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Transferring the purine 2-amino group from guanines to adenines in DNA changes the sequence-specific binding of antibiotics

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ABSTRACT

The proposition that the 2-amino group of guanine plays a critical role in determining how antibiotics recognise their binding sites in DNA has been tested by relocating it, using *tyr*T DNA derivative molecules substituted with inosine plus 2,6-diaminopurine (DAP). Irrespective of their mode of interaction with DNA, such GC-specific antibiotics as actinomycin, echinomycin, mithramycin and chromomycin find new binding sites associated with DAP-containing sequences and are excluded from former canonical sites containing I•C base pairs. The converse is found to be the case for a group of normally AT-selective ligands which bind in the minor groove of the helix, such as netropsin: their preferred sites become shifted to IC-rich clusters. Thus the binding sites of all these antibiotics strictly follow the placement of the purine 2-amino group, which accordingly must serve as both a positive and negative effector. The footprinting profile of the 'threading' intercalator nogalamycin is potentiated in DAP plus inosine-substituted DNA but otherwise remains much the same as seen with natural DNA. The interaction of echinomycin with sites containing the TpDAP step in doubly substituted DNA appears much stronger than its interaction with CpG-containing sites in natural DNA.

INTRODUCTION

Gene targeting via DNA-binding drugs remains a cherished goal of chemotherapy (1–3). If it is to succeed we need a clear understanding of the mechanisms whereby small molecules can recognise and bind to specific nucleotide sequences in DNA. It has long been suspected that the 2-amino group of guanine is the prime element which determines sequence recognition via the minor groove of the helix where most small molecules bind (4). It is the only hydrogen bond donor group exposed there; it impedes access to the floor of the groove and it interferes with the spine of hydration (5). We have tested the importance of the purine 2-amino group by removing it from guanines and relocating it on the adenine residues. Footprinting experiments performed on the *tyr*T DNA fragment thus modified reveal that the binding (recognition) sites for all small sequence-selective ligands tested, including some whose actual specificity is unknown, are profoundly changed.

To accomplish the transfer of the 2-amino group we used the polymerase chain reaction to prepare homologous DNA samples having guanosine nucleotides replaced by inosines, adenine residues replaced by 2,6-diaminopurines (Fig. 1), or both. The modified DNA species, as well as normal DNA prepared by the same route, were then subjected to DNAase I footprinting in the presence of antibiotics known to bind selectively to DNA at particular sequences by disparate mechanisms (Fig. 2). As an example of a well-characterised intercalator we chose actinomycin D and, for comparison, a bis-intercalator of comparable structure and molecular weight, echinomycin: both are cyclic depsipeptides endowed with potent antitumour activity (6). As examples of ligands binding to the minor groove we looked at two antiviral agents, netropsin and distamycin, as well as mithramycin and chromomycin which are also chemotherapeutic drugs used in the treatment of cancer (6). Lastly, we included nogalamycin as a representative of the clinically important anthracycline group of antitumour antibiotics (7).

MATERIALS AND METHODS

Antibiotics

Actinomycin D, nogalamycin, distamycin, chromomycin and mithramycin were purchased from Sigma. Netropsin was from Serva and echinomycin was obtained from Parke-Davis (NJ, USA). Antibiotics were used as supplied without further purification. The tested drugs showed good aqueous solubility except echinomycin which is sparingly soluble in water. Echinomycin was dissolved to a concentration of 100 μM in 10 mM Tris–HCl, pH 7.0, 10 mM NaCl containing 40% (v/v) methanol. The stock solution was diluted to working concentrations with appropriate volumes of 10 mM Tris–HCl, pH 7.0, 10 mM NaCl and methanol so as to yield a final methanol concentration of 10% (v/v) in the footprinting reactions. Under these conditions methanol is known not to affect the nuclease activity (8). Antibiotic concentrations

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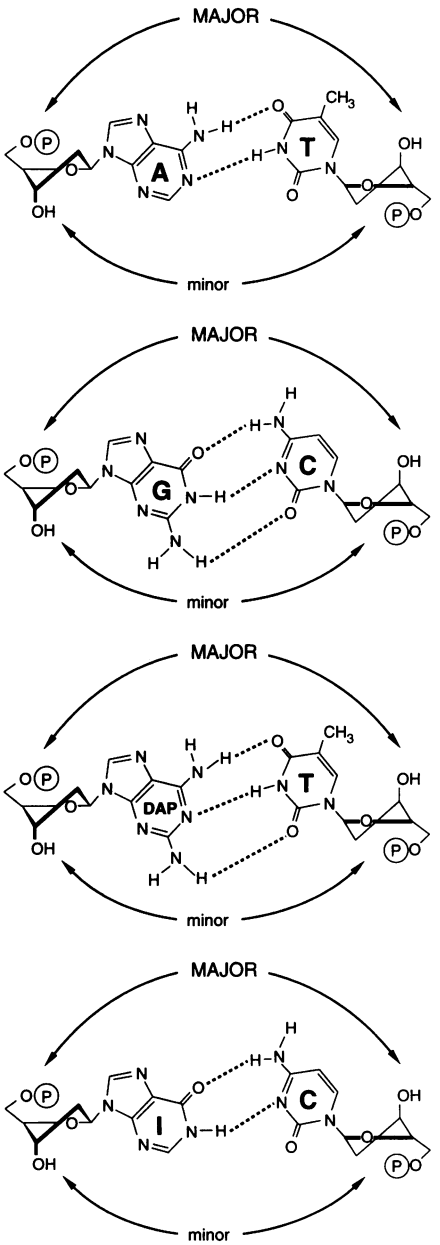

Figure 1. Structures of hydrogen-bonded purine–pyrimidine base pairs. Broken lines represent hydrogen bonds. I represents inosine; DAP represents 2,6-diaminopurine (2-aminoadenine). The major and minor grooves of the helix are indicated.

