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O^6-Methylguanine in Place of Guanine Causes Asymmetric Single-Strand Cleavage of DNA by Some Restriction Enzymes†

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Received July 5, 1989; Revised Manuscript Received September 1, 1989

ABSTRACT: The interactions of restriction enzymes with their cognate DNA recognition sequences present a model for protein-DNA interactions. We have investigated the effect of O^6-methylguanine on restriction enzyme cleavage of DNA; O^6-methylguanine is a carcinogenic lesion and a structural analogue of the biological restriction inhibitor N^6-methyladenine. O^6-Methylguanine was synthesized into oligonucleotides at unique positions. The oligonucleotides were purified and analyzed by high-pressure liquid chromatography to assure that, within the limits of our detection, O^6-methylguanine was the only modified base present. These oligonucleotides were annealed with their complement so that cytosine, and in one case thymine, opposed O^6-methylguanine. DNA cleavage by restriction enzymes that recognize a unique DNA sequence, *Hpa*II, *Hha*I, *Hin*PI, *Nae*I, *Nar*I, *Pvu*II, and *Xho*I, was inhibited by a single O^6-methylguanine in place of guanine (adenine for *Pvu*II) within the appropriate recognition sequences. However, only the modified strand was nicked by *Hpa*II, *Nae*I, and *Xho*I with O^6-methylguanine at certain positions, indicating asymmetric strand cleavage. For all the restriction enzymes studied but *Aha*II, *Ban*I, and *Nar*I, lack of double- or single-strand cleavage correlated with inability of the O^6-methylguanine-containing recognition sequence to measurably bind enzyme. None of the restriction enzymes studied were inhibited by O^6-methylguanine outside their cognate recognition sequences.

The interaction of restriction enzymes with their cognate DNA recognition sequences presents a model system to study protein-DNA interactions; the proteins are relatively small yet have high specificity for simple DNA sequences as short as 4 bases [reviewed in Modrich (1979) and Yuan (1981)]. Such protein-DNA interactions lie at the heart of most biological processes, so the ability to probe such interactions is important.

The simplest type of restriction enzyme, type II, is a single-function enzyme that only requires Mg^{2+} as a cofactor. Type II enzymes recognize sequences with twofold symmetry, and they generally cleave within their cognate recognition sequences. Several approaches have been used to study DNA sequence recognition by type II restriction enzymes including (i) determination of the crystal structure of *Eco*RI complexed with the cognate oligonucleotide duplex TCGC*GAATTC*GCG (McClarin et al., 1986), (ii) chemical substitution of base analogues for bases in the recognition sequence to probe the requirements for specific functional groups (Berkner & Folk, 1979; Dwyer-Hallquist et al., 1982; Bodnar et al., 1983; Brennan et al., 1986; Jiricny & Martin, 1986; Jiricny et al., 1986; McLaughlin et al., 1987), and (iii) chemical protection experiments to indicate where *Eco*RI endonuclease contacts DNA (Lu et al., 1981).

Some points to emerge from the large amount of work represented by these papers, as well as others, include (i) *Eco*RI "hugs" the DNA making contact with phosphates and the DNA major groove. The latter provides the major means of sequence discrimination by specific hydrogen bonding, as proposed by Seeman et al. (1976), and by sensing the presence or absence of a methyl at C-5 of pyrimidines; interactions with the minor groove may be important for recognizing some sequences. *Eco*RI interaction with its cognate recognition sequence is facilitated by kinking at the center of the recognition site, concomitant with enzyme binding, to allow better contact with the major groove (type I neokink) and two more moderate kinks that span the scissile bond (type II neokink). (ii) *Eco*RI contacts at least 10 base pairs, indicating that interactions beyond the recognition sequence are important for stability. (iii) Different restriction enzymes react to the same base modifications differently in terms of binding and cleavage. Thus, the specific modes of enzyme-DNA recognition may be as varied as the sequences to be recognized.

We present studies that place a methyl group at the O^6 position of unique guanines in the recognition sequence of several restriction enzymes. Methylation of guanine at O^6 is the natural product of some chemical carcinogens. This

† This work was supported by USPHS Grant CA46527 and a Scholar Award to M.D.T. from the Leukemia Society of America.
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0006-2960/90/0429-1632$02.50/0

methylation blocks a potential hydrogen-bonding site and results in a decreased T_m regardless of the base opposite the O^6-methylG[1] (Gaffney et al., 1984). Since NMR studies of O^6-methylG-containing oligonucleotide duplexes show the methyl group located in the major groove without significant distortion to helical conformation (Patel et al., 1986), we expected that the O^6-methyl group might interfere with restriction enzyme cleavage because of its blockage of a potential hydrogen-bond-acceptor site. Such disruption could provide information about the specific recognition interactions between restriction enzymes and their cognate recognition sequences. Therefore, we studied the effect of O^6-methylG on sequence recognition by 10 different restriction enzymes.

