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Hydroxyl radical footprinting of the sequence-selective binding of netropsin and distamycin to DNA

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Hydroxyl radicals, generated by allowing an iron (II)·EDTA complex to react with hydrogen peroxide, have been employed to cleave the 160-base pair *tyr*T DNA fragment in the presence and absence of the minor groove-binding antibiotics netropsin and distamycin A. The control DNA cleavage pattern is practically independent of nucleotide sequence, which overcomes certain limitations of other footprinting techniques, so that additional information can be gained about the AT-rich sequence preference of the minor groove-binding ligands.

Antibiotic-DNA binding; Hydroxyl radical footprinting; Iron (II)·EDTA complex; Netropsin; Distamycin A

1. INTRODUCTION

Netropsin and distamycin A are oligopeptide antibiotics exhibiting antitumour and antiviral effects [1,2] which bind to AT-rich regions in double-stranded DNA by nonintercalative means [1–6]. The preference of netropsin for binding to AT residues seems to be determined primarily by van der Waals contacts between the pyrrole rings of the antibiotic and various surfaces on the DNA, while hydrogen bonds from netropsin amides serve only to position the antibiotic within its binding site [7]. We have recently used techniques of DNase I and micrococcal nuclease footprinting [8] to compare netropsin and distamycin binding sites. We found, in agreement with previous solution studies (review [2]), that these antibiotics bind to clusters containing $\geqslant 4$ AT base pairs and that neither guanosine nor cytidine nucleotides seem to be allowed within the preferred binding sites. However, occasionally neighbouring G(C) residues would appear protected from DNase I cleavage, perhaps due to steric occlusion of the minor groove of the helix by bound antibiotic molecules [8] leaving insufficient room for the enzyme to gain access to the hindered G(C) nucleotides sandwiched between two strong antibiotic binding sites. Neither DNase I nor micrococcal nuclease is small enough to cut occluded DNA regions [9,10] though micrococcal nuclease is only about half the size of DNase I.

Hydroxyl radicals attack the deoxyribose sugars along the backbone of the DNA molecule [11]. Subsequent reaction leads to disintegration of the sugar [12] and DNA strand cleavage ensues [13,14]. This process has been used previously to make footprints of the bacteriophage λ repressor and Cro proteins on the operation sequence [14]. All positions along the DNA backbone can be observed since the hydroxyl radical lacks sequence or base specificity [11,13,14].

In this paper, we present the results of hydroxyl radical footprinting applied to investigate the selective binding of netropsin and distamycin to DNA. Because of its small size (cf. DNase I and

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micrococcal nuclease) the OH· radical appears to report some weaker binding sites, and yields a new picture of the nucleotides involved in the strong (preferred) binding sites [8], in some cases discriminating them from those that are only sterically inaccessible to bigger cleavage agents.

2. MATERIALS AND METHODS

2.1. *Antibiotics*

Netropsin was a gift from Dr C. Zimmer (Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, GDR). Distamycin hydrochloride was a gift from Dr F. Arcamone (Farmitalia, Italy). Solutions were prepared freshly for each experiment in 10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl.

2.2. *DNA fragment*

The 160-base pair *tyr*T DNA fragment from *E. coli*, containing the tyrosine tRNA promoter, was isolated as described by Drew and Travers [15] and labelled at one or other of the 3′-ends using reverse transcriptase and [α-^{32}P]dATP or [α-^{32}P]dCTP using standard procedures [6,15,16].

