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Distamycin-induced inhibition of homeodomain–DNA complexes

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The mobility shift assay was used to study the competition of the minor groove binder distamycin A with either an *Antennapedia* homeodomain (*Antp* HD) peptide or derivatives of a *fushi tarazu* homeodomain (*ftz* HD) peptide for their AT-rich DNA binding site. The results show that distamycin and the homeodomain peptides compete under the conditions: (i) preincubation of DNA with distamycin and subsequent addition of HD peptide; (ii) simultaneous incubation of DNA with distamycin and HD peptide; and (iii) preincubation of DNA with HD peptide and subsequent addition of distamycin. There is also competition when using a peptide which lacks the N-terminal arm of *ftz* HD that is involved in contacts in the minor groove. It is proposed that the protein's binding affinity is diminished by distamycin-induced conformational changes of the DNA. The feasibility of the propagation of conformational changes upon binding in the minor groove is also shown for the inhibition of restriction endonucleases differing in the AT content of their recognition site and of their flanking DNA sequences. Thus, it is demonstrated that minor groove binders can compete with the binding of proteins in the major groove, providing an experimental indication for the influence of biological activities exerted by DNA ligands binding in the minor groove.

Key words: Antennapedia/distamycin/DNA-drug/interactions/homeodomain/DNA–protein binding

Introduction

The mutual recognition of molecules is fundamental to all biological processes. For example, it is thought to be decisive in enzyme catalysis and inhibition, gene expression and its control, DNA replication, immune response and drug action. Despite the perception of the importance of this recognition as a whole, little is known about the details of sequence-specific recognition of DNA by drugs such as parameters dictating inhibition of biological processes.

The pyrrole amide antibiotic distamycin A of *Streptomyces distallicus* is an AT directed non-intercalative DNA binder (reviewed in Zimmer and Wähnert, 1986). Distamycin A binds within the minor groove of B-DNA, covering about five AT base pairs and replacing the spine of hydration whereby the NH protons of each of the three pyrrolecarboxamide rings form bifurcated hydrogen bonds with N-3 of adenine or O-2 of thymine in adjacent bases on opposite strands (Kopka *et al.*, 1985; Coll *et al.*, 1987). Further stabilization of the ligand–DNA complex is provided by van der Waals interactions between the CH protons of the pyrroles and the C-2 hydrogens of adenines as well as by electrostatic interactions between the terminal cationic group(s) and phosphates of the DNA backbone (Kopka *et al.*, 1985). In addition it has been suggested that the localization of the deepest electrostatic-molecular potential of double-stranded DNA in the minor groove of its AT sequences may play a fundamental role in the origin of specificity (Pullman, 1990 and references therein).

The AT preference is probably caused by an optimization of van der Waals interactions in the distinct narrow minor groove associated with AT base pairs and by the fact that the exocyclic C-2 amino group of guanine represents a steric hindrance in the minor groove. Distamycin induces local structural distortions of DNA by bending the DNA helix and by inducing conformational changes in the DNA sequence(s) neighbouring its binding site (Kopka *et al.*, 1985). Another reason for the sequence selectivity of distamycin seems to be the larger number of intermolecular bifurcated hydrogen bonds involving the NH protons of distamycin as donors and N-3 of adenine and O-2 of thymine as acceptors for the non-alternating versus alternating AT sequences (Coll *et al.*, 1989). However, the basis of the DNA sequence specificity of distamycin and other ligands is not yet fully understood (Leupin, 1990). In addition, it has already been shown for several systems that the biological activity of a DNA ligand correlates better with the kinetics of its binding rather than with its affinity constant to DNA (e.g. Müller and Crothers, 1968; Feigon *et al.*, 1984; Skorobogaty *et al.*, 1988).

Regulatory proteins help to turn genes on or off as required by selective recognition and binding to specific DNA sequences (Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Struhl, 1989). There can be direct, 'digital' recognition of individual bases or base pairs (Wharton and Ptashne, 1987) and/or an indirect readout (Otwinowski *et al.*, 1988), or 'analogue recognition', of both the local conformation and the local configuration in the DNA target sequence (Drew and Travers, 1985). The marked sequence-dependent variation of the DNA structure is reflected in changes of such parameters as groove width, local twist, displacement of the average base pair plane from the helical axis and the inclination of a base pair plane to its neighbour (Travers, 1989). The details of this local variation (reviewed in Drew *et al.*, 1988) are a major element in the recognition of binding sites by proteins and provide the basis for the direct recognition of DNA by ligands.

The homeodomain is a DNA binding motif that plays a central role in eukaryotic gene regulation (Scott *et al.*, 1989; Gehring *et al.*, 1990). It was first detected in a set of DNA regulatory proteins of *Drosophila* (Laughon and Scott, 1984; McGinnis *et al.*, 1984a), but it belongs to a multigene

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family—found in many other metazoa including vertebrates (McGinnis *et al.*, 1984b)—that regulates transcription by binding *cis*-acting DNA sequences (Affolter *et al.*, 1990b; Hayashi and Scott, 1990).

A 68 amino acid *Antennapedia* homeodomain (*Antp* HD) peptide, a prototypical member of one of the largest sequence subfamilies, has been purified (Müller *et al.*, 1988). *In vitro* DNA binding studies of the *Antp* HD peptide and a 18 bp oligonucleotide containing one recognition sequence showed stable DNA–protein complexes formed with a dissociation constant K_d of about 1×10^{-9} M (Affolter *et al.*, 1990a). The interaction of *Antp* HD with its target DNA has been characterized by ethylation and methylation interference experiments; it has been shown that the 5'-ATTA-3' motif interferes strongly with peptide binding (Affolter *et al.*, 1990a).