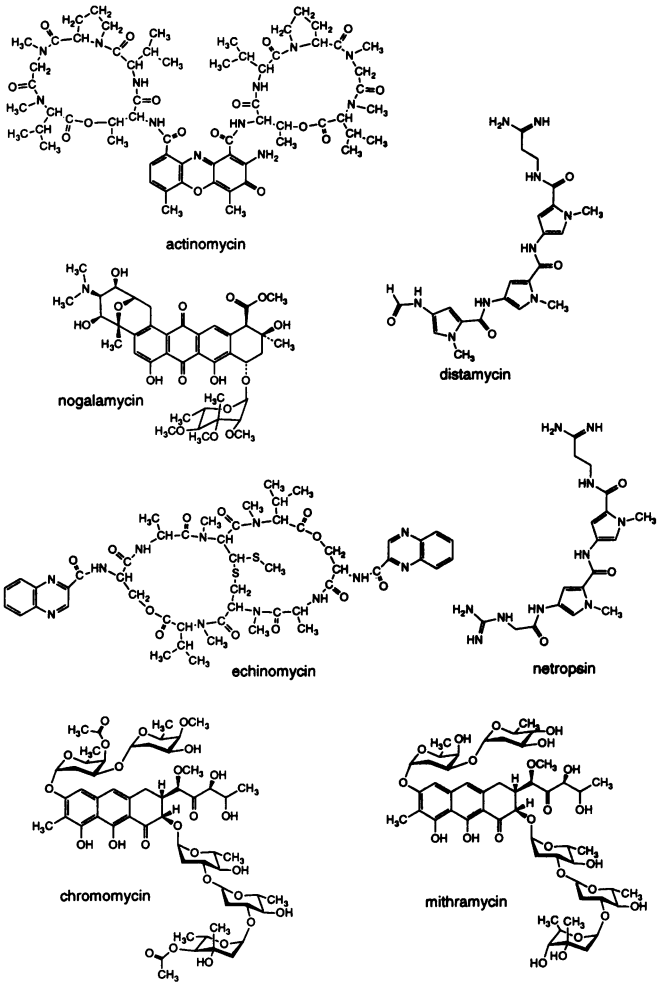

Figure 2. Structures of DNA-binding antibiotics.

were determined spectroscopically in 10 mm pathlength quartz cuvettes through the molar extinction coefficients given in the literature.

Chemicals and biochemicals

Ammonium persulphate, Tris base, acrylamide, bis-acrylamide, ultrapure urea, boric acid, tetramethylethylenediamine and dimethyl sulphate were from BDH. Formic acid, piperidine and formamide were from Aldrich. Photographic requisites were from Kodak. Bromophenol blue and xylene cyanol were from Serva. The nucleoside triphosphate labelled with [γ-^{32}P]ATP was obtained from NEN Dupont. Restriction endonucleases *Eco*RI and *Ava*I (Boehringer), *Taq* polymerase (Promega), DNase I (Sigma) and T4 polynucleotide kinase (Pharmacia) were used according to the supplier's recommended protocol in the activity buffer provided. The primers, 5′-AATTCCGGTTAC-CTTTAATC and 5′-TCGGGAACCCCCACCACGGG having a 5′-OH or 5′-NH_2 terminal group, were obtained from the Laboratory of Molecular Biology, Medical Research Council, Cambridge. Checks were carried out to ensure that the primers blocked with a 5′-NH_2 group were free from contaminants and not labelled by the kinase. All other chemicals were analytical grade reagents, and all solutions were prepared using doubly deionised, Millipore filtered water.

Preparation, purification and labelling of DNA fragments containing natural and modified nucleotides

Plasmid pKMp27 (9) was isolated from *E.coli* by a standard sodium dodecyl sulphate–sodium hydroxide lysis procedure and purified by banding in CsCl–ethidium bromide gradients. Ethidium was removed by several isopropanol extractions followed by exhaustive dialysis against Tris–EDTA buffer. The purified plasmid was then precipitated and resuspended in appropriate buffer prior to digestion by the restriction enzymes. The 160 base

pair *tyrT*(A93) fragment used as a template was isolated from the plasmid by digestion with restriction enzymes *Eco*RI and *Ava*I. It is worth mentioning that this template DNA bore a 5′-phosphate due to the action of *Eco*RI and thus only the newly synthesized DNA (with normal or modified nucleotides) can be labelled by the kinase.

