Tissue-specific Chromosomal Non-histone Protein Interactions with DNA*

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SOPHIA WANG, JEN-FU CHIU,† LEOKADIA KLYSZUKO-STEFANOWICZ,§ HIDEO FUJITANI,‡ AND LUBOMIR S. HNILICA¶

From the Department of Biochemistry, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

ALLEN T. ANSEVIN

From the Department of Physics, The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

A group of chromosomal non-histone proteins with affinity for DNA (NP) was isolated from rat liver and Novikoff hepatoma. This fraction, which represents less than 5% of the total chromatin protein content, binds preferentially to unique, double stranded sequence of fractionated homologous DNA. The interactions are strong at low ionic strength (K_m = 6.7 x 10^{-9} M) and decrease with rising salt concentration. Complexes of the NP protein fraction with homologous DNA are immunologically tissue-specific. As determined by microcomplement fixation, the NP proteins in Novikoff hepatoma are associated with the transcriptionally active, diffuse fraction of chromatin.

Chromosomal non-histone proteins exhibit heterogeneity and tissue specificity which is expected of proteins involved in gene regulatory capacity. Evidence has accumulated in the literature linking changes of chromosomal protein turnover, phosphorylation, specificity, and other properties to the stimulation of genetic transcription by hormones, mitogens, carcinogens, etc. Chromosomal non-histone proteins which can bind to DNA were described by several investigators (1-8). Considerable differences in the DNA binding properties and heterogeneity of these proteins reported in the literature indicate that there may be a family of proteins in the nucleus which can bind to the DNA with a variable degree of specificity. Wakabayashi et al. (7, 9, 10) and Chiu et al. (8) reported a group of non-histone proteins capable of forming complexes with homologous DNA which were tissue specific by immunological criteria. In this paper, we described some additional properties of the DNA-binding chromosomal proteins of rat liver and Novikoff hepatoma.

MATERIALS AND METHODS

Isolation of DNA-binding Proteins—The fractionation scheme developed in our laboratory (8) was followed. This scheme is based on the solubility properties of chromosomal proteins in 5 M urea at three different pH values and salt concentrations. The first extraction is performed at relatively low ionic strength at pH 7.6 and it removes the bulk of proteins which are not firmly associated with DNA in chromatin. The second extraction at high ionic strength and relatively low pH (5.0) takes the advantage of the solubility of histones under these conditions. The DNA binding non-histone proteins are poorly soluble at this pH, which is near their isoelectric point, and remain with the residual chromatin pellet. They can be solubilized by increasing the pH to 8.0 in the final extraction step.

Briefly, isolated rat liver or Novikoff hepatoma chromatin (11) was gently homogenized in 5.0 M urea containing 50 mM sodium phosphate buffer, pH 7.6. After stirring for 2 to 3 hours, the mixture was centrifuged at 20,000 x g for 30 min, and the pellets were extracted once more in the same manner. The combined supernatants contained the bulk of chromosomal non-histone proteins. Histones were removed from the remaining pellets by resuspending them gently in 5.0 M urea/2.5 M NaCl/50 mM sodium succinate buffer, pH 5.0 and centrifuging the viscous solution at 110,000 x g for 30 hours. DNA and associated non-histone proteins pelleted, while histones and small amounts of other proteins remained in the supernatant. Finally, the DNA-binding protein fraction was recovered by dissolving it in 5.0 M urea/2.5 M NaCl/50 mM Tris-HCl buffer, pH 8.0 and centrifugation at 110,000 x g for 48 hours. The NP-containing supernatants were pooled, concentrated by ultrafiltration (Amicon UM-2 membrane), and used in the described experiments.

DNA Protein-binding Studies—DNA was isolated according to the modified procedure of Marmur (12, 13) and iodinated with carrier-free _¹²¹_I using the method of Commerford (14). The chloramine-T technique described by Sonoda and Schlamowitz (15) was used for the iodination of NP proteins.

The binding affinity of NP proteins to unfragmented DNA was studied by reconstituting _¹²¹_I-iodinated NP fraction to DNA and separating free labeled protein from the NP-DNA complex by sucrose density gradient centrifugation.

* The abbreviations used are: NP, DNA-binding chromosomal non-histone protein fraction; UP, chromosomal non-histone proteins soluble in urea at low ionic strength; HP, histones; SSC, 0.15 M NaCl/0.015 M sodium citrate; C_m, concentration (moles/liter) x time (s).
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gradient centrifugation. The samples were layered over a linear 5 to 20% sucrose gradient in 10 mM Tris-HCl buffer, pH 8.0 and centrifuged in a Spinco SW-40 rotor at 38,000 rpm for 6 hours.

