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HhaI methylase and restriction endonuclease as probes for B to Z DNA conformational changes in d(GCGC) sequences

Wolfgang Zacharias, Jacquelynn E.Larson, Michael W.Kilpatrick and Robert D.Wells

Department of Biochemistry, Schools of Medicine and Dentistry, University of Alabama, University Station, Birmingham, AL 35294, USA

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ABSTRACT

The capacity of the modification methylase (MHhaI) and restriction endonuclease (HhaI) from Haemophilus haemolyticus to methylate and cleave, respectively, recognition sites which are in right-handed B or left-handed Z structures was determined in vitro. Plasmids containing tracts of (dC-dG) as well as numerous individual d(GCGC) sites distributed around the vector were studied. Negative supercoiling was used to convert the (dC-dG) tracts (~ 30bp in length) from a right-handed to a left-handed conformation. (Methyl-^{3}H)-SAM was used to localize and quantitate modified d(GCGC) recognition sites, whereas cleavage by HhaI was used to detect unmethylated sites. In the left-handed Z-form, the (dC-dG) blocks were not methylated by MHhaI and not cleaved by HhaI. A two-dimensional gel analysis of a family of 33 topoisomers treated with MHhaI revealed that the lack of methylation in the (dC-dG) blocks was directly correlated to the supercoil-induced B to Z transition in these segments. These results are significant with respect to enzyme-DNA interactions in general and provide the basis for using HhaI and MHhaI as probes for different DNA structures and conformational transitions under physiological conditions.

INTRODUCTION

Left-handed Z-DNA has been found to occur in suitable sequences in recombinant plasmids, restriction fragments, and DNA polymers (reviewed in 1-4). Supercoiling at densities known to exist under physiological conditions induces the B to Z transition (1, 2, 4-13). A variety of other conditions are also effective in stabilizing left-handed structures (1-4, 14-16). The formation of a left-handed helix within a larger DNA which is otherwise right-handed necessitates the formation of two junctions; the properties of these helical interfaces have been investigated by their sensitivity to S_1 and BAL31 nucleases (1, 5, 8, 12, 13, 17). Sequences shown to adopt left-handed structures include (dG-dC)•(dG-dC), (dT-dG)•(dC-dA) (18, 19, 10-12 and reviewed in 1-4), intervening sequences from human fetal globin genes which appear to be recombination hotspots (13), and enhancer sequences in SV40 (20). Thus, the concept of DNA microheterogeneity (reviewed in 21) seems firmly established.

Prior studies with the DNA polymer containing alternating G and 5-methyl C moieties (22-26) and recombinant plasmids and restriction fragments containing (dC-dG) blocks with cytidine replaced by 5-methyl cytidine in the HhaI sites (GCGC) (9) demonstrated the powerful influence of methylation on stabilizing the Z conformation. Complete methylation of the (dC-dG) tracts in restriction fragments and plasmids reduces the concentration of salt ($MgCl_2$ or NaCl) or supercoiling required to induce left-handed Z DNA (9). The unusual concentration dependent dualistic behavior of NaCl on the B-Z transition led to the proposal (9) that methylation may serve as a triggering mechanism for Z DNA formation in supercoiled DNAs. Methylation may be involved in eucaryotic gene regulation (reviewed in 27-29).

Little is known about the biological properties of left-handed Z-DNA; the sole *in vivo* experimental observation (30) is that plasmids containing sequences capable of adopting left-handed Z-structures are prone to deletions and seem to enhance rec A-mediated recombination. Other contributions have proposed a role for left-handed DNA in recombination (13, 31, 32). However, speculations of biological properties of Z-DNA include virtually every function of DNA (reviewed in 1-4).

We have tested the capability of the *Haemophilus haemolyticus* methylase and restriction endonuclease (33, 9) to recognize and methylate or cleave, respectively, GCGC sites in tracts of (dC-dG) in B or Z structures in plasmids. The left-handed conformation is not a substrate for these enzymes. This result is significant from enzyme mechanistic and biological standpoints as well as for establishing a sensitive probe for left-handed helices.

While this manuscript was being completed, a paper appeared (34) containing some of the observations reported herein.

