)

and

3′ AGATCTCAGCTGGAdeoCGATGC 5′ (T20d)

These sequences differ only in the sugar of the nucleotide at position 15 (from the 3′ end) and are designated T20a and T20d. Synthesis of the T20a template required an araC phosphoramidite, which was prepared as described (Beardsley et al., 1988). All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis and checked for purity by electrophoresis after 5′ end labeling with T4 polynucleotide kinase (Maxam & Gilbert, 1980). M13mp19 DNA was isolated in our laboratory by Dr. James Clark.

Enzymes. The large fragment of *Escherichia coli* DNA polymerase I (Klenow fragment), T4 DNA polymerase, avian myeoloblastosis virus (AMV) reverse transcriptase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from International Biotechnologies, Inc. Terminal deoxynucleotide transferase was a gift from Prof. Ronald P. McCaffrey of Boston University. HeLa cell DNA polymerase α_2 holoenzyme (Pol α_2) was a gift from Dr. Earl Baril of the Worcester Foundation for Experimental Biology. Micrococcal nuclease and calf spleen phosphodiesterase (PDE 2) were purchased from Sigma.

DNA Polymerase Reaction Buffers. The following buffer solutions were used for carrying out DNA polymerase reactions: for the Klenow fragment, 150 mM KCl, 300 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, and 1 mM DTT; for T4 polymerase, 66 mM potassium acetate, 30 mM Tris acetate, pH 7.9, 10 mM magnesium acetate, and 0.5 mM DTT; for Pol α_2, 50 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 1 mM DTT, and 1 mg/mL BSA (fatty acid free); for reverse transcriptase, 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, and 2 mM DTT; for terminal deoxynucleotide transferase, 50 mM Tris-HCl, pH 8.3, 2 mM $MnCl_2$, and 4 mM DTT.

DNA Polymerase Reactions: AraC at the Primer Terminus. The standard conditions used in experiments comparing the rate and extent of araC (P16a) and control (P16d) primer extension utilized a total of 50 ng of template/primer. The 16-mer primers were labeled with ^{32}P at the 5′ end prior to annealing to the T20d template. Terminal deoxynucleotide transferase reactions contained 20 ng of end-labeled P16a or P16d primer without template. The reactions also contained 50 μM dATP, dCTP, dGTP, and dTTP, except for the Pol α_2 reactions that contained 250 μM dNTPs and terminal deoxynucleotide transferase reactions that contained 250 μM dGTP alone. Each assay contained the appropriate buffer (see above) for the particular polymerase. Klenow fragment, T4 polymerase, reverse transcriptase, and Pol α_2 assays contained 0.5, 0.25, 4.0, and 0.08 units, respectively, of enzyme (all units are those of the supplier) in a 30-μL reaction volume. Terminal deoxynucleotide transferase assays contained 0.5 unit of enzyme in a 100-μL reaction volume. The reactions were carried out at room temperature (Klenow fragment and T4 polymerase) or 37 °C (reverse transcriptase, Pol α_2, and terminal deoxynucleotide transferase). In experiments that addressed the role of the 3′-5′ exonuclease associated with some of the above polymerases, the DNA synthesis reactions were performed as above except that 10 times the amount of enzyme was added and the reactions were done in the presence and absence of 10 mM dGMP. In experiments that measured the extent of exonuclease-catalyzed hydrolysis of araCMP and dCMP from the primer terminus, the reactions were performed as in the standard assays except that dNTPs were omitted and 10 times the amount of enzyme was added. Aliquots were taken from the reaction mixtures at various times (see figure legends) and added to an equal volume of a dye/EDTA/formamide loading solution to stop the reactions. The samples were then heated at 95 °C for 3 min and immediately loaded onto a high-resolution denaturing polyacrylamide gel (see below) and separated by electrophoresis.

Primer extension utilizing [α-^{32}P]TTP was performed essentially as above except that the reactions contained 0.5 μg of unlabeled template/primer, 25 μM of unlabeled dATP, dCTP, and dGTP (Pol α_2 reactions contained 200 μM of dATP, dCTP, and dGTP), and 20 μM [α-^{32}P]TTP (approximately 270 Ci/mmol). In some of the experiments 10 mM dGMP was also added. Klenow fragment, T4 polymerase, reverse transcriptase, and Pol α_2 assays contained 5.0, 2.5, 25.0, and 0.2 units, respectively, of enzyme. The Klenow fragment, T4 polymerase, Pol α_2, and reverse transcriptase reactions were carried out for 45, 15, 120, and 30 min, respectively, in reaction

volumes of 20 μL (30 μL for Pol α_2). Reactions were then stopped as above and separated on polyacrylamide denaturing gels. The positions of full-length primer extension products were visualized by autoradiography, the bands were cut out, and the DNA was eluted from the gel for subsequent nearest-neighbor analysis.

