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DNA sequence preferences for an intercalating porphyrin compound revealed by footprinting

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ABSTRACT

The DNA sequence preferences of the compound meso-tetra-(4-N-methyl(pyridyl) porphyrin and its nickel complex have been investigated by means of footprinting experiments on several DNA fragments, using DNAase I and micrococcal nuclease as footprinting agents. A complex pattern of both AT and GC-protected sites was found. Ligand-induced long-range conformational changes were inferred in several instances to be related to the observed large-scale blockages of enzymatic cutting.

INTRODUCTION

A number of studies have shown that the cationic porphyrin derivative meso-tetra-(4-N-methylpyridyl) porphyrin (Fig. 1.) binds to double-stranded DNA[1-6]. Both metal-free and nickel-containing compounds are believed to bind by intercalative mechanisms, as evidenced by, for example, characteristic unwinding of supercoiled closed circular duplex DNA, and downfield shifts in the ^{31}P NMR spectrum of DNA. Binding studies with synthetic polynucleotides have shown that there are marked differences in behaviour towards AT and GC sites[7,8]. Interactions at the former have been interpreted in terms of external, non-intercalative binding, while binding at GC sites is suggested to represent intercalation. A recent NMR study of binding to defined oligonucleotide sequences has concluded that this porphyrin selectively intercalates into the dinucleotide CpG.

This paper reports on a series of investigations that probe the sequence selectivity of this porphyrin by means of its effect on the pattern of enzymatic cleavage of several DNA fragments. The porphyrin ligand is especially favourable for footprinting since the kinetics of its interaction with DNA are significantly slower than for many structurally simple intercalators. It has been suggested that compounds such as ethidium do not yield footprinting patterns under standard conditions because they dissociate rapidly from DNA[9]. As a result any blockages are short-lived and are not readily detected by the enzyme.

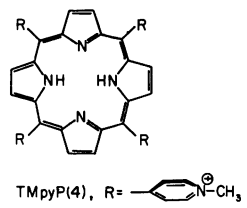


Figure 1 Structural formula of TMPy.

EXPERIMENTAL

Meso-tetra-(4-N-methylpyridyl) porphyrin (TMPy) and its nickel complex (TMPy-Ni) as tosyl and chloride salts respectively, were donated by W.D. Wilson. Stock solutions of them were prepared in 10mM tris,HCl at pH7.5, containing 10mM NaCl. DNAase I was obtained from Sigma and prepared as 7200U/ml stock solution in 0.15M NaCl containing 1mM $MgCl_2$. This was stored at -20°C. Micrococcal nuclease was purchased from Pharmacia and stored at -20°C as a 2500 units/ml stock solution in buffer containing 50mM tris, 2mM $CaCl_2$ pH7.6.

DNA Substrates

The 160 base-pair tyrT fragment was isolated and labelled as previously described[10-12]. Incubation with reverse transcriptase and (α-^{32}P) dCTP led to selective labelling of the 3'-end of the top strand (Fig.5) whereas incubation with dTTP and (α-^{32}P)dATP led to selective labelling at the 3'-end of the bottom strand. The 102 base-pair fragment was obtained from pBR322 by digestion with AatII and Hind III. It was selectively labelled at the 3' end of the Hind III site with reverse transcriptase and (α-^{32}P)dATP. The 166 base-pair ptyr2 DNA fragment was isolated from pMLB1048 as previously described by cutting with ECOR1 and BstE11. It was labelled at the 3'-end of the ECORI site with reverse transcriptase and (α ^{32}P)dATP. Bands in the digestion patterns were assigned by running dimethylsulphate-piperidine markers specific for guanine.

Footprinting

Digestion with DNase I was performed as previously described. Samples of the labelled DNA fragment (about 3 pmol in base-pairs per gel lane) were incubated with porphyrin solution (2-20mM) at 37°C for 30 min, prior to the addition of DNase I dissolved in 20mM NaCl, 2mM $MgCl_2$, 2mM $MnCl_2$, at a final enzyme concentration of 0.01 U/ml. Aliquots (3µl) were removed from the mixture after various times, up to a further 30 mins, and the reaction was stopped by adding 2.5µl 80% of 80% bromophenol blue, 10mM EDTA and 1mM NaOH. Samples were heated at 100°C for at least 3 min prior to electrophoresis.

