Effects of 2-chloroadenine substitution in DNA on restriction endonuclease cleavage reactions

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ABSTRACT

The purine analog, 2-chloro-2'-deoxyadenosine triphosphate (CldATP), was incorporated enzymatically in place of dATP into the minus strand of M13mp18 duplex DNA. Its effect on protein-DNA interactions was assessed by determining the amount of DNA cleavage by type II restriction endonucleases. Substitution of chloroadenine (CIAd) for adenine (Ade) in DNA appreciably decreased the amount and rate of DNA cleavage of the minus strand when the analog was situated within the appropriate endonuclease recognition site. CIAd residues flanking a restriction site had variable effects. *Sma*I cleaved both CIAd-containing and control substrates with equal efficiency. *Nar*I, however, was stimulated 1.5-fold by the presence of CIAd outside its recognition site. The effects of analog incorporation on restriction enzyme cleavage of an opposing unsubstituted strand of duplex DNA was examined by enzymatically incorporating CldATP into the complementary minus strand of a 36-base oligonucleotide. Endonucleolytic cleavage of both plus and minus strands was reduced on 36-mers containing CIAd residues located within only the minus strand. These data suggest that CIAd residues incorporated into a single DNA strand may have an appreciable effect on DNA-protein interactions that involve one or both strands of duplex DNA.

INTRODUCTION

2-Chloro-2'-deoxyadenosine (CldAdo) is a nucleoside analog of deoxyadenosine. The chloro group substitution for hydrogen within the minor groove is conservative in that the C2 position of the purine ring is not directly involved in hydrogen bonding of its complementary base, and the analog cannot form tautomers. Nevertheless, the analog represents a departure from the natural structure of deoxyadenosine. Nucleic acid stability may be compromised by the bulk or electrostatic field of the chloride atom and could lead to significant functional changes. This

structural alteration is sufficient, moreover, to modify several aspects of the compound's cellular metabolism and use during DNA synthesis. The presence of the chloride atom renders the nucleoside resistant to cellular adenosine deaminase (1), but does not inhibit phosphorylation by cellular kinases (2, 3, 4). Intracellularly, CldAdo inhibits ribonucleotide reductase (3, 4, 5, 6), decreases DNA synthesis (2, 6, 7), and is incorporated into DNA (4, 8). The 5'-triphosphate derivative of CldAdo, CldATP, is used less efficiently in place of dATP during DNA synthetic reactions *in vitro* by several bacterial, phage and human DNA polymerases, particularly in regions where consecutive CldATP residues must be inserted (9, 10). This purine analog is of interest clinically as a possible antileukemic drug (11, 12, 13).

The effects of CldAdo, after incorporation into DNA, on other cellular functions such as protein-DNA interactions are unknown. It is possible that the presence of chloroadenine (CIAd) within DNA could alter gene expression by modifying the binding of regulatory proteins to promoter sequences or control regions within DNA. Such changes in gene expression have been observed for other base analogs such as 6-thioguanine (14) and 5-bromo-2'-deoxyuridine (15, 16, 17). Alternatively, CIAd residues within origins of DNA replication might inhibit or delay DNA replication initiation. To assess such changes in protein-DNA interactions, type II restriction endonucleases, in particular, that recognize and catalyze a double-strand DNA cleavage reaction at specific sequences have been used. DNA base substitutions within an endonuclease recognition site have been found to cause a wide range of effects on restriction enzymes, from complete inactivity to enhanced endonucleolytic cleavage of DNA, depending on the base analog substitution and restriction enzyme used (14, 18-23).

In this study, DNA strands substituted with CIAd in place of adenine (Ade) were synthesized enzymatically *in vitro* by using M13mp18 plus strand DNA or a 36-base oligonucleotide as template and replacing dATP in polymerization reactions with CldATP. The resultant double-stranded DNA molecules were used as substrates to determine if the presence of the chloro-substitution within deoxyadenosine disrupted catalysis by various restriction enzymes.

