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Template 2-Chloro-2′-deoxyadenosine Monophosphate Inhibits *In Vitro* DNA Synthesis

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SUMMARY

2′-Chloro-2′-deoxyadenosine triphosphate (cladribine), a purine nucleotide analog and potent antileukemic agent, was enzymatically incorporated into 98-base oligomers in place of dATP to investigate the molecular consequences of 2-chloroadenine (ClAde) in DNA. We have used the resultant oligomers as templates for purified DNA polymerases, to compare the rate and extent of *in vitro* DNA synthesis; the sites of polymerase pausing, if any; and the effects of increasing deoxyribonucleoside triphosphate (dNTP) concentrations on synthetic reactions. Compared with control template, ClAde-containing DNA strikingly reduced the overall amount and rate of chain elongation by human polymerase β and Klenow fragment. Distinct pause sites, which were polymerase dependent, occurred primarily one or two bases before or just after nucleotide incorporation opposite template ClAde. Human polymerase α and phage T4 DNA polymerase likewise exhibited reduced synthesis on ClAde-substituted templates. Bypassing of ClAde residues was possible only at higher dNTP concentrations, with ~20- and 50-fold greater dNTP concentrations being required for synthesis beyond ClAde sites, compared with adenine residues, by polymerase α and β, respectively. These results suggest that ClAde residues located within cellular template DNA may inhibit daughter strand synthesis and thus contribute to the cytotoxic effects of the drug.

CldAdo (cladribine) and similar halogenated purine nucleosides are of interest both therapeutically as anticancer agents and biochemically as modified forms of deoxyadenosine. CldAdo, 2-fluorodeoxyadenosine, and 2-bromodeoxyadenosine effectively inhibit lymphoid L1210 tumor cell growth either *in vivo* or *in vitro*, by themselves (1–3) or in combination with other drugs (3, 4). CldAdo is currently under investigation for use in the treatment of adult (5–8) and pediatric leukemias (9, 10).

Biochemically, the halogenated compounds show altered cellular and metabolic properties, compared with deoxyadenosine. The presence of the halogen atom renders the nucleosides resistant to cellular adenosine deaminase and subsequent inactivation (11) but does not interfere with their cellular uptake and phosphorylation (1, 12, 13). The analogs are highly toxic to T and B lymphoblasts in culture (1, 4, 12, 14–17). In addition, CldAdo inhibits cellular ribonucleotide reductase, decreases DNA synthesis, and is incorporated into cellular DNA (1, 12, 13, 16, 18, 19).

We showed that CldATP was utilized less efficiently than dATP during *in vitro* DNA synthesis by human pol α and pol β (20) and by bacterial and phage DNA polymerases (21). Incorporation of several consecutive CldATP molecules into a replicating DNA strand strikingly reduced the ability of most DNA polymerases to continue strand elongation (20, 21). These findings explain, in part, the observed decrease in cellular DNA synthesis and the accompanying cytotoxicity after exposure to CldAdo. In addition, the presence of ClAde residues within a single DNA strand of restriction endonuclease recognition sequences disrupted double-strand catalysis by enzymes such as *Sal*I, *Pst*I, and *Sph*I, suggesting that ClAde may alter DNA-protein interactions (22).

It is likely that incorporation of CldATP into DNA has significant effects on additional enzymatic processes and rounds of DNA synthesis and that such effects may also contribute to cytotoxicity. Our earlier *in vitro* studies of CldATP as a precursor molecule replacing dATP could not assess the molecular consequences of CldATP incorporation into DNA. Here we have examined whether ClAde residues within DNA affect the extent and rate of *in vitro* DNA synthesis by a number of purified DNA polymerases.

Experimental Procedures

Materials. M13mp18 single-stranded (+) DNA and M13-specific primers 1211 (5′-GTAAAACGACGGCCAGT), 1201 (5′-AACAGCT-

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ABBREVIATIONS: CldAdo, 2-chloro-2′-deoxyadenosine; CldATP, 2-chloro-2′-deoxyadenosine 5′-triphosphate; Ade, adenine; DTT, dithiothreitol; dNTP, deoxyribonucleoside triphosphate; ClAde, 2-chloroadenine; pol β, DNA polymerase β; pol α, DNA polymerase α; PCR, polymerase chain reaction; ara-C, 1-β-D-arabinofuranosylcytosine.

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ATGACCATG), 1212 (5′-GTTTTCCCAGTCACGAC), and 1233 (5′-AGCGGATAACAATTTCACACAGGA) were purchased from New England Biolabs; the dideoxynucleotide sequencing kit and Sequenase II were from United States Biochemical Corp.; T4 DNA polymerase, M13mp18 RF DNA, phenol, and ultra-pure dNTPs were from Bethesda Research Laboratories; *Escherichia coli* polymerase I (Klenow fragment) and T4 polynucleotide kinase were from Promega; human pol α was from Molecular Biology Resources; [γ-^{32}P]ATP was from DuPont-New England Nuclear; Sephadex G-50 was from Pharmacia; and CldATP was kindly provided by Dr. R. L. Blakley, St. Jude Children's Research Hospital, Memphis, TN. Primer RS4 (5′-ACACAGGAAACAG) was synthesized by National Biosciences, Inc. Human pol β was kindly supplied by Dr. Samuel Wilson, University of Texas Medical Branch, Galveston, TX. The PCR kit and native *Thermus aquaticus* DNA polymerase were from Perkin-Elmer Cetus.

