0026-895X/95/050897-08$3.00/0

MOLECULAR PHARMACOLOGY, 48:897–904 (1995).

In Vitro Transcription of DNA Containing 2-Chloro-2′-deoxyadenosine Monophosphate

PATRICIA HENTOSH and MARTIN TIBUDAN

Department of Pharmacology and Molecular Biology, Chicago Medical School, North Chicago, Illinois 60064

Received March 16, 1995; Accepted July 31, 1995

SUMMARY

2-Chloro-2′-deoxyadenosine (cladribine [CldAdo]) represents one of the most promising therapeutic agents for the treatment of pediatric leukemias and adult hairy cell leukemia. We examined whether CldAdo incorporation into DNA inhibited subsequent transcription *in vitro* using purified phage RNA polymerases. Control (Ade-containing) and 2-chloroadenine (ClAde)-substituted DNA strands that contained a RNA polymerase promoter sequence were synthesized by a modified asymmetric polymerase chain reaction. Complementary (+) and (−) strands were annealed, incubated with phage RNA polymerase, and analyzed with denaturing PAGE. When ClAde was present in both strands, the yield of full-length transcripts (≅100 bases) was reduced by ≅90% relative to control DNA. Transcription was also reduced to a slightly lesser degree when substitutions occurred in only one of two strands. The observed low transcript levels on ClAde-containing DNA were due in part to the presence of the analogue within the promoter region. With gel shift binding assays, we demonstrated that RNA polymerase did not bind as well to ClAde-containing promoters. Polymerase/DNA complex formation was decreased by ≅80% compared with that on control unsubstituted promoters. In addition, on binding to the substituted promoter, RNA polymerase had an altered conformation that led to enhanced proteolytic clipping by endoproteinase Glu-C. Transcript sequence analysis indicated that SP6 RNA polymerase read through template ClAde residues with no apparent misincorporation into RNA. Our results provide insight into a novel effect of this nucleoside analogue that may explain its cytotoxicity in nondividing cells.

ClAdo, an analogue of deoxyadenosine, is a clinically important new drug for the treatment of adult hairy cell leukemia (1–3) and a variety of other lymphoid and myeloid malignancies (4–7). ClAdo is unique in its ability to kill both dividing and nondividing cells. The exact biochemical mechanism or mechanisms of action of this compound have been investigated but remain somewhat unclear. In proliferating cells, the drug requires DNA synthesis for its toxicity. Cell culture studies have shown that ClAdo is readily taken up by a specific nucleoside transporter system (8) and is converted to the active triphosphate form, ClATP, by cellular kinases (9–11). The substitution of chlorine for hydrogen at the 2 position of the purine ring renders the nucleoside resistant to cellular adenosine deaminase and subsequent inactivation (12). ClATP inhibits both cellular ribonucleotide reductase and DNA synthesis and is incorporated into cellular DNA (9–11, 13, 14). ClAdo-induced toxicity to resting cells remains unexplained; it has been proposed that ClATP interferes with cellular repair polymerases or is incorporated into DNA during repair processes that occur in response to spontaneous damage (15).

At the molecular level, we and others have shown that ClATP retained base-pairing ability and was used as a substrate during *in vitro* DNA synthesis with human DNA polymerases α and β (13, 16), HIV RT (17), and several bacterial and phage DNA polymerases (17). However, the overall rate and extent of strand elongation were diminished compared with control reactions, with human polymerase β being the most sensitive (16, 17). Incorporation of a single ClATP molecule into a replicating DNA strand strikingly reduced the ability of polymerase β, which functions in DNA repair synthesis, to continue strand elongation, whereas extension by polymerase α, one of the cellular enzymes responsible for DNA replicative synthesis, was inhibited only after consecutive ClATP insertions (16, 17).

Although many DNA-damaging agents have been thought to exert their cytotoxic effects primarily at the level of DNA synthesis inhibition, recent evidence illustrates that altered DNA structure and conformation also affect gene expression and RNA transcription. DNA alkylated by melphalan or ni-

This research was supported by Grant CA55414 from the National Cancer Institute. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

ABBREVIATIONS: ClAdo, 2-chloro-2′-deoxyadenosine; ClAde, 2-chloroadenine; PCR, polymerase chain reaction; DTT, dithiothreitol; DEPC, diethylpyrocarbonate; PAGE, polyacrylamide gel electrophoresis; AMV, avian myeloblastosis virus; RT, reverse transcriptase; GSBA, gel shift binding assay; ara-C, 1-β-D-arabinofuranosylcytosine.

trogen mustard produced truncated RNA molecules *in vitro* (18), as did benzo[a] pyrene-modified DNA (19, 20) and UV-irradiated DNA (21). Because CldATP is not an absolute chain terminator for polymerase α, it is likely that incorporation of this modified nucleotide into cellular DNA will significantly affect other essential biochemical processes. CldAdo has long term effects on cell growth that are expressed only several days after its initial inhibition of DNA synthesis. Huang *et al.* (22) examined cellular recovery after removal of 2-bromo-2′-deoxyadenosine, a related analogue with identical cytotoxicity and antitumor activity. Cells that survived initial treatment with 2-bromo-2′-deoxyadenosine progressed through one or two additional cell divisions before cell death occurred. A possible explanation for such delayed cytotoxicity may be inhibition of RNA transcription due to the presence of incorporated 2-bromoadenine or ClAde residues in template DNA. Likewise in resting cells, CldATP may be incorporated into DNA during repair synthesis at sufficient levels to inhibit cellular protein/DNA interactions and RNA transcription. In support of this proposal, Seto *et al.* reported that RNA synthesis is reduced in resting lymphocytes in response to CldAdo treatment (15). No studies have explored the molecular effects of halogenated nucleoside analogues in template DNA on transcriptional processes. Therefore, we examined whether ClAde-substituted DNA blocks elongation by RNA polymerases using a well-characterized *in vitro* assay.

Experimental Procedures

Reagents. 2-CldAdo was generously provided by Ortho Biotech. CldATP was synthesized from CldAdo by Sierra Bioresearch. PCR kits and *Thermus aquaticus* DNA polymerase were obtained from Perkin Elmer Cetus; T3 RNA polymerase, T4 Gene 32 protein, and ultrapure NTPs from Boehringer Mannheim Biochemicals; SP6 RNA polymerase from Epicentre Technologies; RNasin and *fmol* sequencing kit from Promega; [γ-^{32}P]ATP and [α-^{32}P]CTP from New England Nuclear; M13mp18 RF DNA from Bethesda Research Laboratories; endoproteinase Glu-C (protease *Staphylococcus aureus* V8) from Worthington Biochemicals; and T4 polynucleotide kinase and M13mp18-specific oligonucleotide primers 1201, 1211, and 1212 from New England Biolabs. The following oligonucleotides were synthesized by National Biosciences: primer T3/1201, 5′ CAA TTA ACC CTC ACT AAA GGG AAC AGC TAT GAC CA; primer SP6/1201, 5′ GAT TTA GGT GAC ACT ATA GAA CAG CTA TGA CCA; and primer 1201-C, 5′ TGG TCA TAG CTG TTC.

