Effects of 2-Chloro-2′-deoxyadenosine 5′-Triphosphate on DNA Synthesis in Vitro by Purified Bacterial and Viral DNA Polymerases†

Patricia Hentosh,*,‡,§ J. Clay McCastlain,§ and Raymond L. Blakley§,∥

Department of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, P.O. Box 318, Memphis, Tennessee 38101, and Department of Pharmacology, University of Tennessee College of Medicine—Memphis, Memphis, Tennessee 38163

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ABSTRACT: 2-Chloro-2′-deoxyadenosine 5′-triphosphate (CldATP) was compared with dATP as a substrate for DNA synthesis by bacterial and viral DNA polymerases in vitro. Lengths of chain extension and DNA synthesis pause sites were determined by comparison with products generated by dideoxynucleotide sequencing methods on the same end-labeled primer/template duplex after high-resolution polyacrylamide gel electrophoresis. Reverse transcriptase (RT) from human immunodeficiency virus (HIV-1) and avian myeloblastosis virus (AMV) incorporated CldATP efficiently. DNA strand elongation continued past most chloroadenine (ClA) insertion sites but resulted in shorter chains than when dATP was inserted. Phage T4 DNA polymerase incorporated CldATP least efficiently; Klenow fragment of *Escherichia coli* DNA polymerase I and modified T7 DNA polymerase (Sequenase) showed intermediate ability to utilize the analogue. Incorporation of several consecutive ClA residues into the replicating strand dramatically reduced the ability of Sequenase, Klenow fragment, and T4 DNA polymerases to continue strand elongation. In the absence of the corresponding normal deoxyribonucleoside triphosphate during DNA synthesis, ClA was frequently misincorporated as thymine, cytosine, or guanine by both AMV RT and HIV-1 RT but rarely, if at all, by Klenow fragment, Sequenase, and T4 DNA polymerase. Except T4, for most DNA polymerases, CldATP at 10–20-fold molar excess over dATP was not a strong competitive inhibitor of dATP, as judged by the amount of strand extension and polymerase pause sites during DNA synthetic reactions. Our results indicate that the degree of strand extension in the presence of CldATP, the number and location of polymerase pause sites, and the amount of misincorporation of the analogue are both polymerase- and sequence-dependent.

2-Chloro-2′-deoxyadenosine (ClAdo)[1] is a nucleoside analogue in clinical testing for the treatment of hematologic malignancies (Piro et al., 1988, 1990; Santana & Blakley, 1989). ClAdo has demonstrable cytotoxicity against T and B lymphoblasts in culture (Huang et al., 1981, 1986; Carson et al., 1980, 1983; Blakley et al., 1986; Avery et al., 1989) and tumor cells in vivo (Carson et al., 1980; Huang et al., 1986). The presence of the chlorine group at the C2 position of the purine ring renders the nucleoside resistant to cellular adenosine deaminase (Montgomery, 1982) but does not hinder phosphorylation by deoxycytidine kinase (Carson et al., 1980; Blakley et al., 1986; Griffig et al., 1989). Within whole cells, ClAdo inhibits ribonucleotide reductase (Blakley et al., 1986; Griffig et al., 1989), decreases replicative DNA synthesis to a greater extent than RNA and protein synthesis (Carson et al., 1980), and becomes incorporated into DNA (Carson et al., 1983; Griffig et al., 1989).

The minor structural alteration of ClAdo is clearly adequate to modify many aspects of cellular metabolism and DNA replication. However, the relationship between observed cytotoxicity and biochemical properties of ClAdo is ill-defined. To better understand the molecular basis of the inhibitory effects of ClAdo, we have previously investigated the substrate properties of ClAdTP during DNA synthesis in vitro by using purified human DNA polymerases and M13 phage DNA as template. Our results indicated that ClAdTP retained base-pairing capability and was incorporated into growing DNA strands by human polymerases α and β, although less efficiently than was dATP (Hentosh et al., 1990). Human polymerase β displayed greater difficulty than did polymerase α in continuing the normal polymerization cycle after incorporation of ClA. In addition, DNA synthesis pause sites in the presence of ClAdTP were found to be sequence-specific and occurred not only at regions of two or three consecutive ClA insertions but also at specific single ClA insertion sites. The reduced strand extension by human DNA polymerase α in the presence of ClAdTP could be entirely reversed by equimolar amounts of dATP but not in the case of human DNA polymerase β. Such findings suggest that, despite a common origin, human DNA polymerases with distinct cellular functions and mechanistic properties interact with modified nucleotides in different ways.

The present study has examined the ability of several purified bacterial and phage DNA polymerases as well as the error-prone reverse transcriptases (HIV-1 RT and AMV RT) to utilize ClAdTP as a substrate in place of dATP. Our findings have shown that ClAdTP is a substrate for these enzymes and can be utilized in place of dATP to varying degrees by DNA polymerases. Incorporation of ClAdTP at

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‡Present address: Department of Pharmacology, Chicago Medical School, North Chicago, IL 60064.

§St. Jude Children's Research Hospital.

∥University of Tennessee College of Medicine—Memphis.

[1] Abbreviations: ClAdo, 2-chloro-2′-deoxyadenosine; ClAdTP, 2-chloro-2′-deoxyadenosine 5′-triphosphate; AMV, avian myeloblastosis virus; HIV-1, human immunodeficiency virus 1; RT, reverse transcriptase; ClA, chloroadenine; DTT, dithiothreitol; SE, Sequenase elongated; BrdAdo, 2-bromo-2′-deoxyadenosine; AZT, 3′-azido-3′-deoxythymidine; dNTPs, deoxynucleoside triphosphates; A, adenine; C, cytosine; T, thymine; G, guanine.

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specific DNA sequences, particularly at consecutive CldATP insertion sites, led to DNA polymerization pause sites. In addition, CldATP was misincorporated in the absence of the corresponding nucleotide as dGTP, dCTP, and dTTP by AMV RT and HIV-1 RT.

