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The Antileukemia Drug 2-Chloro-2′-deoxyadenosine: An Intrinsic Transcriptional Antagonist

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ABSTRACT

The nucleoside analog 2-chloro-2′-deoxyadenosine (CldAdo; cladribine) is effective in the treatment of hairy cell leukemia and chronic lymphocytic leukemia. CldAdo is phosphorylated and incorporated into cellular DNA but is not an absolute chain terminator. We demonstrated by in vitro gel-shift assays that binding interactions of the human TATA box-binding protein (TBP) were disrupted on 2-chlorodeoxyadenosine monophosphate (CldAMP)-substituted TATA box consensus sequences. We hypothesized that human RNA polymerase II (pol II) transcriptional processes would therefore be affected by 2-chlorodeoxyadenosine triphosphate (CldATP) incorporation into a promoter TATA element. Double-stranded DNA templates containing the adenovirus major late promoter and coding sequences were enzymatically synthesized as control or with site-specific CldAMP residues, incubated with HeLa extract, and the synthesis of radiolabeled 44-base transcripts was assessed. With increasing amounts of HeLa extract, CldAMP substitution for dAMP within the TATA box decreased in vitro pol II transcription by ~35% compared with control substrates. Time-course studies showed that transcript production increased in a linear fashion on control substrates. In contrast, transcription on CldAMP-substituted TATA sequences reached a plateau after 20 min. Furthermore, CldAMP-substituted promoter sequences trapped or sequestered TBP, preventing its dissociation from DNA and subsequent binding to additional TATA elements to reinitiate transcription. CldAdo thus represents the first example of a nucleoside analog that acts as a transcriptional antagonist. CldATP incorporation into gene regulatory sequences may provide a novel strategy to modulate specific protein/DNA interactions.

The nucleoside analog, 2-chloro-2′-deoxyadenosine (CldAdo; cladribine) represents a highly effective treatment for hairy cell leukemia, chronic lymphocytic leukemia, and several other hematopoietic malignancies (Beutler and Carson, 1993; Tondini et al., 2000; Krance et al., 2001; Robak, 2001) and is in clinical trials for use against rheumatoid arthritis and multiple sclerosis (Beutler et al., 1996; Schirmer et al., 1997; Filippi et al., 2000). CldAdo is distinct among therapeutically beneficial nucleoside analogs because of its cytotoxicity against both dividing and nondividing cells (Seto et al., 1985). The cytotoxic mechanisms of action of CldAdo are multifaceted. Because adenosine deaminase-resistant triphosphate derivatives (CldATP) accumulate intracellularly, ribonucleotide reductase is downregulated, contributing to decreased cellular DNA synthesis (Griffig et al., 1989). CldATP is also an effective substrate for human DNA polymerases and is incorporated into DNA in place of dATP; however, CldAMP is not an absolute chain terminator for DNA polymerase α (Hentosh et al., 1990; Chunduru et al., 1993; Yuh et al., 1998). We have reported that ~2.5 CldATP nucleotides are incorporated into DNA every 1000 bases when cultured leukemia cells were incubated with 300 nM CldAdo for 4 h (Yuh et al., 1998). In patients, plasma CldAdo levels can reach from 200 to 300 nM, and patients are exposed to the drug as a continuous infusion at these levels for 7 days (Liliemark and Juliusson, 1991). Therefore, it is likely that greater CldAMP substitution can occur in patient leukemic cells. When inserted into double-stranded (ds) DNA, the electronegative chlorine substitution for hydrogen at C2 of adenosine extends into the DNA minor groove. Gel-electrophoresis migration patterns and molecular modeling have predicted that the substitution alters normal DNA conformation (Hentosh and McCastlain, 1991, unpublished results). Thus, random incorporation of this nucleotide into DNA of dividing cells may allow insertion into sequences and elements essential for transcriptional regulation and could have delete-

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ABBREVIATIONS: CldAdo, 2-chloro-2′-deoxyadenosine; TF, transcription factor; TBP, TATA box-binding protein; pol II, polymerase II; CldAMP, 2-chlorodeoxyadenosine monophosphate; CldATP, 2-chlorodeoxyadenosine triphosphate; ds, double-stranded; AdML, adenovirus major late; OD, optical density; TBE, Tris/borate/EDTA; bp, base pair(s); fpu, footprinting units; BPDE, benzo[*a*]pyrene diol epoxide; cisplatin, *cis*-diamminedichloroplatinum.

rious effects on DNA binding proteins. In quiescent cells, CldATP could be incorporated during DNA repair processes that occur preferentially in transcribed regions of the genome. CldAdo-treated cells exhibit diminished RNA synthesis (Seto et al., 1985; Carrera et al., 1989, 1990), but the mechanism by which this occurs has not been elucidated.

We reported previously (W. R. Hartman, D. E. Walters, P. Hentosh, submitted) the first evidence that template CldAMP residues interfere with recognition and binding, stability, and conformation of the human TATA box-binding protein (TBP)—a subunit of eukaryotic transcription factor (TF) IID (TFIID)—which binds DNA primarily through minor groove interactions. With the use of gel-shift binding assays, we observed that TBP bound to CldAMP-substituted TATA element less frequently, but once bound, it assumed an altered conformation that increased the stability of the protein/DNA complex compared with control. The altered conformation also prevented TBP from being an efficient nucleus for recruitment and binding of other transcription factors, specifically TFIIB (Hartman et al., submitted). TBP binding to the TATA sequence is the rate-limiting step for the formation of RNA polymerase II (pol II) initiation complex and pol II transcription (Buratowski, 1997). TBP (TFIID), TFIIB, and other factors (TFIIE, TFIIH, TFIIA) form a complex on gene promoters that directs pol II to the correct site for transcription initiation (Nikolov and Burley, 1997).

