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Probing the conformations of eight cloned DNA dodecamers; CGCGAATTCGCG, CGCGTTAACGCG, CGCGTATACGCG, CGCGATATCGCG, CGCAAATTTGCG, CGCTTTAAAGCG, CGCGGATCCGCG and CGCGGTACCGCG

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ABSTRACT

The self complementary DNA dodecamers d(CGCGAATTCGCG), d(CGCGTTAACGCG), d(CGCGTATACGCG), d(CGCGATATCGCG), d(CGCAAATTTGCG), d(CGCTTTAAAGCG), d(CGCGGATCCGCG) and d(CGCGGTACCGCG) have been cloned into the *Sma*I site of plasmid pUC19. Radiolabelled polylinker fragments containing these inserts have been digested with nucleases and chemical agents, probing the structure of the central AT base pairs. The sequences AATT and AAATTT are relatively resistant to digestion by DNase I, micrococcal nuclease and hydroxyl radicals, consistent with the suggestion that they possess a narrow minor groove. Nuclease digestion of TTAA is much more even, and comparable to that at mixed sequence DNA. TpA steps in ATAT, TATA and GTAC are cut less well by DNAse I than in TTAA. DNaseI cleavage of surrounding bases, especially CpG is strongly influenced by the nature of the central sequence.

INTRODUCTION

A great deal of evidence has accumulated over the past few years suggesting that DNA structure is not uniform but is affected by such factors as superhelical stress, drug binding and local DNA sequence. The sequence which has been studied in greatest detail by X-ray crystallography is the 'Dickerson' dodecamer CGCGAATTCGCG, containing a central *Eco*R1 recognition site [1–4]. Although this oligomer adopts a B-like DNA conformation its structure is not uniform along its length. In particular the central AATT possesses a narrow minor groove, associated with higher propeller twist than at the GC ends. The entire molecule is bent by 19°. Attempts to crystallise related DNA fragments containing other central regions, especially TpA steps, have been largely unsuccessful [5], though the structure of CGATTAACG has recently been reported [6]. The structure of CGCGATATCGCG has also been determined, complexed with the minor groove binding drug netropsin [5]. This also possesses a narrow minor groove at the AT base pairs, although this may merely be a consequence of drug binding. The outermost AT base pairs have much lower propeller twist than the central two and the stacking at the two ApT steps is not identical. The helical twist is lower at ApT than TpA. Structures have also been determined for CGCAAATTTGCG, both free [7,8] and complexed with the minor groove binding ligand pentamidine [9] revealing that it too possesses a narrow minor groove in the centre. The crystal structures of several other oligonucleotides have recently been reviewed [10]. The influence of sequence on DNA structure is emphasised for fragments containing repeats of the oligomers CGAAAATTTT and CGTTTTAAAA [11]. The former displays a hydroxyl radical cleavage pattern consistent with a bent structure while the latter presents a more even pattern. This difference may be related to the nature of the central dinucleotide i.e. ApT or TpA.

Lomonosoff *et al.*, [12] and Dickerson & Drew [2] have previously attempted to correlate DNase I digestion of the dodecamer CGCGAATTCGCG with its crystal structure. In this paper we have probed the conformations adopted by closely related sequences by cloning the self-complementary dodecamers CGCGATATCGCG, CGCGTATACGCG, CGCGTTAACGCG, CGCGAATTCGCG, CGCAAATTTGCG, CGCTTTAAAGCG, CGCGGTACCGCG and CGCGGATCCGCG into longer DNA fragments and using these as substrates for enzymic and chemical probes sensitive to DNA structure.

MATERIALS AND METHODS

DNA fragments

The self-complementary oligonucleotides dCGCGATATCGCG, dCGCGTATACGCG, dCGCGTTAACGCG, dCGCGAATTCGCG, dCGCAAATTTGCG, dCGCTTTAAACGC, dCGCGGATCCGCG and dCGCGGTACCGCG were prepared on an Applied Biosystems DNA synthesiser and used without further purification. The dodecamers were treated with polynucleotide kinase and ATP and ligated into *Sma*I (CCC/GGG) cut pUC19, which had been treated with alkaline

