

DNA-PROTEIN BINDING IN INTERPHASE CHROMOSOMES

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ABSTRACT

The metachromatic dye, azure B, was analyzed by microspectrophotometry when bound to DNA fibers and DNA in nuclei with condensed and dispersed chromatin. The interaction of DNA and protein was inferred from the amount of metachromasy (increased β/α -peak) of azure B that resulted after specific removal of various protein fractions. Dye bound to DNA-histone fibers and frog liver nuclei fixed by freeze-methanol substitution shows orthochromatic, blue-green staining under specific staining conditions, while metachromasy (blue or purple color) results from staining DNA fibers without histone or tissue nuclei after protein removal. The dispersed chromatin of hepatocytes was compared to the condensed chromatin of erythrocytes to see whether there were differences in DNA-protein binding in "active" and "inactive" nuclei. Extraction of histones with 0.02 N HCl, acidified alcohol, perchloric acid, and trypsin digestion all resulted in increased dye binding. The amount of metachromasy varied, however; removal of "lysine-rich" histone (extractable with 0.02 N HCl) caused a blue color, and a purplish-red color (μ -peak absorption) resulted from prolonged trypsin digestion. In all cases, the condensed and the dispersed chromatin behaved in the same way, indicating the similarity of protein bound to DNA in condensed and dispersed chromatin. The results appear to indicate that "lysine-rich" histone is bound to adjacent anionic sites of a DNA molecule and that nonhistone protein is located between adjacent DNA molecules in both condensed and dispersed chromatin.

INTRODUCTION

The difference between "active" and "inactive" chromosomal material appears to be correlated with the cytological appearance of chromatin; regions in which DNA is condensed, i.e. in heterochromatin or metaphase chromosomes, indicate, from radioautographic evidence (3, 22), that RNA synthesis is absent or very low in comparison to regions in which DNA is dispersed. In cells that are active in synthetic processes, such as embryonic or glandular cells, the chromatin is usually dispersed, while relatively inactive cells, such as those of the thymus, or degenerating cells, have condensed chromatin. Although a correlation between chromatin morphology and DNA-histone binding

has not been demonstrated by cytochemical methods (1, 4), there is much support from in vitro experiments (2, 6) for the idea that histone is a regulatory factor in DNA-dependent RNA synthesis. Cytochemical information has indicated that the amounts of DNA and histone remain constant in vastly different types of nuclei (1). The possibility that different proportions of the different histone fractions vary within a constant total amount has been investigated (10), but no correlation with tissue activity has been found. Recent evidence has suggested that histones may be dissociated from DNA by acetylation (17) or by the presence of phosphoproteins (12), thus in-

creasing the activity of the DNA. Some indication of differences in DNA-histone binding in condensed and dispersed chromatin of isolated lymphocyte nuclei was presented by Littau et al. (15) who used morphological criteria to study the effect of removing and of restoring histone fractions. The lysine-rich histone fraction seemed involved in the appearance of condensed chromatin observed in the electron microscope. Similar correlations of DNA-histone binding with the condensed appearance of chromatin were sought in the present study by the use of very different methods.

The method used for analyzing DNA-protein binding depends on (a) the hypothesis that protein and cationic dye molecules compete for the same anionic sites on DNA so that removal of protein allows increased cationic dye binding (21) and (b) the selective removal of certain histone fractions according to the methods proposed by Holtzman (11). When a metachromatic cationic dye is used in this way, a change in color may result since different molecular patterns of dye binding may allow different amounts of dye-molecule interaction (8). This phenomenon of metachromasy has been used to analyze the properties of substances to which the dye binds both in solution and in tissue sections (7, 14). Recently, there have been several papers that describe the increased metachromasy that occurs following denaturation of nucleic acids in solution (5, 19). There have also been indications that DNA-dye binding in tissues changes from orthochromasy to metachromasy (or shifts in absorption spectra to shorter wavelengths) following specific treatments (9, 16). In the latter cases, it seems likely that protein dissociation rather than DNA denaturation is the cause of the metachromatic shift, since digestion by trypsin is one of the treatments which clearly affects protein primarily. Further evidence concerning DNA binding to metachromatic dyes when DNA and DNA-histone fibers precipitated from solution are used will be presented. This will indicate the difference between dye-binding sites of DNA available in solution and dye-binding sites of DNA available in a nonrandom, fibrous form, perhaps approximating the condition in chromatin.

Once it is established that metachromasy may be used as a tool to detect the relative proximity of anionic sites of DNA exposed on protein removal, it becomes possible to compare DNA-protein binding in different types of cells. The cells used in this study were erythrocytes and parenchymal

cells of frog liver which were chosen so that the nucleoprotein of relatively inactive cells could be compared with that of active cells.