From our previous experiments, we hypothesized that human RNA pol II transcriptional processes would be modified by CldATP incorporation into DNA promoter regions. We examined this phenomenon in a series of in vitro transcription assays using ds DNA templates containing the adenovirus major late (AdML) promoter with either a control or CldAMP-substituted TATA element and 44 bases of a coding sequence. The AdML promoter was used to crystallize and characterize binding interactions of human TBP and other human transcription factors that participate in the initiation complex (Nikolov et al., 1995, 1996; Weideman et al., 1997). Furthermore, this sequence has been extensively used to characterize initiation and promoter escape by human RNA pol II (Hawley and Roeder, 1987; Kugel and Goodrich, 1998).

Materials and Methods

Oligonucleotides and Reagents. Four single-stranded synthetic oligonucleotides were used in these studies: 1) 5′-GGGGGC-TATAAAAGGGGG-3′; 2) 5′-CCCCCTTTTAT-3′; oligo 3 was an 85-base oligonucleotide described by Pan and Greenblatt (1994) that contained a sequence from −41 to +44 of the AdML promoter region; oligo 4 was a 71-base nucleotide complementary to the 3′ end of oligo 3. Oligonucleotides were synthesized and gel-purified by Integrated DNA Technologies (Coralville, IA). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]GTP (3000 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Molecular-biology grade NTPs were from Roche Applied Science (Indianapolis, IN); CldATP was synthesized from CldAdo by Sierra Bioresearch (Tucson, AZ), and T7 Sequenase version II was from Amersham Biosciences Inc. (Piscataway, NJ). Cladribine (Leustatin) was supplied by Ortho Biotech Products L. P. (Bridgewater, NJ). HeLa nuclear extract (transcription grade) prepared by a modified protocol of Dignam et al. (1983) and purified TBP were purchased from Promega (Madison, WI).

Preparation of Radiolabeled Probes and Synthesis of Double-Stranded DNA. Oligomers 1 and 2 were used to synthesize ds control or CldAMP-substituted 18-mers that contained the AdML promoter TATA element (underlined in the sequence above, see Table 1 [ds oligomers C and D]). Oligos 3 and 4 were used to produce ds 85-mers containing both the AdML promoter and downstream coding region (Table 1; ds oligomers A and B). Single-stranded primer oligonucleotides (oligos 2 and 4) were 5′-^{32}P-end-labeled with T4 kinase and separated from unincorporated nucleotide using QuickSpin G-25 Sephadex columns (Roche Applied Science) as described previously (Hartman et al., submitted). This procedure—prelabeling primer oligos before the synthetic step—prevented any possible differential in the efficiency of the kinase reaction. Labeled oligomers were annealed to unlabeled complementary oligonucleotides as described previously (Hartman et al., submitted). To generate control and CldAMP-substituted ds substrates, annealed oligomers were incubated at 37°C for 2 h in buffer (16 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 40 mM NaCl, 3.2 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin) with 13 units of T7 Sequenase II; 200 μM dCTP, dGTP, and dTTP; and either 200 μM dATP for control DNA or 200 μM CldATP to generate substituted DNA. Synthesis of full-length 18- or 85-mers was assessed by analyzing a small amount of the above reaction mixtures on denaturing 15% acrylamide/bisacrylamide (38:2), 7 M urea gels in 1× Tris/borate/EDTA (TBE) with DNA size markers. Full-length ds DNA was then purified by nondenaturing polyacrylamide (15% in 1× TBE) gel electrophoresis on 30-cm plates at 600 V and eluted overnight from gel slices. The DNA was ethanol-precipitated and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 (Tris-EDTA), at equivalent cpm/μl. In ds competitor 18-mers, the CldAMP substitution was opposite the first thymine in the TATA box (i.e., in the complementary strand; see Table 1). In ds 85-mers, the CldAMP substitution was opposite both of the thymines in the TATA element (Table 1).

Run-Off Transcription Assay Using Transcription-Grade HeLa Nuclear Extract. To determine the effects of CldAMP-substitutions within the TATA element on in vitro RNA pol II transcriptional processes, run-off transcription assays using control and CldAMP-substituted AdML promoter 85-mers were performed. Accurate initiation followed by elongation of AdML promoter-containing templates should produce a run-off transcript of 44 bases. Approximately 100 ng [(1.86 pmol (equivalent cpm)] of ds CldAMP-substituted or control template DNA were preincubated with

TABLE 1
Double-stranded AdML oligonucleotides
The top sequence is the nontranscribed strand, and the bottom sequence is the transcribed strand.

