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SELECTIVE INHIBITION OF RESTRICTION ENDONUCLEASE CLEAVAGE BY DNA INTERCALATORS

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The preferred dye binding sites and the microenvironment of known nucleotide sequences within mitochondrial and plasmid pBR322 DNA was probed in a gross fashion with restriction endonucleases. The intercalating dyes, ethidium bromide and propidium iodide, do not inhibit a given restriction endonuclease equally at all of the restriction sites within a DNA molecule. The selective inhibition may be explained, in part, by the potential B to Z conformation transition of DNA flanking the restriction site and by preferred dye binding sites. Propidium iodide was found to be a more potent inhibitor than ethidium bromide and the inhibition is independent of the type of cut made by the enzyme.

Studies dealing with the elucidation of specific binding sites of intercalating drugs have been conducted primarily with synthetic polynucleotides with the presumed inference that the observed results could be correlated to intact natural nucleic acid molecules. Pack and Loew (1) have demonstrated by model system analysis that the preferred intercalation site of EB (ethidium bromide) is dependent upon the energy of unwinding dictated by the base sequence and not upon the stacking energies between EB and the base pairs. The changes in the conformational energies of unwinding of the dinucleotide sequence comprising the intercalation site in ascending order are: T(3'-5')A; CG; CT; CA; AA; TC; AT; GGl AC; GC. The experimental results of Reinhardt and Krugh (2) and Bresloff and Crothers (3) support the findings that C(3'-5')G and T(3'-5')A were most likely to bind EB. It has also been proposed that after one molecule of EB intercalates between the stacked bases the sites immediately adjacent become excluded due to altered phosphate-phosphate bond lengths (4). Studies performed with fragmented crystalline nucleic acid drug complexes (5) most closely approximates the native state, however, this static model cannot account for actions of solvation and bond oscillations (6).

The relatively recent discovery of Z-DNA in DNA with an alternating CG sequence (7) and the enhanced transition of the B form to the Z form in the presence of high salt (8) or ethanol (9) and the reversed transition in the

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presence of EB (10) adds a new potential complexity to the mechanism of drug-DNA interactions. The Z-DNA conformation can also presumably form in regions of alternating purine-pyrimidine bases (11). The DNA region in the Z conformation presumably cannot undergo normal DNA-drug or DNA-enzyme interactions. The distortion of the helical DNA molecule outside of the Z conformation region is presumed to be minimal - not beyond five base pairs (12).

The preferred binding sites of intercalating drugs and perhaps other compounds that interact with DNA can be analyzed with native DNA in solution if one subjects the DNA-drug complex to various restriction endonucleases. These results can then be compared to those obtained with model systems described above. The endonucleases recognize a unique polynucleotide sequence within the DNA molecule and may act as micro-probes of drug-DNA interactions at these specific sites and the effect of flanking regions.

This report describes the inhibition of restriction endonucleases with some selectivity at particular restriction sites by the phenanthridine dyes EB and PI (propidium iodide) with plasmid pBR322 and hamster mt DNA. The selective inhibition could be explained, in part, by preferential dye binding sites and/or restricted interactions with potential Z-DNA conformation regions. Previous studies with drugs other than EB and PI have also shown partial inhibition of restriction enzymes (13-16).

METHODS

Hamster Mt-DNA Isolation: Mt-DNA was isolated essentially as previously described (17). The lyophilized DNA was suspended in a buffer of 100 mM Tris, 50 mM NaCl and 5 mM $MgCl_2$, pH 7.5 (restriction buffer) at a final concentration of 1 ug/20 ul and stored at -70°C.

Dye Additions: Ten ul of a dye solution was added to 20 ul of the mt-DNA or pBR322 DNA solution (1 ug) to yield the final dye concentrations employed in the various experiments. Samples were incubated for 5 minutes at room temperature followed by the addition of 2 volumes of absolute ethanol. The solution was kept overnight at -20°C and then centrifuged in a Phillips-Drucher table-top centrifuge at 2250 g for 10 minutes. The supernatant was decanted and the pellet lyophilized, dissolved in 20 ul restriction buffer and restricted for 1 hour at 37°C. The reaction was stopped by adding 5 ul of a solution of 5% SDS, 25% glycerol, 0.025% bromophenol blue and heating at 65°C for 10 minutes.

