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DNA methylation can enhance or induce DNA curvature

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Oligomers of different palindromic sequences (*Eco*RI, *Bam*HI, and *Cla*I linkers) were ligated to form distributions of multimers. These long ligation ladders were methylated using corresponding methylases. The migration of the unmethylated as well as the methylated multimer distributions was analysed in 10% polyacrylamide gels. The migration anomaly of these sequences is interpreted in terms of the curvature of the DNA helix axis. The double-stranded oligomer dCGGAATTCCG is considerably curved in its unmodified form. Its curvature is strongly enhanced when the central dAs are methylated. This result is predicted by a model for DNA curvature. Multimers of the closely related sequence dCGGGATCCCG are straight. When methylated at the central dAs or at the most central dCs, a small curvature of the helix axis is induced. The double-stranded oligomer dCCATCGATGG is straight in its unmodified form as well as when it is methylated. Thus, DNA curvature can be induced or enhanced by methylation. However, DNA methylation at palindromic sequences seems not always to influence the linear path of the DNA helix axis.

Key words: DNA methylation/DNA curvature/gel migration anomaly

Introduction

Poly(dA)·poly(dT) has a DNA structure slightly different from standard B-form as detected by various experimental techniques (Peck and Wang, 1981; Arnott *et al.*, 1983; Alexeev *et al.*, 1987; Edmondson and Johnson, 1985; Jolles *et al.*, 1985; Wartell and Harrell, 1986; Taillandier *et al.*, 1987). The structure of this sequence is also recognized by some proteins (Kunkel and Martinson, 1981; Prunell, 1982; Diekmann and Zarling, 1987; Diekmann and Robert-Nicoud, in preparation). Long, as well as short, stretches of $dA_n \cdot dT_n$, with n as small as four to six, seem to adopt the so-called B′-form structure (Diekmann and Wang, 1985; Burkhoff and Tullius, 1987; Diekmann and Zarling, 1987). When this sequence alternates with B-DNA in phase with the turn of the DNA helix, the helical axis of the resultant molecule is curved (Marini *et al.*, 1982; Wu and Crothers, 1984; Hagerman, 1985; Diekmann and Wang, 1985; Hagerman, 1986; Diekmann, 1986; Koo *et al.*, 1986; Ulanovsky *et al.*, 1986; Griffith *et al.*, 1986); for reviews see Trifonov (1985) and Diekmann (1987a).

Recently, von Kitzing and Diekmann (1987) suggested that poly(dA)·poly(dT) might adopt a B′-form structure due to the influence of the chemical groups at the 2- and 6-positions of the adenines. The hypothesis assumes that the bulky NH_2 groups at the 6-position of consecutive dAs interact with one another. As a consequence, the dA·dT base-pairs (bp) close towards the minor groove if the 2-position of the dAs is free. This hypothesis was experimentally tested by Diekmann *et al.* (1987). The central dA·dT base-pair in a run of five dA·dT base-pairs was replaced by chemically related base-pairs to test the influence of the chemical modification at the 2- and 6-position of the purine on the resulting DNA curvature. A DNA curvature of these sequences in a repeat length of 10 bp was found only when the 2-position of the central purine was free. The degree of curvature was modulated by the chemical group at the 6-position of the central purine: The curvature was large when the 6-position was occupied by a NH_2 group, already smaller for an oxygen, and smallest for a free 6-position. The introduction of the same chemical base modifications into the sequence 5′-d(GAATTC)-3′ (S.Diekmann and L.McLaughlin, submitted) display DNA curvature changes in agreement with the results of Diekmann *et al.* (1987). These experimental findings support the hypothesis of von Kitzing and Diekmann (1987) but are in disagreement with others (Chuprina, 1987; Jernigan *et al.*, 1986, 1987).

According to our hypothesis (von Kitzing and Diekmann, 1987) the curvature of the DNA helix axis should increase when the chemical substitution at the 6-position of the dAs becomes larger than the NH_2 group. Thus, methylation of the NH_2 of the dAs to $HNCH_3$ should lead to an enhanced curvature. This prediction was experimentally tested using the self-complementary decanucleotide 5′-dCGGAATTCCG-3′ (*Eco*RI linker). The N(6) of its central dAs were methylated by *Eco*RI methylase. In addition, other self-complementary decanucleotides (*Bam*HI and *Cla*I linkers) were also methylated and analysed for DNA curvature.

