Histone Affinity Chromatography As a Tool for Fractionating Nonhistone Chromatin Proteins and Studying Histone-Nonhistone Protein Interactions*

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The interaction between histone and nonhistone chromatin proteins has been investigated by histone affinity chromatography, a technique that avoids precipitation of histone-nonhistone complexes. When nonhistone proteins labeled with $^{32}$P are loaded directly on histone-Sepharose columns, no binding of radioactivity to the column is observed. However, if labeled nonhistone proteins are mixed with histone-Sepharose in the presence of 4 M urea and 2 M NaCl, and the salt concentration then gradually lowered by gradient dialysis, elution of the histone-Sepharose with increasing NaCl concentration reveals the presence of several peaks of bound radioactivity which are not observed with control columns (blank Sepharose or cytochrome c linked to Sepharose). Columns of histone H2B bind more labeled nonhistones than do columns of histone H1 and the radioactive proteins are eluted at higher salt concentrations. A given peak, rechromatographed on the same histone column, is eluted at the same NaCl concentration originally required for elution. However, when rechromatographed on another type of histone column, the same peak is eluted at an NaCl concentration different from that required for the original elution. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of the radioactive peaks eluted from the histone columns shows a number of highly radioactive proteins, some binding to both histone columns, and others binding preferentially to either histone H2B or H1. These experiments therefore suggest that some nonhistone proteins, particularly the phosphorylated ones, bind to histones in a selective fashion.

Both histone and nonhistone chromatin proteins are thought to be involved in regulating chromatin structure and function. The histones function mainly by packing DNA into compact structures known as $v$ bodies or nucleosomes (1-3). This complexing of DNA with histones generally renders the DNA inefficient as a template for RNA synthesis (4). The phosphorylation of nonhistone chromatin proteins, on the other hand, appear to be responsible for reversing this general template inhibition and for determining which genes are to be expressed (5-7). Recently, it has been demonstrated that the phosphorylation of nonhistone chromatin proteins is intimately involved in this process of regulating gene expression (7-10).

The presence of both histone and nonhistone proteins in chromatin raises the possibility that these two types of protein directly interact with each other. This question was first investigated by Wang and Johns (11) who found that histones and nonhistones precipitate in specific patterns when mixed together. In the present studies we present a new method for investigating histone-nonhistone chromatin protein interactions. To avoid precipitation of basic histones by the acidic nonhistone proteins, we have immobilized the individual histones by attaching them to a Sepharose matrix, which is then used as a matrix for affinity chromatography of nonhistone proteins. By using this approach to fractionate nonhistone proteins on the basis of their affinity for histones H1 and H2B, we have obtained evidence for a class of nonhistone proteins which interact specifically with histones.

EXPERIMENTAL PROCEDURES

Isolation of Histone and Nonhistone Chromatin Proteins—Fresh beef liver was obtained from a local slaughterhouse, cut into small pieces, and kept in homogenization medium (0.32 M sucrose, 3 mM MgCl$_2$, 0.1 mM PMSF) on ice during transport to the lab. The PMSF was used as described by Noodén et al. (12) to inhibit proteolytic activity, especially degradation of the histones. All subsequent operations were conducted at 4°C. After removing the outer fibrous membrane, the pieces of liver were finely minced with scissors. Fifteen grams of the mince were combined with 45 ml of homogenization medium and homogenized in a Potter-Elvehjem homogenizer using three passes. The resulting homogenate was combined with 90 ml of homogenization medium and filtered through six layers of cheesecloth. These steps were repeated 20 to 30 times on separate aliquots of liver. The resulting 2500 to 3000 ml of homogenate was centrifuged for 7 min at 1000 $\times$ g. The resulting crude nuclear pellet was resuspended in 2.6 M sucrose, 1 mM MgCl$_2$, 0.1 mM PMSF using a Potter-Elvehjem homogenizer at slow speed. Additional 1 mM MgCl$_2$, 0.1 mM PMSF solution was added until the sucrose concentration reached 2.15 M, as determined by refractive index measurement. The volume was then brought to 720 to 900 ml with 2.15 M sucrose, 1 mM MgCl$_2$, 0.1 mM PMSF. The nuclear suspension was centrifuged for 65 min at 76,000 $\times$ g in Beckman SW 27 rotors, with 40-ml buckets. After decanting the supernatant, the nuclear pellets were collected and washed twice by resuspension in 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 4 mM MgCl$_2$, 0.1 mM PMSF, followed by centrifugation for 7 min at 1000 $\times$ g.