MATERIALS AND METHODS

The material used for this study was liver of *Rana pipiens* which was fixed by freezing at -180°C , dehydrated at -45°C in absolute methanol for 1 wk (23), and then placed in 70% methanol at 60°C for 40 min. The tissue was again dehydrated, embedded in paraffin, and sectioned at $6\ \mu$. This fixation provided uniform tissue with hepatocyte and erythrocyte nuclei easily identifiable.

Several models were used for dye-binding studies. (a) Fibrous calf thymus DNA (obtained from Worthington) was fixed in Carnoy's (alcohol-acetic acid, 3 to 1), embedded in paraffin, and sectioned to be studied in the same way as the tissue. (b) Fibers of DNA-histone were compressed on a Langmuir trough, after dissolving the DNA and histone (obtained from Worthington Corp., Harrison, N.J.) in $0.1\ \text{N}$ NaCl and "floating" on a surface of saturated $(\text{NH}_4)_2\text{SO}_4$ (8). These fibers were also fixed in Carnoy's, embedded in paraffin, and sectioned. (c) Calf thymus DNA in 0.1% solution in $0.3\ \text{N}$ NaCl was combined with azure B in 1/1 phosphorus/dye ratio, and the precipitating dye-DNA fibers were then air-dried on slides, immersed in matched refractive index oil (18), and analyzed directly. Also, a similar solution of DNA was first combined with an excess of histone in solution and then added to azure B, and the fibrous precipitates which formed were then similarly analyzed.

For all sectioned material, azure B was used at a concentration of $0.3\ \text{mg/cc}$ ($10^{-3}\ \text{M}$) in $0.05\ \text{M}$ citrate buffer at pH 4.0. The slides were stained for 30 min at 25°C , and then they were dipped in water, placed in absolute tertiary butyl alcohol (8) for 10 min, xylol, and mounted in matched refractive index oil. Before staining, the slides were treated for 3 hr at 37°C with ribonuclease (Worthington) ($0.02\ \text{mg/cc}$ H_2O) (8), and then protein was extracted at 25°C with trypsin (Worthington) ($0.01\ \text{mg/cc}$ water) or acid. The acid extractions were those found by Holtzman (11) to give reproducible end points: $0.02\ \text{N}$ HCl for 3-18 hr, $0.25\ \text{N}$ HCl in 80% alcohol for 18 hr, and 6% perchloric acid for 30 min (longer times caused DNA extraction). All acid extractions were carried out at 25°C . In all cases, unextracted controls and ribonuclease controls were stained simultaneously.

Absorption curves of dye bound to DNA were measured by means of a Leitz microspectrophotometer with a tungsten light source, a Leitz prism monochromator, and an oil immersion objective (NA 1.32). Areas $4\ \mu$ in diameter were used for light absorption analysis. Hepatocyte and erythrocyte

nuclei were selected at random, so long as they fulfilled the following conditions: hepatocyte nuclei were cut on upper and lower surfaces, and the major axes of the ellipsoidal erythrocyte were oriented in the plane of the section. The latter condition made it possible to measure extinctions that were not too high for accuracy, since the thickness of the absorbing region was 2-3 μ (the minor axis) rather than the 6 μ length of the erythrocyte nuclei oriented perpendicular to the plane of the section. At least five full absorption curves and ten peak extinctions were determined on each slide. No differences among spectra of dye bound within a slide were found except following trypsin digestion, which will be discussed later.

RESULTS

Metachromatic Staining of DNA and DNA-Histone Fibers

Absorption curves of azure B bound to fibers prepared by fixing, embedding, and sectioning are shown in Fig. 1 *a* and *b*. The dye bound to DNA-histone has the same two absorption peaks that appear in the absorption curves of the dye in water solution alone (8), the α -peak of the dye monomer at approximately 650 $m\mu$ and the dimer, β -peak at 590 $m\mu$. The absorption curve of dye bound to the fixed fiber (curve *a*) shows a broad area of absorption at wavelengths shorter than the β -peak, which has been interpreted as trimer or tetramer absorption (20). This μ -peak absorption is more clearly defined at about 550 $m\mu$ in the absorption