Type II enzymes were used that mainly recognize sequences composed entirely of GC and CG base pairs. To our knowledge, this is the first use of O^6-methylG to systematically probe interactions in the major groove between multiple enzymes and recognition sequences. One of the enzymes studied, *Nae*I, has recently been shown to be regulated in vitro by its interaction with cleavable *Nae*I sites and spermidine, so its mechanism of interaction with DNA is of special interest (Conrad & Topal, 1989). O^6-MethylG is also interesting because it has structural similarity to N^6-methyladenine, one of the biological inhibitors of restriction enzyme cleavage.

We report here that (i) double-strand cleavage by *Ban*I, *Hpa*II, *Hha*I, *Hin*PI, *Nae*I, and *Nar*I, as well as *Pvu*II and *Xho*I, was inhibited by O^6-methylG within the recognition sequence. (ii) *Aha*II and *Hae*II cleaved their cognate recognition sequence with O^6-methylG in place of G at some positions; the rate of cleavage was diminished relative to the unmodified sequence. (iii) *Hpa*II, *Nae*I, and *Xho*I nicked only the modified strand with O^6-methylG at some positions, indicating strand cleavage asymmetry. (iv) For all the restriction enzymes studied but *Aha*II, *Ban*I, and *Nar*I, lack of double- or singl-strand cleavage correlated with an inability of the O^6-methylG-containing recognition sequence to bind enzyme. (v) O^6-MethylG outside the recognition sequences did not inhibit DNA cleavage, irrespective of proximity.

Materials and Methods

Materials. 5′-Dimethoxytrityl cyanoethyl deoxy phosphoramidites were obtained from American Bionetics (Hayward, CA). Anhydrous solvents and other reagents for oligonucleotide synthesis were purchased from Applied Biosystems (Foster City, CA). *Hin*PI, *Hha*I, *Hpa*II, *Nar*I, *Pvu*II, and *Xho*I were purchased from Bethesda Research Laboratories (Gaithersburg, MD). T4 polynucleotide kinase, *Nae*I, *Ban*I, and *Aha*II were obtained from New England Biolabs (Beverly, MA), whereas P1 nuclease was obtained from Boehringer Mannheim (Indianapolis, IN), and *Escherichia coli* alkaline phosphatase and deoxynucleosides were purchased from Pharmacia Inc. (Piscataway, NJ). Adenosine triphosphate and 1,8-diazabicyclo[5.4.0]undec-7-ene were obtained from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]Adenosine triphosphate was purchased from New England Nuclear (Boston, MA).

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized by using an Applied Biosystems Model 380A DNA synthesizer. The purity of each phosphoramidite (including O^6-methylG) was reported by American Bionetics to be greater than 99% as analyzed by ^{31}P nuclear magnetic resonance and high-pressure liquid chromatography. The efficiency of polymerization of each phosphoramidite was reported to be greater than 97%.

Following synthesis, 5′-tritylated oligonucleotides were cleaved from the resin and deprotected by exposure to 10% (v/v) 1,8-diazabicyclo[5.4.0]undec-7-ene in anhydrous methanol for 5 days at 25 °C (Borowy-Borkowski & Chambers, 1987). The tritylated oligonucleotides were purified by high-pressure liquid chromatography (HPLC) on a 4.5 mm × 250 mm C8 reverse-phase column (Alltech Associates, Inc.); a linear gradient of 15–20% acetonitrile in 0.1 M triethylamine acetate, pH 7.0, was used for 10 min, followed by a linear increase to 25% acetonitrile over 40 min. Fractions containing the tritylated oligonucleotides (located by UV absorbance) were evaporated to dryness and the oligonucleotides detritylated in 80% acetic acid for 20 min at 25 °C.

Deprotected, detritylated oligonucleotides were also purified by electrophoresis on 20% denaturing polyacrylamide gels; DNA of the correct length was isolated by electroelution from excised gel slices. The purity of gel- and HPLC-purified oligonucleotides was determined by gel electrophoresis of 5′-^{32}P-labeled oligonucleotides.

