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A MODEL FOR THE SEQUENCE-DEPENDENT DNA BINDING OF 4',6-DIAMIDINO-2-PHENYLINDOLE (DAPI)

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Summary: A study on the sequence dependent DNA binding mode of DAPI has been carried out on pUC8 and the βgal promoter region by restriction endonuclease and DNAase I protection experiments. A molecular model depicting drug interaction at the level of selected palyndromes has also been constructed that confirms the A-T sequence specificity of the compound. Experimental data indicate that the binding sites for RNA polymerase and cyclic AMP receptor protein (CRP) in the βgal gene are privileged locales for DAPI interaction, a feature that explains impairment of transcription at this level. From a stereochemical view point, DAPI binding to DNA minor groove, while being incompatible with promoter unwinding in the open complex, may also disturb optimal contacts with proteins regulating RNA polymerase activity.

The mechanism of action of structurally different molecules having DNA as a target has important implications for a better understanding of the processes of DNA recognition and of gene regulation. In this regard, a number of DNA-interacting agents, characterized for high DNA-affinity, including classical intercalating agents such as ethidium bromide and acridine, and externally binding compounds such as distamycin, netropsin and 4',6-diamidino-2-phenylindole (DAPI) (7, 14, 15, 22, 23), have been studied for their ability to influence DNA structure and template function. While being investigated for their mode of binding to the nucleic acid, these molecules have been also studied to determine whether they influence the regulation of gene expression as a result of an interference with the binding of regulatory proteins to their consensus binding site (1, 2, 3, 5, 6, 12). Recently, we were able to demonstrate that the minor groove binder DAPI can interfere with the expression of pUC8 DNA when given to *Escherichia coli* competent cells in the form of plasmid adduct (16). Moreover, we have shown that DAPI can alter the catalytic function of a number of enzymes which are involved in DNA topology, replication, repair, and transcription (17). In particular, DAPI was capable of operating a preferential inhibition of RNA polymerase and, to a minor extent, of DNA ligase. Since the drug was found to be poorly effective in altering DNA

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structure, the above activity was related to a privileged binding to the DNA locale containing the enzyme recognition sequences. In the case of RNA polymerase, according to Straney and Crothers (20), the strong affinity of DAPI for A-T rich regions might have impaired the enzyme binding to the TAATA box during the reversible phase of transcription initiation. As for the inhibition of DNA ligase, a different explanation should be brought up which considers specific mechanism of minor groove occupancy by the DNA-ligand. In an attempt to gain further insight into the effects produced by DAPI-DNA interaction at the level of gene expression, we have utilized a defined genetic system such as pUC8. DNAase I and restriction endonuclease protection assays were performed on a region which contains the βgal (β-galactosidase) operator-promoter. A computer-based molecular model representing the complex between DAPI and a short stretch of DNA sequences from this region, is also presented.

MATERIALS AND METHODS

Restriction endonuclease protection assay

For this assay, 1 μg pUC8 was incubated with DAPI at molar ratio (P/D) = 10, for a period of 10-15 minutes at room temperature. The complex was then digested with 2U of the following enzymes: *EcoRI*, *BamHI*, *SalI*, *SmaI* and *DraI* at 37°C for 20 minutes up to 5 hours, using appropriate buffer conditions for optimal enzyme activity. The digested product was loaded into 1.2 % agarose gel and electrophoresys was performed at 5 volts/cm/hr in TBE buffer. Gels were stained in ethidium bromide and photographed under UV light (Polaroid ML land camera). Negatives underwent densitometric scanning to quantitate the extent of digestion.

DNAase I protection assay

The 203 bp *lac* promoter fragment was derived by *EcoRI* cleavage of HMW104 plasmid (kindly provided by Dr. D. Crothers) (26) and it is exactly the same as in pUC8. After alkaline phosphatase treatment, the fragment was 5'end-labelled with [^{32}P]-dATP using the T4 Polinucleotide kinase. DAPI/DNA complexes were allowed to form at different P/D values (1, 5, 20, 100) at room temperature for 20 minutes, and subsequently digested with 1U of DNAase I RNAse-free for 20 seconds at 25°C. The products of digestion were resolved on 8% polyacrylamide gels containing 7M urea. Gels were fixed in acetic acid, dried under vacuum at 80°C and exposed to autoradiography at -70°C with an intensifying screen.

Molecular modelling

Molecular modelling was performed on a PS330 Evans and Sutherland graphic system using the program FRODO (11). The starting atomic coordinates were taken from Larsen *et al.* (12) and the DNA sequence appropriately modified. The DNA and the DAPI molecules were considered as rigid groups and only small adjustements were applied manually, without energy minimization.