Methylation of DNA sequences is found *in vivo* in higher and lower organisms (for an overview see Razin *et al.*, 1984). It is generally assumed that the primary biological function of DNA methylation is to affect strongly sequence-specific interactions of proteins with DNA (Engel and von Hippel, 1978; Razin and Riggs, 1980). DNA methylation plays a role in many different biological processes like DNA mismatch repair (Radman *et al.*, 1980; Lu *et al.*, 1983; Pukkila *et al.*, 1983), DNA restriction modification and protection in bacteria (Yuan, 1981; Szyf *et al.*, 1982; Pukkila *et al.*, 1983), and gene regulation (Hattman, 1982; Plasterk *et al.*, 1983; Busslinger *et al.*, 1983; Langner *et al.*, 1984; Cedar, 1984; Doerfler, 1984). In addition, DNA methylation may affect the initiation of replication (Hughes *et al.*, 1984), and seems to be important for chromatin structure and eukaryotic development (Jaehner and Jaenisch, 1984; Reik *et al.*, 1987; Sapienza *et al.*, 1987).

In these processes, the DNA–protein interactions might be influenced by DNA structure changes induced by the chemical modification. Local structure variations might be markers for the DNA–protein recognition. A similar role of methylation on the DNA structure has already been discussed for Z-DNA (Klysik *et al.*, 1983; Nickol and Felsenfeld, 1983). Indeed, methylation affects the double helix stability to melting; the helix is stabilized by methylation of dCs (Gill *et al.*, 1974) but slightly destabilized by methylation of dAs (Engel and von Hippel, 1978; Rinkel *et al.*, 1987a). However, the methylated dAs remain base-paired at temperatures below the melting transition (Engel and von Hip-