Nearest-Neighbor Analysis. Urea and salts were removed from each of the eluted DNA samples by applying the eluate to a Waters C_{18} Sep-PAK and washing first with 25 mM TEAB, pH 7.5. The DNA was then eluted from the column by washing with a 70/30 (v/v) mixture of 100 mM TEAB, pH 7.5, and CH_3CN. Samples were evaporated to dryness and redissolved in 500 μL of a solution containing 0.5 mM Tris and 0.2 mM $CaCl_2$, pH 8.5. Micrococcal nuclease (20–50 units) was added, and the mixtures were incubated for 45 min at 37 °C. The pH was then adjusted to 6 with 1 N HCl, 5 units of calf spleen PDE 2 was added, and the mixture was incubated for 20 min. A second 5 units of PDE 2 was added and the incubation repeated. The protein was removed by phenol/chloroform extraction, and residual phenol was removed by ether extraction. The samples were evaporated and redissolved in a final volume of 50–100 μL of water. Prior to HPLC, the samples were mixed with 3′ nucleotide monophosphate markers for UV visualization. The separation of 3′ nucleotide monophosphates was effected by strong anion exchange using a Waters Radial-Pak SAX column on a Varian Series 5500 instrument outfitted with a Waters Z-module. The mobile phase conditions were 4 mM ammonium phosphate, pH 3.8, isocratic from 0 to 7 min, followed by a 30-min linear gradient from 4 to 175 mM ammonium phosphate, pH 4.3. The flow rate was 3 mL/min. The 3′ nucleotide monophosphate UV elution profile was monitored at 260 nm. Fractions were collected every 0.1 min during the course of each run and mixed with liquid scintillation cocktail, and the ^{32}P activity of each fraction was determined.

Ligation Assay. Ten nanograms of either ^{32}P-labeled P16a or P16d primer was annealed to 5.5 μg of M13mp19 single-strand DNA along with 10 ng of the ^{32}P-labeled L10 fragment that annealed to a site immediately adjacent to the 3′ termini of the 16-mers. The ligation buffer was as described in Maniatis et al. (1982), the reaction volume was 50 μL, and the reactions were carried out at room temperature. T4 DNA ligase (0.005 unit) was added to each tube (units are those of the supplier). Aliquots were removed at various times (see figure legend) and the reactions stopped as above. Samples were denatured prior to electrophoresis. The products were visualized by autoradiography.

Studies with AraC-Containing Templates. DNA synthesis reactions contained 50 ng total of either the annealed T20d template/[^{32}P]P12 primer or the T20a template/[^{32}P]P12 primer as substrate. The standard polymerase conditions were used, and the products were analyzed by electrophoresis as for the end-labeled primer extension assays. In some of the reactions dGTP was omitted.

Electrophoresis and Autoradiography. Products from DNA synthesis and ligation reactions were analyzed by electrophoresis through 1.5 mm 20% polyacrylamide [19:1 (w/w) acrylamide:*N*,*N*′-methylenebis(acrylamide)] gels containing TBE buffer (100 mM Tris, pH 8.3, 100 mM borate, 2 mM EDTA, 8 M urea) at 1000 V for 3–4 h. Autoradiography was performed at −20 °C for 0.5–2 h with an intensifier using Kodak XAR-5 X-ray film.

RESULTS

Effects of AraCMP at Primer Termini. In these experiments, we compared the ability of various DNA polymerases to extend a primer whose 3′-terminal residue was araCMP, with the extension of the analogous dCMP-terminated primer. The ^{32}P-labeled P16a and P16d primers were each annealed to the T20d template to form partial duplexes with a four-nucleotide overhang