The three-dimensional structure of the *Antp* HD peptide in solution has recently been determined by nuclear magnetic resonance spectroscopy (NMR; Qian *et al.*, 1989; Billeter *et al.*, 1990) and has been shown to contain a helix–turn–helix motif as previously found in several bacterial DNA binding factors (for reviews, see Pabo and Sauer, 1984; Johnson and McKnight, 1989). In a further NMR study, Wüthrich and coworkers determined the three-dimensional structure of the complex between the *Antp* HD peptide and a 14 bp DNA fragment in solution (Otting *et al.*, 1990). In this complex the helix–turn–helix motif makes base specific contacts in the major groove and the N-terminal arm reaches into the adjacent minor groove.

Recently, the structure of a complex between the 61 amino acid peptide of an *engrailed* homeodomain (a prototypic member of another major homeodomain subfamily) and a duplex DNA site has been determined at 2.8 Å resolution by X-ray crystallography (Kissinger *et al.*, 1990). This crystal structure compares well with the structure in solution for the *Antp* HD–DNA complex derived by NMR, both in protein structure and overall architecture of the protein–DNA complex. It also shows that the amino acids Arg5 and possibly Arg3 of the N-terminal arm of the *engrailed* homeodomain make direct contacts with DNA bases in the minor groove.

Distamycin A binds best to DNA sites containing at least four consecutive AT base pairs (Zimmer and Wähnert, 1986). Thus the recognition site 5'-ATTA-3' of the *Antp* HD peptide also represents a strong binding site for distamycin. It has been demonstrated previously that distamycin interferes with proteins associated with DNA such as restriction enzymes (Nosikov *et al.*, 1976), topoisomerase II (Fesen and Pommier, 1989), RNA polymerase (Bruzik *et al.*, 1987; Martello *et al.*, 1989), DNA polymerases (Levy *et al.*, 1989) and transcription factors (Broggini *et al.*, 1989). In the study by Broggini *et al.* (1989), the observation of an inhibitory effect of distamycin required a preincubation of the antibiotic with DNA. If the antibiotic was added simultaneously with the protein to DNA, or was added to a solution containing DNA preincubated with protein, little or no inhibition of protein binding to DNA was observed.

The availability of structural information on HD–DNA complexes, the feasibility of the mobility shift assay to study complex formation and the overlap of HD and distamycin binding sites should provide detailed information on how low molecular weight ligands and transcription factor DNA binding domains interact with or compete for shared DNA binding sites.

In our study we demonstrate that distamycin competes with HD peptides for their DNA binding sites under the following experimental conditions: (i) preincubation of DNA with distamycin and subsequent addition of HD peptide; (ii) simultaneous incubation of DNA with distamycin and HD peptides; and (iii) preincubation of DNA with HD peptides and subsequent addition of distamycin.

Results

Competition of Antp HD peptide with distamycin for DNA binding

The antibiotic distamycin A binds double helical DNA rich in adenine (A) and thymine (T) base pairs (Van Dyke *et al.*, 1982) noncovalently. Harshman and Dervan (1985) have shown by footprinting with methidiumpropyl-EDTA-Fe(II) that the size of the binding site of distamycin on DNA amounts to 5 ± 1 bp consisting nearly exclusively of AT. Recently, AT-rich motifs have also been found in recognition sequences of homeodomain containing proteins (Scott *et al.*, 1989; Affolter *et al.*, 1990b).

Specific binding of regulatory factors to their recognition sites can be detected by the electrophoretic mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981; for a phenomenological theory of this method see Cann, 1989; Kleinschmidt *et al.*, 1991). Using the ^{32}P-labelled oligonucleotide BS2-18 wt (see Materials and methods), whose sequence was derived from a DNA binding site of *Antp* HD identified by footprinting analysis (Müller *et al.*, 1988), specific and stable protein–DNA complexes with the 68 amino acid *Antp* HD peptide are formed with an apparent dissociation constant K_d of $\sim 10^{-9}$ M at 25°C (Müller *et al.*, 1988; Affolter *et al.*, 1990a). Under the experimental conditions and the low DNA concentrations employed, these complexes decay with a half life of ~90 min (data not shown; Affolter *et al.*, 1990a).

Preincubation of the labelled oligonucleotide BS2-18 wt with distamycin A clearly inhibits the formation of the *Antp* HD–DNA complex (Figure 1). This effect was already

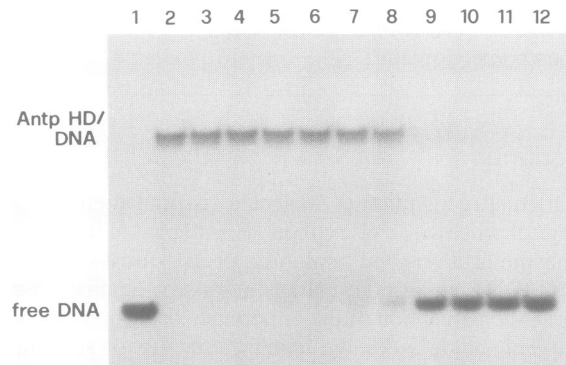

Fig. 1. Effect of distamycin on *Antp* HD binding to BS2-18 wt oligonucleotide; the DNA was preincubated with increasing concentrations of distamycin: 0.12 (lane 3), 0.24 (lane 4), 0.48 (lane 5), 0.75 (lane 6), 1.2 (lane 7), 2.4 (lane 8), 4.8 (lane 9), 7.5 (lane 10), 12 (lane 11) and 30 μM (lane 12) for 20 min before addition of *Antp* HD (~80 nM). Lane 1 contains BS2-18 wt oligonucleotide only, lane 2 is *Antp* HD and BS2-18 wt with no distamycin.