MATERIALS AND METHODS

DNAs

Plasmids pRW451 (gift of G. Staffeld, this lab) (35), pRW751 (35,30) and pRW756 (gift of D. Kang, this lab) (30) were isolated according to published procedures (35, 30). Topoisomer families with average negative superhelical densities of 0.09 ± 0.01 for the highly supercoiled samples and of 0.02 ± 0.01 for the low supercoiled samples (hereafter designated High and Low, respectively) were prepared by the topoisomerase/ethidium bromide technique (36).

Enzymes

The restriction enzymes BamHI, AvaI, PstI, and HhaI were from Bethesda

Research Labs. The MHhaI was isolated by J. Klysik in this lab (9). In addition, the MHhaI from New England Biolabs was used in some of the experiments. Calf thymus topoisomerase I (gift of N. P. Higgins, this department) and the wheat germ topoisomerase I (gift of R. Burgess, Univ. of Wisconsin) were used as described (36).

Chemicals

S-(methyl-^{3}H)-adenosyl-L-methionine (^{3}H-SAM) (specific activity 80 Ci/mMol) and Aquasol-2 were obtained from New England Nuclear, whereas the unlabeled SAM and chloroquine were from Sigma. Low melting temperature agarose was obtained from Seaplaque.

Kinetics of MHhaI Reaction

75µl reaction mixtures at 22°C contained 100mM Tris-HCl (pH 7.8), 10 mM EDTA, 20 mM dithiothreitol, 0.3 µg DNA, 108 pmoles ^{3}H-SAM, and 22 U of MHhaI. 5 µl samples were taken at intervals and the incorporation of radioactive methyl groups was monitored by acid insolubility (37).

Quantitation of Methylated Sites in Segments of pRW751 and pRW451

112 µl reaction mixtures were as described above. 7 µl samples were taken at time intervals and heated to 55°C for 10 minutes to inactivate the methylase. These 7 µl samples were brought to 28 µl for the BamHI-AvaI double digest with a final concentration of $MgCl_2$ at 20 mM and NaCl at 50 mM. Each sample contained 2 U AvaI and 6 U BamHI and, as markers for the gels, 0.6 µg of either pRW451 or pRW751 which were both unmethylated and unlabeled. After 2 hrs. at 37°C, the reactions were stopped with a bromophenol blue mixture containing 2% SDS and were electrophoresed on 2% agarose gels at 120 V in buffer containing 40 mM Tris-acetate, 20 mM sodium acetate and 2.2 mM EDTA (pH 8.3). The marker bands were visualized with ethidium bromide and ultraviolet light. The gel slices were excised, melted, solubilized in Aquasol-2, and counted in a scintillation counter to determine the incorporation of tritiated methyl groups in each fragment.

HhaI Digests after MHhaI Reactions

54 µl reaction mixtures at 22°C contained 100 mM Tris-HCl, pH 7.8, 10 mM EDTA, 20 mM dithiothreitol, 4 µM SAM, 17 pmoles ^{3}H-SAM, 30 U of MHhaI, and 0.8 µg of pRW756. Incorporation of ^{3}H-labeled methyl groups was monitored to ensure that the reactions were complete. Reaction mixtures were heated to 55°C for 10 min. to inactivate the methylase. Both mixtures were made 20 mM in $MgCl_2$. The highly supercoiled DNA was relaxed with calf thymus topoisomerase I for 10 min. at 22°C. Portions of each reaction mixture were adjusted to 125 mM Tris-HCl, 25 mM $MgCl_2$, 50 mM NaCl, and either 6 U of HhaI

or 6 U of HhaI plus 9 U of PstI per 0.12 µg of DNA. After digestion for 0.5 hrs. at 37°C, electrophoresis was performed on a 6 x 8 cm 1% agarose gel at 50 V for 70 min.

Two-dimensional Gel Electrophoresis of Topoisomers

A family of topoisomers was generated as described (36). These topoisomers were subjected to methylation essentially as described above. The methylation reaction contained 5 µg DNA, 1 µM ^{3}H-SAM, 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, 20 mM DTT, pH 8.2, and was carried out at 37°C for 6 hrs. 2 µg of the family of methylated topoisomers was electrophoresed in two dimensions essentially as described (13) but using Tris-borate buffer to ensure consistency between the methylation and electrophoresis conditions.