This washed nuclear pellet served as the starting material for isolation of nonhistone chromatin proteins by a procedure derived from van den Broek et al. (10). The pellet was washed twice in 75 mM

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The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.
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NaCl, 0.1 mM PMSF, 25 mM EDTA (pH 8) by resuspension with a Potter-Elvehjem homogenizer and centrifugation for 7 min at 10,000 x g. The resulting pellets were resuspended and centrifuged four times in 0.1 mM PMSF, 10 mM Tris-HCl (pH 8) for 10 min at 10,000 x g, then twice for 15 min at 15,000 x g. The small opaque layer between the supernatant and the precipitate was removed. The supernatant was carefully removed. The chromatin pellet was resuspended in 3 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl (pH 8) using a Potter-Elvehjem homogenizer. Additional 3 M NaCl solution was added to give an A260 of 20 to 23 cm⁻¹ and the preparation was stirred for 30 min. The dissociated chromatin was centrifuged in a Beckman 60 Ti rotor for 13 h at 140,000 x g.

The supernatant from this centrifugation was separated by volume into an upper two-thirds and a lower one-third, the latter of which contained DNA. The DNA in the lower one-third was separated from protein by gel filtration chromatography on Bio-Gel A-15m columns (100 to 200 mesh, equilibrated with 3 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl, pH 8.0), each 4 x 80 cm. The protein peak from the columns was combined with the upper two-thirds fraction from the supernatant and dialyzed against 10 volumes of 0.1 mM PMSF, 10 mM Tris-HCl (pH 7) for 2 h, against 10 volumes of 65% saturated (NH₄)₂SO₄, (334 g/milliter at 0°C), 0.1 mM PMSF, 30 mM Tris-HCl (pH 7.0) for 8 h, and finally against 10 volumes of 34% saturated (NH₄)₂SO₄, (481 g/milliter at 0°C), 0.1 mM PMSF, 30 mM Tris-HCl (pH 7.0) overnight. The precipitated proteins were collected by centrifugation, dissolved in 3 M NaCl, 0.1 mM Tris-HCl (pH 7) at a concentration of about 2 mg/ml, and dialyzed against 25 volumes of 0.4 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl (pH 7.0) overnight, with one change of buffer. After removal of a small precipitate by centrifugation, the protein solution was applied to a Bio-Rex 70 column (100 to 200 mesh), precyvled with 3 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl (pH 7) and equilibrated with 0.4 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl (pH 7.0). The histone chromatin proteins were eluted with the 0.4 M NaCl buffer, while the histones were retained and could be eluted with 3 M NaCl buffer. The histone and nonhistone solutions were quick frozen in dry ice/ethanol and stored at −70°C.

Phosphorylation of Nonhistone Chromatin Proteins—Isolated nonhistone chromatin proteins from beef liver contain endogeneous protein kinase activity, which catalyzes the phosphorylation of histone phosphoproteins in vitro in the presence of [γ-³²P]ATP and Mg²⁺ (17). Frozen solutions of nonhistone chromatin proteins, purified as above, were thawed and dialyzed against 30 volumes of 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5) overnight, with one change of buffer. The small amount of precipitate that formed was removed by centrifugation for 10 min at 20,000 x g. Labeling with [³²P]ATP was typically carried out in an incubation mixture containing 0.15 ml of 1 M MgCl₂, 0.8 to 1.3 mg of nonhistones (in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5), 1 to 6 nmol of [γ-³²P]ATP (specific activity, 10 to 30 Ci/mmole), and excess 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5) to give a volume of 6 ml. The reaction mixture was incubated at 37°C for 30 min, then stopped by the addition of 1/4 volume of a 50% trichloroacetic acid, 10% (v/v) acetic acid, and 10% (v/v) 0.1% SDS, 10 mM sodium phosphate, pH 7.2) by heating in a boiling water bath for 5 min. A small amount of bromphenol blue tracking dye was added to each sample and the samples were then pipetted into the wells of the slab gel. The proteins were electrophoresed at 100 V for 6 to 8 h on the short slab or 16 to 18 h on the long slab. The gels were stained with 0.25% Coomassie Blue G-250 (w/v) in 10% (v/v) acetic acid for 2 h at 37°C and destained in ethanol/acetic acid at 37°C. A molecular weight calibration curve was obtained by electrophoresing five proteins with known molecular weights: chymotrypsin (26,000), ovalbumin (43,000), bovine serum albumin (66,000), lactalbumin (14,000), and ovalbumin (66,000).
albumin (67,000), phosphorylase a (94,000), and β-galactosidase (130,000). The calibration curve was then obtained by plotting the log of the molecular weight of the proteins versus their mobilities relative to the tracking dye front.