observed at ~2 μM distamycin A. Broggini *et al.* (1989) described the inhibition of binding of the ubiquitous octamer binding factor (OTF-1) and of the erythroid specific GATAAG protein (NFE-1) to their conserved DNA elements by distamycin. Preincubation of distamycin with the labelled DNA fragment diminished the DNA binding of OTF-1 and NFE-1 in a concentration-dependent manner. However, preincubation of distamycin with DNA was essential for this effect; addition of the drug to the OTF-1/NFE-1–DNA complex neither inhibited nor influenced the peptide binding.

In order to investigate whether binding inhibition in principle requires the previous presence of distamycin, we added the antibiotic to the preformed *Antp* HD–DNA complex. With increasing concentrations of distamycin the intensity of the DNA–protein band was clearly lowered; this effect becomes observable at 3 μM distamycin and at 12 μM, no more bound DNA can be detected (Figure 2). Simultaneous incubation of protein and distamycin with DNA also resulted in a binding inhibition of the *Antp* HD–DNA complex at a distamycin concentration of 2 μM (data not shown).

Close inspection of the DNA sequence of BS2-18 wt reveals that in addition to the 5′-ATTA-3′ motif there is a second distamycin binding site d-AAAAA. Mutating this site (BS2-18c to BS2-18e; see Materials and methods) had no measurable effect on distamycin inhibition of the *Antp* HD–DNA complex (Figures 2 and 3). This suggests that the additional binding site for distamycin does not influence the inhibition of the *Antp* HD–DNA complex and that the inhibitory effect of distamycin is exerted by its binding to the ATTA motif.

Netropsin is a close relative of distamycin containing two methylpyrrolecarboxamide rings and a guanidinium group in place of the third methylpyrrole moiety of distamycin (Zimmer, 1975) which exhibits very similar specificities for AT-rich regions of double helical DNA to distamycin (Van Dyke *et al.*, 1982; Lane *et al.*, 1983). In order to check whether distamycin and netropsin inhibit the specific *Antp* HD–DNA binding equally well we performed the same competition experiments with netropsin. Preincubation of netropsin with DNA prevented protein binding at a concentration of 5 μM. Similar effects are obtained by a simultaneous incubation of netropsin and protein with DNA as well as preincubation with *Antp* HD peptide (data not shown).

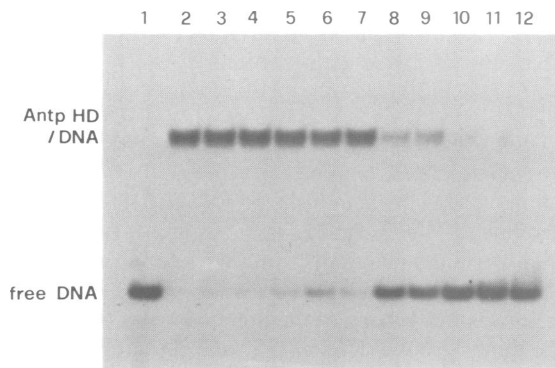

Fig. 2. Effect of distamycin on the *Antp* HD–DNA complex. The BS2-18 wt oligonucleotide was preincubated with *Antp* HD (~80 nM) for 20 min, then increasing concentrations of distamycin were added; the distamycin concentrations in lane 3–12 were 0.24, 0.48, 0.75, 1.2, 2.4, 4.8, 7.5, 12, 30 and 60 μM, respectively. Lane 1 and 2 are controls containing BS2-18 wt only and the *Antp* HD–DNA complex, respectively.

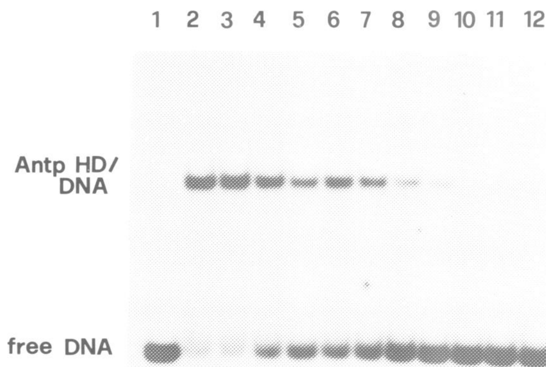

Fig. 3. Effect of distamycin on *Antp* HD binding using the mutated oligonucleotides BS2-18c–BS2-18d (see Materials and methods). As an example the results with BS2-18d are shown. In this oligonucleotide a second distamycin binding site d-AAAAA (BS2-18 wt) is mutated to d-AGGAA. The DNA–*Antp* HD complex was incubated with increasing concentrations of distamycin as described in Figure 2.