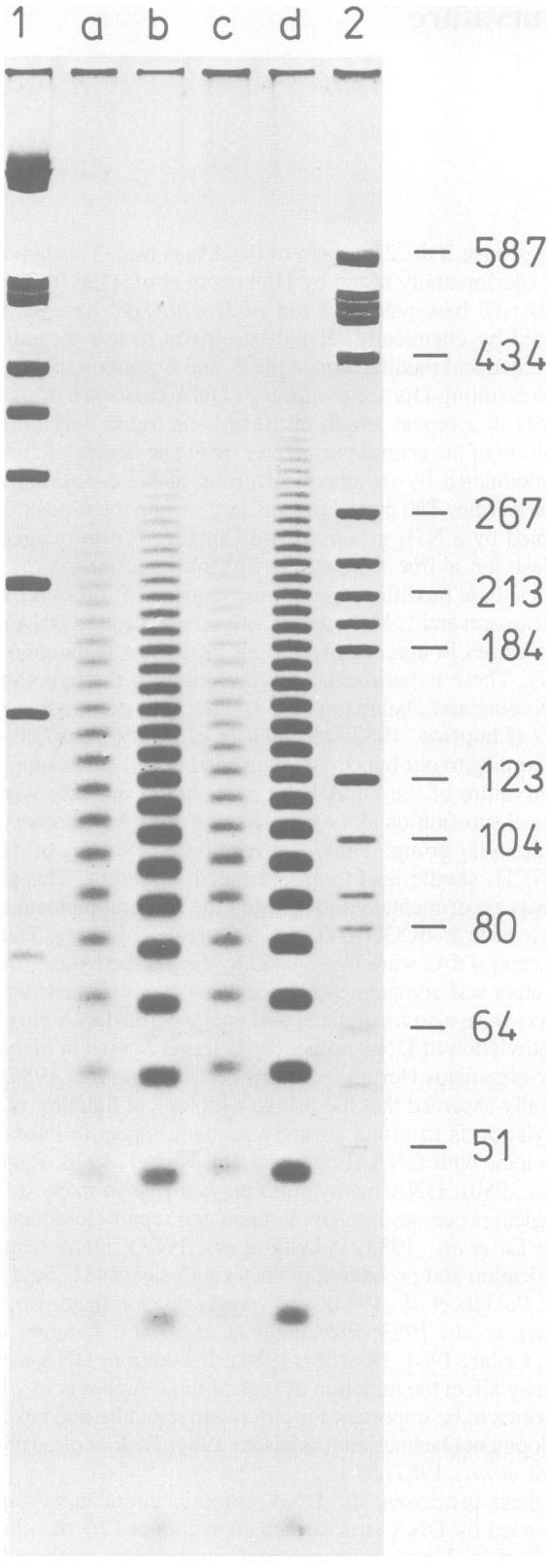

Fig. 1. Comparison of the migration of methylated to unmethylated multimers. **Lane a**: 10 bp *Bam*HI oligomers ligated to multimers, methylated by *Bam*HI methylase; **lane b**: same *Bam*HI sequences, unmethylated; **lane c**; 10 bp *Cla*1 oligomers ligated to multimers, methylated by *Cla*I methylase; **lane d**: same *Cla*I sequences, unmethylated; **lane 1**: *Hinf*1, digest of pBR322 (Sutcliffe, 1979); **lane2**: *Hae*III digest of pBR322 with lengths of fragments indicated. The DNA fragments of 50 bp and shorter migrate according to their length and can easily be identified.

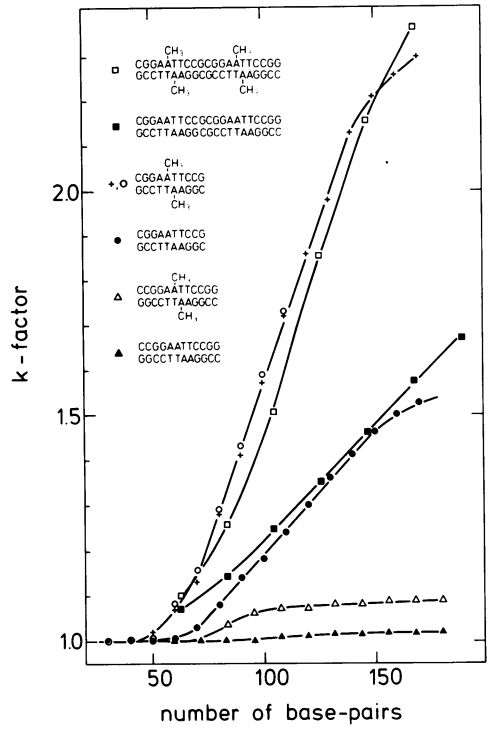

Fig. 2. Apparent multimer length in the 10% polyacrylamide gel divided by multimer sequence length ('*k*-factor') versus number of base-pairs of 10 bp, 12 bp, and 21 bp *Eco*RI linker multimers. (●) 10 bp unmethylated, (+) 10 bp methylated by *Eco*RI methylase and cut by *Eco*RI, (○) 10 bp methylated by *Eco*RI methylase but not cut by *Eco*RI endonuclease, (▲) 12 bp unmethylated, (△) 12 bp methylated by *Eco*RI methylase and cut by *Eco*RI, (■) 21 bp unmethylated, (□) 21 bp methylated by *Eco*RI methylase and cut by *Eco*RI. The experimental error of the *k*-factor is ±0.02.

pel, 1978). Recently, the sequence dCCGAATTCGG and its methylated form (at the central dAs) were analysed by NMR spectroscopy (Rinkel *et al.*, 1987b). The authors concluded that the influence of the methylation on the local structure of the duplex must be small. A detectable but small structural perturbation associated with methylation was also observed by Cheng *et al.* (1985). These authors calculated that the DNA helix unwinds by 0.5°/methyl group.

In the present work I have tested whether the methylation of different DNA sequences induces a curvature of the DNA helix axis.

Results

Decanucleotides of three different sequences were ligated to multimer distributions. These molecules were then methylated using commercially available DNA methylases. The electrophoretic migrations of the methylated, as well as the unmethylated, multimer distributions were determined in 10% polyacrylamide gels. Since the repeat length of the oligomer sequence is close to the repeat length of the DNA double helix (~10.4 bp; Fratini *et al.*, 1982; Diekmann and Wang, 1985), this technique allows sensitive measurement of the local curvature of the oligomer helix axis (Hagerman, 1985; Koo *et al.*, 1986). Thus, the sequence-directed curvature, as well as the influence of the chemical modification of the bases on the local curvature, can be detected.