Synthesis of control Ade or ClAde-substituted DNA templates. Double-stranded DNA molecules that corresponded to a segment of phage M13mp18 DNA sequence were generated enzymatically by a series of exponential PCR techniques. Resultant linear DNA duplexes were subsequently used as substrates for asymmetric PCR to synthesize single-stranded (+) or (−)DNA (control or ClAde-substituted) with an intact T3 or SP6 promoter. All oligonucleotide primers used in PCR (shown in Fig. 1) were gel-purified. Exponential PCR mixtures consisted of the following in a final volume of 50 μl: 2.5 ng M13mp18 RF DNA (final concentration 0.01 nM); 200 μM concentrations of dATP, dCTP, dGTP, and dTTP; 1× PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin); and 2.5 units native *Taq* DNA polymerase. To produce a double-stranded 139-bp oligomer that contained a T3 RNA polymerase promoter, exponential PCR was conducted using M13mp18 RF DNA as template plus 10 pmol each of primers 1212 and T3/1201. A 98-bp oligomer was likewise synthesized with M13mp18 RF DNA and 10 pmol each of primers 1201 and 1211. SP6 promoter-containing double-stranded 137-mers were synthesized in separate reactions using primers 1212 and SP6/1201. *Taq* DNA polymerase was added, reaction mixtures were overlaid with mineral oil, and DNA was amplified during 30 cycles consisting of denaturation at 94° for 1 min, reannealing at 45° for 2 min, and synthesis at 72° for 3 min. After PCR, double-stranded 98-, 137-, and 139-mers were extracted once with chloroform and once with phenol/chloroform and subsequently purified through a Qiagen column followed by centrifugation through a Centricon 100 unit. These oligomers were tested further for the absence of residual primers or dNTPs as described (23) before use in asymmetric reactions.

Asymmetric PCR mixtures were conducted in 50 μl of 1× PCR buffer; 200 μM concentrations of dTTP, dGTP, and dCTP; and either 200 μM dATP or 350 μM CldATP as the fourth nucleotide. Oligonucleotide primers used in asymmetric PCR were 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase by standard procedures. To synthesize (+) strand 119-mers (control or ClAde-substituted) that contained a T3 RNA promoter, double-stranded template 98-mers were incubated with 20 pmol unlabeled primer T3/1201 as the sole primer and ≅1 pmol 5′-^{32}P-labeled T3/1201 to aid in visualizing full-length products on polyacrylamide gels. Complementary (−) strand 119-mers with the T3 promoter were produced using only primer 1211 (20 pmol unlabeled and 1 pmol ^{32}P-labeled) and double-stranded 139-mers in an asymmetric PCR. Likewise, SP6 promoter-containing 117-mers [(+) strand] were synthesized by asymmetric

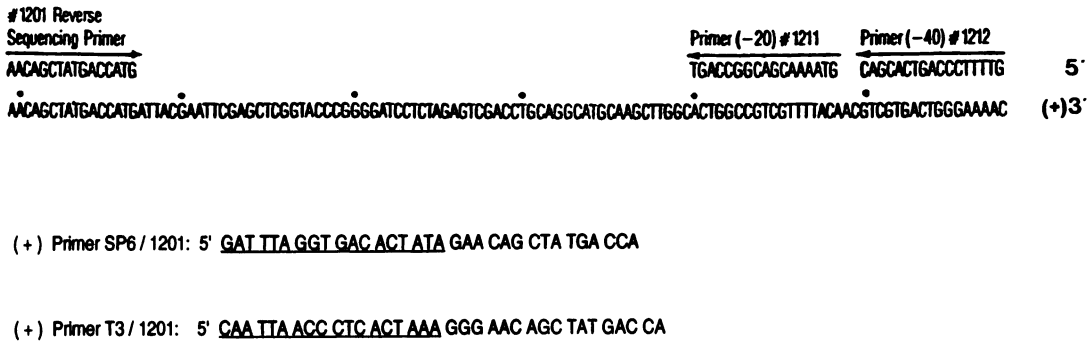

Fig. 1. Oligonucleotide primers and DNA template (+ strand only) used for synthesis of control and ClAde-substituted strands. *Underlined regions* indicate SP6 or T3 RNA polymerase promoter sequences. Double-stranded 137-mers were generated from PCR primers 1212 and SP6/1201, which contains the SP6 promoter. Double-stranded 98-mers were produced from primers 1201 and 1211. Control Ade and ClAde-substituted single-stranded (−) 117-mers were then generated with primer 1211 by asymmetric PCR. Single-stranded (+) 117-mers containing the SP6 promoter were subsequently synthesized with primer SP6/1201 by asymmetric PCR. The (+) and (−) strands were annealed and used for *in vitro* transcription reactions.

PCR with primer SP6/1201 and double-stranded 98-mers; (−) strand synthesis used primer 1211 and double-stranded 137-mers. For all reactions involving CldATP, T4 Gene 32 protein was included at 0.9 μg/reaction to enhance the yield of ClAde-substituted oligomers. Fifty asymmetric PCR cycles identical to the exponential PCR program were carried out; an additional 2.5 units of *Taq* DNA polymerase were added after 25 cycles. Resultant single-stranded 119- or 117-mers were extracted with chloroform and phenol/chloroform, heat denatured, and electrophoresed through a denaturing 8% polyacrylamide/7 M urea gel. After a 60-min exposure to XAR-5 film, full-length oligomers were excised from the gel, eluted overnight, and centrifuged through a Spin-X column. DNA was then ethanol-precipitated, dried, and resuspended in DEPC-treated water at equivalent cpm/μl.

***In vitro* transcription.** Equivalent amounts of (+) and (−) strands (either 119- or 117-mers) were heated to 90° for 4 min and annealed at room temperature for 1 hr. RNA transcription reactions consisted of the following in 15 μl final volume: 1× RNA transcription buffer (40 mM Tris·HCl, pH 8.0; 6 mM $MgCl_2$; 2 mM spermidine; 10 mM NaCl [SP6 polymerase only]; 500 μM concentrations each of ATP, UTP, and GTP; 200 μM CTP; 10 μCi [α-^{32}P]CTP (800 Ci/mmol); 20 units RNasin; 10 mM DTT; and either 40 units of T3 or 25 units of SP6 RNA polymerase in DEPC-treated water). Reaction mixtures were incubated at 37° for 1 hr; samples were then extracted once with phenol/chloroform (1:1), ethanol-precipitated, and resuspended in DEPC-treated H_2O and formamide stop solution (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF). Products were heat denatured at 90° for 4 min, quickly cooled on ice, and analyzed on denaturing 8% polyacrylamide/7 M urea gels. Autoradiograms were obtained by exposing XAR-5 film with or without an intensifying screen at −70°; they were then scanned and analyzed with a LKB laser densitometer. To examine the effects of ClAde in the promoter region on RNA polymerization, transcription reactions were conducted as above but without UTP; transcripts were analyzed by 12% or 15% PAGE.