Materials and Methods

Materials. AMV RT, phage T4 DNA polymerase, dideoxynucleotide sequencing reagents, formamide stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% Xylene cyanol FF), and Sequenase were purchased from United States Biochemical Corp. *Escherichia coli* DNA polymerase I (Klenow fragment), M13mp18 (+) strand DNA, primer 1 [a 17-base deoxynucleotide (5′-GTTTTCCCAGTCACGAC-3′) complementary to positions 6327–6311 of M13mp18 (+) strand], and primer 2 [5′-GTAAAACGACGGCCAGT-3′, which anneals to positions 6307–6291 of M13mp18] were obtained from New England Biolabs. Molecular biology grade dNTPs, primer 3 [5′-GTCATAGCTGTTTCCTG-3′, complementary to bases 6221–6205 of M13mp18], and acrylamide were from Bio-rad, and Kodak XAR and Dupont Cronex film were from Med Cor X-ray Systems. Nensorb columns, [α-^{32}P]dATP, and [γ-^{32}P]ATP were from New England Nuclear. T4 polynucleotide kinase was from Bethesda Research Laboratories. M13mp18-specific primers were also produced in the Molecular Resource Center at St. Jude Children's Research Hospital on an Applied Biosystems 380B DNA synthesizer. CldAdo was synthesized as described (Huang et al., 1981), and triphosphate derivatives were produced from it by standard techniques. The purity of CldATP was assessed by analytical HPLC. No contaminating normal dNTPs were detected, and very little or no mono- and diphosphate forms were detected (<1%). HIV-1 RT was the gift of Dr. Hiroaki Mitsuya, National Cancer Institute, Bethesda, MD.

In Vitro DNA Synthesis Reactions Using 5′-End-Labeled Primers. M13mp18-specific primers 2 and 3 were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP by the forward kinase reaction as reported (Hentosh et al., 1990). Labeled primer was annealed to M13mp18 (+) strand DNA and used in primer extension reactions in the presence of three normal dNTPs and either CldATP or dATP as the fourth nucleotide as described (Hentosh et al., 1990). Final primer extension reaction buffers for each polymerase contained the following in 6.5 μL: for AMV RT, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, and 2–4 units of AMV RT (48 245 units/mg); for Klenow fragment, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM DTT, and 0.05–0.1 unit of Klenow fragment (15 000 units/mg); for Sequenase, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 35 mM NaCl, 3.5 mM DTT, and 0.013 unit of Sequenase (30 000 units/mg); for T4 DNA polymerase, 67 mM Tris-HCl (pH 8.8). 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, and 0.6 unit of T4 DNA polymerase (33 300 units/mg); for HIV-1 RT, 50 mM Tris-HCl (pH 8.), 100 mM NaCl, 8 mM DTT, and 0.4 μg of HIV-1 RT. The unit definitions for each enzyme were as stated by the manufacturer: for Sequenase, Klenow fragment, and T4, 1 unit of enzyme converted 10 nmol of total dNTPs into an acid-insoluble form in 30 min. One unit of AMV RT incorporated 1 nmol of [^{3}H]TTP into nucleic acid product in 10 min at 37 °C. HIV-1 RT at 4 μg incorporated $\approx 3 \times 10^6$ cpm of [^{3}H]TMP in 1 h at 37 °C. In addition, each reaction mixture contained 0.32 mg/mL bovine serum albumin, 10–20 μM unlabeled dCTP, dGTP, and dTTP, and 0.023 μM primer annealed to 0.015 μM M13mp18 (+) strand DNA. CldATP or dATP was added as the fourth nucleotide at 0.5–20 μM. Reactions were performed for 5–30 min at 37 °C for all enzymes except Klenow fragment, which was reacted at 25 °C. Procedures for termination of primer extension reactions, subsequent gel electrophoresis of reaction products, and autoradiography followed those described previously (Hentosh et al., 1990). Dideoxynucleotide sequencing reactions (Sanger et al., 1977) were performed with T7 Sequenase.

Misincorporation of CldATP by DNA Polymerases. Misincorporation of CldATP as dTTP, dCTP, and dGTP was determined in the absence of the corresponding deoxynucleoside triphosphate with SE primers of various lengths. Reactions to generate SE primers with primer 1 have been reported previously (Hentosh et al., 1990) and were modifications of procedures described by Topal et al. (1982) and Toorchen and Topal (1983). After purification of SE primers on a Nensorb column and subsequent evaporation, duplex DNA was redissolved in polymerase reaction buffer (see above) at 3 times the final buffer concentration. To investigate misincorporation, final reaction mixtures contained (in a volume of 8 μL) 0.02–0.025 μM primer, 0.015–0.02 μM template, 1× concentration of reaction buffer, and three of the four normal dNTPs (including dATP) at 20 μM but lacked either dGTP (minus G), dTTP (minus T), or dCTP (minus C). CldATP was added at 7.5 μM in place of the missing nucleotide in separate minus reactions. Thus, the minus G reaction consisted of dATP, dCTP, and dTTP without or with CldATP. DNA polymerase was added as above, and mixtures were incubated at 25 °C (Klenow fragment) or 37 °C (all others) for 30 min.

Results

Comparison of CldATP and dATP as Substrates for DNA Polymerases. To examine the ability of CldATP to replace dATP as a substrate for DNA polymerase, we annealed end-labeled primer 2 or 3 to M13mp18 (+) strand DNA, which served as a template for strand extension in the presence of three normal dNTPs and either dATP or CldATP as the fourth nucleotide. Figure 1 (B and C) shows DNA strands produced by AMV RT extension of primer 2. With 0.5 μM dATP (panel B), chain extension by AMV RT produced a range of DNA fragment sizes, many of which terminated one nucleotide before an A insertion site (e.g., T-6261, T-6229, A-6228, and C-6183) most likely because dATP was present in limited concentration compared with the three other dNTPs. Other pause sites, however, appeared at specific positions distributed nonuniformly along the template (e.g., G-6205, T-6204, and T-6139; see arrows, panel B). At higher concentrations of dATP, chain extension was greater, and the shorter DNA fragments, that were prevalent when dATP was limiting, disappeared. Distinct pause sites were still observed even with 5 μM dATP, however. Some of these occurred near A insertion sites (6145, 6092), but the pattern of non-A pause sites persisted (6205, 6204, and 6139).

