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Footprinting studies on the sequence-selective binding of pentamidine to DNA

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The sequence-selective binding of pentamidine, an antimicrobial aromatic diamidine, has been investigated by footprinting studies on two different DNA fragments using DNase I, micrococcal nuclease and hydroxyl radical as probes. Each probe reveals drug-induced protection from cleavage in AT-rich regions. The best binding sites consist of at least 5 consecutive AT base pairs. Three or less AT pairs do not constitute a pentamidine binding site.

Pentamidine; Drug-DNA interaction; Minor groove binding

1. INTRODUCTION

Pentamidine is an antimicrobial agent useful for the treatment of various protozoal infections [1]. Attention has recently been focused on this compound as it is effective in treating *Pneumocystic carinii* pneumonia, a recurring opportunistic infection in patients with AIDS, proving fatal in about 20% of cases in each cycle of infection [2]. Although the mode of action of pentamidine is unknown, several lines of evidence suggest that DNA may be its molecular target [1]. It is a member of the class of aromatic diamidines, several of which have been shown to bind to DNA. Both circular dichroism and melting point studies have shown that pentamidine forms complexes with AT-rich DNAs [3,4]. Recent molecular mechanics modelling studies, based on the crystal structure of pentamidine isethionate [5], have suggested that the drug binds to the DNA minor groove spanning 4 base pairs with the charged amidinium groups forming hydrogen bonds to O2 of thymine or N3 of adenine [6].

In this study we examine the sequence-selective binding of pentamidine to two DNA fragments using a variety of footprinting techniques.

2. MATERIALS AND METHODS

2.1. *Drugs*

Pentamidine isothionate was purchased from Sigma and stored as a 10 mM solution in 10 mM Tris-HCl, pH 8.0, containing 10 mM NaCl.

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2.2. *DNA fragments*

The 160 base pair tyrT DNA fragment (Fig. 2) was isolated and labelled at the 3'-end of either the *Eco*RI site with [α-^{32}P]dATP (bottom strand) or the *Ava*I site with [α-^{32}P]dCTP (top strand) using reverse transcriptase [7]. The 270 base pair *Eco*RI-*Hin*dIII kinetoplast DNA fragment [8] (Fig. 2) was isolated from plasmid pK201/CAT (a gift from Dr T. Tullius) and labelled at the *Eco*RI end with [α-^{32}P]dATP using reverse transcriptase.

2.3. *Footprinting*

Footprinting reactions were performed as previously described using DNase I [7], micrococcal nuclease (MNase) [9] and hydroxyl radicals [10]. The products of the reactions were resolved on 6–8% polyacrylamide gels containing 8 M urea. Gels were fixed in 10% acetic acid before drying under vacuum at 80°C and subjecting to autoradiography at −70°C using an intensifying screen. Bands in the autoradiographs of DNase I and hydroxyl radical digests were assigned by comparison with Maxam-Gilbert dimetylsulphate-piperidine markers specific for guanine. Since MNase cuts the *O*5'–P bond, in contrast to the *O*3'-bond which is cleaved by DNase I, the bands do not comigrate with the G-marker lanes. Bands in MNase digests were therefore assigned using the observation that MNase only cuts pA and pT bonds [11] and by comparison with previous work [9].

3. RESULTS

3.1. *tyrT DNA*

Fig. 1 presents the results of DNase I digestion of tyrT DNA in the presence and absence of pentamidine. Drug concentrations of 5 μM and above have altered the nuclease digestion pattern, confirming that pentamidine possesses some sequence selectivity. Even at 100 μM many regions are still cleaved by the enzyme, suggesting that the drug does not bind to certain sequences. The clearest regions of protection, visible with 5 μM drug on both DNA strands, can be seen around positions 28, 45 and 130. Other sites around positions 65, 80 and 110 are evident with concentrations of

