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THE BINDING OF NUCLEAR NON-HISTONE PROTEIN TO DNA

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Summary

The binding of rat liver nuclear non-histone proteins to DNA has been detected using a membrane filter technique. Binding is optimal at 0.05 M NaCl, increases with decreasing pH, and is enhanced in the presence of Mg^{2+} . About 50% of the DNA is bound reversibly. Histone contamination of non-histone preparations contributes little, if at all, to the observed binding while cytoplasmic proteins, isolated like non-histones, have no ability to bind DNA. The binding activity, which is unstable, can be destroyed by heat or pronase.

Introduction

Nuclear non-histones have recently been implicated in cellular regulation, being capable of specifically controlling DNA template capacity [1–7]. These findings have spurred interest in the development of fractionation techniques and methods of analysis for non-histones. Some fractionation has been achieved, utilizing gel electrophoresis for analysis [8–11]. Other assays for non-histones take advantage of their ability to interact with DNA. Binding has been demonstrated by co-sedimentation of ^{32}P -labelled non-histone phosphoproteins and DNA [12] or by DNA-cellulose columns [10,13]. However, such assays are time consuming and may require large amounts of DNA and non-histone. It would be desirable to have an assay for DNA–non-histone interaction which is rapid, reliable, and uses small amounts of material.

This paper describes such a system for non-histone binding to DNA, based on a nitrocellulose membrane filter assay [14]. In this procedure, labelled native DNA passes through the filter while protein is retained. In a solution of the two, DNA bound by non-histone is also retained.

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Rat liver non-histone was isolated by a relatively gentle procedure in which chromatin DNA is removed by ultracentrifugation and non-histones are separated from histones by ion-exchange chromatography. Non-histones were free of histone contamination and capable of binding to ^{125}I -labelled DNA. Basic parameters affecting the binding, such as salt and Mg^{2+} concentrations, pH dependence, and non-histone stability were determined.

Materials and Methods

DNA purification

Rat liver DNA was exhaustively purified by a combination of previously described methods (refs 15 and 16 and Hamilton, F. and Simpson, M.V., unpublished). Additionally, after the ribonuclease step, the DNA solution was treated with α -amylase (Worthington, hog pancreas, 665 $\mu\text{g}/\text{mg}$) at 20 $\mu\text{g}/\text{ml}$ and 37°C until clearing was complete. The DNA contained < 0.03% protein [17] and < 0.13% RNA [18]. An $S_{20,w}$ of 37.6 ± 0.9 was determined by velocity sedimentation in a Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics, monochromator, scanner, and multiplexer.

DNA labelling

Aliquots (50 μg) of rat DNA were labelled with Na^{125}I [19]. Sephadex columns and ammonium acetate— NH_4OH were freshly prepared for each experiment. Stock Na^{125}I (New England Nuclear, 50 Ci/l, carrier free) was replaced every month, while iodinated DNA was used within 4 days after labelling.

Preparation of nuclei

Nuclei were isolated from livers of male Sprague—Dawley rats (150–250 g), essentially as described by Blobel and Potter [20]. The procedure was modified for larger quantities of tissue by centrifuging in a Beckman SW 25.2 rotor at $75\,000 \times g$ for 50 min. Nuclei were washed twice in 0.15 M NaCl (30–40 ml per 15 g liver), with centrifugation at $1000 \times g$ for 10 min in the Sorvall SS-34 rotor. The pellet was suspended twice in 0.01 M Tris (pH 8.3) and centrifuged at $3000 \times g$ for 10 min. The swollen gel was compacted by a final centrifugation at $27\,000 \times g$ for 10 min.

Preparation of non-histone

Non-histone was prepared by chromatography in urea, employing the method of Richter and Sekeris [9], with minor modifications. Chromatin (from 15 to 30 g of liver) was dissolved in high salt and urea to give a DNA concentration of about 1 mg/ml. After centrifugation for 18 h in a Beckman Type 65 rotor ($269\,000 \times g$), the supernatant salt concentration was lowered to 0.05 M NaCl by chromatography on a 2.5 cm \times 43 cm Sephadex G-25 column at room temperature. Desalted samples were applied to a 2 cm \times 5 cm column of Bio Rex 70* (Bio Rad) precycled according to Bonner et al. [21]. Flow rates were 0.3–0.5 ml/min; protein was detected by $A_{230\text{nm}}$ or colorimetric assay [17].