Production of single-stranded DNA substituted with ClAde and *in vitro* DNA synthesis. ClAde-containing or control 98- and 122-base DNA fragments, complementary to (+)-strand M13mp18 DNA from position 6210 to 6307 and from position 6186 to 6307, respectively, were produced by exponential and asymmetric PCR, using *T. aquaticus* polymerase and a combination of primers 1201, 1233, 1212, and/or 1211, and gel purified as reported previously (23). The substituted 98-mer consisted of 17 putative ClAde residues (11 single sites and three sets of two consecutive ClAde residues); no ClAde sites occurred in the first 20 bases of the 5′ end. Oligonucleotide primers were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP by the forward kinase reaction, as described previously (20). *In vitro* DNA synthesis reactions using ClAde-substituted or control DNA as template DNA contained ~0.2 pmol of 5′-^{32}P-labeled primer 1201 or RS4, four dNTPs at 5–25 μM each, and 0.32–0.5 mg/ml bovine serum albumin, in a final volume of 6.5 μl/reaction. Each mixture also contained the following: for Klenow fragment, 50 mM Tris·HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM DTT, and 0.06–0.12 units of enzyme; for T4 DNA polymerase, 33 mM Tris·HCl, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM DTT, and 0.25 units of enzyme; for pol α, 60 mM Tris·HCl, pH 8.0, 5 mM magnesium acetate, 1 mM DTT, 0.1 mM spermine, and 0.6–1.5 units of enzyme; for pol β, 60 mM Tris·HCl, pH 7.5, 90 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 87.5 ng (~0.9 unit) of enzyme. The unit definition for each DNA polymerase was the amount of enzyme that incorporated 10 nmol (1 nmol for pol α) of total nucleotide into acid-insoluble material in 30 min at 37°. DNA synthetic reactions were incubated at 37° for various times and inactivated with an equivalent amount of stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF). Reaction products were heat denatured at 90° for 4 min and analyzed on denaturing 8–15% polyacrylamide/7 M urea gels. Autoradiograms were obtained by exposing XAR-5 film overnight at −70°; they were then scanned and analyzed with a LKB laser densitometer and data system to determine the distribution of DNA.

Results

Template ClAde effects on amount and rate of DNA synthesis. Single-strand DNA fragments substituted at Ade sites with ClAde were generated by asymmetric PCR. ^{32}P-labeled primer 1201 was annealed to control and ClAde-substituted 98-mers, and an *in vitro* DNA synthetic assay was then used to investigate the amount and rate of synthesis on ClAde-containing DNA templates, to identify sites of pausing by polymerases, and to determine the effects of increasing dNTP concentrations on synthetic reactions. Human pol β (Fig. 1) synthesized nearly full length DNA strands on control templates within ~15 min; the lack of distinct dense bands throughout the autoradiogram indicated little or no pausing by pol β at specific positions along the template. The faint band present in all lanes near the top of the autoradiogram represented end-