The base content of each purified oligonucleotide was analyzed as described by Eadie et al. (1987). Briefly, oligonucleotides were digested with P1 nuclease for 3 h at 37 °C and with *E. coli* alkaline phosphatase for 4 h at 37 °C. The resulting nucleosides were separated by HPLC on two 4.5 × 250 mm C18 Macrosphere 300 reverse-phase columns connected in series. Samples were injected, and the column was washed with equilibration buffer (50 mM potassium phosphate, pH 7.0, containing 5% methanol) for 30 min. The methanol content was then increased linearly in turn from 5% to 8% over 15 min, from 8% to 45% over 20 min, from 45% to 70% over 20 min, and finally from 70% to 90% over 20 min. Product elution was monitored by UV absorbance. Following integration of peak areas, the number of moles of each eluted nucleoside was determined from standard curves generated by injection of known amounts of authentic nucleoside standards. This method accurately detected a single O^6-methylG in a 50-base oligonucleotide.

Restriction Enzyme Cleavage Assays. Complementary oligonucleotides, in a volume of 65 μL containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM $MgCl_2$, were heated at 55 °C for 5 min and annealed by cooling slowly to 25 °C over 30 min. The annealed DNA was desalted by using Sephadex G-25 resin into 10 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$.

Annealed DNAs (20 ng) were incubated, in a total volume of 20 μL, with a 4–6-fold excess of restriction enzyme over that necessary to completely digest the control duplex in 1 h at 37 °C under the conditions recommended by the supplier. Reactions were stopped by heating in a boiling water bath after addition of 10 μL of stop mix (10 M urea, 10 mM EDTA, 0.025% bromphenol blue, and xylene cyanol for denaturing gels or dyes only for native gels). Digestion products were separated on 20% polyacrylamide urea gels or nondenaturing gels in the absence of urea. The gels were autoradiographed by using a Du Pont Cronex intensifying screen; cleavage of duplexes was quantitated by densitometric scanning of autoradiograms.

Restriction Enzyme Binding Assays. Restriction enzyme binding to O^6-methylG-containing oligonucleotide substrate was assayed by competition between this modified substrate and unmodified substrate for enzyme. Eighty nanograms of duplex DNA containing O^6-methylG at the site to be tested

[1] Abbreviations: O^6-methylG, O^6-methylguanine; HPLC, high-pressure liquid chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance.

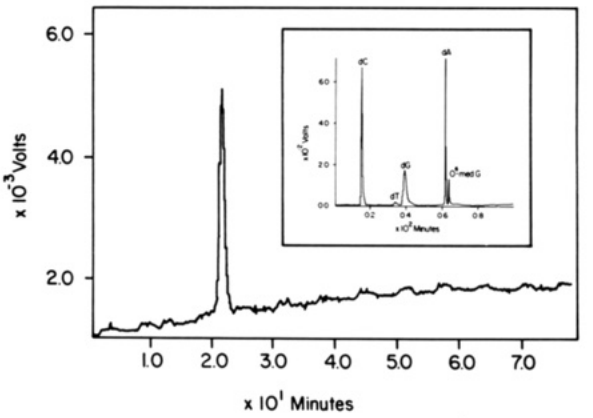

FIGURE 1: HPLC purification and analysis of an O^6-methylG-containing oligonucleotide. Chromatograph of purified, deprotected oligonucleotide (CACCAGCTGCCCACACCGCCGGCG^{me}CCCACCACCAG) is shown. The sample was chromatographed on a linear gradient of 10–15% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0, over 50 min. The inset shows the separation of nucleosides generated by nuclease and phosphatase treatment of the purified oligonucleotide as described under Materials and Methods.

and 10 ng of 5′-^{32}P-labeled control duplex were incubated together in 20 μL of reaction buffer for 30 min with an amount of enzyme insufficient to completely cleave labeled duplex. Reactions were terminated and then electrophoresed on denaturing polyacrylamide gels, as described above. Restriction enzyme binding to O^6-methylG-containing oligonucleotides was judged by inhibition of cleavage of unmodified, radiolabeled DNA, relative to controls.

