Study of the Loosely Bound Non-Histone Chromatin Proteins

STIMULATION OF DEOXYRIBONUCLEIC ACIDTEMPLATED RIBONUCLEIC ACID SYNTHESIS BY A SPECIFIC DEOXYRIBONUCLEIC ACID-BINDING PHOSPHOPROTEIN FRACTION*

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SUMMARY

The loosely bound chromatin proteins of Ehrlich ascites hyperdiploid cells have been prepared by extraction of chromatin with 0.35 M NaCl. Sodium dodecyl sulfate gel electrophoresis of the 0.35 M NaCl-soluble chromatin proteins reveals high heterogeneity with a molecular weight range of 10,000 to 170,000. The 0.35 M NaCl-soluble chromatin proteins contain many components similar to the more tightly bound non-histone chromatin proteins that are released by stronger salt solutions.

When histones and DNA formed a complex with the loosely bound chromatin proteins by gradient dialysis, the inhibitory effect of histones on transcription of DNA in vitro was reduced. The reconstituted complex manifested a level of template activity similar to that of native chromatin as measured in an Ehrlich ascites tumor RNA polymerase reaction.

The loosely bound chromatin proteins contain RNA as well as phosphoproteins. Phenol extraction or DNA affinity chromatography of these proteins yielded fractions enriched 25- to 30-fold in phosphorus which were capable of enhancing RNA synthesis in vitro. The loosely bound non-histone chromatin proteins contain a fraction that specifically binds to Ehrlich ascites tumor RNA polymerase.

Another fraction, rich in phosphoproteins, binds specifically to DNA of the tissue of origin (5-0) and stimulates DNA-templated RNA synthesis in vitro (6, 10). Hence, non-histone proteins contain at least two potential regulatory fractions operative in positive control of gene activity.

Removal of loosely bound non-histone proteins from chromatin by 0.35 M NaCl alters neither the structure nor the template activity of the chromatin (11). Thus, these loosely bound chromatin non-histone proteins appear to be nonfunctional in gene regulation. However, Fujitani and Holoubek (12) have shown that the gel electrophoretic pattern of loosely bound non-histone proteins is similar to that of the tightly bound non-histone proteins. This suggests that non-histone proteins may be in a dynamic state within the chromatin and that the loosely bound non-histone proteins may contain functional fractions similar to the more tightly bound non-histone chromatin proteins. In this report, we describe the isolation of a specific DNA-binding phosphoprotein fraction from the loosely bound non-histone proteins which stimulates transcription of DNA in vitro.

EXPERIMENTAL PROCEDURE

Preparation of Chromatin—Male Albany Swiss mice, weighing 30 to 40 g, were each inoculated with 10⁷ Ehrlich ascites tumor cells. The mice were killed 13 days postinoculation by cervical dislocation, and the ascites fluid was harvested from the abdominal cavity. All subsequent steps were carried out at 4°C. The ascitic fluid was passed through two layers of cheesecloth and whole cells were collected by centrifugation at 800 × g for 10 min in a Sorvall refrigerated centrifuge. The packed cells were washed twice in 10 volumes of each of 0.14 M NaCl-0.01 M Tris-HCl, pH 7.0, and collected by centrifugation. The washed cells were osmotically ruptured by suspension in 10 volumes of distilled water and gently homogenized by 30 up-and-down strokes in a loose fitting Dounce homogenizer. The resulting crude nuclei were sedimented by centrifugation at 1000 × g for 10 min. Contaminating cytoplasm and the outer nuclear membrane were removed by suspending the crude nuclear pellet in 5 volumes of Buffer A (0.14 M NaCl-0.01 M Tris-HCl, pH 8.0) 0.001 M MgCl₂), after which an equal volume of 2% Triton X-100 in Buffer A was added. The protease inhibitor, phenylmethylsulfonyl fluoride was added to all of the solutions used throughout this study at a final concentration of 0.1 mM. The nuclear suspension was stirred for 3 min and then centrifuged at 1400 × g for 10 min. The nuclei were washed free of Triton X-100 with 10 volumes of 0.14 M NaCl-0.01 M Tris-HCl, pH 8.0, and pelleted at 1400 × g for 10 min. This washing was performed three times. The washed nuclei were suspended in 10 volumes of 0.05 M EDTA-0.005 M Tris-HCl, pH 8.0, and collected by centrifugation.