Polymerase chain reaction (PCR). The protocol used to incorporate inosine and/or 2,6-diaminopurine residues into DNA is comparable to those previously used to incorporate 7-deazapurine or inosine residues with only a few minor modifications (10,11). PCR reaction mixtures contained 10 ng of *tyrT*(A93) template, 1 μM each of the appropriate pair of primers (one with a 5′-OH and one with a 5′-NH_2 terminal group) required to allow 5′-phosphorylation of the desired strand, 250 μM of each appropriate dNTP (dTTP, dCTP plus dATP or dDTP and dGTP or dITP according to the desired DNA), and 5 U of *Taq* polymerase in a volume of 50 μl containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 0.1% Triton X-100 and 1.5 mM $MgCl_2$. To prevent unwanted primer–template annealing before the cycles began, the reactions were heated to 60°C before adding the *Taq* polymerase (12). Finally, paraffin oil was added to each reaction to prevent evaporation. After an initial denaturing step of 3 min at 94°C, 20 amplification cycles were performed, with each cycle consisting of the following segments: 94°C for 1 min, 37°C for 2 min and 72°C for 10 min. After the last cycle, the extension segment was continued for an additional 10 min at 72°C, followed by a 5 min segment at 55°C and a 5 min segment at 37°C. The purpose of these final segments was to maximize annealing of full-length product and to minimise annealing of unused primer to full-length product. The reaction mixtures were then extracted with chloroform to remove the paraffin oil, and parallel reactions were pooled. Several extractions with water-saturated *n*-butanol were performed to reduce the volume prior to loading the samples on to a 6% non-denaturing polyacrylamide gel. After electrophoresis for ~1 h, a thin section of the gel was stained with ethidium bromide so as to locate the band of DNA under UV light. The same band of DNA free of ethidium was excised, crushed and soaked in elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) overnight at 37°C. This suspension was filtered through a Millipore 0.22 μm filter and the DNA was precipitated with ethanol. Following washing with 70% ethanol and vacuum drying of the precipitate, the purified DNA was resuspended in the kinase buffer.

DNA labelling and purification. The purified PCR products were 5′ end-labelled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase according to a standard procedure for labelling blunt-ended DNA fragments (13). After completion the labelled DNA was again purified by 6% polyacrylamide gel electrophoresis and extracted from the gel as described above. Finally, the labelled DNA was resuspended in 10 mM Tris–HCl, pH 7.0 buffer containing 10 mM NaCl.

DNase I footprinting

DNase I experiments were performed essentially according to the original protocol (8). The digestion of the samples (6 μl) of the labelled DNA fragment dissolved in 10 mM Tris buffer pH 7.0 containing 10 mM NaCl was initiated by the addition of 2 μl of a DNase I solution whose concentration was adjusted to yield a final enzyme concentration of ~0.01 U/ml in the reaction mixture. The extent of digestion was limited to <30% of the starting material so as to minimize the incidence of multiple cuts in any strand ('single-hit' kinetic conditions). Optimal enzyme dilutions were established in preliminary calibration experiments. After 3 min, the digestion was stopped by freeze drying, samples were lyophilized, washed once with 50 μl of water, lyophilized again and then resuspended in 4 μl of an 80% formamide solution containing tracking dyes. Samples were heated at 90°C for 4 min and chilled in ice for 4 min prior to electrophoresis.

Electrophoresis and autoradiography

DNA cleavage products were resolved by polyacrylamide gel electrophoresis under denaturing conditions (0.3 mm thick, 8% acrylamide containing 8 M urea) capable of resolving DNA fragments differing in length by one nucleotide. Electrophoresis was continued until the bromophenol blue marker had run out of the gel (~2.5 h at 60 W, 1600 V in TBE buffer, BRL sequencer model S2). Gels were soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at –70°C with an intensifying screen. Exposure times of the X-ray films (Fuji R-X) were adjusted according to the number of counts per lane loaded on each individual gel (usually 24 h).

Quantitation by storage phosphorimaging

A Molecular Dynamics 425E PhosphorImager was used to collect data from storage screens exposed to the dried gels overnight at room temperature (14). Base line-corrected scans were analyzed by integrating all the densities between two selected boundaries using ImageQuant version 3.3 software. Each resolved band was assigned to a particular bond within the *tyr*T(A93) fragment by comparison of its position relative to sequencing standards generated by treatment of the DNA with formic acid followed by piperidine-induced cleavage at the purine residues (G+A track).

RESULTS

Strong footprints were produced by each ligand on the normal and doubly substituted DNAs (Fig. 3). Echinomycin is a bis-intercalator (8,15), and actinomycin a mono-intercalator (16,17), which normally bind to CpG and GpC steps respectively: the footprints for both antibiotics are radically altered by the nucleotide substitution. The same is true for netropsin (18) and distamycin (19,20) which are AT-specific minor groove-binders, as well as for mithramycin and chromomycin (21) which bind in dimeric form to GC-rich sequences within the minor groove (22–24). Even the binding of nogalamycin (25–27), a 'threading' intercalator which interacts with a puzzling variety of sites in natural DNA (28), is changed.