Transcript sequencing. After electrophoresis and autoradiography, full-length 98- or 100-base transcripts were cut from the gel, eluted overnight in DEPC-treated water containing 0.3 M sodium acetate and 1.25 mM EDTA, centrifuged through a Spin-X column, extracted once in phenol/chloroform, and precipitated in ethanol. Transcripts were then dried, resuspended in DEPC-treated water, and digested at 37° for 30 min with 10 units RNase-free DNase I in 50 μl final volume that contained 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, and 0.05 mg/ml bovine serum albumin. Samples were phenol-extracted and precipitated in ethanol, and cDNA was generated with 25 units AMVRT. Reaction mixtures (20 μl) also contained 2.8 ng (0.5 pmol) of primer 1211, 500 μM concentrations each of four dNTPs, 1 unit/μl RNasin, 1 mM DTT, and 1× PCR buffer. Reactions were incubated at room temperature for 10 min, followed by 42° for 60 min. AMVRT was denatured by heating at 99° for 5 min, and 2 μl of the cDNA was removed, diluted in 1 ml of water, and centrifuged through a Centricon-100 to remove excess dNTPs and primer. The remaining volume (50 μl) was dried, resuspended in 9 μl of water and then sequenced by an *fmol* sequencing reaction using ^{32}P-labeled primer 1201 and 2.5 units *Taq* DNA polymerase. Sequencing reactions were conducted in 50 mM Tris·HCl, pH 9.0, and 2 mM $MgCl_2$ for one cycle at 95° for 4 min, followed by 30 cycles at 95° for 30 sec, 42° for 30 sec, and 70° for 1 min. Stop solution was added, and samples were heat-denatured before loading on an 8% denaturing polyacrylamide gel.

RNA polymerase/GSBA. Double-stranded control and ClAde-substituted SP6 promoter-containing oligomers (33-mers) were enzymatically synthesized essentially as described by Hentosh *et al.* (24). Approximately 23 pmol each of ^{32}P-labeled primers SP6/1201 and 1201-C were annealed together and incubated at 37° for 4 hr in a buffer containing 16 mM Tris·HCl, pH 7.5, 8 mM $MgCl_2$, 40 mM NaCl, 3.2 mM DTT, 0.5 mg/ml bovine serum albumin, and 13 units Sequenase II in the presence of four normal dNTPs (200 μM concentrations for each) or CldATP in place of dATP as the fourth nucleoside triphosphate. This synthetic reaction resulted in a double-stranded 33-bp oligonucleotide that contained 18 bases of the SP6 promoter and an additional 15 template bases downstream. Full-length control or ClAde-containing DNA substrates were purified by nondenaturing 12% PAGE, autoradiography, and subsequent elution out of a gel slice. Gel shift binding reactions with the above generated promoter-containing oligonucleotides were conducted in 1× transcription buffer. Varying amounts of SP6 RNA polymerase (20–100 ng) were preincubated with or without unlabeled competitor oligomers that had an intact promoter (0.5–3.5 pmol) at room temperature for 3 min. ^{32}P-Labeled Ade or ClAde-substituted double-stranded SP6 promoter oligomers (≅0.15 pmol/μl) were then added and incubated at room temperature for 5 min in a final volume of 5 μl. Gel loading buffer (0.5 μl) was added to a final concentration of 50 mM Tris·HCl, pH 7.5, 0.02% bromphenol blue, 0.02% xylene cyanol, and 4% glycerol. The entire volume was electrophoresed at 350 V on a nondenaturing 6% polyacrylamide gel in Tris-glycine buffer (40 mM Tris·HCl, pH 8.5, 260 mM glycine). Gels were subjected to autoradiography, and the relative amount of bound polymerase/DNA complex was quantified by densitometric scanning and subsequent integration of peak areas.

Protease clipping band shift assay. Endoproteinase Glu-C was resuspended in water to 2 mg/ml and stored as aliquots at −70° until needed. Proteolytic clipping was carried out essentially as described by Schreiber *et al.* (25) with the following modifications. RNA polymerase gel shift binding reactions were conducted as above with 50 ng of SP6 RNA polymerase and ≅0.15 pmol ^{32}P-labeled Ade or ClAde-substituted double-stranded SP6 promoter oligomers. DNA/protein complexes were then incubated with varying amounts of endoproteinase Glu-C for 7 min at room temperature before electrophoresis through a 6% nondenaturing gel and subsequent autoradiography.

Results

***In vitro* transcription reactions.** Control Ade and ClAde-substituted, complementary DNA strands designated (+) and (−) were generated by exponential and asymmetric PCR using combinations of primers shown in Fig. 1. The (+) strand sequence of the resultant amplified DNA is also illustrated. Single-stranded DNA fragments were subsequently annealed together in various combinations to examine ClAde effects either in the template or nontemplate strands on *in vitro* transcription with phage SP6 RNA polymerase (Fig. 2, *top*). The (−) strand was always the template strand for RNA transcription. Conditions used in this series of experiments permitted multiple rounds of initiation and elongation by RNA polymerase. The top band in each lane (*small arrow*) indicates the position of 117-base-long template DNA. On control (A+/A−) DNA, SP6 RNA polymerase produced high levels of the expected 98-base-long transcripts (*bottom band, large arrow*). In contrast, when ClAde was substituted for Ade within either (+) or (−) strands, or both, transcript amounts were dramatically reduced. Autoradiograms from three such experiments were scanned by laser densitometry. Resultant transcript levels were corrected for small differences in template amounts and plotted relative to control (A+/A−) transcription, which was set at 100% in each experiment (Fig. 2, *bottom*). ClAde in only the (−) strand (A+/N−) or (+) strand (N+/A−) reduced transcription levels to 22 ± 14% and 24 ± 19%, respectively, of control levels. When ClAde was in both strands, full-length transcripts represented only 7 ± 5% of control. We observed similar trends for T3 RNA polymerase (data not shown).