phosphatase, and transformed into *E.coli* TG2. Transformants were picked from X-gal, IPTG plates containing 100μg/ml ampicillin. The insertion of a 12 base pair fragment preserves the reading frame and is not large enough to inactivate the *lacZ* gene product; as a result successful full length monomeric clones were picked off as blue colonies (except for CGCGTTAACGCG and CGCTTTAAAGCG which contain in-frame stop codons (TTA) and so yield white colonies). All the other white colonies consisted of either multiple insertions (although trimers still gave blue colonies) or contained one or two base deletions around the *SmaI* site. Some digestion experiments were also performed on the fragments containing deletions in the bases surrounding the inserts to confirm the digestion pattern of the central base pairs. The sequences of the plasmids were confirmed by dideoxy sequencing using a T7 sequencing kit (Pharmacia).

DNA fragments containing the dodecamer inserts were obtained by cutting with *Hin*dIII, labelling at the 3'-end with α-[^{32}P]dATP using reverse transcriptase and cutting again with *Sac*I. In some instances the DNA was labelled at the opposite end by cutting with *Eco*R1 and *Pst*I. The radiolabelled DNA fragments were separated from the rest of the plasmid on 8% polyacrylamide gels.

DNA cleavage

The fragments were digested with DNase I [13,14], micrococcal nuclease [15] and hydroxyl radicals [16,17] as previously described. The products of digestion were resolved on 12% polyacrylamide gels containing 8M urea and 20% formamide. We found that the formamide was essential to prevent unacceptable band compressions in the GC regions. Gels were run at 1500V for about 2 hrs, fixed in 10% acetic acid, transferred to Whatmann 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at −70°C with an intensifying screen. Autoradiographs were scanned with a Joyce-Loebl chromoscan III microdensitometer.

RESULTS

Four central AT base pairs

DNase I cleavage. Figure 1 presents DNase I digestion patterns for DNA fragments containing the inserts CGCGATATCGCG, CGCGTATACGCG, CGCGTTAACGCG and CGCGAATTCGCG. A quantitative representation of the cleavage of the central dodecamers is shown in Figure 2. It is immediately apparent that these cleavage patterns are very different. For ATAT the strongest cleavage products correspond to the second ApT step and the first CpG on the 3'-side of the centre. Three strong products are evident with TATA, corresponding to cleavage of GpT, ApT and ApC. Cleavage of TTAA is more even with seven strong bands evident for cutting at each of the steps in the central sequence GpTpTpApApCpGpC. In contrast cleavage of the central region of AATT is poor; the only strong band corresponds to cutting of the TpC step. This is similar to the pattern reported by Lomonosoff *et al.* [12], working with the isolated dodecamer, except that we observe weaker cleavage at the GpC step in the second half of the sequence. This difference most likely arises from constraints imposed as a result of cloning the DNA into a longer fragment. The short oligonucleotide may suffer from end-effects in which the DNA structure will be less rigidly constrained. Also shown in Figure 2 is the DNase I cleavage pattern of a trimer insert of CGCGAATTCGCG

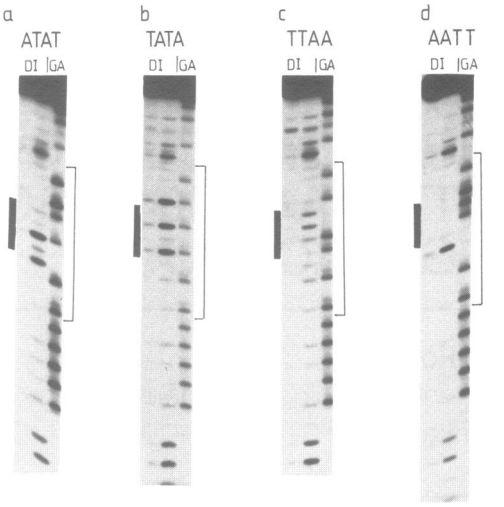

Figure 1. DNase I cleavage patterns for fragments containing the inserts a) CGCGATATCGCG (ATAT); b) CGCGTATACGCG (TATA); c) CGCGTTAACGCG (TTAA) and d) CGCGAATTCGCG (AATT). Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. The tracks labelled 'GA' are Maxam−Gilbert sequencing lanes for purines (G+A). The brackets indicate the position and length of the dodecamer inserts, the boxes show the position of the four central AT base pairs.