RESULTS

Restriction endonuclease protection assay

The ability of DAPI to interfere with the activity of several restriction endonucleases has been assayed. The examined enzymes (see table 1) recognized a stretch of sequences at the βgal promoter/operon boundary (26). DAPI/pUC8 complexes, which were allowed to form at phosphate to drug molar ratios (P/D) = 10, were digested for variable time periods following which an electrophoretic and densitometric analysis allowed to calculate the relative amount of undigested DNA. *DraI* did not cleave the substrate even after five hours of incubation, as reported in fig.1 and in

Table 1. Restriction endonuclease protection assay

Enzymes	Sequences	Digestion Time (hours)	
		2	5
BamH I	GGATCC CCTAGG	50	0
Sma I	GGGCCC CCCGGG	80	20
EcoR I	GAATTC CTTAAG	100	55
Dra I	TTTAAA AAATTT	100	100
Sal I	GTCGAC CAGCTG	50	10

Densitometric analysis following restriction endonuclease assay (P/D=10). The values reported represent the amount (%) of undigested DAPI/DNA complex with respect to the control.

table 1 (100% of the complex remaining undigested). *EcoRI* showed a similar pattern (table 1 and data not reported), since 50% of the DAPI/DNA complex still remained undigested even after 5 hours of incubation. *SmaI*, *BamHI* and *SalI*, on the other hand, exhibited a reduced catalytic activity, but were able to digest the substrate nearly to completion over a period of incubation longer than two to three hours (table 1 and data not shown).

DNAase I protection assay

DAPI-DNA complexes were initially allowed to form at different P/D values and then characterized by DNAase I footprinting. As reported in fig.2, DAPI was capable to protect selected segments of the βgal promoter/operon from DNAase I digestion. In particular, clear-cut footprints appeared at the level of sequences that are involved in specific interactions with RNA polymerase, namely position -14-33, which includes the TAATA box. This phoenomenon was observed up to a P/D value of 5. At higher P/D, no protection was noticeable. A minor degree of protection was observed for region -49-59, that includes the CRP (cyclic AMP receptor protein) binding site and for regions +23+34 and +18+7, encompassing part of the operator and the mRNA initiation site.

Molecular modelling

All the models proposed to explain the mechanistics aspects of DAPI-DNA interaction must refer to the crystal structure of DAPI bound to DNA (12). In the model shown in fig. 3A, it appears clearly that the charged aminic nitrogen of DAPI (from both sides) is quite close (about 3A°) to the N1 of G and that some repulsion between positively charged groups must be operating. The same obviously applies for GGGCCC and GTCGAC sequences (*SmaI* and *SalI*, respectively). The model of the complex between DAPI and *EcoRI* sequence (GAATTC, fig. 3B) is adapted from that reported in the literature. It can be seen that DAPI is in contact with three base pairs, since the electropositive -NH2 group of G is at about 7 A° from the aminic nitrogen of DAPI. A better accomodation of the ligand in the nucleic acid pocket is apparent in this case. In the *DraI* model (AAATTT, fig. 3C) the absence of the guanine -NH2 groups provides, even more strongly, a steric advantage.