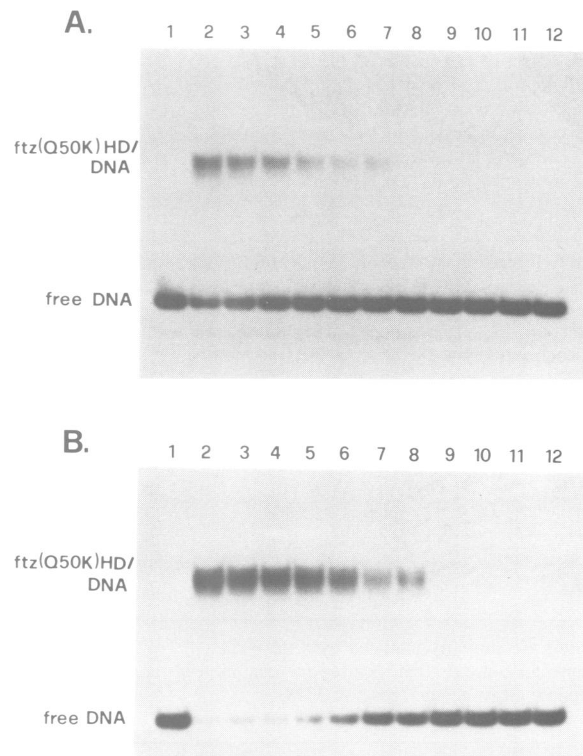

Fig. 4. (**A**) Effect of distamycin on *ftz* (Q50K) HD binding to BS2-18 wt oligonucleotide. Preincubation of BS2-18 wt with *ftz* (Q50K) HD (~100 nM) and subsequent treatment with increasing concentrations with distamycin were as described in Figure 2. (**B**) Effect of distamycin on *ftz* (Q50K) HD binding to BS2-18 mut oligonucleotide. The DNA–*ftz* (Q50K) HD complex was incubated with increasing concentration of distamycin as described in Figure 2.

Competition experiments with the ftz (Q50K) HD

In order to investigate whether the concentration of distamycin required for the inhibition of the HD–DNA complexes depends on the binding affinity of the HD–DNA complex, we employed mutant *fushi tarazu* homeodomain (*ftz* HD) peptides that were previously found to bind with reduced affinity to the ATTA site in BS2-18 wt (Percival-Smith *et al.*, 1990). The DNA binding properties of the *ftz* HD and those of the *Antp* HD have been analysed in detail and found to be very similar (Percival-Smith *et al.*, 1990).

The *fushi tarazu* (Q50K) homeodomain [*ftz* (Q50K) HD] has an identical amino acid sequence to the *ftz* HD, except that the glutamine at position 50 (position 9 of helix III) of the homeodomain has been changed to lysine (Percival-Smith *et al.*, 1990), as found in the *bicoid* homeodomain at position 50 (Berleth *et al.*, 1988). It has been demonstrated that position 50 of the homeodomain is an important determinant of DNA specificity (Hanes and Brent, 1989; Treisman *et al.*, 1989). The binding affinities of *Antp* HD and *ftz* (Q50K) HD to different double-stranded oligonucleotides follow an almost reciprocal order: for *Antp* HD they are BS2-18 wt > BS2-18 b >> BS2-18 mut; for *ftz* (Q50K) HD they are BS2-18 mut >> BS2-18 b = BS2-18 wt (see Materials and methods). Results from competition experiments with distamycin and *ftz* (Q50K) HD binding to its high affinity site (BS2-18 mut; Figure 4b) are not different from the data obtained for the same assays with *Antp* HD binding to its high affinity site (BS2-18 wt) (Figure 2). However, lower concentrations of distamycin are required for *ftz* (Q50K) HD binding to the low affinity binding site BS2-18 wt (Figure 4a) if compared with *Antp* HD binding to the same site (Figure 2). This suggests that in this particular case the inhibitory effect caused by distamycin depends on the protein's affinity for its binding site. In line with the *Antp* HD–DNA complexes, distamycin is able to compete for the DNA binding site of *ftz* (Q50K) HD even after preincubation of DNA with protein.

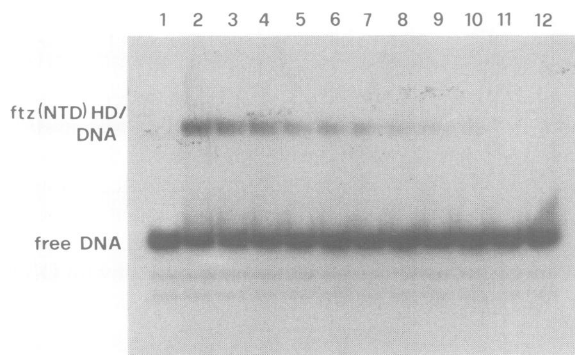

Fig. 5. Effect of distamycin on the *ftz* (NTD) HD–DNA complex. BS2-18 wt was incubated with *ftz* (NTD) HD (~1 μM) for 20 min; then increasing concentrations of distamycin were added. The concentrations in lanes 3 to 12 were 0.24, 0.48, 0.75, 1.2, 2.4, 4.8, 7.5, 12, 30 and 60 μM, respectively. Lane 1 contains BS2-18 wt only, lane 2 shows the *ftz* (NTD) HD–DNA complex.

Table I. Protection of recognition sites of various restriction endonucleases of pBR322 by distamycin A

Restriction endonucleases	Recognition site	Distamycin A (μM)		Cleavage sites on pBR322
		Partial inhibition	Complete inhibition	
*Dra*I	5'-TTTAAA-3'	3[a]	6	3
*Eco*RI	5'-GAATTC-3'	3	6	1
*Eco*RV	5'-GATATC-3'	40	300	1
*Avi*II	5'-TGCGCA-3'	13[b]	500	4
*Nru*I	5'-TCGCGA-3'	300	700	1
*Sal*I	5'-GTCGAC-3'	300	>1300	1
*Ecl*XI	5'-CGGCCG-3'	700	1300	1

[a]All sites are similarly inhibited by distamycin.
[b]Distamycin concentration required for inhibition of the most susceptible cleavage site (position 1358).

