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RELAXATION OF CHROMATIN STRUCTURE INDUCED BY ETHIDIUM BINDING : 1 - MICROCOCCAL NUCLEASE DIGESTION OF THE ETHIDIUM - CHROMATIN COMPLEX

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SUMMARY : Degradation of chromatin by micrococcal nuclease is activated in the presence of ethidium bromide. This activation is shown by an increase in the rate of appearance of acid soluble material and by a more rapid conversion of chromatin toward mononucleosomes. Relaxation of chromatin structure is suggested as the mechanism responsible for this activation.

INTRODUCTION : The dye ethidium bromide binds to chromatin DNA mainly through intercalation (1,2,3) and this binding is located, at least for values of a dye to DNA nucleotide ratio smaller than .05 , in the internucleosomal DNA of chromatin as demonstrated by fluorescence depolarisation measurements (3). It has been assumed that the binding of ethidium in this internucleosomal DNA induces a conformational change of chromatin. It is clear that if such a change takes place, resulting from a modification of the DNA duplex, this change could play an important role in the binding of enzymes involved in the regulation of the replication or the transcription of the eukariotic genome. Up to now, the relaxation of chromatin structure induced by ethidium binding has been examined only by viscosimetry and histone dissociation studies and only at high concentrations of bound ethidium (4). This paper describes a new approch to study the effects of ethidium on chromatin structure based upon micrococcal nuclease digestion.

Micrococcal nuclease digestion has been widely used to demonstrate and study the repetitive structure of chromatin (see 5 and references therein). The DNA fragments generated from this type of digestion are multiple of a basic size (corresponding to the nucleosome) and lead to a limit digest (corresponding to the core particle). These DNA fragments reflect a regular arrangement of the chromatin which evolves from a basic unit. We have therefore studied the effects of ethidium on micrococcal nuclease digestion of chromatin, by looking at the rate of degradation and at the resulting products. Of particular interest is the extent to which ethidium binding modifies the overall structure of chromatin. Any chromatin structural changes which are induced by ethidium binding can then be examined in view of the currently well known binding process of ethidium (6,9).

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MATERIAL AND METHODS : chromatin was extracted from Erlich ascites tumor cells. The ascite fluid (20ml) was collected 7 days after the transplantation and the cells suspended in a large volume of ice cold solution of .25 M sucrose containing 5 mM EDTA pH 8 and centrifuged for 5 min at 5000 rpm in the JA 20 rotor of the J 21 Beckmann centrifuge. All subsequent operations, up to the detergent treatment, have been previously described (10). The cells were then washed three times in .25 M sucrose, .1 mM EDTA pH 8. The final pellet was suspended in 100 ml of buffer A (15 mM Tris-HCl ph 7.4, 60 mM KCl, 1.5 mM NaCl, .15 mM spermine, .5 mM spermidine, 15 mM β-mercaptoethanol, 67 mM phenyl methyl sulfonyl fluoride). Nonidet P40 was added at a final concentration of .5 %. After incubation for 5 min at 0° the suspension was centrifuged and resuspended in .34 M sucrose - buffer A.

Nuclei digestion : After a wash in .34 M sucrose - buffer A, the nuclei were suspended in the same buffer and the suspension made 1 mM in $CaCl_2$. The digestion of the nuclei was made at 37° for 2 min in presence of 15 units/ml of micrococcal nuclease (Worthington) and stopped by addition of EDTA to a final concentration of 2 mM followed by chilling on ice. After centrifugation the nuclear pellet was gently resuspended with a pasteurpipet in.2 mM EDTA and centrifuged at 4000 rpm for 10 min. 95 % of the chromatin, is greater than ten nucleosomes as determined through migration of its DNA on polyacrylamide gel electrophoresis, while observations under electron microscopy gives a size of about 20 nucleosomes for 90 % of this chromatin.

Chromatin digestion : Chromatin in Tris - HCl 10^{-2}M pH 7.6, EDTA .2 mM and $CaCl_2$ 1 mM (100 µg/ml of DNA) was incubated at 37° in presence of 15 units/ml of micrococcal nuclease and the required amount of ethidium bromide. At different times, an aliquot (.2 ml) of the incubation mixture was transfered in .25 ml of an albumine solution (3 mg/ml in EDTA 10 mM) at 0° and precipitated with 50 µl of 10 N perchloric acid. After centrifugation at 5000 rpm the absorbance at 260 nm of the supernatant was determined and divided by 1.68 to correct for the hyperchromic effect (11).