CldATP was also incorporated by AMV RT (panel C) with chain elongation continuing after its incorporation, but the overall amount of extension was clearly less than that obtained with dATP, especially at low concentrations. Moreover, chain extension in the presence of CldATP paused at positions similar to those when dATP was limiting (e.g., sites 6261, 6229, and 6228). Additional pause sites, not detected in reactions with dATP present, occurred primarily before or at ClA insertion sites (A-6287, C-6272, G-6242, G-6236), indicating that AMV RT paused either before inserting CldATP or after analogue incorporation. However, many potential ClA insertion sites caused little or no inhibition of strand extension by AMV RT at any concentration of CldATP used (e.g., 6255,

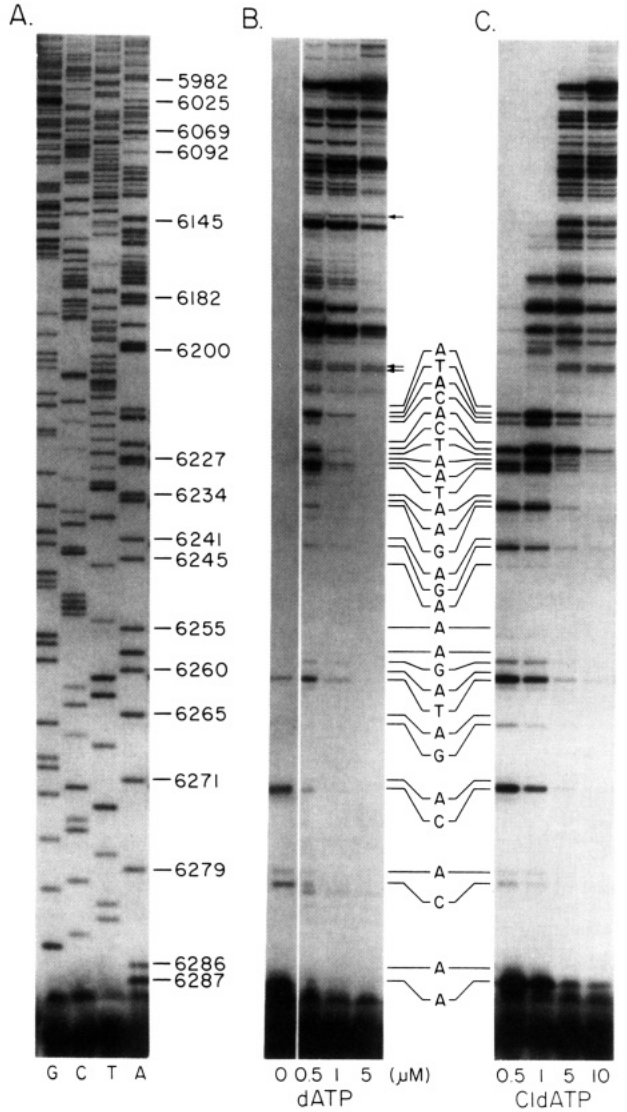

FIGURE 1: Extension of M13mp18-specific primer 2 by AMV reverse transcriptase in the presence of normal substrates (B) or with CldATP in place of dATP (C). The concentration of dCTP, dGTP, and dTTP was 10 μM; that of dATP or CldATP is indicated below each lane. Dideoxynucleotide sequencing results (Sanger et al., 1977) are indicated (A). Other conditions were as stated under Materials and Methods.

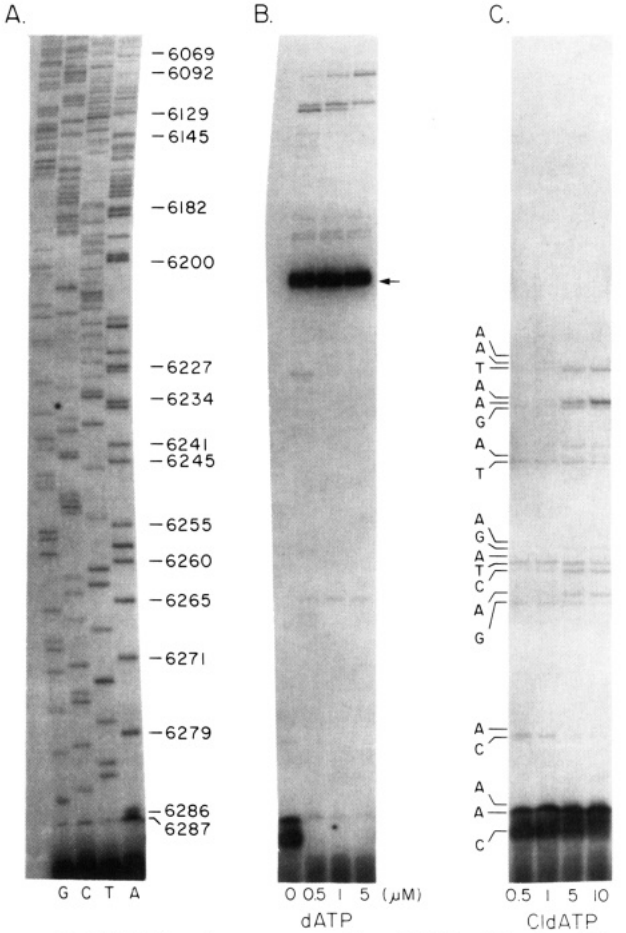

FIGURE 2: T4 DNA polymerase extension of M13mp18 primer 2 in the presence of normal substrates (B) or with CldATP replacing dATP (C). The concentration of dGTP, dCTP, and dTTP was 10 μM. dATP and CldATP concentrations are shown below each lane. The sequence of the newly synthesized strand is shown in panel A.

6245). As the CldATP concentration was increased, pause sites below position 6227 disappeared, and longer DNA strands were synthesized. In addition, the amount of extension and pattern of pause sites with 5 μM CldATP resembled those detected in reactions with 0.5 μM dATP. Similar trends for CldATP incorporation and subsequent strand extension were observed when primer 3 was extended (data not shown).