A similar procedure was employed for footprinting with micrococcal nuclease using a final enzyme concentration of 0.2-1 unit/ml in buffer containing 50mM tris-HCl, 2mMCaCl$_2$pH7.6. It should be noted that micrococcal nuclease cleaves the 05'-P bond whereas DNAase I and piperdine both cut the 03'-P bond. As a result, labelled fragments in the G tracks, carry an extra phosphate group and so run between 0.3-1.5 bands faster than corresponding bands in the micrococcal digest.

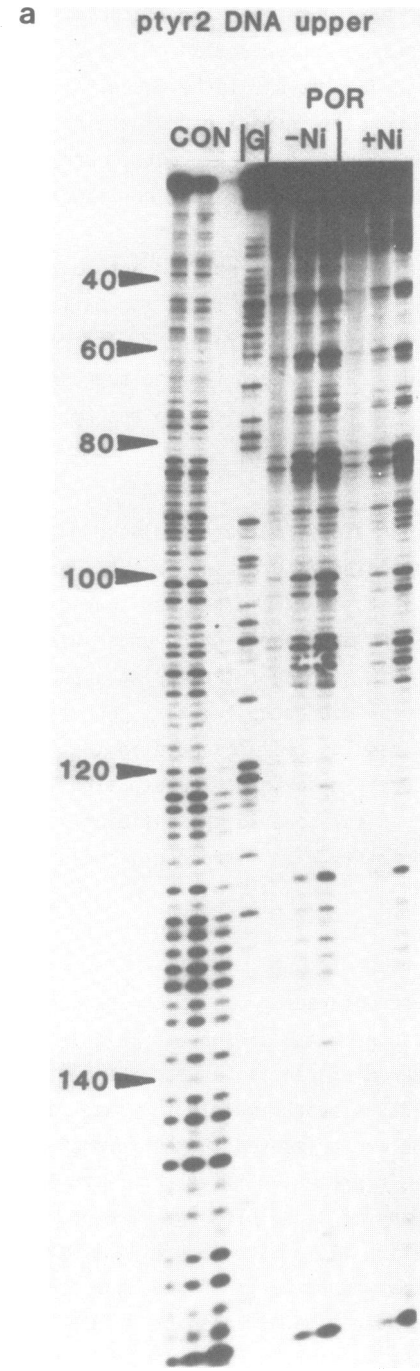
A comparison of cleavage patterns from both enzymes for both the 1 min and 30 min digestion periods (representing total incubation periods of 31 and 61 min respectively), show no significant differences with respect to positions of enhancement or protection. This would tend to suggest that the initial incubation period of 30 min was sufficient to ensure an equilibrium distribution of the ligand.

Gel Electrophoresis

The products of DNA digestion were analysed on 0.3mm 8% (w/v) polyacrylamide gels containing 8M urea and Tris-borate-EDTA buffer (pH 8.3). After 2h electrophoresis at 1500 V the gel was soaked in 10% acetic acid for 10 min, transferred to Whatman 3MM paper, dried under vacuum at 80^{o}C and subjected to autoradiography at -70^{o}C with an intensifying screen. Autoradiographs were scanned using a Joyce-Loebl microdensitometer. For experiments involving digestion with DNAase I results are presented in the form of differential cleavage plots as previously described, representing ln fa/fc where fa and fc are the fractional cleavage of any bond in the antibiotic treated and control lanes respectively. For experiments with micrococcal nuclease blockage was assessed by visual inspection.

RESULTS

Figure 2a shows an autoradiograph of the products of digestion of ptyr2 DNA by DNAase I in the absence and presence of either TMPy or TMPy-Ni. It can be seen that there are clear differences between the control lanes and those for the two compounds, which produce approximately the same gel patterns. The data for the nickel-free compound are presented in the form of a differential cleavage plot in Fig. 1b. The main protected sites occur around bases 76-80, 104-107, 114-128 and 131-149. Many of these blockages are broad and it is especially striking that from position 110 onward much of the DNA is protected. This might suggest that DNA conformation rather than sequence per se is an important determinant of binding, but it should also be pointed out that this end of the