Competition of an N-terminal-deleted ftz HD with distamycin for DNA binding

The amino acid sequence of the *ftz* (NTD) HD is identical to that of the *ftz* HD except that amino acids 1–6 are deleted (NTD, N-terminal deletion) fusing the starting methionine to position 7 of the *ftz* HD (Percival-Smith *et al.*, 1990). This removes the portion of the homeodomain that reaches into the minor groove (Kissinger *et al.*, 1990; Otting *et al.*, 1990; Percival-Smith *et al.*, 1990).

In order to investigate if the competition of distamycin with the homeodomain depends on its direct interference with the peptide through the amino acids of its N-terminal arm reaching into the minor groove, we employed the *ftz* (NTD) HD to examine the inhibition of protein–DNA interaction by distamycin. Electrophoretic mobility shift assay shows that the affinity of the *ftz* (NTD) HD to the BS2-18 wt oligonucleotide is reduced—at least by an order of magnitude—compared with that of *Antp* HD and *ftz* HD (Percival-Smith *et al.*, 1990). Thus the minor groove contacts made by the peptide's N-terminal arm seem to be important for stable complex formation. Nevertheless, incubation of the *ftz* (NTD) HD–DNA complex with increasing concentrations of distamycin abolished the DNA–peptide band at a concentration of 12 μM as shown in an electrophoretic mobility shift assay (Figure 5). The results obtained with *ftz* (NTD) HD are similar to those observed with *Antp* HD peptide. This also applies for simultaneous incubation of

Table II. Flanking sequences of the recognition sites of *Avi*II and *Nru*I

Restriction enzyme	pBR322 sequence	Position of cleavage sites
*Nru*I	5'-GTCTTGCTGGCGTTCGCGACGCGAGGCTGG-3'	974
*Avi*II	5'-GATGCAATTTCTATGCGCACCCGTTCTCGG-3'	262
	5'-GGAGAACTGTGAATGCGCAAACCAACCCTT-3'	1358
	5'-TCCTGGCCACGGGTGCGCATGATCGTGCTC-3'	1456
	5'-CCAGTTAATAGTTTGCGCAACGTTGTTGCC-3'	3590

distamycin with *ftz* (NTD) HD and DNA as well as for preincubation of distamycin with oligonucleotides. Therefore distamycin's interference is not only based on a possible direct interference with the N-terminal arm.

Sequence-selective inhibition of restriction endonucleases

The results presented so far suggest that the inhibition of the formation of HD–DNA complexes may arise through the presence of the common DNA sequence motif ATTA, recognized by distamycin and HD. To test the dependence of this motif on distamycin inhibition, the AT stretch would have to be disrupted by a GC base pair. However, such mutations also destroy the HD binding site, thus making competition studies impossible (data not shown). A suitable system for studying the distamycin binding site dependence on the biological activity of proteins is provided by restriction endonucleases. We used different restriction endonucleases which cleave the DNA at AT- or GC-rich sites (Table I). Type II resriction endonucleases recognize short nucleotide squences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequences. The recognition sequences are generally, but not always, four to six nucleotides in length and are usually characterized by a dyad symmetry.

Distamycin's high sequence selectivity for AT can be observed in the selective protection of the cleavage sites of different restriction enzymes which have recognition sites of variable AT content. The results of our studies are summarized in Table I, indicating the concentrations of distamycin producing partial and complete inhibition of digestion by seven different restriction endonucleases. The extent of inhibition by the different restriction enzymes with distamycin is in the order *Dra*I = *Eco*RI > *Eco*RV > *Avi*II > *Nru*I > *Sal*I = *Ecl*XI. This order reflects fairly well the relative AT content of the recognition sequences for these restriction enzymes. Interestingly, there is a slight difference in distamycin concentrations necessary to inhibit the activity of *Avi*II and *Nru*I, which have the same relative AT content but differ only in the flanking sequences of their recognition sites (see Discussion and Table II).

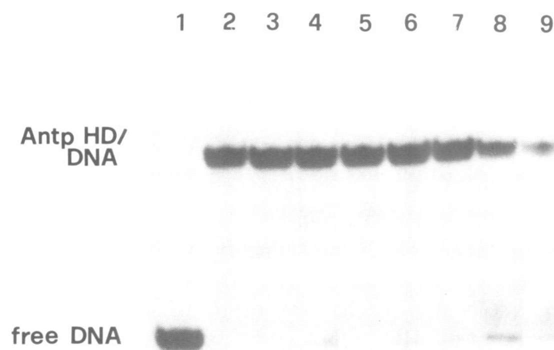

Fig. 6. Effect of berenil on *Antp* HD binding to BS2-18 wt oligonucleotide; the DNA was preincubated with increasing concentrations of berenil; 1.7 (lane 3), 3 (lane 4), 7 (lane 5), 70 (lane 6), 130 (lane 7), 330 (lane 8), 660 μM (lane 9) for 20 min before addition of *Antp* HD (~80 nM). Lane 1 contains BS2-18 wt oligonucleotide only, lane 2 is *Antp* HD and BS2-18 wt without berenil.