Results

Construction of Duplex DNA Containing Restriction Sites with O^6-MethylG at Unique Positions. The effect of O^6-methylG on DNA cleavage by restriction enzymes was studied by using oligonucleotides of defined sequence synthesized to contain multiple recognition sequences. Five oligonucleotides of identical sequence were synthesized, each containing O^6-methylG at a different unique position. The oligonucleotides were isolated, purified, and characterized as described under Materials and Methods (Figure 1). The oligonucleotides were greater than 98% free of incorrect length sequences, and compositional analysis showed that each contained the expected number of bases (±0.1 bases) and a single O^6-methylG (±0.1).

Each oligonucleotide duplex was stable under our experimental conditions whether or not it contained O^6-methylG. Stability was defined by gel mobility (Figure 2), T_m determination (T_ms of representative O^6-methylG-containing duplexes were greater than 50 °C; not shown), and by cleavage at a second independent restriction site that did not contain O^6-methylG. For example, *Pvu*II was able to cleave duplexes containing O^6-methylG at site 1, 2, 3, or 4 (not shown), indicating that, at least during the time *Pvu*II was bound, the duplex was intact.

Effect of O^6-MethylG on Restriction Enzyme Cleavage of Recognition Sequence. Eight restriction enzymes were tested for their ability to cleave the oligonucleotide duplex with O^6-methylG at sites within and near their cognate recognition sequences. Six of these restriction enzymes could not cleave double-strand DNA when O^6-methylG was present within the recognition sequence at site 3 (Figure 3). The other two, *Aha*II and *Hae*II, weakly cleaved both strands of the double-strand oligomer (Figure 4).

The products of cleavage reactions were separated by electrophoresis on denaturing polyacrylamide gels. Higher

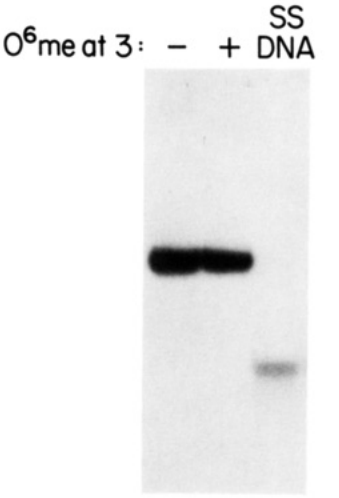

FIGURE 2: Electrophoresis of duplex DNA containing O^6-methylG at site 3. Oligonucleotides containing G or O^6-methylG at site 3 were annealed to a 5′-^{32}P-labeled 45-base complementary oligonucleotide. The duplex DNA was incubated under conditions of restriction enzyme cutting reactions, and single-strand DNA was separated from duplex DNA by electrophoresis on a 20% polyacrylamide nondenaturing gel. Product bands were visualized by autoradiography. (−) and (+) indicate the absence and presence of O^6-methylG, respectively.

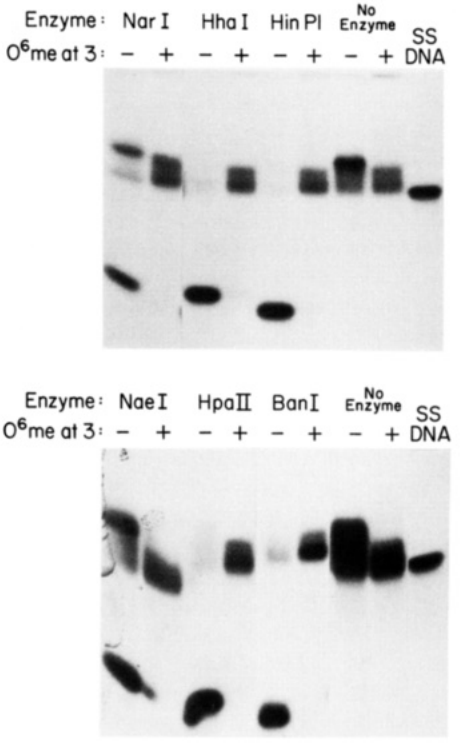

FIGURE 3: Inhibition of restriction enzyme cutting by O^6-methylG at site 3. Oligonucleotides containing G (− lanes) or O^6-methylG (+ lanes) at site 3 were annealed to a 5′-^{32}P-labeled 45-base complementary oligonucleotide. Duplexes were incubated for 1 h at 37 °C with *Nar*I, *Hha*I, *Hin*PI, *Nae*I, *Hpa*II, *Ban*I, and without enzyme. Restriction enzyme cleavage products were separated by electrophoresis on 20% polyacrylamide denaturing gels and identified following autoradiography.