Because the fragmented (sonicated) DNA would not sediment under these conditions, the binding of ^14[^H]-NP proteins to fractionated DNA was investigated by reconstituting the labeled NP fraction to DNA and filtering the complexes through nitrocellulose filters (Schleicher & Schuell, B-6) according to the method of Riggs et al. (18).

Fragmentation and Fractionation—DNA was isolated by the modified procedure of Marmur (12, 13) and fragmented by ultrasonad. DNA solution (0.5 mg/ml) in ice-cold 0.3 M NaCl/0.03 M sodium citrate (2 × SSC) was sonicated for 3 min (10 to 15-s bursts followed by 1-min cooling intervals) with a microtip-equipped Branson Sonifier cell disruptor, model W 185, at a setting of 6. Before sonication, the DNA solution was purged with nitrogen for 10 min. The DNA, sheared to 400 to 500 base pairs as detected by ultracentrifugation, was concentrated in a rotary evaporator. The fragmented DNA was dissolved in 0.12 M sodium phosphate buffer (pH 6.8), dialyzed against the same buffer for 6 hours with two changes, and finally passed through a hydroxylapatite column at 60°C (17). The double-stranded DNA was eluted with 0.46 M sodium phosphate buffer (pH 7.0), dialyzed against the same buffer for 6 hours with two changes, and finally passed through a hydroxylapatite column at 60°C (17). The double-stranded DNA was eluted with 0.48 M phosphate buffer (greater than 96% recovery), and characterized by thermal denaturation in a buffer containing 5 mM caccyclate, 0.15 mM EDTA, and 3.6 M urea, pH 7.0 (18).

Fractionation of Chromat—in the distribution of immunologically active DNA-binding proteins in chromat was investigated by chromatofractionation. To separate active (diffuse) and inactive (condensed) chromatin, a modified method of McCarthy et al. (19) was employed. Novikoff hepatoma chromat was suspended in 10 mM Tris-HCl buffer, pH 8.0 and sheared in an Omni-Mixer with a microtip (MicroTip) set at 10,000 rpm for 210 s (in 30-s bursts separated by 60-s intervals). After shearing, the chromatin was centrifuged at 12,000 × g for 10 min. The supernatant was layered over a linear 0.17 to 1.7 mM sodium phosphate buffer (greater than 96% recovery), and characterized by thermal denaturation in a buffer containing 5 mM caccyclate, 0.15 mM EDTA, and 3.6 M urea, pH 7.0 (18).

In Vitro Transcription of Chromatin—The ability of the chromatin fractions to support in vitro RNA transcription was assayed using RNA polymerase from Escherichia coli. (20). The reaction mixture (0.05 mol final volume) consisted of 20 mM Tris-HCl buffer, pH 8.0; 120 mM KCl; 0.1 mM EDTA; 2.5 mM MgCl2; 0.1 mM dithiothreitol; 0.8 mM each ATP, GTP, and CTP; and 0.2 mM [32P]UTP (specific activity 100 µCi/mol). The concentration of chromatin in each assay was 25 to 75 µg with respect to DNA. To each assay 15 units of E. coli RNA polymerase (specific activity 100 units/mg of protein) were added and the assay mixtures were incubated at 37°C for 10 min. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid/1% sodium pyrophosphate solution.

Immun assay Procedure—The DNA- and protein-containing complexes were solubilized in 1.5 mM/0.15 mM sodium citrate, pH 7.0 and dialyzed extensively against the same solution. After dialysis, the material was mixed with an equal volume of complete Freund's adjuvant and injected into male New Zealand rabbits; 1 mg of the DNA mixture was injected weekly into each rabbit for 1 week (17, 21). The antisera, collected 1 week after the last injection, were purified by chromatography on DEAE cellulose. The antigenicity of the individual samples was determined by the complement fixation method of Wasserman and Levine (22). The antigens and antisera were reacted at 2-4°C overnight.

Phosphorylation of Chromosomal Proteins—The nuclear proteins were labeled in vivo by injecting rats with 2 mCi/100 g of carrier-free [32P]orthophosphate intraperitoneally. The rats were killed 1 hour after the injection, and their livers were excised and used for the isolation of chromosomal protein fractions. The in vitro labeling of chromatin was achieved by incubation in 16 µM [γ-32P]ATP (0.23 Ci/mmol), 20 mM MgCl2, and 100 mM NaCl in 80 mM Tris-HCl buffer, pH 7.5 at 37°C for 10 min (23). The reaction was terminated by addition of 1% sodium pyrophosphate. The phosphorylated chromatin samples were extensively washed with 1% sodium pyrophosphate in 0.01 × SSC, pH 7.5, to remove free [γ-32P]ATP. After washing, the chromatin was fractionated into UP, HP, and NP fractions.

