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DAPI - pUC8 COMPLEX: A TOOL TO INVESTIGATE BIOLOGICAL EFFECTS OF NUCLEIC ACID-DRUG INTERACTION

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SUMMARY: A complex consisting of pUC8, a 1.8 Md plasmid, and 4'-6-diamidino-2-phenylindole, a DNA-binding agent, has been preformed in vitro under different conditions of ionic strength, and used to transform competent cells. A strong interference with the plasmid-coded activities, related to the P/D ratio whereat the DNA-drug complex was formed, was shown to occur. Since the compound does not inhibit the uptake process neither affects plasmid activity once dissociated at high ionic strength, it is likely to be acting from inside the cell while still in the form of a DNA-adduct. This system is proposed as a useful tool to investigate the effects on target genes of drugs endowed with DNA sequence specificity.

The action of molecules that have DNA as a target can be studied either in functionally intact or isolated systems. In the latter case, the objects of study are the structural and functional modifications induced on the nucleic acid by changes in several physico-chemical parameters or by the addition of enzymes recognizing it is a substrate. The major drawback of this simplified approach is the loss of cellular compartimentation and microenviromental organization where DNA and the DNA-ligand complex naturally would interact. To couple the advantage of having natural conditions and a simplified system to work with, we have devised to preform a plasmid-drug complex and subsequently introduce it in the host cell with the aim to follow how and where the presence of the drug affects plasmid expression. As a DNA model we have used pUC8, a 1.8 Md plasmid that contains two easily selectable determinants, the gene for ampicillin resistance (amp^r) and the NH_2 terminus of beta-galactosidase (1,2).

As a drug we used 4'-6-diamidine-2-phenylindole (DAPI), a fluorescent compound which has been biologically used as a chromosomal staining agent and

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as a trypanocide substance (3-10), and deeply investigated for its mode of binding to DNA from a biophysical point of view (11-15).

MATERIALS AND METHODS

Drugs: Ethidium bromide (EB) and DAPI were purchased from Boehringer.
Bacteria and DNA: The **E. coli** strain K12 JM83 (ara, Δlac-pro, Stra, thi, Φ80 lacZ Δ M15) was utilized, together with plasmid pUC8, in all the experiments reported in this paper. pUC8 was propagated on the same cells and purified by means of conventional techniques (16).
Drug-DNA interaction and transformation of **E. coli**: To an aqueous solution of DNA variable amounts of drug were added so as to obtain different DNA/ligand ratios (P/D). In some experiments, ionic strength was varied by adding to the mixture increasing concentrations of NaCl up to a maximum of 1M. After incubation at room temperature for 30 min., the reaction mixture was utilized to transform JM 83 cells made competent by $CaCl_2$ (17). Transformant clones were selected on agar plates containing 50 μg/ml ampicillin and 5 μg/ml 5'-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Boehringer).
Labelling of plasmid and uptake studies: One μg of pUC8 was labelled with ^{32}P dCTP (Amersham, S.A. 3000 Ci/nmol) by the nick translation reaction and freed by unincorporated nucleotides essentially as reported (18). This DNA was incubated with $CaCl_2$-treated competent cells as described above for transformation with the only exception that bacteria were not grown after the period at 4 ^{0}C, but extensivelly washed in isotonic water and spotted onto GF/C filters (Watman) to count TCA precipitable radioactivity.
Circular dichroism measurements: CD measurements were performed in a JascoJ500 A dicrograph equipped with a unit for spectra accumulation. All the samples were dissolved in an aqueous buffered solution of 0.01 M NaCl, 0.01 M Tris, pH 7.4. Titrations were carried out either by addying increasing amounts of the polymer to a solution of DAPI, whose concentration was kept constant (15), or aliquots of DAPI to a fixed quantity of DNA.