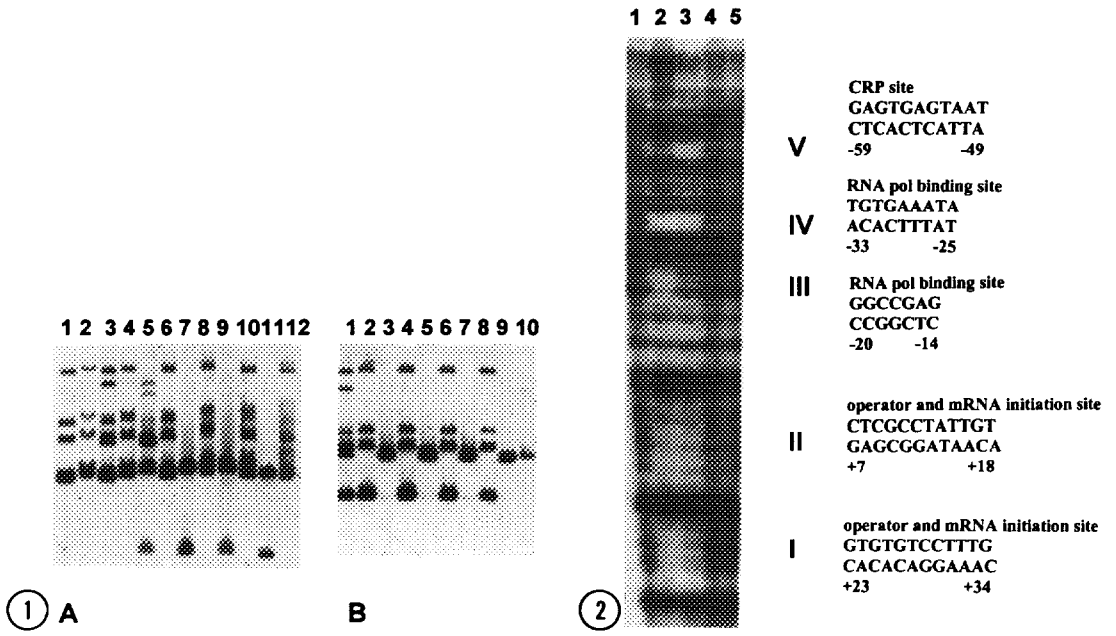

Fig.1. Electrophoretic profiles of pUC8 (1 μg) after digestion at 37°C with *DraI* (panel A) and *SmaI* (panel B) endonucleases. DNA/DAPI complexes (at P/D = 10) were allowed to form at room temperature before digestion. Panel A : incubations with *DraI* were carried on for 40 (3,4); 60 (5,6); 90 (7,8); 210 (9,10) and 300 (11,12) minutes both in the case of DNA/DAPI complexes (4,6,8,10,12) and of controls (3,5,7,9,11). Lanes 1 and 2 represent pUC8 and pUC8/DAPI complex, respectively. Panel B : incubations with *SmaI* were carried on for 30 (1,2); 60 (3,4); 90 (5,6); 120 (7,8) and 300 (9,10) minutes both in the case of DNA/DAPI complexes (2,4,6,8,10) and of controls (1,3,5,7,9).

Fig.2. Left panel: DNAase I footprinting of the 203 bp *lac* promoter fragment was performed in the absence (lane 1) and in the presence of DAPI (lanes 2 to 5). DAPI/DNA complexes were allowed to form at different P/D values: 1, 5, 20 and 100 (lanes 2, 3, 4, and 5, respectively). The DNAase I-protected regions are schematically drawn in the right panel and are represented in bold. Protected regions were defined by G-A Maxam and Gilbert sequencing of the *lac* promoter fragment.

DISCUSSION

In the present paper, working with a defined genetic system (represented by pUC8 and its βgal promoter region) we have tried to dissect the molecular basis of the specific interaction between DAPI and DNA, as an attempt to explain some of the drug effects at the level of the nucleic acid. A restriction endonuclease protection analysis performed with pUC8, has produced clear-cut evidence that DAPI strongly prevented cutting by *DraI* and *EcoRI*, which recognize AAATTT and GAATTC sequences respectively. A minor degree of protection was instead found in the case of *BamHI*, *SalI* and *SmaI* sequences (GGATCC, GTCGAC and GGGCCC, respectively) that, like the *EcoRI* site, are located at the boundary between the βgal promoter and structural gene and are present only in one copy in the plasmid. The above effect seems to be directly related to the A-T contents of the palyndrome as expected for the known ability of DAPI to bind selectively to the DNA minor groove, preferentially at three or more consecutive A-T base pairs (8, 14, 18, 21). In our *EcoRI* molecular model, DAPI is in contact with three successive AT base pairs, with the indole nitrogen of the dye making bifurcated hydrogen bonds to the O_2 atoms of the central Ts, as described by Larsen (12).