Oligonucleotide	Sequence
A	5′GAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCACTCTCTTCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGCTGC
	CTTCCCCCCCGNTNTTTTCCCCCACCCCCGCGCAAGCAGGAGTGAGAGAAGGCGTAGCGACAGACGCTCCCGGTCGACAACCGACG
	-30 -20 -10 +1 +10 +20 +30 +40
B	5′GAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGTTCGTCCTCACTCTCTTCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGCTGC
	CTTCCCCCCCGATATTTTCCCCCACCCCCGCGCAAGCAGGAGTGAGAGAAGGCGTAGCGACAGACGCTCCCGGTCGACAACCGACG
C	5′GGGGGCTATAAAAGGGGG
	CCCCCGNTATTTTCCCCC
D	5′GGGGGCTATAAAAGGGGG
	CCCCCGATATTTTCCCCC

varying concentrations (8–32 units) of transcription-grade HeLa nuclear extract for 20 min at 30°C to allow the protein complex to assemble. Transcription was initiated by adding 10 μCi [α-^{32}P]GTP (final concentration, 0.13 μM) and unlabeled rNTPs in a volume of 25 μl (final concentrations were 0.4 mM each of ATP, UTP, and CTP, and 0.016 mM GTP). Final concentrations of buffer reagents were 8.8 mM Hepes, pH 7.9, at 25°C; 44 mM KCl; 0.088 mM EDTA; 0.22 mM dithiothreitol; 8.8% glycerol, 1.2 units/μl RNasin; and 6.5 mM $MgCl_2$. Reactions were incubated at 30°C for 10 to 60 min and terminated by adding 175 μl of stop solution (0.3 M Tris-HCl, pH 7.4, at 25°C, 0.3 M NaOAc, 0.5% SDS, 2 mM EDTA, 3 μg/ml tRNA). RNA was extracted with phenol/chloroform (50:50), ethanol-precipitated, dried in the speed-vacuum concentrator, and reconstituted with 10 μl of nuclease-free water and an equal volume of formamide loading solution (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue). Samples were denatured at 90°C for 10 min and electrophoresed on a denaturing 8% polyacrylamide/bisacrylamide (38:2) urea (7 M) gel (30 cm) in 1× TBE, pH 8.4, at 1000 V. Transcripts were visualized via overnight autoradiography. Quantitative analysis of transcript production was done by scanning 44-mer bands with a molecular imager (Bio-Rad, Hercules, CA) to produce mean optical density (OD) values.

In Vitro Transcription Time Course with Supplementation of Transcription Factors. The above protocol was modified to test whether transcript synthesis could be reconstituted on ClAMP-substituted and control templates after supplementation of transcription factors. A master reaction mix was made and preincubated as described above. Subsequently, [α-^{32}P]GTP and unlabeled rNTPs were added to initiate transcription. After 20 min, aliquots were removed and added to fresh tubes containing either 4 units of HeLa extract or 2 footprinting units (fpu) of purified TBP. One tube with no additional factors served as control. Reactions were then incubated at 30°C for various times and analyzed as described above.

In Vitro Transcription/Competition Assay. The transcription assay was also used to assess transcript production on ds control (dAMP-containing) 85-mers in the presence of competitor control or ClAMP-substituted ds AdML promoter 18-mers; these 18-mers cannot be transcribed. The ClAMP substitution was opposite only one thymine in the TATA element (in the bottom strand). Preincubation of ~1.86 pmol ds control DNA 85-mer only was conducted as described above to allow assembly of the initiation complex. Subsequently, 3 pmol of ^{32}P-labeled control or ClAMP-substituted 18-mers was added along with rNTPs as above; reactions were incubated at 30°C and then terminated at various times. In some experiments, the effects of transcription factor supplementation were examined on DNA transcription from control 85-mer templates in the presence of competitor DNA. After a 30-min reaction incubation, either 2 units of purified TBP or 4 units of HeLa extract was added to separate reaction tubes containing control ds 85-mer templates and either control or ClAMP-substituted competitor ds 18-mer. Transcription assay buffer was supplemented to maintain the appropriate concentrations, and samples were incubated again at 30°C. Reactions were terminated after an additional 20 min; samples were then analyzed as described above.

Statistics. Experiments were performed in duplicate or triplicate. Data are expressed as an average of two experiments or as a mean ± S.D..

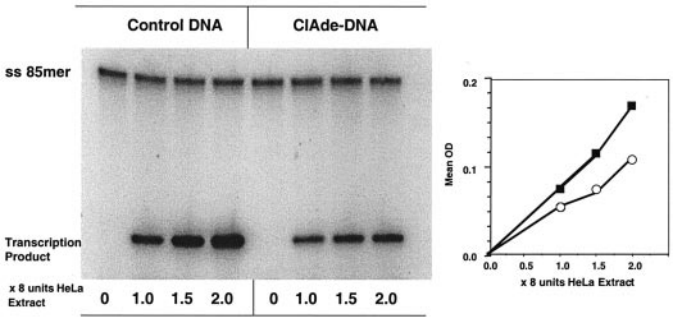

Fig. 1. In vitro transcription with increasing HeLa extract concentrations. In vitro pol II transcript synthesis on control or ClAMP-substituted AdML promoter ds 85-mers (one strand was labeled to control for equal template amounts) was compared (autoradiograph, left). Approximately 1.8 pmol (equivalent amounts of radioactivity) duplex DNA was incubated with increasing concentrations of HeLa extract for 30 min, and reaction products were electrophoresed. Transcription product (44-mers) bands were scanned, and OD values were determined and plotted versus extract concentration (line graph, right; ■, control; ○, ClAMP-substituted DNA).