Electrophoresis: Gel electrophoresis was carried out on a 1% or 1.5% horizontal agarose slab gel with a 2.5 cm 0.5% agarose stacking gel, and run at 100 to 125 mAmps for 5 to 6 hours in a buffer of 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA and 1.5% glycerol (w/v), pH 7.3. After electrophoresis the gel was stained in a 2 ug EB/ml solution and photographed with Type 55 black/white Polaroid film through a red filter.

RESULTS

Studies employing the pBR322 plasmid DNA, with a known sequence (18), were conducted to probe the mechanism(s) of dye inhibition of the restriction enzymes. The phenanthridine dyes EB and PI were used at initial concentrations varied between 25 and 600 ug per ml. The methods employed

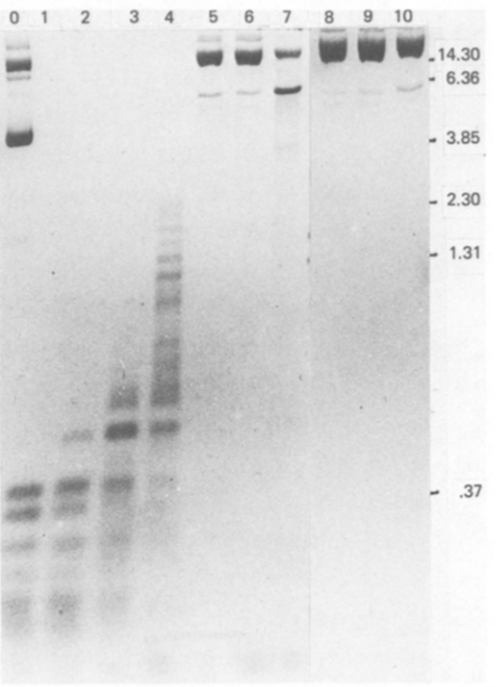

Figure 1. Digestion of plasmid pBR322 DNA with Hpa II (for 1 hour at 37°C) incubated with increasing concentrations of propidium iodide (PI). Lane 0 is unrestricted DNA; Lane 1 - 0; Lane 2 - 25; Lane 3 - 50; Lane 4 - 100; Lane 5 - 200; Lane 6 - 250; Lane 7 - 300; Lane 8 - 325; Lane 9 - 350; and Lane 10 - 400 ug/ml PI added initially. Molecular weight standards on right of figure are X 10^{-6}.

resulted in an unknown final concentration of dye bound. These methods were employed to obviate the question of the effect of large quantities of dye interacting directly with the restriction enzymes. Restriction enzymes were selected on the basis of which nucleotide sequences they recognized so as to allow the assessment of dye binding specificities on combinations of dinucleotide sequences.

Approximately 1 ug of DNA was used per gel lane and the DNA to enzyme ratio required to achieve complete restriction in the absence of the dye was maintained throughout the series of experiments. The restriction of pBR322 with Hpa II in the presence of increasing concentrations of PI (0 to 400 ug/ml) is shown in Figure 1. Inhibition was complete at a concentration of 200 ug/ml. Inhibition of Hpa II by EB showed the same inhibitory trends as with PI, however, PI clearly inhibited at lower dye concentrations (data not shown). The complete inhibition of Hpa II by both PI and EB appears to go through a partial release of inhibition at very high dye concentrations with a return to complete inhibition as more dye is added. Only one new band appears at 25 ug PI or 50-100 ug EB with a molecular weight of 5.5×10^5 derived by the selective inhibition of restriction sites at coordinates 2120