Non-histone proteins contain two fractions involved in activation of RNA synthesis. One activates transcription of chromatin, resulting in tissue-specific RNA synthesis (1-4). This research was supported by Grant GM-11698 from the United States Public Health Service.

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This EDTA-Tris treatment was repeated once. The resulting pellet was washed three times with 10 volumes of 0.05 M Tris-HCl, pH 8.0, to remove EDTA. Chromatin was isolated according to the methods of Alberts and Herrick (18). Briefly, 24 ml of Ehrlich ascites tumor cells were homogenized in 0.25 m sucrose, 0.002 M CaCl₂. The homogenate was passed through two layers of cheesecloth and centrifuged at 14,000 × g for 15 min. This purified chromatin was the starting material for the isolation of the various chromatin fractions. Freshly prepared chromatin was used throughout the present work, because extraction of frozen chromatin resulted in the release of DNA in the 0.35 M NaCl extract of chromatin.

For quantitative determination of chromatin histones and non-histone proteins, the chromatin was extracted with 0.4 M H₃BO₃ to yield histones and then the non-histone proteins were obtained by dissolving the H₃BO₃-insoluble chromatin residue in 0.1 M NaOH.

Preparation of 0.35 M NaCl-soluble Chromatin Proteins—The isolated chromatin was extracted with 1,000 parts (w/w) of Buffer B (0.35 M NaCl-0.02 M Tris-HCl, pH 7.5) by suspending the chromatin in Buffer B with a loose fitting Donnell homogenizer and stirring for 20 min. The extract was collected by centrifugation at 105,000 × g for 2 hours. The resulting pellets were cut into small pieces and reextracted three more times with 1,000 parts (w/w) of Buffer B. The extracted chromatin was saved for differential salt extraction. The next supernatant was pooled, lyophilized, taken up in a small volume of water, and dialyzed against 2 liters of Buffer C (0.4 M NaCl-0.01 M Tris-HCl, pH 7.0) with three changes of the buffer. The dialyzed sample was clarified by centrifugation at 20,000 × g for 15 min, and applied to a Bio-Rex 70 (Na⁺) column previously equilibrated with Buffer C. For each 10 mg of protein, 1 ml of Bio-Rex 70 was used. The column was eluted with Buffer C. Fractions of 0.6 ml were collected. The absorbance of the fractions were monitored at 280 nm. The 0.35 M NaCl-soluble chromatin acidic proteins were thus eluted and pooled, dialyzed against 20 volumes of 0.05 M NaCl-0.01 M Tris-HCl, pH 7.0, for 4 hours, and lyophilized until use.

Differential Salt Extraction of Chromatin—The chromatin after extraction with 0.35 M NaCl as described above was homogenized in 0.04 M Tris-HCl, pH 8.0. An aliquot of 1.25 M NaCl was added dropwise into the suspension and stirring was continued for 30 min. The DNA concentration of the chromatin suspension was adjusted to 100 µg of DNA per ml with 0.6 M NaCl-0.02 M Tris-HCl, pH 8.0. The chromatin suspension was centrifuged at 165,000 × g for 48 hours (15). The resulting pellet was extracted overnight with 0.54 volume of isopropyl alcohol, washed in ethanol, and dissolved in 0.01 X SSC. Once in solution, the salt concentration of the solution was raised to 2 X SSC by addition of 10 X SSC.

Reconstitution of Chromatin—Reconstitution of chromatin was performed according to the method described by Bekhor et al. (16). Various combinations of Ehrlich ascites chromosomal components were mixed and dialyzed against 2.0 M NaCl-5.0 M urea-0.02 M Tris-HCl, pH 8.0, for 14 hours. The samples were subsequently dialyzed for 4 hours, each against decreasing salt concentrations consisting of 1.5 M, 1.0 M, 0.8 M, and 0.6 M NaCl in 5.0 M urea and 0.2 M Tris-HCl. The above treatment was repeated until dialyzed against buffered 0.4 M NaCl for 14 hours, followed by 0.01 M NaCl-0.01 M Tris-HCl, pH 8.0. Prior to use, the various samples were dialyzed against de-ionized distilled water and the DNA concentration of each reconstituted chromatin was adjusted to 500 µg per ml. The following combinations of Ehrlich ascites chromosomal components were used in preparing the reconstituted chromatin complexes: (a) whole histone and DNA in a ratio of 1:1.21, (b) 0.35 M NHP and DNA in a ratio of 0.94:1; (c) whole histone, 0.35 M NHP and DNA in ratios of 1.12:1:0.4.