Results

ClAMP Residues in a TATA Element Decrease in Vitro Transcript Synthesis by RNA Pol II. We first investigated transcript production by RNA pol II from templates containing a ClAMP-substituted TATA sequence (ds 85-mers) using an in vitro transcription assay. The 85 base pair DNA fragment contained 41 bases of the AdML promoter (including the TATA box) and 44 bases of downstream coding region DNA. Under the assay conditions established, multiple rounds of initiation and elongation were possible. To assess transcription from ClAMP-substituted or control AdML 85-mer templates, we first conducted runoff transcription assays with increasing concentrations of HeLa extract for one time period. Equivalent DNA amounts of either substrate were ensured by using an equal quantity of radioactivity in reaction mixtures and loading. Both DNA substrates had been 5′-end labeled on the transcribed "primer" strand only before annealing and synthesis of ds templates.

The resulting autoradiograph (Fig. 1) shows two bands. The top band represents single-stranded 85-mers; the bottom band is a single-stranded 44-mer that represents full-length synthesized transcripts. On earlier gels, a labeled single-stranded 25-mer was included as a DNA size marker (data not shown). At each HeLa extract concentration, 44-mer transcripts were produced in reactions containing either control or ClAMP-substituted 85-mers. No shorter transcripts were observed with either template. As the extract concentration increased, transcript production increased likewise on both templates. This result also indicated that ds template DNA was in excess over pol II transcription factors. In contrast to control reactions, reduced transcript synthesis occurred in each reaction containing a ClAMP-substituted TATA box compared with control. The 44-mer transcript bands were scanned and plotted versus HeLa extract concentration (Fig. 1, line graph). Control OD readings were set to 100% for each extract concentration, and the amount of transcripts produced on ClAMP-substituted 85-mers was calculated relative to the control. This experiment was performed four times; mean and S.D. values of transcript synthesis on ClAMP-substituted templates at each HeLa extract concentration were determined and plotted (Fig. 2).

Transcript synthesis on both ClAMP and control templates occurred in a linear fashion dependent on HeLa extract concentration. However, RNA synthesis was reduced by 35% on ClAMP-substituted TATA element templates at each HeLa extract concentration compared with control. We concluded, therefore, that transcript synthesis from ClAMP-substituted promoter templates could occur and that the amount of transcripts produced from ClAMP-con-

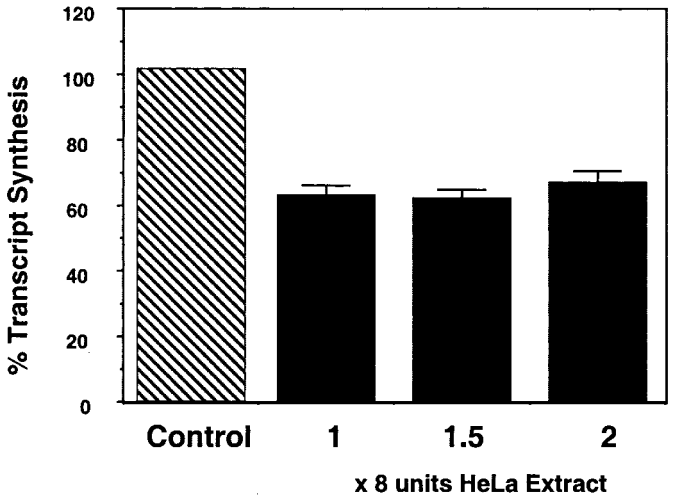

Fig. 2. Quantitative analysis of in vitro RNA pol II transcript synthesis with increasing HeLa extract concentrations. The in vitro transcription assay shown in Fig. 1 was performed four times. Transcript band intensities were scanned, and control synthesis at each extract concentration was set to 100%. OD values for 44-mer bands on CldAMP-containing templates were then calculated as a percentage of control values in each experiment and plotted as a mean and S.D. ▧, control templates; ■, CldAMP-substituted templates.

taining templates was reduced compared with control synthesis, but as the HeLa extract concentration was increased, so did the amount of transcript synthesis.

Time Course of RNA Synthesis. We next assessed the time course of pol II transcription from CldAMP-substituted templates using a single concentration of HeLa extract. HeLa extract (8 units) was incubated with ds control or CldAMP-substituted 85-mers for various periods before termination (Fig. 3). At each time point, 44-mer transcripts were produced in reactions containing either control or CldAMP-substituted TATA boxes. However, less transcript synthesis occurred in reactions containing a CldAMP-substituted TATA 85-mer. Furthermore, whereas the intensity of 44-mer bands increased with time in the control lanes, the band intensity in CldAMP-containing DNA lanes seemed to reach a plateau after 20 min. The 44-mer transcript bands from a lesser-exposed version of the autoradiograph were scanned to generate mean OD values, which were plotted versus time. The control plot was fit with a "best fit" line with a correlation coefficient of 0.984, indicating that transcript synthesis on control templates occurred in a linear fashion over time. A linear line could not be fit to OD values representing RNA synthesis on CldAMP-substituted templates. Transcript production on CldAMP-substituted AdML templates did not increase after 20 min. From three experiments, transcript bands at 10, 20, and 30 min were scanned, and control synthesis was set to 100%. At each time point, a mean ± S.D. of transcript synthesis on CldAMP-substituted DNA was calculated as a percentage of the control and plotted (Fig. 4). Synthesis was decreased by 35% on CldAMP-substituted 85-mer templates compared with control 85-mers at 10 min. After a 30-min incubation, however, this difference increased to 60%. An identical trend was observed after longer (60-min) incubations also (data not shown). The percentage of transcript synthesis on CldAMP-substituted templates decreased with time because transcription plateaued on CldAMP templates, whereas it increased on control templates.