Gel electrophoresis of the digest : When digestion was followed by gel electrophoresis of the DNA, 1 ml of the incubation mixture was made 10 mM in EDTA, chilled on ice and extracted three times with phenol (12). After dialysis against the electrophoresis buffer (Tris .04 M, Na acetate .02 M, EDTA 2 mM pH 7.8), 5µg of the extracted DNA were loaded on 3.6 % polyacrylamide gels and run as described (13). The gels were stained with ethidium bromide (.5µg/ml) and photographed under UV light. Scanning of the negatives were performed with a Joyce Loebel microdensitometer.

Ethidium bromide was purchased from Sigma.
Ellipticine derivatives were a generous gift from Dr Dat Xuong.
All other chemicals were reagent grade.

RESULTS : The effects of ethidium bromide upon the micrococcal nuclease digestion of DNA and chromatin are shown in figure 1.
Ethidium inhibits the rate of appearance of acid soluble material following the digestion of DNA with micrococcal nuclease. This inhibition has been shown for other nucleases such as DNase 1, DNase 2, and RNase (14). In contrast, ethidium activates the digestion of chromatin with micrococcal nuclease, as evidenced in Figure 1 by the increasing rate of appearance of acid soluble material as a function of the increasing number of bound ethidium. When studied as a function of the bound ethidium to DNA-phosphate ratio (r), the rate of degradation passes through a maximum for a ratio equal to.05 (figure 2). This value of r, for which the activation is maximum, corresponds to the saturation of the internucleosomal

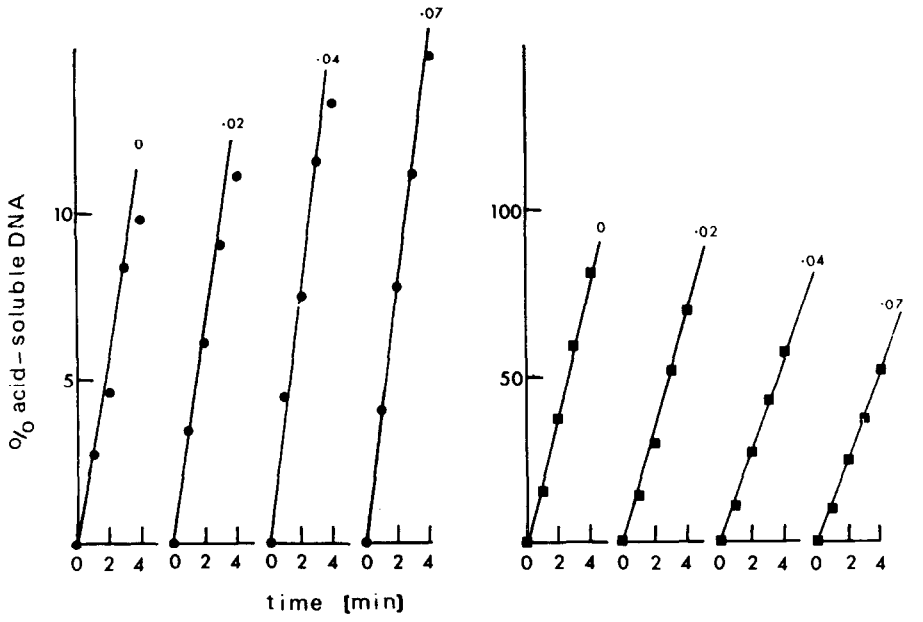

Figure 1 : Kinetics of acid soluble DNA release during digestion of DNA or chromatin by micrococcal nuclease, for different values of bound ethidium/DNA ratio (r)
—●— chromatin, —■— DNA.
The value of r is indicated at the top of each curve. For clarity, the kinetic experiments at different r values have been graphically separated.

DNA with the drug (3) and no release of histones is observed (4). This last point suggests that the activation of the chromatin degradation in presence of ethidium (at low concentrations of bound ethidium) is not due to a demasking of protected DNA by the release of histones.