Reconstitution of chromatin with phenol-soluble proteins was carried out in the same manner.

Preparation of Phenol soluble 0.35 M Proteins—Phenol soluble proteins were isolated from the 0.35 M NHP following the procedures of Teng et al. (6). Briefly, the lyophilized 0.35 M NHP were separately treated with Bio-Rex 70. The unadsorbed proteins were dialyzed against 20 volumes of 0.05 M Tris-HCl, pH 8.0, for 6 hours and lyophilized. The proteins were extracted by adding an equal volume of buffer-saturated phenol and allowing to stand at 4°C for 10 to 12 hours. The resulting suspension was centrifuged at 12,000 × g for 10 min. The aqueous phase was re-extracted with an equal volume of buffer-saturated phenol and pH 8.4, containing 0.01 M EDTA and 0.14 M β-mercaptoethanol. The proteins were isolated from the 0.35 M NHP following the procedure described in the next section, whereas the four supernatants were pooled, dialyzed against 20 volumes of 0.05 M Tris-HCl, pH 8.0, for use.

Preparation of DNA—DNAs were isolated from the nuclei of Ehrlich ascites cells, rat liver, calf thymus, and chicken erythrocytes by a modification of the method of Marmur (17). The nuclei were suspended in 0.25 M sucrose containing 0.1 M EDTA, pH 8.0, and 0.14 M NaCl. SDS was added to the suspension to 3%, and the suspension was extracted twice with a chloroform-isomyl alcohol (94:1) mixture. After centrifugation, the nucleic acid precipitated from the aqueous phase was re-extracted with an equal volume of buffer-saturated phenol and pH 8.0. The two phenol extracts were pooled and dialyzed against 0.1 M acetic acid containing 0.14 M β-mercaptoethanol until the volume of the phenol extract was reduced by 80%. The phenol extract was subsequently dialyzed against 0.05 M acetic acid-9.0 M urea-0.14 M β-mercaptoethanol for 24 hours, followed by 0.01 M Tris-HCl (pH 8.4)-8.0 M urea-0.01 M EDTA-0.14 M β-mercaptoethanol for 24 hours.

Preparation of DNA-cellulose Chromatography—DNA-cellulose was prepared by the methods of Alberts and Herrick (18). Briefly, 24 ml of a DNA solution (3 mg per ml of native DNA in 0.01 M Tris-HCl (pH 7.4)-0.001 M EDTA) was added to 8.0 g of washed cellulose (Munktell 410 cellulose) and the paste was spread on a watch glass, covered with cheesecloth, and allowed to air-dry for 24 hours. The mixture was ground to a fine powder and dried to a lyophilized form. The above treatment was repeated three times. The dry DNA-cellulose powder was then suspended in 100 volumes of the Tris-EDTA and left at 4°C for 24 hours with occasional swirling. The Tris-EDTA was decanted and the DNA-cellulose was washed free of unbound DNA by suspending it in Tris-EDTA and collected by centrifugation. This was repeated as necessary.

¹ The abbreviations used are: SDS, sodium dodecyl sulfate; SSC, 0.15 M sodium chloride-0.015 M sodium citrate.

¹ The 0.35 M NaCl-soluble chromatin acidic proteins is referred to as 0.35 M NHP, and the phenol-soluble fraction of 0.35 M NHP is referred to as PS-0.35 M NHP.
To determine the amount of DNA bound to the cellulose, an aliquot of the DNA cellulose in Buffer D (0.05 mM NaCl-0.001 mM EDTA-0.01 M Tris-HCl, pH 7.4) was boiled for 20 min and centrifuged. The amount of DNA released in the supernatant was estimated from the absorbance of the supernatant solution at 260 nm against a buffer blank.