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MATERIALS AND METHODS

T7 Sequenase II and a dideoxynucleotide sequencing kit were obtained from United States Biochemical Corp., M13mp18 plus strand DNA, M13 specific primer 2 (5' GTAAAACGACGGCC-AGT) and formamide stop solution from New England Biolabs; molecular biology grade normal dNTPs and acrylamide from Biorad; G50 Sephadex from Pharmacia; Kodak XAR and Dupont Cronex film from Med Cor X-ray Systems; [γ - 32 P]ATP from ICN and T4 polynucleotide kinase from Bethesda Research Laboratories. M13 specific primers 2B (5' GGCCAGTGCC-AAG) and 2C (5' GGCCAGTGCCAAGCTTGC) and a 36-base oligonucleotide were synthesized on an Applied Biosystem 380B DNA synthesizer in the Molecular Resource Center, St Jude Children's Research Hospital. Restriction endonucleases were purchased as follows: *Aha*II, *Ava*II, *Eco*RI, *Pst*I, and *Hind*III from New England Biolabs, *Nar*I and *Bam*HI from Pharmacia, *Sal*I, *Sma*I and *Sph*I from IBI.

Enzymatic synthesis of Ade- or ClAde-containing minus strand DNA

M13mp18 specific primer 2 (which anneals to positions 6291–6307) and primer 2C (which anneals to positions 6280–6297) were 5'-end-labeled by using T4 kinase in the forward kinase reaction as previously reported (9, 10). Labeled primer (16 ng, 2.7 pmol) was annealed to M13mp18 plus strand DNA (5 μ g, 2.0 pmol) by heating the mixture at 90°C for 4 min and slowly cooling to room temperature for 30 min. The final synthetic reaction mixtures of 50 μ l contained 40 nM M13 template annealed to 54 nM primer, 50–75 μ M each dCTP, dGTP, and dTTP, 10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 35 mM NaCl, 3.2 mM dithiothreitol, and 50–75 μ M CldATP or dATP as the fourth nucleotide. T7 Sequenase was added (1 μ l, 16 units) and each mixture was incubated at 37°C for 30–45 min, before inactivation by adding Na₂EDTA to a final concentration of 20 mM. The partial double-stranded DNA was separated from unincorporated nucleotides by centrifugation through G-50 Sephadex spin columns. DNA synthetic products were routinely analyzed for size distribution by electrophoresis on a 5% denaturing polyacrylamide gel. Most DNA fragments did not enter the gel indicating a size range greater than 600 bases. Typically, however, a small amount (<2%) of DNA synthesized in the presence of CldATP was less than 600 bases in length due to the reduced efficiency of DNA polymerases to incorporate consecutive (>3) CldATP residues into a nascent DNA strand (9, 10). The first of such sites occurs near position 5890 in M13mp18 DNA which is 417 bases upstream of the primer annealing site.

Restriction endonuclease digestion reactions

During timed reactions, 40 μ l (~1.6 pmol) of duplex DNA synthesized as above, which contained either four normal bases in the minus strand or a ClAde-substituted minus strand, were suspended in the appropriate endonuclease assay buffer. Ten units of restriction enzyme were added in the presence of 100 μ g/ml bovine serum albumin in a total volume of 50 μ l. The unit definition for all enzymes was the amount of enzyme required to hydrolyze 1 μ g of DNA to completion in 60 min at optimum temperature in 50 μ l. The final glycerol concentration in all reactions was less than 8%. Incubations were at 37°C; at various intervals, 6- μ l-aliquots were removed and added to a stop solution of deionized formamide containing 0.3% xylene cyanol, 0.3%

bromophenol blue, and 0.37% Na₂EDTA (pH 7.0). For reactions in which the endonuclease concentration was modified, approximately 0.2 pmol of DNA was incubated with various amounts of enzyme in a final volume of 8–9 μ l for 1 hr at 37°C. Cleavage of the minus strand preceded denaturation and electrophoresis of cleavage products on 8% polyacrylamide, 7 M urea gels before autoradiography on XAR or Cronex film at –70°C. Positions of cleavage products were identified by comparison with standard dideoxynucleotide sequencing reactions (24) conducted on the same primer/template DNA.

