The effect of *Hha*I methylation on DNA local structure

Keith R. FOX

University of Cambridge Department of Pharmacology, Medical School, Hills Road, Cambridge CB2 2QD, U.K.

The effect of methylating the central cytosine in the sequence GCGC on the sensitivity to digestion by deoxyribonuclease I was investigated. Examination of five such sites shows that the extra methyl group renders the bond on its 5' side more susceptible to cleavage, and this is interpreted by suggesting that it causes small changes in the local DNA phosphate orientation.

INTRODUCTION

There is a considerable amount of evidence that methylation of the 5-position of cytosine in the nucleotide pair CpG may be one of the signals regulating gene expression in eukaryotic DNA (Razin & Friedman, 1981; Doerfler, 1983; Razin & Riggs, 1980). In general there appears to be a correlation between hypomethylation and gene activity (Razin & Szyf, 1984), although a loss of methylation does not necessarily result in expression (Stein *et al.*, 1983). Although this represents an elegant model for describing one method by which gene activity might be controlled and inherited, there is little or no information concerning the mechanism by which these changes in DNA activity occur. Does the presence of the extra methyl group in the DNA major groove directly affect the binding of certain proteins, thereby altering the complex secondary and tertiary folding of eukaryotic chromosomes, or does it alter the local DNA structure, producing a form that is less easily expressed?

Previous studies have suggested that methylation of the 5-position of cytosine can indeed affect DNA structure. At a gross level it is known that the transition of poly(dG-m⁵dC) from B to Z forms occurs at close to physiological salt concentrations whereas the conversion of the unmethylated polymer requires very high salt concentrations (Behe & Felsenfeld, 1981). Although it seems unlikely that these dramatic changes are relevant to the DNA conformation around the step CpG, they do serve to demonstrate that the methyl group can have a profound effect on the conformations accessible to the DNA. It has also been demonstrated that the *lac* repressor binds about 20 times better to poly(dA-dU) than to poly(dA-dT) (Lin & Riggs, 1971). Although this might be explained by a favourable hydrophobic interaction between the 5-position of the uracil and the repressor, the protein still binds well to various mercaptan derivatives of the type poly(dA-dU-HgX), in which the major groove is sterically blocked (Richmond & Steitz, 1976), so that the increased affinity must be due to some alteration in the backbone structure of the DNA (Klug *et al.*, 1979).

In the present study the sensitivity of DNAase I to the precise local DNA structure (Drew & Travers, 1984; Fox & Waring, 1984) was exploited to investigate the small changes that occur on methylating the central cytosines of the sequence GCGC. This enzyme is especially useful since it is known to cut DNA from the minor groove

(Drew, 1984; Drew & Travers, 1984), whereas the 5-position of cytosine lies in the major groove. Any changes in cleavage rate must therefore be due to DNA structural changes rather than altered hydrophobic contacts between the endonuclease and the DNA.

MATERIALS AND METHODS

DNA

The 160-base-pair *tyrT* DNA fragment containing the tyrosine tRNA promoter was isolated and labelled as previously described (Drew & Travers, 1984). Incubation with reverse transcriptase, dGTP and [α -³²P]dCTP led to selective radioactive labelling of the 3' end of the 'Watson' (sense) strand (upper sequence in Fig. 2). Incubation with reverse transcriptase, dTTP and [α -³²P]dATP led to selective labelling of the 3' end of the 'Crick' strand (lower sequence in Fig. 2).

The 166-base-pair *pTyr2* fragment (Fig. 4) was cut from plasmid pMLB1048 between the unique *Eco*RI and *Bst*EII sites and was a gift from Dr. C. M. L. Low. The *Eco*RI end (top strand) was selectively labelled with dTTP and [α -³²P]dATP, and the *Bst*EII end (bottom strand) was labelled with dGTP and [α -³²P]dTTP.

*Hha*I methylation

*Hha*I methylase, which methylates the internal cytosine residue in the sequence GCGC (Smith, 1979), was purchased from New England Biolabs and used according to the supplier's instructions. The extent of methylation was assessed by the Maxam–Gilbert sequencing method (Maxam & Gilbert, 1977) since 5-methylcytosine does not appear in the pyrimidine cleavage patterns on reaction with hydrazine. In all cases the DNA used was at least 95% methylated.

Deoxyribonuclease I digestion

Samples (8 μ l) of the labelled DNA fragment (9 pmol in base-pairs) were digested with 2 μ l of DNAase I (final concentration 0.01 unit/ml) as previously described (Drew & Travers, 1984; Fox & Waring, 1984). Portions (3 μ l) were removed from the mixture after 1 min, 5 min and 30 min digestion, and the reaction was stopped by adding 2.5 μ l of 80% (v/v) formamide containing 0.1% Bromophenol Blue. The products of digestion were analysed on 0.3 mm 8% (w/v) polyacrylamide gels containing 7 M-urea and Tris/borate/EDTA buffer,

pH 8.3. After 2 h electrophoresis at 1500 V the gel was soaked in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, dried under vacuum at 80 °C and subjected to autoradiography at -70 °C with an intensifying screen.