For autoradiography, the gels were first dried on filter paper as follows. Each slab was soaked in a 10% acetic acid, 1% glycerol solution for 1 h and then placed on pieces of heavy filter paper already saturated with the acetic acid/glycerol solution. The paper-gel complex was placed in a slab gel dryer (Bio-Rad) covered with a layer of Saran Wrap. Alternatively, the gel, after soaking in acetic acid/glycerol, was sandwiched between two pieces of cellophane dialysis membrane (Bio-Rad), and then placed in the gel dryer. The gel was dried for 6 h under aspirator vacuum and heat. After drying, the slab was exposed to Kodak Industrial grade AA x-ray film for a period ranging from 8 h to 2 weeks. After exposure, the x-ray film was developed in Kodak x-ray developer and replenisher for 4 min, and fixed for 15 min with Kodak fixer.

RESULTS

Top-loading Affinity Chromatography—The technique of top-loading nonhistone chromatin proteins on histone-Seph-}

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FIG. 1. Top-loading affinity chromatography of whole nonhistone proteins on Sepharose 4B columns with histone H1 and histone H2B as bound ligands. Approximately 2 mg of nonhistone proteins, isolated as described under "Experimental Procedures," were equilibrated as bound ligands. Approximately 2 mg of nonhistone proteins, isolated and phosphorylated with [γ-32P]ATP as described under "Experimental Procedures," were equilibrated as bound ligands. Approximately 2 mg of nonhistone proteins, isolated and phosphorylated with [γ-32P]ATP as described under "Experimental Procedures," were equilibrated as bound ligands.

Gradient-Dialysis Affinity Chromatography—Because gradient dialysis from high salt/urea is required to reconstitute chromatin from isolated DNA, histones, and nonhistones (5, 20), we reasoned that a similar approach might be useful in studying interactions between nonhistones and histone-Sepharose. When 32P-labeled nonhistone chromatin proteins are similarly tested by top-loading on histone-Sepharose columns, no binding of radioactivity is detected at all (Fig. 2). The top-loading procedure without the urea produced identical results except that removal of the urea from the sample before loading caused a substantial loss of protein due to precipitation.

Fig. 1. Top-loading affinity chromatography of whole nonhistone proteins on Sepharose 4B columns with histone H1 and histone H2B as bound ligands. Approximately 2 mg of nonhistone proteins, isolated as described under "Experimental Procedures," were equilibrated as bound ligands. Approximately 2 mg of nonhistone proteins, isolated as described under "Experimental Procedures," were equilibrated as bound ligands. Approximately 2 mg of nonhistone proteins, isolated as described under "Experimental Procedures," were equilibrated as bound ligands.

Fig. 2. Top-loading affinity chromatography of 32P-labeled nonhistone proteins on a Sepharose-histone H1 affinity column. Nonhistone proteins were isolated and phosphorylated with [γ-32P]ATP as described under "Experimental Procedures." After equilibration with 4 mM urea, 0.1 mM PMSF, 10 mM sodium phosphate (pH 6.7), the radioactive nonhistone proteins were top-loaded onto the column, washed through with 4 mM urea, 0.1 mM PMSF, 10 mM sodium phosphate (pH 6.7), and eluted as described in Fig. 1. After collecting 1.5-ml fractions, 0.1-ml aliquots of each fraction collected were dried on filter paper (Whatman No. 3MM) and counted by liquid scintillation methods. Note that the profiles of the histone affinity column and the blank Sepharose column are identical.
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Sepharose run under the same conditions. Likewise, little radioactive nonhistone chromatin protein binds to columns of Sepharose-bovine serum albumin or Sepharose-cytochrome c, even though cytochrome c is a small, basic protein like histone.

The behavior of the radioactive nonhistone chromatin proteins on the two histone affinity columns suggests that significant differences exist in the interactions between these two histones and the nonhistone chromatin proteins. Not only does the H1 affinity column bind less total radioactivity (39%) than the H2B affinity column (65%), but the labeled nonhistone proteins are also eluted from the H2B column at higher salt concentrations (Table 1). The largest peak of 32P is eluted from the H1 column by 0.3 M NaCl, but is not eluted from the H2B affinity column until 0.5 M NaCl. Finally, most of the radioactivity can be removed from the H1 affinity column with the 0.3 M NaCl wash, while a large amount of radioactivity still remains bound to the H2B affinity column after the 0.3 M NaCl wash.