The canonical and newly created binding sites in the two types of DNA can be identified in Figure 4 where the near-inversion of the footprinting pattern for most ligands is clearly evident, especially in the central portion of the sequence extending from position 60 to 100 of the *tyr*T fragment. Irrespective of their mode of binding, the normally GC-selective antibiotics are displaced from their clustered sites lying between positions 70 and 80 in natural DNA, to pick up new sites in the DAP•T-rich sequences either side (panels A, B and D). By contrast, the normally

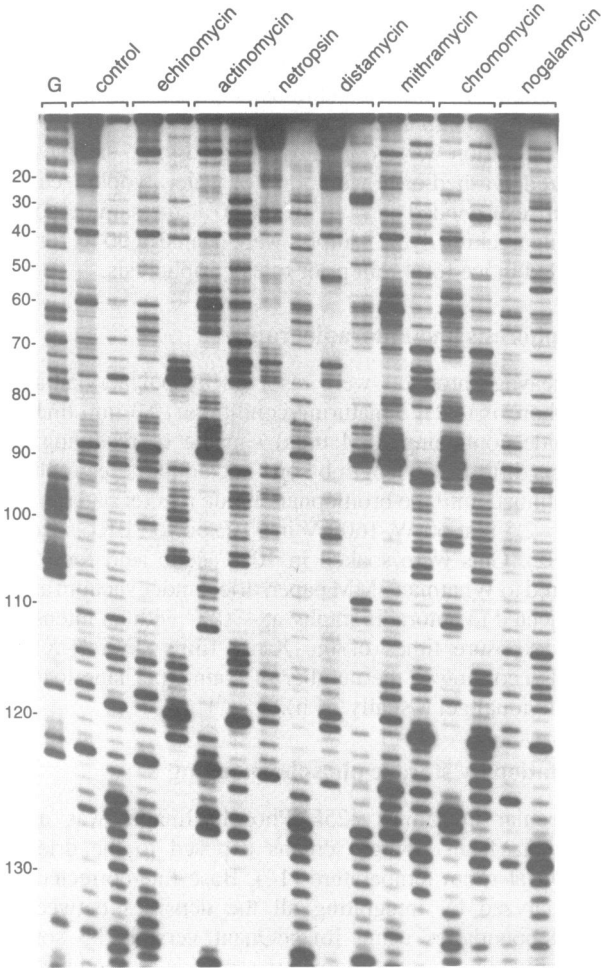

Figure 3. Autoradiograph of a high-resolution denaturing gel showing DNAase I footprinting of antibiotics on the Crick (sense) strand of normal and inosine plus DAP *tyr*T(A93) DNA. Each pair of lanes corresponds to digestion of normal (left) and I + DAP DNA (right). The products of DNAase I digestion were resolved on an 8% polyacrylamide gel containing 8 M urea. Their identities were assigned by reference to the Maxam–Gilbert guanine markers (lane G), taking into account the difference in mobility of the fragments due to the presence or absence of a 3′-phosphate group. Lanes marked control refer to the DNAase I digests of the normal and doubly substituted DNA in the absence of antibiotic. The other lanes contained 20 μM actinomycin, 10 μM netropsin, 10 μM distamycin, 10 μM mithramycin, 10 μM chromomycin or 5 μM nogalamycin. With echinomycin, 20 μM was used to footprint normal DNA and 5 μM for inosine plus DAP-DNA. The scale on the left corresponds to the standard numbering of the *tyr*T(A93) sequence (9) as represented in Figure 5.