A very different result occurred with T4 DNA polymerase, which has an active 3′→5′ exonuclease. Reactions in which dATP was included as the fourth nucleotide yielded primarily large DNA strands (80 to ≥300 bases) (Figure 2B). However, a strong pause site occurred at position 6206 (see arrow), near a region of potential secondary structure formation within template DNA as determined by computer-derived predictions. This site was not an absolute block to synthesis, since a small amount of extension by T4 DNA polymerase continued beyond the region at all concentrations of dATP. By contrast, in the presence of CldATP (Figure 2C), only limited extension resulted (≈63 bases maximum). Most of the extended primer accumulated as a 20-mer at a position (C-6288) just before the first of two consecutive ClA insertion sites or as a 21-mer after the first CldATP was inserted (6287), particularly at low CldATP concentrations. At higher CldATP concentrations, some elongation past the first strong pause site took place. In such reactions, T4 polymerase paused one or two nucleotides before a potential ClA insertion site (e.g., G-6266, C-6262, T-6261, T-6246), or at the site of CldATP incorporation (6265, 6235). In reactions with primer 3, T4 polymerase extension was absolutely blocked at a region of three consecutive ClA residues that occur four bases beyond the primer (data not shown).

Comparison of Pause Sites and Extent of Elongation. Figure 3 illustrates that CldATP was used as a substrate by HIV-1 RT, Klenow fragment, and T7 Sequenase, in addition to AMV RT, although less efficiently than was dATP. Standard primer extension reactions were conducted in the presence of three normal dNTPs at 10 μM each and CldATP or dATP at 1 μM. Within the group of five polymerases studied, the pattern of pause sites and extent of elongation in the presence of either dATP or CldATP varied. Figure 3 summarizes the positions of observed pause sites within the newly synthesized (−) strand of M13mp18 after extension of either primer 2 (panel A) or primer 3 (panel B) by each polymerase. Extension of primer 2 in the presence of dATP resulted in very few pause sites for most DNA polymerases within the sequence illustrated, but considerable arrest of synthesis by T4 polymerase occurred at position 6206 at the beginning of a region of potential secondary structure within template DNA. Extension of primer 2 in the presence of CldATP produced many pause sites that occurred primarily

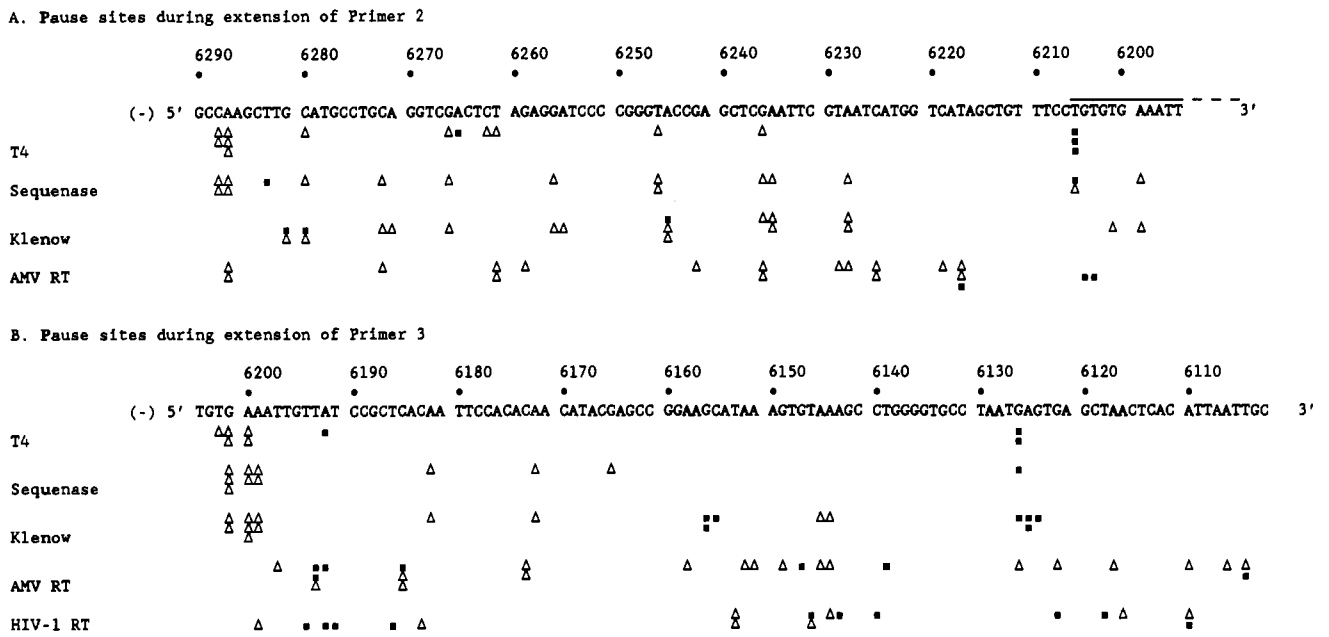

FIGURE 3: Pause sites for chain extension of primer 2 (A) or primer 3 (B) in the presence of either dATP (■) or CldATP (Δ) by DNA polymerases. Reaction mixtures contained 10 μM each of dTTP, dGTP, and dCTP, 15 nM M13mp18 DNA, and 25 nM ^{32}P-end-labeled primer 2 or 3 in the appropriate polymerase reaction buffer. dATP or CldATP was present at 1 μM. Incubation times and temperatures were as under Materials and Methods. The relative intensity of bands on autoradiograms has been indicated as heavy (three stacked symbols), moderate (two stacked symbols), or light (single symbol). Numbering of the newly synthesized minus strand shown is 5′ to 3′ from the terminus of each primer. In panel A, the line above the DNA sequence indicates a region of potential secondary structure within template DNA.

one nucleotide before or at the ClA insertion site, and frequently both. However, there was variability in the intensity of pause sites, and primer elongation did not pause equally at all template T residues. Moreover, different polymerases paused at different sites. Klenow fragment and T7 Sequenase, both of which have low levels of 3′→5′ exonuclease activity, expressed similar patterns of pause sites in CldATP extension reactions.