Determination of the Relative Radioactivity of Restriction Fragments from Individual Methylated Topoisomers

Individual methylated topoisomers separated in the two-dimensional electrophoresis system were excised from the gel, and the DNA extracted essentially as described (38). DNA recovered by ethanol precipitation was redissolved in 50 µl of 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.5, and digested with AvaI and BamHI. The resulting fragments were separated on a 2% agarose gel. Each fragment was cut from the gel, solubilized, and its activity determined by liquid scintillation counting.

RESULTS

Strategy of the Study

The tetranucleotide sequence d(GCGC) in its right-handed B-conformation is the substrate for MHhaI which attaches a methyl group to the 5-position of the internal cytosine in each strand. Thus, the DNA of Haemophilus haemolyticus is protected from cleavage by its own restriction enzyme HhaI, which cleaves the same sequence at the 3' side of the internal guanosine residue.

It has been shown (8) that the alternating sequence (dC-dG) of 10bp or longer has the potential to exist in a left-handed helix conformation in a recombinant plasmid. However, it is uncertain if a short tetramer, like this HhaI recognition site, can adopt a Z-form when imbedded in a right-handed B-helix. For this study, we used stretches of (dC-dG) ~ 30bp in length incorporated into plasmid vectors (Fig. 1). These (dC-dG) blocks can be converted into a Z-form by the torsional stress of supercoiling (1-13) without requiring chemical agents or salt/solvent conditions that interfere with the activity of the enzymes used. Extensive studies in this lab with supercoil

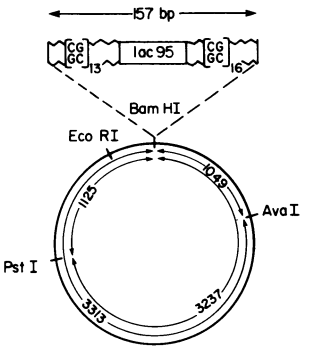

Figure 1: Sequence features of the recombinant plasmids used: pRW751: shown above (35); pRW451: same as pRW751, except that the 157bp BamHI insert was replaced by a 174bp HhaI fragment from pBR322 (35); pRW756: a part of the 157bp insert in pRW751 (95bp lac region plus 32bp dC-dG block) was inserted into the EcoRI - BamHI linearized pBR322 vector. Therefore, the EcoRI - BamHI segment and the 26bp dC-dG block were eliminated (30).

relaxation in one (39, 9) and two dimension electrophoresis (12, 13), S_1 nuclease cleavage (1-3, 8, 12, 13, 17), and antibody binding experiments (13, 19), have revealed that the (dC-dG) blocks in pRW751 and pRW756 are in a left-handed conformation at the natural superhelical density of the plasmid and under physiological conditions.

The reaction of MHhaI with these plasmids was quantitated and localized by two different methods: A) The use of ^{3}H-SAM as substrate allows quantitation and, after digestion with a suitable restriction enzyme, localization of methylated sites. B) The modification with methyl groups of the recognition site makes it resistant to HhaI; therefore, any inhibition of cleavage by HhaI indicates methylation.

Kinetics of Methylation

The kinetics of methylation of four DNA samples (pRW751 High and Low, pRW451 High and Low as defined in Materials and Methods) with MHhaI and ^{3}H-SAM were determined (Fig. 2). Table 1 compares the total number of d(GCGC) sites present in each of the two plasmids (left column) with the number of susceptible sites expected, assuming that the d(GCGC) sites in a left-handed region are not a substrate for MHhaI (right column).