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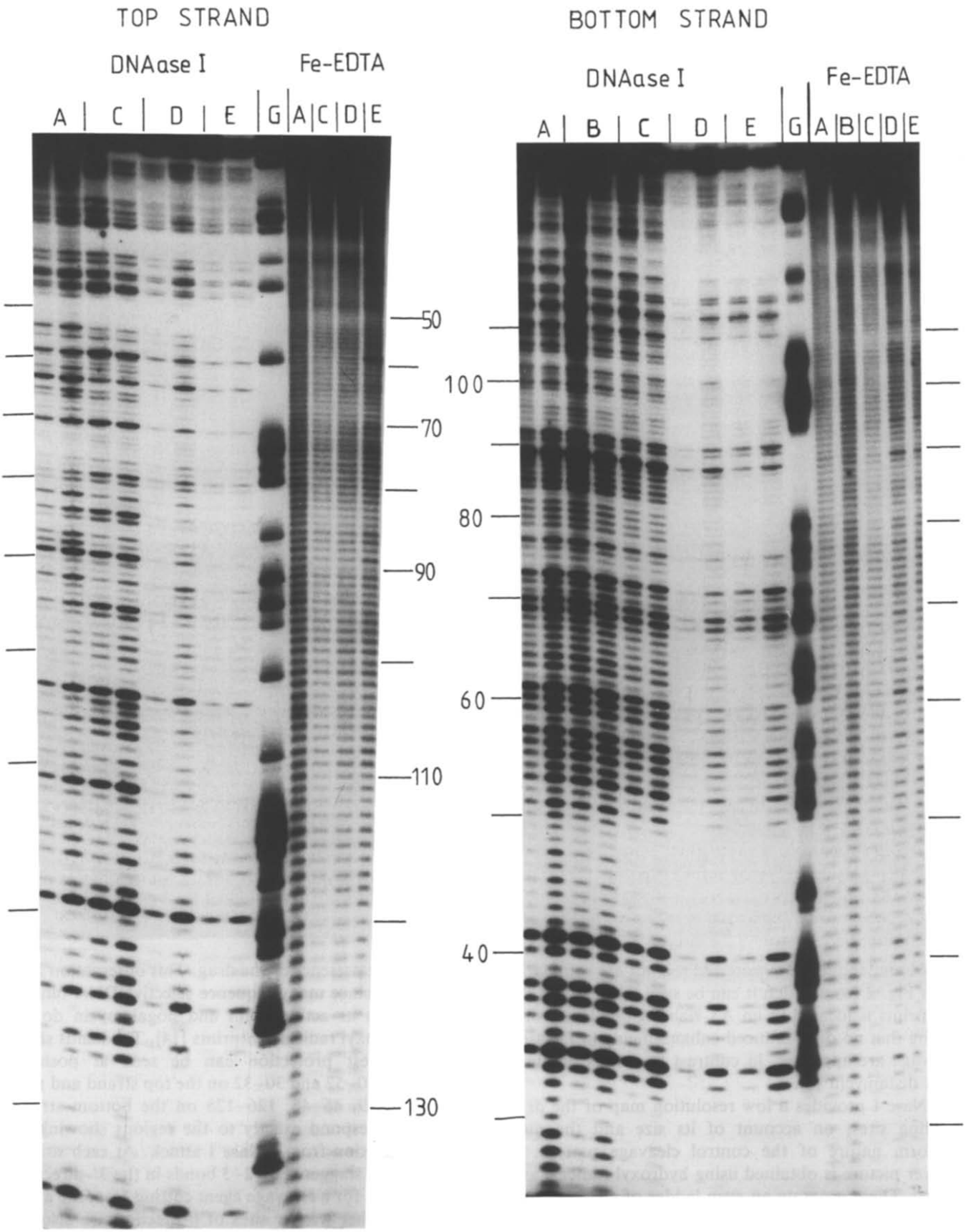

Fig. 1. DNase I and hydroxyl radical (Fe-EDTA) footprinting of pentamidine on tyrT DNA. For DNase I cleavage each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The sequence numbering and strand designation correspond to the sequence shown in Fig. 2. The track labelled 'G' is a Maxam-Gilbert dimethylsulphate-piperidine marker specific for guanine. The pentamidine concentrations were: (A) control 0 μM; (B) 1 μM; (C) 5 μM; (D) 25 μM; (E) 100 μM.