Phosphorylation of isolated UP, HP, and NP protein fractions was performed with partially purified rat liver phosphoprotein kinase (24, 25). The reaction mixture contained in a 0.25 ml final volume 20 µmol of Tris-HCl, pH 7.4; 4 nmol of [γ-32P]ATP (0.23 Ci/mmol); 5 µmole of MgCl2; 25 µmole of NaCl; 50 µg of protein fraction, and 10 µg of partially purified rat liver phosphoprotein kinase. The reaction was terminated after incubation at 37°C for 10 min by adding ice-cold 10% CCl4/COOH.

RESULTS

The amounts of NP protein fraction which binds to the DNA was found to increase with the protein concentration until it reached a maximum at which no NP protein was accepted by the DNA (Fig. 1). The binding ratio at the saturation level of NP proteins was found to depend on the salt concentration (Fig. 2). When the interactions were performed at 10 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0, the binding sites available on homologous DNA (rat spleen) were saturated at the NP protein/DNA ratio of approximately 1.5/100 (w/w). The reciprocal plot of the saturation curve resulted in a straight line intercepting the abscissa at the Ks value of about 6.7 × 10-4 M (Fig. 3). The binding of NP proteins is species-specific. As can be seen in Table I, both rat spleen and liver DNA bind the liver NP protein fraction equally well. Calf thymus DNA exhibits a small but significant binding, while the affinity of rat liver NP proteins to chicken erythrocyte or Escherichia coli DNA is negligible.

The binding of NP proteins to single- and double-stranded DNA was determined using the unique sequence DNA fraction. This DNA remained single-stranded for a long time; renaturation was performed in 0.12 M sodium phosphate buffer, pH 6.8 at 65°C for 90 hours (Cm = 1.4 × 104). Under these conditions most of the DNA became double-stranded. As can be seen in

![Fig. 1. Saturation of rat spleen native unfractionated DNA with rat liver NP proteins (sucrose density gradient data). The DNA concentration was kept at 100 μg/ml at each experimental point.](http://www.jbc.org/)

![Fig. 2. Effect of ionic strength on rat spleen native total DNA binding of rat liver NP proteins.](http://www.jbc.org/)
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**FIG. 3.** Reciprocal plot of rat spleen DNA (native, unfractionated) and rat liver NP binding. The calculations are based on the assumed molecular weight of 12,000. All experimental points were derived from sucrose density gradient centrifugation.

**TABLE I**

*Interactions of rat liver NP fraction with homologous and heterologous DNA*

The formation of DNA-protein complexes was assayed by sucrose density gradient centrifugation using \(^{3} \text{H}\)-labeled NP protein. The binding ratios represent weight percentages of protein retained by the DNA.

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>DNA applied (μg)</th>
<th>Protein bound (μg)</th>
<th>Protein/DNA binding ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat spleen</td>
<td>400</td>
<td>5.8</td>
<td>0.0145</td>
</tr>
<tr>
<td>Rat liver</td>
<td>400</td>
<td>5.6</td>
<td>0.0141</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>400</td>
<td>1.0</td>
<td>0.0025</td>
</tr>
<tr>
<td>Chicken erythrocyte</td>
<td>400</td>
<td>0.3</td>
<td>0.0008</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>400</td>
<td>0.1</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

**TABLE II**

*Rat liver NP protein binding to single- and double-stranded unique sequences of DNA*

<table>
<thead>
<tr>
<th>DNA fraction</th>
<th>DNA applied (μg)</th>
<th>Protein bound (μg)</th>
<th>Protein/DNA binding ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-stranded unique sequences</td>
<td>200</td>
<td>1.8</td>
<td>0.0069</td>
</tr>
<tr>
<td>Double-stranded unique sequences</td>
<td>200</td>
<td>3.7</td>
<td>0.0185</td>
</tr>
</tbody>
</table>

Table II, the rat liver NP proteins showed a significant preference for double-stranded DNA.

It was shown by several investigators (7, 8, 21) that certain chromosomal non-histone proteins are tissue-specific by immunological criteria. Wakabayashi et al. (10) pointed out that the immunological tissue specificity resides with a small protein fraction which associates strongly with homologous DNA. More recently, Chiu et al. (8) localized these tissue-specific non-histone proteins in the NP fraction of chromatin. In agreement with these findings, Fig. 4 shows that the Novikoff hepatoma residual chromatins fixed the complement in the presence of Novikoff hepatoma dehistonized chromatin antiserum as long as the NP protein fraction remained associated with chromatin DNA.

The sucrose density gradient fractionation of sheared Novikoff hepatoma chromatin resulted in a clear separation of two fractions. The light material (peak II in Fig. 5) represented the open fraction, while the more abundant and heavier material (peak I) represented the heterochromatic fraction. This conclusion is supported by the incorporation of labeled UTP into acid insoluble precipitate templated by the isolated chromatin fractions in the presence of *E. coli* RNA polymerase. Complement fixation assay of these fractions revealed that essentially all the immunological reactivity was associated with the diffuse and transcriptionally active chromatin (Fig. 6).