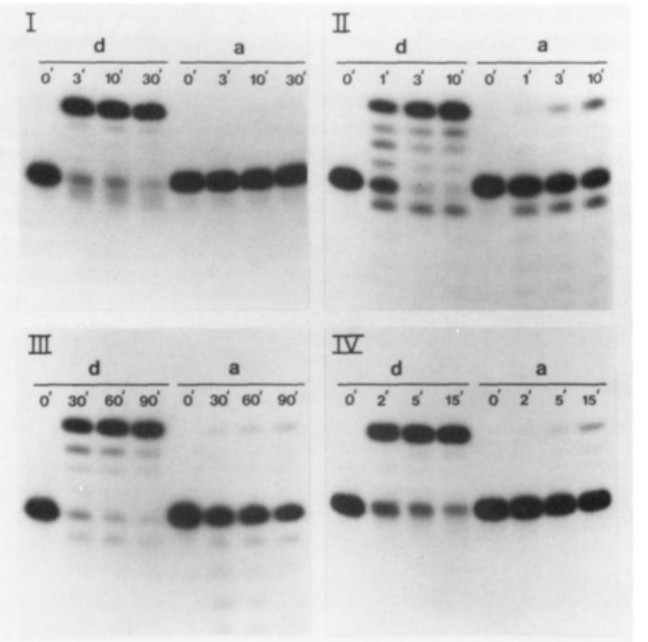

FIGURE 2: Autoradiograms showing the rate and extent of primer elongation by several polymerases for the P16a and P16d primers annealed to the 20-mer template (T20d). The primers were labeled with ^{32}P at the 5′ end. DNA synthesis reactions were carried out with the Klenow fragment (I), T4 polymerase (II), HeLa cell Pol α_2 (III), and AMV reverse transcriptase (IV). Reaction times (in minutes) are indicated above the lanes for each panel. The lanes labeled d in each panel indicate P16d primer extension reactions. The lanes labeled a indicate the P16a primer extension reactions. The 0 time lane serves as an internal size marker. DNA synthesis reactions and gel electrophoresis were carried out as described under Materials and Methods.

5′ [^{32}P]TCTAGAGTCGACCTGC* 3′
3′ AGATCTCAGCTGGACGATGC 5′

where C* is either araCMP (P16a) or dCMP (P16d). Figure 2 shows the rate and extent to which the P16a and P16d primers were extended by various polymerases. Since the primers were 5′ end labeled, the band intensities on the autoradiograms are directly proportional to the number of primers extended. Panel I shows the results obtained with the Klenow fragment. Extension of the control primer (P16d) to the full length of the template was essentially complete by the final time point (30 min). During the same period, the araC-terminated primers (P16a) remained largely unextended. Similar results were obtained with T4 polymerase (panel II), Pol α_2 (panel III), and AMV reverse transcriptase (panel IV). Small amounts of unextended control primer were observed in all cases, and for the polymerases with associated 3′–5′ exonuclease activity, some partially degraded primers were observed for both the P16d and P16a primers.

In order to estimate the quantitative difference between the rates of P16a and P16d primer utilization, the above reactions were repeated and the products studied at an early time point when the rates of addition to the two primers were approximately linear. The products were then separated electrophoretically, the full-length extension products (20-mers) were excised from the gel, and the amount of radioactivity was determined. This method allowed estimation of the relative rate of product formation for the P16a primer. Expressed as

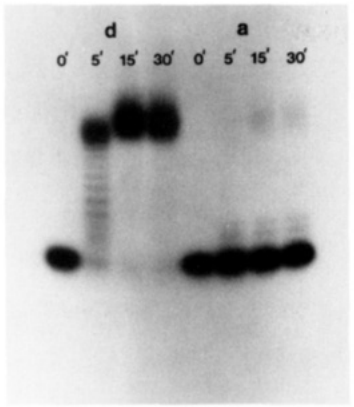

FIGURE 3: Autoradiogram of terminal deoxynucleotide transferase catalyzed addition to 5′ end labeled P16d (d) and P16a (a) primers. Primer addition experiments and gel electrophoresis were carried out as described under Materials and Methods.

a percent of the control (P16d) product formed under the same conditions, the amount of product formed from the P16a primer was ~0.5% with the Klenow fragment, ~5% with T4 polymerase, ~1% with Pol α_2, and ~3% with reverse transcriptase.

Figure 3 shows the results of P16a and P16d primer extension by terminal deoxynucleotide transferase. This enzyme catalyzes non-template-directed nucleotide addition to the 3′ end of single-stranded DNA; thus, end-labeled primers alone were used with this enzyme. As the figure shows, extensive addition to the P16d primer was observed, while the P16a primer remained largely unextended. Thus, terminal deoxynucleotide transferase, like the template-directed polymerases, failed to catalyze nucleotide addition to the araC-terminated primer to any appreciable extent.

Role of 3′–5′ Exonuclease Activity in Primer Extension. Since a small but measurable fraction of the araCMP-terminated primers was extended by each of the polymerases studied, we investigated whether this extension represented direct addition to araCMP or whether polymerases with associated 3′–5′ exonuclease activity excised araCMP from the primer terminus prior to elongation of the primer. DNA synthesis reactions were performed with the above primer/template substrates except that the primers were not end-labeled and the reactions were carried out in the presence of [α-^{32}P]TTP. TMP is the first nucleotide added to the primer terminus in the absence of excision. It is inserted only once in the four-base overhang of the template and therefore has only one 5′ neighbor. Upon digestion of the extended P16a primer to 3′ nucleotide monophosphates, the incorporated radiolabel is passed to its 5′ neighbor. The radiolabel appears as 3′-[^{32}P]araCMP if direct addition occurs or as 3′-[^{32}P]-dCMP if excision of araCMP occurs prior to extension. This allows for the determination of the relative frequency of each event among the primers that were extended.