2.3. *Hydroxyl radical footprinting*

The hydroxyl radical cutting reaction was carried out essentially as described in [14]. Samples (1 μl) of the radioactively labelled DNA (3 pmol in base pairs) were incubated with 5 μl of the antibiotic solution (5–40 μM) at 37°C for 30 min. A stock solution of iron (II)·EDTA was prepared immediately before use by mixing 10 μl freshly prepared 0.2 mM $Fe(NH_4)_2 \cdot (SO_4)_2$ (Sigma) with 10 μl of 0.4 mM EDTA (pH 7.4) and 20 μl freshly prepared 10 mM ascorbic acid. Hydroxyl radicals were generated by allowing that mixture to react with 20 μl of 0.3% H_2O_2. DNA backbone cleavage was initiated immediately by adding 7.5 μl of the final mixture to the DNA-antibiotic complex. Aliquots (6 μl) were removed after 1 and 5 min digestion and the reaction quenched by adding 5 μl of 0.1 M thiourea. Samples were then made up to 0.3 M with sodium acetate, ethanol precipitated, washed with cold 70% ethanol, dried and dissolved in 3 μl of 80% formamide containing 0.1% bromophenol blue and 10 mM EDTA. They were heated at 100°C for 2 min prior to electrophoresis.

2.4. *Gel electrophoresis and analysis of data*

Products of footprinting were analysed on 0.3 mm, 8% (w/v) polyacrylamide gels containing 8 M urea and Tris-borate-EDTA buffer, pH 8.3. After 2 h electrophoresis at 1500 V (40 W for 40 cm gels or 45 W for 50 cm gels) the gel was fixed, dried, developed and scanned as described in [16].

3. RESULTS

In fig.1 the effects of 30 μM netropsin or 20 μM distamycin on the pattern of fragments produced by hydroxyl radical attack on *tyr*T DNA are illustrated. The most obvious change in the presence of either ligand is a decrease in the intensity of several bands with respect to the otherwise almost even ladder in the control lanes, although sharply defined gaps similar to those reported by DNase I footprinting [8] are not usually observed. Further analysis of the footprinting patterns requires detailed comparison of densitometric scans, permitting better definition of several antibiotic-protected sites (fig.2). These are summarised in the form of a cleavage map in fig.3. Using this approach it is possible to assign the binding sites affecting each strand over a region of about 90 base pairs. The analysis is limited by smearing towards the top of the gel lanes, where the longer labelled fragments migrate, most probably caused by the formation of some precipitate due to the presence of Fe(II)·EDTA in the reaction. Such smearing is not peculiar to our experiments: it can be seen in the work of others where OH· radicals were employed but no antibiotic was present [17].

With netropsin, the most obvious protected zones on the Watson (upper) strand occur around positions 34, 43, 52, 60, 69, 88, 111 and 127. On the labelled Crick (lower) strand similar protected regions can be identified but staggered by 2–3 bonds towards the 3′-end. This is usual, because the shortest distance across the minor groove of the B-DNA helix does not lie along a single base pair but between positions approx. 3 base pairs apart in the primary sequence [15,16].

It is noteworthy that except in the vicinity of positions 65 and 87, the zones protected by netropsin are less well defined on the Crick strand than on the Watson strand. Such is not the case for

