Supercoiling-dependent sequence specificity of mammalian DNA methyltransferase

Timothy Bestor

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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SUMMARY

Negative supercoiling of substrate DNA dramatically alters the in vitro sequence specificity of mammalian DNA methyltransferase (DNA MeTase). This result suggests that in vivo site selection by DNA MeTase could be regulated by conformational information in the form of alternative secondary structures induced in DNA by local supercoiling or by the binding of specific nuclear proteins. DNA in the left-handed Z-form is shown not to be a substrate for mammalian DNA MeTase. The sensitivity of DNA MeTase to DNA structure may also make it useful as a probe for sequences which undergo supercoiling-dependent structural transitions in vitro.

INTRODUCTION

Tissue-specific patterns of methylated CpG dinucleotides, which are thought to be involved in gene regulation, are faithfully transmitted to daughter cells through the action of DNA (cytosine-5)-methyltransferase (DNA MeTase). This enzyme is strongly stimulated by the hemimethylated CpG sites which result from semiconservative DNA replication (1). Methylation patterns can be altered and repaired (2,3) by sequence-specific de novo methylation; both de novo and maintenance activities appear to reside in the same enzyme (4). De novo methylation is directed in part by cis-acting sequences, as shown by the imposition of a typical Thy-1 methylation patterns on transfected Thy-1 DNA in transgenic mice (5). However, the de novo activity of DNA MeTase appears to have little dependence on the sequence context of CpG sites in vitro (4,6). Additional factors must be involved to direct the action of DNA MeTase in vivo since reconstitution of homologous DNA MeTase and cellular DNA results in the methylation of CpG sites which were maintained in the unmethylated state in vivo (8).

The major form of DNA MeTase in log-phase murine erythroleukemia (MEL) cells has a relative mass of 190,000 and has been termed DNA MeTase III (6). All preparations of DNA MeTase examined to date are strongly stimulated by

hemimethylated substrates (4, 8-11); for DNA MeTase III, activity on unmethylated substrates amounts to about 3% of that seen with the homologous unmethylated substrate (8).

Negative supercoiling and the binding of specific nuclear factors has been shown to induce sequence-specific alternative secondary structures in duplex DNA both in vivo (12) and in vitro (13). The ability of such structural polymorphism to influence the sequence specificity of DNA MeTase has been tested in a reconstituted system involving purified DNA MeTase III from MEL cells and pBR322, a prokaryotic plasmid which has been especially well characterized in terms of supercoiling-induced structural transitions (14-16). The results suggest a mechanism that may reconcile the lack of sequence specificity displayed by DNA MeTase in vitro with the observed ability of this enzyme to carry out site specific DNA methylation in vivo.

EXPERIMENTAL PROCEDURES

DNA MeTase III was purified from log-phase MEL cells through the blue agarose chromatography step as described (6), at which point DNA MeTase represents about 60% of total protein, as determined by densitometry of Coomassie blue stained electrophoregrams. Traces of contaminating topoisomerase I were removed by chromatography on phosphocellulose equilibrated with buffer A (20 mM tris HCl (pH 7.4), 5 mM Na₃EDTA, 0.5 mM dithiothreitol, and 2.7 M glycerol). DNA MeTase was eluted, free of topoisomerase I activity, with buffer A containing 0.25 M NaCl; active fractions were made 50% in glycerol and stored at -20°C.

DNA topoisomerase co-extracts from MEL cell nuclei with DNA MeTase III and is eluted from DEAE sephacel at a slightly higher NaCl concentration (0.2 vs. 0.15 M). Active fractions were identified by their ability to relax supercoiled pBR322 DNA and were stored in 50% glycerol at -20°C.

Plasmid topoisomers were produced by relaxation to completion in the presence of DNA topoisomerase I and ethidium bromide as described (17). At the end of the reaction, ethidium was removed by extracting three times with water-saturated n-butanol, twice with phenol, and ethanol precipitation. Superhelical densities were determined by the band counting method (17) after electrophoresis in 1.2% agarose gels containing 0-500 μ M chloroquine (19). Superhelicity is expressed as the mean superhelical density ($\bar{\sigma}$), which is in units of superhelical turns per helical repeat. The values of $\bar{\sigma}$ given throughout the text refer to electrophoresis conditions (90 mM tris-borate, 2.5 mM Na₃EDTA) because it was not possible to obtain satisfactory resolution of topoisomers in gels run in buffer A.