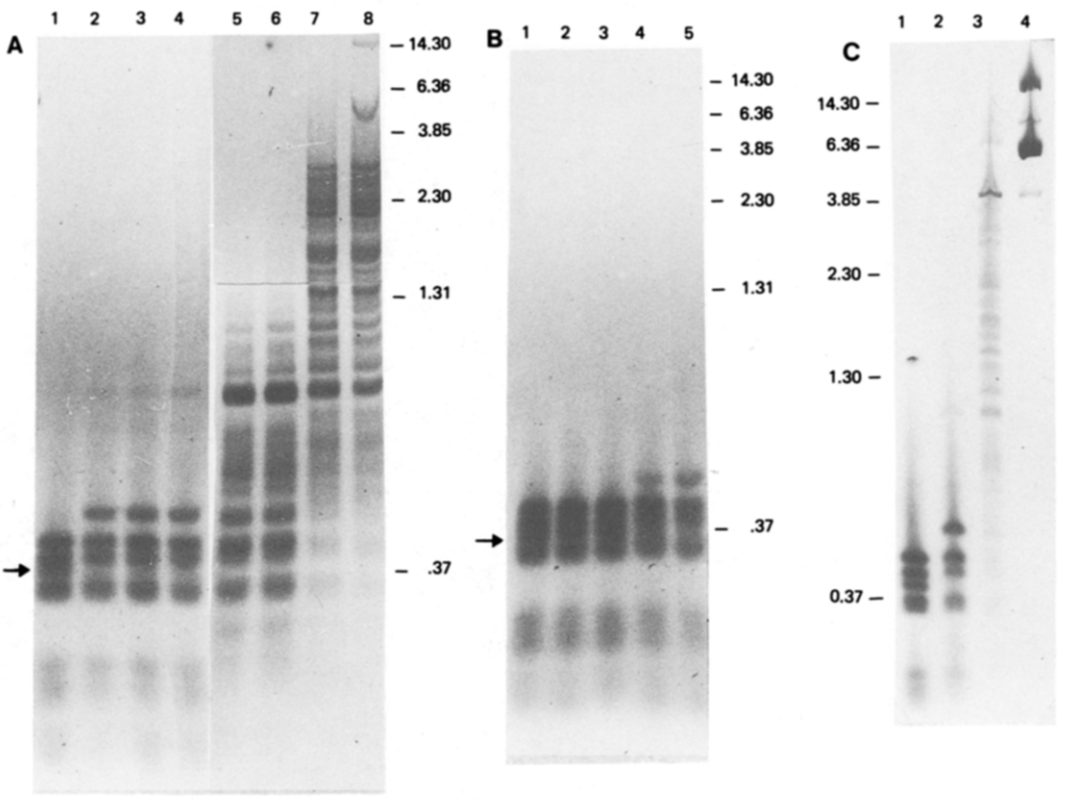

Figure 2. Digestion of plasmid pBR322 DNA with Hae III (for 1 hour at 37°C) incubated with increasing concentrations of dye. (A) With propidium iodide: Lane 1 - 100; Lane 2 - 200; Lane 3 - 250; Lane 4 - 300; Lane 5 - 340; Lane 6 - 400; Lane 7 - 500; and Lane 8 - 600 ug/ml PI added initially. (B) With ethidium bromide (EB): Lane 1 - 0; Lane 2 - 50; Lane 3 - 100; Lane 4 - 400; and Lane 5 - 500 ug/ml EB added initially. (C) With ethidium bromide and non-ethanol precipitated plasmid pBR322 DNA:Lane 1 - 0; Lane 2 - 50; Lane 3 - 100; and Lane 4 - 300 ug/ml EB added to reaction solution. Molecular weight standards on right of figure are X 10^{-6}. Arrow indicates the loss of the 0.312 x 10^6 restriction fragment.

and 2154 (joining restriction fragments of 3.27, 1.92 and 0.21 x 10^5). The cleavage of pBR322 by Hae III in the presence of increasing concentrations of PI (100-600 ug/ml) and EB (0-500 ug/ml) are shown in Figure 2. In both instances one restriction site (at coordinate 1445) involved in generating two fragments of molecular weight 1.14 and 3.12 x 10^5, is the most sensitive to the presence of the dye (yielding a new fragment of 4.27 x 10^5). In the presence of EB it appeared to be the only site inhibited (Figure 2 A and B - arrow). All of the subsequent experiments were conducted with only PI at 50 and 200 ug PI/ml to conserve plasmid and restriction enzyme. Figure 3 demonstrates that Hinf I was also selectively inhibited by 50 ug PI at a single site (coordinate 3362) generating a new fragment of molecular weight 1.33 x 10^6 (joining

