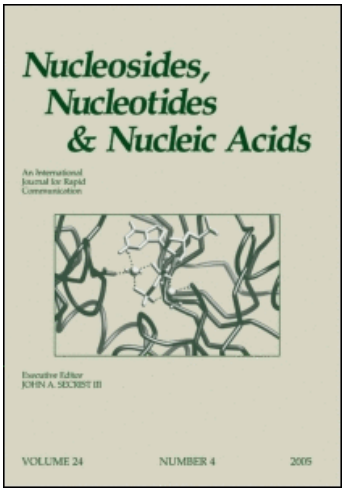

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DNA ACCESSIBILITY TO MINOR GROOVE LIGANDS IN CORE NUCLEOSOME AND CHROMATOSOME

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ABSTRACT

Using the circular dichroism spectra induced in the visible by the binding to the minor groove of DNA, we found that Hoechst 33258 is able to occupy its specific sites even when these are located inside the nucleosome structure. This high accessibility of the DNA in the nucleosome is not modified by the removal of the amino-terminal domains of the octamer histones and is not reduced by the presence of linker histone. Interesting and reasonable differences were found in the association constants.

The processes of eukaryotic gene expression must be considered in the light of the intrinsically dynamic properties of chromatin, and, in particular, of the basic level of it which is the nucleosome. It is now clear that in the process of chromatin condensation two structures have a prominent role: the amino-terminal domains of the histone octamer and the linker histones (H1, H5 and the other isoforms). Both act by favoring the coming-near of two adjacent nucleosomes, forming the high order structure of 30 nm fibers, the model of which (solenoid or zigzag) is not yet clearly assessed. The role of the amino-terminal domains in gene expression processes is firmly established; in fact these domains act as regulators of transcription with negative and positive effects either by restricting DNA accessibility to transcription factors[1] or through interactions with specific transcriptional regulatory proteins[2]. Much less is known about the role of linker histones. Nucleosomes containing linker histones (H1-H5) and about 20 bp of linker DNA are called chromatosomes and could be considered as the fundamental units of the condensed chromatin fibers. The exact position of the linker histone in the chromatosome is not yet

defined[3], but it is known to "lock" two complete turns of DNA on the histones octamer; so its presence, preventing DNA mobility, seems to reduce also its accessibility toward outer molecules.

The molecules used to study the structure and the accessibility of the DNA belong to many classes, including minor groove binding ligands that, in recent years, have attracted much interest because of their diverse antimicrobial and antitumor activities[4-6]. Within this class, much used are the fluorochromes 4,6-diamidino-2-phenylindole (DAPI) and 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bis-1H-bis-benzimide (Hoechst 33258), particularly well suited for comparative studies on natural DNA and DNA in the chromosome. These ligands interact with 3-4 bp of DNA in AT-rich sequences, as seen also from the crystal structures of their complexes with oligonucleotides[7,8]. Their preference for AT sites depends on the narrow minor groove that characterizes these sequences and from the bend of their axis that fit the curve of the groove. However, they substantially differ in overall size and charge. Hoechst 33258 is more tolerant of G-C base pairs, it contains one puckered piperazine ring that cannot fir within the narrow A-T region of the minor groove, and the phenol hydroxyl group can provide additional hydrogen-bonding potentials in analogs where it is moved from the para position. From the model of nucleosome positioning proposed by Travers[9] it is known that the AT sequences are preferentially positioned inward in the nucleosome structure, where the DNA is facing towards the histones, in a way that could inhibit the ligands access.

This situation encourages the use of the AT specific ligands to investigate the accessibility of the DNA in the nucleosome, as a model of the interactions between DNA sequence elements and protein regulatory factors. In a previous work we found that DAPI is able to recognize its DNA sequence targets even when these are constrained in the nucleosome[10]. This finding suggests that the capacity for specific interactions of the minor groove may well be exerted in chromatin *in vivo*. We also investigated whether minor groove specific ligands can recognize DNA regions having different intrinsic curvature, with the aim to explain at the molecular level their different interactions with different chromosomal domains[11]. We found that DAPI does not discriminate between intrinsically curved and non curved sequences (CA_4T_4G and CT_4A_4G tracts, respectively) as to the high affinity minor groove bindings, but Hoechst 33258 does, disfavoring non-curved sequences by a factor of about seven with respect to curved sequences (and with

respect to DAPI). On this ground, and with the understanding that Hoechst 33258 is one of the best basic models for designing DNA-binding drugs, we focused on chromatin accessibility to that ligand.