If the two (dC-dG) blocks of the 157bp insert in pRW751 were not methylated when in a left-handed conformation, the total incorporation into pRW751 High and pRW451 High should be the same, whereas the total incorporation into pRW751 Low relative to pRW451 Low should be increased by a

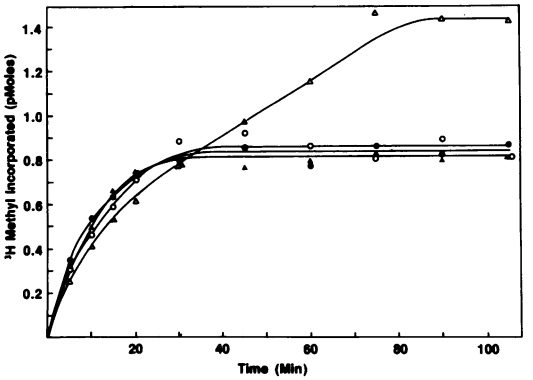

Figure 2: Influence of supercoil density on the kinetics of methylation of pRW751 and pRW451 by MHhaI as determined by the incorporation of ^{3}H-methyl groups from SAM as a function of time into the following DNAs: pRW451 High, ●; pRW451 Low, ▲; pRW751 High, o; and pRW751 Low, Δ.

Table 1: Number of HhaI sites available in segments of pRW451 and pRW751, assuming that the recognition sites in a left-handed region do not serve as substrates.

DNA	Superhelical Density	
	-0.02 ± 0.01 (Low)	-0.09 ± 0.01 (High)
pRW451	31	31
pRW751	59	31
pBR322 vector	31	31
174bp BamHI insert (in pRW451)	0	0
157bp BamHI insert (in pRW751)	28	0
3313bp fragment (AvaI - BamHI)	20	20
1049bp fragment (BamHI - AvaI)	11	11

factor of 59/31=1.9 (Table 1). If the (dC-dG) blocks were methylated independent of their helix sense, the incorporation into pRW751 High relative to pRW451 High should be increased by the same factor of 1.9, and the incorporation should be the same for both pRW751 High and pRW751 Low.

Fig. 2 shows that the total incorporation into pRW751 Low was increased by approximately 90% relative to all other DNAs. The final level of incorporation into pRW751 High was the same as for pRW451 High. This indicates that the number of modified recognition sites in this topological form of pRW751 was the same as for pRW451 (Table 1). The amount of incorporation of ^{3}H-methyl groups is in excellent agreement ($\pm<10\%$) with the theoretically expected values based on the available number of substrate sites (Table 1), thus indicating that the plasmids were fully methylated. These results are in agreement with the interpretation that the left-handed blocks in pRW751 High were not methylated.

In addition, an increased superhelicity of the plasmids by itself did not have any detectable effect on the efficiency of the MHhaI reaction since the incorporation into pRW451 High and pRW451 Low was the same.

Localization of Modified Sites

For a more specific quantitation as well as localization of the ^{3}H-methyl groups at the HhaI sites in these plasmids, the radioactivity was determined individually in the 157bp or 174bp BamHI inserts of pRW751 and pRW451, respectively, and the vector DNAs. However, since the two BamHI sites in pRW751 High are strongly inhibited because of the influence of the neighboring Z-form blocks (8, 40), it was necessary to linearize this plasmid first with a single cutting enzyme (AvaI) after complete methylation and before BamHI cleavage. The three DNA components resulting from this double digest (see also Table 1) were separated by gel electrophoresis, and the ^{3}H-incorporation in each of these fragments was determined. This procedure provides a measure of ^{3}H-incorporation exclusively into the two (dC-dG) blocks of the 157bp fragment in pRW751 (the central 95bp segment between the blocks does not contain any HhaI sites) as well as into the 174bp insert in pRW451 (which also does not have any HhaI sites) as background control (Fig. 1 and Table 1).

Fig. 3 shows that, as expected, no incorporation was observed into the 174bp insert of pRW451 at both superhelical densities, since no HhaI sites are present in this fragment. The ratio of incorporation into the two large vector fragments (3313bp versus 1049bp) was found to be the same for pRW751 and pRW451 both at high and low superhelicity, reflecting the ratio of HhaI

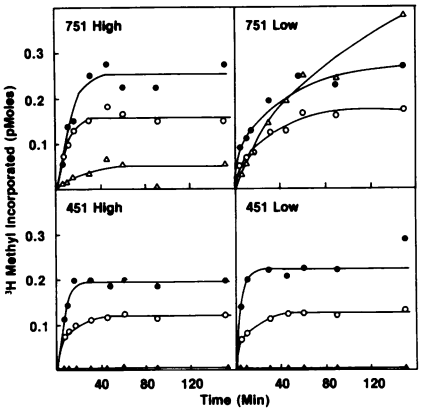

Figure 3: Quantitation of methylated sites in different segments of pRW751 and pRW451 at low and high superhelical densities. The following fragments were generated by AvaI - BamHI double digests of the plasmids (see also Fig.1, Table 1, and Materials and Methods): ●, 3313bp fragment containing 20 HhaI sites; ○, 1049bp fragment containing 11 HhaI sites; Δ, 157bp fragment from pRW751 containing 28 HhaI sites; and Δ, 174bp fragment from pRW451 containing no HhaI sites.