(lacking one of the central cytosines). This again shows that the only bond which is efficiently cleaved is the TpC step. Experiments on fragments labelled at the opposite (*Eco*R1) end or with inserts lacking one or two GC base pairs from the *Sma*I site gave similar cleavage patterns.

Cleavage in the GC-portions of the inserts is fairly constant throughout the fragments. At the boundary with the AT-base pairs the GT bonds of TATA and TTAA are cut much better than the GA bonds of ATAT and AATT; cleavage of GT is better in TATA, possessing a strictly alternating sequence of purines and pyrimidines than in TTAA. In general cleavage of GpC is better than CpG as previously observed with alternating GC [18]. The one exception is at the first CG step in the 3'-half. This is cut poorly in TATA and AATT, but is one of the strongest cleavage products in ATAT. Cleavage of this bond in TTAA is intermediate intensity.

Micrococcal nuclease cleavage. Figure 3 presents patterns of micrococcal nuclease digestion of the four DNA fragments. Histograms of the cleavage pattern are also presented in Figure 2. This nuclease cuts almost exclusively at pA and pT bonds, especially in regions of alternating AT, and is presumed to be sensitive to the DNA local breathing motions [15,19]. As a result the strong bands arise from cleavage of the four central AT base pairs. The relative efficiency of cleavage in the various central regions can be seen by comparison with the other pA and pT bonds located towards the bottom of the gel. Once again the cleavage patterns are different for the four inserts. Micrococcal nuclease digestion of ATAT is weak, the three strongest products correspond to cutting of pTpApT; the first of these is slightly stronger. Cleavage of TATA is stronger and again is best at the three bonds on the 3'-side of the central AT-stretch. TTAA is cut poorly; the strongest band corresponds to cutting of the central TpA step. AATT is cut poorly, the strongest bond corresponds to cleavage of the TpT step.

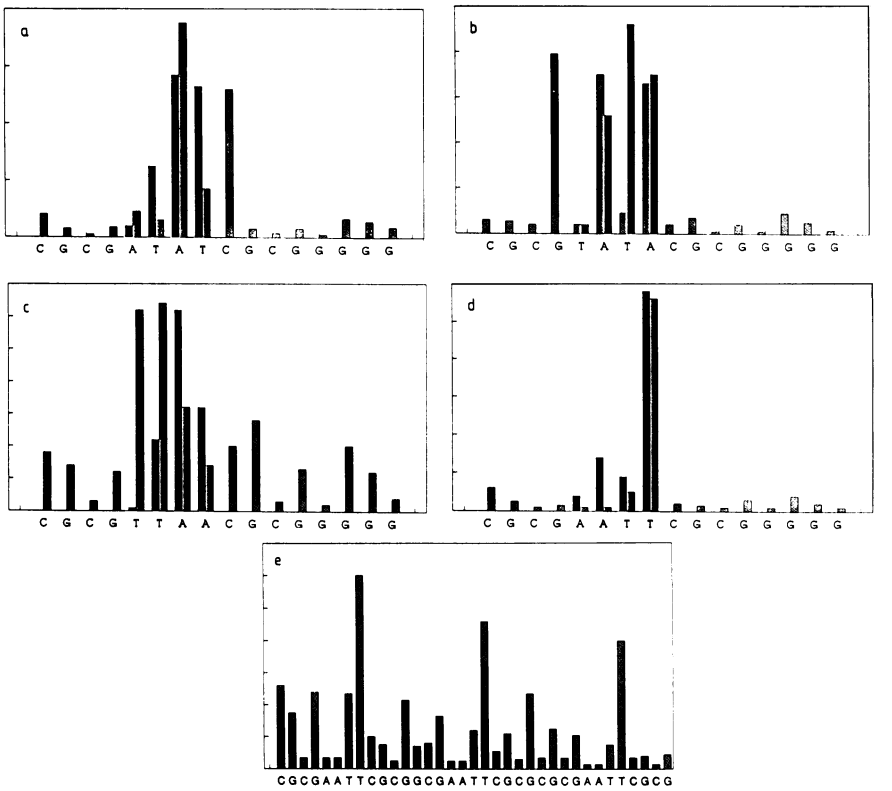

Figure 2. Histograms representing the relative cleavage at each bond in the inserts by DNase I (hatched boxes) and micrococcal nuclease. a) CGCGATATCGCG, b) CGCGTATACGCG, c) CGCGTTAACGCG, d) CGCGAATTCGCG, e) Trimeric insert of CGCGAATTCGCG. The values were calculated from densitometer traces of the data presented in Figures 1 and 3. Note that the values are relative within each sequence and for the specified probe. For DNase I each bar corresponds to cleavage of the bond on the 3'-(right hand) side. For micrococcal nuclease each bar corresponds to cleavage of the bond on the 3'-(left hand) side.