As a control for this method, reverse transcriptase was used since it lacks an associated 3′–5′ exonuclease and is therefore unable to excise araCMP from the primer terminus. The full-length extension products were isolated and then digested enzymatically to 3′-monophosphates. These were then separated by HPLC. Fractions were collected at intervals, and the amount of ^{32}P label in each was determined. Figure 4 shows the HPLC elution profile and radioactivity distribution for the digested P16a and P16d primers previously extended by reverse transcriptase. As expected, the radiolabel was found exclusively in the 3′-araCMP fraction for the P16a material and in the 3′-dCMP fraction for the P16d material.

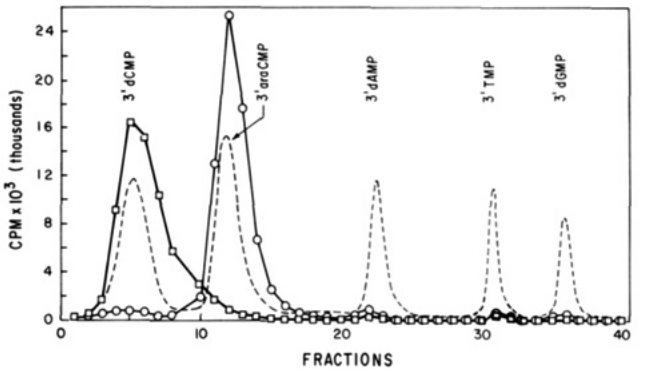

FIGURE 4: Nearest-neighbor analysis of P16d and P16a primers annealed to T20d template and extended in the presence of [α-^{32}P]TTP (the first nucleotide incorporated at the primer terminus) by reverse transcriptase. Experimental conditions were as described under Materials and Methods. The figure shows the elution profile (---) for 3′ nucleotide monophosphate markers and the distribution of ^{32}P collected over the course of the separation of the digested P16d (□) and P16a (○) primer extension products. Injections volumes were unequal, with the P16a material in this experiment being approximately 10 times that of the P16d.

Table I: ^{32}P Label Distribution (%) among 3′-Deoxynucleotides[a]

	−10 mM dGMP	+10 mM dGMP
Klenow fragment		
3′-araCMP	30	60
3′-dCMP	70	40
T4 DNA polymerase		
3′-araCMP	<5	<5
3′-dCMP	>95	>95
polymerase α_2		
3′-araCMP	<5	ND[b]
3′-dCMP	>95	ND[b]

[a] Nearest-neighbor analysis of P16a and P16d primers annealed to the T20d template and extended in the presence of [α-^{32}P]TTP by the Klenow fragment, T4 polymerase, and Pol α_2. DNA synthesis reactions in both the preseence and absence of 10 mM dGMP, digestion to 3′-monophosphates, separation and radiolabeled quantification were as described under Materials and Methods. Percentages were calculated by setting the sum of the cpm totals for the 3′-dCMP and 3′-araCMP fractions equal to 100%. [b] Value not determined.

The results of the experiments with polymerases that have an associated 3′–5′ exonuclease activity were more complex and are shown in Table I. The primer extension reactions were also performed in the presence of 10 mM dGMP, to partially suppress the exonuclease activity (Byrnes et al., 1977). In all reactions, the large majority of P16a primers remained unextended, and the values shown refer only to that small fraction of primers which were extended to full length. For the Klenow fragment, analysis of the digested P16a primers extended in the absence of added deoxynucleotide monophosphate showed that araCMP was excised from 70% of these primers prior to chain elongation. In the presence of 10 mM dGMP this value fell to 40%. For T4 polymerase, which has a highly active exonuclease (Kornberg, 1980), araCMP was excised from all P16a primers that were extended. Addition of 10 mM dGMP had no detectable effect on the radiolabel distribution of the extension products analyzed. Pol α_2, which exhibited moderate 3′–5′ exonuclease activity (Skarnes et al., 1986), also excised araCMP from all P16a primers extended. Addition of 10 mM dGMP resulted in an insufficient amount of extended P16a primers for product analysis.

Since removal of araCMP occurred prior to next nucleotide addition for the majority of the araCMP-terminated primers that were extended, partial suppression of 3′–5′ exonuclease activity by the addition of 10 mM dGMP should further reduce the overall rate of primer elongation. This was indeed

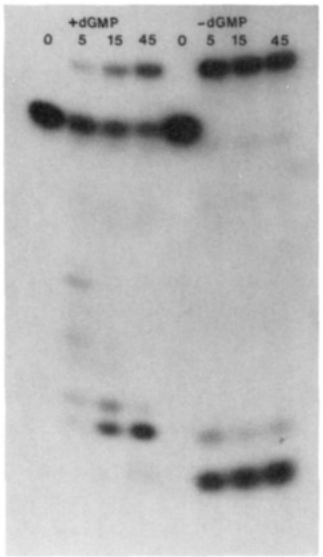

FIGURE 5: Autoradiogram showing the rate and extent of end-labeled P16a primer extension by T4 polymerase in the presence (+) and absence (−) of 10 mM dGMP. Incubation times are indicated above the lanes. Reaction conditions and gel electrophoresis were as described under Materials and Methods.