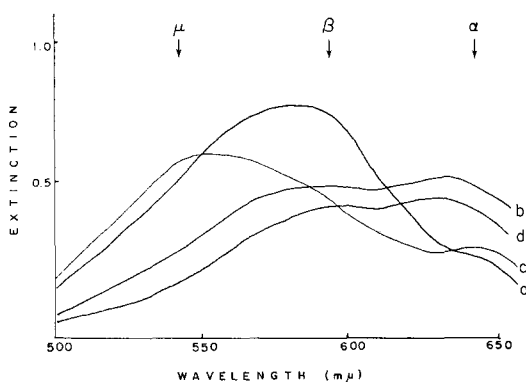


FIGURE 1 Absorption curves of azure B bound to DNA and DNA-histone fibers. *a*, DNA fiber fixed in Carnoy's. *b*, DNA-histone fiber fixed in Carnoy's. *c*, DNA precipitated from solution with azure B. *d*, DNA and histone precipitated from solution with azure B.

curve of dye bound to DNA during precipitation from solution (curve *c*). When azure B is permitted to bind in solution after DNA and histone are mixed, the absorption curve of the resulting precipitate is similar to that of the stained DNA-histone fiber and also similar to that of azure B bound to nuclear DNA (Fig. 2 curves *a*, *b*, and *c*). Carnoy fixation does not appear to alter the metachromatic staining of DNA bound to histone although DNA in solution may be denatured followed Carnoy fixation (14), and cause increased "stacking" of the metachromatic dye molecules bound. The difference in absorption curves *a* and *c* (Fig. 1) when no histone is present is probably not due to Carnoy fixation, since more "dye stacking" or "polymer formation" occurs in the unfixed DNA. These results offer strong support to the idea that metachromasy depends on protein association rather than on the single (denatured) or double strandedness of the DNA molecule when the DNA is not in solution.

Nuclear DNA Staining

The curves, presented in Figs 2 and 3 of dye bound to DNA of hepatocyte and erythrocyte nuclei are consistent under the staining conditions used. The relative heights of the α -, β -, and μ -peaks are a much more accurate indication of spectra than is possible by visual judgment of color because there is a great (about fourfold) difference in concentration of DNA in the two kinds of nuclei studied. The morphology of the hepatocyte nuclei is heterogeneous after the freeze-substitution method of fixation, with about five to ten clumps of chromatin scattered in diffuse chromatin. Areas of predominately diffuse chromatin were chosen, but it was impossible to exclude all the condensed chromatin. The erythrocyte nuclei appeared homogeneously condensed. The nuclei maintained their morphological appearance after all of the treatments except prolonged trypsin digestion when nuclear dissolution seemed to occur. This effect of trypsin was variable; nuclei at the surface of the section seemed more affected than others; also, within a nucleus the diffuse chromatin occasionally was affected before the condensed clumps of chromatin.

The similarity in the shape of the absorption curves of dye bound to DNA of erythrocytes and hepatocytes is evident following each of the methods of histone extraction in all the curves of Figs. 2 and 3. This is also indicated in Table I, in

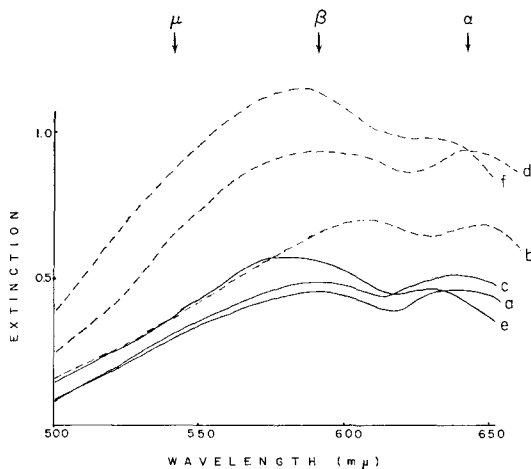


FIGURE 2 Absorption curves of azure B bound to DNA of *Rana pipiens* liver nuclei. Hepatocytes, —; erythrocytes, ---- a, b. RNase, no protein extraction. c, d. RNase followed by acidified alcohol extraction. e, f. RNase followed by 0.02 N HCl extraction.

which the extinctions at the β - and α -peak are compared. Following perchloric acid extraction and acidified alcohol extraction, there may be increased dye binding but the shape of the absorption curve shows no change. Extraction with 0.02 N HCl does cause a change in dye binding, with a relatively higher amount of β -peak absorption (indicating dimer formation), but no distinct μ -peak is seen (Fig. 2, curve e and f). The difference in azure B absorption after prolonged trypsin digestion is clear in Fig. 3, in which a distinct μ -peak is present.