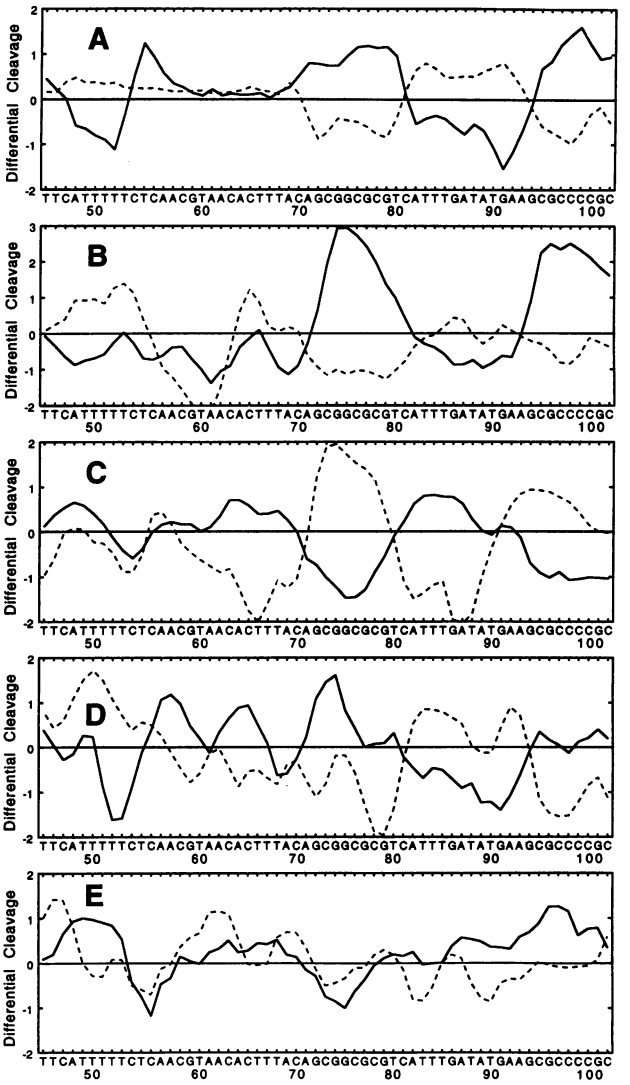

Figure 4. Differential cleavage plots comparing the DNAase I-mediated cleavage of the Watson (antisense) strand of normal and I + DAP *tyr*T(A93) DNA in the presence of actinomycin (**A**), echinomycin (**B**), netropsin (**C**), chromomycin (**D**) and nogalamycin (**E**). Data for distamycin and mithramycin are not included; they were much the same as those shown for netropsin and chromomycin respectively. The plots drawn as continuous lines refer to the modified *tyr*T(A93) DNA fragment containing inosine and DAP residues. The plots indicated by dashed lines refer to normal *tyr*T(A93) DNA. Antibiotic concentrations used to generate these plots were identical to those specified in Figure 3. Positive and negative values correspond, respectively, to enhanced or diminished DNAase I cutting at each internucleotide bond. The values plotted compare the measured probabilities of cleavage expressed in logarithmic units and are smoothed by taking a three-bond running average.

AT-selective netropsin is displaced from its canonical sites in those flanking sequences (positions 60–69 and 82–90) to bind decisively to the intervening IC-rich cluster created in the doubly substituted DNA (panel C). Essentially similar results were obtained with distamycin and with the synthetic minor groove-binding drugs DAPI and berenil (29,30).

The case of echinomycin is examined in greater detail in Figure 5 which shows the complete footprinting pattern on both strands of the *tyr*T(A93) DNA over the whole length of sequence accessible to analysis. As previously reported with this restriction fragment (8) the canonical sites in natural *tyr*T DNA are squarely located around the CpG steps, marked by open rectangles in the illustration. In the doubly inosine plus DAP-substituted DNA every region protected from DNAase I attack occurs at a TpDAP step and all such steps (shown as black rectangles) constitute part of a ligand binding site. The radically changed pattern of binding sites for echinomycin, reflecting its faithful recognition of the displaced 2-amino groups, is emphasised by an examination of the antibiotic concentration-dependence of the footprinting profile (Fig. 6). Over at least two orders of magnitude the footprinting pattern remains firmly locked to the sites surrounding the TpDAP steps at positions 61, 69, 89, 111, 127 and 137 on the Crick strand of the *tyr*T fragment. Only at the TpI step at