FIGURE 1. Effect of supercoiling on the sequence specificity of DNA MeTase III. Plasmide pBR322 topoisomers at $\sigma = 0$ (lane A) and $\sigma = -0.1$ (lane B) were methylated by incubation with DNA MeTase III and [^3H -methyl] S-adenosyl L-methionine, cleaved with *Msp*I, and analyzed by electrophoresis and fluorography. Equal amounts of radioactivity were loaded onto each lane. Fragment lengths are in base pairs, and are derived from the sequence of ref. 30.

Methylation reactions contained 5 μg DNA, 0.5 μg DNA MeTase III (50 units, where 1 unit catalyzes the incorporation of 1 pmol $\text{CH}_3 \text{ hr}^{-1} \mu\text{g DNA}^{-1}$ with poly(dG·dC) as substrate), and 1.2 μM [^3H -methyl] S-adenosyl L-methionine (ICN or Amersham, 12-15 Ci mmol^{-1}) in a total volume of 100 μl buffer A. Reactions were terminated by phenol extraction when analysis of aliquots showed methylation of 2-5% of total CpG sites; incubations were normally about 2 hr at 37°C. To ensure that nicking or relaxation of the DNA had not occurred during methylation, portions were analyzed by electrophoresis on 1% agarose gels containing 5 μM chloroquine. Digestion with restriction endo-

nucleases was as recommended by the vendor (New England Biolabs). DNA fragments were analyzed on 40 cm-long 6% polyacrylamide gels in 90 mM tris-borate buffer. Fluorography was performed as described (7).

Equal amounts of radioactivity, rather than equal masses of DNA, were analyzed. Since intermediate levels of supercoiling (ca. $\bar{\sigma} = -0.05$) increase the net initial rate of methylation by about 2 fold, the data presented here depict relative changes in rates of methylation of pBR322 sequences rather than absolute rates.

RESULTS

Supercoiling-dependent changes in initial rates of methylation of different plasmid sequences were detected by gel electrophoresis and fluorography of small DNA fragments derived from methylated pBR322 topoisomers (6). Figure 1 shows that supercoiling has a striking effect on the sequence specificity of DNA MspI; different plasmid sequences display increases (notably the fragments of 404, 190, and 122 base pairs) or decreases (309, 242, and 110 base pairs) in rate of methylation as the DNA becomes increasingly negatively supercoiled.

Supercoiling-induced structural transitions are often cooperative processes which occur at characteristic levels of supercoiling (17,18). To determine whether changes in rates of methylation show similar behavior, populations of topoisomers spanning a range of superhelicity were analyzed as in figure 1. As can be seen in figure 2, changes in rate of methylation of different

FIGURE 2. Effect of supercoiling different among pBR322 sequences. In panel A, an abrupt increase in rate of methylation of the 404 base pair MspI fragment can be seen to occur at $\bar{\sigma} < -0.06$; in panel B, the rate of methylation of the 122 base pair fragment increases when $\bar{\sigma} < -0.015$ while methylation of the 110 base pair fragment increases when $\bar{\sigma} < -0.014$. Conditions were as in figure 1. Portions of fluorograms were taken from experiments which gave best resolution in the areas of interest.

erent sequences can be abrupt or gradual and can occur over a wide range of superhelical densities. The 404 bp fragment, which shows an especially large and abrupt increase in rate of methylation at high negative superhelical densities, encompasses a cluster of inverted repeat sequences which have been shown to form cruciform structures at superhelical densities very close to those which cause enhanced methylation of this region (16). Other plasmid sequences which undergo marked changes in rate of methylation do not correspond to sequences known to undergo structural transitions in supercoiled pBR322 DNA, as shown in figure 3. While the lack of detailed structural information about most types of alternative secondary structures prevents the identification of the structures which are responsible for enhancing or suppressing methylation of the affected sequence, the results suggest that DNA

FIGURE 3. pBR322 sequences which show supercoiling dependent-sensitive initial rates of methylation are shown in relation to supercoiling dependent structural features. *Msp*I fragments of pBR322 have been ordered along the horizontal axis according to the sequence and numbering convention of Sutcliffe (30); the sequence is shown as linearized at nucleotide 402. Gaps indicate positions of fragments which were too small for analysis. Vertical axis indicates supercoiling-induced changes in initial rates of methylation, as determined by quantitative densitometry of fluorograms such as those shown in figures 1 and 2. The symbols indicate the positions of: ■, Z-forming sequences (15,17,32); Δ, *S*₁ nuclease-hypersensitive sites (16); and ▲, Mung bean nuclease-hypersensitive sites formed at low ionic strength in the absence of metal ions (14). Data shown are means of three determinations.