Extension of primer 3 with dATP as the fourth nucleotide resulted in a number of pause sites that occurred nonrandomly at specific DNA sequences. No consensus sequence for such pauses could be detected, and one region at 6126–6124 was a strong pause site for the three polymerases but not the reverse transcriptases. Extension in the presence of CldATP also produced a varied pattern of pauses. The strongest pause site of Klenow fragment, Sequenase, and T4 DNA polymerase occurred at positions 6201–6199 in a region that contains a sequence where three consecutive ClA residues (6200–6198) must be inserted; the two reverse transcriptases paused only slightly in this area. These sites were an absolute block to chain extension by T4 DNA polymerase over the time course studied, regardless of the CldATP concentration. As the CldATP concentration was increased or the incubation time lengthened, there was no longer pausing by the other DNA polymerases at sites early in chain extension, but new sites appeared at later locations in chain extension (data not shown).

In Table I, the extent of chain elongation by each DNA polymerase on either primer was determined under the same reaction conditions as for Figure 3 in which dATP or CldATP was present at 1 μM along with the three normal dNTPs at 10 μM. The maximum length of DNA fragments synthesized was assessed by gel electrophoresis of reaction products. In general, extension of either primer 2 or primer 3 in the presence of 1 μM dATP produced maximum DNA strands of 200 or more bases. Although CldATP could substitute for dATP as a substrate, the maximum DNA fragment length synthesized by each DNA polymerase studied decreased in the presence of 1 μM CldATP as the fourth nucleotide. The greatest

Table I: Comparison of DNA Strand Elongation in the Presence of dATP or CldATP[a]

	primer 2		primer 3	
enzyme	dATP	CldATP	dATP	CldATP
T4 polymerase	200	56	>300	5
T7 Sequenase	310	127	300	75
Klenow	280	91	194	47
AMV RT	300	141	>225	100
HIV-1 RT	ND[b]	ND[b]	225	110

[a] Maximum chain length synthesized in the presence of either 1 μM dATP or CldATP as analyzed by gel electrophoresis. Conditions were identical with those stated in Figure 3. [b] ND, not determined.

extension with CldATP occurred with AMV RT and HIV-1 RT, the two polymerases that lack proofreading activity; the least amount was with T4 DNA polymerase. When the CldATP concentration was increased, longer strand lengths were synthesized (data not shown).

The extent of elongation in the presence of CldATP also varied with the primer used (Table I). Although some chain extension of primer 2 by T4 polymerase could be detected in the presence of CldATP, very little occurred when primer 3 was used owing to arrest near the first incorporation site of three consecutive ClA residues (Figure 3B). A decrease in maximum chain length synthesized on primer 3 was similarly observed with each of the four other polymerases studied, due to reduced extension past the same triple ClA site (6200–6198). Unlike reactions with T4 polymerase, this site was not an absolute block to further synthesis by reverse transcriptase, Klenow fragment, and Sequenase.

CldATP as a Competitive Inhibitor of dATP. The ability of CldATP to act as a competitive inhibitor of dATP was investigated by primer extension reactions in which dATP and CldATP were both included at various concentrations (Figure 4). In the presence of 1.0 μM dATP, strand extension of annealed primer 3 by Klenow fragment was not significantly altered by the inclusion of 10 μM CldATP (Figure 4A). Although extension in the presence of 10 μM CldATP alone

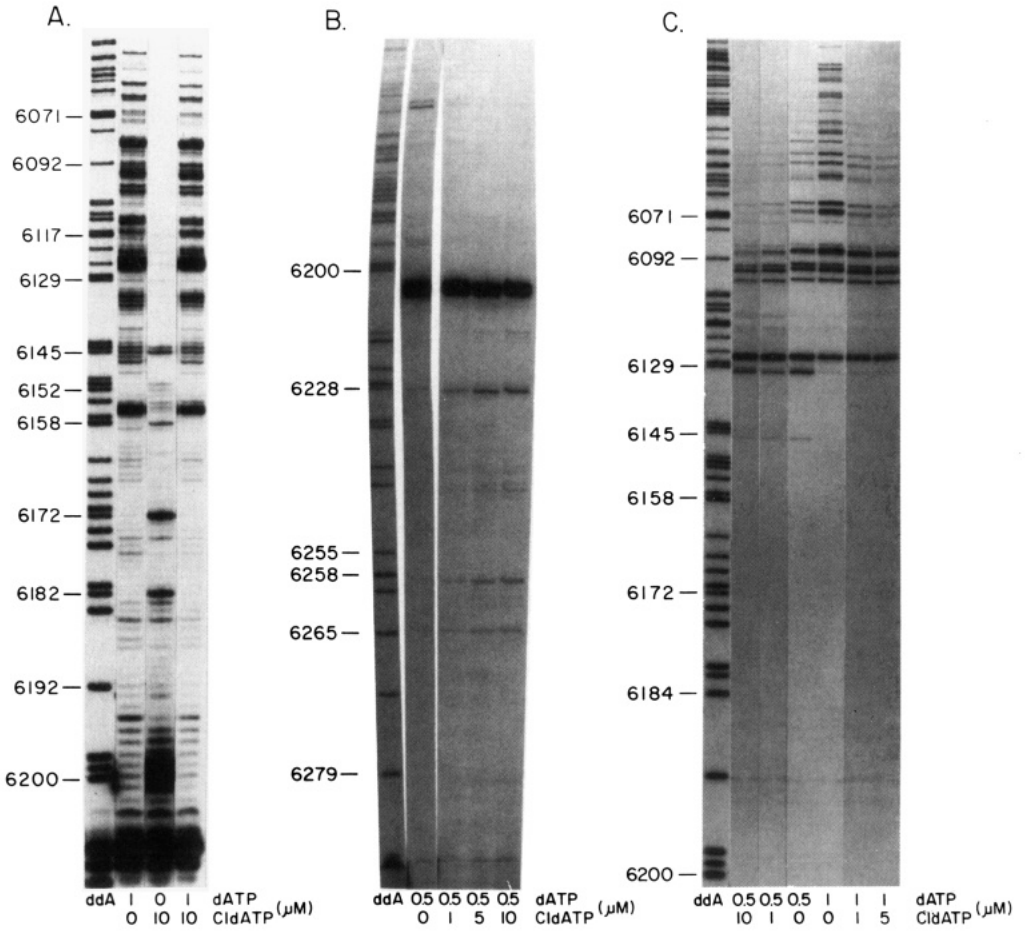

FIGURE 4: ClдATP as a competitive inhibitor of dATP. (A) Extension of primer 3 by Klenow fragment. (B) Extension of primer 2 by T4 DNA polymerase. (C) Extension of primer 3 by T4 DNA polymerase. Three normal dNTPs were present at 10 μM. Concentrations of ClдATP and dATP are indicated below each lane. Sanger–Coulson sequencing results with 2′,3′-dideoxyATP are in lanes marked ddA.