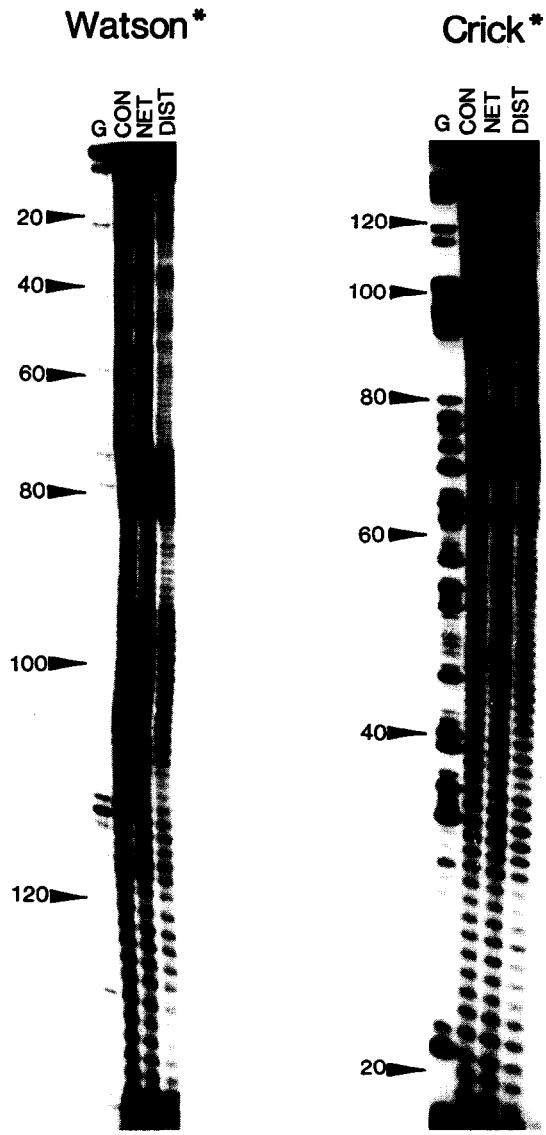

Fig.1. Hydroxyl radical footprinting of netropsin (NET, 30 μM) and distamycin A (DIST, 20 μM) on the *tyr*T DNA fragment. The asterisk indicates which strand bears the 3'-end label (Watson = top strand in fig.3; Crick = bottom strand). For each strand a control sample (CON) is shown to which no drug had been added, and a track labelled 'G' representing a dimethylsulphate piperidine marker specific for guanine.

distamycin, which often produces better-defined regions of protection on the lower strand (fig.3).

As regards the size of the protected regions they are by and large similar in length (fig.3) and all of

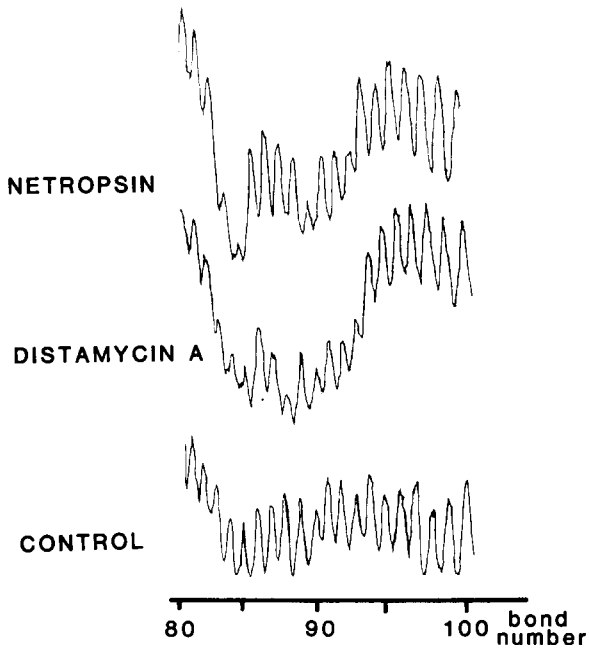

Fig.2. Densitometer scans of autoradiographs showing the region between bonds 80 and 100 of the Watson strand. Bands were assigned by reference to a dimethylsulphate piperidine marker track for guanine. The relative intensities of individual maxima in the presence of each antibiotic can be compared.

them contain several adenine and thymine nucleotides. Most have already been identified as preferred binding sites by DNase I or micrococcal nuclease footprinting [6,8]: those located around positions 33, 51, 68, 88 and 127 correspond well with known sites. However, certain new regions appear which are only protected in the hydroxyl radical footprinting profiles, e.g. in the vicinity of positions 43 and 60. These regions, where protection is admittedly rather weak, most probably represent secondary binding sites which only become occupied at high antibiotic concentrations [8].