**DISCUSSION**

Chromosomal non-histone proteins which can bind DNA with some specificity were reported by several investigators (1-10). In general, these proteins represent only a small part (less than 10%) of the total chromatin protein content, and some of them can selectively recognize DNA of the same species (homologous DNA).

Allfrey et al. (6) described a comprehensive technique for the fractionation of calf thymus nuclear non-histone proteins which employs affinity chromatography on aminoethyl Sepharose 4B columns charged with fragmented and fractionated DNA.
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A considerable variety of chromosomal non-histone proteins can be separated by this method into groups with preferences for native or denatured DNA, unique sequence or repetitive DNA, homologous or heterologous DNA, etc. Some nuclear DNA-binding proteins also interact selectively with adenosine 3':5'-cyclic phosphate or guanosine 3':5'-cyclic phosphate. Most of the DNA-binding proteins described by these authors registered molecular weights higher than 20,000. The molecular weight range determined electrophoretically for the major bands of DNA binding proteins in NP is lower, between 12,000 and 15,000 (8, 10).

van den Broek et al. (4) isolated a fraction of chromosomal non-histone proteins from rat liver which interacted preferentially with rat DNA. This fraction represented about 4% of the total rat liver chromosomal non-histone protein content, and in its molecular weight, amino acid composition, and other properties it resembled the rat liver DNA-binding proteins of our NP fraction. In their recent communication, Sevall et al. (5) extended the studies on rat liver chromosomal proteins to include binding specificity to various DNA preparations. At a relatively low ionic strength (110 mM), the interactions were strong but nonspecific. At this ionic strength, the authors found very little difference in binding of the rat liver non-histone protein fraction to the DNA from rat liver, calf thymus, and Drosophila melanogaster. However, the DNA-binding proteins described by these authors registered molecular weights higher than 20,000. The molecular weight range determined electrophoretically for the major bands of DNA binding proteins in NP is lower, between 12,000 and 15,000 (8, 10).

Profiles of chromatin proteins phosphorylated either in in vivo or in vitro by endogenous phosphoprotein kinases were similar. After in vivo phosphorylation, the phosphoprotein-containing non-histone fraction UP, which represents almost one-half of all the chromatin protein content (Table I), was extensively phosphorylated (about 90% of all 32P counts). However, the DNA-binding NP fraction showed only background incorporation (Table III). The histones accounted for about 10% of the total 32P incorporation. The distribution of 32P incorporated into the proteins was considerably changed when the individual isolated fractions UP, HP, and NP were phosphorylated in vitro by exogenous kinase. Under these conditions, the histones were phosphorylated almost as extensively as the phosphoprotein-containing fraction UP. Again, the DNA-binding non-histone protein fraction NP was not phosphorylated. Although it cannot be excluded that the NP fraction may contain phosphorus which does not turn over at appreciable rates, such irreversibly bound phosphorus would probably have little biological significance. By its lack of appreciable phosphorus turnover, the non-histone NP fraction differs from DNA binding proteins reported by other investigators (1-3).

In his recent review, Altfrey (26) points out that the positive and selective aspects of genetic control in eukaryotes are most likely operated by chromosomal non-histone proteins, and biological activity, which can be followed and quantitatively assayed, is a most valuable asset in purification and identification of chromosomal non-histone proteins. We have reported earlier (7-10) that a fraction of chromosomal non-histone proteins which bind strongly to the DNA is immunologically tissue-specific as long as it remains associated with homologous DNA. Complement fixation performed with the NP fraction employed in the DNA binding experiments described in this paper confirmed that this fraction also contains the immunologically active complexes. Although the biological fraction of the NP fraction is presently unknown, it is of interest that these proteins are preferentially accumulated in the transcriptionally active and loosely packed part of sheared and fractionated chromatin.

Unfortunately, the techniques for fractionation of chromatin into its transcriptionally active and heterochromatic parts are still equivocal. Macromolecular rearrangements can occur during the shearing process with possible accumulation of the immunoreactive DNA binding NP proteins in the slowly sedimenting chromatin fraction. According to some investigators, this fraction may be impoverished in some histones,

**Table III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Chromatin protein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total protein</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
</tr>
<tr>
<td>UP</td>
<td>44-46</td>
</tr>
<tr>
<td>HP</td>
<td>50-52</td>
</tr>
<tr>
<td>NP</td>
<td>2-3</td>
</tr>
</tbody>
</table>

especially the H1, and therefore enriched in available DNA (27). However, considering the DNA binding properties of NP proteins described previously (7-10) and in this paper, it is very unlikely that the NP proteins could become dissociated from DNA in the shearing buffer (10 mM Tris-HCl, pH 8.0). Preliminary experiments on the distribution of labeled NP proteins during chromatin fractionation support this conclusion.

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