A typical polyacrylamide gel is shown in Figure 1. By comparing the mobility of the different multimers with the mobility of pBR322 restriction fragments (Sutcliffe, 1979), the apparent

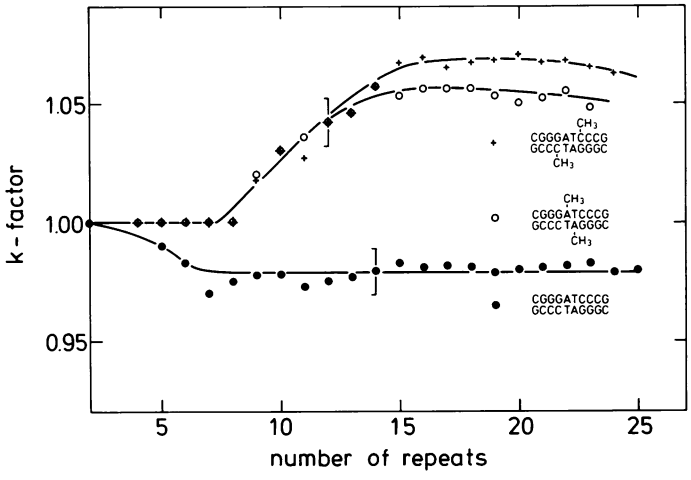

Fig. 3. *k*-factor versus number of repeats of the 10 bp *Bam*HI oligomer. (●) unmethylated, (○) methylated by *dam* methylase and cut by *Mbo*I, (+) methylated by *Bam*HI methylase and cut by *Bam*HI. The experimental error is indicated by a vertical bar.

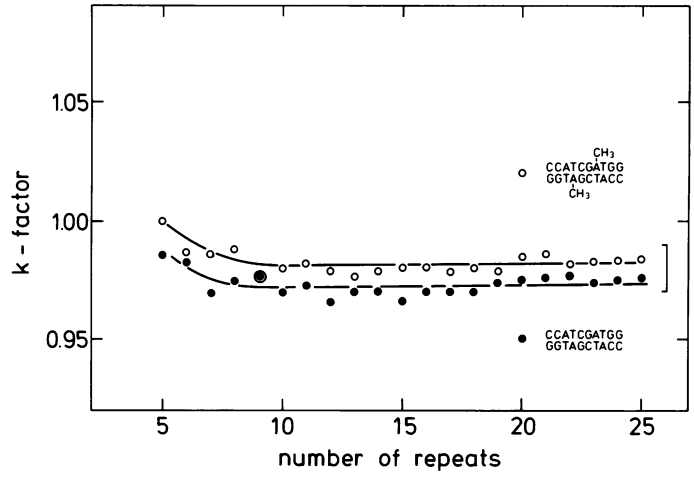

Fig. 4. *k*-factor versus number of repeats of the 10 bp *Cla*I oligomer. (●) unmethylated, (○) methylated by *Cla*I methylase and cut by *Cla*I. The experimental error is indicated by a vertical bar.

length of every multimer can be determined. The migration data obtained from this and similar gels are presented by plotting the apparent length of every multimer relative to its sequence length ('*k*-factor') versus the sequence length or versus the number of repeats of the oligomer sequence in the multimer under consideration (see Figures 2, 3 and 4).

The results indicate that the methylation of the DNA sequences was complete. When the methylated multimers were digested with the restriction endonuclease against which the sequence became protected by methylation, neither the distribution of the multimers changes nor the mobility of any single multimer (see Figure 2). For incomplete methylation the appearance of additional bands was observed (data not shown).

The sequence dCGGAATTCCG

The 10 bp long self-complementary *Eco*RI linker dCGGAATTCCG shows a gel migration anomaly for multimers with more than a 60 bp length (see Figure 2) and thus, is intrinsically curved. Since the migration of curved molecules through the gel pores is hindered, these molecules display a reduced mobility yielding an increased apparent length and a *k*-factor larger than one. This effect of the similar sequence dGGGAATTCCC has already been observed earlier (Hagerman, 1987) and was assigned to the central sequence element dAATT. The crystal analysis of the dodecamer dCGCGAATTCGCG also showed curvature (Fratini *et al.*, 1982).

*Eco*RI methylase recognizes this sequence. The enzyme replaces one of the hydrogens of the NH_2 group at the 6-position of the central dA on both strands, with a CH_3 group. This chemical modification has a marked influence on the mobility of the DNA molecules in polyacrylamide gels. Multimers of the methylated decanucleotides show a strongly enhanced migration anomaly. The mobility change relative to the normal, non-methylated curved sequences is about tripled (see Figure 2).