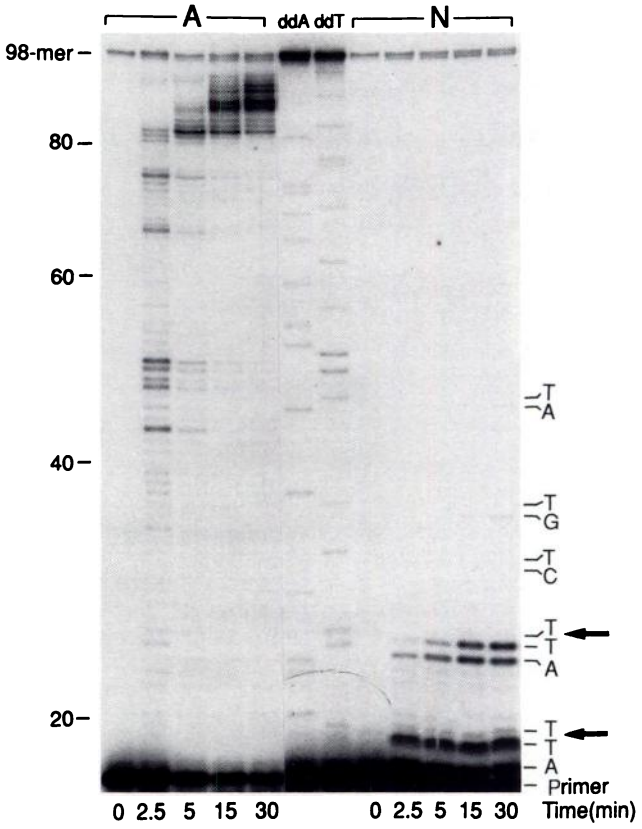

Fig. 1. Extent of replication by human pol β on ClAde-containing template DNA. The ^{32}P-labeled primer 1201 was annealed to either control (*A*) or ClAde-substituted (*N*) 98-mers and incubated with 87 ng (0.3 μM) of pol β in the presence of four dNTPs at 25 μM each. Aliquots were removed at various times, and reaction products were analyzed on an 8% polyacrylamide gel. *Lanes ddA* and *ddT*, dideoxynucleotide sequencing reactions conducted on control 98-mer with primer 1201, representing the sequence of the nascent strand. *Numbers on the left*, nucleotide positions from the 5′ terminus of the primer. *Sites labeled T on the right*, positions where dTTP would be incorporated into the nascent strand opposite a template Ade or ClAde. *Arrows*, strong pause sites near regions of two consecutive template ClAde residues.

labeled 97-mer template DNA that was generated by including a small amount of ^{32}P-labeled primer 1211 during the asymmetric PCR synthesis; this provided a control for the amount of template DNA (normal and ClAde-containing) in each polymerase reaction.

In contrast, when ClAde-substituted DNA was used as template (Fig. 1), extension by pol β was greatly reduced over the same time period, as indicated by the absence of full length 97-mers, and a varied pattern of pause sites resulted. The strongest pause sites, determined by relative band intensities, occurred near the primer opposite two regions of consecutive template ClAde residues (Fig. 1, *arrows*) and resulted in DNA strands of eight or nine nucleotides in length. These sites were very effective blocks to further chain elongation by pol β, because only a few slightly longer strands (maximal lengths of 45–50 bases) were synthesized with time. Comparison of band positions with dideoxynucleotide sequencing reactions conducted on control template with primer 1201 showed that pol β always paused one nucleotide before incorporating a dNTP opposite template ClAde residues. At sites with two consecutive ClAde residues, pol β also paused upon incorporation of a deoxynucleotide opposite the first ClAde residue (i.e., before the second

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template ClAde). The absence of bands 3′ to the deoxythymidine-chloroadenine pair indicated that strand elongation easily continued past the analog upon deoxynucleotide incorporation. With greater amounts of pol β, longer strands were synthesized and pause sites near the primers decreased, but new sites appeared opposite later ClAde template sites (data not shown).

Template ClAde residues were blocks to DNA synthesis not only for human pol β but also for *Escherichia coli* Klenow fragment, which is slightly processive in nature and has an associated 3′→5′ exonuclease. Full length 97-mers were produced on control templates within the first 30 sec, with few or no polymerase pause sites (Fig. 2). In contrast, the use of ClAde-containing DNA as template greatly decreased the rate of extension by Klenow fragment, many truncated DNA strands were detected after 15 min of incubation, and defined pause sites were apparent. Sites of pausing by Klenow fragment differed from those observed with pol β. In general, Klenow fragment paused one or two nucleotides (Fig. 2, *asterisks*) before incorporating a dNTP and sometimes paused at the site of dNTP incorporation opposite the analog. At certain sites (Fig. 2, *arrow*), Klenow fragment extended a dX-chloroadenine pair by one nucleotide before pausing. Unlike reactions with pol β, however, full length DNA strands were synthesized by Klenow fragment during the 5- and 15-min incubation periods. In a similar manner, both human pol α and phage T4 DNA polymerases showed reduced ability to elongate DNA strands past template ClAde residues (data not shown). Furthermore, their respective pause site patterns resembled that of Klenow fragment, rather than that of pol β.

To rule out the possibility of decreased or inefficient primer 1201/template renaturation due to the presence of three ClAde residues within the 16-base template annealing site, we annealed a 122-base control or ClAde-containing DNA fragment also produced by PCR to primer RS4. RS4 is complementary to a region of template containing only thymine, cytosine, and guanine, with no Ade or ClAde residues. The nascent strand sequence is 5′-primer RS4+CTATGAC .... 3′, and there are only singly occurring template Ade or ClAde sites in a stretch of 12 bases. With use of this primer/template, *in vitro* DNA synthesis by pol β was likewise drastically reduced on ClAde-substituted DNA, compared with control DNA, and a similar pattern of pause sites was evident (Fig. 3). DNA elongation