Perhaps more interesting are the instances where G(C) nucleotides are located between two closely spaced strong binding sites, as at position 86. Densitometer tracings for this region are shown in fig.2. They reveal how little the region around the central 'sandwiched' guanosine nucleotide is protected by binding of netropsin, whereas it appears substantially protected in the distamycin footprinting pattern. This is direct evidence that occa-

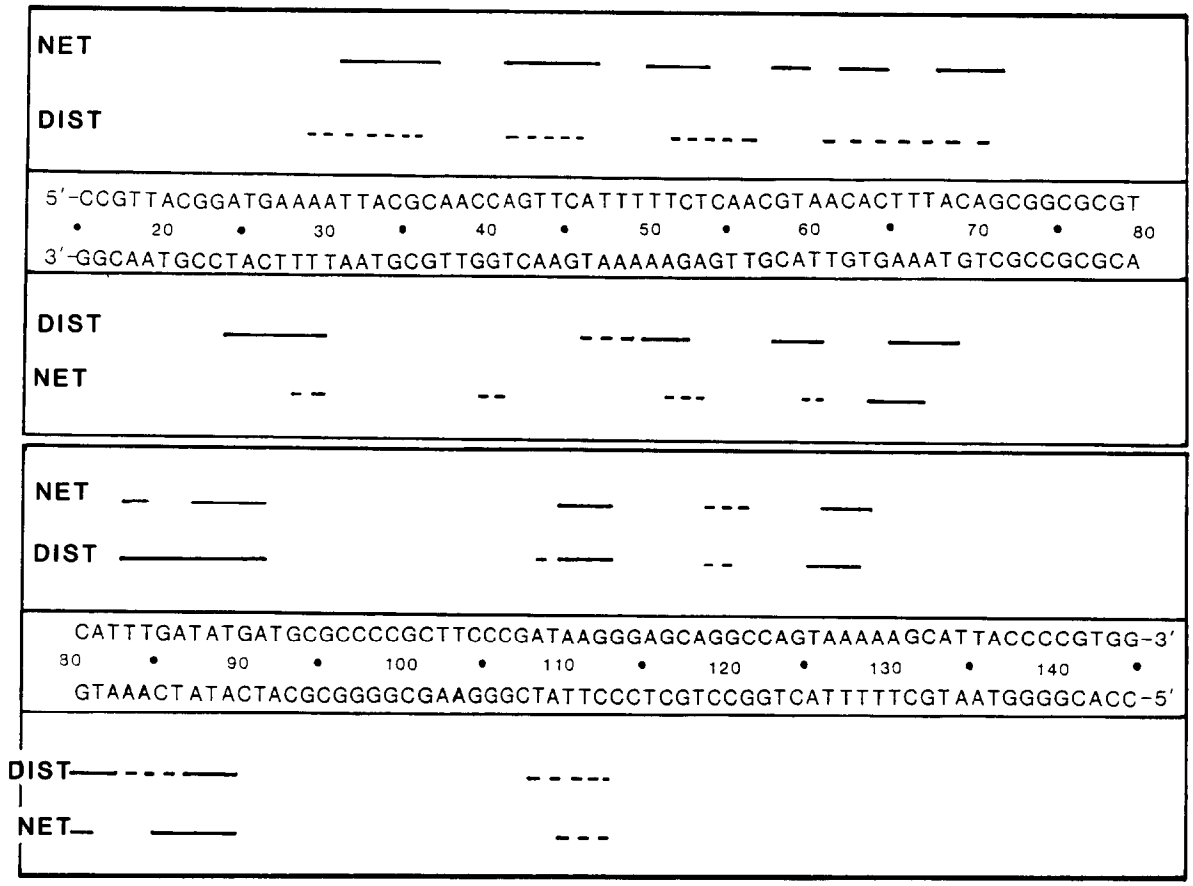

Fig.3. A protection map showing the effects of distamycin A (DIST) and netropsin (NET) on hydroxyl radical cleavage of each strand of *tyr*T DNA. Protected regions were assessed from densitometric scans such as those illustrated in fig.2. Bars indicate regions which are protected from cleavage. Broken bars represent zones where the extent of protection is either weak or doubtful.

sional GC base-pairs are acceptable at or even within the ends of preferred binding sites for distamycin. This result accords with previous experiments performed using quite different techniques (review [2]), which led to a suggestion that the lack of one cationic end in the distamycin molecule is the feature which allows the antibiotic to move away from the floor of the groove so as to accommodate the presence of a guanine NH_2 group at the end of the binding site, or even to permit an isolated GC base pair to intrude into the binding site itself [7,8].