Preparation of cytoplasmic proteins and histones

Cytoplasmic proteins were prepared from an aliquot of the high-sucrose supernatant from the nuclear preparation after centrifugation at $300\,000 \times g$ in a Beckman SW 65 L rotor for 1 h at 4°C . The supernatant was treated with high salt and urea, centrifuged, and chromatographed as described for non-histone isolation.

Histones were isolated from nuclei washed, successively, first in 0.05 M sodium cacodylate (pH 7.5), 0.015 M KCl, 0.005 M MgCl_2 , and 0.25 M sucrose, and then in 0.02 M sodium cacodylate (pH 7.5), 0.005 M MgCl_2 , and 0.25 M sucrose. Nuclei were treated three times with cold 0.25 M H_2SO_4 and the acid extract was precipitated overnight in the freezer with 2 vol. of 95% ethanol. Histones were stored frozen in 0.01 M HCl.

Binding of non-histones to DNA

Non-histone from the Bio Rex column in urea and 0.05 M NaCl was dialyzed overnight at 4°C against 0.05 M NaCl and 0.01 M Tris-HCl (pH 8.3), unless otherwise stated. After a further 2-h dialysis against fresh buffer, samples were centrifuged at $27\,000 \times g$ for 10 min to remove a slight precipitate and the supernatant protein was quantitated [23].

Non-histone (5–100 μl) was mixed with ^{125}I -labelled DNA and unlabelled DNA to give the final desired concentrations, generally 1 $\mu\text{g}/\text{ml}$ DNA (about 50 000 cpm/ml) and 0–20 $\mu\text{g}/\text{ml}$ non-histone in 0.8 ml. After 20 min at room temperature, triplicate 0.2-ml samples were filtered. Magnesium acetate was included where indicated. Non-histone was used within 24 h after the end of dialysis.

Filtration methods

The filtration method used was essentially that of Riggs et al. [14]. Nitrocellulose membrane filters (Type B6, Schleicher and Schuell) were individually wetted and treated with 0.5 M KOH [23] for 20 min at room temperature. They were rinsed in distilled water and soaked in several buffer changes for at least 30 min before use.

Up to nine samples were filtered simultaneously by use of a porous plastic plate to hold the filters. The vacuum was adjusted so that a 0.2-ml sample passed through the filter in 30–60 s. The filters were then washed with 0.5 ml buffer and counted for radioactivity. Unless otherwise indicated, all results are averages from triplicate filters.

Liquid scintillation counting

Aqueous samples or wetted filters were solubilized in 5 ml of scintillation fluid [24] and counted (with external standard quench correction) in a Beckman LS-230 scintillation counter.

Urea purification

Urea solutions (about 8 M) were deionized by passage over a column of AG-501 \times 8 (Bio Rad), followed by decolorization with Norit A and filtration through Whatman No. 1 filter paper, a Millipore prefilter, and a 0.65- μm pore-diameter Millipore filter. The final urea concentration was determined refractometrically.

Gel electrophoresis

Acid-urea gels were run according to Panyim and Chalkley [25]. Samples were dialyzed into 6.25 M urea, 1% 2-mercaptoethanol, 0.9 M acetic acid. Under these conditions, histone F3 is reduced, and virtually all of the protein enters the gel [26]. Histone contamination of non-histone preparations was determined by quantitative densitometry of gels containing non-histone alone or non-histone with added rat liver histones.

Results

Preparation of non-histones

Recovery of total proteins (histones and non-histones) from the Bio Rex 70⁺ column was approx. 90%. The minimum recovery of non-histones applied to the column was 75%. The non-histone fraction which eluted unretarded from the Bio Rex column in 0.05 M NaCl constituted about 20% of the total recovered non-histones. This unretarded non-histone fraction consisted of over 98% non-histone proteins; less than 2% of the material exhibited the mobility of histones when examined by quantitative gel electrophoresis. The remaining recovered non-histones can be eluted in 0.35 M NaCl, but exhibited definite histone contamination. Histones could be recovered by elution of the Bio Rex column with 15% guanidine · HCl.

Binding of non-histone to DNA

Fig. 1 shows that the binding of non-histone to DNA is quite rapid, and appears to be stable for at least 50 min. The very early time points are only approximate since filtration takes 30–60 s. A standard incubation time of 20 min was chosen for further studies.

The influence of filtration speed is shown in Fig. 2. As the speed of filtration increases, more counts are retained. Because the flow characteristics differ among filters, rapid filtrations (< 10 s) can lead to variable results. All assays reported here are from 30–60-s filtrations.