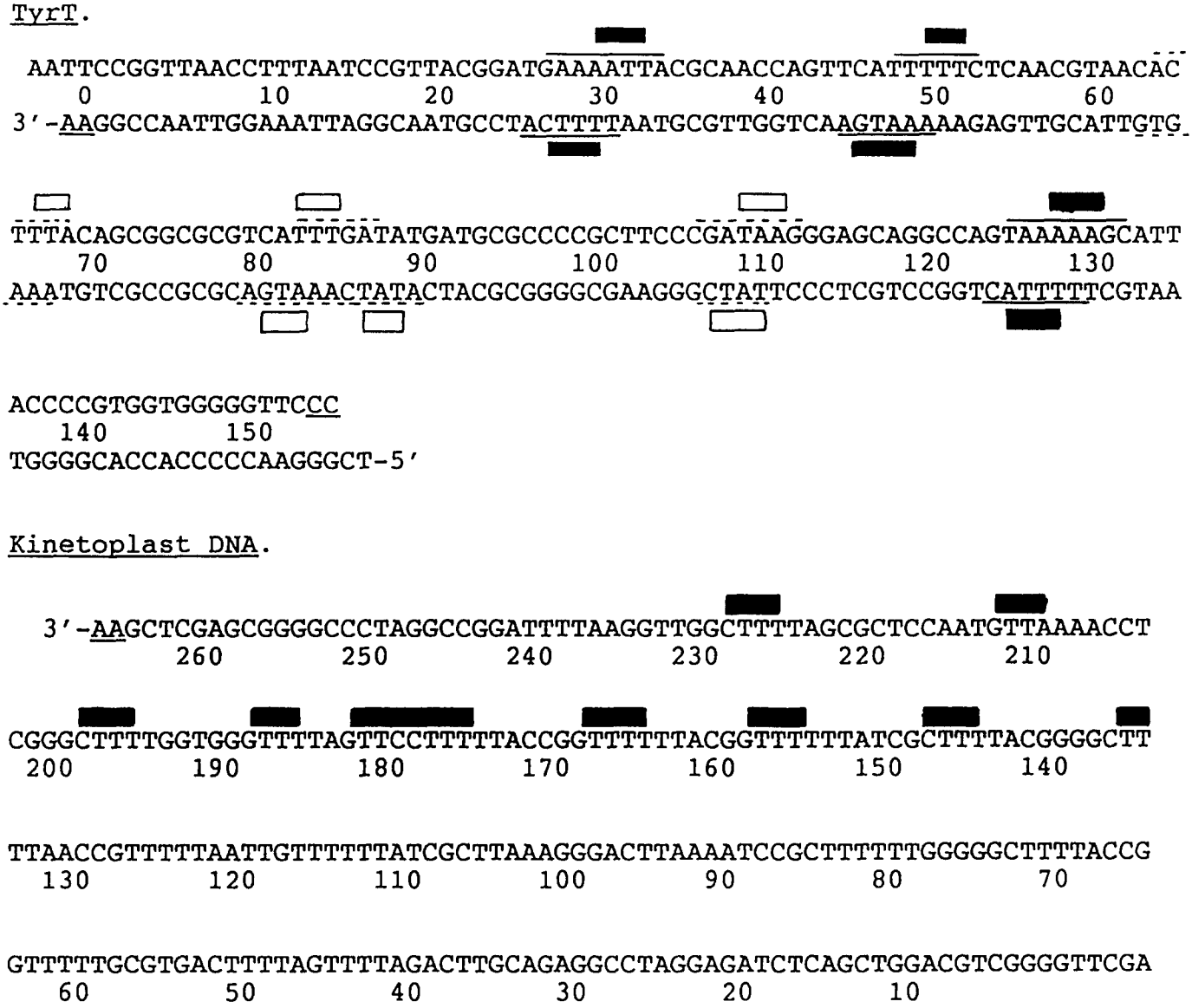

Fig. 2. Sequences of the tyrT and kinetoplast DNA fragments and the protections from DNase I and hydroxyl radical cleavage afforded by pentamidine. For kinetoplast DNA only the strand bearing the radioactive label is shown. The radiolabelled bases are underlined. Boxes indicate regions protected from hydroxyl radical attack, the filled boxes show the stronger binding sites. The lines indicate protection from DNase I, the broken lines correspond to the weaker sites. For kinetoplast DNA binding sites were resolved between 230 and 130.

25 μM and above. The protected regions are summarised in Fig. 2 from which it can be seen that each of the footprints is located in an AT-rich region. It is worth noting that no drug-induced enhancements in DNase I cleavage are apparent, in contrast to results obtained with distamycin [7].

DNase I provides a low resolution map of the drug binding sites, on account of its size and the non-uniform nature of the control cleavage pattern. A clearer picture is obtained using hydroxyl radicals as a probe. These generate an even ladder of cleavage products and have previously been used to map drug binding sites at high resolution [10,12,13]. Hydroxyl radical footprints of pentamidine on tyrT DNA are presented in Fig. 1 and reveal attenuated cleavage patterns in the presence of the drug. This observation is informative since many sequence selective DNA-binding drugs such as actinomycin and nogalamycin do not yield hydroxyl radical footprints [14]. The bands showing the best protection can be seen at positions 129–131, 50–52 and 30–32 on the top strand and positions 27–29, 46–48, 126–128 on the bottom strand. These correspond exactly to the regions showing the best protection from DNase I attack. At each site the blockage is staggered by 2–3 bonds in the 3'-direction, as expected for a cleavage agent cutting from the minor groove. Other weaker sites of protection are also evident on both strands; these correspond to the weaker sites of protection seen with DNase I. Both the weak and strong cleavage sites are summarised in Fig. 2. In-