a
ptyr2 DNA upper
POR
CON
G
-Ni
+Ni
40
60
80
100
120
140

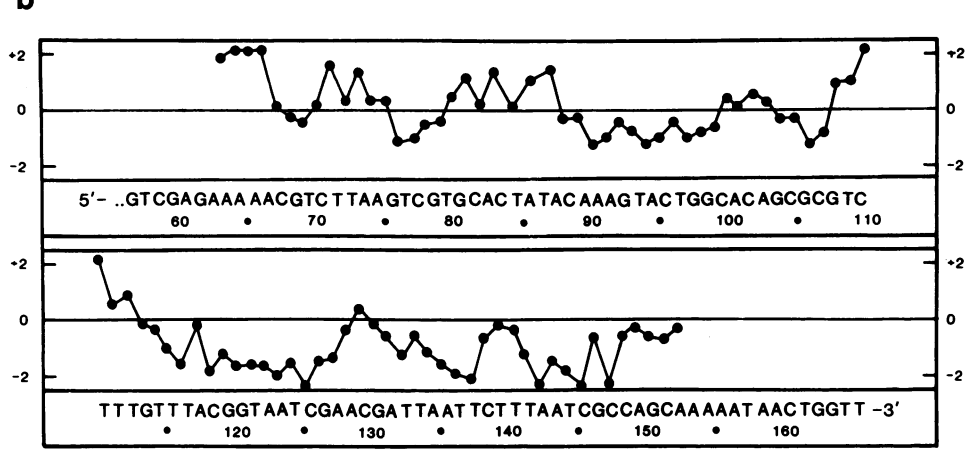
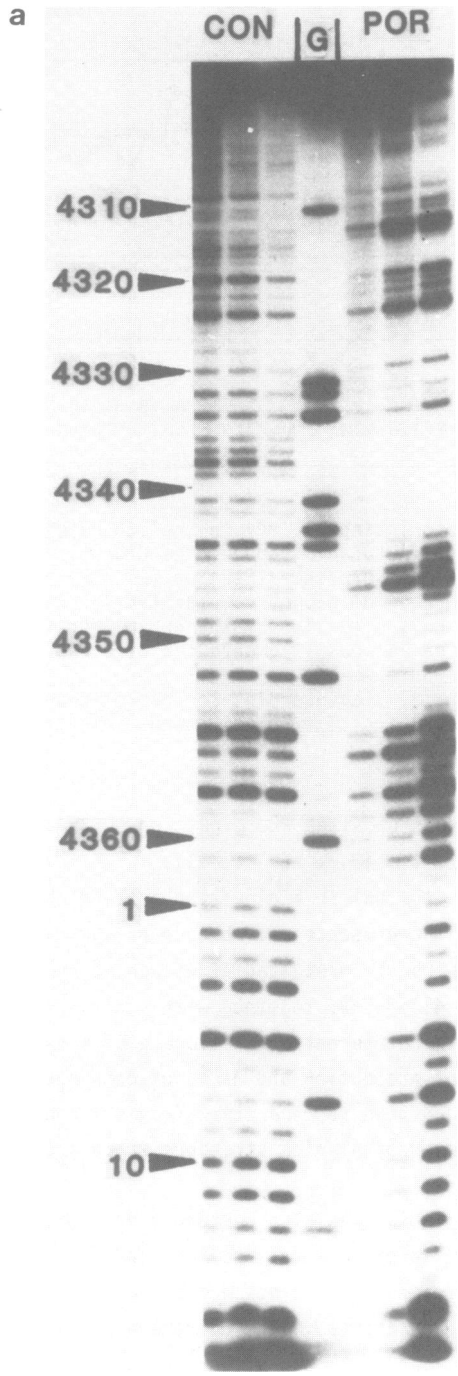


Figure 2 DNAase I footprinting of TMPy on the 166 base-pair ptyr2 DNA fragment. (a) Autoradiograph of the digestion pattern in the absence (CON) and presence (POR) of 5μM TMPy in the nickel-free form (-Ni) or as the nickel complex (+Ni). Each set of three lanes represents digestion by the enzyme for 1, 5, 30 mins. The track labelled 'G' has fragments cut at G residues by the Maxam & Gilbert procedure.
(b) Differential cleavage plot for nickel-free TMPy on ptyr2 DNA. Positive values indicate relative enhancements, negative values indicate blockages.

DNA is AT-rich. A number of sites where cutting by DNAase I is enhanced are also evident at positions 63-68, 83-88 and 108-111.