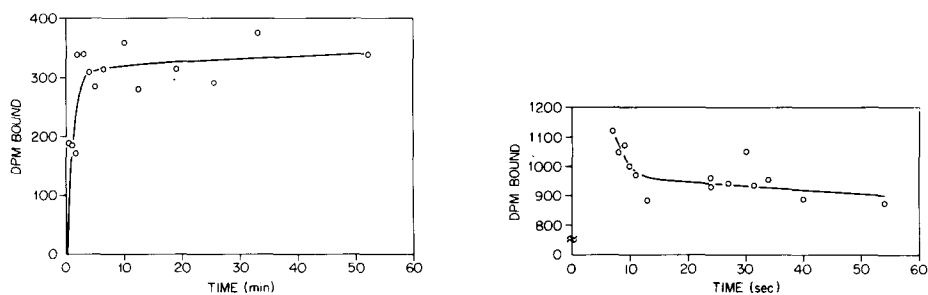


Fig. 1. Time dependence of DNA–non-histone interaction. Non-histone and DNA (0.4 and 1.0 $\mu\text{g}/\text{ml}$, respectively) in 0.05 M NaCl and 0.01 M Tris (pH 8.3) were filtered after the indicated incubation time and counted.

Fig. 2. Effect of filtration rate on complex retention. Complexes formed at about half saturation were assayed with 1.0 $\mu\text{g}/\text{ml}$ DNA in 0.05 M NaCl and 0.01 M Tris (pH 8.3). The filtration rate is defined as the time required for a 0.2-ml sample to pass through a filter. Each point was determined from a single filter.

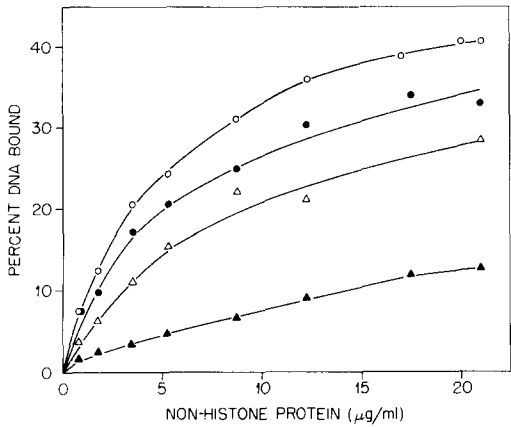


Fig. 3. Salt dependence of DNA-non-histone interaction. Non-histone in 0.05 M NaCl and urea was dialyzed against, and assayed in, the indicated NaCl concentrations (○—○, 0.05; ●—●, 0.01; △—△, 0.10; ▲—▲, 0.15 M) in 0.01 M Tris (pH 8.3). The DNA concentration was 5 μg/ml.

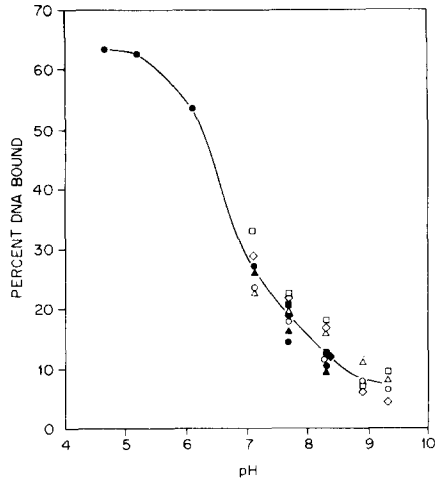


Fig. 4. Effect of pH on binding. Samples dialyzed from urea into 0.05 M NaCl and 0.01 M Tris-HCl (pH 8.3) were diluted into Tris-HCl (○), or Tris-maleate (●); Tris-HCl (△), or Tris-maleate (▲), each containing 50 μg/ml bovine serum albumin. Another group of samples were dialyzed from urea into 0.05 M NaCl, 0.01 M Tris (-HCl or -maleate), at the indicated pH. These were then diluted into the same buffer in the absence of bovine serum albumin in Tris-HCl (□) or Tris-maleate (◆), or in the presence of bovine serum albumin (50 μg/ml) in Tris-HCl (◇) or Tris-maleate (◆). The indicated pH is the final pH in the binding mixture which contained 1 μg/ml DNA.

Salt dependence of non-histone and histone binding

As indicated in Fig. 3, non-histone binding is optimal at 0.05 M NaCl. Lower salt concentrations (0.01 M) reduce binding about 20% while high salt (0.15 M) results in a 90% decrease in binding, as measured by the amount of protein required to bind the same quantity of DNA.