produced unique pause sites (e.g., 6200, 6182, 6172), in the presence of both nucleotides the degree of strand elongation and pattern of pause sites resembled those resulting from reactions with only dATP as the fourth nucleotide. Likewise, insignificant changes in strand elongation and pause sites were observed in such reactions when HIV-1 RT, AMV RT, and T7 Sequenase were used, even at 10–20-fold molar excess ClдATP and at several concentrations of dATP (data not shown).

However, ClдATP caused observable inhibition of primer 2 extension by T4 DNA polymerase (Figure 4B). Without ClдATP, chains with a maximum length of about 170 bases were synthesized in the presence of 0.5 μM dATP, with major pausing at position 6206. When ClдATP was added at 1, 5, and 10 μM, both the synthesis of DNA fragments beyond position 2606 (Figure 4B) and the amount of chains accumulating at position 6206 (evident on less exposed autoradiograms) diminished. Concomitantly, a larger number of shorter DNA chains accumulated as the ClдATP concentration was increased, and new pause sites occurred just before or after ClдATP incorporation (positions 6265, 6259, and 6229). Strand elongation was similarly diminished in the presence of ClдATP when T4 DNA polymerase reactions used primer 3 and when higher concentrations of dATP were tested (Figure 4C). Although the maximal strand length decreased from ≈400 bases in the presence of 1 μM dATP alone to ≈190 bases when 5 μM ClдATP was included with dATP, no accumulation of shorter DNA chains or of polymerase pause sites was detected as compared to those observed in reactions with primer 2. Thus, except for T4, ClдATP appears to be an ineffective inhibitor of dATP incorporation by most DNA polymerases.

Misincorporation of ClдATP as Other Deoxynucleotides. Misincorporation of ClдATP as dGTP, dCTP, or dTTP was assessed by incubating SE primers (Hentosh et al., 1990; also see Materials and Methods) in extension reactions that contained three normal dNTPs including dATP but which were missing dGTP, dCTP, or dTTP (–G, –C, or –T reaction mixtures, respectively). ClдATP was included in separate minus reactions as the fourth nucleotide along with three normal dNTPs. In the absence of a fourth nucleotide and ClдATP, most SE primers were extended by AMV RT (Figure 5) to a position just before the missing nucleotide insertion site (evident as dark bands in –G, –T, and –C lanes). After misincorporation of ClдATP by DNA polymerase at various sites, longer chains were synthesized, and were detected as a corresponding change in band migration and intensity; most often a band shift upward on the gel, equivalent to the addition of one nucleotide (ClA) to DNA fragments followed by immediate chain termination, was observed (Figure 5, solid arrows). At other positions, however, the changes in DNA strand length were more complex and suggestive of a small amount of extension by AMV RT following ClA incorporation (i.e., –T + ClдATP lane, between open arrows). Variation in the degree of misinsertion for a given base at different sequence positions was evident, and misinsertion of ClдATP was usually incomplete (i.e., –G + ClдATP lane, top three arrows).

Table II summarizes the misincorporation results for all the polymerases studied. AMV RT misincorporated ClдATP as

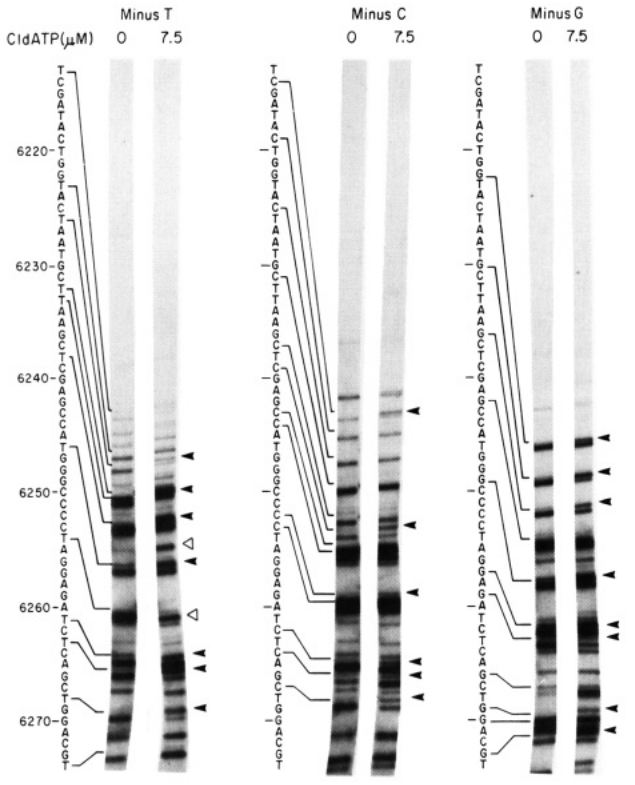

FIGURE 5: Misincorporation of CldATP by AMV RT. SE primers were incubated with AMV RT in reaction mixtures containing three normal dNTPs (including dATP) at 20 μM but lacking dGTP (minus G), dTTP (minus T), or dCTP (minus C). In separate minus reactions, CldATP was included as the fourth nucleotide at 7.5 μM. The sequence of the newly synthesized minus strand of M13mp18 DNA is shown.