Construction and digestion of duplex oligonucleotide

An oligonucleotide of 36 bases, representing a portion of the plus strand sequence of M13mp18 DNA from positions 6262–6297, was used as a template that contained restriction enzyme sites for *Sal*I, *Pst*I, and *Sph*I. The 5' end of either a complementary 13-base primer (primer 2B, which anneals to positions 6285–6297) or the 36-mer template plus strand was labeled with 32 P using T4 kinase as described above. To examine cleavage of the minus strand (the enzymatically synthesized strand), 32 P-end-labeled primer 2B (1 pmole) was annealed to an equimolar amount of unlabeled plus strand 36-mer. Cleavage of the plus strand was monitored by annealing unlabeled primer 2B to 32 P-labeled 36-mer template. Synthesis of the minus strand to generate a duplex molecule was initiated by adding 3 normal dNTPs (dGTP, dCTP, and dTTP) to a final concentration of 150 μ M, either dATP or CldATP as the fourth nucleotide at 150 μ M, and 13–26 units of Sequenase II in 25 μ l of the same reaction mixture as stated above for the M13mp18 synthetic mixtures. Greater amounts of DNA polymerase and dNTPs were added in these synthetic reactions because preliminary experiments indicated that a 36-base DNA template was used less efficiently than M13 DNA was by Sequenase. After overnight incubation at 37°C, reactions were inactivated with Na₂EDTA, and the DNA was centrifuged through a G-50 Sephadex column. A portion of duplex DNA generated was phenol extracted and analyzed by non-denaturing polyacrylamide gel (10%) electrophoresis. Approximately 0.17 pmole of duplex 36-mer was then incubated for 1 hr with 10–20 units of restriction enzyme in a final reaction volume of 10 μ l. Analysis of cleavage products was on 20% polyacrylamide denaturing gels.

Densitometry

Autoradiograms were scanned with a Hoefer Scientific densitometer to determine the distribution of DNA. Densitometer curves were analyzed with a Hoefer GS 370 data system by

Figure 1. Structure of CldATP.

integrating the area under the absorbance curve for each band above a baseline drawn manually at points of minimum absorbance.

RESULTS AND DISCUSSION

Synthesis of 'CIAdé'-containing DNA

The structure of the purine analog, CldATP, is shown in Figure 1. Total replacement of Ade by CIAdé in one strand of M13mp18 DNA was achieved by enzymatically synthesizing a partial minus DNA strand using M13mp18 plus strand DNA as template with T7 Sequenase. Previously, we showed that synthesis of an M13 minus strand by T7 Sequenase was slightly less efficient in the presence of CldATP as the fourth nucleotide compared with that in the presence of dATP (10). However, with adequate enzyme and long incubation periods, DNA strands longer than 600 bases were typically generated that contained CIAdé in place of all Ade residues, and all primed DNA molecules were used to the same degree during both synthetic reactions (data not shown). The restriction enzymes chosen for the study all have only one cleavage site on M13mp18 DNA; most (except *AvaII*) recognize sequences of 6 nucleotides in length, and all sites were within 75 bases or less of the primer sequence, except those cleaved by *AvaII*, *AhaII*, and *NarI*, which were less than 380 bases upstream of the primer annealing site.