In contrast, the interaction of histone with DNA is not significantly affected in the range 0.05–0.20 M NaCl, but is reduced about 4-fold at 0.40 M NaCl.

Effect of pH on binding

Fig. 4 illustrates that apparent binding increases as the pH is lowered. It seems to make no difference whether non-histone is diluted or dialyzed into buffer, whether bovine serum albumin is present, or if Tris-HCl or Tris-maleate is the buffer. Fig. 5 shows complete titration curves at pH 7.0 and pH 8.3; the curves differ in final saturation values, but do not differ significantly in the protein concentration required to reach half saturation.

The effect of pH on the dissociation of complexes is shown in Fig. 6. The complex is stable at pH 8.3 for 90 min (Fig. 6A), whereas at pH 7.0 (Fig. 6B) there is a time-dependent decrease in binding. Fig. 6C shows that the rate of dissociation is the same at both pH values; about 50% of the DNA is bound reversibly by non-histone.

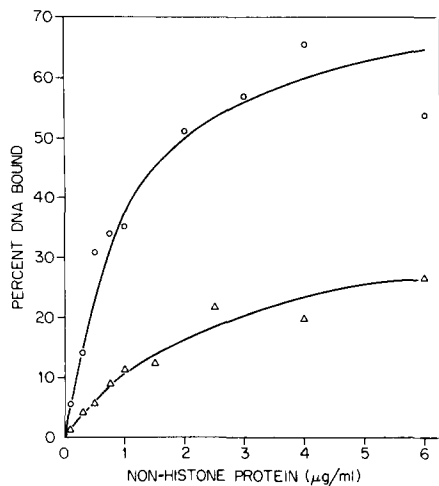


Fig. 5. Titration of DNA with non-histone at pH values 8.3 and 7.0. Non-histone was diluted from 0.05 M NaCl and 0.01 M Tris (pH 8.3), into DNA solutions (1.0 $\mu\text{g/ml}$) containing 0.05 M NaCl and 0.01 M Tris, to give a final pH of 7.0 (\circ — \circ) or 8.3 (Δ — Δ) in the binding mixture.

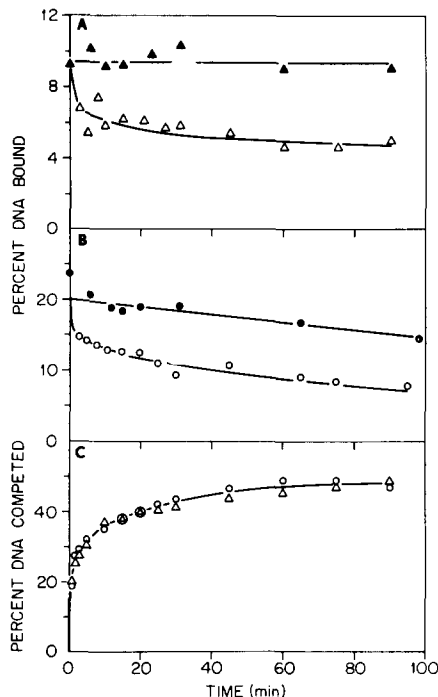


Fig. 6. Reversibility of DNA—non-histone binding at pH values 8.3 and 7.0. Complexes were formed at 1 $\mu\text{g/ml}$ DNA and 0.5 $\mu\text{g/ml}$ non-histone. After a 20-min incubation, a 14.6-fold excess of unlabelled DNA (competition) or the corresponding volume of buffer (control) was added. Samples were removed and filtered at the indicated times after addition of excess DNA. (A) pH 8.3: competition (Δ — Δ), control (\blacktriangle — \blacktriangle); (B) pH 7.0: competition (\circ — \circ), control (\bullet — \bullet); (C) percent of DNA competed at both pH values.

The effect of Mg^{2+} on binding

Mg^{2+} apparently increases the retention of protein on nitrocellulose filters [14,27]. In preliminary experiments, binding of non-histone to DNA increased with Mg^{2+} concentration, plateauing at 10 mM. The salt dependence of binding in the presence of 10 mM Mg^{2+} is similar to that shown in Fig. 3, although the optimum is shifted downward, probably due to the increased ionic strength.

Titration curves extrapolate to 100% of the input DNA on double-reciprocal plots under certain conditions. At pH 8.3, Mg^{2+} is required for binding of all of the input DNA, whereas at pH 7.0, 100% binding will occur in the presence or absence of Mg^{2+} . In all cases, however, the half-saturation values are virtually identical.