RESULTS

Inhibitory action of DAPI and EB on E. coli transformation by pUC8: The ability of pUC8 to transform competent cells was assayed after direct incubation of plasmid with varying concentrations of DAPI and EB. The number of transformed clones was evaluated by scoring Amp^r/gal^+ colonies on H agar plates.

Inspection of Fig. 1 clearly shows different patterns of inhibition by the studied compounds. EB is almost devoid of activity, while DAPI is strongly inhibitory on the transformation process. It can be shown that the number of transformed clones is reduced by more than 50% over the control value even at a P/D ratio of 100. This effect disappears when DNA is in large excess over the ligand (P/D equal to 1000, not shown).

DAPI effect on plasmid uptake: Fig. 2 depicts the number of plasmid molecules internalized by E. coli during the transformation process, after interaction with varying amounts of the dye. It can be noticed that DAPI inhibits DNA uptake to some extent (20%) only at low P/D ratios. On the contrary, at high values of P/D, either no effect or a slight enhancement of the internalized copy numbers is observed.

In order to better characterize the physico-chemical nature of DNA-DAPI interaction, and the role played by this binding in the series of events

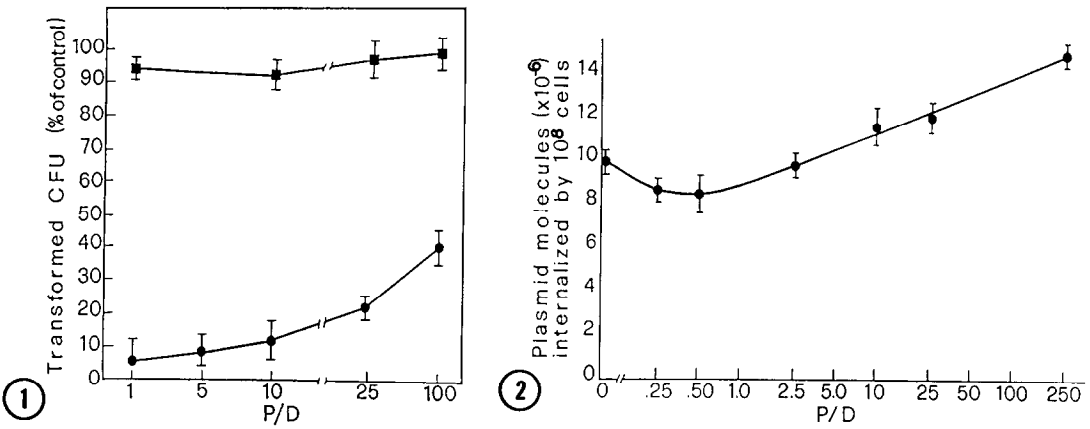

Fig. 1 Effect of varying concentrations of DAPI (●) and ethidium bromide (■) on the transformation of recipient JM83 cells by pUC8. Values represent efficiency of transformation of the drug-DNA adduct vs control (%) and reproduce means ± SD of five separate experiments.

Fig. 2 Incorporation of radiolabelled pUC8 in JM83 cells. One µg of nick translated plasmid (see Materials and Methods) was allowed to interact with DAPI at different P/D ratios. The cell complex was followed by measuring TCA precipitable radioactivity. Values represent means ± SD of three separate experiments.

leading to transformation, we have used different conditions of ionic strength during incubation.

Effect of ionic strength variation on the transforming ability of pUC8: Appropriate molar concentrations of the ligand and the plasmid were used to obtain the two different DNA-binding modes that are exhibited by DAPI at high and low P/D ratios: a strong one and a weak one respectively, none of which seems to be intercalative (15,19).

Fig. 3 shows the influence of ionic strength variation on the transforming ability of pUC8 after incubation with DAPI at a P/D of 1 and 10 respectively.