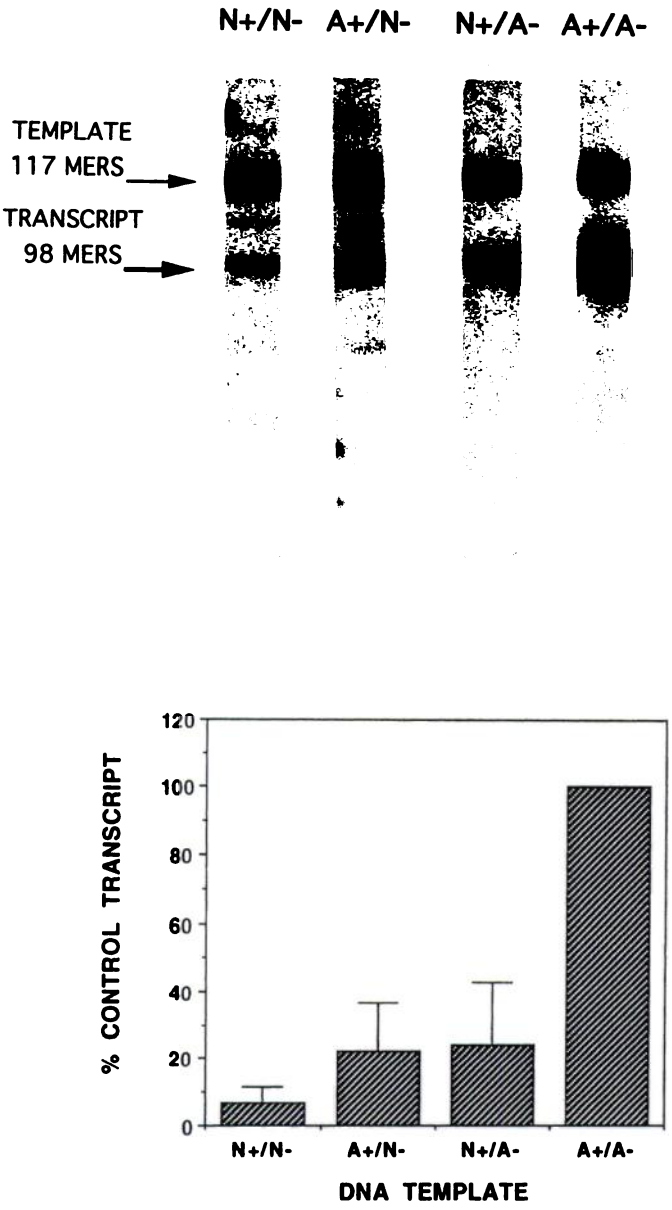

Fig. 2. SP6 RNA transcription of ClAde-substituted DNA. *Top*, The (−) and (+) strand Ade (A) or ClAde-containing (N) 117-mers were annealed and incubated with SP6 RNA polymerase in the presence of four normal NTPs and [α-^{32}P]CTP. Reaction conditions were such that multiple rounds of transcription occurred. Reaction products were electrophoresed on an 8% denaturing polyacrylamide gel and autoradiographed. *Top arrow* indicates DNA template 117-mers. *Bottom arrow* shows full-length 98-base transcripts. *Bottom*, Both template and transcript bands from three separate experiments were scanned by laser densitometry. Reactions were corrected for differences in template amounts, and the relative amount of transcription on each DNA template was quantified as percentage (mean ± standard deviation) of control transcription on A+/A− duplex. Control transcript levels were set at 100% in each experiment.

Reduced transcript levels seen with ClAde-containing DNA led us to hypothesize that RNA polymerase stalled on encountering ClAde residues and dissociated from template DNA. On dissociation, polymerase would be free to reinitiate additional rounds of transcription as reported for other DNA lesions. Therefore, we expected to see truncated transcripts on autoradiograms, corresponding to ClAde sites, but no bands unique to ClAde-substituted templates were detected in Fig. 2, on overexposed films, or after electrophoresis on high percentage gels. In addition, to rule out possible inefficient reannealing between ClAde-containing strands, we examined duplex formation for various combinations by nondenaturing gel electrophoresis. We did not detect any differences in the ability of ClAde-containing strands to anneal to control or other ClAde-substituted strands (data not shown).

To determine whether ClAde residues located within SP6 or T3 promoter regions were responsible in part for decreased transcription, we conducted RNA polymerase reactions in the absence of UTP. As illustrated in Fig. 3, two of the four T3 duplex combinations, A+/A− and N+/A−, have an intact promoter on both strands (*underlined regions*); there are no ClAde residues in the (+) N strand until +20; and no ClAde residues in the template (−) strand. In contrast, both A+/N− and N+/N− duplexes have ClAde residues in the (−) strand promoter and in the coding region at +10 and beyond but otherwise resemble the former duplexes. SP6 templates were similarly constructed (not shown) but lacked two cytosines at the +1 start site. In the absence of UTP, T3 and SP6 transcription on all four duplexes should stop at +9 and +7, respectively, before the first Ade or ClAde site in the (−) strand. We speculated that if ClAde had no effect on promoter function, transcript synthesis by RNA polymerase should be equal on all four duplexes. However, if ClAde in the promoter region interfered with or modified polymerase recognition and/or binding, transcription should be equivalent on both A+/A− and N+/A− combinations but reduced to the same degree on A+/N− and N+/N−. Fig. 4 shows a representative autoradiogram from one experiment with densitometric scanning results from two experiments. For both SP6 and T3 RNA polymerases, RNA synthesis on duplexes with an intact promoter was approximately equivalent (results are reported relative to the level of control A+/A− transcripts, which was set at 100%). When ClAde was located in promoter regions, however, production of nine base transcripts by T3 polymerase decreased to 30–45% of the intact promoter and SP6 transcripts to ≅60% of intact duplexes. Thus, there was an inhibitory effect of ClAde within promoter regions on polymerase function that was particularly strong for T3 RNA polymerase. However, as illustrated (Fig. 2) by the decreased amounts of full-length transcripts on N+/A− duplex DNA, which has an intact promoter, there was also a component contributed by the presence of this unusual nucleotide within the template or nontemplate strand.

A+/A-, N+/A- (Intact T3 Promoter)

```
                    +1→                ▼
(+) 5' CAA TTA ACC CTC ACT AAA GGG AAC AGC TAT GAC CA....
(-)    GTT AAT TGG GAG TGA TTT CCC TTG TCG ATA CTG GT....5'
```

A+/N-, N+/N- (ClAde T3 Promoter)

```
                    +1→                ▼
(+) 5' CAA TTA ACC CTC ACT AAA GGG AAC AGC TAT GAC CA....
(-)    GTT NNT TGG GNG TGN TTT CCC TTG TCG NTN CTG GT.... 5'
```

Fig. 3. Comparison of T3 promoter regions of various template combinations. *Underlined regions* represent T3 RNA polymerase promoter sequences; +1 is the start of transcription. *N* represents ClAde substitutions for Ade residues. *A* represents control Ade-containing strands.