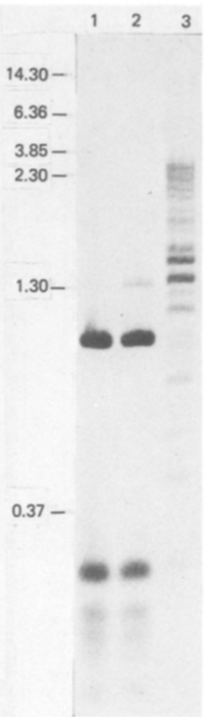

Figure 3. Digestion with Hinf I (for 1 hour at 37°C) of plasmid pBR322 DNA incubated with increasing concentrations of propidium iodide. Lane 1 - 0; Lane 2 - 50; and Lane 3 - 200 ug/ml PI added initially. Molecular weight standards on left side of figure are X 10^{-6}.

fragments of 1.01 and 0.32 x 10^6). Table I summarizes the results of dye inhibition of all of the restriction endonucleases employed with plasmid DNA and with hamster mt-DNA at two dye concentrations. The mt-DNA restriction patterns, in the absence of dye, were the same as those previously reported (19).

A final set of experiments were conducted with pBR322 DNA to determine if ethanol, a known inducer of the B to Z transition (9), when used to precipitate the DNA played any role in the observed dye inhibition of restriction enzymes. We modified the above methods by omitting the alcohol precipitation of the DNA and added Hae III directly to the dye-DNA solution (all in restriction buffer) in a final volume of 25 ul. Figure 2C demonstrates that at 50 ug EB/ml the enzyme is inhibited primarily at coordinate 1445. At 300 ug EB/ml there is virtually complete inhibition of the enzyme. Similar studies with PI demonstrated that at 50 ug PI/ml enzyme inhibition was so extensive that the selective inhibition at coordinate 1445 was not observed and at 300 ug PI/ml total enzyme inhibition occurred (data not shown). This same experiment was repeated with pBR322 DNA that had

been linearized by digestion with Eco RI in order to determine if the covalently closed circular molecule was required for the observed selective inhibition. The electrophoresis patterns were virtually identical to those in Figure 2C except that at 300 ug EB/ml the only band observed corresponded to linear pBR322 DNA (data not shown).

DISCUSSION

This study presents data that indicates that certain restriction endonucleases, employed in conjunction with intercalating dyes, can be used as sensitive probes to analyze nucleotide sequences and loci within the macromolecule that may limit or enhance the structural alterations allowed by the drugs. Several conclusions can be drawn from the results. PI is a more potent inhibitor of the endonucleases than EB. Inhibition of the enzyme by the dyes appears to be independent of whether the cut made by the endonuclease is staggered or straight through. The degree of inhibition is clearly not equal at all of the recognition sites with a given endonuclease. A comparison of results in Figures 2A,B and 2C would indicate that our final reaction mixture in the experiments depicted in Figures 1-3 and Table I contained less than one tenth the initial dye concentration.

Two aspects of dye inhibition of restriction enzymes must be considered: (1) a general inhibition of restriction endonucleases at all sites that may be accounted for by a single mechanism and (2) a selective inhibition of one or a few sites in a DNA molecule which contains multiple restriction sites that can only be explained by a heterogenous micro-environment with potentially different mechanisms of inhibition.

General Inhibition: Our results with both plasmid pBR322 DNA and mt DNA-dye complexes essentially conform to an expected pattern of preferred binding sites as defined by Pack and Loew (1). The exceptions may be due to the ability of some enzymes to be more or less tolerant to distortions of the nucleotide sequence recognized. Other workers have also found Eco RI to be relatively insensitive to the intercalating dye EB (20,21) while drugs binding to the DNA externally were potent inhibitors of Eco RI (20).