sites (20 versus 11) in these fragments. This again shows that the superhelical density itself in this range does not have any effect on the reactivity of individual HhaI sites in the vector.

The highest incorporation was found in the 157bp fragment of pRW751 Low; approximately 1.4 times more ^{3}H-label than in the 3313bp segment was detected. The corresponding ratio of HhaI sites (157bp versus 3313bp) is 28 versus 20, or 1.40. Similarly, about 2.5 times more ^{3}H-label than in the 1049bp segment was found; the ratio of sites in this case (157bp versus 1049bp) is 28 versus 11, or 2.54.

A low but reproducible incorporation into the 157bp fragment of pRW751 High was found which was not expected. The reason for this incorporation could be nicking of the supercoiled plasmid during the reaction, the influence of B-Z junctions, or a slow B-Z equilibrium (see Discussion).

In summary, the good agreement between the ratios of HhaI sites and the experimentally determined ratios of incorporation (Fig. 2) indicates that all sites in pRW751 Low including the (dC-dG) blocks of the insert were completely methylated. The results of this experiment confirm that the lack of methylation in pRW751 at high superhelicity is located in the left-handed blocks of the 157bp insert, whereas all other recognition sites in the vector segments and in pRW451 are not inhibited by high supercoil densities.

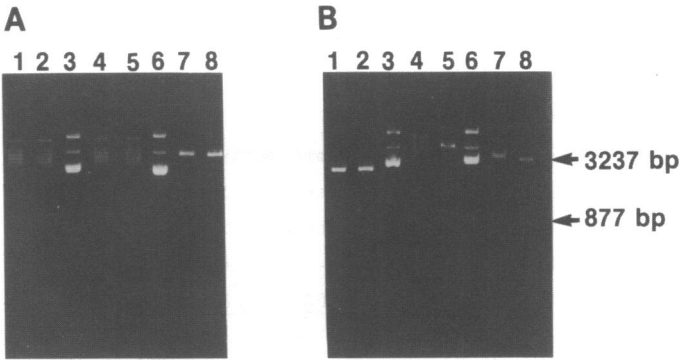

Figure 4: HhaI restriction enzyme susceptibility as a probe for resistance to methylation of the left-handed (dC-dG) block in pRW756.
A. Low supercoiled pRW756 (lane 1), which is methylated (lane 2), adjusted to 20 mM in $MgCl_2$ (lane 4), treated with HhaI (lane 5) and treated with both HhaI and PstI (lane 7). Lanes 3 and 6 are markers of pRW751 showing, from top to bottom, the nicked, linear, and supercoiled forms. Lane 8 shows the original DNA treated with PstI to indicate the gel pattern that would result if the DNA were insusceptible to HhaI.
B. High supercoiled pRW756 (lane 1), which is methylated (lane 2), adjusted to 20 mM $MgCl_2$ and relaxed with calf thymus topoisomerase I (lane 4), treated with HhaI (lane 5), and treated with both HhaI and PstI (lane 7). Lanes 3 and 6 are as above. Lane 8 is relaxed and unmethylated pRW756 treated with BamHI and PstI to indicate the gel pattern that would result if the (dC-dG) block in pRW756 were susceptible to HhaI.

Resistance to HhaI Cleavage after Methylation

Digestion of a plasmid with HhaI after complete methylation with MHhaI should reveal the presence of non-methylated d(GCGC) recognition sites in this plasmid, since only these sites can be cleaved by HhaI. This test was performed with pRW756 which contains a single (dC-dG) tract (see Fig. 1).