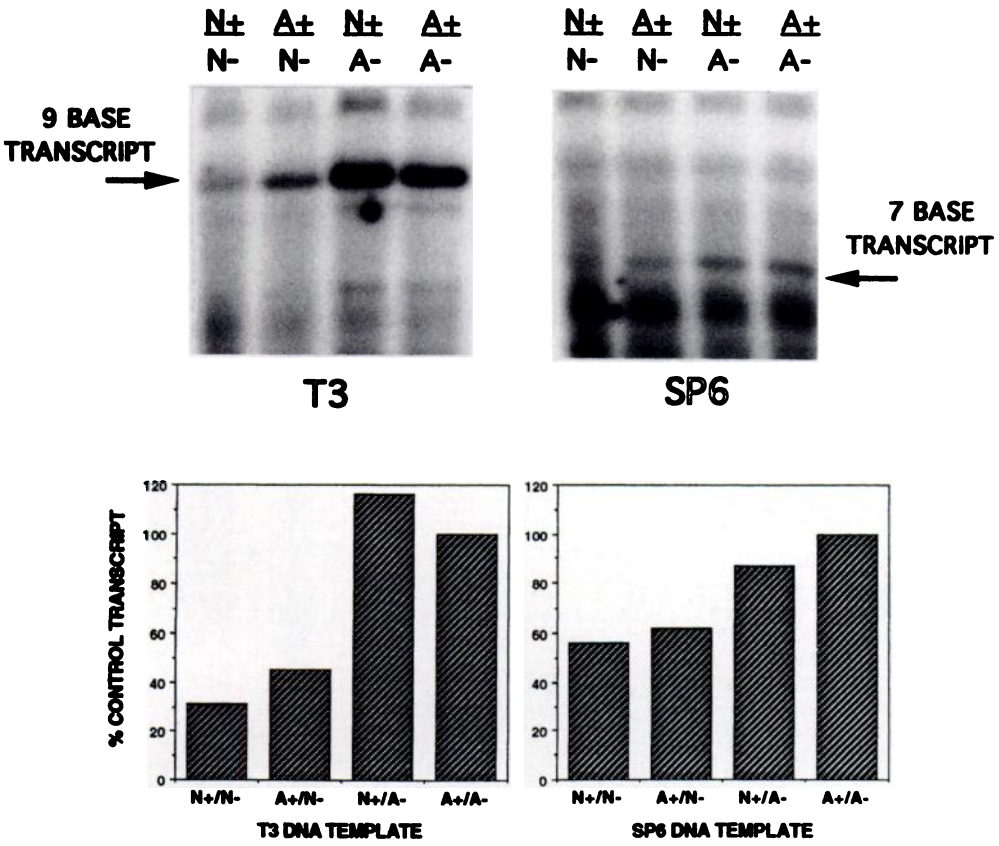

Fig. 4. RNA transcription in the absence of UTP. Top, The (−) and (+) strand control (*A*) or ClAde-containing (*N*) 119- or 117-mers were annealed and incubated at 37° for 1 hr with T3 (*left*) or SP6 (*right*) RNA polymerase in the presence of GTP, ATP, CTP, and [α-^{32}P]CTP. Reaction products were analyzed by 12% denaturing PAGE and autoradiography. *Arrows* indicate expected 9- and 7-base transcript products. *Bottom*, Both template (not shown) and transcript bands were scanned by laser densitometry. Reactions were corrected for differences in template amounts, and the relative level of transcription on each DNA duplex was quantified as percentage of control transcription on A+/A− duplex. The average value from two experiments is shown.

Promoter binding reactions. The above results suggested that RNA polymerase did not recognize and bind to ClAde-substituted promoters, was bound less tightly to the promoter, or was bound to the promoter in a conformation that did not lead to initiation of transcription. To distinguish among these possibilities, we conducted GSBAs with double-stranded ^{32}P-labeled DNA probes containing the SP6 promoter sequence that were synthesized as described in Experimental Procedures (Fig. 5). DNA probes also contained 15 additional M13 sequence-specific bases downstream of the promoter sequence to ensure that DNA termini were not frayed and that the substrate was a suitable length for polymerase binding. The SP6 promoter sequence has six Ade sites within the (−) strand. In the absence of RNA polymerase, ^{32}P-labeled promoter-containing oligomers migrated near the bottom of the gel as free probe (Fig. 5, *lanes 1 and 6, bottom arrow*). Incubation of DNA with SP6 RNA polymerase produced a polymerase/DNA complex on both control and ClAde-substituted DNA (*lanes 2 and 7, top arrow*). However, SP6 RNA polymerase binding to ClAde-containing promoter sequences was decreased by 80.9 ± 9.6% (mean ± standard deviation) compared with control binding, as determined by densitometric scanning of autoradiograms from five experiments. Competitive binding experiments in which we included nonradiolabeled control (Ade-containing) SP6 promoter oligomers before the addition of ^{32}P-labeled probes (*lanes 3–5 and 8–10*) illustrated that the protein bound to ClAde-containing oligomers was specific for promoter sequences and not a contaminating protein specific for the analogue itself. Specificity was also demonstrated by incubating SP6 oligomers with T3 RNA polymerase. T3 RNA polymerase, which should not recognize SP6 promoter sequences, did not cause a protein/DNA complex shift (not shown).

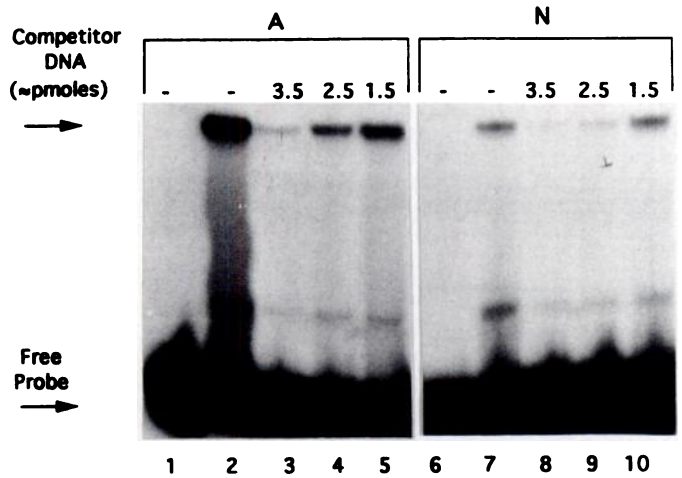

Fig. 5. Gel shift analysis of SP6 RNA polymerase binding to promoter-containing oligomers. Approximately 0.15 pmol of ^{32}P-labeled double-stranded control Ade (*A*) or ClAde-substituted (*N*) SP6 promoter-containing oligomers were incubated in the absence (*lanes 1 and 6*) or presence (*lanes 2–5 and 7–10*) of 25 units (50 ng) SP6 RNA polymerase for 5 min at room temperature in 1× transcription buffer before analysis by nondenaturing 6% PAGE. *Top arrow* indicates position of bound polymerase/DNA complex. To illustrate binding reaction specificity, increasing concentrations of nonradiolabeled control SP6-containing oligomers (*top of autoradiogram*) were added as competitor DNA for 3 min before the addition of radiolabeled probes (*lanes 3–5 and 8–10*).