Hydroxyl radical cleavage. Hydroxyl radicals are generally used as sequence neutral DNA probes which produce an even ladder of bands which is almost independent of the local sequence [16,17]. However several studies have shown that bent DNA fragment, possessing a narrow minor groove show a reduced sensitivity to hydroxyl radical attack [11,20]. Repeated blocks of oligodA.oligodT show a sinusoidal cleavage pattern which gets weaker towards the 3'-end of each oligodA tract [11,20]. Figure 4 presents hydroxyl radical cleavage of the four fragments. At first glance each of these is an even ladder of bands. However, close inspection and careful analysis of densitometer traces reveals that there are fluctuations in cleavage intensity within the inserts. Histograms of the cleavage are presented in Figure 5. These show an even pattern of products for ATAT but reveal that cleavage of the other three fragments decreases in the central AT region. This is most pronounced for AATT. Also included in Figure 5 is a histogram of hydroxyl radical cleavage of the trimeric insert of AATT which again displays a reduced cleavage around each AATT. Experiments on fragments labelled at the opposite (*Eco*R1) end or with inserts lacking one or two GC base pairs from the *Sma*I site gave similar cleavage patterns.

Two central AT base pairs

DNase I cleavage. Figure 6a,b presents DNase I digestion patterns for DNA fragments containing the inserts CGCGGATCCGCG, and CGCGGTACCGCG. A quantitative representation

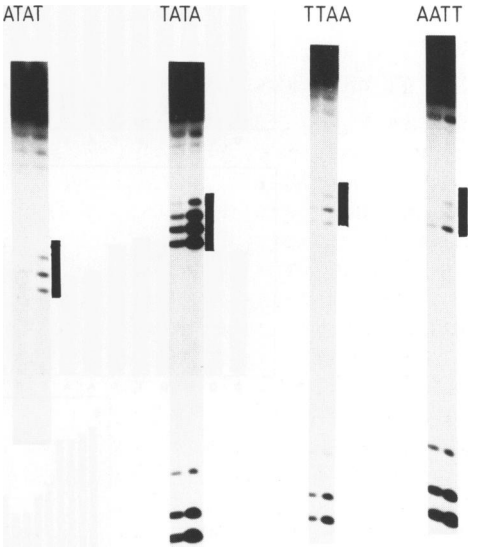

Figure 3. Micrococcal nuclease cleavage patterns for fragments containing the inserts CGCGATATCGCG (ATAT); CGCGTATACGCG (TATA); CGCGTTAACGCG (TTAA) and CGCGAATTCGCG (AATT). Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. The boxes show the position of the four central AT base pairs.

of the cleavage of the central dodecamers is shown in Figure 7a,b. Comparing ATAT with GTAC, which retain the same order of purines and pyrimidines, we can see that replacing ApT with ApC has drastically reduced the cleavage, though the anomalously good cleavage of the distal CpG is retained. Similarly comparing AATT with GATC it can be seen that the TpC and CpC steps in identical positions are cut with similar efficiency. The greatest difference between these DNAs it at the distal CpG step which is cut much better in GATC than AATT.

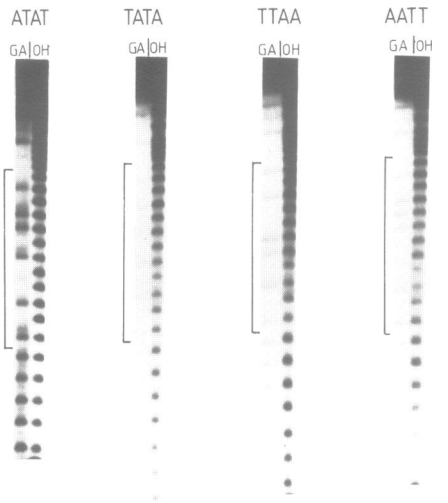

Figure 4. Patterns of hydroxyl radical cleavage (OH) for fragments containing the inserts CGCGATATCGCG (ATAT); CGCGTATACGCG (TATA); CGCGTTAACGCG (TTAA) and CGCGAATTCGCG (AATT). The lanes labelled 'GA' are Maxam Gilbert G+A tracks. The brackets indicate the position and length of the dodecamer inserts.