Densitometry

Autoradiograms were scanned with a Joyce-Loebl microdensitometer to produce profiles from which the relative intensity of each band was measured. The results are expressed as fractional cleavage $f = A_i/A_t$ as previously described (Low *et al.*, 1984a,b; Fox & Waring, 1984), where A_i is the area under band i and A_t is the sum of the intensity under all bands in any gel lane. When comparing different digestion patterns care was taken to ensure that the extent of digestion was similar and limited to 20–40% of the starting material, so as to minimize the incidence of multiple cuts in any strand. Data from this analysis are presented in the form of $\ln(f_{\text{methylated}}) - \ln(f_{\text{unmethylated}})$, representing differential cleavage at each bond relative to the control. The results are displayed on a logarithmic scale for the sake of convenience so that positive values indicate an enhanced rate of cleavage.

RESULTS

tyrT DNA

The *tyrT* DNA fragment contains two *HhaI* methylation sites, at positions 76 and 95. Fig. 1 shows the DNAase I digestion patterns of the methylated and unmethylated DNA labelled at the 3' end of the top strand. The cytosine residues that have been methylated are indicated by the arrows. It can be seen that at both positions the bond on the 5' side of the methylated cytosine (GpC) is cleaved better (2–3-fold) than in the control DNA. The results are presented in a more quantitative form for both strands in Fig. 2, representing the difference in cleavage at each bond between the control and the methylated DNA. At each site the only

Fig. 1. DNAase I footprinting of *HhaI*-methylated *tyrT* DNA: digestion pattern of *tyrT* DNA labelled at the 3' end of the top strand in Fig. 2

Time in minutes (1, 5, 30) after the addition of enzyme is shown at the top of each gel lane. Numbers refer to the numbering scheme of Drew & Travers (1984) used in Fig. 2. Tracks labelled 'C' are hydrazine/piperidine marker lanes specific for cytosine. The arrows indicate those cytosine residues that have been methylated.

Fig. 2. DNAase footprinting of *HhaI*-methylated *tyrI* DNA: differential cleavage plot for methylation-induced changes in susceptibility to DNAase I digestion

Vertical scales are in units of $\ln(f_m) - \ln(f_c)$, where f_m is the fractional cleavage at any bond in the methylated DNA and f_c is the fractional cleavage of the same bond in the control. Positive values indicate enhanced cleavage. The four bars represent those cytosine residues that have been methylated.

Fig. 3. DNAase I footprinting of *HhaI*-methylated *pTyr2* DNA: digestion pattern of *pTyr2* DNA labelled at the 3' end of the bottom strand of Fig. 4

Details are similar to those given in Fig. 1 legend.

bond affected is the GpC immediately on the 5' side of the methylated cytosine. The complementary GpC on the opposing strand, which remains unmethylated, is unaffected, as too is the central CpG on both strands. The structural changes that could be responsible for these changes are considered in the Discussion section.

The effect of distamycin and actinomycin D on the DNAase I cleavage of the methylated DNA was also

investigated and found to be identical with that of the control DNA. Both methylated regions form part of actinomycin D-binding sites, as revealed by DNAase I footprinting (Fox & Waring, 1984), and these blockages are retained in the methylated fragment, as expected, since this ligand binds to the DNA minor groove. In contrast, distamycin has been shown to protect from cleavage at position 96 but enhance cleavage at position 75 (Fox & Waring, 1984). Both of these effects are still evident in the methylated DNA, the enhancement at position 75 being more pronounced in the methylated DNA. This last effect is interesting since it demonstrates that the small structural changes produced by methylation can be superimposed on the much larger changes in groove width induced by ligand binding.

pTyr2 DNA

In order to extend these observations, the effect of methylation on the DNA fragment *pTyr2* was also investigated. This piece of DNA contains three methylation sites, two of which overlap in the sequence GCGCGC around position 43. Typical digestion patterns for the 3'-end-labelled bottom strand of this DNA are shown in Fig. 3 and the differential cleavage plot for both strands is presented in Fig. 4. Once again the GpC bonds on the 5' side of the methylated cytosine residues show an increased rate of DNAase I cleavage whereas the CpG steps remain unaffected. The changes are also restricted to the strand bearing the methylated residues, and are not apparent in the opposing strand, except where the two sites overlap. The same changes on the 5' side alone are also evident for the sequence GCGCGC, which is doubly methylated, suggesting that the conformational changes are restricted to the bond immediately adjacent to the methylation site and do not appear to be propagated to more distant regions.

DISCUSSION

The results presented in this paper clearly demonstrate that methylation of the central cytosine in the sequence GCGC does produce small but consistent changes in local DNA conformation as defined by the susceptibility to cleavage by DNAase I. This altered rate of cleavage cannot be attributed to any changes in hydrophobic interaction between the enzyme and DNA, since the additional methyl group lies in the major groove whereas DNAase I cuts from the minor groove (Drew, 1984; Drew & Travers, 1984). At all five sites investigated the presence of a methyl group on the 5-position of cytosine induces an enhanced rate of DNAase I cleavage of the bond (GpC) on the 5' side of the methylcytosine. What, then, is the structural basis of this change?