Figure 3a shows the DNAase I digestion pattern observed with the 102 base-pair fragment in the presence and absence of 5μM nickel-free porphyrin (TMPy). A number of strongly protected sites can be seen around positions 4304-4307, 4316-4319, 4335-4344, together with other weaker sites of protection which are evident in the differential cleavage plot shown in Fig. 3b. Both sites of protection and areas of enhanced DNAase I cleavage are rather more well-defined than for the ptyr2 fragment, possibly because the 102-mer contains a number of AT-rich stretches which are more evenly distributed throughout the fragment.

DNAase I footprinting was also performed on samples of the tyrT fragment having either upper or lower strands labelled, and the autoradiographs are shown in Fig. 4. This DNA fragment contains several large GC clusters in contrast to both the other DNA sequences used. The differential cleavage plot (Fig. 5) illustrates that the major protected sites for the top strand occur around


a
CON
G
POR
4310
4320
4330
4340
4350
4360
1
10

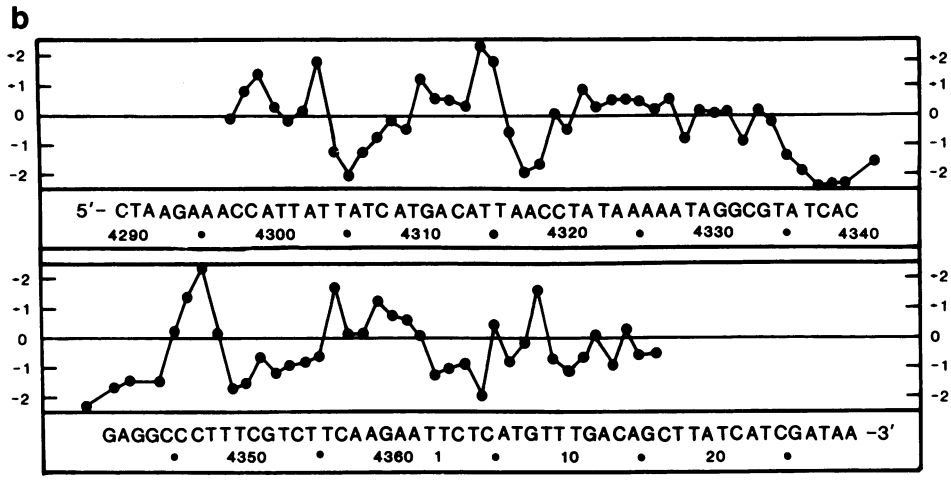


Figure 3 DNAase I footprinting of TMPy on the 102 base-pair AatII-HindIII fragment from pBR322. (a) Autoradiograph of the digestion pattern in the absence (CON) and presence (POR) of 5μM nickel-free TMPy. Each set of three lanes represents digestion by the enzyme for 1, 5, 30 mins. The track labelled 'G' is a dimethylsulphate-piperidine marker specific for guanine. (b) Differential cleavage plot for the data presented in Fig.2(a). Positive values indicate relative enhancements, negative values indicate blockages.

positions 87-90, 108-113, 129-131 and 135-142. Most of these sites are small and span 3-4 bases. Enhancements of cutting by DNAase I can again be seen at several positions. It is noteworthy that the GC-rich regions between 71-77 and 91-100 are not significantly affected by the ligand. Analysis of the bottom strand reveals discordant results at some positions. The discrepancy is most obvious around position 88 which corresponds to a protection of the top strand, with no corresponding inhibition on the lower one. Likewise positions 77-81 are protected on the lower strand, yet enhanced on the upper one. There is, however, excellent agreement between the two strands around position 108. Protected regions on the lower strand are found at positions 73-84 (the first part of which is very GC rich), 55-60 (having a high AT content) and 105-110. Various enhancements are also apparent, the most prominent located around position 48-52 in the polydA stretch. The discrepancies between the two strands are unusual and will be considered further in the discussion.

The effect of the porphyrin compound on the pattern of cutting by micrococcal nuclease is shown in Fig. 6, for both strands of tyrT DNA and the 102 base-pair fragment. This enzyme cuts on the 5' side of T and A nucleotides with