Hydroxyl radical cleavage. Hydroxyl radical cleavage patterns for CGCGGATCCGCG and CGCGGTACCGCG are presented in Figure 6a,b and are illustrated as histograms in Figure 8a,b. It can be seen that cleavage of GTAC is fairly even throughout the fragment, whereas GATC shows a significant decrease towards the centre. This is similar to the patterns found for ATAT and AATT though the attenuated cleavage in the centre of G-ATC is less pronounced than for AATT.

Six central AT base pairs

DNase I cleavage. Figure 6c,d presents DNase I digestion patterns for DNA fragments containing the inserts CGC-AAATTTGCG, and CGCTTTAAAGCG. A quantitative representation of the cleavage of the central dodecamers is shown in Figure 7c,d. It is evident that cleavage of the central AT-region is very different for these two fragments. Looking first at A_3T_3 the strongest cleavage products correspond to the first ApA and the second TpT. The latter is in a similar position to the TpC bond which was cut best in AATT (Figure 2) and the CpC bond of GATC (Figure 7), which contains the same order of pyrimidine and purine residues. However this is cut much less efficiently than in AATT and is now of comparable intensity to the cleavage in the GC-regions. It appears that increasing the length of the AT-stretch from AATT to AAATTT has drastically reduced its sensitivity to DNAse I. Comparison of T_3A_3 with TTAA is less easy since the precise order of purine and

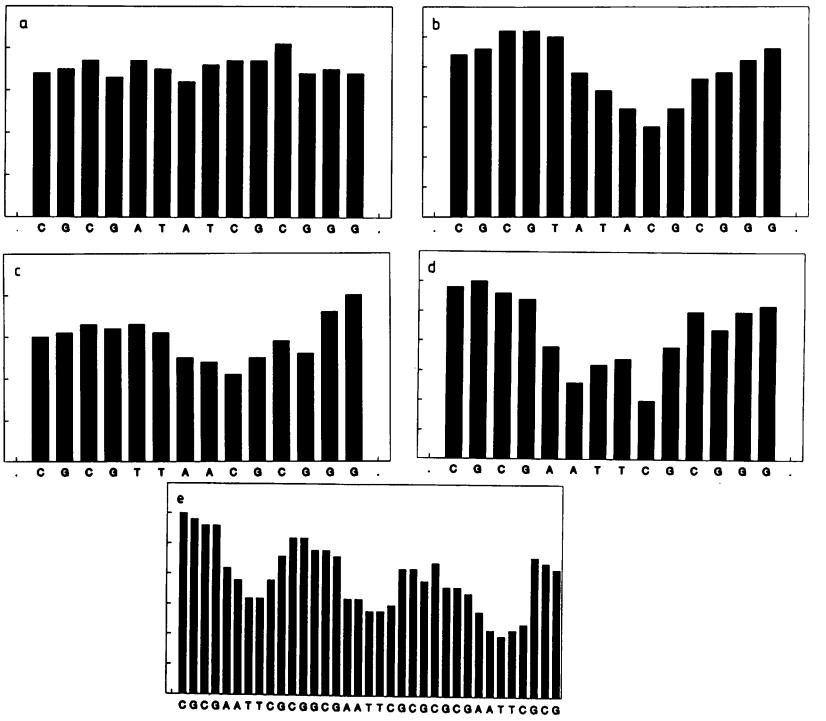

Figure 5. Histograms representing the relative efficiency of hydroxyl radical cleavage at each bond in the inserts. a) CGCGATATCGCG, b) CGCGTATACGCG, c) CGCGTTAACGCG, d) CGCGAATTCGCG, e) trimeric insert of CGCGAATTCGCG. The values were calculated from densitometer traces of the data presented in Figure 4.

pyrimidine bases has been changed, however it can be seen that the second TpT step is cut much better than all the other bonds in the insert. This compares with the good cleavage found at the TpT in TTAA. However whereas the TpA step was cut well in TTAA it is a much poorer DNase I substrate in T_3A_3. The GpC steps on the 3'-side of the centre are cut poorly in both T_3A_3 and A_3T_3 in both instances CpG is cut with slightly greater efficiency. The TpG step in A_3T_3, which replaces the CpG step in AATT and GATC which was cut surprisingly well, is cut poorly.