TBP Is Trapped on CldAMP-Containing TATA Elements and Inhibits Initiation Complex Formation. The above data and plateau effect suggested the possibility that CldAMP-substituted TATA sequences were sequestering or trapping transcription factor(s). As a result, the substitutions interfered with reinitiation of transcription on itself or other available DNA templates, abating transcript synthesis and producing the plateau in transcript synthesis that we observed. We thus predicted that RNA synthesis would resume on CldAMP-substituted templates if transcription factors were replenished during the plateau phase. We assessed this by performing an in vitro transcription assay similar to that described above, but after 20 min of incubation, one reaction was stopped, and to two other tubes, either nothing or 4 units of HeLa extract was added. Reactions were then incubated for an additional 20 min (Fig. 5, autoradiograph). Subsequent scanning of 44-mer transcript bands generated mean OD values that were plotted versus time (Fig. 5, bar graph). In

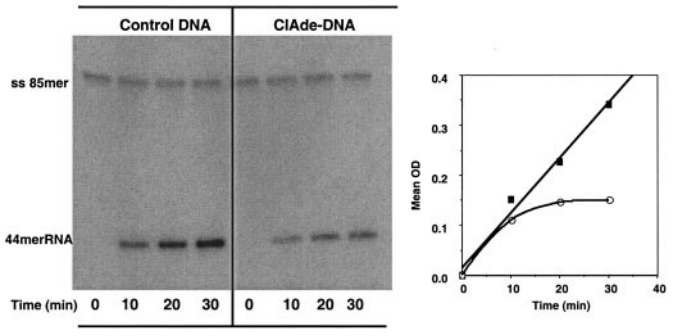

Fig. 3. In vitro transcription assay with constant amount of HeLa extract and varying reaction times. Control or CldAMP-substituted 85-mers (~1.8 pmol) were incubated with 8 units of HeLa extract for various times before termination and subsequent gel electrophoresis (autoradiograph). Resulting single-stranded 44-mer bands from this autoradiograph were scanned, and the extent to which transcripts were synthesized was expressed as a mean OD (line graph; ■, control TATA sequence; ○, CldAMP-substituted TATA box).

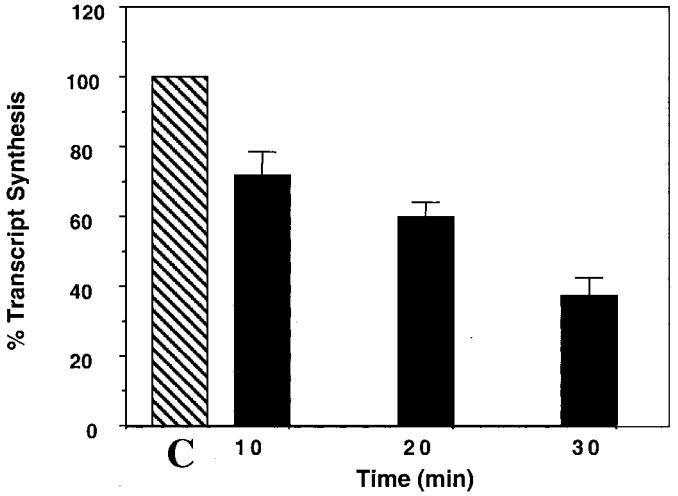

Fig. 4. Quantitative analysis of transcript synthesis from templates with constant amount of HeLa extract and increasing reaction times. The reactions represented in Fig. 3 were performed and quantified three times. Transcript band intensities were scanned, and control synthesis (C) was set to 100%. At each time point, an average and S.D. of transcript synthesis on CldAMP-substituted DNA were calculated as a percentage of control and plotted. ▧, control transcript synthesis; ■, transcript synthesis on CldAMP-containing TATA element.

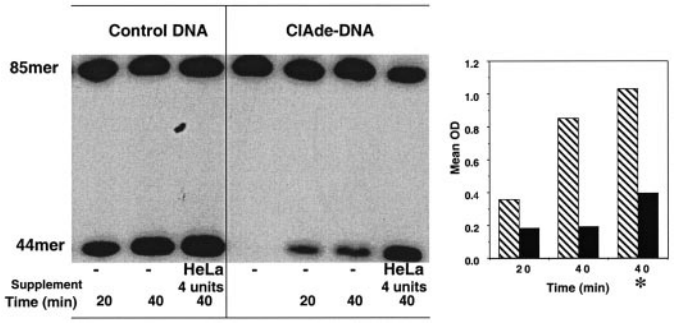

Fig. 5. In vitro transcription assay with HeLa extract supplementation. Transcript synthesis over a 40-min period on control or a ClAMP-substituted AdML promoter was assessed by an in vitro transcription assay. Control or ClAMP-substituted ds 85-mers were incubated with 8 units of HeLa extract for 20 min, at which time one reaction was terminated. A second reaction was incubated for an additional 20 min (no additional extract), and to a third reaction, 4 units of HeLa extract was added and incubated for 20 min. The 44-mer transcription product band intensities were scanned to generate mean OD values, which were plotted versus time (right). ▧, control; ■, ClAMP-DNA; *, reactions supplemented with HeLa extract.