Fig. 4 shows that after methylation (Panel A, lane 2) of pRW756 Low (Panel A, lane 1), complete resistance to HhaI was found (Panel A, lane 5). Methylation of pRW756 High (Panel B, lane 2) left the left-handed (dC-dG) block unmodified and therefore susceptible to restriction by HhaI as expected. After relaxation of pRW756 High with topoisomerase I in order to revert the (dC-dG) block back to a right-handed form (Panel B, lane 4), a HhaI digest generated a vector fragment of 4082bp (Panel B, lane 5) as expected.

In order to map the regions in pRW756 High that were susceptible to HhaI cutting after methylation, a second digest with the single cutting restriction enzyme PstI was done. For pRW756 Low, only one band corresponding to the PstI-linearized plasmid was observed (Panel A, lane 7). For pRW756 High, two fragments were generated whose sizes indicated that the initial cleavage by

HhaI (Panel B, lane 5) was located approximately 850bp from the PstI site, i.e., at or inside the (dC-dG) block (Panel B, lane 7). The occurrence of only two fragments with the expected sizes after HhaI-PstI double digestion showed that the left-handed block in pRW756 High was the only region that was cleaved by HhaI and therefore initially not methylated by MHhaI.

Thus, these results confirm the interpretations from the other determinations that the left-handed tracts of (dC-dG) are not methylated by MHhaI whereas the right-handed GCGC sites are methylated.

Lack of Cleavage by HhaI of Left-handed (dC-dG) Blocks

The types of studies described in Fig. 4 enabled the determination of the inability of HhaI to cleave (dC-dG) blocks in a left-handed Z-helix. It was shown in the previous section that pRW756 High, after methylation and relaxation with topoisomerase I, could be linearized by HhaI at the (dC-dG) block (Fig. 4B, lane 5). However, when the same DNA sample, again after methylation, was digested with HhaI without prior relaxation, no cleavage of the highly supercoiled DNA was observed (data not shown); the gel pattern was identical to Fig. 4B, lane 2. The same results were obtained when pRW751 High was used instead of pRW756 High.

It was shown in the previous and subsequent sections that the left-handed (dC-dG) blocks were not methylated. Therefore, we conclude that the (dC-dG) segments are not cleaved by HhaI as long as they are maintained in a Z-form by the high superhelicity of the plasmid.

Analysis of Individual Methylated Topoisomers of pRW751

The ability to separate topoisomers of pRW751 in a two-dimensional gel electrophoresis system (4) enabled the study of methylation of the plasmid at the level of the individual topoisomer. A family of topoisomers of pRW751 was methylated (under electrophoresis buffer conditions) using MHhaI and ^{3}H-SAM, then separated in the two-dimensional gel electrophoresis system (Fig. 5). Two distinct relaxations correponding to the B to Z transitions of the 32bp (dC-dG) block and the 26bp (dC-dG) block were seen. The relaxation of about 5 supercoils observed for topoisomers -15 through -19 agrees well with the 5.7 superhelical turns that should be relaxed when 32bp of alternating (dC-dG) undergo a B to Z transition, and the relaxation of an additional 4.5 supercoils observed for topoisomers -22 onward agrees well with the relaxation of 4.7 superhelical turns expected when a 26bp block of alternating (dC-dG) undergoes a B to Z transition. This assumes that the methylated (dC-dG) region is in a 10.4bp/turn B helix when right-handed and in a 12.0bp/turn Z helix when left-handed.

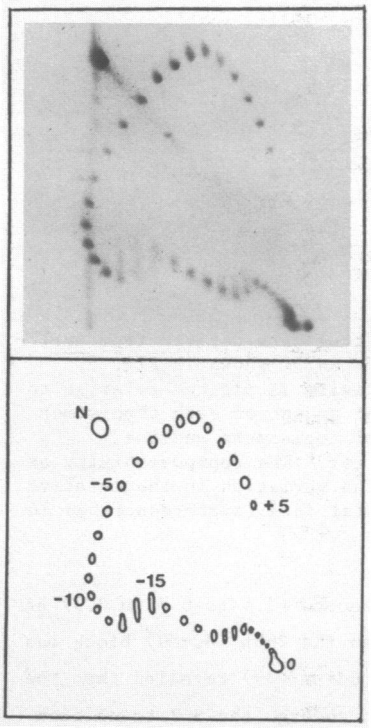

Figure 5: Two-dimensional gel electrophoresis of methylated topoisomers of pRW751. The top panel shows individual topoisomers visualized under UV light after staining with ethidium bromide. The first dimension is from top to bottom, the second dimension from left to right. The bottom panel is the key to the top panel; the numbers designate the number of superhelical turns in each topoisomer (τ). N = nicked plasmid. Experimental details are in Materials and Methods.