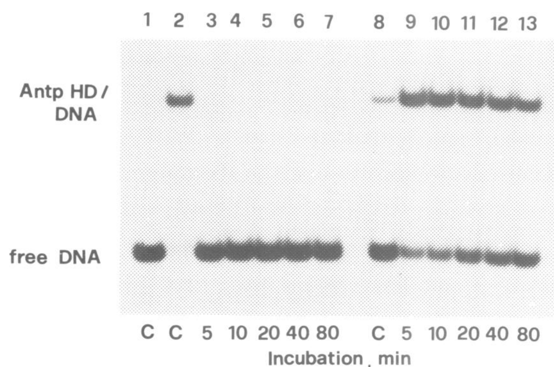

Fig. 7. Kinetic studies on *Antp* HD–DNA interaction. *Antp* HD and labelled BS2-18 wt were incubated for 20 min at room temperature. Then 30 μM of distamycin (lane 3–7) or a 6000-fold excess (50 nM) of unlabelled BS2-18 wt (over labelled BS2-18 wt) (lane 9–13) was added and incubation proceeded for the indicated time period. Controls (C) are free BS2-18 wt (lane 1), *Antp* HD–BS2-18 wt complex (lane 2) and labelled BS2-18 wt oligonucleotides mixed with 6000-fold excess of unlabelled BS2-18 wt before adding of *Antp* HD peptide (lane 8).

Discussion

The possibility of selective protein release from its DNA recognition site upon incubation with DNA ligands has been demonstrated for different types of antiviral and antitumour drugs. Specific protein release could be achieved with DNA intercalating agents (Schröter *et al.*, 1985), covalently binding drugs (Ushay *et al.*, 1981) and groove binding ligands (Broggini *et al.*, 1989; Woynarowski *et al.*, 1989a; Käs *et al.*, 1989; Reeves and Nissen, 1990; Mortensen *et al.*, 1990; Selby and Sancar, 1991). However, DNA–antibiotic complexes were competitively inhibited by addition of increasing amounts of proteins or peptides (Broggini *et al.*, 1989; Reeves and Nissen 1990; Mortensen *et al.*, 1990) whereas it was not possible to dissociate a preformed DNA–protein complex by an antibiotic (e.g. Broggini *et al.*, 1989). Our study demonstrates that distamycin interferes with DNA binding of certain HD peptides *in vitro*. The inhibition of binding of HD peptides is also observed upon addition of distamycin to preformed HD–DNA complexes, suggesting that the low molecular weight antibiotic binding in the minor groove can effectively displace peptides bound in the major groove of DNA.

The competition between the two DNA ligands—HD peptide and distamycin—seems not to be the result of distamycin inhibition of minor groove contacts by Arg3 and Arg5 in the N-terminal arm of the HD (Kissinger *et al.*, 1990; Otting *et al.*, 1990). This conclusion is based on the finding that the extent of inhibition of the protein–DNA interaction by distamycin is similar in the presence or absence of the N-terminal amino acids, although the binding affinity of the homeodomain peptides clearly increases in the presence of minor groove contacts made by the N-terminal arm (Percival-Smith *et al.*, 1990). As a possible explanation for the observed competition we assume that the binding of distamycin in the minor groove of the d(ATTA)·d(TAAT) motif of the homeodomain recognition site induces a change of the local DNA conformation which diminishes binding affinity of the peptide for the major groove. This implies

that the specificity of binding to DNA by these homeodomains is affected not only by the sequence of their DNA recognition site but also by the local DNA conformation at that site (see also McCarthy *et al.*, 1990 and references therein).

This assumption is further substantiated by our experimental finding that the minor groove ligand, berenil, does not compete with *Antp* HD for DNA binding up to an antibiotic concentration of 660 μM (Figure 6); it is known from an X-ray crystallographic study that the structure of a dodecanucleotide duplex is hardly disturbed upon binding of berenil (Brown *et al.*, 1990). We have further found that intercalating molecules (e.g. derivatives of 9-amino acridine) which are known to perturb the conformation of the DNA, also compete with *Antp* HD binding (unpublished results).

The hypothesis that the distamycin-induced change of the local DNA conformation is responsible for the competition with the homeodomains for their recognition site is supported by similar findings of other groups. It has been shown for calf thymus DNA that the binding of distamycin induces a cooperative transition to a new DNA form with altered structural properties and this allosteric effect can be transmitted over ~100 bp (Hogan *et al.*, 1979). That the distortions introduced by distamycin might propagate along the DNA helix was also shown in a study by Bruzik *et al.* (1987). They demonstrated that distamycin can induce a specific activation of transcriptional initiation by binding to non-alternating AT sequences in the spacer DNA (P_{RM} promoter of phage λ) separating the −10 and −35 regions which are contacted by the *Escherichia coli* RNA polymerase, indicating that the polymerase and distamycin binding sites are distinct. A subsequent study (Martello *et al.*, 1989), using mutated spacer DNA and distamycin and derivatives thereof, led to the hypothesis that the different ligand-induced structural changes in the non-alternating AT sequences of the spacer DNA are responsible for the observed differential stimulation. Another study showed that distamycin stimulates the copying of oligo(dA)·poly(dT) by DNA polymerase through increasing the rate of initiation of oligo(dA) primers by drug induced stabilization of the oligo (dA)·poly(dT) complexes (Levy *et al.*, 1989). In all these studies, the evidence for distamycin-induced changes of local DNA structure follows indirectly from analysis of a variety of experiments. However, direct evidence for the existence of conformational changes of DNA induced by distamycin has been obtained by two-dimensional ^{1}H-NMR analysis (Klevit *et al.*, 1986; Pelton and Wemmer, 1989, 1990) and by X-ray crystallography (Coll *et al.*, 1987) of distamycin−oligonucleotide complexes. With the excess of distamycin used in our competition studies, it is safe to assume that distamycin also binds within the A-tract d-AAAAA of BS2-18 wt. This DNA motif can introduce bends into DNA and in particular can change the conformations of the nucleotides at the junctions between the A-tract and the flanking DNA sequences (Celda *et al.*, 1989 and references therein). But the distamycin-induced variation of the conformation within this motif, as observed in longer A-tract containing DNA fragments (Wu and Crothers, 1984; Martello *et al.*, 1989; Barcelo *et al.*, 1991), does not change the binding parameters of the homeodomains, as must be concluded from the finding that the introduction of GC base pairs within this AT stretch (yielding in the mutants BS2-18 c to BS2-18 e) does not show an effect on the competition