In the present report we investigated the interactions of Hoechst 33258 in the nucleosome and in two substantially opposite situations of the mono-nucleosome: the one in which the linker histone is present together with two complete turns of the double helix (chromatosome), and that in which the amino-terminal domains of the histone octamer are removed from core nucleosome by digestion with trypsin (a model of hyperacetylated chromatin)[12]. The high accessibility of the minor groove we found in all of the nucleosome situations examined seems to us rather surprising, and deserving discussion both in terms of a less rigid structure than deduced from crystallography[13] and in terms of rotational variations of the double helix on the octamer surface. The second hypothesis is supported by evidence of a DNA rotation induced by Hoechst 33258 in the nucleosome[14,15] and is also in agreement with the model of a dynamic nucleosome proposed by Widom[16].

Methods and Materials

Preparation of Core Particles, Chromatosomes and Trypsinized nucleosomes

Mononucleosome core particles and chromatosomes were prepared from chick erythrocytes according to Kornberg[17] and Simpson protocols[18] respectively, with minor modifications. The trypsinized nucleosomes were obtained by using free trypsin in a ratio of approximately 1:100 to nucleosomes (1.2 μg of trypsin for 100 μg of nucleosomes). Nucleosomes were digested at room temperature. After 30 min the reaction was stopped by adding the nucleosome solution to the immobilized trypsin-inhibitor (inhibitor bound to DITC glass, from Sigma) in a ratio 1μg of trypsin/1000 μg of glass beads. The immobilized inhibitor was preconditioned in 25 mM NaCl, 10mM Tris-HCl pH 7.5 under mild rotary shaking at 4°C for 24h, and washed thoroughly with TE buffer (0.01M Tris, 0.001M EDTA) pH 7.5 prior to use. As inhibitor binds trypsin in a covalent way, the enzyme-inhibitor complex is removed by centrifugation of the glass beads (12.000 rpm for

10 min). Finally, PMSF was added to a final concentration of 0.3 mM. The trypsinized nucleosomes were stored at 0-4°C.

Preparation of nucleosomal and chromatosomal DNA

DNA was obtained by adding TE buffer to 100 μg of nucleosomes (or chromatosomes) to a final volume of 200 μl. Then 1800 μl of 1% CTAB, NaCl 0.3 M were slowly added, shaking continuously by vortex. After 30 min at room temperature, the sample was centrifuged for 15 min at 15.000 rpm and the pellet was resuspended in 500 µl of a TE, 1 M NaCl. After 45 min at room temperature, 1ml of absolute EtOH was added and the solution was put on ice for 15 min. After centrifugation at 15.000 rpm for 15 min, the pellet was resuspended in 70% EtOH and washed twice or more with the same. Finally, the DNA was dried, resuspended in TE and stored at 4°C.

Electrophoretic Mobility Assay

Nucleosomal and chromatosomal DNA were run on a native 10% polyacrylamide gel (20 x 20 x 0.5 cm) in 1x TBE buffer (90 mM Tris-Borate, 2.5 mM EDTA) at a constant voltage of 200 V. The gel was then colored for 30 min with an EtBr solution and washed with distilled water. The electrophoretic mobility of the samples was compared with that of a molecular weight standard. Chromatosomes, mononucleosomes and trypsinized nucleosomes were run on a 1% agarose gel in 1% TBE buffer at a constant voltage of 70V for about 2h. After running, the gel was colored with EtBr solution for 10 min and then washed with distilled water. To be sure that Hoechst 33258 does not induce the dissociation of the DNA-histones complex, electrophoretic mobility essays were repeated comparing samples with and without the drug. The drug/DNA ratio used was 1/5.

Circular Dichroism measurements

CD spectra were recorded at room temperature on a Jasco 715 spectropolarimeter. Starting samples were prepared with DNA-drug concentration ratio ([bp]/[D]) of about 50. Subsequently [DNA]/[Drug] rations were made to decrease by sequential substitu-

tion of volumes with drug at the same concentration. Spectra were recorded for each [bp]/[D] ratio thus obtained.

Results

It is known that the binding of the minor groove ligands to DNA can cause little distortion in the double helix and so it could have an effect on nucleosome stability. To be sure that Hoechst 33258 and DAPI bind to nucleosomes, chromatosomes and trypsinized nucleosomes without causing the dissociation of these DNA-histones complexes, we compared the electrophoretic migration of the complexes with and without drugs, obtaining no differences (data not shown). This result demonstrate that this ligands do not cause the disruption of the nucleosomes and of the other DNA-histones complexes used in our experiments, even if they seem to inhibit the nucleosome assembly[19].