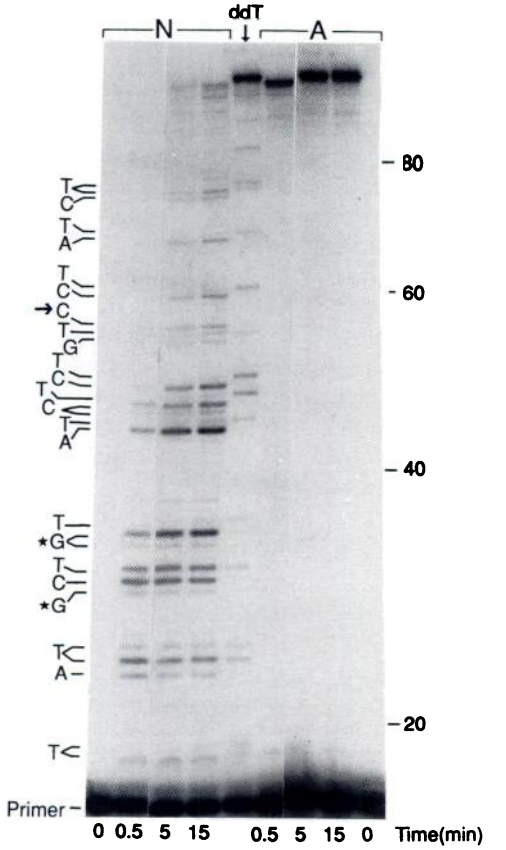

Fig. 2. Extent of replication by Klenow fragment on ClAde-substituted template DNA. Primer 1201 was annealed to control (*A*) or ClAde-containing (*N*) 98-mer DNA and incubated with 0.06 units (~7.5 nM) Klenow fragment in the presence of four dNTPs at 25 μM each. *Lane ddT*, dideoxynucleotide sequencing reaction conducted on control 98-mer with primer 1201, included to depict sites of dTTP incorporation opposite template Ade or ClAde residues. Other reaction conditions were as described for Fig. 1. *Nucleotides shown at left*, sequence of the nascent strand synthesized from primer 1201. *Arrow*, a site where DNA polymerase extended a dX-chloroadenine pair by one nucleotide before pausing. ★, Sites where polymerase paused one or two nucleotides before a template ClAde site.

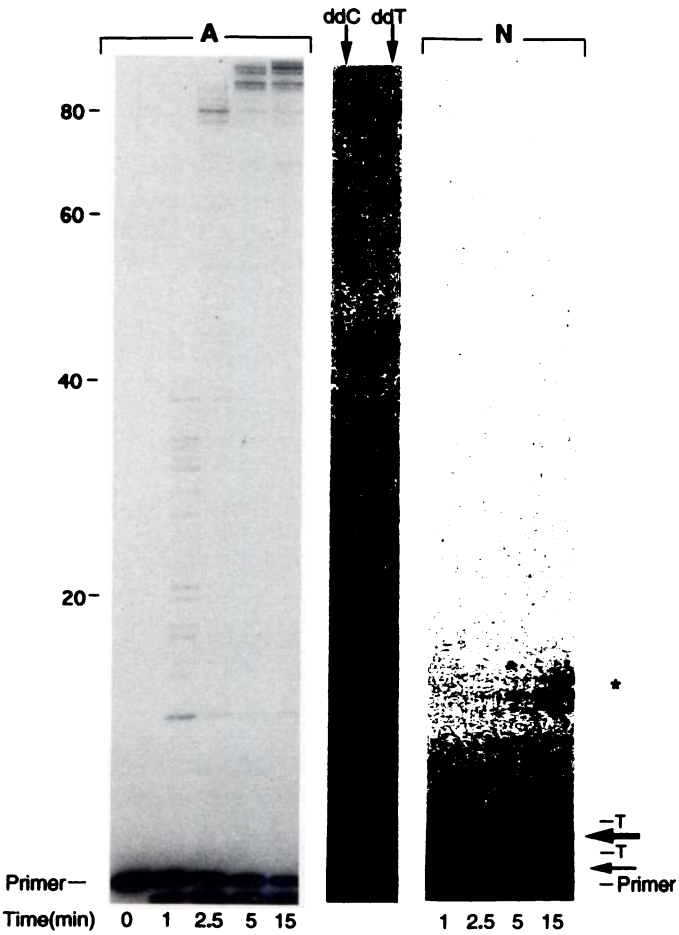

Fig. 3. Time course of human pol β replication on primer RS4/122-mer template DNA. The ^{32}P-labeled primer RS4 was annealed to either control (*A*) or ClAde-substituted (*N*) 122-mers and incubated with 87 ng (0.3 μM) of pol β in the presence of four dNTPs at 25 μM each. *Lanes ddC* and *ddT*, dideoxynucleotide sequencing reactions conducted on control 122-mers annealed to primer RS4. Other reaction conditions were as described for Fig. 1. *Arrows*, DNA strands blocked after dCTP (*small arrow*) and dATP (*large arrow*) incorporation just before single dTTP insertion sites on the nascent strand. *, Maximal DNA lengths synthesized on ClAde-containing templates.