The size of the DNA pices generated by the micrococcal nuclease with and without ethidium are shown on figure 3 and 4. For a 1 minute digestion period (corresponding to 3 % of the total DNA released as acid soluble material), the control without ethidium shows a classical pattern of DNA pieces whose sizes are multiple of 190 base pairs. When chromatin is digested in the presence of ethidium, we observe on the electrophoretic pattern a disappearance of the pieces of DNA corresponding to particles greater than the dinucleosomes. This result indicates that ethidium does not destroy the regularly organised structure of chromatin since mononucleosomes and dinucleosomes are still generated. At very high concentrations of dye (total ethidium to DNA-phosphate ratios greater than or equal to 1, we observe a disappearance of the bands on gel and the appearance of a smear corresponding to a destruction of the chromatin structure (result not shown).

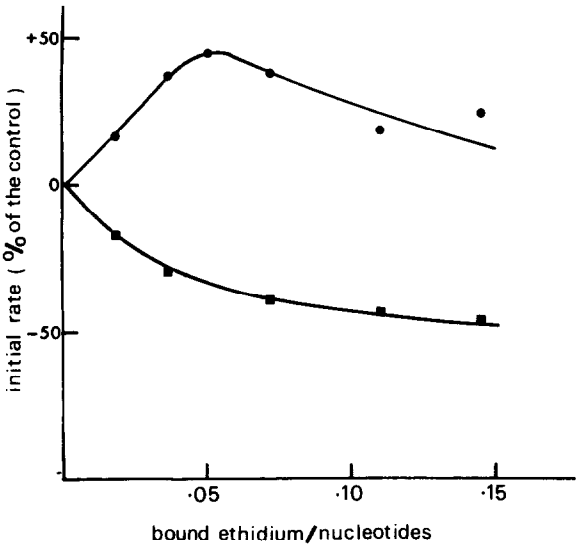

Figure 2 : Percent of increase of the initial rates of degradation of chromatin or DNA by micrococcal nuclease, as a function of r
—●— chromatin, —■— DNA.
The 0 % value on the ordinate axis represents the initial rate of degradation of the control (DNA or chromatin) without ethidium.

For a greater time of digestion (10 min), the control is extensively digested. We observe the appearance of two types of monomeric DNA whose length are respectively 140 and 160 base pairs. In the ethidium treated samples, most of the DNA has a size of 140 base pairs. The DNA size in the ethidium treated sample corresponds to a more rapid degradation of the chromatin toward the core particle, without any apparant change in the structure of this particle. Furthermore, the gels are in good agreement with the activation of micrococcal nuclease digestion observed by acid soluble release.

All these results indicate that the binding of ethidium to the internucleosomal DNA favors the action of micrococcal nuclease upon chromatin, without disrupting the over-all chromatin structure.

DISCUSSION : In the range of ethidium concentrations which lead to a maximum activation of the micrococcal nuclease digestion of chromatin, the dye binds chromatin through the internucleosomal DNA where it is intercalated (1,3). The sites of action of micrococcal nuclease are also located in the same region of chromatin (5). Therefore, we would expect to observe inhibitory effects during nuclease action in the presence of ethidium, due to a competitive process between the enzyme and the dye. On the contrary, the nuclease is activated by ethidium and this activation is correlated to the amount of dye bound to chromatin. It has been shown, by gel electrophoresis studies at 4°C and by