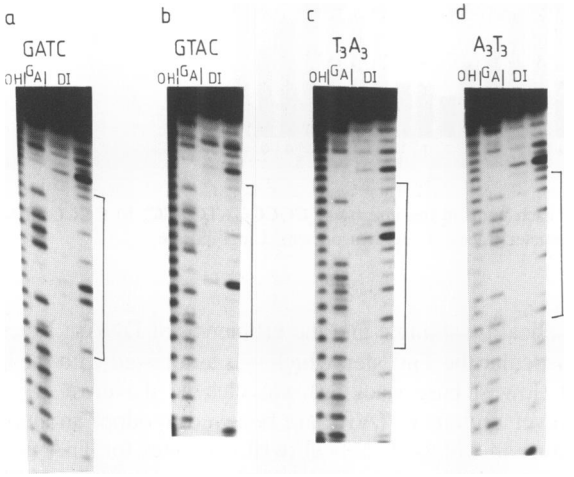

Figure 6. DNase I (DI) and hydroxyl radical (OH) cleavage patterns for fragments containing the inserts a) CGCGGATCCGCG (GATC); b) CGCGGTACCGCG (GTAC); c) CGCTTTAAAGCG (T_3A_3) and d) CGCAAATTTGCG (A_3T_3). For DNase I each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. The tracks labelled 'GA' are Maxam–Gilbert sequencing lanes for purines (G+A). The brackets indicate the position and length of the dodecamer inserts.

Hydroxyl radical cleavage. Hydroxyl radical cleavage patterns for CGCAAATTTGCG and CGCTTTAAAGCG are presented in Figure 6c,d and are illustrated as histograms in Figure 8c,d. Both of these show decreased cleavage in the central AT-region which is much more pronounced for A_3T_3 than T_3A_3.

DISCUSSION

Global structure at the central AT base pairs

Several studies have suggested that local variations in the hydroxyl radical cleavage pattern arise from changes in the minor groove width [11,20]. If we accept this explanation then the results presented in this paper suggest that the central portions of AATT and AAATTT adopt a narrower minor groove than the surrounding GC regions, as demonstrated in crystal structures of these sequences. The effect is more pronounced in the latter sequence. The fragment containing only a single AT step (GATC) also revealed a small decrease in hydroxyl radical cleavage. It therefore appears that the presence of an isolated AT step is sufficient to cause a change in the DNA structure but that this is most pronounced within a longer stretch of $A_n.T_n$. This decreased cleavage may be caused by either a local narrowing of the minor groove or a bend in the helix axis, or some combination of both. It is worth remembering hydroxyl radicals act by extracting a hydrogen atom from deoxyribose, probably attacking C4'[21] so that a decrease in cleavage reflects reduced accessibility of this position, not necessarily a decrease in groove width. Small decreases in hydroxyl radical attack are also found in the centre of TATA, TTAA and TTTAAA. In contrast ATAT and GTAC show an even ladder of hydroxyl radical cleavage products. Of these dodecamers only AATT and AAATTT have been successfully crystallised [1–4], possibly because they alone possess a narrow groove width in this region, which might maximize the phosphate-phosphate distances between adjacent molecules in the crystal packing [5]. Of the other dodecamers ATAT has been crystallised in the presence of the minor groove