Although RNA polymerase binding to ClAde-substituted sequences was greatly reduced, enzyme/DNA complexes

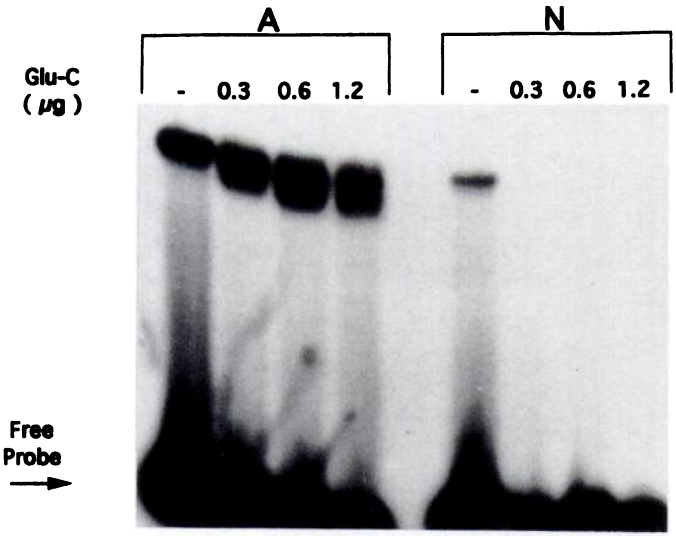

Fig. 6. Proteolytic clipping analysis of bound SP6 RNA polymerase to promoter sequences. Standard gel shift binding incubations were conducted as in Fig. 5. Control Ade (*A*) or ClAde-substituted (*N*) ^{32}P-labeled SP6-promoter-containing oligomers were incubated with 50 ng SP6 RNA polymerase. Protein/DNA complexes were then treated without or with increasing amounts of endoproteinase Glu-C for 7 min at room temperature before nondenaturing gel electrophoresis and autoradiography. Shown is a representative autoradiogram from three experiments in which a range of Glu-C concentrations was used.

were observed with the altered promoter. We therefore investigated a possible unusual or modified conformation of SP6 RNA polymerase on binding to ClAde-substituted promoters by the protease clipping band shift assay (25) (Fig. 6). Protein conformational changes are often reflected in increased proteolytic cleavage, indicative of altered protease accessibility to specific amino acids within a protein. In this series of experiments, SP6 RNA polymerase was incubated with control Ade and ClAde-containing promoter sequences as above in GSBAs. After limited treatment of bound polymerase/DNA complexes with endoproteinase Glu-C, which cleaves peptide bonds of either aspartic or glutamic acids, we saw that the RNA polymerase/ClAde-substituted promoter complex was completely cleaved at much lower Glu-C concentrations than required for control complexes. This indicated that RNA polymerase aspartic or glutamic acid sites involved in binding to or near ClAde residues were more accessible to proteolysis. The absence of smaller bound peptide fragments on the gel suggested that the amino acid cleavage sites in RNA polymerase were critical for DNA binding.

Identification of nucleotides inserted opposite ClAde residues. Although RNA synthesis was severely hampered on ClAde substrates, low levels of transcripts were produced on all three substituted duplexes, indicating that SP6 RNA polymerase bypassed ClAde residues. The base-pairing capabilities during transcription of DNA-containing ClAde residues were addressed by sequencing resultant RNA transcripts. To avoid possible introduction of errors by PCR amplification techniques, we eluted full-length transcripts from the gel and directly sequenced the RNA using AMVRT and dideoxynucleotides. These procedures were unsuccessful (data not shown) most likely due to limited transcript quantities. We subsequently synthesized a cDNA copy of DNase-digested transcripts by reverse transcription and sequenced

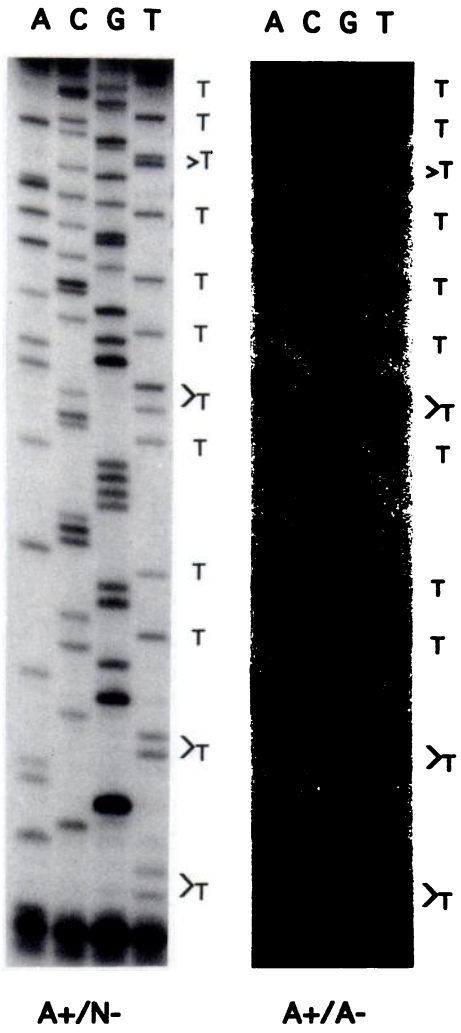

Fig. 7. Transcript sequence analysis by the *fmol* protocol. RNA transcripts were cut out of the gel and eluted overnight, treated with RNase-free DNase, reverse transcribed, and sequenced by standard *fmol* dideoxynucleotide (A, C, G, and T) methods. Results correspond to the + strand from cDNAs of full-length transcripts produced by SP6 RNA polymerase. DNA templates were composed of + and − control (*A*) or ClAde (*N*) strands (*bottom*). The − strand was always the template for *in vitro* transcription. If uracil were incorporated opposite a template ClAde residue, the correct nucleotide in the cDNA + strand will be a T, shown at the *right* of each autoradiogram.

resultant products by the *fmol* method (Fig. 7). The data shown represent the (+) strand cDNA sequence and correspond to the RNA sequence itself (see also Fig. 1 for entire sequence of the (+) strand). If RNA polymerase inserted uracil opposite template ClAde, the correct corresponding nucleotide in the (+) strand cDNA would be a T. Within the detection limits of our assay, a comparison of transcripts generated from control A+/A− and A+/N− duplexes revealed no misinsertion opposite (−) strand ClAde residues by SP6 RNA polymerase at any thymine site within the 98-base transcript. Similarly, no misincorporation was observed on N+/N− and N+/A− duplexes (not shown). Sequence information on the first 19 bases of RNA transcripts, the region of primer annealing that contains the first three potential ClAde substitutions, cannot be determined in the *fmol* procedure with primer 1201. However, to overcome this limitation, we sequenced the complementary (−) cDNA strand in

the opposite direction with primer 1211 and likewise detected no misincorporation in that region of 19 bases (data not shown).

Discussion

This is the first report describing template ClAde effects on RNA transcription. Our study demonstrated that *in vitro* RNA transcription by phage polymerases was severely reduced on ClAde-substituted DNA. Such effects must be considered because CldAdo and several other nucleoside analogues are not absolute chain terminators for replicative DNA polymerases. Results from the Huang *et al.* study (22) suggest that CldAdo has long term effects on cell growth that are expressed only several days after their initial inhibition of DNA synthesis. Moreover, CldAdo is also active against nondividing lymphocytes. It has been proposed that CldATP is incorporated into cellular DNA during repair processes (15) that are invoked in response to spontaneous damage. In light of our results, a possible explanation for both observations, the delayed cytotoxicity and the response in nondividing lymphocytes, is inhibition of cellular transcription and subsequent protein synthesis by CldAdo. Repair of most forms of DNA damage occurs preferentially in actively transcribed genes (26) and in the transcribed DNA strand (27, 28). In view of the similar K_m values for CldATP and dATP for several human polymerases (13), CldATP could be incorporated preferentially into active, essential genes during repair processes, subsequently inhibit gene transcription, and lead to cytotoxicity. In support of this, Seto *et al.* (15) reported decreased RNA synthesis in resting lymphocytes after CldAdo treatment.