molecular weight, uncleaved material ran not as a single band, as it did under native conditions (Figure 2), but as a closely spaced multimer. The multimer is caused by the high GC content of the oligomer duplex; it is not completely melted under electrophoresis conditions. Substitution of O^6-methylG for a single G in the oligomer lowers the T_m and so shifts the equilibrium toward the single-strand conformation. Cleavage of the duplex to smaller size also lowers the T_m, so cleaved

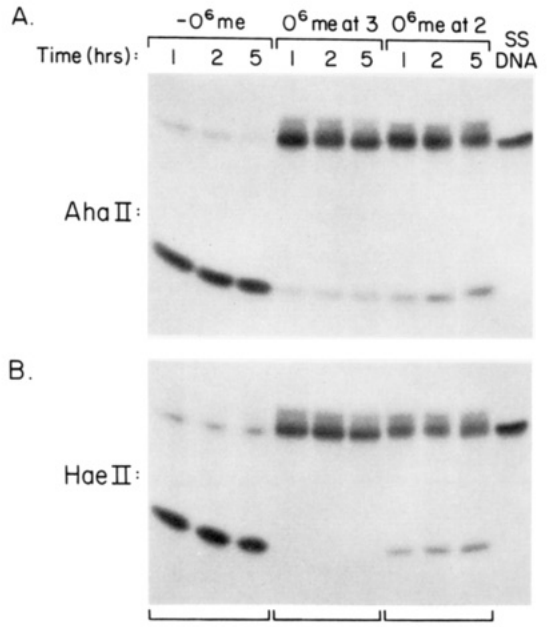

FIGURE 4: *Aha*II and *Hae*II cutting of duplexes containing O^6-methylG at site 2 or 3. Oligonucleotides containing G or O^6-methylG at site 2 or 3 were annealed to a 5′-^{32}P-labeled 45-base complementary oligonucleotide. Duplexes were incubated with *Aha*II or *Hae*II for 1, 2, or 5 h at 37 °C. Restriction products were identified following electrophoresis on 20% polyacrylamide denaturing gels and autoradiography.

products run as unique bands.

O^6-MethylG outside the recognition sequence did not interfere with restriction enzyme cleavage (only cleavage of the unmodified strand was determined for these sites). For example, although an O^6-methylG at site 2 lies 5′ adjacent to the recognition sequences for *Hin*PI and *Hha*I, these restriction enzymes cleaved their recognition sequences whether or not O^6-methylG was present at site 2 (not shown).

Removal of the methyl group at O^6 with O^6-alkylguanine–DNA alkyltransferase, a repair protein specific for O^6-methylG (Ada protein, a gift from Tomas Lindahl), restored cleavage by the appropriate restriction enzymes [for example, see Figure 6, Voigt et al. (1989)]. Therefore, inhibition of restriction enzyme cleavage was caused by O^6-methylG and not another modification resulting from oligonucleotide synthesis chemistry. Identical results were observed by using either HPLC or gel-purified oligonucleotides.

Single-Strand Nicking of the Recognition Sequence. Since recognition sequences containing mismatches can be nicked by restriction enzymes (Shenoy et al., 1986), we radiolabeled the modified (top) strand and the unmodified (bottom) strand in separate experiments and used polyacrylamide gel electrophoresis under denaturing conditions (see Materials and Methods) to determine if either strand was nicked in the presence of O^6-methylG and enzyme (Figure 5). *Hpa*II, unable to cleave DNA modified at either site 2 or 3 within its recognition sequence (see Figure 3), partially nicked the strand (about 20%) modified at site 3. *Nae*I also nicked the modified strand (about 40%) with O^6-methylG at one of three positions studied within the recognition sequence (Figure 5). In contrast, neither enzyme cleaved unmodified strand. *Xho*I showed a marked bias for nicking the modified strand at all modification sites studied (Figure 6).

To confirm that nicking was biased for one particular strand because of the O^6-methylG modification rather than other influences such as DNA sequences flanking the recognition sequence, the O^6-methylG was switched to the corresponding G of the *Xho*I recognition sequence in the other strand.