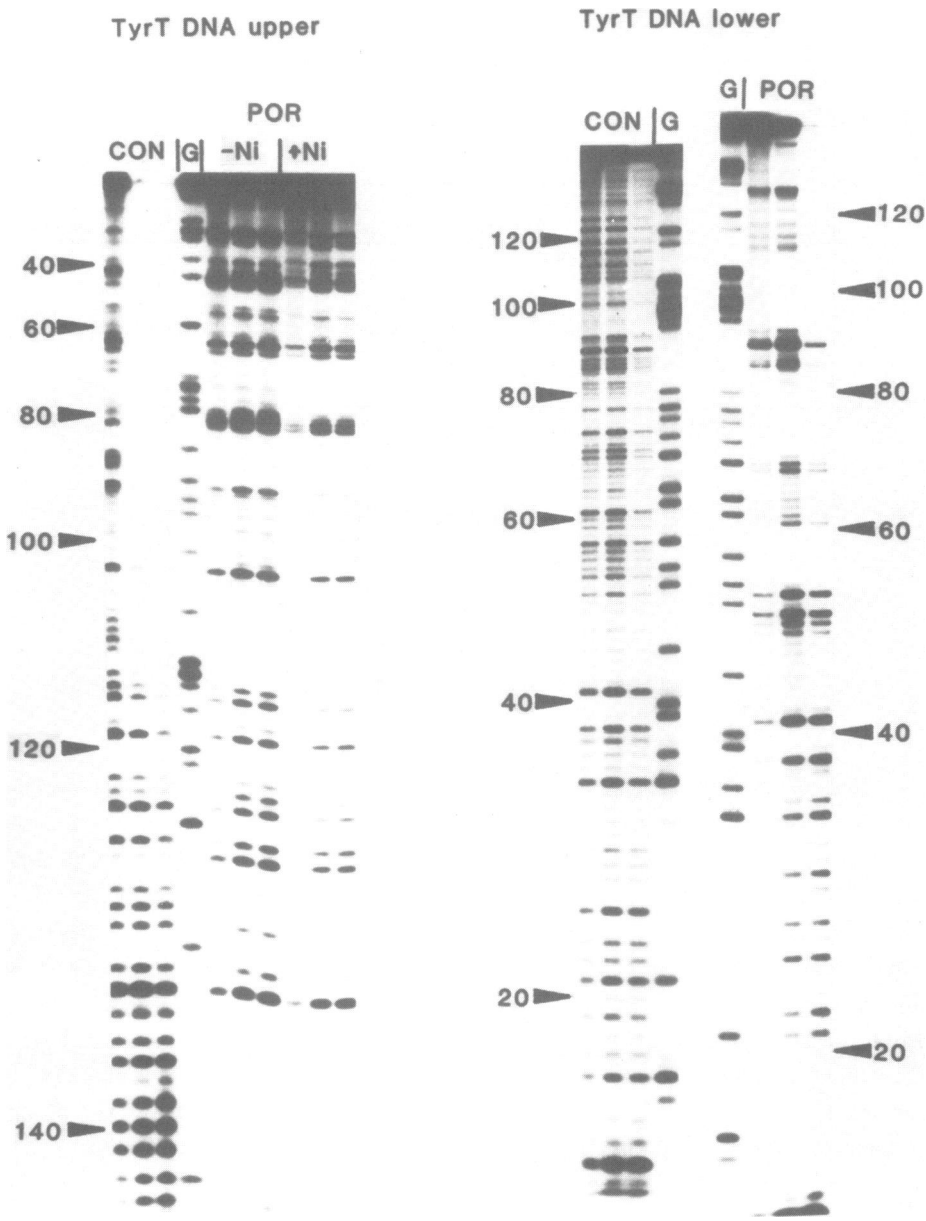


Figure 4 DNAase I footprinting of 5μM TMPy on the 160 base pair tyrT DNA fragment. Left hand panel: autoradiograph of the digestion pattern of the fragment labelled at the 3'-end of the upper (Watson) strand in the absence (CON) and presence (POR) of the nickel-free form (-Ni) or nickel complex (+Ni) of TMPy.

Right-hand panel: autoradiograph of the digestion pattern of the fragment labelled at the 3'-end of the lower strand in the presence (POR) and absence CON) of nickel-free TMPy. Each set of three lanes represents digestion by the enzyme for 1, 5, 30 mins. Tracks labelled 'G' are dimethylsulphate-piperidine markers specific for guanine.

low specificity[13,14] presumably because of their greater conformational mobility. For the lower strand of tyrT DNA the most obvious sites of protection appear at positions 19-21, 29-34, 60-61 and around 110. These correspond to blockages produced in the digestion with DNAase I, except at position 29-34. This slight discrepancy could be related to the recent observation that micrococcal nuclease can sometimes detect weaker ligand binding sites which are not apparent with DNAase I[15].