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products accumulated primarily after dCTP incorporation (Fig. 3, *small arrow*) (one nucleotide before the first dTTP insertion site opposite template ClAde) and after incorporation of three nucleotides (Fig. 3, *large arrow*) before the second dTTP insertion site. During a 15-min incubation, the maximal DNA strands produced were only ~13 nucleotides in length, with synthesis halted near two consecutive template ClAde sites (Fig. 3, *asterisk*). In contrast, extension on control 122-mers resulted in nearly full length DNA strands within 5 min. These findings indicated that decreased DNA synthesis on ClAde templates was not due to inefficient renaturation of primer 1201 and 98-mer DNA or a "frayed" primer terminus. In addition, the use of the RS4/122-mer combination showed that pol β extension past singly occurring template ClAde residues was as strongly inhibited as when two consecutive ClAde residues occurred in the template. This rules out the possibility that multiple ClAde residues have an unusually strong inhibitory effect on pol β synthesis.

To limit overall synthesis and obtain a more direct and accurate measurement of the relative rates of elongation on normal and ClAde-substituted DNA, we conducted pol β extension reactions at short incubation times in the presence of only three dNTPs. The sequence of the nascent strand 3′ to annealed primer 1201/98-mer is 5′-primer+ATTACG. . .3′. Thus, in the presence of dATP, dTTP, and dCTP, a maximum of five nucleotides would be added onto primer 1201 (a 16-mer) to produce a 21-mer, and DNA polymerase would insert two consecutive dTTP molecules opposite two template ClAde residues. A gel autoradiogram from such a study and the accompanying scanning densitometry results (Fig. 4) revealed only slight accumulation of 17–20-mers on control DNA over a 6-min period and a steady increase in the formation of 21-mers. The bands at 17–20-mer sites represent flow into and out of these four sites as synthesis progresses; 21-mers accumulate because flow occurs into but not out of the last site. On ClAde-containing DNA, in contrast, there was rapid and steady accumulation of 17-base products (up to 31% of the total radioactivity) that were blocked one nucleotide before a template ClAde residue. Incorporation of a dNTP opposite ClAde to produce an 18-mer was extremely slow and, after 6 min, represented only 5% of the total radioactivity in the lane. Moreover, no bypass of ClAde sites occurred during the incubation period.

Bypass of ClAde residues with increasing dNTP concentrations. We assessed the effect of dNTP concentration on incorporation and subsequent elongation of a nucleotide opposite ClAde by examining pol β and pol α extension of primer 1201 in the presence of only two dNTPs; a maximum of four nucleotides should be added under these conditions. On control 98-mers (Fig. 5I) full length 20-mers were synthesized by pol β at low dNTP concentrations (<5 μM), suggesting that elongation of the primer was very rapid and efficient. Synthesis of intermediate-length products (17-, 18-, and 19-mers) was also evident at detectable levels, but with greater concentrations of dNTPs these bands disappeared and the production of 20-mers increased. In the presence of 54 μM dNTPs, pol β misincorporated either dATP or dTTP opposite template guanine residues, evident as an additional band on the autoradiogram. In contrast, pol β extension of primer annealed to ClAde-containing template was inhibited one nucleotide before dTTP incorporation, resulting in an accumulation of 17-mers that increased with greater dNTP concentrations up to 5 μM. At concentrations above 5 μM, the 17-mer band decreased concomitantly with the appearance of an 18-mer band corresponding to incorporation of the first dTTP opposite template ClAde. There was little accumulation of 19-mers (primer+ATT) with increasing dTTP concentration, and full length 20-mers were not visible on the autoradiogram until dNTP concentrations reached ~54 μM.

The quantitative distribution of DNA products as a function of dNTP concentration was determined by densitometric analyses of the experiment described above and others in which greater dNTP amounts were used for pol β extension on ClAde-containing templates (Fig. 6). Full length 20-mers were synthesized in linear fashion on control template up to 5 μM dNTPs, with peak synthesis at 20 μM (~80% of total radioactivity in