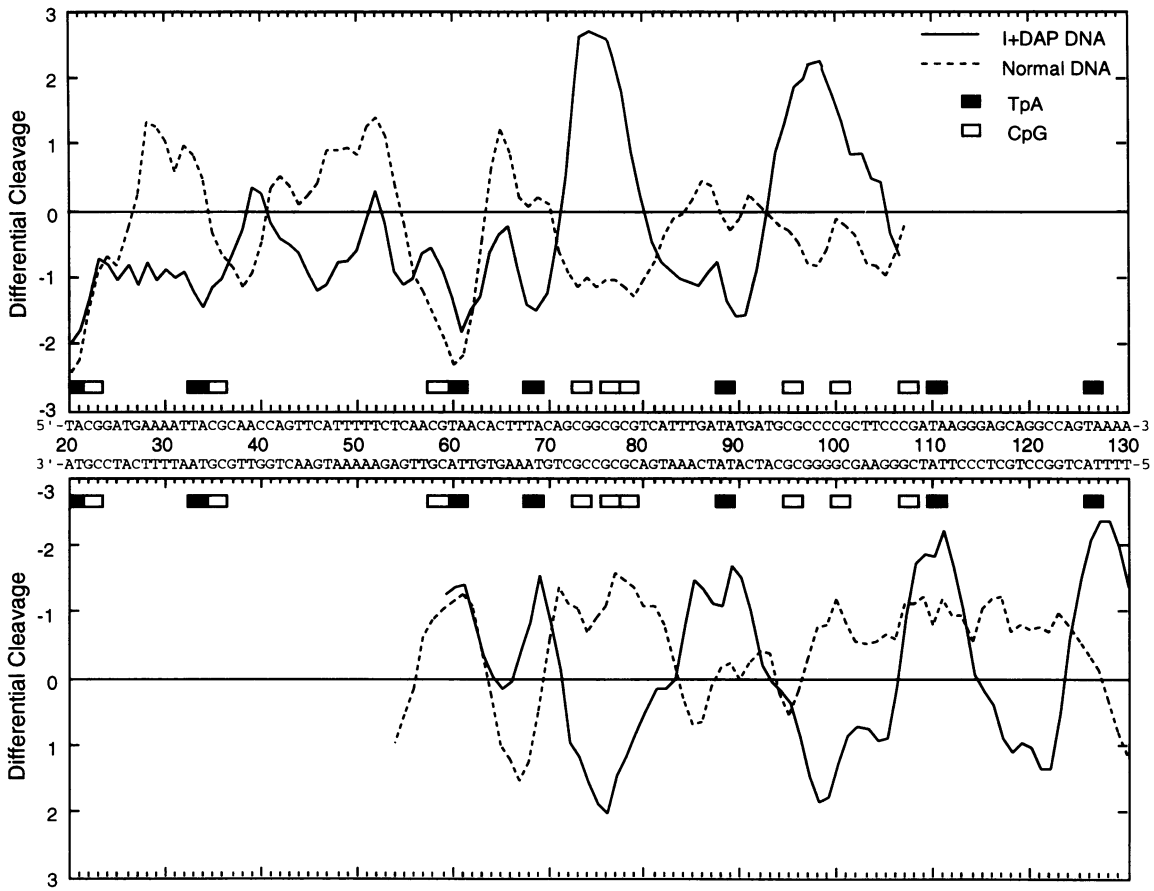

Figure 5. Differential cleavage plots comparing the susceptibility of *tyr*T(A93) DNA to cutting by DNAase I in the presence of 10 μM echinomycin. The dashed and continuous curves refer to natural and inosine plus DAP-substituted DNA respectively, as for Figure 4. The upper panel shows differential cleavage of the Watson strand, the lower panel that of the complementary Crick strand. The ordinate scales for the two strands are inverted, so that deviation of points towards the lettered sequence (negative values) corresponds to a ligand-protected site and deviation away (positive values) represents enhanced cleavage. The filled rectangles near the indicated dinucleotide steps show the positions of the TpA steps and the open rectangles show the positions of the CpG steps. Other details as for Figure 4.

position 82 can any departure from strict adherence to this rule be discerned: at very low echinomycin concentrations (≤1 μM) the band corresponding to cutting at this step is clearly enhanced but by 10–20 μM the enhancement is lost, suggestive of incipient protection (footprinting) at this site. This looks like a classic instance of secondary binding, characterised by diminished specificity, to a site for which the antibiotic has lower affinity (and which lacks one critical 2-amino recognition element) such as is commonly seen with many DNA-binding drugs (6).

A full analysis of the behaviour of actinomycin D has yielded equivalent results (31): the binding sites in doubly inosine plus DAP-substituted DNA lie squarely over the DAPpT steps and all such steps form part of a binding site for the antibiotic, whereas the GpC step is the essential component of canonical recognition sequences in natural DNA. Not so for nogalamycin, however: the sequences to which this antibiotic binds in the two types of DNA are not greatly different though the intensity of the footprinting profile is markedly enhanced at specific sites particularly on the lower (Crick) strand (Fig. 7). A previous exhaustive footprinting study with several DNA restriction fragments (28) concluded that most nogalamycin binding sites are located near regions of alternating purine–pyrimidine sequence, most commonly associated with the dinucleotide steps TpG (CpA) and GpT (ApC), suggesting that the preferred antibiotic binding sites may contain all four nucleotides and/or that peculiarities of the dynamics of DNA conformation at alternating sequences may be critical for nogalamycin binding. Given its unusual mode of 'threading' intercalation which probably involves local disruption of base-pairing and results in conspicuously slow association and dissociation kinetics (32,33) the results in Figure 7 are perhaps unsurprising. It is likely that the binding of this ligand is dominated by the structural and dynamic features of DNA which may well be broadly similar for natural and doubly-substituted DNA molecules. For example, the alternation of purines and pyrimidines at particular sites remains the same in both types of DNA. At all events, there is rather poor correspondence between the sites protected from nuclease cleavage by nogalamycin and the TpG (CpA) and GpT (ApC) steps in natural DNA or TpI (CpDAP) and IpT (DAPpC) steps in substituted DNA, so the process of site recognition by nogalamycin appears to proceed to a large extent independently of the placement of the purine 2-amino group. It is tempting to speculate that the preferred nogalamycin binding sites are determined chiefly by substituents lying in the major groove of the DNA helix, which is where one of the bulky sugar substituents of the antibiotic must come to lie (27), and consequently the best sites are to be found at the same places in both types of DNA in Figure 7 although the ease of binding to such sites reflected in the binding kinetics may be