FIGURE 4. Supercoiling-sensitive methylation of the *lac* UV5 promoter. Plasmid pLP332, a pBR322 derivative containing the UV5 allele of the *lac* promoter (20), was methylated at $\bar{\sigma}=0$ (lane A) or $\bar{\sigma}=-0.06$ (lane B). Digestion with *Msp*I and *Bam*HI was used to excise the 55 bp fragment containing the promoter. Densitometry revealed that supercoiling enhanced methylation by a factor of 20. Lane A received slightly more total radioactivity than did lane B.

MeTase may find use as a reagent for detecting structural transitions in defined DNA sequences, as in the following example.

Rates of *in vitro* transcription of the wild-type and P_S alleles of the *lac* promoter have been shown to be strongly stimulated by negative supercoiling, while transcription of the UV5 allele is relatively insensitive (21). As shown in figure 4, the rate of methylation of the *lac* UV5 promoter in plasmid pLP332 (20) increases by a factor of 20 when $\bar{\sigma} < -0.04$. A test for a possible structural basis for the differences in rates of *in vitro* transcription would involve comparing rates of methylation among the three alleles as a function of superhelical density; in cases such as this, DNA MeTase may be useful as a probe for detecting conformational features in DNA which might not be reactive towards extant chemical or enzyme probes.

A prominent supercoiling-induced structural transition in supercoiled DNA is the flipping of $(Pu \cdot Py)_n$ sequences from the B to the Z helical forms (17).

FIGURE 5. $(dC \cdot dG)_{16}$ in the left-handed Z form is not a substrate for DNA MeTase III. Plasmid pLP32 (19) was methylated at $\bar{\sigma}=0$ (lane A) and $\bar{\sigma}=-0.07$ (lane B) and cleaved with *Msp*I and *Bam*HI to release the $(dC \cdot dG)$ insert flanked by remnants of *Bam*HI linkers. The insert is unmethylated in lane B, where supercoiling had caused it to adopt the Z configuration (17). Equal amounts of radioactivity were analyzed in each lane. Film darkening below the 67 base pair band in lane A is an artifact caused by labeling of RNA in the plasmid preparation by RNA methyltransferases present as minor contaminants in the DNA MeTase preparation.

The ability of DNA MeTase III to methylate Z-form $(dG \cdot dC)_n$ was evaluated through the use of pLP32, a pBR322 derivative which contains a $(dG \cdot dC)_{16}$ insert flanked by *Bam*HI linkers (20). Supercoiling has been shown to convert the insert to the Z form quantitatively (19). As shown in figure 5, superhelical densities sufficient to convert the insert to the Z configuration completely prevent its methylation. Previous reports indicated that mammalian DNA MeTase can methylate poly $(dG \cdot dC)$ that had been alkylated (22) or treated with $(NH_4)_6Co$ (10), treatments which favor the B-Z conversion. However, Z formation may not have been complete, while negative supercoiling causes quantitative conversion of $(dG \cdot dC)_n$ to the Z form (19) under conditions optimal for enzyme activity in the absence of agents which bind to DNA. The bacterial DNA methyltransferase *M.Hha*I has also been reported to be inactive on Z-DNA (23,24).

DISCUSSION

The present findings suggest that the sequence specificity of DNA MeTase may be conditional on the physical conformation of the DNA substrate. This in turn suggests a mechanism for directing site-specific DNA methylation in vivo that is in agreement with what is known of the physical state of DNA in nuclei. First, it is well established that unusual structural features (observed as nuclease-hypersensitive sites) appear in the vicinity of active genes, usually in the promoter region 5' to coding sequences (12), and that corresponding features are often formed in the cloned genes when present in supercoiled plasmids (25). Methylation of 5' sequences has been shown to correlate most closely with gene activity (26). Second, while it remains impracticable to measure levels of supercoiling in specific sequences within intact cells, it is possible that a fraction of mammalian DNA bears unconstrained supercoils (27,28). Binding of specific protein factors can induce structural transitions in adjacent sequences (13) which differ in location from those induced by supercoiling in the corresponding cloned gene (29). Last, the suggestion that DNA structural polymorphism directs de novo methylation is consistent with the results of Groudine and Conklin (31), who report that areas of local undermethylation were specifically associated with nuclease-hypersensitive sites in two constitutive genes in sperm DNA. These nuclease-hypersensitive sites may function to inhibit methylation of local CpG dinucleotides, protecting them from de novo methylation during spermatogenesis. Further testing of the possible role of conformational information in directing DNA methylation will require the use of authentic mammalian DNA sequences which have been shown to undergo de novo methylation or demethylation in vivo to determine whether supercoiling and the addition of appropriate nuclear fractions can reproduce cell-type specific methylation patterns in vitro.

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