Transcription reactions conducted without UTP revealed that reduced transcription is attributed, at least in part, to an effect of ClAde residues within the promoter. Our results extend the observations of Stahl and Chamberlin (29) that base analogues such as 2,6-diaminopurine or hypoxanthine, which have alterations within the DNA helix minor groove, led to reduced *in vitro* transcript levels. However, unlike their experiments, ClAde substitution in our study occurred within the coding strand. Although Stahl and Chamberlin demonstrated reduced transcription on DNA with a modified promoter, they did not assess mechanistically the cause. We speculated that ClAde within the promoter coding strand prevented recognition and/or binding of RNA polymerase to its cognate sequence or, alternatively, allowed polymerase to bind but in a conformation that prevented its movement or translocation along the template and would not lead to the initiation of transcription. It was also possible that a modified base that projected into the minor groove could prevent the putative protein conformational change that was proposed by Ikeda and Richardson (30) as necessary for RNA polymerase to switch from a binding to initiation mode. By GSBAs, we demonstrated that the observed low transcript levels on ClAde-containing DNA were due in part to limited polymerase binding to its promoter. In addition, we have shown that when RNA polymerase does bind to ClAde-substituted promoter sequences, it has an altered conformation that leads to greatly enhanced proteolytic clipping by endoproteinase Glu-C. We cannot rule out, however, the possibility that RNA polymerase binds to its promoter sequence but with reduced affinity, thereby leading to its dissociation from DNA during gel electrophoresis. Further experiments are necessary to reveal if and how an altered conformation may affect transcriptional processes.

Conditions used in the present study permitted multiple rounds of initiation and elongation by RNA polymerase. Our attempts to determine whether RNA polymerases stalled or dissociated at coding strand ClAde residues by including a heparin trap that limits transcription to a single round (31) have been unsuccessful due to the modest amounts of modified DNA template available for transcription. The only other study on the transcriptional effects of a nucleoside analogue has been with ara-C, another potent antileukemic drug, which has an intact base but an arabinose sugar moiety. The triphosphorylated form of ara-C exhibits similar properties to CldATP during *in vitro* DNA strand elongation (32), but considerably different effects on the transcriptional process have been noted. In contrast to ClAde residues, ara-C substitution in a promoter region had no apparent effect on RNA polymerase binding and the overall amount of *in vitro* transcription. However, ara-C located within the first 10 bases of the DNA coding strand led to transcription miss-starts and primer slippage and prevented RNA polymerase from shifting to a processive elongation mode (33).

Because of the asymmetric PCR technique we must use to generate ClAde-containing DNA strands, we cannot easily eliminate the analogue from template promoter sequences to address separately ClAde effects on transcript elongation. Two lines of evidence, however, suggest that there is also a component contributed by the presence of this unusual nucleoside within template and nontemplate strands that is unrelated to promoter effects. Reactions conducted with N+/A− duplex DNA, which has an intact promoter, illustrated that ClAde residues downstream from the promoter also inhibit RNA polymerization. However, when we terminated transcription before the first ClAde residue by eliminating UTP, transcript levels on N+/A− duplexes were approximately equivalent to those on control duplex. Decreased transcription with N+/A− duplex was also noteworthy because the ClAde substitutions occurred only in the nontemplate (+) strand. Such effects are in contrast to several other DNA modifications including pyrimidine dimers (21), 2-aminofluorene- and *N*-acetyl-2-aminofluorene-modified guanines (34), and cisplatin-modified bases (35), all of which do not inhibit transcription when located only in nontemplate strands. Our results suggest that minor groove contacts on ClAde-containing nontemplate strands are important during the processive elongation stage. Previous footprinting analyses (36) and recent crystallization of phage T7 RNA polymerase (37) have shown that during elongation, RNA polymerase wraps itself around duplex DNA and makes contact with both strands. Future studies will allow identification of specific interaction sites between RNA polymerase and DNA.

Transcript sequence analysis indicated that SP6 RNA polymerase prefers to insert uracil opposite template ClAde residues. To the best of our knowledge, this is the first time that the transcriptional coding capability of a nucleoside analogue has been determined. Based on other studies that have used DNA sequencing gels to detect misincorporation events (34), the sensitivity of such assays is in the range of ≅2.5–5%, that is, one misincorporated base in 20–40 correct bases can be observed. Thus, unlike abasic sites (31, 34) and

8-oxoguanine (34), ClAde residues may not be highly mutagenic lesions at a transcriptional level.

Acknowledgments

We thank Ortho Biotech for their generous gift of CldAdo. We also thank Dr. Dennis Peffley for helpful discussions and advice and Ms. Deborah Rubinstein for expert artwork.