Alternatively it should be noted that this sequence occurs in a region where cutting by DNAase I is poor in the drug-free control, so that drug-induced protection in this position is not easy to assess. For the labelled upper strand

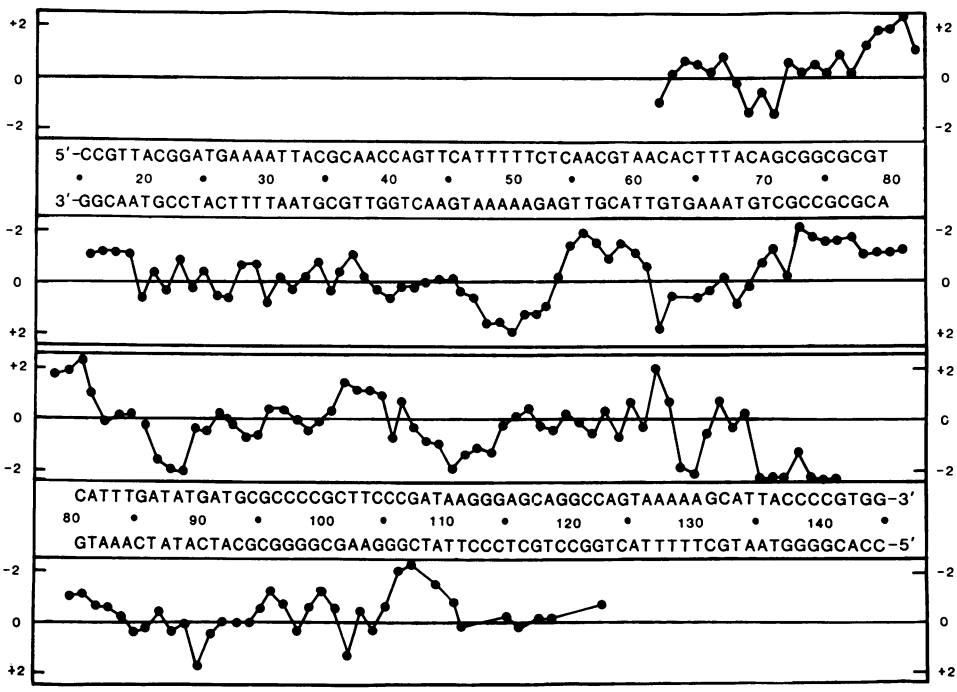
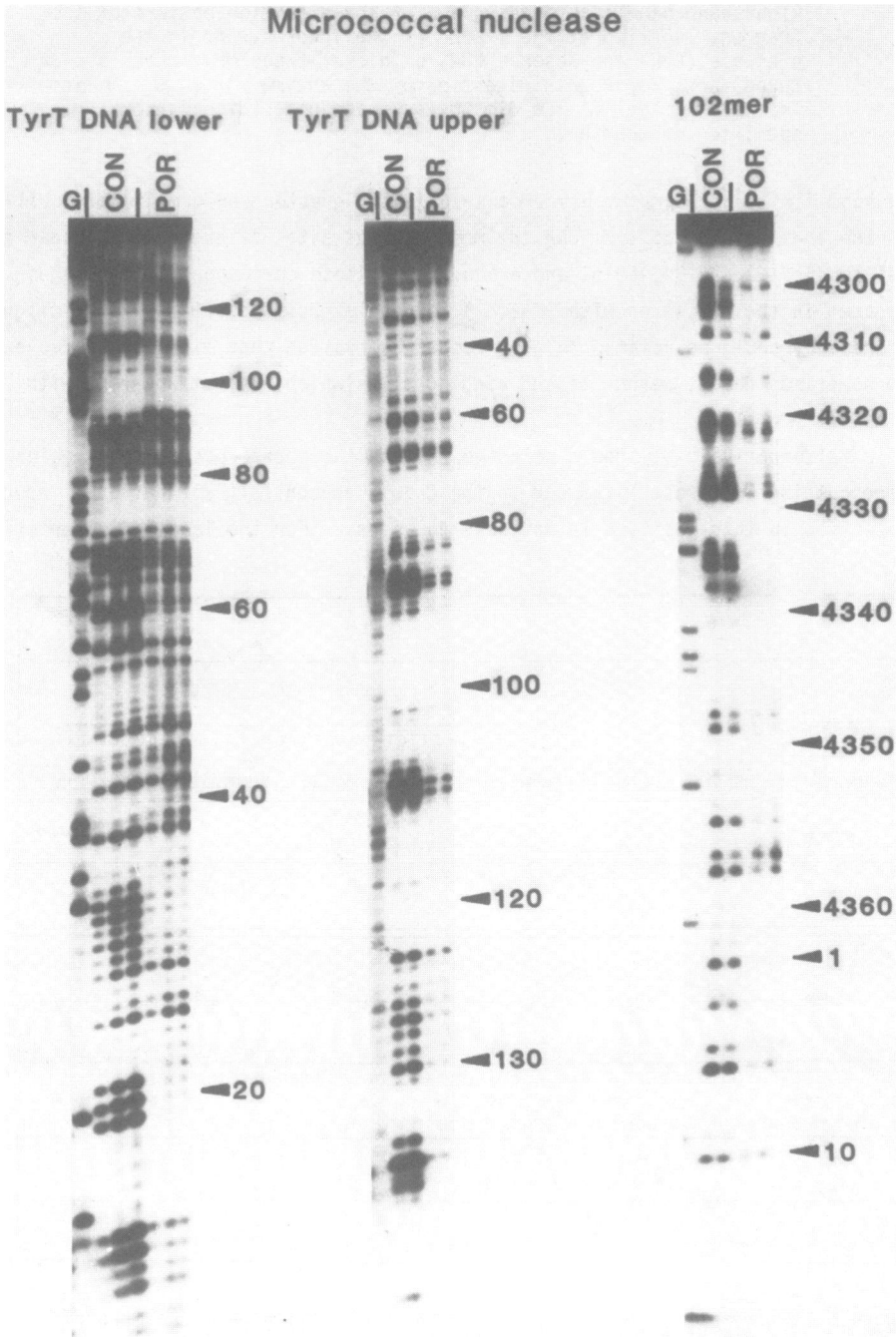