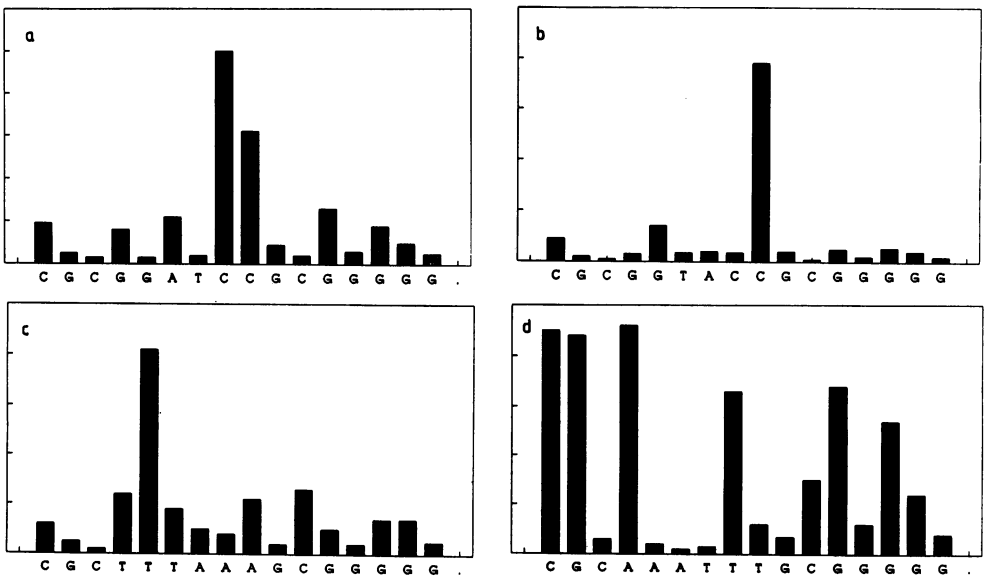

Figure 7. Histograms representing the relative cleavage at each bond in the inserts by DNase I. a) CGCGGATCCGC, b) CGCGGTACCGC, c) CGCTTTAAACGC, d) CGCAAATTTCGC. The values were calculated from densitometer traces of the data presented in Figure 6. Each bar corresponds to cleavage of the bond on the 3'-(right hand) side.

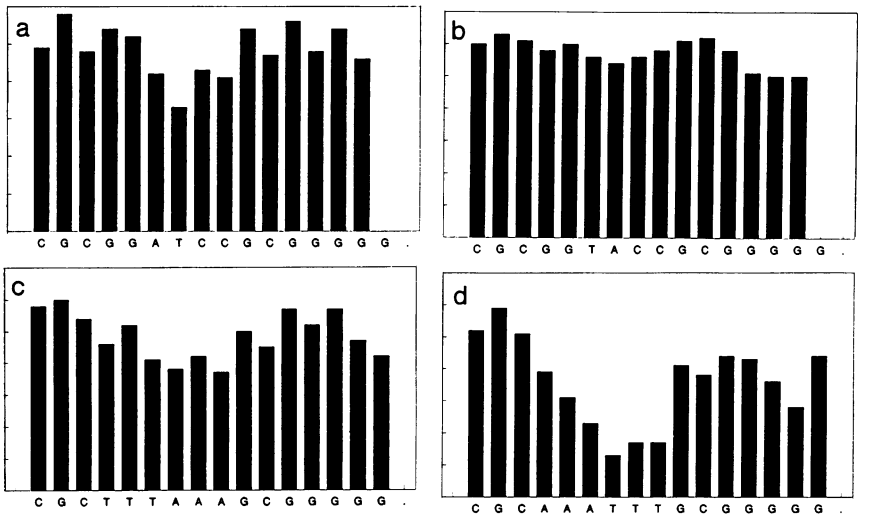

Figure 8. Histograms representing the relative efficiency of hydroxyl radical cleavage at each bond in the inserts. a) CGCGGATCCGC, b) CGCGGTACCGC, c) CGCTTTAAACGC, d) CGCAAATTTCGC. The values were calculated from densitometer traces of the data presented in Figure 6.

binding drug netropsin, but could not be crystallised alone [5]. This structure also revealed a narrow minor groove in the central portion, though it might be argued that this arises as a consequence of ligand binding and would not be present in the native dodecamer. The only other closely related DNA sequence which has been crystallised is the octanucleotide GGTATACC [22] which adopted an A-like conformation. This sequence has also been crystallised with DNase I [23], and shown to be resistant to this nuclease, revealing a structure with several of the characteristics of A-DNA.