Endonuclease activity on substituted M13mp18 DNA

Digestion of M13mp18/primer 2 duplex DNA with *SmaI*, which recognizes the sequence 5' CCC↓GGG (+), produces a 57-base DNA fragment. The nearest flanking CIAdé residues in the minus strand are two bases 3' and three bases 5' to the recognition site. Figure 2A illustrates that cleavage of either Ade- or CIAdé-containing DNA by *SmaI* (1.4 units/reaction) was comparable even after a brief incubation period. Densitometer scans of the autoradiogram confirmed these observations and revealed no increase in the amount of cleavage products between 2 and 20 minutes, suggesting that 1.4 units of *SmaI* was a saturating concentration. In the absence of enzyme, primarily large molecular weight DNA that did not enter the gel was observed (not shown). It is also evident that the electrophoretic mobility

of CIAdé-containing fragments was slightly reduced compared with the normal strand (Figure 2A). This altered mobility provides a convenient marker for CIAdé-containing DNA. Digestion reactions with less *SmaI* present (from one-half to one-tenth the standard concentration, e.g., 0.7 to 0.14 units of enzyme) demonstrated that 0.14 and 0.28 units of *SmaI* produced 50 and 65%, respectively, of the maximal amount of cleavage achieved within one hour with 1.4 units (data not shown). Moreover, at either of these non-saturating concentrations, cleavage of CIAdé-containing DNA was equivalent to that of control DNA. The ability of *SmaI* to cleave CIAdé-containing and control DNA with equal efficiency and rate also provided evidence that both types of synthesized DNA were in a stable duplex form.

By contrast, most enzymes had reduced or no ability to cleave a CIAdé-substituted minus strand if CIAdé was within the recognition sequence. *EcoRI* cleaves within the sequence 5' G↓AATTC (+) and generates a DNA fragment of 62 bases when primer 2C is used in the extension reaction. Control Ade-containing minus strand DNA was maximally digested by *EcoRI* within 2 to 5 min (Figure 2B), but only a small amount of the CIAdé-containing strand was cut to completion even after a 60-min incubation. The digested product, however, was the correct size. The rate of *EcoRI* hydrolysis of the CIAdé strand was linear with time, determined by densitometer scans, but resulted in only 10% of the cleavage attained on the normal Ade-containing strand after 60 min. When the *EcoRI* concentration was increased, greater hydrolysis of CIAdé-containing DNA was observed (Figure 3), but even at 10–20 times (14–28 units/reaction) the normal amount of enzyme used, cleavage of substituted DNA was only ~50% that of the control amount which reached maximum cleavage at about 5.6 units of enzyme. The glycerol concentration in such reactions was always <8%. Approximately 10 times more enzyme (14 units) was required to achieve a similar degree of digestion on DNA with the incorporated analog compared with the normal substrate by using only 1.4 units.

Table I summarizes results obtained from cleavage reactions with various restriction endonucleases on the M13 partial duplex. *HindIII*, which had to cleave between two CIAdé residues, did

Figure 2. Restriction endonuclease cleavage of control (A) and CIAdé-substituted (CIA) minus strand DNA. M13mp18 partial duplex DNA (40 ng) was incubated with 10 units of either *SmaI* (Panel A) or *EcoRI* (Panel B) at 37°C for the times indicated. Hydrolysis products were denatured and analyzed by polyacrylamide gel electrophoresis and subsequent autoradiography. Uncleaved DNA was very high molecular-weight DNA that appeared as intense radioactive bands in the wells at the top of the gel.

Figure 3. Effect of endonuclease concentration on the amount of *EcoRI* cleavage of Ade and CIAdé-containing M13mp18 minus strand DNA. DNA was incubated at 37°C for 1 hr with increasing amounts of enzyme. After gel electrophoresis and exposure to X-ray film, autoradiograms were scanned by densitometry.

Table I. Cleavage by restriction endonucleases of M13mp18 minus strand DNA containing ClAde in place of Ade