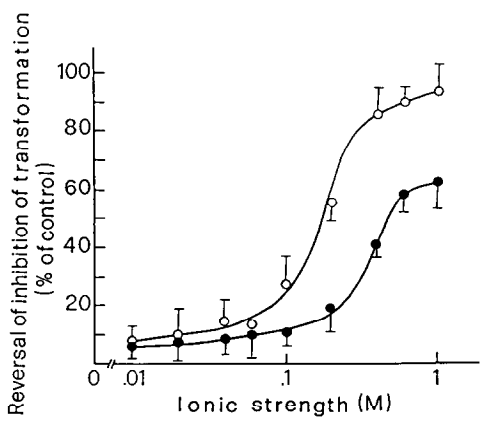

Fig. 3 Effect of ionic strength variation on the efficiency of transformation of JM83 cells by two types of pUC8-DAPI complexes: one taking place at a P/D ratio of 1 (○), the other at a P/D ratio of 10 (●). Values represent means ± SD of three separate experiments.

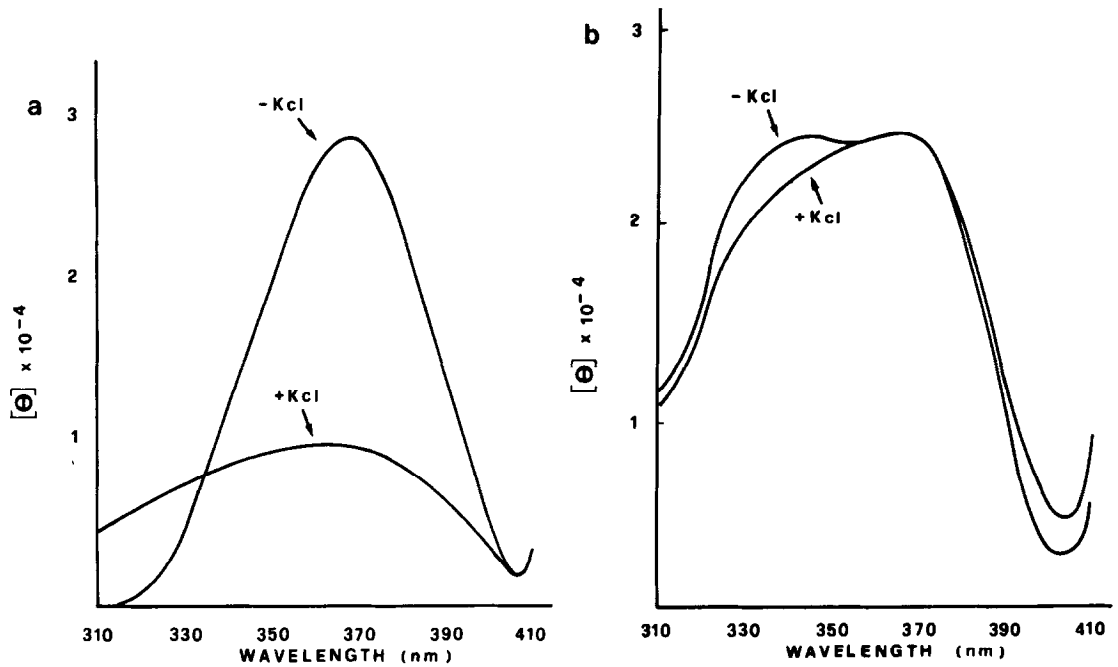

Fig. 4 a) CD spectra of DAPI-pUC8 complex at P/D ratio = 0.48, KCl was 0.83 M.
b) CD spectra of DAPI-pUC8 complex at P/D ratio = 3.6, KCl was 0.60 M.

It can be noticed that the effect induced at a either one stoichiometric ratio is quickly reversed by the change of the ionic strength between 0.2 and 0.3M. Instead, a quite different response is observed at a P/D ratio of 10 since reversion of the inhibitory activity of DAPI is not complete even by increasing ionic strength up to 1M. However, at this molarity, the shape of the curve resembles the one observed at a P/D of 1.