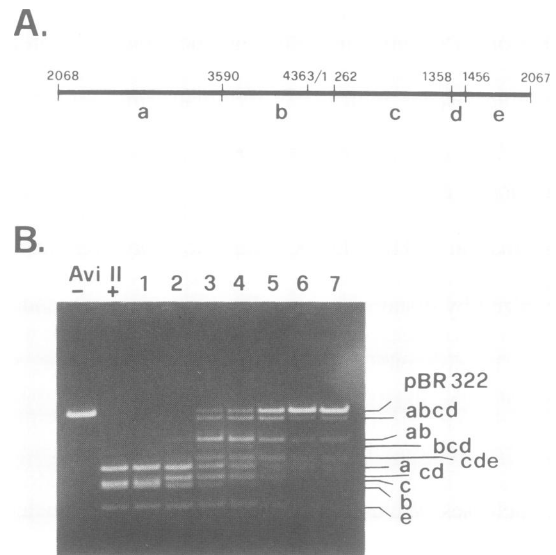

Fig. 8. Effect of distamycin in *Avi*II activity. (**A**) pBR322 linearized with *Pvu*II (position 2068). The four *Avi*II cleavage sites and position 4363/1 are indicated. The DNA fragments are designated by letters a-e. (**B**) *Pvu*II-linearized pBR322 without (−) and with (+) *Avi*II. Lane 1−7 are *Avi*II digestions of pBR322 fragments obtained in the presence of distamycin. The distamycin concentrations in lanes 1−7 were 6, 13, 30, 60, 80, 100 and 120 μM, respectively.

involving distamycin, homeodomains and these mutant DNA duplexes.

The molecular mechanism of the distamycin-induced inhibition of the HD−DNA complexes was evaluated by a mobility shift assay in which the preincubated *Antp* HD−BS2-18 wt complex was incubated either with distamycin or an excess of unlabelled BS2-18 wt DNA for various times (Figure 6). From these experiments we conclude that the lifetime of the *Antp* HD−BS2-18 wt complex in the presence of 30 μM distamycin is less than 5 min (Figure 7). This is in contrast to the long lifetimes of 80−100 min in the presence of 50 nM (in duplex) unlabelled BS2-18 wt DNA which agrees well with the value of 90 min determined previously (Affolter *et al.*, 1990a). Based on the results of these measurements and: (i) the experimentally determined lifetimes of distamycin of ~0.1−2 s in various AT-containing binding sites (Feigon *et al.*, 1984; Pelton and Wemmer, 1989, 1990); and (ii) the ability of distamycin to induce conformational changes of the DNA upon binding, we propose the following mechanism. Distamycin binds in the minor groove of the DNA adjacent to *Antp* HD thus forming a ternary complex and simultaneously changing the local conformation of the DNA. This conformational change drastically diminishes the peptide's affinity constant for the ATTA site, concomitant with a decrease of the residence time on the ATTA site for *Antp* HD in the ternary complex and an increase of the peptide's dissociation rate constant, which leads to the release of *Antp* HD from the ternary complex. Thus, the ability of distamycin to form a ternary complex and to induce conformational changes of the DNA upon binding is probably responsible for its inhibitory effect. This mechanism also suggests that the comparison of dissociation rates of low molecular weight ligands and peptides from binary DNA−ligand complexes may lead to incorrect assessments of an

antibiotic's ability to inhibit protein–DNA complex formation.