Table II: DNA Polymerase Misincorporation of CldATP[a]

	relative incorporation[b] in place of		
enzyme	G	C	T
AMV RT	+++	++	++
HIV-1 RT	+++	+	+
Klenow	--	--	±
T4	--	--	--
Sequenase	--	--	--

[a] Relative incorporation based upon the number of sites where CldATP has been inserted in the absence of the corresponding dNTP, compared with the total number of G, C, or T sites within a 120-base segment of M13mp18 DNA. [b] (−), no misincorporation; (±), 5–15% misincorporation; (+), 20–40%; (++), 45–65%; (+++), 70–90%. CldATP was present at 7.5 μM; other dNTPs were present at 20 μM.

dGTP at 8 of 11 G insertion sites, as dCTP at 6 of 13 C insertion sites, and as dTTP at 8 of 15 T insertion sites. A similar pattern of ClA misincorporation was observed for HIV-1 RT and AMV RT, both of which lack a 3′→5′ exonuclease, but Klenow fragment, T4 DNA polymerase, and Sequenase rarely, if at all, inserted ClA in place of T, G, or C.

Discussion

DNA Synthesis Pause Sites in the Presence of Four Normal dNTPs. Potential secondary structure within the M13mp18 DNA (+) strand caused three DNA polymerases to pause at the beginning of this region consistent with reports that such structures hinder strand extension by several purified DNA polymerases in vitro (Weaver & DePamphilis, 1982; Myers & Romano, 1988; Abbotts et al., 1988). However, even in the absence of secondary structure, others have observed nonrandom pause sites along the template during normal DNA synthesis in vitro (Abbotts et al., 1988; Huber et al., 1989; Bebenek et al., 1989; Hentosh et al., 1990). Moreover, the location and intensity of pause sites appear to be characteristics of the DNA polymerase as well as the nucleotide sequence of the template (LaDuca et al., 1983; Abbotts et al., 1988; Hentosh et al., 1990). The results presented here for bacterial and viral DNA polymerases are also indicative of a unique nonrandom pattern of pause sites during DNA synthesis in the presence of the four normal dNTPs. In general, few pause sites were common to all polymerases studied. A region near position 6126 (primer 3) was a distinct pause site for T4, Sequenase, and Klenow fragment, but not the reverse transcriptases. This pause site was also detected in our previous studies of human polymerase α (Hentosh et al., 1990).

Despite variation in assay conditions in vitro (i.e., polymerase concentrations, incubation times, and DNA templates), similarities in pause sites in our study as well as others were evident. With Klenow fragment, Abbotts and co-workers (Abbotts et al., 1988) saw that the highest frequency of pause sites occurred with a purine as the newly inserted nucleotide (3′-Pur) and a pyrimidine as the incoming substrate. In addition, the combination -A-Pur-dXTP (where dXTP is an incoming substrate other than dATP) was also a particularly frequent pause site. In Figure 3, it can be seen that the main pause sites with Klenow fragment in our study followed these patterns with both primer 3 (positions 6156 and 6124) and primer 2 (positions 6245 and 6281). In similar studies, Bebenek et al. (1989) have indicated that pause sites within the M13 lacZα gene sequence for AMV RT included positions -32, +14, and +15 (numbering scheme for lacZα sequence: position +1 is the first transcribed base), whereas HIV-1 RT paused at positions +13, +14, and +16 among others. These particular sites are identical with pause sites identified in this study on the M13mp18 DNA (−) strand at positions 6147, 6192, and 6193, respectively, for AMV RT and positions 6191, 6192, and 6194 for HIV-1 RT (Figure 3, primer 3).

The relationship, if any, between such pause sites and DNA polymerase fidelity within the cell is currently under study (Abbotts & Loeb, 1984; Bebenek et al., 1989; Roberts et al., 1989). No significant correlation has been found between the spectrum of single base mutations for either AMV RT or HIV-1 RT and termination sites on the M13mp2 template (Bebenek et al., 1989; Roberts et al., 1989). With HIV-1 RT, however, there was a strong correlation between termination sites at runs of three or more identical nucleotides and the occurrence of frameshift mutations in such runs (Bebenek et al., 1989). In this study, HIV-1 RT paused during normal DNA synthesis at positions 6146 and 6143 near a run of three A's. Moreover, the latter base corresponds to a site within the lacZα gene sequence (−36), identified as a major hot spot for base substitutions by HIV-1 RT (Bebenek et al., 1989). This A-T-rich area within the M13mp18 DNA sequence from 6142 to 6152 lies within the *lac* promoter and is thought to be an important site for RNA polymerase interaction (Reznikoff & Abelson, 1978).

Pause Sites in the Presence of CldATP. Pause sites observed during DNA synthesis in the presence of CldATP as the fourth nucleotide (in place of dATP) also appeared to be both sequence- and polymerase-dependent. In general, however, such sites occurred near ClA insertion sites, either one nucleotide before CldATP was to be inserted or at the site of incorporation. The strongest and most frequent pause sites occurred where two or three consecutive ClA's were to be inserted as was the case in our earlier studies with human polymerase α (Hentosh et al., 1990). These findings suggest

that sequential incorporation of CldATP leads to decreased stability of base pairing or fraying of the primer terminus, in much the same manner as that postulated for pause sites observed after incorporation into DNA of consecutive cytosine arabinoside (Townsend & Cheng, 1987; Ohno et al., 1988) or dihydrothymidine (Ide & Wallace, 1988) residues. The greatest degree of similarity in the pattern of pause sites in CldATP reactions was observed for T7 Sequenase and Klenow fragment. Whether the similarity in chain extension pause sites between those two polymerases is due to similar low level amounts of a 3′→5′ exonuclease (Tabor & Richardson, 1987), to the significant amino acid sequence homology within the polymerization domains (Ollis et al., 1985; Bernad et al., 1987), or to some other factor is unknown.