Electrophoretic Analysis of Eluted Fractions—SDS-polyacrylamide gel electrophoresis and autoradiography of the gels were performed on aliquots of 32P-labeled nonhistone protein fractions eluted from the histone-Sepharose columns after gradient dialysis. Photographs of a typical gel and autoradiogram are shown in Fig. 4. Several points should be noted. First, there are significant differences in the behavior of individual nonhistone proteins in binding to histones. For example, band 5 binds to histone H2B and is eluted primarily at 0.5 M NaCl, while bands 1, 2, and 4 are more tightly bound, not being eluted until the salt concentration is raised to 2.0 M NaCl. Furthermore, the various nonhistone proteins exhibit different affinities for the two types of histone affinity columns. Thus, band 5 can be seen to bind strongly to histone H2B, not being eluted until the salt concentration reaches 0.5 M NaCl. In contrast, this same band does not bind to histone H1 at all, appearing in the runoff.

Second, comparison of the autoradiograms with the stained

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**Table 1**

<table>
<thead>
<tr>
<th>Sepharose-bound ligand</th>
<th>Runoff</th>
<th>0.15 M</th>
<th>0.3 M</th>
<th>0.5 M</th>
<th>2.0 M</th>
<th>pH 2</th>
</tr>
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<tr>
<td>H1</td>
<td>736</td>
<td>61</td>
<td>136</td>
<td>258</td>
<td>40</td>
<td>66</td>
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<tr>
<td>H2B</td>
<td>418</td>
<td>35</td>
<td>100</td>
<td>206</td>
<td>265</td>
<td>22</td>
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</tbody>
</table>

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**Fig. 3.** Gradient-dialysis affinity chromatography of 32P-labeled nonhistone proteins on Sepharose 4B columns with cytochrome c (cyto c), histone H1, or histone H2B as the bound ligand. Nonhistone proteins were phosphorylated in vitro, combined with histone-Sepharose affinity material via gradient step dialysis, and eluted from the columns with steps of increasing NaCl as described under "Experimental Procedures." Radioactive profiles were determined by counting 0.1-ml aliquots of each 2.0-ml fraction by liquid scintillation methods. Each eluted peak was then expressed as a percentage of the total counts per min recovered. The experiment was repeated four times and the data below, taken from one experiment, are typical of the pattern obtained. The total counts per min recovered from an affinity column ranged from 45 to 65% of the total counts per min added before starting gradient dialysis.
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FIG. 4. Autoradiograms and SDS-polyacrylamide electrophoresis gels of eluted peaks of $^{32}$P-labeled nonhistone proteins from histone affinity columns. Details of the methods are given under "Experimental Procedures." The left-hand member of each pair of strips is a photograph of a slab gel channel stained with Coomassie blue, while the right-hand member is a photograph of the corresponding autoradiogram. Molecular weights are determined as described under "Experimental Procedures." The runoff from the control column is the unbound peak from the blank affinity column. Band 2 is a highly labeled protein with a molecular weight of 93,000. Bands 1 and 3 are examples of the numerous bands that are common to both histone affinity columns, and Bands 4 and 5 appear to bind to the histone H2B column only. As the stained stripes indicate, the runoffs of the histone columns contained most of the protein applied.

Rechromatography of Eluted Peaks—In order to investigate the specificity of binding between the various eluted peaks of $^{32}$P-labeled nonhistone chromatin proteins and histones H1 and H2B, peaks of radioactivity eluted from one histone column were rechromatographed on either the same or another type of histone affinity column. The results of a typical experiment are shown in Fig. 5 (peaks from the H1 column) and Fig. 6 (peaks from the H2B column). These experiments indicate there is a high degree of cross-reaction between the two histone affinity columns. Most of the radioactive nonhistone chromatin proteins that bind to one histone also show affinity for the other histone. Thus, overall, there appears to be only a limited degree of binding specificity of the in vitro $^{32}$P-labeled nonhistone chromatin protein to one histone to the exclusion of the other. However, in all cases, any particular peak of radioactive nonhistone chromatin protein must be eluted from the H2B column with a higher NaCl concentration than from the H1 column. For example, the rechromato-

FIG. 5. Rechromatography of $^{32}$P-labeled nonhistone proteins on histone affinity columns. The peaks of radioactivity originally eluted at either 0.3 or 0.5 M from an initial histone H1 affinity column (see Fig. 3) were divided in half. One-half of each peak was rechromatographed on a histone H1 affinity column (---) while the other half was rechromatographed on a histone H2B affinity column (---) (see "Experimental Procedures" for details). After collecting 1.5-ml fractions, radioactivity profiles were obtained as described in Fig. 2. The data are plotted as a per cent of the total counts per min eluted from each column. Gradient dialysis techniques were used in all chromatographic runs.
Histone Affinity Chromatography

These proteins to histones versus cytochrome c.