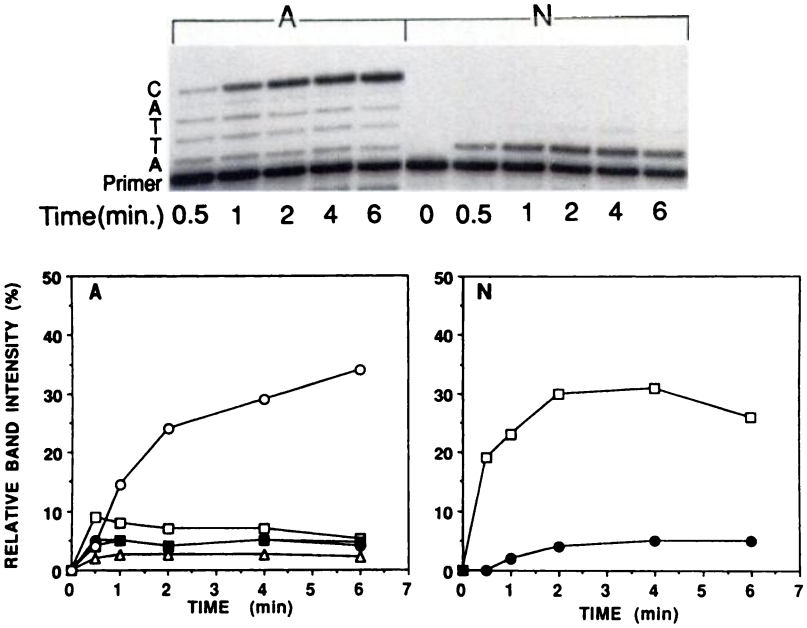

Fig. 4. Pol β bypass of template ClAde sites as a function of time. *Upper*, the ^{32}P-labeled primer 1201 was annealed to either control (*A*) or ClAde-substituted (*N*) 98-mer and incubated with ~1 unit of pol β in the presence of 5 μM each dATP, dTTP, and dCTP, to limit extension to five nucleotides. Aliquots were removed at various times and analyzed on a 20% polyacrylamide gel. *Sequence shown on left*, that of the primer-extended (nascent) strand. *Lower*, results from scanning densitometry. Relative intensity values for each band are indicated as a percentage of the integrated intensity of all bands in the lane. □, Primer+A; ●, primer+AT; ■, primer+ATT; △, primer+ATTA; ○, primer+ATTAC.

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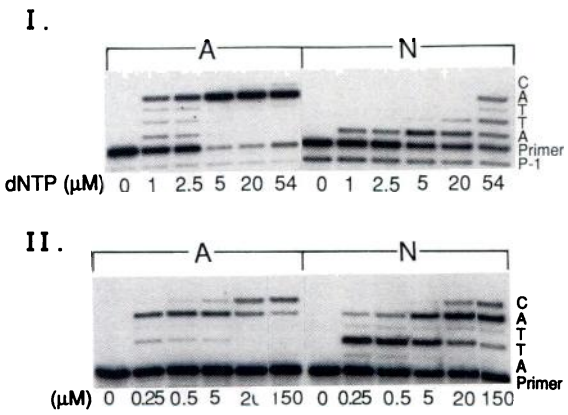

Fig. 5. Concentration-dependent bypass of template Ade or ClAde residues by human pol β and pol α. The ^{32}P-labeled primer 1201 was annealed to control 98-mer (*A*) or ClAde-substituted 98-mer (*N*) and incubated with ~1 unit of pol β (I) for 10 min at 37°, with increasing molarities of dATP and dTTP, as indicated below each lane. Reactions with pol α (1.5 units) were for 20 min (II). *Sequence shown on right*, that of the primer-extended strand. Two consecutive ClAde residues in template DNA were located opposite the extended strand thymine sites.

the lane). Relatively small quantities (<15% of total radioactivity) of intermediate products were evident only at concentrations of <5 μM dNTPs. On ClAde-containing template, however, elongation of annealed primer to full length 20-mers was not evident with 20 μM dNTPs, and with 54 μM dNTPs the density of the 20-mer band represented only ~20% of the total radioactivity. As the dNTP concentration was increased to 125 μM the yield of 20-mers doubled (to 40% of the total radioactivity), but it never reached the level of extension achieved on control DNA. Comparison of the relative amounts of full length products produced on either template revealed that equivalent amounts (20% of the total radioactivity) were produced in the presence of 1 μM dNTPs for control templates and 54 μM dNTPs for ClAde-containing DNA. Thus, an approximately 50-fold greater concentration of dNTPs was necessary to bypass two template ClAde residues, compared with synthesis on normal DNA. Moreover, identical results were obtained when we examined pol β synthesis with annealed primer RS4 and 122-mers as a function of dNTP concentration (data not shown). Bypass of singly occurring ClAde sites within this template sequence also required a 50-fold greater dNTP concentration, compared with control DNA. Upon incorporation of dTTP, synthesis readily continued, with no accumulation of primer+CT products. This experiment confirmed that the rate-limiting step for pol β elongation was insertion of dTTP opposite ClAde but that extension of the deoxythymidine-chloroadenine pair was not appreciably impaired (see also Fig. 3).