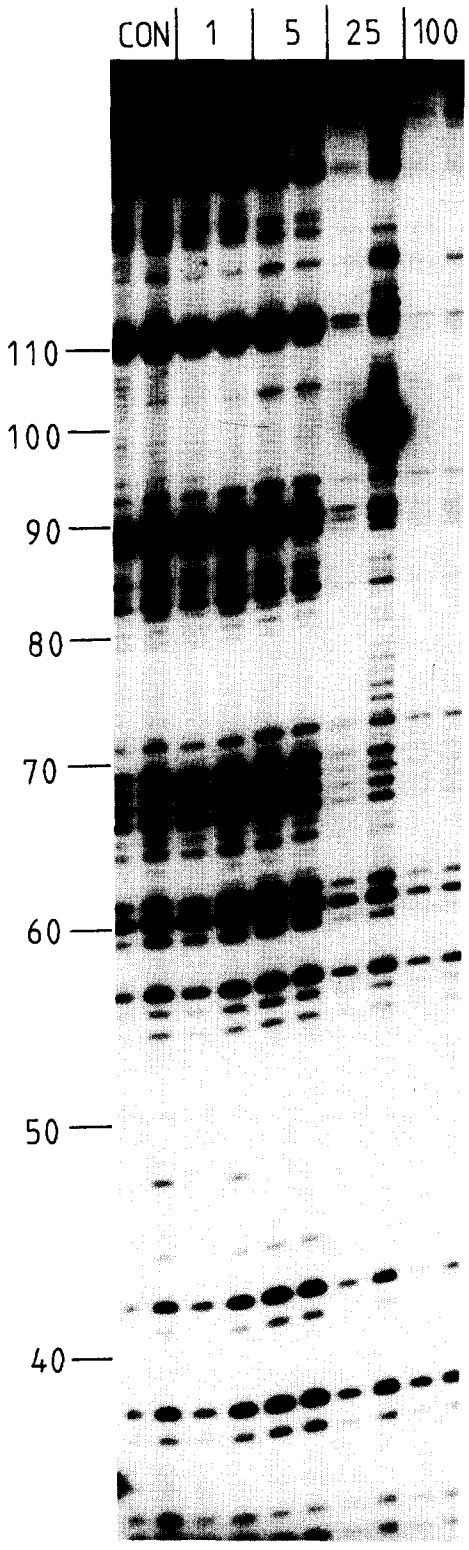

Fig. 3. Micrococcal nuclease footprinting of pentamidine on the tyrT DNA fragment labelled on the bottom strand of the sequence shown in Fig. 2. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The drug concentrations (μM) are shown at the top of each pair of lanes.