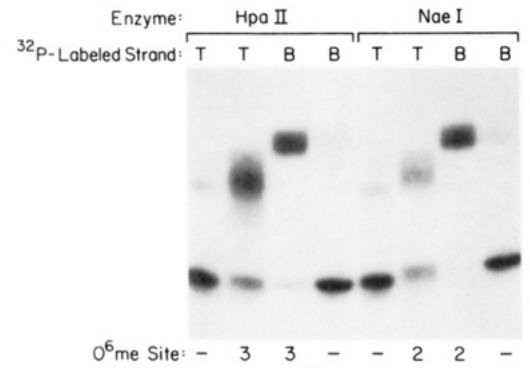

FIGURE 5: Asymmetric cleavage of O^6-methylG-containing oligonucleotides by *Hpa*II and *Nae*I. Oligonucleotide duplexes containing G or O^6-methylG at site 2 or 3 were incubated with *Hpa*II (site 3) or *Nae*I (site 2) for 1.5 h at 37 °C. Restriction products were identified following electrophoresis on polyacrylamide denaturing gels and autoradiography. Cleavage of each DNA strand was studied by using duplexes in which either the top (T) or the bottom (B) strand was 5′-^{32}P-labeled. The top and bottom strands, as well as the O^6-methylG sites, are shown in Table I.

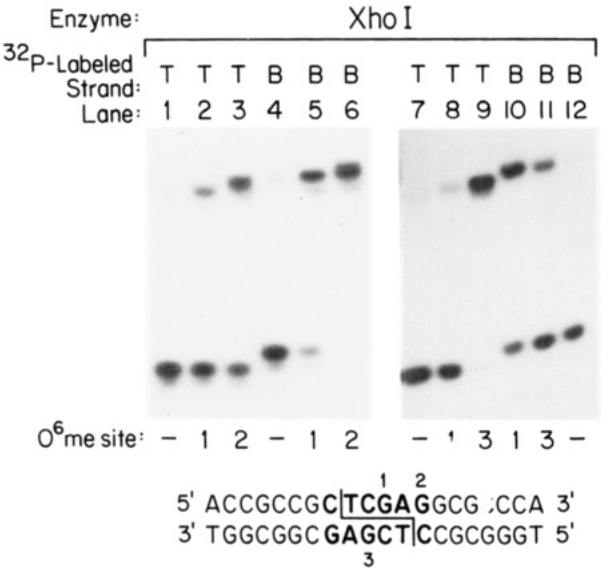

FIGURE 6: Asymmetric cleavage of O^6-methylG-containing oligonucleotides by *Xho*I. Oligonucleotide duplexes containing G or O^6-methylG at site 1, 2, or 3 were incubated with restriction enzyme *Xho*I for 1.5 h at 37 °C. Restriction products were identified following electrophoresis on polyacrylamide denaturing gels and autoradiography. Cleavage of each DNA strand was studied by using duplexes in which either the top (T) or the bottom (B) strand was 5′-^{32}P-labeled.

Nicking by *Xho*I followed the O^6-methylG modification to the opposite strand, demonstrating that the bias was dependent only on placement of the O^6-methylG modification (Figure 6).

Enzyme–Substrate Recognition. The inhibition of either double-strand cleavage or single-strand nicking at some sites could result from interference with bond scission or interference with enzyme binding. Enzyme binding to O^6-methylG-containing duplexes was determined by monitoring the ability of O^6-methylG-containing recognition sites to compete with unmodified recognition site for enzyme and thus reduce the amount of cleavage of the unmodified site (Figure 7). The results indicated that all enzymes, except *Nar*I, *Ban*I, and *Aha*II, unable to cleave their modified recognition site were also unable to bind it. However, low levels of binding may have gone undetected. *Nar*I, *Ban*I, and *Aha*II showed significant amounts of binding even though they could not cleave DNA (Figure 7).

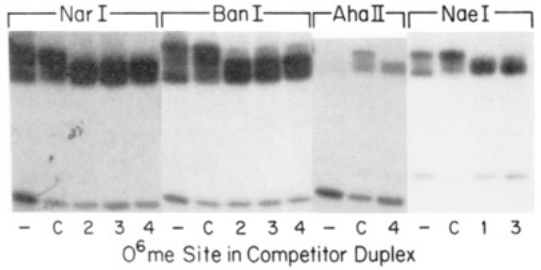

FIGURE 7: Binding of *Nar*I, *Ban*I, *Aha*II, and *Nae*I to duplexes containing O^6-methylG. Control oligonucleotide without O^6-methylG was annealed to a 5′-^{32}P-labeled 45-base complementary oligonucleotide. This duplex was incubated with *Nar*I, *Ban*I, *Aha*II, or *Nae*I for 30 min at 37 °C in the absence of unlabeled competitor duplex (−), in the presence of unlabeled control duplex (C), or in the presence of unlabeled duplex containing O^6-methylG at site 2, 3, or 4. Restriction products were identified following electrophoresis on 20% polyacrylamide denaturing gels and autoradiography. The decrease in intensity of (−) band by competition with control (C) and modified DNAs (lanes 1–4) indicates binding of *Nar*I, *Ban*I, and *Aha*II enzymes to modified DNA; no attenuation of (−) lane band intensity is seen by modified DNAs (lanes 1 and 3) for *Nae*I.