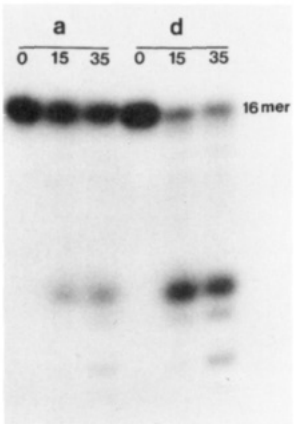

FIGURE 6: AraCMP at 3′ terminus partially suppresses primer degradation by the Klenow fragment associated 3′–5′ exonuclease. End-labeled primers were annealed to the T20 template and the reactions carried out as in Figure 2 except that dNTPs were omitted and 10 units of enzyme was added to each reaction. Incubation times are indicated above the lanes. Electrophoresis was as described under Materials and Methods.

observed and is illustrated for T4 polymerase in Figure 5. Similar observations were made for the Klenow fragment and Pol α_2 (data not shown).

Effect of AraCMP at Primer Termini on 3′–5′ Exonuclease. In view of the important role of polymerase-associated exonuclease activity in the extension of araCMP-terminated primers, we investigated comparative rates of excision of araCMP and dCMP from primer termini. These assays were carried out in the absence of dNTPs, eliminating the polymerization reaction and allowing for direct comparison of the rates and extent of 3′ degradation. The autoradiogram in Figure 6 shows the results for the [^{32}P]P16a/T20d and [^{32}P]P16d/T20d substrates incubated with the Klenow fragment. The P16a primer was more resistant to degradation than the P16d primer, indicating that the 3′–5′ exonuclease associated with the Klenow fragment hydrolyzes araCMP more slowly than dCMP under similar conditions. However, in similar experiments with T4 polymerase and Pol α_2, no difference in the rate of araCMP versus dCMP removal was observed with either of these enzymes (data not shown).

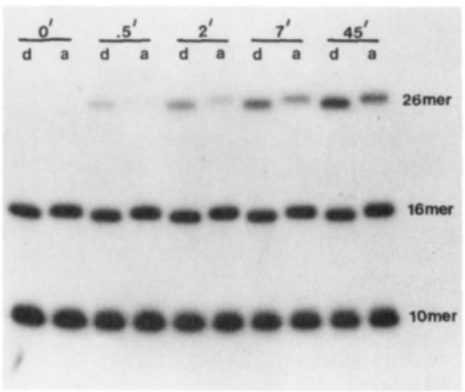

FIGURE 7: AraCMP at the 3′ terminus of a ligation junction affects the efficiency of fragment ligation. Equimolar amounts of end-labeled P16d and P16a primers were separately annealed to m13mp19 single-strand DNA. An end-labeled 10-mer fragment (L10) was annealed to a site immediately adjacent to the 3′ termini of the 16-mers. These substrates were each incubated with T4 DNA ligase as described under Materials and Methods. The autoradiogram shows the time course and extent of ligation product (26-mer) formation. Incubation times are indicated above each set of two lanes. The lanes labeled d refer to the P16d/L10 reaction; those labeled a refer to the P16a/L10 reaction, indicating that dCMP and araCMP, respectively, are the 3′ residues at the ligation junction.

Effect of AraCMP on Fragment Ligation. DNA ligase also acts at 3′ termini in the processing of DNA during replication and repair. In order to determine whether araCMP at the 3′ terminus affects the efficiency of fragment ligation, the end-labeled P16a and P16d primers were each annealed to a site on M13mp19 DNA along with the end-labeled 10-mer fragment (L10) to produce substrates for fragment ligation

5′[^{32}P]T-C-T-A-G-A-G-T-C-G-A-C-C-T-G-C*[^{32}P]A-G-G-C-A-T-G-C-A-A 3′
3′—A-G-A-T-C-T-C-A-G-C-T-G-G-A-C-G—T-C-C-G-T-A-C-G-T-T–5′ (M13)

where C* is either araCMP (P16a) or dCMP (P16d). The two substrates were then incubated with T4 DNA ligase in the presence of ATP. Aliquots from the reaction mixtures were removed at various times, and the material was analyzed by gel electrophoresis. The autoradiogram in Figure 7 shows the early time course of fragment ligation for these two substrates. As the figure shows, the 10-mer fragment is ligated to the P16a fragment more slowly than it is to the P16d fragment. This difference was reproducible over many repetitions of this assay and was estimated to be approximately 3-fold.