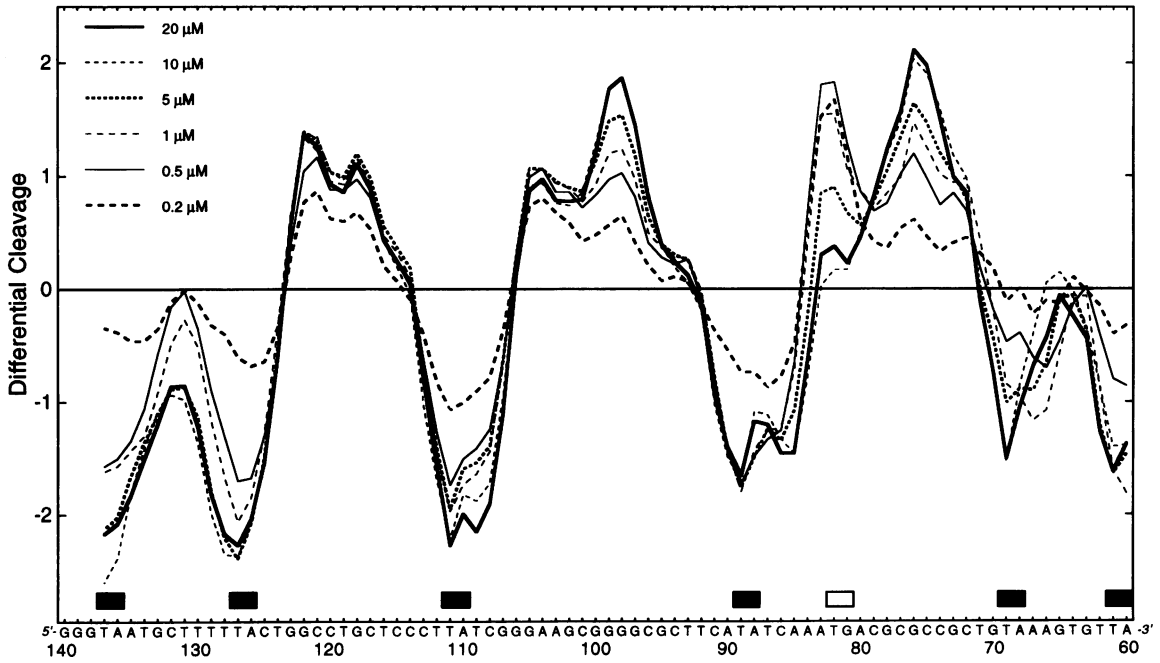

Figure 6. Differential cleavage plots showing the concentration-dependence of footprinting by 0.2–20 μM echinomycin on the Crick (sense) strand of *tyr*T(A93) DNA containing inosine plus DAP residues. Positive and negative values represent, respectively, enhanced or diminished DNAase I cutting efficiency at each internucleotide bond. Note that the sequence shown corresponds to that of natural DNA, though here the A residues are replaced by DAP and the G residues by inosine. Black boxes show the locations of the TpDAP steps. A white box is drawn over the TpI step at position 82.

much affected, witness the exaggerated excursions of the differential cleavage plot for the substituted DNA.

The findings summarised in Figures 3 and 4 establish that the purine 2-amino group characteristic of guanine nucleotides serves as both a positive and negative effector to determine ligand binding sites, and thereby dominates the sequence-recognition process. This interpretation is amplified by parallel observations on the interaction of the same antibiotics with singly inosine (11) or DAP-substituted (34) DNA molecules. The positive role of the 2-amino group as a necessary component of binding sites for GC-selective antibiotics is confirmed by the failure of echinomycin, actinomycin, chromomycin and mithramycin to footprint on inosine-containing DNA, plus their redistribution on to new sites in DAP-substituted DNA. Conversely, the negative signal given by the 2-amino group as a marker of sites unavailable for binding AT-selective antibiotics is rendered obvious by the failure of netropsin and distamycin to footprint on DAP-containing DNA, whereas they seem to bind all over the inosine-substituted polymer.

Quantitative analysis of ligand-site interactions backs up these conclusions. The full concentration-dependence profile (35) for each antibiotic has been determined at all binding sites and a few examples are illustrated in Figure 8. With echinomycin, the footprinting on natural DNA at the canonical CpG steps and the corresponding enhancement of DNAase I cleavage at TpA steps occur with half-maximal effect at concentrations (C_{50}) ~2–5 μM. By contrast, the footprinting at TpDAP steps in inosine plus DAP-substituted DNA, and enhancement of cutting at CpI steps, take place at much lower concentrations: C_{50} = 1 μM or below. The same is true for DNA substituted only with DAP. We must conclude that the new DAP-containing binding sites are superior to the canonical CpG-containing sites. Under the conditions of these footprinting experiments a large fraction of the added ligand is likely to remain free, such that C_{50} values will approximate to thermodynamic dissociation constants for binding to individual sites (35). On this basis we estimate that the binding constant for echinomycin at several DAP-containing sites must be enhanced by a factor of ten or more. With netropsin and distamycin no such potentiation of effect occurs: C_{50} values for footprinting at the newly created IC-containing binding sites, and for enhanced nuclease cleavage at DAP•T-rich clusters, are generally somewhat higher than those required to produce equivalent effects at the canonical sites in normal DNA (Fig. 8). With actinomycin and the other antibiotics the apparent affinity for new binding sites in doubly-substituted DNA is generally little different from that measured for the natural *tyr*T fragment.

DISCUSSION

What is the mechanism by which the purine 2-amino group dictates where sequence-selective small molecules bind to DNA? It could be mediated via direct contact between the interacting species involving the formation of hydrogen bonds together with elements of steric complementarity—the so-called 'digital' readout (36). Alternatively it could occur by a kind of 'analogue' readout in which the favoured binding sites are recognised by virtue of some conformational property such as groove width which is only indirectly influenced by the location of the critical 2-amino group. We have evidence from comparing the reactivity of inosine and DAP-substituted DNAs towards structure-sensitive probes that groove width is a relevant parameter which can be strongly affected by re- positioning the purine 2-amino group (37). Deformability of the helix is another property which is likely to be affected. Either or both of these features of DNA helical structure could conspire to determine what nucleotide sequences,