Enzyme	Recognition Sequence ^a	% Yield of Product ^b
<i>Hind</i> III	3' <i>ACG TTCGA</i> !A CCG(-) (N) (N N)	(0,0)
<i>Eco</i> RI	3' <i>ATG CTAA</i> !G CTC (N) (NN)	6 ± 7
<i>Pst</i> I	3' <i>CTG G</i> !ACGTC CGT (N)	7 (11,2)
<i>Sph</i> I	3' <i>GTC C</i> !GTACG TTC (N)	23 (12,33)
<i>Bam</i> HI	3' <i>GCC CCTAG</i> !G AGA (N) (N N)	22 ± 9
<i>Sal</i> I	3' <i>TCT CAGCT</i> !G GAC (N) (N)	50 (49,51)
<i>Sma</i> I	3' <i>CAT GGG</i> !CCC CTA (N) (N)	102 ± 7
<i>Nar</i> I ^c	3' <i>GGA CCGC</i> !GG GTT (N)	147 ± 7
<i>Aha</i> II ^c	3' <i>GGA CCGC</i> !GG GTT (N)	1 (0,2)
<i>Ava</i> II	3' <i>GCA CCTG</i> !G CGA (N) (N)	68 (90,46)

^aItalicized bases lie outside actual recognition site and are included to demonstrate location of flanking ClAde residues (designated as N) within M13mp18 DNA.

^bRelative to control (Ade-containing) DNA. Yield (%) is the mean calculated from at least two experiments for each enzyme. Numbers in parentheses show the range of two determinations. The average of three or more determinations is shown with its standard deviation. DNA (0.25 pmol) was incubated with 2 units of enzyme for 1 hour at 37°C.

^cBoth *Nar*I and *Aha*II recognize the same sequence.

Figure 4. DNA sequence of the 36-base oligonucleotide and location of restriction enzyme sites within the sequence. Underlined area indicates primer 2B used in extension reactions with Sequenase to synthesize the minus strand. Positions of ClAde residues within the minus strand are designated as (N).

not hydrolyze the substituted strand within one hour. In general, the more distant the substitution occurred relative to the actual site of cleavage, a greater amount of DNA hydrolysis was detected. Both *Eco*RI and *Pst*I produced similar degrees of cleavage adjacent to a ClAde residue. As the ClAde substitution moved one and two bases away from the cleavage site, increased incision occurred with *Sph*I and *Bam*HI. The amount of cleavage by these two enzymes was similar despite the presence of two ClAde residues in the 5' flanking sequence of the *Bam*HI site. *Sal*I, which incised a site three bases from the ClAde substitution, cleaved the modified strand half as well as it did the normal strand. Our results are similar to those by Bodnar and co-workers (21) in which structural alterations in a base pair immediately adjacent to bonds cleaved by an enzyme reduced the rate of cleavage most dramatically. Only one enzyme examined thus far, *Nar*I, consistently hydrolyzed ClAde-containing duplex DNA to a greater extent than it did Ade-containing sites. As seen in Table I, the *Nar*I restriction sequence contains no Ade or ClAde residues within the cognate cleavage site. The adjoining ClAde residues within flanking sequences may act to stabilize the enzyme to the

Figure 5. Stability of duplex DNA substituted with ClAde in the minus strand. Duplex DNA was synthesized enzymatically with T7 Sequenase. 5'-³²P-labeled primer 2B was annealed to plus strand 36-mer and extended in the presence of 4 normal dNTPs (A) or CldATP (N) in place of dATP. DNA was phenol extracted, and its mobility through a nondenaturing 10% polyacrylamide gel was compared to that of 5'-³²P-labeled single-stranded (SS) 36-mer (plus strand).

DNA fragment leading to increased hydrolysis. Such a mechanism of action has been postulated also for the reported 5-fold stimulation in the amount of cleavage by *Mbo*I of 5-bromodeoxyuridine-substituted DNA (25).

In contrast to *Nar*I, however, *Aha*II, which recognizes the degenerate sequence 3'-CPyGCPuG-5' (Py=pyrimidine, Pu=purine), exhibited very little DNA cleavage, despite relaxed sequence requirements and the absence of ClAde residues within its specific recognition site. Both *Aha*II and *Nar*I hydrolyze a common sequence in M13mp18 DNA with a single Ade or ClAde residue immediately adjacent to the cognate site in the minus strand (see Table I). Hydrolysis of the *Ava*II site, which also contains ClAde residues only within flanking sequences, was slightly reduced on the substituted minus strand. The contrasting cleavage patterns exhibited by *Ava*II, *Sma*I, *Aha*II, and *Nar*I suggested that adjacent bases can influence enzyme activity differently, but a correlation between the presence of flanking ClAde residues and the degree of cleavage has not been established. These findings, that ClAde outside a cognate recognition sequence altered restriction endonuclease activity, also differed from studies in which nucleotide analogs, such as 2-aminopurine, deoxyinosine, and 5-bromodeoxycytidine, located within only flanking sequences were found to have little or no effect on cleavage by most restriction enzymes (21).