The protein sample to be chromatographed on DNA-cellulose was dialyzed against three changes of Buffer D and clarified by centrifugation at 13,000 X g for 10 min. The sample was then passed through a DNA-free cellulose column to remove nonspecific adsorption and applied to the Ehrlich ascites tumor DNA-cellulose column (2.2 X 3.0 cm). The nonadsorbed proteins were collected and loaded onto an Ehrlich ascites tumor DNA-cellulose column. After washing the tumor DNA-cellulose column with Buffer D, the adsorbed proteins were eluted with 0.6 M NaCl in 0.01 M Tris-HCl (pH 7.4)-0.001 M EDTA. Elution with salt solutions greater than 0.6 M did not release any detectable proteins. Generally, 10 to 15 mg of proteins were applied to the column, equivalent to approximately one-half the amount of DNA present in the DNA-cellulose column. The columns were eluted at a flow rate of 1 ml/3 min and 1 ml fractions were collected.

Analytical Gel Electrophoresis—Polyacrylamide gel electrophoresis of the protein fractions was performed by the method of Laemmli (10). The samples, dissolved in 1.0% SDS-0.065 M Tris-HCl (pH 6.8)-5.0% β-mercaptoethanol, were dialyzed at room temperature against 1.0% SDS-0.065 M Tris-HCl (pH 6.8)-0.1% β-mercaptoethanol-15% glycerol. The proteins were completely dissociated by immersing the samples in a boiling water bath for 2 min prior to their application onto the gels. Approximately 60 to 100 µg of proteins were applied to each gel. The separating gels were prepared by adding 2.5 ml of a final solution of 10% acrylamide, 0.07% bis-acrylamide, 0.375% ammonium persulfate, 0.125% N,N',N'-tetramethylethylenediamine, 1.0% urea, 0.15% ammonium persulfate to the gel tubes. The acrylamide solution was overlaid with 30 µl of isobutyl alcohol. The stacking gels were allowed to polymerize for 6 hours before use. The upper and lower buffer reservoirs of the electrophoresis apparatus were filled with a running buffer (pH 8.3) of 0.025 M Tris-HCl, 0.192 M glycerine, and 0.1% SDS. Electrophoresis was carried out at 0.5 ma per gel tube for 24 hours unless otherwise specified. At the end of electrophoresis, the gels were stained for 12 hours with 0.1% Coomassie brilliant blue in 9.0% acetic acid-50% methanol, and destained by horizontal diffusion in 7.5% acetic acid-5% methanol at 40°. When densitometric tracing was desired, the stained gels were scanned at 620 nm in a spectrometer with a Giford linear transport. Molecular weight markers used consisted of trichloroacetic acid (molecular weight 165,000), β-galactosidase (molecular weight 130,000), phosphorylase A (molecular weight 94,000), bovine serum albumin (molecular weight 67,000), ovalbumin (molecular weight 45,500), and trypsin (molecular weight 26,000).

Preparation and Assay of RNA Polymerase—DNA-dependent RNA polymerase II was isolated from 7-day inoculated Ehrlich ascites tumor cells by a modified procedure of Natori et al. (20). The glycerol gradient centrifugation in Natori's final purification procedure of Lowry et al. (21), RNA by orcinol reaction (23), and DNA by the method of Burton (24). Phosphoprotein was analyzed by alkali-Jabohsophorus, determined by the method of Berenblum and Chain (25). Specific activity of the enzyme was 550 units per mg of protein. One unit of enzymic activity is defined as the amount of RNA polymerase that catalyzes the incorporation of 1 nmol of labeled nucleotide into acid-insoluble material under the assay conditions as described by Nakamoto et al. (21).

Other Methods—Protein concentration was determined by the procedure of Lowry et al. (22), RNA by orcinol reaction (23), and DNA by the method of Burton (24). Phosphoprotein was analyzed by alkali-Jabohsophorus, determined by the method of Berenblum and Chain (25).