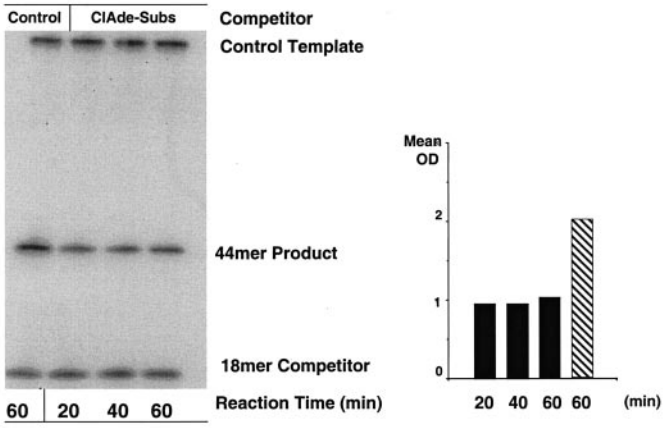

Fig. 6. Competitive in vitro transcription. Control AdML promoter ds 85-mers (1.8 pmol) were preincubated for 20 min with HeLa extract. Subsequently, 3 pmol ds control or ClAMP-substituted 18-mer competitor DNA, rNTPs, and ^{32}P-GTP were added. Transcription reactions were stopped at indicated times and electrophoresed (autoradiograph). The 44-mer bands were scanned, and the extent to which transcripts were synthesized was plotted versus time. ▧, control competitor 18-mer TATA sequence; ■, ClAMP-substituted competitor 18-mer TATA box.

the absence of supplemented HeLa extract, transcript production on control templates more than doubled between 20 and 40 min (2.36-fold increase). With extract supplementation, transcript synthesis on control templates increased an additional 1.2-fold between 20 and 40 min. When transcript synthesis was assessed on ClAMP-containing DNA templates, there was little or no increase in transcript synthesis between 20 and 40 min, which is identical with the plateau observed in Fig. 3. However, with HeLa extract addition, transcript synthesis increased by ~2.1-fold.

We concluded from these results (three experiments) that transcription on ClAMP-substituted templates, which reached a plateau after 20 min, could be reconstituted with supplements of HeLa extract. We hypothesized, therefore, that after initial complex assembly and one round of transcription on ClAMP-substituted 85-mers (with no factor replenishment), TBP is "trapped" on a substituted TATA element and cannot dissociate. More importantly, it is trapped in an altered conformation that does not efficiently recruit or bind transcription-initiation factors necessary to form another preinitiation complex. If this were the case, we predicted that transcript synthesis on control templates would decline if TBP were competed out by an excess of competitor DNA with a ClAMP-substituted TATA element. We assessed this using an in vitro transcription competition assay.

Approximately 1.8 pmol ds control AdML promoter 85-mers only were preincubated with HeLa extract to allow complex formation. Subsequently, 3 pmol ds control or ClAMP-substituted 18-mer competitor DNA, rNTPs, and [^{32}P]GTP were added to initiate transcription. Competitor DNA contained only 18 bp of AdML promoter consensus TATA sequence but had no coding region sequences. These DNA fragments were substituted with only one ClAMP residue within the TATA element (Table 1). Transcription reactions were stopped at various times and electrophoresed, and gels were exposed to film (Fig. 6). The autoradiograph showed three bands: the top and middle bands were single-stranded control 85-mer template DNA and 44-mer transcripts, respectively; the bottom band was control or ClAMP-substituted ds 18-mer competitor DNA (labeled on one strand to ensure equivalent amounts per reaction). The 44-mer transcript product bands were scanned, and the extent to which transcripts were synthesized was plotted (Fig. 6, bar graph). Quantitation after 60 min revealed that transcription on control templates in the presence of ds ClAMP-substituted 18-mers was 50% lower than it was in the presence of ds adenosine-containing 18-mers. Furthermore, transcript synthesis in the presence of ClAMP-substituted competitor did not significantly increase between 20 and 60 min. This experiment was performed three times with similar results (data not shown).

We concluded that preincubation of control ds 85-mers and HeLa extract allowed the formation of the initiation complex and subsequent transcription. As TBP dissociated from control templates, it became competitively bound to the excess control or ClAMP-substituted ds 18-mers. When TBP bound ClAMP-substituted competitor DNA, however, it could not dissociate to bind control 85-mer templates and initiate another round of transcription. We therefore predicted that supplementing com-