Endonuclease activity on ClAde-substituted 36-base oligonucleotides

To determine if restriction enzymes nicked the unsubstituted opposing strand preferentially but could not cleave double-stranded ClA-substituted DNA, we constructed a 36-base oligonucleotide duplex that contained three restriction enzyme sites (Figure 4), the minus strand of which was enzymatically synthesized in the presence or absence of CldATP. The use of short oligonucleotides also provided a DNA substrate that would more likely lack non-specific recognition sites compared to complex molecules such as M13 DNA, and as such, could reveal subtle differences in binding that might be masked on long substrates. Duplex DNA containing either Ade or ClAde had similar mobilities on a non-denaturing polyacrylamide gel (Figure 5), indicating that ClAde-substituted DNA was as stable as control duplex. Cleavage of either minus or plus strands was then followed simultaneously by using duplexes that were preferentially ³²P-labeled at either 5'-end. Enzymatic synthesis of the minus strand in the presence of either dATP or CldATP

Figure 6. Inhibition of restriction enzyme cutting of both strands of the 36-base oligonucleotide by CIAdes residues within the minus strand. Duplex 36-mer containing Ade (A) or CIAdes (N) residues within the minus strand was incubated for 1 hr at 37°C with or without restriction enzymes. Endonucleolytic cleavage products were analyzed by 20% polyacrylamide denaturing gel electrophoresis. Panel I: Cleavage of ³²P-labeled minus strand. Panel II: Cleavage of ³²P-labeled plus strand.

(Figure 6, panel I) produced primarily DNA strands 37 and 38 bases in length. Synthesis of longer minus DNA strands than that encoded by the template occurred to the same extent in the presence or absence of CldATP, demonstrating that the analog alone was not perturbing synthetic processes. Non-template directed incorporation of extra nucleotides has been observed and reported previously (14, 26, and 27) and is thought to be due to the formation of a loopback structure by terminal nucleotides (27). In addition, a small amount of less than full length DNA was synthesized in both reactions most likely because of diminished stability of the polymerase-DNA complex near the end of the 36-base template.

As was observed in reactions with M13mp18 duplex DNA, CIAdes residues decreased *SphI*, *PstI* and *SalI* cleavage within the ³²P-labeled substituted minus strand to 21, 16, and 26%, respectively, relative to control minus strand cleavage (Figure 6, panel I). Reactions to investigate nicking of an unsubstituted plus DNA strand opposite control DNA or CIAdes residues demonstrated several points (Figure 6, panel II). Compared to the degree of hydrolysis observed on the minus strand, *SalI* hydrolysis of plus strand from control DNA occurred at only low levels, and cleavage of substituted DNA was not detectable. Decreased *SalI* hydrolysis may be attributed to proximity of the plus strand cleavage site to the 5' terminus of the oligonucleotide (See Figure 4). DNA contact sites for restriction enzymes other than *EcoRI* (28) and *EcoRV* (20) are not well defined, but it is likely that at least 10 base pairs or more are involved in a stable, symmetric endonuclease/DNA complex. Thus the *SalI*/36-base oligonucleotide interaction may be marginally or less than favorable for efficient scission.