Discussion

We have placed a methyl group at the O^6 position of single guanines in the recognition sequences of several restriction enzymes to probe their interaction with enzyme. The ability of enzyme to bind and cleave hemimethylated cognate recognition site was determined, and the results are summarized in Table I.

O^6-MethylG Inhibits Restriction Enzyme Cleavage. All enzymes studied that recognized a unique sequence (*Hpa*II, *Hha*I, *Hin*PI, *Nae*I, *Nar*I, and *Pvu*II) were unable to their recognition sites containing O^6-methylG in place of any one G. *Pst*I (Green et al., 1984; Wu et al., 1987) and *Pvu*II (Wu et al., 1987) are also inhibited by O^6-methylG, at least at the one position tested in their recognition sequences.

*Aha*II, *Hae*II, and *Ban*I each recognize multiple recognition sequences. The relaxed recognition specificity of *Aha*II and *Hae*II allowed for slow cleavage of several O^6-methylG-containing duplexes. This relaxed specificity may also be reflected in the finding that both *Aha*II and *Ban*I were able to bind but not cleave O^6-methylG-containing duplexes. Restriction enzymes with relaxed recognition specificities can also cleave when a mismatch is present within the recognition sequence (Jiricny & Martin, 1986). This ability to recognize mismatch and O^6-methylG-containing DNA may result from a decreased potential for hydrogen-bond formation between enzyme and recognition sequence.

Differences in cleavage also appeared dependent on relative placement of O^6-methylG within the recognition sequence. For example, *Aha*II was able to partially cleave its recognition sequence containing O^6-methylG at site 3 (site adjacent to cleavage site) less efficiently than when it contained O^6-methylG at site 2 (Figure 4). These findings support those of Bodnar et al. (1983), who found that structural alterations in base pairs immediately adjacent to the phosphodiester bonds cleaved by the enzymes reduced the rate of cleavage most dramatically.

Enzyme–DNA Binding. Our qualitative studies of the ability of the enzymes to bind to their modified cleavage sites indicated, on the one hand, that *Hin*PI and *Hha*I, which both recognize GCGC, could not measurably bind their recognition sites containing O^6-methylG at any position. This explains the lack of enzymatic cleavage of these recognition sites when containing O^6-methylG; apparently these enzymes use G-O^6 as a recognition signal.

Table I: Summary of Restriction Enzyme Binding and Cleavage of Duplexes Containing O^6-MethylG in Place of G at Positions 1–5

enzyme (recog seq)[a]	cleavage activity[b]					
O^6methylG position[c]:	none	1	2	3	4	5
Hpa II C/CGG (23[d])	+	+	-/-[e]	+/-	+	n.d.
Hin PI G/CGC (3 4)	+	+	+	-/-	-/-	n.d.
Hha I GCG/C (3 4)	+	+	+	-/-	-/-	n.d.
Nae I GCC/GGC (1 23)	+	-/-	+/-	-/-	+	n.d.
Nar 1 GG/CGCC (23 4)	+	+	-/-	-/-	-/-	n.d.
Pvu II CAG/CTG (5)	+	+	+	+	+	-
Aha II GU/CGYC (23 4)	+	+	w/w	w/w	-/-	n.d.
Ban I G/GYUCC (2 3 4)	+	+	-/-	-/-	-/-	n.d.
Hae II UGCGC/Y (23 4)	+	+	w/w	-/-	-/-	n.d.