Multimers of the 21 bp sequence 5'-d(CGGAATTCCGCGGAATTCCGG)-3' were also analysed which contain the *Eco*RI recognition site alternating in a 10 bp and 11 bp repeat distance (average repeat distance 10.5 bp). Unmethylated multimers of this sequence show a migration anomaly slightly larger than that for the corresponding decanucleotides (see Figure 2). The migration anomaly of the methylated *Eco*RI recognition site is slightly smaller when arranged in an average repeat distance of 10.5 bp compared to 10.0 bp (Figure 2).

In general, a gel migration anomaly of curved DNA sequences is observed only when the repeat length of the sequence creating the effect (in bp) is close to the helical repeat (or its multimers, in bp/turn; Hagerman, 1985; Koo *et al.*, 1986). In this way, the small local curvature adds up to a macroscopically detectable (close to) planar curvature of the DNA molecule. When the sequence is not repeated in phase with the helix turn, the curvature of each short sequence is not confined to the same plane. This strongly reduces the migration anomaly or leads to normal migration behaviour (Hagerman, 1985; Koo *et al.*, 1986; Diekmann, 1987b). Thus, by varying the length of the oligonucleotide (i.e. varying the relation between sequence and helix repeats) we can test whether the observed migration anomaly originates from sequence-directed curvature.

The migration anomaly nearly vanishes when the repeat length of the *Eco*RI linker is changed from 10 bp to 12 bp. The 12 bp oligomers migrate almost normally although the central curvature inducing sequence is unchanged. The same effect is observed for methylated sequences. The migration anomaly of the methylated 10 bp linker is reduced > 10-fold for the methylated 12 bp linker (see Figure 2). A similar reduction in migration anomaly had already been observed before for another curved DNA sequence when the repeat length was changed from 10 to 12 bp (Koo *et al.*, 1986). In general, these results allow us to interpret the observed gel migration anomaly in terms of DNA curvature.

The self-complementary molecules could form cruciforms (Rinkel *et al.*, 1987a). DNA molecules with stable cruciforms also show gel migration anomalies (Gough and Lilley, 1985; Diekmann and Lilley, 1987). However, the nearly normal behaviour of the methylated and the unmethylated 12 bp *Eco*RI linkers strongly argues against this alternative explanation.

The sequence dCGGGATCCCG

The *Bam*HI linker sequence dCGGGATCCCG is closely related to the *Eco*RI sequence discussed above. The purine−pyrimidine sequence is identical in both sequences; however, the central dAATT is replaced by dGATC. The gel migration of these multimers is not hindered; instead, these molecules migrate slightly faster than expected for their length (*k*-factor < 1, see Figure 3). This small *k*-factor can be easily explained. Each of the marker fragments obtained from the pBR322 plasmid (Sutcliffe, 1979)

contain a few curved sequence elements. Thus, the marker fragments are not perfectly straight. However, the oligomer dCGGGATCCCG does not seem to contain any curved sequence element. Thus, its multimers migrate slightly faster than expected for 'normal' behaviour from the comparison with pBR322 marker fragments.

The sequence dCGGGATCCCG is recognized by two different methylases. *Bam*HI methylase replaces the hydrogen at the 5-position of the most central dC on both strands by a CH_3 group, while *dam* methylase methylates the central dA (see above) on both strands. Both chemical modifications of this sequence lead to a comparable but small retardation in polyacrylamide gels (see Figure 3). Thus, although different nucleotides are methylated and the position of the modified base relative to the centre of symmetry of the sequence is different, they seem to deflect the helix axis by about the same amount. This effect cannot be due to a change in the DNA helix stability, since the helix is stabilized by the methylation of the dCs (Gill *et al.*, 1974) but destabilized by the methylation of the dAs (Engel and von Hippel, 1978; Rinkel *et al.*, 1987a).

The small amount and the similarity of the gel retardations of these two modifications might suggest that the observed retardation is an artifact due, for example, to chemical impurities in the solutions loaded on the gel or to proteins bound to the DNA. To avoid these problems, special care was taken. Further, these experiments were carried out in parallel with the modification of the *Cla*I sequence. For that sequence no retardation was observed (see below).