The large differences between the distamycin concentrations needed for partial and complete inhibition of *Dra*I and *Eco*RI on one side and *Eco*RV on the other side, can be correlated with the high selectivity for non-alternating AT sites of the type dATTT compared with alternating AT sites dATAT, as derived from calorimetric (Breslauer *et al.*, 1987), footprinting (Schultz and Dervan, 1984; Fish *et al.*, 1988) and X-ray crystallographic studies (Coll *et al.*, 1987). It is known that rates of enzymatic cleavage of DNA can vary widely (Nosikov *et al.*, 1976; Kuroyedov *et al.*, 1977; Malcolm and Moffatt, 1981), e.g. the difference between the most and the least susceptible *Eco*RI sites in bacteriophage λ DNA is ~10-fold (Thomas and Davis, 1975). Similarly, the *Eco*RI sites in adenovirus DNA are hydrolysed at different rates (Forsblom *et al.*, 1976). It is assumed that such variations in restriction digestion are a reflection of the sequences flanking the recognition site. Distamycin also does not reduce the rate of restriction digestion equally at each site, which is probably also a consequence of varying DNA sequences around the recognition site (Tables I and II). The restriction enzymes *Nru*I and *Avi*II were chosen since they recognize similar cleavage sites (5'-TCGCGA-3' and 5'-TGCGCA-3' respectively, see Table I) but differ in the base sequences adjacent to their recognition sites (Table II). Digestion of pBR322 with these two restriction enzymes showed that distamycin inhibits the endonucleolytic cleavage at different concentrations (Table I), sugesting that the bases flanking the recognition sites are important for inhibition. This can also be demonstrated for the four *Avi*II cleavage sites (Table II) for each of which the enzyme activity is inhibited to a different extent (Figure 8). A careful titration with distamycin shows that the inhibition of *Avi*II digestion takes place first at position 1358 (Table II, Figure 8 and data not shown) which might be because of the AAT and AAA sequences flanking the enzyme's recognition site. The other cleavage sites are poorly protected by distamycin. This is in agreement with the findings of Nilsson *et al.* (1982) who showed that the most protected cleavage sites of *Mbo*I have distamycin binding sites within 2 bp of, or immediately adjacent to, the enzyme's recognition sequence, whereas less protected sites have distamycin binding sequences 4–7 bp away. One can speculate that the slight difference in distamycin concentrations necessary to inhibit the enzyme activity of *Nru*I and *Avi*II results from differences of their flanking sequences and that the inhibition of the enzyme-catalysed cleavage reaction is caused by an alteration of the conformation at and around the recognition site by distamycin.

Although these experiments demonstrate the occurrence of distamycin-induced structural changes of DNA, a direct link between these effects and the antibiotic's influence on biological processes has not been found yet. However, it has been shown that distamycin can act as a factor in the regulation of the activity to topoisomerase I (McHugh *et al.*, 1989) and topoisomerase II (Woyonarowski *et al.*, 1989a,b). Similar behaviour has also been demonstrated recently for the minor groove-binding ligand Hoechst 33258 (Finlay and Baguley, 1990). Whether distamycin interferes with the control of gene expression via HD proteins upon administration of the antibiotic *in vivo* can now be tested in other assay systems.

Materials and methods

Distamycin

Distamycin A was purchased from Sigma. 20 mM stock solutions of distamycin were dissolved in 50% dimethyl sulphoxide (DMSO). Further dilutions were with distilled water.

Oligonucleotides

Purified single-stranded oligodeoxynucleotides (MedProbe) were radioactively labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase at the 5' termini, annealed and purified on a 15% polyacrylamide gel as described (Dorn *et al.*, 1987). The sequences of the radiolabelled double-stranded oligonucleotides used are (5'–3'): BS2-18 wt, GAGAAAAAGCCATTAGAG; BS2-18 mut, GAGAAAAAGGGATTAGAG; BS2-18 b, GAGAAAAAGCCCTTAGAG; BS2-18 c, GAGAAGAAGCCATTAGAG; BS2-18 d, GAGAGGAAGCCATTAGAG; and BS2-18 e, GAGAGAAAGCCATTAGAG.

Mobility shift assay

The mobility shift assay was performed in a total volume of 18 µl and consisted of ^{32}P-labelled DNA (800–1000 c.p.m.), purified peptides [*Antp* HD; *ftz* (NTD) HD; *ftz*Q50K; final concentrations are ~80–100 nM for *Antp* HD and *ftz*Q50K and 800–1000 nM for *ftz* (NTD) HD], in an HD buffer containing 20 mM Tris–HCl, pH 7.6; 75 mM KCl; 1 mM dithiothreitol; 10% glycerol; the mixture was incubated for 20 min at room temperature; then 1 µl of the appropriate aqueous distamycin solutions (final concentrations 0–60 µM) was added, incubated for another 20 min at room temperature and loaded onto a 10% polyacrylamide gel (acrylamide: bisacrylamide ration of 30:1) in 1 × TE buffer, pH 7.4. The gel was electrophoresed for 2.5 h at 35 mA per gel at room temperature, soaked for 15 min in 5% glycerol, then dried and autoradiographed.

The same assay conditions were used or simultaneous incubation of *Antp* HD and distamycin or preincubation of distamycin with DNA.

Expression and purification of peptides

All peptides were expressed in *E.coli* BL21 (De3) lysogen and purified as described in Müller *et al.* (1988) and Percival-Smith *et al.* (1990).

The *Antp* HD peptide contains a serine instead of a cysteine at residue 39 in order to prevent oxidative dimerization of the protein (Müller *et al.*, 1988). In the *fushi tarazu* Q50K homeodomain [*ftz* (Q50K) HD] the glutamine 50 at the ninth position of the third helix is exchanged for lysine (Percival-Smith *et al.*, 1990). In the *ftz* (NTD) HD the first six amino acids of the homeodomain are deleted (Percival-Smith *et al.*, 1990).

Restriction endonuclease inhibition assay

pBR322 (100 ng), linearized with *Pvu*II, was preincubated for 10 min at 37°C with distamycin in the respective incubation buffers of the restriction enzyme nucleases used (Boehringer, Mannheim). Then the enzyme (*Eco*RI: *Eco*RV; *Dra*I; *Sal*I; *Nru*I;*Avi*II) was added (3–7 U) to the samples and incubated for 30 min at 37°C. The incubation with the restriction enzyme *Ecl*XI was carried out over night at 37°C. After electrophoresis [0.7% agarose in 1 × TAE (40 mM Tris–acetate; 33 mM sodium acetate; 1 mM EDTA)] the DNA fragments were visualized by staining with ethidium bromide followed by UV exposure.

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