[a] Recognition sequences are for the enzymes listed and are found within the sequence in *c* below. [b] +, complete cleavage; w, weak cleavage, less than 10% cleavage (relative to control DNA); −, no cleavage; n.d., cleavage not determined. Nicking of modified strand by *Hpa*II with O^6-methylG at site 3 and by *Nae*I with O^6-methyl G at site 2 was 20% and 40%, respectively. [c] The position numbers indicated in the table refer to the positions of individual bases replaced by O^6-methylG as indicated in

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          5              1  23 4
    5'CACCAGCTGCCCACACCGCCGGCGCCCACCACCAG 3'
3' TCGAGTGGTCGACGGGTGTGGCGGCCGCGGGTGGTGGTCGATC 5'
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[d] Sequence numbers correspond to methylation positions; multiple-numbered positions refer to multiple oligonucleotide duplexes with only one modified site each. [e] To determine if both strands were nicked within the enzyme recognition sequence, each of the strands of the O^6-methylG-containing duplexes was labeled in turn and tested for restriction enzyme nicking. Before the slash indicates relative amount of cleavage of the top, modified strand (see sequence in *c*); after the slash indicates same for bottom, unmodified strand. Only nicking of the unmodified strand was tested for O^6-methylG outside the enzyme recognition sequence. Circled entries indicate modified sites that did not measurably bind enzyme, as defined in Figure 7; binding of *Hpa*II to its recognition site with O^6-methylG at position 2 was not determined (indicated by broken circle).

On the other hand, *Ban*I and *Nar*I, which both recognize GGCGCC in the oligonucleotide duplexes studied, bound substrate without cleavage with O^6-methylG in place of any G in the recognition sequence. This suggests that these two enzymes do not probe G-O^6 for recognition even though GCGC is contained within their recognition sequence. Between these two extremes fell *Nae*I, *Aha*II, and *Hae*II. These enzymes appear to probe O^6 at some positions but not at others (Table I).

In addition to blocking a potential hydrogen-bond acceptor, the methyl groups at O^6 causes a small local structural distortion to the oligonucleotide duplex (Patel et al., 1986). This distortion may also contribute to the inability of enzyme to bind or cleave substrate. Either way, it is interesting that several restriction enzymes, recognizing sequences that overlap within the same oligonucleotide duplex, respond so differently to an O^6-methylG within that overlapping region. Thus, in agreement with Bodner et al. (1983), different sequences apparently call for different strategies for proteins to recognize and to distinguish them from all other sequences.

Strand Cleavage Asymmetry. *Hpa*II, *Nae*I, and *Xho*I nicked the modified strand, but not the unmodified strand,

when O^6-methylG replaced G at some positions within their recognition sequences (both possible positions for *Xho*I). When the O^6-methylG was switched to the same G position in the complementary strand, nicking by the enzyme also switched to the complementary strand. Thus, the bias for nicking a particular strand observed here is unrelated to strand preferences caused by flanking sequences (Baumstark et al., 1979).

The molecular basis for this cleavage asymmetry, which varied not only with the enzyme tested but also with the position of the O^6-methylG, is not clear since the functional groups in the major and minor grooves that are important for binding and cleavage by each of these enzymes have not been identified.

Registry No. *Hpa*II, 81295-25-2; *Hin*PI, 95229-16-6; *Hha*I, 81295-20-7; *Nae*I, 90463-50-6; *Nar*I, 93586-00-6; *Pvu*II, 81295-34-3; *Aha*II, 92228-42-7; *Ban*I, 85876-07-9; *Hae*II, 81295-17-2; 5′-tritylated oligonucleotide, O^6-methylG at site 1, 124441-21-0; 5′-tritylated oligonucleotide, O^6-methylG at site 2, 124441-22-1; 5′-tritylated oligonucleotide, O^6-methylG at site 3, 124441-23-2; 5′-tritylated oligonucleotide, O^6-methylG at site 4, 124441-24-3; 5′-tritylated oligonucleotide, O^6-methylG at site 5, 124441-25-4; oligonucleotide, O^6-methylG at site 1, 124441-26-5; oligonucleotide, O^6-methylG at site 2, 124441-27-6; oligonucleotide, O^6-methylG at site 3, 124441-28-7; oligonucleotide, O^6-methylG at site 4, 124441-29-8; oligonucleotide, O^6-methylG at site 5, 124441-30-1; 5′-CTAGCTGGTGGTGG-GCGCCGGCGGTGTGGGCAGCTGGTGAGCT, 124441-31-2; duplex, O^6-methylG at site 1, 124441-32-3; duplex, O^6-methylG at site 2, 124441-33-4; duplex, O^6-methylG at site 3, 124441-34-5; duplex, O^6-methylG at site 4, 124441-35-6; duplex, O^6-methylG at site 5, 124441-36-7.

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