Figure 5 Differential cleavage plot for the interaction of nickel-free TMPy with tyrT DNA labelled on either strand. Positive values indicate relative enhancements, negative values indicate blockages.


Micrococcal nuclease
TyrT DNA lower
G
CON
POR
120
100
80
60
40
20
TyrT DNA upper
G
CON
POR
40
60
80
100
120
130
102mer
G
CON
POR
4300
4310
4320
4330
4340
4350
4360
1
10

of tyrT DNA there is strong protection around 125-135, 109-112 and 81-86, each of which corresponds to a region of blockage in the DNAase I footprinting experiment. With the 102 base-pair fragment the principal regions protected from micrococcal nuclease occur around positions 4335 and 1. Weaker blockages are also evident, which generally correlate with the footprints seen with DNAase I.

DISCUSSION

The results presented in this study show that TMPy and its nickel derivative induce distinct changes in susceptibility to both DNAase I and micrococcal nuclease cleavage. The two ligands produce identical results in this respect, indicating that the metal ion itself plays no role in the sequence selectivity. The differential cleavage plots reveal a wide variation in the length of DNA sequence protected from enzymatic cleavage. Intercalative binding of TMPy must involve initial breakage of hydrogen bonds so as to accommodate the bulky meso substituents, as a result of which we would expect to see structural perturbations throughout a significant length of DNA. Other studies have demonstrated the existence of two distinct binding modes for porphyrin ligands which have been interpreted as intercalation which occurs at CpG sites and external (presumably groove) binding at AT rich regions[4,5,7,8]. Footprinting studies cannot distinguish between these two binding modes and consequently we might anticipate that many different types of sequence will be protected. With regard to the large structural transition required before intercalative binding can occur, it is worth noting that the footprinting patterns observed in this paper are very different from those induced by nogalamycin, for which prior rupture of hydrogen bonding is also presumed to be necessary[16,17].

What then are the characteristics of the sequences protected by the porphyrin? Inspection of the differential cleavage plots reveals that the steps most closely associated with protection are CpG and TpA, which are either within

Figure 6 Autoradiographs showing the effect of 5μM nickel-free TMPy on the digestion of three DNA fragments by micococcal nuclease. Left-hand panel: TyrT DNA labelled at the 3'-end of the lower strand. Centre panel: TyrT DNA labelled at the 3'-end of the upper strand. Right-hand panel: 102 base-pair HindIII-AatII fragment from pBR322. In each case (CON) indicates the control, (POR) is in the presence of 5 M TMPy. Each set of lanes represents digestion by the enzyme for 1, 5, 30 mins (left-hand panel) or 1, 5 mins (centre and right-hand panels). Tracks labelled 'G' are dimethylsulphate-piperidine markers specific for guanine, which are 0.3 - 1.5 bond faster than corresponding fragments in the nuclease digest (see text).