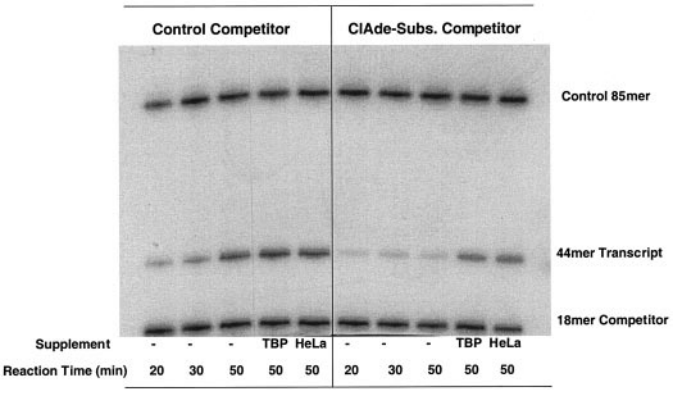

Fig. 7. Reconstitution of transcription in the presence of competitor DNA with purified TBP or HeLa extract. HeLa extract was preincubated with 1.8 pmol control ds 85-mers for 20 min before the addition of NTPs and control or ClAMP-substituted 18-mer competitor DNA. Reactions were stopped after 20 or 30 min. Separate samples were then not reconstituted or were reconstituted after 30 min with 2 fpu purified TBP or 4 units of HeLa extract. Reactions were then incubated for an additional 20 min before subsequent analysis.

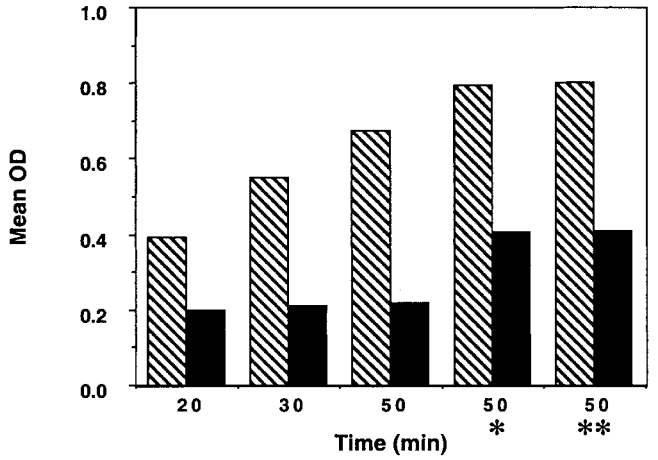

Fig. 8. Quantitative analysis of competitive in vitro transcription assay with supplements of TBP or HeLa extract. The 44-mer transcript bands in Fig. 7 were scanned to generate mean OD values and were plotted versus time. The bar graph shows a comparison of relative transcript synthesis between reactions with control or CldAMP-competitor DNA. ▨, control competitor; ■, CldAMP competitor; *, supplement of 2 fpu TBP; **, supplement of 4 units of HeLa extract.

petition reactions with additional HeLa extract or purified TBP would restore transcription on control templates.

We then performed a competition reaction identical with that described above, but reactions were supplemented after 30 min of synthesis with either TBP or HeLa extract and incubated for an additional 20 min (Figs. 7 and 8). Between 20 and 50 min of incubation, transcription on control templates in the presence of control competitor increased in a linear fashion by ~70% (1.7-fold increase). Supplements of either TBP or HeLa extract after 30 min (total incubation time, 50 min) increased transcript synthesis by 20% over control amounts at 50 min. In contrast, transcript synthesis on control 85-mers in the presence of CldAMP-substituted competitor DNA remained stationary between 20 and 50 min; no appreciable increase was detected. However, supplements of either TBP or HeLa extract after 30 min increased transcription ~2-fold at 50 min compared with synthesis in reactions lacking supplements. However, transcription under these conditions did not reach control levels.

Discussion

Previous results suggested that transcriptional processes would be affected by the presence of CldAMP residues in DNA templates. Studies here have addressed the effects of CldAMP substitutions on steps involved in transcription initiation by human RNA pol II, specifically when the analog was placed within a eukaryotic promoter region. We found that transcript production on a CldAMP-substituted TATA element was mitigated by two mechanisms. First, the frequency of formation of transcription initiation complexes on CldAMP-substituted TATA elements compared with an unmodified sequence was less. This was shown in both Figs. 1 and 3, in which 30 to 35% less DNA transcription occurred in the presence of increasing amounts of HeLa extract (Fig. 1) and at relatively short incubation periods (10 min) (Fig. 3). It has been shown that transcription from promoters with low-affinity TATA boxes is limited both by how much TBP is bound and by the ability of TBP to assume a conformation on the TATA element and function as a nucleus for preinitiation complex assembly (Hoopes et al., 1998). Because the CldAMP substitutions in the AdML 85-mers mimicked the substitutions used previously in gel-shift assays, we conclude that decreased transcript synthesis resulted from diminished TBP recognition of and binding to the CldAMP-substituted TATA sequence and therefore reduced formation of transcription-initiation complexes. Thus a CldAMP-substituted TATA sequence must be categorized as a low-affinity TATA box that limits how much TBP becomes bound.

Our result contrasts with that found for another potent antileukemia agent and nucleoside analog, cytosine arabinoside, which has an intact base with an unusual sugar (arabinose) ring. Cytosine arabinoside substitutions in the central promoter region for phage T7 RNA polymerase did not modify polymerase binding, initiation, or transcriptional output (Mikita and Beardsley, 1994). However, phage RNA polymerases do not rely on the formation of a multiprotein initiation complex involving TBP but rather are sequence-dependent. On the other hand, the presence of random nitrogen mustard (bis[2-chloroethyl]methylamine)-induced alkylations of guanines within a promoter region reduced the amount of elongated transcripts by *Escherichia coli* RNA polymerase (Gray et al., 1991). Moreover, numerous forms of DNA damage have been shown to prevent specific transcription factor binding interactions with their consensus sequences including 8-oxoguanine and benzo[*a*]pyrene diol epoxide (BPDE)-modified AP-1 binding sites (Parsian et al., 2002; Persson et al., 1996), BPDE-modified Sp-1 binding sequence (MacLeod et al., 1996), and nitrogen mustard-damaged Sp-1 and AP-2 consensus sites (Chen et al., 1999).