In order to study the extent of methylation of the topoisomers, each was eluted from the gel and restricted with BamHI and AvaI. The resulting fragments were then separated on a 2% agarose gel, excised and the radioactivity determined. Fig. 6 is a plot of the activity of the 157bp BamHI-BamHI fragment and the 1049bp BamHI-AvaI fragment relative to the 3313bp BamHI-AvaI fragment. The 1049bp fragment shows the same level of methylation, throughout the range of topoisomers studied, of around 0.55 relative to the 3313bp fragment, as expected from the ratio of HhaI sites (11 versus 20) in these fragments. For the 157bp fragment however, the initial level of methylation of around 1.4 drops to about 0.7 for topoisomers -19 to -24 and then to a level of approximately 0.3 for topoisomers -25 and higher. The initial level of 1.4 is that which would be expected from the ratio of the HhaI sites in the 157bp and 3313bp fragments (28 to 20) (see also Fig. 3). The biphasic drop in extent of methylation of the 157bp fragment reflects well the behavior that would be expected if firstly the 32bp (dC-dG) block, and then the 26bp (dC-dG) block is undergoing the B to Z transition and consequently being rendered insusceptible to methylation by MHhaI. The

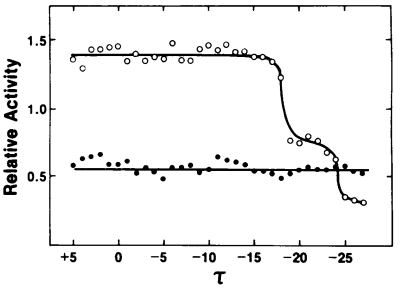

Figure 6: Relative radioactivity of restriction fragments derived from AvaI - BamHI digestion of the methylated topoisomers of pRW751 shown in Fig. 5. o, 157bp fragment; •, 1049bp fragment. Radioactivity is plotted relative to the 3313bp fragment to compensate for the unequal amount of each topoisomer due to the method of preparation of the family of topisomers and the possibility of unequal recovery of DNA from the gel. The reproducibility of the data is estimated as ± 0.1 as deduced from the variation in the relative radioactivity of the 1049bp fragment. Experimental details are described in Materials and Methods.

initial drop in methylation is larger than the second (1.4 to 0.7 to 0.3) as expected if first the 32bp (dC-dG) block and then the 26bp (dC-dG) block was rendered insusceptible to methylation. Prior studies (39) revealed that the 32bp (dC-dG) segment required less perturbant to undergo the B-Z transition than the 26bp segment in pRW751.

The low but significant level of methylation observed even for topoisomers - 25 and higher could be a reflection of the B to Z equilibrium in the (dC-dG) blocks of the supercoiled plasmid (see Discussion). Interestingly, the biphasic drop in the extent of methylation is offset by about 5 topoisomers from the transitions observed on the two-dimensional gel (Fig. 5). This was expected from the previous observation (8) that methylation of pRW751 using MHhaI lowers the superhelical density required for the B to Z transition of the (dC-dG) blocks by about 4 superhelical turns.

DISCUSSION

These results demonstrate that the modification enzyme MHhaI has a very strong preference for the right-handed helix conformation of its recognition sequence d(GCGC) as shown by two independent methods: (A) use of ^{3}H-SAM to localize and quantitate methylated Hha sites; and (B) use of the HhaI restriction enzyme to detect unmethylated recognition sites. Thus, the left-handed Z-form structure of the same sequence is not a substrate for either the HhaI methylase or the HhaI restriction endonuclease.