**RESULTS**

**Extraction of Chromatin with 0.35 M NaCl**—The composition of Ehrlich ascites tumor chromatin with respect to DNA, RNA, histones, and non-histone proteins is shown in Table I. Acid extraction of chromatin, used to isolate histones, also releases some non-histone proteins (26, 27). To correct for the excess histone value due to acid-soluble non-histone proteins, the acid extract of chromatin was dialyzed against 0.4 M NaCl-0.01 M Tris-HCl, pH 7.0, and chromatographed on Bio-Rex 70. As shown in Table I, the histone to DNA ratio changes from 1.22 to 1.12, whereas the non-histone protein to DNA ratio changes from 0.84 to 0.94, following Bio-Rex 70 treatment.

Release of chromatin from loosely bound protein by extraction with 0.35 M NaCl-0.02 M Tris-HCl, pH 7.5, was near complete after four extractions. The extracted 0.35 M NHP represented 45% of the total non-histone proteins of chromatin. Further extractions of the chromatin with 0.6 M, 1.0 M, and 2.0 M NaCl removed additional 32%, 3.6%, and 2.4% of non-histone proteins, respectively. Nineteen per cent of the non-histone chromatin proteins remained in close association with DNA, and could not be removed by exhaustive extraction of chromatin with 2.0 M NaCl-0.02 M Tris-HCl, pH 8.0. Most of these tightly bound chromatin proteins may be released by 3 M NaCl-7 M urea (15, 28, 29).

Electrophoretic Analysis of the 0.35 M Proteins—Fig. 1 shows SDS polyacrylamide gel electrophoretic patterns of the 0.35 M NHP compared to cytoplasmic and nuclear sap proteins and non-histone proteins subsequently released by extraction with 0.6 M, 1.0 M, and 2.0 M NaCl. The heterogeneity of the 0.35 M NHP is apparent. Approximately 40 to 50 components are discernible with molecular weight range from 10,000 to 170,000. Comparison of the loosely bound non-histone proteins with cytoplasmic and nuclear sap proteins reveals equivalent as well as different protein species. Higher salt concentrations appear

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Relative composition of Ehrlich ascites chromatin</th>
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<td>Average of three determinations.</td>
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<table>
<thead>
<tr>
<th>DNA/DNA</th>
<th>0.082 ± 0.003</th>
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<tbody>
<tr>
<td>NHP/DNA</td>
<td>0.84 ± 0.02</td>
</tr>
<tr>
<td>Histone/DNA</td>
<td>1.22 ± 0.03</td>
</tr>
<tr>
<td>Protein/DNA</td>
<td>2.11</td>
</tr>
</tbody>
</table>

Corrected for acid-soluble NHP

| 0.94 | 1.12 |
to remove selectively higher molecular weight proteins from chromatin than do 0.35 M and 0.6 M NaCl. The differences among these selectively extracted protein fractions are apparently more quantitative than qualitative. Bekhor et al. (29) have compared selective salt-extracted non-histone proteins of salivary glands and showed quantitative as well as qualitative differences between the loosely and tightly bound non-histone proteins. Fujitani and Holoubek (12), however, found no differences between these two groups of non-histone proteins isolated from rat hepatoma.

It should be emphasized that gross electrophoretic analysis of proteins as shown in Fig. 1 does not distinguish subtle differences in molecular weight, nor permit qualitative distinctions to be made between components with similar molecular weights. The complexities of nuclear and chromatin proteins are apparently of a higher order than are indicated here as demonstrated by Yeoman et al. (30, 31) and MacGillivray and Rickwood (32) who showed greatly expanded complexity of nuclear and chromatin proteins by two-dimensional gel electrophoresis.

Reconstitution of Chromatin with 0.35 M NHP—One way of determining whether the 0.35 M NHP have properties ascribed to non-histone proteins is to determine their ability to reduce histone inhibition of DNA transcription. For this purpose, reconstituted “chromatins” were prepared from DNA and histones, with and without the 0.35 M NHP. The template activities of these reconstituted chromatins were assayed in the Ehrlich ascites tumor RNA polymerase system and compared with that of the native chromatin. The results are shown in Fig. 2. In contrast to reconstituted DNA-histone complex, reconstituted chromatin composed of DNA, histones, and 0.35 M NHP manifests a template activity quantitatively similar to that of native chromatin indicating that the 0.35 M NHP can counteract the inhibitory effect of histones on DNA.