There is little or no evidence of antibiotic-induced increase in the cutting of certain bonds in the gels illustrated in fig.1. Such band-enhancement effects are tentatively attributed to changes in the width of the minor helical groove, to which the nucleases are sensitive [6,8,16,18]. It appears that hydroxyl radicals, which are in any case much smaller and are believed to react with rather exposed elements of the sugar-phosphate backbone, do not sense such induced conformational changes.

4. DISCUSSION

In hydroxyl radical footprinting, the maxima and minima of the cleavage patterns in the presence of netropsin or distamycin are spanned by bands that vary smoothly in intensity (fig.1), showing that some internucleotide bonds are only partially blocked by the bound ligand. Because of this

it is sometimes difficult to assess the true extent of protected regions, but by and large they correspond with the binding sites on *tyr*T DNA as determined in [6,8]. The additional, presumably secondary, sites detected by hydroxyl radical footprinting are a new feature which may be peculiar to the use of this probe. We already know that DNase I and the synthetic probe methidiumpropyl-EDTA·Fe(II) [MPE-Fe(II)] provide different information about DNA-antibiotic interactions [19]. If MPE-Fe(II) footprints are rightly considered more accurate for determining the actual size of binding sites it will be interesting in the future to compare the binding sites reported by MPE-Fe(II) cleavage with those observed using hydroxyl radical cleavage, since MPE-Fe(II) footprints might simply represent regions of the DNA where a bound molecule directly inhibits intercalation by the cleavage agent. Unlike the hydroxyl radical, MPE-Fe(II) is not entirely sequence neutral, perhaps because its central ethidium-like portion [19] may be expected to act in a similar way to the parent compound as regards discriminating between different nucleotide sequences [20]. Using the protocol described in this paper we can produce footprints with concentrations of iron (II) so low that the conformation of the DNA molecule should not be altered by binding of the ion [14], while the hydroxyl radical, by abstracting a hydrogen atom from the sugar ring, mediates the cleavage of DNA [11]. Clearly the reaction breaks the DNA backbone with no regard to sequence as is evident in the control lanes in fig.1. These features of hydroxyl radical footprinting render it attractive as an additional method to complement experiments employing the other probes.

A certain asymmetry is sometimes apparent in the protection of the complementary DNA strands by netropsin and distamycin (figs 1 and 3). This could provide some clues as to whether netropsin is centred symmetrically in the minor groove as suggested by the disposition of the antibiotic within the AATT site of a dodecamer polynucleotide seen by crystallography [7] or NMR in aqueous solution [21], or, as in an alternative model of binding [22], the netropsin molecule faces asymmetrically towards the adenosine-rich strand. In the cleavage map (fig.3) it is evident that in general the Watson strand of *tyr*T DNA is better protected than the Crick strand (e.g. around positions 30, 42, 50, 61 and 110, though not around 83 and 88). However, the sequence near position 30 contains a polyd(A) run, but at about position 50 the protected zone overlaps a polyd(T) tract. It seems, therefore, that although hydroxyl radical footprinting indicates the existence of asymmetry in some protected regions our results cannot be used to support or reject either the symmetric [7,21] or the asymmetric [22] binding models for netropsin and distamycin. It is, however, to be noted that the alternating region 5′-ATAT-3′ around bond 88 appears equally protected on both strands, perhaps as a consequence of symmetric disposition of the antibiotic molecule in the alternating regions, in accordance with the crystal structure model [7].

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