Effect of AraCMP in the Template. In order to assess the ability of a primer to extend past an araCMP lesion in the template, the end-labeled 12-mer primer (12P) was annealed to the control T20d template and the araCMP-containing T20a template to form a partial duplex with an eight nucleotide overhang

5′ [^{32}P]TCTAGAGTCGAC 3′
3′ AGATCTCAGCTGAC*GATGC 5′

where C* is either araCMP (T20a) or dCMP (T20d). The end-labeled primers were then extended under identical conditions by DNA polymerases utilizing unlabeled dNTPs. Since the primer was 5′ end labeled, the band intensities on the autoradiograms (Figure 8) are directly proportional to the number of molecules that terminated synthesis at any given position along the template. These experiments were carried out with equimolar concentrations of all four dNTPs to avoid any effects of nucleotide pool imbalance on the pattern of synthesis arrest.

The autoradiograms in Figure 8 show the products of the DNA synthesis reactions. Panel I shows the results for the Klenow fragment. Synthesis on the T20a template was par-

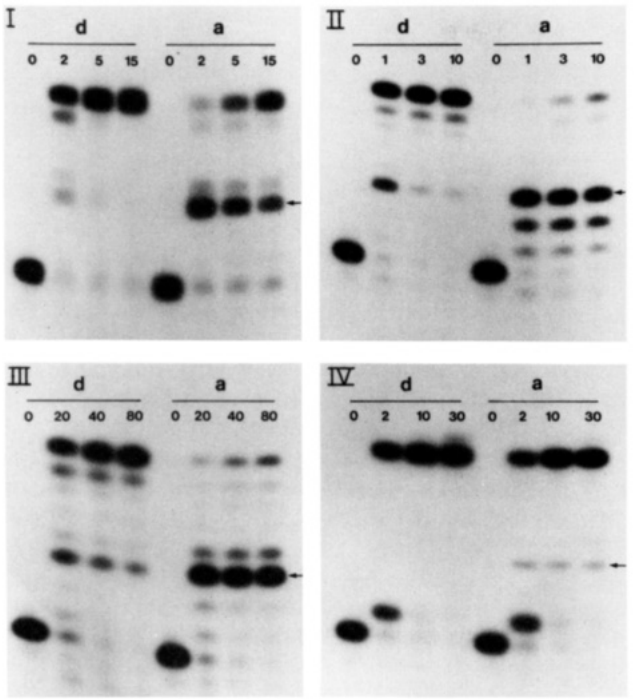

FIGURE 8: AraCMP in the template shows replicative bypass. The end-labeled 12-mer primer (P12) was separately annealed to equimolar amounts of the T20a and T20d 20-mer templates to give the duplexes below, where C* is either dC (T20d) or araC (T20a). These template primers were then incubated with Klenow fragment (I), T4 polymerase (II), Pol α_2 (III), or reverse transcriptase (IV) as described under Materials and Methods. The autoradiograms show the time course, sites of synthesis arrest, and extent of product formation for the above polymerase reactions. Incubation times are indicated above the lanes. The first four lanes in each panel refer to synthesis using the P12/T20d primer/template; the second set of four lanes refers to synthesis using the P12/T20a primer/template. AraCMP is located at position 15 in the T20a template. The arrow indicates the position corresponding to a 15-mer. The 0 time points serve as an internal size marker (12-mer).

TCTAGAGTCGAC
AGATCTCAGCTGGAC*GATGC

tially arrested at the site of araCMP in the template (see arrow). While a strong arrest band was observed initially, a significant degree of lesion bypass had occurred by the final time point. For T4 polymerase (panel II) and Pol α_2 (panel III) a strong arrest band occurred at the lesion site, and overall synthesis to full-length extension products was markedly slowed compared to synthesis on control templates. T4 polymerase also exhibited a weaker arrest band one nucleotide before the lesion site. Pol α_2 exhibited an additional, weaker, arrest band one nucleotide after the lesion site. Reverse transcriptase (panel IV) exhibited only a very weak band at the lesion site, and its effect on the overall rate of synthesis to the full-length product appeared negligible. For all the polymerases, synthesis on control templates showed essentially complete elongation of the primer to the full length of the template strand by the final time point. Both T4 polymerase and Pol α_2 exhibited a weak arrest band at position 15 (see arrow) on the control template. This may be a sequence-specific phenomenon or may be inherent to the size of the substrate; as such, this must also be considered to make a contribution to the arrest pattern on the T20a template since it occurs at the same position as the lesion site.

Figure 9 shows the products of the above polymerase reactions when dGTP was deleted from the reaction mixture. Under these conditions, synthesis terminates one nucleotide before the araC site (T20a) or dC site (T20d) indicated by the arrow in Figure 9. Thus, there is no detectable nucleotide incorporation opposite araC in the absence of dGTP, while there is incorporation opposite the lesion when dGTP is present (Figure 8). These results indicate that the correct nucleotide, dGMP, is inserted opposite araC.