the centre of each site or positioned nearby. In a number of instances, these sites are found in clusters such as between positions 70-80 and 95-102 in tyrT DNA. In the ptyr2 DNA sequence there is an almost continuous blockage between positions 115-150; this stretch contains a high concentration of CpG and TpA steps, many of which are in close proximity. A notable exception is the lack of protection around position 130 (CpG), an effect which may be related to large-scale conformational perturbations on either side. Looking at the top strand of tyrT DNA there is a close correspondence between blockage and the occurence of the dinucleotide TpA, found at positions 68,88,110, 126 and 136. Such a correlation is less evident on the bottom strand in which the GC rich region from 75-85 is well protected. Some interesting subtleties in the interaction can be noted with the 102 base-pair fragment. The tetranucleotide ATTA centred around position 4301 is largely unaffected by the ligand (as too is position 4315) while the same sequence at 4304 forms the centre of a strongly protected site. Surprising too is the lack of any detectable inhibition in the sequence TATA at position 4322. It thus appears that while there may be an slight preference for TpA and CpG, the binding to these dinucleotides is strongly affected by the sequences in neighbouring regions, and predictions about binding based on local environment alone are not valid.

The poor cross-strand correlation observed in the DNAase I footprinting experiments is unusual and merits further consideration. On theoretical grounds such a descrepancy is unexpected since DNAase I binds across the minor groove of the helix so that altering the binding (and cutting) on one strand should have an equal effect on the opposing strand. The differences might arise from some (subtle) changes in conditions between the two experiments. We can envisage two possibilities. Firstly, the differences may be due to some kinetic factor(s), so that in one instance the footprinting was performed under equilibrium conditions while non-equilibrium binding determinated the second experiment. Alternatively, changes in the ionic strength could have significant effects on the observed sequence selectivity. The precise ionic conditions of these experiments are difficult to control rigorously since they involve manipulations with small volumes and require several precipitation steps. It has recently been suggested that the sequence-selectivity of these ligands varies as a function of salt concentration, favouring GC rich regions at low ionic strength, yet AT sequences at elevated ionic strengths[18]. In order to clarify the unusual behaviour of this ligand, future experiments should be directed at examining the effect of both ionic strength and equilibration time on the footprinting pattern.

The observation that TMPy is often bound to TpA sites and other T-rich

regions is in agreement with data on the free-radical DNA strand cleavage caused by several TMPy metal complexes[19]. These studies have suggested that such free-radical activation can only occur when the metal centre is located on the exterior of the DNA duplex. The CpG preference is in agreement with NMR data on the binding of TMPy to oligonucleotides[20].

Another feature of the footprinting patterns observed with TMPy is the appearance of regions of enhanced cleavage. These are generally, though not exclusively, located in oligo(dT) stretches. As previously suggested, enhanced cutting at particular bonds must be due to drug-induced changes in the DNA structure in regions surrounding the ligand binding site, thereby rendering it more susceptible to nuclease attack. Not all runs of A and T are affected, possibly because the ligand has some affinity for the dinucleotide step ApA.

Preliminary molecular modelling studies currently being performed (Ford, Pearl & Neidle, to be published) have indicated that only a partial intercalative mode is possible for TpA sites, the binding being principally electrostatic in nature. For CpG sites however, full intercalation is possible, resulting in a stable overall energy for the complex. Its geometry indicates that subsequent ligand dissociation would be unfavourable. These two models suggest two differing kinetic effects, whereby presumably binding of porphyrin to a TpA sequence is more readily dissociable than bound to CpG regions, in broad agreement with the known kinetic behaviour of TMPy[5]. Thus the digestion patterns may be complicated by the rapid exchange of ligands between TpA sites.

Recent studies on TMPy interaction with Z form DNA have indicated that one porphyrin molecule on average can convert 10-20 base pairs of Zform DNA to the B form[21]. If these extensive deformational abilities also apply to other DNA forms such as B form itself, then this must also be considered when regarding the results from the DNA digestion patterns.

However, there is no evidence as yet to suggest that this is the case, althoug it does seem likely that deformation of DNA occurs upon ligand binding, at least to some extent.

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