Specificity in the interaction between 32P-labeled beef liver proteins were labeled with 32P under the same conditions used to determine whether the preferential binding to histones HI and H2B occurs under gradient dialysis conditions, and there are no signs of a preferential binding of these proteins to histones versus cytochrome c.

Our results indicate the existence of a limited degree of specificity in the interaction between 32P-labeled beef liver nonhistone chromatin proteins and histones H1 and H2B. Four lines of evidence lend support to this conclusion. First, no binding of 32P-labeled nonhistone proteins to serum albumin or to cytochrome c, a protein similar to histones in size and charge, is seen. Second, the 32P elution profiles of the two histone affinity columns (H1 and H2B) differ, suggesting a difference in the affinity of these two histones for phosphorylated nonhistones. Third, polyacrylamide gel profiles reveal the existence of certain nonhistone proteins which preferentially bind to one histone versus the other. And fourth, a given peak of eluted nonhistone proteins elutes at the same NaCl concentration when rechromatographed on the same type of histone column, but not when rechromatographed on another type of histone column.

Although it is attractive to assume that these fractionation results are based upon biologically relevant interactions between histones and nonhistones, one must consider the possibility that histone-Sepharose is simply acting as an ion exchanger. Second, gradient dialysis is required to obtain preferential binding of nonhistones to the histone-Sepharose, while the classic top loading technique used for ion exchange chromatography does not work (Fig. 2). The technique of gradient dialysis is well known to be required for reconstituting functional interactions between isolated chromatin components (5, 20). And finally, 32P-labeled proteins present in the cytoplasm do not preferentially bind to histones versus cytochrome c, indicating that our observed results are selective for nonhistone phosphoproteins.

The existence of specific interactions between histone and nonhistone proteins is a question that has not received much consideration, although the idea that these interactions play a role in gene regulation is not new (21, 22). Wang and Johnson (11) showed that histone-nonhistone complexes precipitate out of solution in a specific manner, while Kaplowitz et al. (23) showed that histone-nonhistone interaction greatly enhanced the rate of nonhistone protein phosphorylation, with histone H1 stimulating phosphorylation the most and H2B stimulating the least. In studies on DNA-cellulose columns, Noodén et al. (2) also found that a group of nonhistone chromatin proteins which do not bind to DNA-cellulose alone (13) do bind when the DNA is previously loaded with histone. The present demonstration of selective interactions between individual nonhistone chromatin proteins and histone H1 and H2B using gradient dialysis affinity chromatography further supports these earlier indications that nonhistone-histone interactions may be important in chromatin structure and function. In addition, this technique provides a new method for fractionating nonhistone chromatin proteins on what could be a functional basis and may prove useful in purifying nonhistone chromatin proteins for further study.

Acknowledgments—We thank Patricia Wander for preparing the illustrations and David Bay for making the photographs.

REFERENCES


Fig. 6. Rechromatography of 32P-labeled nonhistone proteins on histone affinity columns. Peaks of radioactivity originally eluted at 0.3, 0.5, or 2.0 M from an initial histone H2B affinity column (see Fig. 3) were divided in half and treated as described in Fig. 5, except that here: — —, H2B affinity column; — —, H1 affinity column.

Table II

<table>
<thead>
<tr>
<th>Sepharose-bound ligand</th>
<th>Per cent radioactivity in:</th>
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<tbody>
<tr>
<td></td>
<td>Runoff</td>
</tr>
<tr>
<td></td>
<td>NHP C</td>
</tr>
<tr>
<td>HI</td>
<td>57%</td>
</tr>
<tr>
<td>H2B</td>
<td>92%</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>89%</td>
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</tbody>
</table>

* NHP, 32P-labeled nonhistone proteins; C, 32P-labeled cytoplasmic supernatant proteins.
Histone Affinity Chromatography

Histone affinity chromatography as a tool for fractionating nonhistone chromatin proteins and studying histone-nonhistone protein interactions.
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