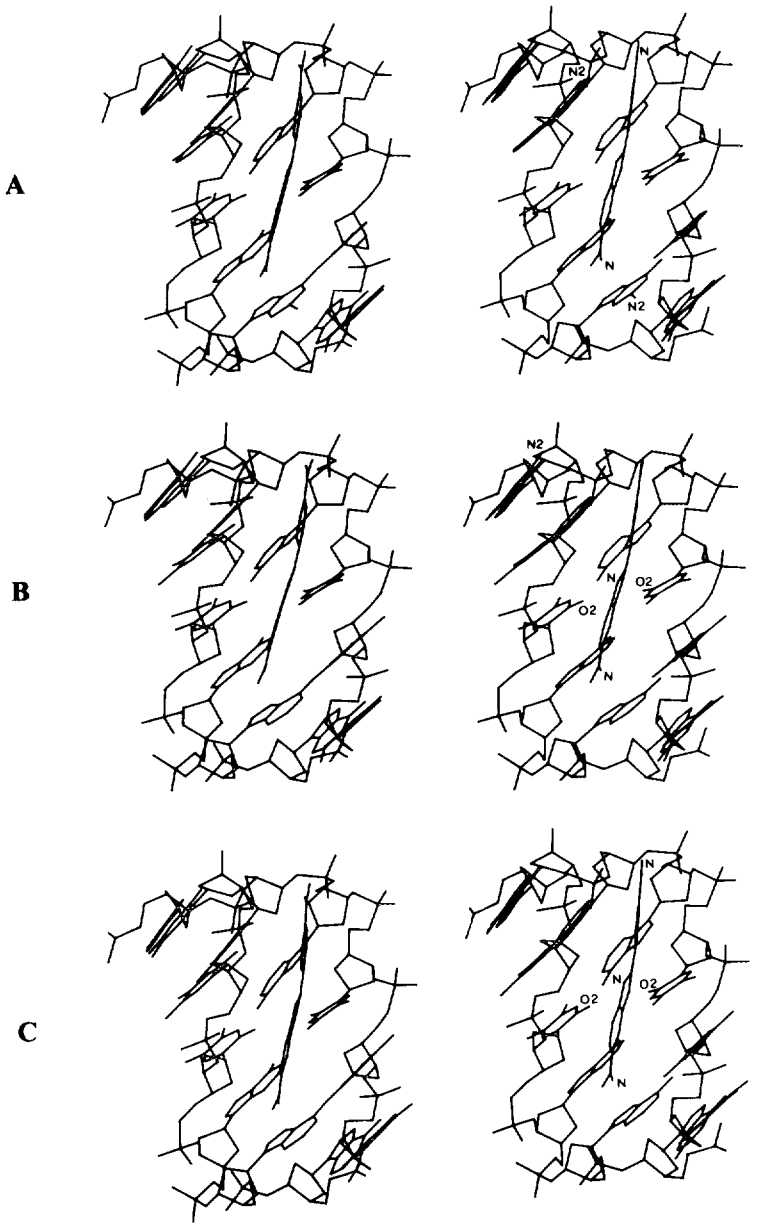

Fig. 3.Stereo drawing of the models of polynucleotides GGATCC (3A), GAATTC (3B) and AAATTT (3C) with the DAPI molecule bound. Some of the atoms possibly involved in specific interactions are labelled (see text for details).

Such a finding would fit with the experimental evidence indicating that three AT base pairs are the minimum requirement for specificity (10, 14, 19, 24, 25). However, ^{1}HNMR spectroscopy studies of $[d(GCGATCGC)]_2$ and its interaction with DAPI have very recently indicated that only two AT base pairs can promote a specific binding to the minor groove (21). Thus, to understand the weakness of the interaction between DAPI and the *BamHI, SmaI* and *SalI* sequences, some stereochemical constraints have to be taken into account that fit with our models. The intrinsically narrower minor

groove in the A-T regions with respect to the G-C regions and the steric advantage of the absence of the guanine -NH2 groups are certainly crucial for high binding affinity (12, 21). Since only three base pairs can be occupied by DAPI, it is quite plausible, in the case of the GAATTC sequence, that the electropositive -NH2 group of G lies at some distance from the aminic nitrogen of DAPI and does not interfere with the Van der Walls forces that keep the binder between the two sugar-phosphate walls of the minor groove. The fact that the AAATTT sequence (*DraI* palyndrome) is better protected by DAPI than the GAATTC sequence (*BamHI* palyndrome), may simply rest on the relative higher electronegative potential of the former palyndrome granting a relatively higher binding affinity. Since A-T sequences are notably part of the promoter consensus structure, template occupancy at this level could explain the effects on transcription and gene expression (16). In this regard, previous reports have indicated that DAPI was able to inhibit RNA transcription initiation by clumping DNA in the B form, that is incompatible with promoter unwinding in the open complex. (20). Moreover, Chiang et al. (4) have shown that DAPI and other minor groove binders can prevent complex formation between tha TATA box and TBP (TATA box binding protein) a general transcription factor required for proper initiation of gene expression by RNA polymerase II. Our DNAase I footprinting results are in line with these findings showing marked protection at the level of the RNA polymerase binding site, with a more prominent effect around the TATA box. However, protection applies also to other segments of the βgal promoter-operator such as region -49-59. By occupying this site, DAPI can affect the access of the CRP-cAMP complex to the promoter region. Protein-protein contacts that optimize RNA polymerase function will then be impaired and under these conditions, processivity of RNA polymerase would be reduced. Binding of DAPI at the operator-mRNA initiation site could further affect transcription through a different mechanism which includes inhibition of elongation. It has to be noticed that, under the conditions reported in the footprinting experiments, DAPI binds to both AT and GC sequences. Therefore, the prevalence of one effect over the others, may simply rest on the relative ratio of ligand to DNA that is adopted. All together these observations can explain the previously reported interference of DAPI with pUC8 expression (16) and the inhibition of RNA polymerase and DNA ligase *in vitro* (17). Although other mechanisms might have a complementary role *in vivo*, due to its characteristics and the above described properties, DAPI can be viewed as a model compound to study the activity of transcription factors in the context of DNA interaction.

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