Phenol-soluble 0.35 M Proteins—It has been shown that phosphoproteins, prepared by extraction of nuclei either with phenol (6) or with 1 M NaCl (10), bind preferentially to homologous DNA, resulting in activated transcription of the DNA. In view of the above result, the question arises as to whether the 0.35 M NHP may contain phosphoproteins that stimulate transcription from DNA. To test this, the alkali-labile phosphorus content of the 0.35 M NHP was determined and a phenol-soluble fraction was isolated from the 0.35 M NHP. This fraction was equivalent to 5.9% of the total 0.35 M NHP, was electrophoretically heterogeneous, and contained 0.9% (w/w) alkali-labile phosphorus, representing a 30-fold enrichment of phosphoprotein from the 0.35 M NHP. The binding of the PS-0.35 M NHP to DNA and its effect on transcription was investigated. As shown in Fig. 3, the PS-0.35 M NHP is capable of stimulating transcription of DNA in the Ehrlich ascites RNA polymerase system and such stimulation is proportional to the
Fig. 2 (left). Template activity of Ehrlich ascites tumor native chromatin and nucleoproteins reconstituted from various Ehrlich ascites tumor chromatin components. Reconstitution was performed as described under "Experimental Procedure." The various templates used were: DNA-histone (O-O), Ehrlich ascites tumor DNA and whole histone reconstituted in a ratio of 1:1.12; native chromatin from Ehrlich ascites tumor (O); DNA-histone-0.35 M NHP (V-V), DNA, whole histone and 0.35 M NHP from Ehrlich ascites tumor reconstituted in ratios of 1:1.12:0.94, DNA-0.35 M NHP (Δ-Δ), DNA and 0.35 M NHP from Ehrlich ascites tumor reconstituted in a ratio of 1:0.94; DNA (O-O), Ehrlich ascites tumor native DNA. Abscissa indicates amount of DNA present in each template. Template activities were assayed with Ehrlich ascites tumor nucleoplasmic RNA polymerase as described under "Experimental Procedure." Fig. 3 (center). Effect of PS-0.35 M NHP on template activity of DNA in Ehrlich ascites tumor nucleoplasmic RNA polymerase reconstituted with tumor DNA, as described under "Experimental Procedure," in the following protein to DNA ratios: 0:1 (O-O); 0.25:1 (A-A); 0.5:1 (Δ-Δ); 1:1 (O-O). The abscissa indicates amount of DNA present in the reconstituted nucleoprotein complex being assayed and the ordinate indicates counts per min of [3H]GMP incorporation. Template activities of the reconstituted nucleoprotein complexes assayed at 24 μg of DNA concentration are replotted as a function of protein to DNA ratio as shown in the inset.

Fig. 4 (right). Affinity chromatography of Ehrlich ascites tumor 0.35 M NHP on heterologous Escherichia coli DNA-cellulose and Ehrlich ascites tumor DNA-cellulose. The run-off fraction from E. coli DNA-cellulose column (top left peak) was chromatographed on Ehrlich ascites tumor DNA-cellulose (lower curves). The tumor DNA binding protein fraction (lower right peak) was eluted by 0.6 M NaCl.

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Bound to E. coli DNA</th>
<th>Bound to tumor DNA</th>
</tr>
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<tr>
<td>Cytoplasmic soluble protein</td>
<td>18.7</td>
<td>0</td>
<td>5.0</td>
</tr>
<tr>
<td>Total non-histone proteins</td>
<td>18.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Total 0.35 M NHP</td>
<td>18.0</td>
<td>1.5</td>
<td></td>
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Analysis of Ehrlich ascites DNA binding proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Weight</th>
<th>Alkali-labile phosphorus in protein (μg/g)</th>
</tr>
</thead>
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<tr>
<td>Total non-histone protein</td>
<td>97.1</td>
<td>0.5</td>
</tr>
<tr>
<td>DNA-binding fraction</td>
<td>96.8</td>
<td>0</td>
</tr>
<tr>
<td>Total 0.35 M NHP</td>
<td>97.2</td>
<td>0</td>
</tr>
<tr>
<td>DNA-binding protein</td>
<td>95.0</td>
<td>0</td>
</tr>
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</table>

amount of the protein used in the complex formation. These data are consistent with the results obtained with chromatin reconstitution, suggesting that the 0.35 M NHP have a regulatory function in gene activity.