Similarly, we assessed pol α extension on ClAde-containing 98-mers and primer 1201 as a function of dNTP concentration (Fig. 5II). Synthesis of full length 20-mers on control template was nearly complete during a 20-min incubation with 0.25 μM dNTPs. In the presence of greater dNTP concentrations, misincorporation opposite template guanine was apparent. In contrast, extension on ClAde-containing template at 0.25 μM dNTPs was inhibited to a large extent just after incorporation of dTTP opposite the first ClAde (~30% of the total radioactivity). A small amount of 20-mer (4% of the radioactivity), corresponding to bypass of the ClAde sites, was also produced. At dNTP concentrations of >0.5 μM, DNA elongation beyond

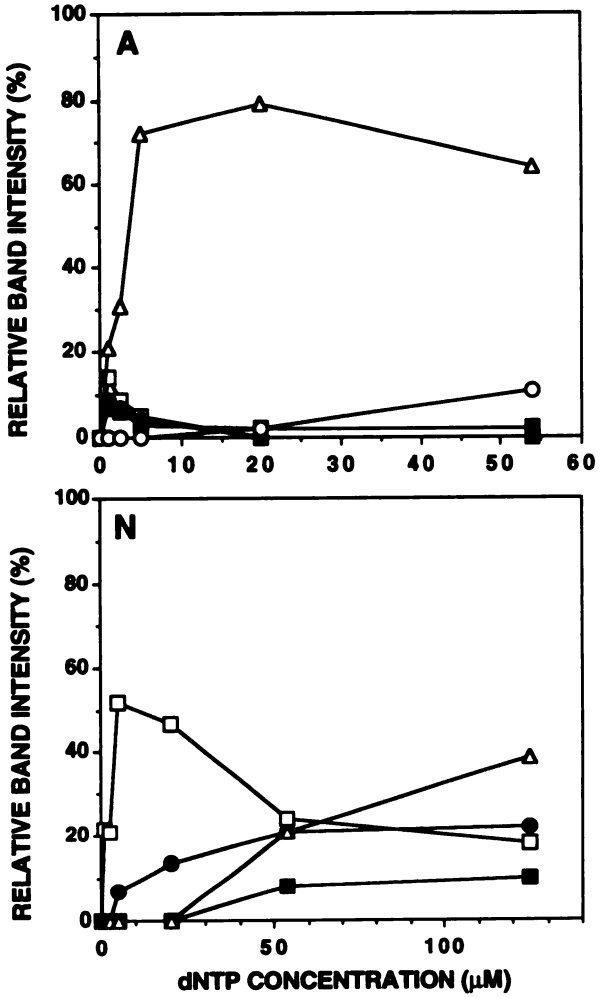

Fig. 6. Gel band intensity changes as a function of dNTP concentration. Extension by pol β of ^{32}P-labeled primer 1201 annealed to control (*A*) or ClAde-substituted (*N*) 98-mer template DNA was conducted in the presence of only dATP and dTTP, to limit extension to four bases. The density of each band on the autoradiograms was quantitated by scanning densitometry; addition of nucleotides to the primer is shown as a percentage of the total radioactivity in the lane. Data points represent the average of at least two experiments. Note the higher dNTP concentrations on the *x*-axis for ClAde experiments. □, Primer+A; ●, primer+AT; ■, primer+ATT; △, primer+ATTA; ○, primer+ATTAC.

ClAde sites continued more readily, the 18-mer band intensity decreased, and full length 20-mers and 21-mers (due to misincorporation) were synthesized. Densitometric analysis indicated that ~20-fold greater concentrations of dNTPs were required to produce a similar amount of full length 20-mers on ClAde-substituted templates, compared with control DNA. However, a partial block to continued elongation at the first deoxythymidine-chloroadenine pair was still evident with 150 μM dNTPs. Thus, in contrast to the pattern of pol β pausing during elongation past the same ClAde-containing sequence, pol α exhibited little difficulty in incorporating dTTP opposite a template ClAde residue; instead, its ability to continue elongation beyond the deoxythymidine-chloroadenine pair was reduced (compare Fig. 5, I and II).

Discussion

Several potent antineoplastic nucleoside analogs that have been postulated to act solely at the level of DNA synthesis

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inhibition upon incorporation into DNA are not absolute blocks to DNA synthesis. Compounds such as CldAdo, 9-β-D-arabinofuranosyladenine, ara-C, and certain arabinofuranosyl nucleoside analogs, such as 1-β-D-arabinofuranosyl-5-azacytosine and 9-β-D-arabinofuranosyl-2-fluoroadenine, only partially inhibit strand elongation *in vivo* or *in vitro* (20, 24–26). Furthermore, these analogs cause appreciable arrest of DNA synthesis primarily upon incorporation into multiple consecutive sites. As a consequence of strand elongation beyond a single incorporation site, altered bases most likely have an impact on additional cellular functions, particularly on further rounds of DNA synthesis. Such effects may contribute to cytotoxicity and cellular mutagenesis as much as a total blockade of DNA replication does.