The binding of the ligand to DNA (naked and organized with the histones) was studied by circular dichroism spectroscopy, using the spectra induced in the visible range by the specific interactions of DNA with the drug (fig. 1). A further control of the integrity of the complexes through the experiment was indeed provided by the CD spectra in the UV (not shown). The CD spectrum of naked DNA is strongly different from that of the DNA-histones complexes. This situation is substantially conserved also in the presence of the ligand; furthermore, the DNA molar ellipticity spectra are almost independent on [D]/ [bp] ratios in the range from 0 to 0.1. These features confirm that the binding of the drug does not cause dissociation.

No isodichroic point is found with Hoechst 33258 in all complexes, suggesting that the types of strong bindings are either one or more than two. It can be reasonably affirmed that these strong bindings are conserved in all forms of nucleosome examined.

In order to construct the binding isotherms, we considered the variations of molar ellipticity as a function of DNA/ligand molar ratios at a fixed wavelength. A suitable wavelength is the λ_{max} of the visible absorption spectrum of the ligands. Direct isotherms are shown in the fig. 2, where r (the bound ligand molecules per DNA base pair) is plotted against C/[D] (ligand free concentration relative to total concentration). The plots of free DNA and of purified mono-nucleosome devoid of linker DNA and of H1-

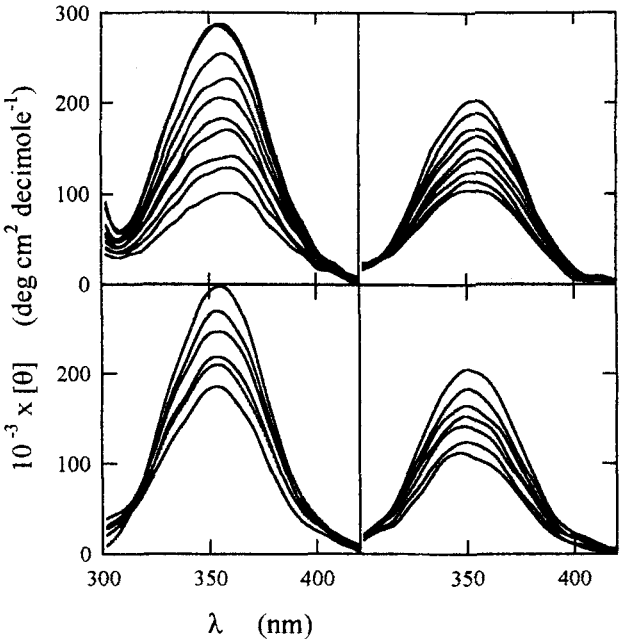

FIG. 1 – CD spectra induced by the bindings of Hoechst 33258 to DNA at [bp]/[D] ratios decreasing from 50 to 1. Left panel: complexes with free nucleosomal DNA (top) and core nucleosome (bottom). Right panel: complexes with trypsinized core nucleosome (top) and chromatosome (bottom).

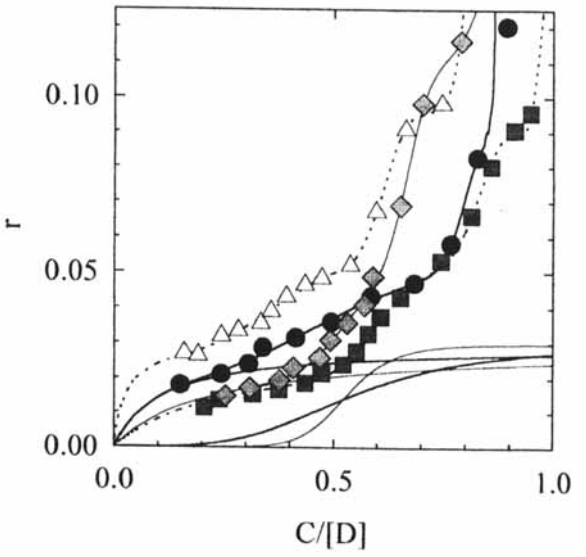

FIG. 2 – Direct binding isotherm of Hoechst 33258 complexes with DNA. Points are means of at least three experiments, with standard deviations in the order of 10%. Deconvolutions of the first two bindings are reported as continuous lines only for trypsinized core nucleosome (diamonds and thin lines) and chromatosome (circles and thick lines). Dotted lines refer to cumulative bindings of free nucleosomal DNA (triangles) and core nucleosome (squares).

H5 histones (core nucleosome) are almost parallel along the abscissa. This strongly suggests that the number of sites for the ligand be conserved for each binding type, while the association constants are the most affected parameters. The plots of Hoechst 33258 complexes with trypsinized nucleosomes and chromatosomes lay within the limits defined by those of free DNA and core nucleosome. It appears that following partial trypsinization a core nucleosome maintains the strong modes of Hoechst binding, while the weaker bindings are barely distinguishable from those of free DNA. On the contrary, the chromatosome displays features of the strong bindings that are intermediate between those of free DNA and those of the core nucleosome, while joining the latter as regards the weaker bindings.