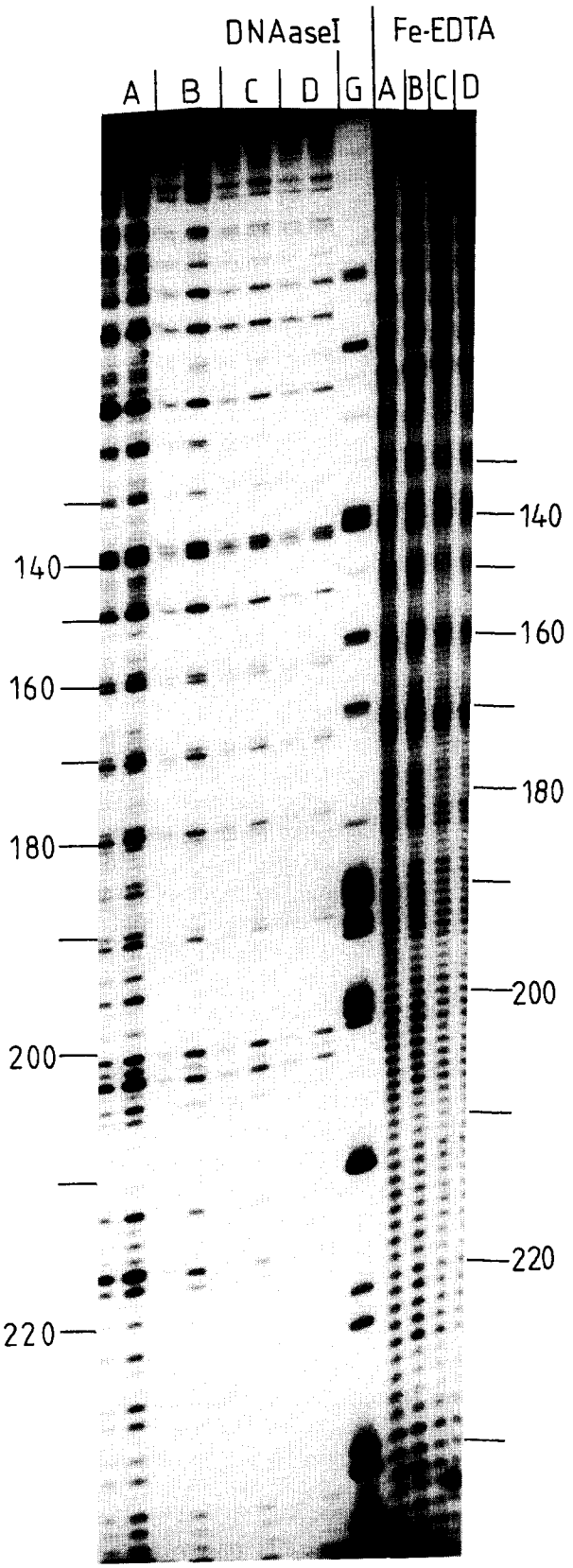

Fig. 4. DNase I and hydroxyl radical (Fe-EDTA) footprinting of pentamidine on the kinetoplast DNA fragment. For DNase I cleavage each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. The numbers correspond to the sequence shown in Fig. 2. The track labelled 'G' is a Maxam-Gilbert dimethylsulphate-piperidine marker specific for guanine. The pentamidine concentrations were: (A) control 0 μM; (B) 1 μM; (C) 5 μM; (D) 25 μM.

spection of Fig. 2 reveals that although all the sites are in AT-rich regions those best protected contain runs of 6 or more AT base pairs whereas the weaker ones all consist of 4 AT pairs. Three contiguous AT pairs do not appear to constitute a binding site since no protection is apparent around position 60 (TAA).

In order to confirm these observations we have performed footprinting experiments on the same DNA fragment using micrococcal nuclease as a probe. This enzyme cuts exclusively at pT and pA bonds and is especially useful for investigating ligands which bind to AT-rich regions. The results are presented in Fig. 3 and reveal that 5 μM pentamidine inhibits MNase attack specifically at positions 47 and 126/127, regions which correspond to the best sites seen with DNase I and hydroxyl radicals. At concentrations of 25 μM and above many more protections are evident, so that by 100 μM few cleavage products remain as would be expected for a ligand with broad AT-selectivity.

3.2. *Kinetoplast DNA*

Fig. 4 presents the results of DNase I and hydroxyl radical digestion of the kinetoplast DNA fragment in the presence and absence of pentamidine. This DNA contains runs of adenines spaced at 10 base pair intervals and so adopts an unusual bent structure [15]. As a result, the control digestion patterns are uneven displaying a phased pattern of bands for both DNase I and hydroxyl radicals as previously noted [15]. The minima in these cleavage patterns are found at the 3′-end of each of the blocks of A and T. In the presence of pentamidine this phasic cleavage pattern is accentuated so that for DNase I weaker cleavage products in between the maxima are no longer visible. With hydroxyl radicals the troughs in the cleavage pattern are more pronounced. The protections observed with hydroxyl radicals are summarised in Fig. 2 and confirm that each is located towards the 3′-end of each of the runs of A and T. The results with tyrT DNA suggested that good pentamidine binding sites consist of runs of over 4 AT base pairs; it is therefore not surprising that kinetoplast DNA presents many excellent binding regions.

4. DISCUSSION

The results presented here demonstrate that pentamidine is an AT-selective DNA binding ligand. The best binding sites contain runs of 5 or more AT base pairs, though runs of 4 AT pairs are protected from cleavage at higher concentrations. Three consecutive AT pairs do not constitute a binding site. This is consistent with previous molecular mechanics studies which revealed a binding site size of 4 base pairs [6]. However, the modelling studies suggested that the drug could tolerate a single GC pair within the binding site; this is not confirmed with the footprinting data with DNase I and hydroxyl radicals, though the abolition of MNase activity at high concentrations of pentamidine may reveal weaker binding to other DNA regions. The drug does not appear to distinguish between homopolymeric and alternating regions of A and T. In contrast to distamycin, no enhancements in DNase I activity sites were observed suggesting that pentamidine causes few changes in DNA structure. The footprinting data together with the molecular mechanics calculations suggest that pentamidine binds in the DNA minor groove forming hydrogen bonds between its amidinium groups and adenine N3 or thymine O2 bridging 4 or 5 base pairs. Selectivity for A or T in the intervening pairs is achieved by exclusion of GC as a result of the guanine 2-amino group, protruding into the minor groove, interrupting the mainly hydrophobic interaction between the drug molecule and the exposed DNA surface.

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