References

1. Piro, L. D., C. J. Carrera, D. A. Carson, and E. Beutler. Lasting remissions in hairy cell leukemia induced by a single infusion of 2-chlorodeoxyadenosine. *N. Engl. J. Med.* **322:**1117–1121 (1990).
2. Tallman, M. S., D. Hakimian, D. Variakojis, D. Koslow, G. S. Sisney, A. W. Rademaker, E. Rose, and K. Kaul. A single cycle of 2-chlorodeoxyadenosine results in complete remission in the majority of patients with hairy cell leukemia. *Blood* **80:**2203–2209 (1992).
3. Estey, E. H., R. Kurzrock, H. M. Kantarjian, S. M. O'Brien, K. B. McCredie, M. Beran, C. Koller, M. J. Keating, C. Hirsh-Ginsberg, Y. Huh, S. Strass, and E. J. Freireich. Treatment of hairy cell leukemia with 2-chlorodeoxyadenosine (2-CdA). *Blood* **79:**882–887 (1992).
4. Santana, V. M., J. Mirro, F. C. Harwood, J. Cherrie, D. Kalwinsky, and R. L. Blakley. A phase I clinical trial of 2-chlorodeoxyadenosine in pediatric patients with acute leukemia. *J. Clin. Oncol.* **9:**416–422 (1991).
5. Santana, V. M., J. Mirro, Jr., C. Kearns, M. J. Schnell, W. Crom, and R. L. Blakley. 2-Chlorodeoxyadenosine produces a high rate of complete hematologic remission in relapsed acute myeloid leukemia. *J. Clin. Oncol.* **10:**364–370 (1992).
6. Piro, L. D., C. J. Carrera, E. Beutler, and D. A. Carson. 2-Chlorodeoxyadenosine: an effective new agent for the treatment of chronic lymphocytic leukemia. *Blood* **72:**1069–1073 (1988).
7. Kay, A. C., A. Saven, C. J. Carrera, D. A. Carson, D. Thurston, E. Beutler, and L. D. Piro. 2-Chlorodeoxyadenosine treatment of low-grade lymphomas. *J. Clin. Oncol.* **10:**371–377 (1992).
8. King, K. M., and C. E. Cass. Membrane transport of 2-chloro-2′-deoxyadenosine and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine is required for cytotoxicity. *Biochem. Cell. Biol.* **71:**Abstract XVII (1993).
9. Carson, D. A., D. B. Wasson, J. Kaye, B. Ullman, D. W. Martin, Jr, and R. K. Robins. Deoxycytidine kinase-mediated toxicity of deoxyadenosine analogs toward malignant human lymphoblasts *in vitro* and toward murine L1210 leukemia *in vivo*. *Proc. Natl. Acad. Sci. USA* **77:**6865–6869 (1980).
10. Blakley, R. L., M.-C. Huang, R. A. Ashmun, and R. Koob. Mechanism of cytotoxicity of 2-chloro and 2-bromodeoxyadenosine for a human lymphoblastic cell line, CCRF-CEM, in *Purine and Pyrimidine Metabolism in Man* (W. L. Nyhan, L. F. Thompson, and R. W. E. Watts, eds.). Plenum Publishing Corp., New York, Vol. V, 589–593 (1986).
11. Griffig, J., R. Koob, and R. L. Blakley. Mechanisms of inhibition of DNA synthesis by 2-chlorodeoxyadenosine in human lymphoblastic cells. *Cancer Res.* **49:**6923–6928 (1989).
12. Montgomery, J. A. Has the well gone dry? The First Cain Memorial Award Lecture. *Cancer Res.* **42:**3911–3917 (1982).
13. Parker, W. B., A. R. Bapat, J. Shen, A. J. Townsend, and Y. Cheng. Interaction of 2-halogenated dATP analogs (F, Cl, Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. *Mol. Pharmacol.* **34:**485–491 (1988).
14. Carson, D. A., D. B. Wasson, R. Taetle, and A. Yu. Specific toxicity of 2-chlorodeoxyadenosine toward resting and proliferating human lymphocytes. *Blood* **62:**737–743 (1983).
15. Seto, S., C. J. Carrera, M. Kubota, D. B. Wasson, and D. A. Carson. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. *J. Clin. Invest.* **75:**377–383 (1985).
16. Hentosh, P, R. Koob, and R. L. Blakley. Incorporation of 2-halogeno-2′-deoxyadenosine 5-triphosphates into DNA during replication by human polymerases α and β. *J. Biol. Chem.* **265:**4033–4040 (1990).
17. Hentosh, P., J. C. McCastlain, and R. L. Blakley. Effects of 2-chloro-2′-deoxyadenosine 5′-triphosphate on DNA synthesis *in vitro* by purified bacterial and viral DNA polymerases. *Biochemistry* **30:**547–554 (1991).
18. Pieper, R. O., B. W. Futscher, and L. C. Erickson. Transcription-terminating lesions induced by bifunctional alkylating agents *in vitro*. *Carcinogenesis* **10:**1307–1314 (1989).
19. Leffler, S., P. Pulkrabek, D. Grunberger, and I. B. Weinstein. Template activity of calf thymus DNA modified by a dihydrodiol epoxide derivative of benzo[a] pyrene. *Biochemistry* **16:**3133–3136 (1977).
20. Nath, S. T., M.-S. Lee, and L. J. Romano. Effect of carcinogenic adducts on transcription by T7 RNA polymerase. *Nucleic Acids Res.* **15:**4257–4271 (1987).
21. Selby, C. P., and A. Sancar. Transcription preferentially inhibits nucleotide excision repair of the template DNA strand *in vitro*. *J. Biol. Chem.* **265:**21330–21336 (1990).
22. Huang, M. C., R. A. Ashmun, T. L. Avery, M. Kuehl, and R. L. Blakley. Effects of cytotoxicity of 2-chloro-2′-deoxyadenosine and 2-bromo-2′-deoxyadenosine on cell growth, clonogenicity, DNA synthesis, and cell cycle kinetics. *Cancer Res.* **46:**2362–2368 (1986).
23. Hentosh, P., J. C. McCastlain, P. Grippo, and B. Y. Bugg. Polymerase chain reaction amplification of single-stranded DNA containing a base analog, 2-chloroadenine. *Anal. Biochem.* **201:**277–281 (1992).
24. Hentosh, P., M. Tibudan, and P. Grippo. A human factor that recognizes DNA substituted with 2-chloroadenine, an antileukemic purine analog. *Mol. Carcinog.* **13:**245–253 (1995).
25. Schreiber, E., P. Matthias, M. Muller, and W. Schaffner. Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping band shift assay (PCBA). *EMBO J.* **7:**4221–4229 (1988).
26. Mellon, I., V. A. Bohr, C. A. Smith, and P. C. Hanawalt. Preferential DNA repair of an active gene in human cells. *Proc. Natl. Acad. Sci. USA* **83:**8878–8882 (1986).
27. Mellon, I., G. Spivak, and P. C. Hanawalt. Selective removal of transcription blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell* **51:**241–249 (1987).
28. Venema, J., A. van Hoffen, V. Karcagi, A. T. Natarajan, A. A. van Zeeland, and L. H. F. Mullenders. Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes. *Mol. Cell. Biol.* **11:**4128–4134 (1991).
29. Stahl, S. J., and M. J. Chamberlin. Transcription of T7 DNA containing modified nucleotides by bacteriophage T7 specific RNA polymerase. *J. Biol. Chem.* **253:**4951–4959 (1978).
30. Ikeda, R. A., and C. C. Richardson. Interactions of the RNA polymerase of bacteriophage T7 with its promoter during binding and initiation of transcription. *Proc. Natl. Acad. Sci. USA* **83:**3614–3618 (1986).
31. Zhou, W., and P. W. Doetsch. Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA polymerases. *Proc. Natl. Acad. Sci. USA* **90:**6601–6605 (1993).
32. Mikita, T., and G. P. Beardsley. Functional consequences of the arabinosylcytosine structural lesion in DNA. *Biochemistry* **27:**4698–4705 (1988).
33. Mikita, T., and G. P. Beardsley. Effects of arabinosylcytosine-substituted DNA on DNA/RNA hybrid stability and transcription by T7 RNA polymerase. *Biochemistry* **33:**9195–9208 (1994).
34. Chen, Y.-H., and D. F. Bogenhagen. Effects of DNA lesions on transcription elongation by T7 RNA polymerase. *J. Biol. Chem.* **268:**5849–5855 (1993).
35. Corda, Y., C. Job, M.-F. Anin, M. Leng, and D. Job. Transcription by eukaryotic and prokaryotic RNA polymerase of DNA modified at a d(GG) or a d(AG) site by the antitumor drug cis-diamminedichloroplatinum (II). *Biochemistry* **30:**222–230 (1991).
36. Shi, Y., H. Gamper, and J. E. Hearst. Interaction of T7 RNA polymerase with DNA in an elongation complex arrested at a specific psoralen adduct site. *J. Biol. Chem.* **263:**527–534 (1988).
37. Sousa, R., Y. J. Chung, J. P. Rose, and B.-C. Wang. Crystal structure of bacteriophage T7 RNA polymerase at 3.3 A° resolution. *Nature (Lond.)* **364:**593–599 (1993).

Send reprint requests to: Dr. Patricia Hentosh, Department of Pharmacology and Molecular Biology, Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064.