Stability of binding activity

About 50% of non-histone binding activity is lost in 12 h. The presence of Mg^{2+} (35 mM) greatly accelerates this loss; in 2 h about 75% of the activity is lost. Thus far, no conditions tested have stabilized binding activity.

The binding activity is destroyed by boiling for 5 min or by pronase

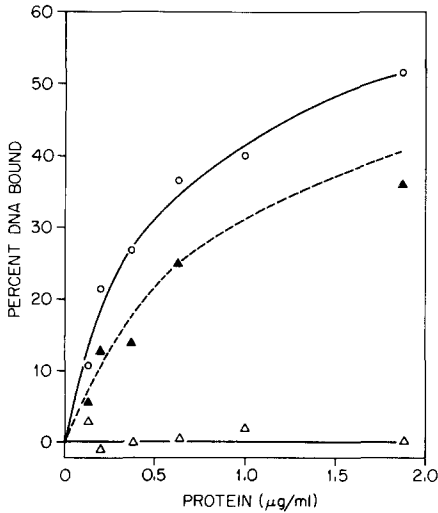


Fig. 7. Binding of non-histone and cytoplasmic protein to DNA. Non-histone protein (○—○), cytoplasmic protein (△—△), and a mixture of the two (▲—▲) were dialyzed to remove urea as described in Materials and Methods. Assays were performed at 1 μg/ml DNA in 0.05 M NaCl, 0.01 M magnesium acetate, and 0.01 M Tris (pH 8.3). The points for the mixture were experimentally determined, while the dashed line represents expected non-histone activity in the mixture.

treatment. Activity is not due to trapping of DNA by protein at the filter surface: filtration of protein alone, followed by DNA, results in only background retention. The time-dependent association of DNA and non-histone also argues against a filter artifact.

Binding of other proteins to DNA

Titration curves show that bovine serum albumin exhibits 10^{-2} – 10^{-3} as much binding activity as non-histone. Similarly, cytoplasmic proteins, isolated in the same way as non-histone, are unable to bind DNA (Fig. 7). A mixture of cytoplasmic proteins and non-histone exhibits the level of activity expected with non-histone alone. Thus the cytoplasmic protein preparation contains no factor which inhibits or abolishes binding activity.

Discussion

The non-histone preparations used in this work are freely soluble and essentially devoid of histone contamination, both of which are necessary in the assay system. From studies of the salt dependence of non-histone and of histone binding to DNA, we estimate that if all non-histone binding to DNA in 0.05 M NaCl were due to histone proteins, then histone contamination could contribute a maximum of 10% to the observed binding activity; i.e. non-histone binding to DNA in 0.15 M NaCl is about 10% of that in 0.05 M NaCl. We consider this very unlikely, however, because quantitative gel electrophoresis indicated that our non-histone preparation consisted of > 98% non-histone protein. This, together with the response to boiling and pronase, makes it virtually certain that binding activity is a function of non-histone, and not of a contaminant.

The salt optimum (0.05 M NaCl) found with this non-histone preparation is higher than that described for phenol-extracted phosphoprotein [12] (0.01 M NaCl). The salt dependence of other preparations [10,13] has not yet been characterized.

The pH curve may reflect the efficiency of protein retention on the filter (similar to the Mg^{2+} effect) or may indicate an effect on protein-nucleic acid interaction. The finding of similar half-saturation values and rates of dissociation at pH values 8.3 and 7.0 suggest that the pH effect is primarily on retention. It certainly appears that retention is incomplete at pH 8.3 in the absence of Mg^{2+} . A similar pH effect with nitrocellulose filters has been found by Lever [28].

The largest variation of binding data from different non-histone preparations is about 2-fold: much or all of this variation can be ascribed to loss of binding activity with time.

The capacity to interact with DNA is not a general property of proteins: neither bovine serum albumin nor cytoplasmic proteins are effective at binding to DNA. At least one component in non-histone preparations is essentially irreversibly bound to DNA, a finding consistent with data on non-histone binding to DNA-cellulose columns [10,13].

Several groups have reported that at least some non-histones are capable of interacting specifically with the DNA of origin [10,12,13]. Preliminary results with this system have shown such specificity, and work is continuing in this direction.

The virtues of the assay described in this paper are speed, simplicity, sensitivity, and reproducibility, which should make it a useful tool in the study of DNA-non-histone interaction.

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