In previous publications drug-induced enhanced rates of DNAase I cleavage have been attributed to changes in the width of the minor groove (Low *et al.*, 1984a,b; Fox & Waring, 1984). This seems an unlikely explanation of the changes observed here, for several reasons. Firstly, changes in groove width should affect the rate of cleavage of two bonds, so that an enhanced rate of cutting should be observed for the opposing phosphate across the minor groove, staggered two or three bonds in the 3' direction. This is not found to be the case; for example, cleavage of bond 77 on the bottom strand of the *tyrT* fragment is enhanced by methylation, but no such change is apparent at bonds 79 or 80 on the opposing strand. A second factor

Fig. 4. DNAase footprinting of *HhaI*-methylated *pTyr2* DNA: differential cleavage plot for methylation-induced changes in susceptibility to DNAase I digestion

Details are similar to those given in Fig. 2 legend.

is that only one bond is affected by methylation, whereas changes in conformation would be expected to extend over a much wider region.

It seems likely that the altered rate of cleavage is produced by changes in the precise orientation of the phosphodiester bond on the 5' side of the methylcytosine. DNAase I cuts the 3'-O-P bond by nucleophilic attack (Laskowski, 1971), so that when this bond points outward DNAase I should have easy access, whereas the approach of the enzyme will be hindered when it has the opposite orientation (see Fig. 8 of Drew & Travers, 1984). This argument involving the precise orientation of the phosphodiester bond was used by Drew & Travers (1984) to explain why the local cutting efficiency of DNAase I varies from the global cutting function. From a structural point of view the phosphate orientation will depend on some combination of base-pair-step stacking parameters. This is best illustrated by reference to the crystal structure of the B-DNA dodecamer dCGCGAATTCGCG (Dickerson & Drew, 1981), in which the orientation of the 3'-O-P bond varies along the length of the helix and is affected by the degree of the base overlap. This is especially relevant for the two GpC steps, which have quite different accessibilities for nucleophilic attack at the 3'-O-P bond, step (j) being more accessible than step (b). A possible suggestion for the structural basis of the changes induced by methylation consistent with this crystal structure is that the guanine on the 5' side of the methylcytosine moves slightly so as to cover over the exposed methyl group, rotating about the GpC bond on the opposite strand.

It is worth noting that changes observed here are not dissimilar to the differences between crystal structures of the brominated and native forms of a dodecamer (Fratini *et al.*, 1982). In these structures the only bond significantly affected (CpT) was the 5' side of the bromocytosine and involved a decrease in the roll angle at this dinucleotide step. It remains to be seen how these

small structural changes affect gene activity, and whether similar investigations at methylation sites involved in gene control will yield similar results.

This work was performed in Dr Michael Waring's laboratory and was supported by grants to him from the Cancer Research Campaign, the Medical Research Council and The Royal Society. I thank Dr. H. R. Drew and Dr. C. M. L. Low for helpful discussion and advice.

REFERENCES

- Behe, M. & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1619-1623
- Dickerson, R. E. & Drew, H. R. (1981) *J. Mol. Biol.* **149**, 761-786
- Doeffler, W. (1983) *Annu. Rev. Biochem.* **52**, 93-124
- Drew, H. R. (1984) *J. Mol. Biol.* **176**, 535-557
- Drew, H. R. & Travers, A. A. (1984) *Cell (Cambridge, Mass.)* **37**, 491-502
- Fox, K. R. & Waring, M. J. (1984) *Nucleic Acids Res.* **12**, 9271-9285
- Fratini, A. V., Kopka, M. L., Drew, H. R. & Dickerson, H. R. (1982) *J. Biol. Chem.* **257**, 14686-14707
- Klug, A., Jack, A., Viswamitra, M. A., Kennard, O., Shakked, Z. & Steitz, T. A. (1979) *J. Mol. Biol.* **131**, 669-680
- Laskowski, M. (1971) *Enzymes* 3rd Ed. **4**, 289-311
- Lin, S. & Riggs, A. D. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1542-1547
- Low, C. M. L., Drew, H. R. & Waring, M. J. (1984a) *Nucleic Acids Res.* **12**, 4865-4879
- Low, C. M. L., Olsen, R. K. & Waring, M. J. (1984b) *FEBS Lett.* **176**, 414-420
- Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 560-564
- Razin, A. & Friedman, J. (1981) *Prog. Nucleic Acid Res. Mol. Biol.* **25**, 33-52
- Razin, A. & Riggs, A. D. (1980) *Science* **210**, 604-610
- Razin, A. & Szyf, M. (1984) *Biochim. Biophys. Acta* **782**, 331-342
- Richmond, T. & Steitz, T. A. (1976) *J. Mol. Biol.* **103**, 25-28
- Smith, H. O. (1979) *Science* **205**, 455-462
- Stein, R., Sciaky-Gallili, N., Razin, A. & Cedar, H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2422-2426