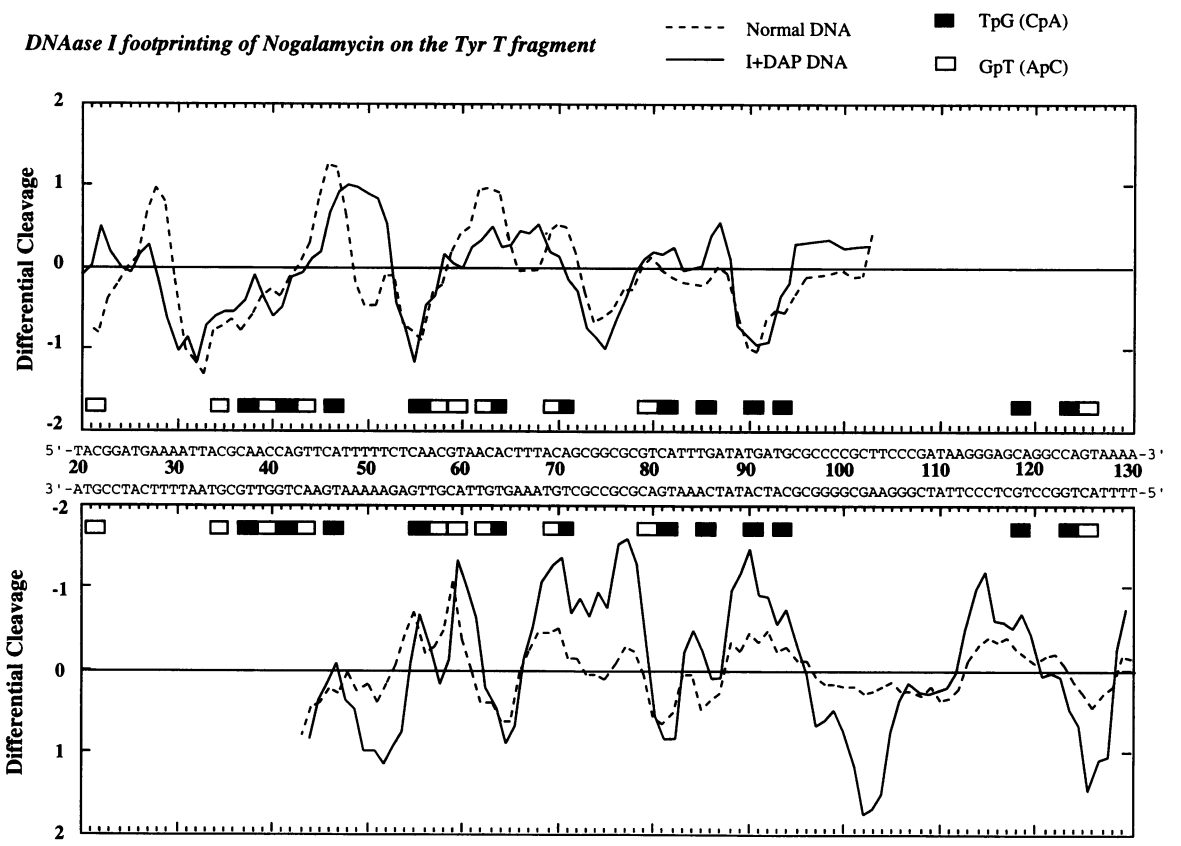

Figure 7. Differential cleavage plots comparing the susceptibility of *tyr*T(A93) DNA to cutting by DNAase I in the presence of 5 μM nogalamycin. Details as for Figures 4 and 5. Black rectangles indicate the TpG (CpA) steps, open rectangles the GpT (ApC) steps.

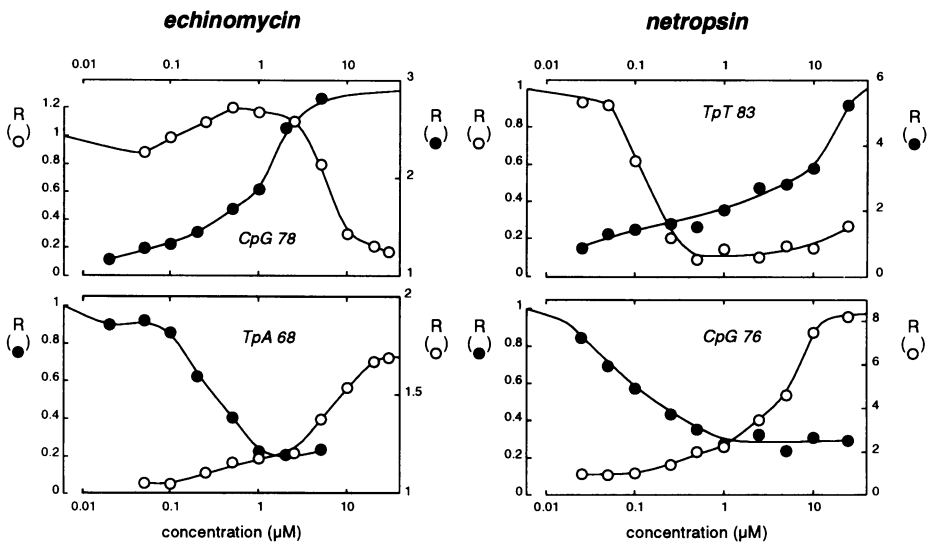

Figure 8. Footprinting plots (35) for selected bonds in the normal *tyr*T(A93) fragment (open circles) and its inosine plus DAP-substituted homologue (filled circles). The relative band intensity R corresponds to the ratio I_c/I_o where I_c is the intensity of the band at a ligand concentration c and I_o is the intensity of the same band in the absence of antibiotic. Ligand concentrations are plotted on a logarithmic abscissa and each plot is linked to the appropriate scale on the left or right ordinate.

in a particular context, might constitute an acceptable ligand binding site. Indeed, for a given ligand the part played by any single factor ultimately under the control of the purine 2-amino group could vary at different potential binding sites. At all events, based on our present findings we are confident that efforts to design DNA sequence-specific ligands using various motifs, all crucially dependent upon exploiting principles of molecular recognition, can be placed on a firmer footing.

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