CD titration: Fig. 4a shows the appearance, at low P/D ratios, of a dichroic band centered near 370 nm, which arises from the weak, electrostatic binding (15). Addition of KCl abolishes it as expected.

At high P/D ratios, a system of two bands appears, resulting from the strong mode of binding, which is partially electrostatic in nature (15): one band is centered near 340 nm and the other near 365 nm. Addition of KCl, as shown in fig. 4b has virtually no effect but for a slight decrease of the intensity of the 340 nm band, representative of the strong type of interaction.

DISCUSSION

The direct dependence of DAPI concentration on the inhibition of plasmid activity is a safe indication that the dye can alter this function by the nature and strength of its binding to the nucleic acids.

The absence of interference in this same model by EB can be explained by differences on the stereochemistry of binding to the macromolecule. These are quite obvious when considering the intercalation process which is typical of EB and the external binding of DAPI.

The observation that the yield of transformants is reduced by more than 50% when DNA and DAPI are incubated at a P/D ratio of 100 suggests that the ligand is not merely interfering with DNA uptake by the cells. At this molar ratio the drug is all bound to its target and is not substantially altering the disposition of the charged residues of the macromolecule neither its net charge that could be critical for the uptake process (19). In fact, when using radiolabelled pUC8 we have observed that the intracellular access of the plasmid-dye complex was virtually unaffected at a P/D ratio of about 5, with an average of 0.1 plasmid copies being internalized per cell (see Fig. 2). It is therefore apparent that DAPI is not acting from without. Such a conclusion is further substantiated by the increased uptake which is observed at P/D ratios of 25 and 250. This increase could be explained by a hydrodynamic effect of the ligand upon the macromolecule which eventually facilitates its permeability across the membrane.

Experiments on inhibition of transformation by ionic strength, produce evidence that such a phenomenon occurs in a different manner at the two P/D ratios, representative of the weak and the strong binding process respectively. This observation points to the crucial role played by the complex structural organization and the ligand mode of interaction in inhibiting plasmid expression. From our data, it is reasonable to assume that the complex which is less affected by ionic strength variations is largely conditioning the biological response. Besides, the observation that DAPI has no effect on plasmid activity once dissociated from the weak complex (P/D 1) by increasing the ionic strength (1 M), is indicating that the compound is functioning within the cell, while still in the form of a DNA-adduct (strong complex). These results quite agree with the physico-chemical data previously published (15,19) that indicate the occurrence of a prevalent non-electrostatic interaction responsible for the strong binding mode (P/D 10 or higher). It has been proposed that such a bond involves hydrogen bond formation between the indol moiety of the dye, which is buried in the minore groove, and the aromatic ring of A-T base pairs (15).

It is likely that the neighboring DNA conformation, that follows such an interaction, is less compatible with DNA function than the one induced by EB which involves unwinding of the nucleic acid. The intercalating agent EB, in fact, has been shown to be uncapable of interfering with "active" DNA regions (21,22) and relatively uneffective in inhibiting transcription initiation (Crothers D.M., personal communication).

Altough several methods, such as the use of phage induction (23) and the study of mutagenesis in **Salmonella** strains (24) or shuttle vectors (25), have been extensively employed to monitor the biological effects of DNA-ligands, they are somewhat technically complicated. Furthermore, they suffer from the

limitation of being only partially informative when used as a screening system.

The plasmid-drug complex and the relative modalities of use which are reported in this paper represent a quite simple, unexpensive and not-time consuming tool for investigating the modification of gene expression induced by drugs and their specificity of action on DNA as a target. This approach, while allowing to draw relationships between biological activity, ligand structure and binding stereochemistry, can also be useful in monitoring drug affinity to selected DNA sequences in a more physiological way. More insight into the nature of inhibition of pUC8 activity by DAPI and similar compounds may be eventually reached by investigating the in vitro processes of DNA transcription and replication and analyzing nucleotide sequences after plasmid growth in bacteria.

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