The sequence dCCATCGATGG

Multimers of the *Cla*I linker have a *k*-factor < 1 (see Figure 4). Thus, this sequence seems to have a straight helix axis (see above). This sequence is recognized by the *Cla*I methylase, which methylates the most central dA on both strands (see above). Within experimental error, the methylation of this sequence has no effect on the migration in polyacrylamide gels (see Figure 4). These results indicate that the effect of methylation on electrophoretic mobility observed for the other sequences is not due to the increase in mol. wt of the DNA which accompanies methylation.

Discussion

In general, large DNA curvature is observed only for stretches of dA_n with $n \geq 4$ in a repeat length close to the turn of the helix (for a review see Diekmann, 1987a). Hagerman (1987) showed that the sequences dA_iT_i with $i = 2-5$ in a repeat length of 10 bp show electrophoretic properties similar to dA_n sequences with $n \geq 4$ while dT_iA_i sequences do not (Hagerman, 1986). The dA_iT_i sequences seem to be curved in a similar way and with similar properties as dA_n sequences with n slightly smaller than $2i$. This view is also supported by molecular mechanics calculations (von Kitzing and Diekmann, in preparation) and chemical modification studies (S.Diekmann and L.McLaughlin, submitted).

It is predicted by the hypothesis of von Kitzing and Diekmann (1987; Diekmann *et al.*, 1987) that a bulky chemical group at the N(6) position of dAs within stacked dAs enhances the curvature of this sequence. Indeed, this is observed for the *Eco*RI oligomer. The strong increase in curvature of the sequence dCGGAATTCCG upon methylation of the central dAs supports this hypothesis.

For the methylation of dAs in the sequences dGATC and dGAATTC (using *dam* and *Eco*RI methylase, respectively; Cheng *et al.*, 1985) an unwinding of the DNA double helix was observed. For the methylation of the self-complementary sequence dCGGAATTCCG the total unwinding angle is 1°. This corresponds to a change in the helical repeat of +0.03 bp/turn. Thus, the unwinding increases the difference between the helical repeat of this sequence (Fratini *et al.*, 1982; Diekmann and Wang, 1985) and the sequence repeat length (10.0 bp). This larger difference is expected to lead to a reduced migration anomaly (Hagerman, 1985; Diekmann, 1987b). However, the observed migration anomaly increases due to methylation. Thus, the increase in migration anomaly cannot be explained by the modified phase relation betweeen sequence and helix repeat. This conclusion is further supported by the data obtained for the *Eco*RI recognition site arranged in an average repeat distance of 10.5 bp. This sequence repeat length closely matches the number of bp/helical turn of these molecules (Fratini *et al.*, 1982; Diekmann and Wang, 1985). When methylated, the migration anomaly of the 21 bp multimers increases similarly to the 10 bp multimers.

According to our hypothesis (von Kitzing and Diekmann, 1987, Diekmann *et al.*, 1987), the stacking of purines with free 2-positions (for example dAs) are essential for the formation of DNA curvature. Sequences without stacked dAs, for example dA_iT_i sequences with $i = 1$, were found to be straight, in agreement with the hypothesis and with data of Hagerman (1987). When these dAT sequences are methylated at the dAs, no overall curvature (for the *Cla*I sequence) or only small curvature is induced (for the *Bam*HI sequence). Also in the *Cla*I sequence 5′-dCCATCGATGG-3′, the methylation of the dATs might induce a small degree of curvature as detected for the *Bam*HI sequence 5′-dCGGGATCCCG-3′. However, in the *Cla*I sequence the influence of the first dAT might be counter-balanced by the influence of the second dAT, since the distance between these sequences is about half a helix turn.

A small degree of curvature is also found for the *Bam*HI sequence when the most central dCs but not the dAs, are methylated. Thus, the helix axis of the methylated *Bam*HI oligomers might become slightly curved for different structural reasons.

For one of the sequences (the *Eco*RI oligomer), methylation has a marked effect on the DNA curvature. This structural feature might be used *in vivo* to recognize the methylated site. The presence of such a structural feature might significantly increase the rate of the recognition process (Diekmann and McLaughlin, submitted). Curvature, however, is not a general property of methylated DNA sequences. The straight helix axis of the *Bam*HI sequence is only slightly affected by methylation, that of the *Cla*I sequence apparently not at all. Thus, curvature at methylated sites might be an additional recognition feature of some sequences; however, such a mechanism would not be a general phenomenon.