The studies reported here demonstrate that template ClAde residues indeed hinder DNA elongation with all of the polymerases examined, regardless of the presence or absence of 3′→5′ exonuclease. When total DNA synthesis in the presence of four dNTPs was examined over long incubation periods, there was dramatically less overall elongation on ClAde-containing templates, as measured by the relative quantities and lengths of DNA strands. In this regard, the inhibition of *in vitro* strand elongation by template ClAde residues is similar to effects observed with the nucleoside analog ara-C (27) and with modified bases such as alkylation products (28–30), thymine dimers (31), and thymine glycols (32, 33). Polymerase pause sites on substituted templates were both polymerase and sequence dependent. The slow rate of synthesis by human pol β was due almost exclusively to inefficient and slow incorporation of dTTP opposite ClAde residues. This step required longer times and greater concentrations of dTTP than did extension on normal templates. However, extension from a deoxythymidine-chloroadenine pair was subsequently efficiently catalyzed, because little accumulation of DNA fragments at those sites was observed. The pattern of pause sites for Klenow fragment was varied and suggestive of neighboring sequence effects. Pause sites both before and directly opposite ClAde residues were most common, indicating that both incorporation of dTTP and extension beyond a deoxythymidine-chloroadenine pair were inefficient. In addition, a few pause sites occurred one base beyond the ClAde residue; thus, elongation slightly downstream of a deoxythymidine-chloroadenine pair was also impaired. Human pol α inserted dTTP opposite template ClAde with relative ease but showed reduced ability to extend beyond the deoxythymidine-chloroadenine pair.

The very strong pause sites observed with pol β near the first several template ClAde sites in Fig. 1 severely limited the overall amount of DNA synthesis, compared with synthesis with Klenow fragment and human pol α. These results parallel the relative efficiency of *in vitro* elongation by these three enzymes in the presence of CldATP, where pol β was also the most sensitive to incorporation of the triphosphate (20, 21). In those studies (20, 21), we demonstrated that pol β readily inserted CldATP into a nascent DNA strand, but incorporation of additional deoxynucleotide triphosphates was extremely slow and the overall amount of extension was drastically reduced, compared with Klenow fragment and pol α. In a similar manner, Abbotts *et al.* (34) showed that pol β poorly incorporated a nucleotide opposite the modified bases N^3-methyl-deoxythymidine and O^6-methyl-deoxyguanosine and that there was no appreciable synthesis beyond the modified site. Compared with Klenow fragment and pol α, both of which are large proteins with several functions and/or subunits, pol β is a small (38-kDa) monomeric polymerase that is distributive in nature (35). After dissociating from DNA during a nucleotide incorporation cycle, pol β may not be able to rebind to ClAde-substituted templates or CldAdo monophosphate-containing nascent strand termini, due to distortion in the DNA and altered protein contact sites.

Others have also noted differences in the ability of DNA polymerases to synthesize beyond various modified template nucleosides. *In vitro* synthesis with avian myeloblastosis virus reverse transcriptase was not inhibited by template ara-C residues, whereas replicative bypass for T4 DNA polymerase and human pol α was markedly slowed (27). Purine and pyrimidine monoadducts induced by cisplatin or by furocoumarin photoaddition inhibited eukaryotic enzymes such as *Drosophila* pol α more than prokaryotic DNA polymerases such as Klenow fragment, but both species of polymerases were strongly blocked by UV-induced pyrimidine dimers (36). These studies suggest that not only the size and chemical nature of a nucleotide modification but also the type and origin of DNA polymerase determine bypass capability.

Our finding that template ClAde residues severely limit *in vitro* DNA polymerization is significant in light of *in vivo* studies by Huang *et al.* (15), in which they examined resumption of cell growth after exposure to 1.5 or 5 μM 2-Bromo-dAdo, a related halogenated nucleoside analog with cytotoxicity and antitumor activity virtually identical to those of ClAdo. These concentrations reduced viability of cultured CCRF-CEM cells to ~12 and 4%, respectively. After a long initial lag period and considerable cell killing, surviving cells exhibited growth through only approximately two more divisions; proliferation then ceased, and the cell number began to decrease. This delayed cytotoxicity suggests that there are long term effects due to 2-Bromo-dAdo and ClAdo treatment that are exhibited only when surviving cells attempt to go through more rounds of DNA synthesis. Such a finding may be explained by the inability or reduced ability of DNA polymerases to bypass template ClAde residues, leading to enhanced cytotoxicity. It will be of interest to assess the presence of ClAde residues in DNA from cells that survive the initial exposure to ClAdo.

Considered together, the results described above suggest that the polymerase/template complex near a ClAde residue is somewhat unstable and unsuitable for positioning incoming dNTPs, despite the availability of the appropriate hydrogen bonding sites in ClAde. Moreover, incorporation of dTTP opposite ClAde apparently causes sufficient distortion to decrease the efficiency of phosphodiester bond formation downstream of the deoxythymidine-chloroadenine pair. It is not known from our experiments whether processive polymerases dissociate from the primer/template near ClAde residues or merely pause within the region. Our *in vitro* results emphasize the diverse effects a nucleoside analog such as ClAdo may have within a cell. Cellular replication may be delayed or inhibited at one level upon incorporation of the analog into DNA, but persistence of the modified nucleoside in DNA may affect not only further rounds of replication but also other cellular metabolic processes. Thus, the multipotent nature of these analogs may account for their efficacy as cytotoxic agents against leukemic cells.

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