The binding isotherms were reduced to putative components by simultaneous fittings to one hyperbolic and one or more logistic functions, that are representative, respectively, of the first, strongest binding to a class of independent sites, and of a number of weaker bindings to co-operative sites (fig. 2). The parameters calculated from these deconvolutions are reported in table I. The analysis confirms the number of bindings suggested by the spectra. In particular, only three specific bindings are found for Hoechst. A less specific, weaker binding of both ligands is found only for free DNA. This means that the minor groove of free DNA can be saturated by Hoechst 33258 about every 6 bp with a relative affinity of 1.2.

The main result of this analysis is that all the specific sites are "seen" by the ligand in the core nucleosome. Hoechst 33258 recognizes them also in the chromatosome and in the partially trypsinized nucleosome; actually, in the latter structure the third binding of free DNA is substituted by a couple of bindings with higher frequency. It is noteworthy that the first two specific bindings take place with the same periodicity, that is every 33 bp for Hoechst 33258. The second important result is that only the association constant for the first, strongest binding is drastically reduced, by an order of magnitude. This effect is partially moderated by the removal of amino-terminal domains of octamer histones, but, surprisingly, even more so by the presence of the linker histone.

Discussion

The organization of the DNA around the histones octamer seems to preclude the

Table I - Equilibrium binding properties of Hoechst 33258 to nucleosomes and free DNA.

	n (bp)				K_a			
	I	II	III	IV	I	II	III	IV
146 bp nucleosomal DNA	33	33	25	6	15.6	1.0	0.6	0.5
chromatosome	33	33	25	-	3.6	0.6	0.5	-
trypsinized core nucleosome	33	33	17	17	1.7	0.8	0.6	0.5
core nucleosome	33	33	25	-	1.4	0.7	0.5	-

The parameters are derived from the equations used to deconvolute the binding isotherms (FIG. 2). n is the number of bp per bound ligand molecule (or the mean periodicity of the binding site in bp), and is calculated as the recirpocal of the r asymptote. K_a is the apparent binding constant, and is calculated as the reciprocal of the free ligand concentration at half the value of n^{-1}; it is expressed in 10^8 M^{-1} units.

accessibility of external molecules toward sequences positioned inside the nucleosome structure. From this assumption rise the needs to know how processes like DNA transcription and replication may occur, and if a nucleosome disassembly would be indispensable to make them possible.

From our results we can assert that small molecules like DAPI and Hoechst 33258 are able to bind their specific sites also when these are organized in a nucleosome. In fact, in presence of the histones we found a decrease in binding affinity, but the number of binding sites remain unmodified with respect to free DNA. To explain this unexpected result we can draw two hypotheses. First, minor groove ligands could have the capacity to follow the groove and so to arrive at their sites without suffer the steric inhibition of the histones. This situation implies a consideration of a "relaxed" nucleosome

structure, in which the contacts between histones and DNA leave the possibility for specific interactions with other molecules. The second hypothesis is supported by the model of a dynamic nucleosome proposed by Widom[16]. According to this model, the DNA around the histones could change his rotational and translational phases, exposing the internal sites to the outside of the structure, thus making them available for the contacts with the ligands. This DNA mobility is confirmed by Fox[15], who found a DNA rotation of 180° on the histones surface, after its interactions with Hoechst 33258. He interprets this rotation as an effect inducted by Hoechst, but it could also be explained by the intrinsic DNA mobility founded by Widom; both explanations can account for the high accessibility of the nucleosome found in our experiments. In the case of trypsinized nucleosome, the removing of the amino-terminal domains increases the affinity of the specific binding and allows for the presence of a couple of bindings with lower specificity. This is a reasonable result if we consider that the amino-terminal domains may stabilize the DNA around the octamer. What is more surprising is the behavior of the chromatosomes. In fact the linker histone should lock the DNA on the octamer surface, thereby condensing the nucleosome structure. In this way, each DNA movement should be inhibited and so the binding of the ligands remains possible only if they can arrive inside the nucleosome. With these premises, it should be interesting to verify if we can obtain the DNA rotation found by Fox in the nucleosome also in presence of the linker histones, thus gaining a possibility to discriminate between our two initial hypothesis. In conclusion, we can say that the organization of the DNA in chromatin structures such as core nucleosome and chromatosome does not reduce the possibility of contacts with outer molecules, and that our results permit us to investigate about the mechanism that allows this high accessibility.

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