Materials and methods

Three different DNA linkers of the sequences 5′-d(CGGGATCCCG)-3′ (*Bam*HI), 5′-d(CCATCGATGG)-3′ (*Cla*I) and 5′-d(CGGAATTCCG)-3′ (*Eco*RI) were purchased from New England Biolabs. These non-phosphorylated linkers are 10 bp in length. Their sequences are self-complementary. The lyophilized samples were dissolved in water to yield DNA concentrations of 0.1−1.0 μg/μl. The 12 bp long self-complementary oligomers 5′-d(CCGGAATTCCGG)-3′ and the 21 bp long oligomers 5′-d(CGGAATTCCGCGGAATTCCGG)-3′ and 5′-d(GAATTCCGCGGAATTCCGCCG)-3′ were synthesized on an Applied Biosystems synthesizer. These oligomers were purified by HPLC on a reverse-phase column (Ott and Eckstein, 1984).

In separate reactions, ~3 μg of each self-complementary linker were phosphorylated using T4 Polynucleotide Kinase (10 U, New England Biolabs, 2 h, 37°C). The reaction mixtures were cooled slowly to 0°C so that blunt-ended double-stranded oligomers are formed. Then, T4 DNA Ligase (400 U, New England Biolabs) was added. The ligase reaction was carried out at 16°C for 1 h followed by another hour at 37°C. The reaction mixtures were ethanol precipitated. The ligase reactions yield a multimer distribution for each DNA

oligomer. The complementary 21 bp oligomers were phosphorylated separately. The reaction solutions were mixed, and after another 15 min at 37°C slowly cooled to 0°C. The two oligomers anneal to double-stranded molecules with overhanging ends. After the addition of 200 U of T4 DNA Ligase the solution was incubated at 16°C. Four aliquots of equal volume of the reaction mixture were taken at different times (for example at 4 min, 10 min, 20 min and 40 min). The reactions were stopped by ethanol precipitation. Multimer distributions of different incubation times were mixed to give a more homogeneous multimer distribution.

About 0.5 μg of the linkers ligated to multimers were methylated using *Bam*HI and *dam* methylase for the *Bam*HI sequence and the *Cla*I- and *Eco*RI methylase for the *Cla*I and *Eco*RI sequences, respectively (all methylases from New England Biolabs). The methylation reactions (10 μl) were carried out in the buffers indicated by New England Biolabs using between 8 U (*Bam*HI methylase) and 30 U (*dam* methylase) per reaction. After ~3 h at 37°C the reaction mixtures were ethanol precipitated and the methylation reactions were repeated as described above. After this second methylation reaction the solutions were phenol and chloroform extracted once, ether extracted three times and ethanol precipitated. After only one methylation reaction, the distribution of ligated multimers of the *Eco*RI oligomer was found not to be completely methylated (data not shown), while after two methylation reactions complete methylation was observed.

The methylation multimers were incubated in the required buffers with restriction endonucleases against which the sequences became protected by the methylation: *Bam*HI (8 U) for the *Bam*HI linker methylated by *Bam*HI methylase, *Mbo*I (10 U) for the *Bam*HI linker methylated by *dam* methylase, *Cla*I (10 U) for the *Cla*I linker methylated by the *Cla*I methylase, and *Eco*RI (20 U) for the *Eco*RI linkers methylated by *Eco*RI methylase (*Eco*RI from Boehringer Mannheim, all other restriction endonucleases from New England Biolabs). After 3 h of reaction, the solutions were ethanol precipitated and loaded on a 10% (w/v) polyacrylamide gel (29:1 acrylamide to bis, 4 V/cm at room temperature in 45 mM Tris-borate, 1.25 mM Na–EDTA, pH 8.6). The mobilities of these DNA fragments were compared to those of DNA fragments obtained from a *Hae*III and a *Hin*fI digest (both endonucleases from New England Biolabs) of pBR322 (Sutcliffe, 1979). A typical gel is presented in Figure 1. The corresponding unmethylated multimer distributions were digested under the same conditions. The unprotected multimer distributions were completely cut down to monomers (for the *Eco*RI and *Cla*I sequences) or to a distribution up to trimers (for the *Bam*HI sequence, data not shown).

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