Furthermore, despite the lack of CIAdes residues within plus strand DNA, *SphI*, *PstI*, and *SalI* all hydrolyzed the plus strand of substituted substrates much less relative to control DNA plus strand (compare A and N lanes, panel II). *SphI* cleavage of CIAdes-containing substrate DNA (Figure 6 and additional experiments not shown) was roughly equivalent on both minus and plus strands indicating that the duplex was incised in a symmetrical manner. *PstI* hydrolysis of plus strand (6% of control amount) was slightly less than that observed for minus strand DNA (16% of control cleavage), but an evaluation of several similar experiments demonstrated no appreciable difference in the amount of plus and minus strand cleavage by *PstI* on CIAdes-substituted 36-mers. These findings indicated that CIAdes residues in one strand also affected enzyme catalysis of the opposing unsubstituted DNA strand, but in the case of *SphI* and *PstI*, preferential strand cleavage was not evident.

Because of the limited availability or lack of crystal structures for most restriction enzyme-DNA complexes, these experiments with CIAdes-substituted DNA cannot definitively address the cause of reduced endonucleolytic cleavage. Although extensive T_m studies have not been conducted, the similarity in gel mobility under non-denaturing conditions of DNA with and without CIAdes residues suggests that no major perturbation in helical stability has occurred. The unattenuated activity of several enzymes such as *SmaI* and *NarI* on CIAdes-containing DNA also rules out the possibility of appreciable distortion in the DNA backbone; however, slight or localized alterations in DNA conformation may exist near or opposite CIAdes residues. The presence of CIAdes within DNA could alter the recognition and subsequent binding of enzyme and substrate, inhibit the actual hydrolytic

event, or modify the stability of the enzyme-DNA complex. The bulk of the chloro group compared with that of the 2'-H may hold the nuclease away from the site of DNA cleavage or create sufficient local distortion of the DNA duplex to prevent the enzyme from binding and cleaving the substrate. Such a situation would be similar to the slight distortion caused by base pair mismatches (29, 30) which leads to reduced cleavage (31, 32). This may be unlikely, however, for at least *EcoRI* and perhaps other proteins because of evidence that both *EcoRI* and *EcoRV* interact with their recognition sequences only through functional groups within the major groove of DNA (18, 20, 33). The 2'-H group of Ade is thought to be uninvolved in either sequence recognition or catalysis by *EcoRI* (33).

Conversely, after binding of an endonuclease, DNA is thought to undergo a conformational change (33). It has been suggested by Brennan and co-workers that minor groove substituents, such as 2,6-diaminopurine, disrupt the spine of hydration that is important for stability or alternatively, interfere with the conformation transition and thereby prevent optimal interactions of the endonuclease and its contacts in the major groove (18). Thus, it is more likely that ClAde prevents or causes conformational changes in the DNA that are required for stable binding and catalysis. Inhibition of the appropriate DNA conformational change could also account for the similar but diminished cleavage on both plus and minus strands by *SphI* and *PstI*. However, because the DNA minus strand has been substituted at all Ade sites with ClAde residues, we cannot ignore the possible modifying effects of ClAde-containing flanking sequences on the DNA-enzyme complex. As illustrated by our contrasting results with *AhaII*, *SmaI*, and *NarI*, ClAde residues outside a restriction site may have a role in the binding or catalysis of specific enzymes but not others.

Although we have conducted our studies on both macromolecular DNA (M13) and short oligonucleotides, neither system adequately addresses the cellular environment in which substitution of Ade by ClAde within DNA will be incomplete and much less frequent (4). With this caveat in mind, our data have demonstrated that the substitution of a chloro group for hydrogen in deoxyadenosine clearly modifies the activity of a specific set of proteins (restriction endonucleases) with their respective substrate molecules. It is unknown whether other DNA binding proteins are affected by the presence of ClAde residues. It will be of interest to examine the interaction of non-catalytic proteins with ClAde-containing DNA.

CONCLUSIONS

The presence of ClAde within a given recognition sequence reduced the amount of cleavage of substituted strands by most restriction enzymes studied. Similar trends were observed on both a macromolecular substrate (M13 DNA) and short oligonucleotides. Studies with ClAde-containing oligonucleotides demonstrated that cleavage of an unsubstituted strand was likewise diminished.

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