There was very little extension by T4 DNA polymerase in the presence of CldATP in our in vitro synthesis assay. This finding parallels that observed in studies with purified human polymerase β in which very limited strand extension in the presence of CldATP occurred due to several pause sites that were strong blocks to continued synthesis (Hentosh et al., 1990). Under our assay conditions, we were unable to determine if CldATP was indeed a poor substrate for T4 DNA polymerase or if CldATP was inserted efficiently only to be excised by the vigorous 3′→5′ exonuclease activity of T4 polymerase. Although the primary structure of T4 DNA polymerase is significantly homologous to human polymerase α (Spicer et al., 1988; Wong et al., 1988), a completely different pattern of CldATP incorporation and extension was found in our in vitro studies with purified polymerase α that lacks 3′→5′ exonuclease (Hentosh et al., 1990).

CldATP as a Competitive Inhibitor of dATP. For most polymerases investigated except T4 polymerase, the decreased strand extension and distinctive pause sites resulting from reactions with only CldATP present as the fourth nucleotide were completely reversed in reactions with equimolar amounts of CldATP and dATP. Even a 5–10-fold excess of CldATP had little or no appreciable effect on strand elongation, suggesting that CldATP does not compete effectively for incorporation into DNA and is similar to the result obtained in studies with human polymerase α (Hentosh et al., 1990). The two enzymes that elongated primers the least in the presence of CldATP, T4 polymerase and human polymerase β (Hentosh et al., 1990), did result in reduced strand extension and the appearance of pause sites unique to CldATP reactions. However, the inhibitory effects of CldATP on strand elongation in T4 polymerase extension reactions were not as dramatic or severe as those observed in studies with human polymerase β (Hentosh et al., 1990). This reduced competitive inhibition by CldATP in the presence of dATP may be due to the exonucleolytic removal of inserted ClA residues by T4 polymerase and the subsequent insertion of dATP opposite template T residues. Thus, strong pause sites would occur less frequently when both nucleotides were present.

CldATP Misincorporation as dGTP, dTTP, or dCTP. The highest degree of ClA misincorporation of T, G, and C (in the absence of the corresponding nucleotide) was observed with both AMV RT and HIV-1 RT. A similar trend has been observed with Moloney murine leukemia virus RT (unpublished data). A common feature of retroviral RTs is the virtual absence of a 3′→5′ proofreading exonuclease activity (Battula & Loeb, 1976; Roberts et al., 1988) to correct errors made during DNA synthesis. Consequently, RTs exhibit low accuracy of base incorporation and are considered to be the most error-prone polymerases (Battula & Loeb, 1974, 1976; Roberts et al., 1988, 1989; Preston et al., 1988; Weber & Gross, 1989). Misincorporation of CldATP by human DNA polymerases α and β, both of which lack exonuclease activity, has also been found during DNA strand extension reactions in vitro in the absence of the corresponding nucleotide. Human polymerases misincorporated CldATP much less frequently than did reverse transcriptase and only as dGTP and dTTP (Hentosh et al., 1990). The site specificity of misincorporation of CldATP as dGTP, dTTP, and dCTP, however, was very different for RTs compared with both human DNA polymerases despite the common absence of exonuclease.

In contrast to the relatively high degree of CldATP misincorporation by viral RTs, the analogue was rarely, if ever, inserted as other bases by those DNA polymerases containing either low-level (T7 Sequenase and Klenow fragment) or vigorous (T4 DNA polymerase) 3′→5′ exonuclease activity, supporting observations that the exonuclease functions to maintain genomic integrity and to prevent the incorporation of a mismatched base. The lack of CldATP misincorporation as other bases by these three polymerases also provides enzymatic evidence against possible contaminating normal dNTPs within the CldATP solution. Incorporation of normal precursors in place of CldATP would have resulted in uninterrupted DNA elongation rather than in the appearance of distinct pause sites one base prior to the missing nucleotide. In a similar manner, immediate chain termination rather than continued strand extension was observed after misincorporation of CldATP by human DNA polymerases α and β (Hentosh et al., 1990). These results are likewise incompatible with dNTP contamination.

In our misincorporation study, ClA was inserted by AMV RT and HIV-1 RT most frequently as G opposite a template C, producing a ClA·C mispair. This result is similar to that of a recent report of RT misincorporation of normal dNTPs. Under similar conditions (i.e., in the absence of the correct nucleotide), both HIV-1 RT and AMV RT formed A·C mismatches much more frequently than they mispaired A·G or A·A (Preston et al., 1988). Perhaps the similarity in size and structure of the three purine nucleotides (A, ClA, and G) encourages misinsertion of A or ClA in place of G. We have shown that human polymerases α and β also form ClA·C mispairs more readily than the other two combinations, albeit less often than the RTs (Hentosh et al., 1990).

CldATP Interaction with HIV-1 RT. Our studies with HIV-1 RT indicated that very few strong DNA synthesis pause sites occurred in the presence of CldATP and that strand elongation was not effectively terminated. In addition, extremely high concentrations of CldATP relative to dATP were only slightly inhibitory to the incorporation of normal dNTPs and to the elongation of DNA by HIV-1 RT. We have not, however, examined the effects of incorporated ClA residues within template DNA on further rounds of synthesis by HIV-1 RT. The DNA polymerase activity of HIV-1 RT has been the focus of attempts to identify drugs such as AZT that block HIV-1 virus replication (Mitsuya et al., 1985). AZT-triphosphate has been shown to selectively inhibit HIV-1 RT as a competitive inhibitor with respect to dTTP (Cheng et al., 1987; St. Clair et al., 1987). Thus, although CldAdo has demonstrable potential as an antileukemic agent (Piro et al., 1988, 1990; Santana & Blakley, 1989), its effectiveness against AIDS may be limited. Indeed, preliminary evaluation of a similar halogenated compound, BrdAdo, for anti-HIV activity disclosed an insignificant response against HIV-1-infected human cells (B. J. Abbott, personal communication, Biological Testing Branch, NCI–FCRF). These observations demonstrate the utility of screening, at the molecular level, new

antiviral nucleoside analogues for incorporation or specificity differences between host cell DNA polymerases and HIV-1 RT.

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Registry No. dATP, 1927-31-7; dGTP, 2564-35-4; dCTP, 2056-98-6; dTTP, 365-08-2; CldAdo, 4291-63-8; CldATP, 106867-30-5; DNA polymerase, 9012-90-2; reverse transcriptase, 9068-38-6.

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