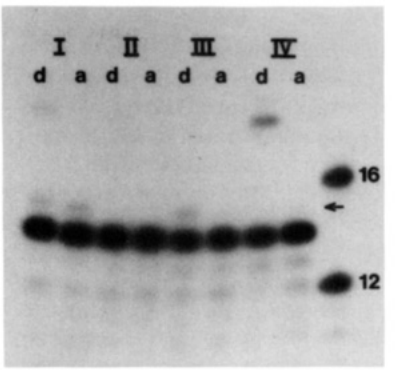

FIGURE 9: Primer extension in the absence of dGTP. The end-labeled 12-mer primer (P12) was annealed to the T20a (a) and T20d (d) templates and extended by the Klenow fragment (I), T4 polymerase (II), Pol α_2 (III), and reverse transcriptase (IV). Conditions were as in Figure 8, with the exception that dGTP was omitted from the reactions, and the reactions were stopped at 10 (I), 5 (II), 30 (III), and 10 min (IV). The arrow indicates the site of dC (d) or araC (a) in the template. The two nucleotides incorporated before the lesion site are C followed by T (see Figure 8 legend). The 12- and 16-mer bands in the far right lane serve as size markers.

DISCUSSION

AraC differs structurally from deoxycytidine in only a minor way. The cytosine base of araC is structurally normal, and the sugar differs only in the presence of an additional hydroxyl group at the 2′ carbon located trans to the 3′ hydroxyl. Crystallographic (Sherfinski & Marsh, 1973; Sundaralingam, 1976) and NMR (Dalton et al., 1977) studies with the free nucleoside have shown that the arabinose sugar does not significantly alter the possible conformations of the araC nucleoside from those that are adopted by deoxyC. The present studies demonstrate, however, that when this analogue is introduced into DNA oligomers, this small structural difference has a profound effect on the utilization of these template/primers as substrates for DNA polymerases.

AraCMP at the primer terminus dramatically reduced the rate of primer extension for all polymerases tested. In the case of DNA polymerase α_2, this difference in rate is more than 100-fold. The analysis of the reaction products clearly demonstrates that this results from failure to add the succeeding nucleotide to the araCMP residue at the 3′ terminus.

The precise basis for this inhibition is not clear. We have found in preliminary studies that the araC–G base pair has comparable stability to the C–G base pair in duplex DNA (Beardsley et al., 1988). It seems unlikely that poor base pairing and consequent "fraying" at the primer terminus could be responsible for the severe inhibition of the 3′ addition reaction. It is noteworthy in this respect that terminal deoxynucleotide transferase, which does not require a template (or paired terminus) to catalyze nucleotide addition, was also dramatically inhibited by the presence of araCMP at the primer terminus. It is possible that the presence of the additional hydroxyl group, while not affecting the stability of the primer terminus, interferes directly with the mechanism by which the polymerase catalyzes phosphodiester bond formation between the 3′-hydroxyl of the terminal sugar moiety and the α phosphorus of the incoming nucleotide. Structural models of araCMP in B-form DNA show that the additional hydroxyl group on the arabinose sugar projects into the major

groove of the duplex. In this position it might interefere with the binding of the polymerase to the DNA substrate and/or its catalytic function.

All of the polymerases tested extended only a small fraction of the araC-terminated primers under conditions where dC-terminated primers are essentially all extended to full length. Analysis of these small amounts of araC primer extension products showed that for polymerases with 3′–5′ exonuclease activity excision of araCMP was strongly preferred over addition to araC in all cases. For Pol α_2 and T4 polymerase, extension occurred only after prior excision, as no direct addition to araC could be detected. The results as shown in Figure 2 therefore greatly underestimate the actual difference between the rates of nucleotide addition to araC and dC at primer termini. For these polymerases, the rate of exonucleolytic excision of araCMP probably determines the rate of overall primer extension. This is supported by the finding that the rate of primer extension is decreased further by addition of deoxynucleoside monophosphate, which partially suppresses the exonuclease activity.

A measurable level (30%) of direct addition to araC occurred with the Klenow fragment. The apparent increase (to 60%) in the presence of 10 mM dGMP actually reflects the decrease in the amount of primers extended through prior excision of araCMP. The total amount of product formed through direct addition to 3′-araC in both assays was roughly the same, while the amount of primers extended through prior excision of araCMP was significantly less in the presence of 10 mM dGMP. It is clear from these data that decreased excision of araCMP lowers the overall rate of primer extension for this enzyme.

Since excision of araCMP from 3′ termini is an important determinant in its overall effect on DNA synthesis, it was of interest to know if the excision process was inhibited by the lesion. The rates of excision of araCMP and dCMP from the primer termini were approximately equal for T4 polymerase and also for Pol α_2. However, for the exonuclease associated with the Klenow fragment, araCMP was relatively more resistant to hydrolysis than dCMP. For this enzyme, increased resistance to hydrolysis contributes to the overall inhibition of chain growth.