Precise interpretation of the DNase I and micrococal nuclease digestion patterns is not easy since too little is known about the exact structural requirements of these enzymes. Micrococcal nuclease is presumed to be sensitive to local DNA structure. The crystal structure of this enzyme reveals that it possesses a cleft which can accommodate a single strand of DNA, suggesting that the enzyme senses the local breathing motions of the DNA helix [24]. If this is the case then we can correlate micrococcal nuclease digestion with the local stacking patterns. Interestingly the sequence which shows best MNase cleavage is TATA, containing two TpA steps which are known to be poorly stacked. Cleavage of the other fragments by this enzyme is poor and in order ATAT > TTAA > AATT. This suggests that not only are the average structures different for each of the AT sequences but their dynamic breathing motions are also affected. It seems that alternating AT is cut better than homopolymeric AT and that the presence of an TpA step increases the efficiency of MNase attack.

The factors affecting DNase I cleavage efficiency have been the subject of much debate. It is accepted that the enzyme is sensitive to the width of the DNA minor groove [13,25–27]; explaining why sequences such as polydA.polydT are resistant to enzyme attack. However this global parameter does not explain why certain regions with an apparently wide minor groove (such as GC-rich regions) are often poor substrates for the enzyme. Variations in this global parameter can not explain the large differences observed in the cleavage of adjacent phosphodiester bonds. By comparing the crystal structure of the dodecamer CGCGAATTCGCG with its DNase I cleavage pattern Dickerson & Drew [2] suggested that there was a good correlation between the helical twist angle and the efficiency of DNAse I cleavage. In particular the TpC step which was associated with the highest twist (lowest base stacking) was cleaved the most efficiently. However regions of $(AT)_n$ are believed to adopt an alternating structure in which the helical twist is greater for TpA than ApT [28], the opposite of the DNase I cleavage preference [29]. The relationship with helical twist also seems unlikely since in the crystalline complex of DNase I with dGCGATCGC the CpG bond (with low twist) was preferentially cleaved [27]. It has been noted that in each of the crystal structures of DNase I with oligonucleotides the DNA is bent towards the major groove, opening up the roll angles of the bases in the minor groove. It therefore seems likely that DNA flexibility is another factor contributing to the cleavage efficiency. However this again complicates the interpretation since in the crystal structure the base steps showing the largest bends are two or three bases removed from the actual cleavage site [27]. A further complicating factor is that there may be unfavourable steric interaction between the enzyme and the 2-amino group of guanine in bases within the six base pair binding domain, but remote from the cleavage site [27]. Indeed DNase I cleavage efficiency may be affected by structural alterations at any step along its six base pair binding site. However we would not expect this to affect the relative cleavage patterns in the central regions of these four dodecamers since they are each located in identical GC-rich environments.

Cleavage at identical dinucleotide steps

Looking only at the central AT base pairs and their immediately adjacent GC pairs there are examples of six ApT steps (two in ATAT, and one each in TATA, AATT, GATC and A_3T_3) and six TpA steps (two in TATA, one each in ATAT, TTAA, GTAC and T_3A_3). How does DNase I cleavage of each of these steps compare in the different sequence environments? In every case the ApT bonds are cut better than corresponding TpA steps. However there is considerable variability in the cutting at each identical step. For example the two ApT steps in ATAT are cleaved at very different rates and the steps in the centre of AATT and A_3T_3 are hardly cut at all. The opposite is found for

cleavage of TpA; this is very weak at both steps in TATA and in GTAC, but is relatively strong in TTAA. The cleavage of TpC is also very different in ATAT and AATT, being much stronger in the latter.

The bond showing the most dramatic changes in cleavage efficiency is the first CpG step on the 3'-side of the centre. This is cut poorly in AATT and TTAA but one of the most sensitive cleavage sites in ATAT and GTAC; cleavage of TTAA and GATC is intermediate. This base step is part of the same tetranucleotide (TCGA) in both ATAT and AATT yet is cleaved with very different efficiencies. Whatever may be the structural features responsible for efficient DNase I cleavage it appears that (at least for CpG) even four base pairs is not sufficient to define the local structure.

DNAse I cleavage at certain positions appears to be influenced by the surrounding sequences, rather than the dinucleotide step itself. For example good cleavage products are found in identical positions for A_3T_3, AATT and GATC (i.e. the last TT step in A_3T_3, TC step in AATT and the CC step in GATC). It appears that, within the context of the sequence RRATYY, the YY step will be cut well by DNase I. Similarly the distal CG step is cut well in ATAT and GTAC. However in other instances changing one purine for another changes the cleavage pattern. For example the distal CG step is cut well in GATC, but hardly at all in AATT.

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