Selective Inhibition: A difference in dye binding and inhibition would most likely result from the adjacent nucleotide sequences and/or a difference in the micro-conformational environment. Similar conclusions have been drawn, in the absence of drugs, to explain variations in cleavage efficiency within a DNA molecule with Eco R1 (22) and in the presence of anthramycin or mitomycin C with a variety of restriction enzymes (16). While EB intercalation has been shown to induce a Z to B transition in synthetic dG-dC polymers (10), it may have other effects at low concentrations, on native DNA. The only Hae III restriction site in pBR322 inhibited by low concentrations of PI or EB is at coordinate 1445, the

Table I: The general inhibition of restriction endonuclease digestion of plasmid pBR322 DNA and mt DNA by ethidium bromide or propidium iodide.

Enzyme - Sequence		Dye	Initial Dye Concentration 50 ug/ml	200 ug/ml	Selective Inhibition Observed
pBR322:					
Alu I	5'-AGCT-3'	PI	++	++++	
Taq I	TCCA	PI	++++	++++	
Hpa II	CCGG	PI	++	++++	yes
		EB	+	+++	
Hae III	GGCC	PI	0	+ (a)	yes
		EB	0	± (a)	
Hinf I	GANTC	PI	±	+++	yes
	A				
Cla II	GG T CC	PI	0	+++	
Eco RI	GAATTC	PI	N.D.	+	
Bam HI	GGATCC	PI	N.D.	+ ±	
Sal I	GTCGAC	PI	N.D.	+++	
Hinc II	GTPyPuAC	PI	0	+++ ±	
mt DNA:			100 ug/ml	200 ug/ml	
Alu I	AGCT	PI	0	++	
Hpa II	CCGG	PI	+++	++++	
		EB	N.D.	++	
Hind III	AAGCTT	PI	+	+++	yes
		EB	+	++	
Eco RI	GAATTC	PI	+	+++	
		EB	0	±	
Bgl II	AGATCT	PI	0	±	yes
Pst I	CTGGAG	PI	0	+	
Hpa I	GTTAAC	PI	0	0	
		EB	0	0	

Note: ++++ equals complete inhibition; ± equals an intermediate inhibition level. Ethidium bromide - EB and Propidium iodide - PI. N.D. - not determined. (a) - reactions conducted at an initial dye concentration of 400 ug/ml.

same site previously shown to be partially inhibited in the absence of drug and to be flanked by Z DNA (23). This is the only Hae III site flanked up- and down-stream by nucleotide sequences with the potential to undergo a B to Z conformation transition. The six nucleotide sequence flanking both sides of the restriction site at 1445 also has the lowest likelihood of binding dye as compared to similar regions surrounding all of the other Hae III sites. This could increase the probability of dye intercalation pair at restriction site 1445. It is also possible that base modification may play a role in selective inhibition. Selective inhibition of Hpa II restriction occurs at 2120 and 2154. These sites are again the only two Hpa II sites surrounded by potential nucleotide sequences that can undergo a B-Z transition. The 2154 site is 20 nucleotides removed from the nearest 6 base alternating sequence. However, the 2120 and 2154 sites are within a region of the greatest number of potential base sequences that can undergo a B to Z transition - 7 sets of alternating purine pyrimidine bases

between 2070 and 2310. The most dye sensitive Hinf I restriction site is at coordinate 3362, however, this site only has one possible nucleotide sequence near it that can form the Z-conformation. This is also true for one of the other Hinf I sites - at 1524. The difference between these two sites is that the 3362 restriction sequence has a C for its variable nucleotide while the 1524 site has an A. The CT sequence at 3362 has a higher propensity to bind PI than AT at 1524 which could account for the greater sensitivity at 3362. Base modification at 3362 could also play a role.

The extension of these studies with other restriction endonucleases and other synthetic and native DNA species with known nucleotide sequence should help to establish rules of DNA-drug interactions.

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