Second and perhaps more importantly, our results indicate that fewer 44-mer transcripts were synthesized on ds 85-mers with a CldAMP-substituted promoter because, once bound to the analog-containing TATA element, TBP became trapped or sequestered, thus preventing its dissociation and further rounds of initiation. To the best of our knowledge, this is the first report of a nucleoside analog that acts as a transcription antagonist by sequestering a key transcription factor on DNA. This conclusion is supported by several experiments, particularly the time-course study (Fig. 3) in which transcript synthesis on unmodified promoters led to the linear production of 44-mer transcripts over a 30-min incubation period. In contrast, 44-mer production reached a plateau after ~20 min on DNA templates with the altered TATA element. Studies shown in Fig. 5, in which HeLa nuclear extract addition during the plateau phase increased synthesis of 44-mers in reactions with CldAMP-substituted TATA elements, also support this finding. As mentioned earlier, in vitro transcription assays with HeLa nuclear extract can undergo multiple rounds of initiation and elongation (Hawley and Roeder, 1987). Some studies indicate that complete assembly and disassembly of the transcription initiation complex occur during each round of transcription (Kadonaga, 1990). Alternatively, Hawley and Roeder (1987) have argued that at least certain transcription components remain committed to a portion of initiated promoters after formation and transition to an elongation complex. Our competition results in Figs. 6 and 7 favor the former and suggest that TBP dissociates from its DNA site. However, upon interacting with a CldAMP-containing TATA element, TBP was unable to dissociate to re-enter the pool of transcription factors necessary to assemble and form the core initiation complex. Furthermore, competition experiments in which only control (adenosine-containing TATA element) ds 85-mers were incubated with an

excess of either ds control or singly CldAMP-substituted competitor 18-mers (Fig. 7) also strongly support the notion that CldAMP-containing competitor sequences are capable of trapping TBP after it dissociates from 85-mers in the first round of transcription. When transcription factors (TBP) were replenished (Fig. 7), more TBP was available to bind to unoccupied control DNA templates, and transcript production increased.

The terms "molecular decoy" and "hijacking" have been used to describe this phenomenon by which various forms of DNA damage or adducts trap or sequester transcription factors (Treiber et al., 1994; Butler et al., 1997; Zhai et al., 1998). *cis*-Diamminedichloroplatinum (cisplatin)-, UV-, and *N*-2-acetylaminofluorene–damaged DNA are all very effective competitor binding sites for TBP because of distortion or kinks introduced into DNA molecules by these forms of damage (Vichi et al., 1997; Coin et al., 1998). Cisplatin adducts situated near a TATA box enhanced TBP binding to its consensus sequence; interestingly, however, they also caused a much slower dissociation rate (Cohen et al., 2000) similar to our finding with CldAMP residues. Cisplatin-DNA adducts also trap the human upstream binding factor needed for ribosomal RNA transcription (Treiber et al., 1994; Zhai et al., 1998). Likewise, BPDE adducts were found to sequester Sp-1 and disrupt in vitro transcription (Butler et al., 1997). However, in all of these examples, transcription factors misrecognize a distortion in DNA as an appropriate binding site; that is, these distortions mimic the DNA kink observed in a normal TATA box configuration. In our study, the CldAMP substitution is within the complementary strand of the consensus TATA sequence. Our previous work showed that the altered site allows binding by TBP, which then assumes an altered and more stable conformation. Here, we demonstrated that these effects cause diminished in vitro transcription by human RNA pol II. Thus, under either condition, the consequences would be similar. Essential transcription factors, particularly TBP, would be titrated or depleted out by CldAMP residues or DNA adducts to reduce quite effectively transcription of other genes that rely on RNA pol II. The "hijacking" phenomenon was observed in our study with either singly or doubly CldAMP-substituted TATA elements. More importantly, because TBP is also used by both RNA pol I and III (Goodrich and Tjian, 1994), sequestration onto CldAMP-containing TATA elements could prevent TBP interactions with other natural promoter binding sites on rRNA and tRNA genes.

Our findings have important implications for cellular transcriptional effects of CldAdo. In a cell containing both CldAMP-substituted and normal (unsubstituted) promoter sequences, RNA synthesis could be reduced for three reasons. First, TBP does not readily recognize and bind a CldAMP-substituted promoter; it would therefore initiate transcription at lower levels compared with a normal promoter. Second, once bound, the protein would remain trapped on CldAMP-containing TATA elements such that TBP could not dissociate and bind unsubstituted promoters. Third, despite a stable interaction between TBP and the substituted TATA element, the protein complex would not function as an initiation nucleus site to allow further transcription after an initial round. Even if these events only partly inhibited cellular RNA synthesis, certain proteins, particularly those with short half-lives, would not be produced that are essential for cell survival. Thus, template CldAMP residues may contribute to reduced RNA synthesis observed in treated cells.

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