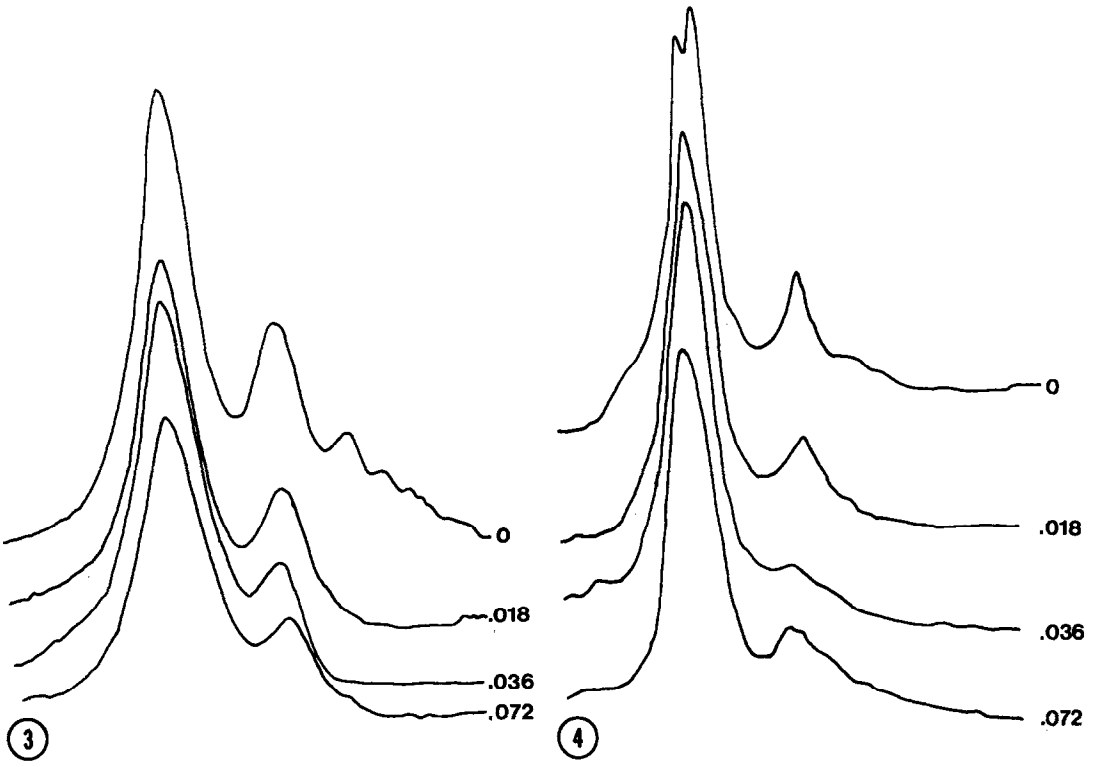

Figure 3 : Gel electrophoresis pattern of digested chromatin (1 min digestion). The gels are read from right to left and the numbers on the right represent the different values of r.

Figure 4 : Gel electrophoresis pattern of digested chromatin (10 min digestion). Same conventions as in figure 3.

viscosimetric measurements at 24°C, that ethidium which binds to the chromatin DNA does not release any histones, at least in the range of salt and ethidium concentrations studied here (4). To exclude the possibility of a temperature dependant histone release at 37°C, under the conditions of our present experiments, we have performed the same experiments at 20°C with identical results. We can thus assume that the nuclease action is enhanced by a conformational change of the chromatin due to the binding of the dye. Since this binding corresponds to intercalation, this conformational change could be due to the unwinding of the DNA duplex by ethidium. This last point should be discussed in detail.

When ethidium is intercalated into the DNA, there is both an unwinding of the DNA helix (15-17) and at the same time an introduction along the DNA of a positive charge (quaternary nitrogen in position 5 of the ethidium

molecule). This charge could interfere with the binding of histone H_1 thus labilising the H_1-DNA link, causing an activation of the degradation of the DNA by nuclease. In order to differentiate between the relative importance of these two factors. We have studied the effects of a non-intercalating derivative of ethidium (tetramethyl ethidium) and of molecules of the ellipticine series (manuscript in preparation). The results indicate that intercalation alone is able to induce an activation of the nuclease digestion of chromatin. Furthermore, this activation is due to a relaxation of chromatin structure as shown by electron microscopy (manuscript in preparation).

A recent work (18) compares the accessibility to the micrococcal nuclease of the ovalbumin and globin genes in chick oviduct nuclei. The expressed ovalbumin genes are more rapidly digested than the globin genes (not expressed), suggesting that an active gene does not have the same structure as an inactive one. This difference of succeptibility to the nuclease is comparable to the difference we observe after treatment of chromatin with intercalative drugs.

Our results suggest that intercalative drugs, which are known to affect biological process, could act at the level of chromatin by inducing a conformational change of its structure.

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