Interestingly, a low but significant amount of methylation in the (dC-dG) blocks of pRW751 High was observed under conditions where these (dC-dG) tracts are known to be completely left-handed (1, 5, 8, 12, 13). This amount of methylation was higher than the incorporation into the 174bp region of pRW451 High or Low which served as a background control. This effect is not due to a slow reaction between MHhaI and these Z form blocks for the following reasons: (A) ^{3}H-incorporation into the 157bp fragment of pRW751 High levels off after 40 to 45 minutes. Control experiments (data not shown) showed that the methylase maintained its activity over a period of several hours. Therefore, this kinetic profile reflects a true saturation of incorporation; it excludes a slow but steady reaction between MHhaI and the Z-form (dC-dG) blocks. (B) The incorporation data in Fig. 3 were corrected for small amounts of nicked or relaxed molecules which were present in the highly supercoiled samples or which were generated during the reaction. Therefore, this unexpected incorporation cannot be explained by a contribution of nicked molecules which are completely right-handed.

We suggest that the low extent of methylation in the 157bp region of pRW751 High is due to the B-Z dynamic equilibrium. In the topoisomer family of highly supercoiled pRW751, all (dC-dG) blocks are predominantly in a Z-form which makes them insusceptible to methylation. However, since the B-Z transition is reversible, each molecule also spends some limited time with the blocks in a right-handed form. At this stage, MHhaI binds and methylation can be initiated. The lifetime of the (dC-dG) blocks in a right-handed form is very short, since the conditions strongly favor the Z-form. Therefore, after methylation of one or a few C-residues in a block, this segment reverts back to a Z-form. The methylated C-residues contribute substantially to the stability of the Z-form in these blocks (9, 22, 23). Consequently, the transition back to a B-form again is very much slowed down or completely blocked under these conditions. Thus, a gradual and stepwise methylation of the whole block is not possible because of this mechanism. The products of this dynamic equilibrium are (dC-dG) blocks which are left-handed but are methylated to a small extent.

This mechanism explains the low but significant amount of incorporation into the Z-form blocks. It also explains why there was a limited methylation of the 157bp insert, although no cleavage of the parent pRW751 High by HhaI restriction enzyme was observed.

Azorin *et al.* (41) reported that a short segment in supercoiled pBR322 DNA binds antibodies specific for left-handed DNA. They concluded that a 14bp

alternating purine-pyrimidine sequence including a HhaI site d(GCGC) adopts a left-handed form at natural supercoil density. This result is in disagreement with the work presented here, since we were not able to detect left-handedness in any of the isolated d(GCGC) sites of the pBR322 vector DNA. Moreover, even with other techniques used in this and other labs [S_1 cleavage of B-Z junctions (1, 5, 8, 12, 13), supercoil relaxation analyses (4-12)], a Z-form segment in pBR322 vector DNA was not found up to a superhelical density of -0.1, although our methods allowed the detection of a left-handed (dC-dG) block as short as 10bp in length that was cloned into pBR322 (8). This lack of agreement of the antibody binding result (41) with other more informative analyses may be related to the procedures employed as emphasized by Hill and Stollar (42).

The results of this study demonstrate that the primary base sequence, at least in the case of HhaI and MHhaI, is not the only basis for recognition and association in a DNA-protein complex. The secondary conformation of the helix also has an important influence on the complex formation and/or reactivity of an enzyme with a DNA segment. However, it is possible but less likely that the methylase enzyme can recognize and bind even to the left-handed (dC-dG) sequence, but that in the geometry of the Z-conformation, the reaction center, i.e. the 5-position of cytosine, is not accessible for the transfer of a methyl group by the enzyme.

Previous studies in this lab revealed similar structure-reactivity correlations. It was observed that a conformational change in a small region of a DNA duplex can decrease or completely inhibit the reactivity of an enzyme that recognizes this region [S_1 cleavage at B-Z junction regions (8), inhibition of BamHI cleavage at a d(GGATCC) site that is flanked by left-handed (dC-dG) blocks (8)]. Thus, the reactivity or inhibition of an enzyme at a specific site can be used to determine if a conformational transition has occurred.

Finally, it should be noted that this method of using enzymes as conformational probes for various DNA structural forms can be extended to other recognition sequences and enzymes. It may be applied to other conformational features such as cruciforms, helical junction regions, unpaired helix segments, strand slipped structures, or various helical forms of DNA duplexes like A-, C-, or D-form helices (reviewed in 21).

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