DNA ligase, another enzyme necessary for the overall processes of DNA replication and repair, catalyzes 3′–5′ phosphodiester bond formation between adjacent DNA fragments aligned on a template strand. While the details of the mechanism for phosphodiester bond formation as catalyzed by DNA polymerases and DNA ligase are quite different, there is formal similarity between the two reactions, as nucleophilic attack by the 3′-hydroxyl terminus on an activated 5′-phosphate group occurs in both cases. These similarities prompted us to study the consequences of araCMP at 3′ termini on fragment ligation. Our results show an approximately 3-fold decrease in ligation rate for the araCMP-terminated fragment in comparison with the dCMP-terminated fragment. This effect is small compared to the more dramatic effect of araCMP on next nucleotide addition by DNA polymerase. Although at a reduced rate, araCMP clearly can be incorporated into DNA at internucleotide positions via the ligase reaction.

When araCMP is located at the 3′ terminus of a primer or fragment, the aberrant sugar moiety of the nucleotide is directly involved in the success or failure of either chain elongation or fragment ligation. When araCMP occupies an internucleotide position in the template, however, the sugar moiety serves only as a structural component of the template sugar-phosphate backbone and is not directly involved in phosphodiester bond formation. Nevertheless, our results show clearly that the araC lesion in the template constitutes a significant block to DNA synthesis for the Klenow fragment, T4 polymerase, and Pol α_2. These results demonstrate that an anomaly in the sugar moiety of the template backbone, which does not disrupt the thermodynamic stability of base pairing or introduce a bulky side group into the DNA, can effect replication bypass.

A striking feature of this partial replication block is that it occurs at, rather than before, the template lesion site. There appears to be no impediment to addition of the correct nucleotide, dGMP, opposite araCMP. Most other template lesions, such as those resulting from various anomalies in the structure of the base or those that occur at abasic sites, result in synthesis arrest one nucleotide before the lesion site because of gross distortion in the DNA structure and/or loss of the base-coding property (Moore & Strauss, 1979; Moore et al., 1981; Sagher & Strauss, 1983; Takeshita et al., 1987). An exception is the thymine glycol lesion, which arrests synthesis at the site of the lesion (Clark & Beardsley, 1987). In the case of thymine glycol, modeling and molecular mechanical studies provide a rationale for this arrest pattern. The altered base structure disturbs the position of its 5′ neighbor in the template, while leaving its own base-pairing properties intact (Clark et al., 1987). The structure of araC, however, has less apparent potential for such steric perturbations, and it is unclear how this small structural modification in the DNA backbone could affect the catalytic function of the enzyme and/or its binding affinity for the template/primer.

One hypothesis is that there are important polymerase–DNA contacts along the sugar–phosphate groups of the DNA which are required for normal binding and activity of the enzyme but which are somehow perturbed by the presence of the arabinose sugar. This might explain how araCMP when situated in the template could have very little effect on the thermodynamic stability of base pairing and yet still result in partial synthesis arrest. Such sugar–phosphate contacts might be critical for the processive functioning of the enzyme. The recently determined high-resolution crystal structure for the Klenow fragment (Ollis et al., 1985), together with footprinting and amino acid substitution studies (Joyce et al., 1986) with this same enzyme, suggests that such contacts within the cleft of the enzyme are quite probable. More direct investigation of this possibility must await more detailed information about the structural and functional domains of DNA polymerase when it is bound to DNA.

Unlike the other polymerases, reverse transcriptase was markedly less sensitive to the presence of araCMP in the template. This permissivity may be related to the much higher spontaneous error rate this enzyme exhibits in vitro compared to that of the other polymerases (Loeb & Kunkel, 1982). It is also possible that lack of an associated 3′–5′ exonuclease could be responsible for the insensitivity to araC in the template observed for this enyzme. According to this hypothesis, polymerases with associated exonuclease activity are inhibited in their progression past the lesion site because of increased nucleotide turnover, or "idling", at the site of the lesion. Synthesis by reverse transcriptase would be unaffected at the lesion site because it lacks an exonuclease. Direct measurement of nucleotide turnover opposite the lesion site will be required to assess the contribution of idling to overall synthesis arrest.

The in vitro experiments described here constitute a greatly simplified and limited representation of DNA replication

processes as they occur in vivo. Our results do not address the contribution to overall synthesis inhibition by the competition between araCTP and dCTP nor to the effect of misincorporated araC on other mammalian cellular polymerases such as the β and δ polymerases. Nevertheless, our results clearly demonstrate important ways in which the araC lesion may compromise DNA synthesis and suggest a complex mechanism for the inhibition of DNA replication that occurs in cells which have been treated with this nucleoside analogue.

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