DISCUSSION

The metachromatic color changes of a cationic dye bound to nucleic acids in solution are clearly correlated with the relative amounts of dye and substrate present and with the physical condition of the nucleic acid. More dye aggregation or "stacking" will occur following denaturation of DNA when concentrations are kept constant (5,14). The difficulties in comparing DNA in solution with nuclear DNA in tissue sections are mainly due to two variables; one, the proportions of dye to substrate cannot be controlled when the substrate is solid, and two, the competition between dye and protein amino groups for nucleic acid anionic sites must be considered. In the work presented here, the dye concentration, pH, temper-

ature, and time have been kept constant so that protein interference could be investigated. It is possible that some of the protein-extraction procedures used might also denature DNA and cause increased metachromasy for this reason, but this seems unlikely since without protein extraction neither Carnoy fixation nor heat appeared to have any effect on the spectra of dye subsequently bound to nuclear DNA. However, the possibility cannot be ruled out that the native conformation of DNA is stabilized by its associated protein and that extraction of this protein leaves the DNA in a form that is then readily denatured. Native DNA in solution (which does not shift azure B absorption to lower wavelengths) differs markedly from solid, native DNA which binds azure B with μ -peak absorption (Fig. 1, curve c). This is probably caused by the nonrandom position of adjacent molecules of fibrous DNA so that dye molecules attached to phosphate sites of different neighboring molecules are close enough to interact (13). The only possible way to approximately duplicate for nuclear DNA the dye-binding capacity of DNA fibers was by prolonged trypsin digestion, which removes histone and nonhistone protein. This removal of protein presumably allows more dye molecules to bind in positions which permit greater metachromasy to occur. A similar result was found in acridine orange binding (16), in which the ribonuclease which had blocked dye binding was removed. No effect of ribonuclease blocking was encountered in any experiments in the present

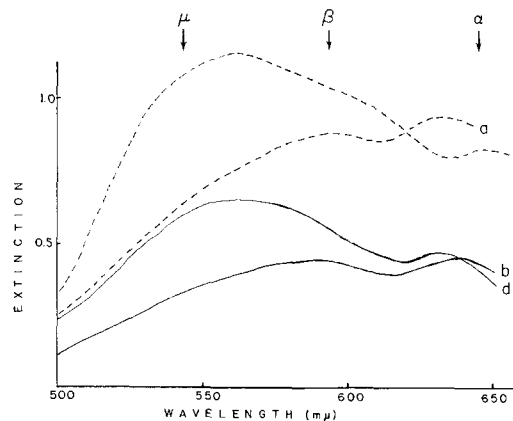


FIGURE 3 Absorption curves of azure B bound to DNA of *Rana pipiens* liver nuclei. Hepatocytes, —; erythrocytes, ---- a, b. RNase followed by trypsin, 10 min c, d. RNase followed by trypsin, 1 hr.

TABLE I
Metachromatic Changes in Azure B

Method of extraction	Hepatocyte β/α E ₅₅₀ /E ₆₄₀	Color	Erythrocyte β/α E ₅₅₀ /E ₆₄₀
Untreated	0.94	Blue-green	1.04
Perchloric acid	0.98	"	0.95
Acidified alcohol	1.02	"	1.04
0.02 N HCl	1.38	Blue	1.45
Trypsin, 10 min	1.00	Blue-green	1.02
Trypsin, 2 hr	μ -peak absorption	Purple	μ -peak absorption
Azure B in water 10 ⁻³ M	1.44		
" 10 ⁻⁴ M	0.88		

work. The μ -peak absorption of azure B bound to nuclear DNA after trypsin (Fig. 3) suggests that nonhistone protein must be removed before dye molecules can bind to adjacent DNA molecules, and must therefore, be bound in cross-linkage either directly to DNA or to histone. It is impossible to distinguish between these two possibilities since it is impossible to extract nonhistone protein without also extracting histone.

Since no differences in DNA-protein linkages in condensed and dispersed chromatin were demonstrated, as indicated by the similar absorption curves through erythrocyte and hepatocyte nuclei stained with azure B, one may conclude that (a) the differences are too small to be detected by these methods, or (b) of a nature not revealed by the extraction procedures, (c) the mild fixation may nonetheless have altered the DNA-protein binding,

or (d) there are no differences, in which case the degree of condensation of the DNA may be independent of its association with histone. The author favors the last hypothesis.

The methods of histone extraction may distinguish between "lysine-rich" histone (extraction with 0.02 N HCl) and "arginine-rich" histone (perchloric acid-extractable) (11), although chemical analysis of these fractions has not yet been performed. The evidence presented here suggests that "arginine-rich" histones are bound on the DNA at distant sites, where dye molecules that replace them are too far apart to interact, while the "lysine-rich" histones are bound on adjacent sites, since dye molecules replacing them show greater metachromasy.

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