Binding of 0.35 μM Proteins to DNA—If the above deduction is correct, binding of the 0.35 M NHP by DNA should be specific. To test whether the 0.35 M NHP contain protein(s) showing preferential affinity towards homologous DNA, these proteins were initially passed through a heterologous DNA (Escherichia coli DNA)-cellulose column. The unadsorbed proteins were collected and applied to an homologous DNA (Ehrlich ascites DNA)-cellulose column. As shown in Fig. 4, a small amount of the unadsorbed fraction from E. coli DNA-cellulose is bound to the homologous DNA-cellulose. This fraction is eluted with 0.6 M NaCl and considered as the specific DNA-binding fraction.

To verify that the bound proteins were specific for homologous DNA, the proteins eluted from the tumor DNA-cellulose were again applied to the E. coli DNA-cellulose column. The proteins passed through unbound. However, these proteins were retained when reloaded onto the homologous DNA-cellulose column. The retained proteins, therefore, represent the fraction of the 0.35 M NHP that bind selectively to native homologous DNA. Table II summarizes the results on the binding of the 0.35 M NHP to E. coli native DNA and Ehrlich ascites native tumor DNA, as compared to DNA binding by cytoplasmic soluble proteins and total tumor non-histone proteins. Thus, the basic affinity of native DNA, the 0.35 M NHP may be classified into three groups: heterologous and homologous DNA binding proteins, representing 15% and 1.5% of the total 0.35 M NHP, respectively, and 80.5% proteins (by difference) that do not bind to native DNA. The relative amount of the heterolo-
gous DNA-binding proteins of Ehrlich ascites tumor is much lower than that of total non-histone proteins isolated from rat liver, but the percentage of 0.35 M NHP bound to homologous DNA is comparable to that reported for rat liver non-histone proteins (33).

Table II also shows the alkali-labile phosphorus and RNA contents of the 0.35 M NHP and the total non-histone proteins of Ehrlich ascites tumor chromatin before and after binding to homologous DNA. The specific binding to homologous DNA resulted in 25- and 30-fold enrichment of the alkali-labile phosphorus of the 0.35 M NHP and total non-histone proteins, respectively. The RNA contents of the 0.35 M NHP and total non-histone proteins were increased to 1.8 and 1.3 fold, respectively. The presence of 3 to 5% RNA in the specific DNA-binding proteins, either of 0.35 M NHP or of total non-histone proteins, is in good agreement with the values of $A_{260}/A_{450}$ ratio of 1.22 to 1.28 determined on rat liver DNA-binding non-histone proteins by Van den Broeck et al. (33).

It may be noted that soluble cytoplasmic proteins show considerable binding affinity toward E. coli DNA (13.7% bound). Vaughan and Comings (34) have previously reported DNA binding of soluble cytoplasmic proteins prepared from Chinese hamster. However, in the present work, the run-off fraction of the tumor-soluble cytoplasmic proteins from E. coli DNA-cellulose did not bind to tumor DNA. Specific DNA binding of soluble cytoplasmic proteins of Ehrlich ascites tumor is therefore not detected in this study.

The specific DNA-binding fraction of the 0.35 M NHP contained components mostly of low molecular weight of 30,000 or less as compared with the unfractionated proteins and are also heterogeneous as seen in Fig. 5.

**Fig. 5.** Densitometer tracing of SDS polyacrylamide gel electrophoresis patterns of total 0.35 M NHP (top) and specific DNA binding 0.35 M NHP fraction (bottom) from Ehrlich ascites tumor chromatin. Approximately 100 μg of protein were applied to each gel and electrophoresis was carried out as described under “Experimental Procedure.” Molecular weights are indicated in lower margin.
The loosely bound proteins of Ehrlich ascites tumor chromatin were released by extraction with 0.35 M NaCl. Such treatment apparently does not change the structure of the chromatin nor the template activity of the chromatin in RNA synthesis in vitro (11). Doubt has been raised that these loosely bound proteins may be cytoplasmic protein contamination (35, 36). However, it has been shown by gel electrophoresis that the loosely bound non-histone proteins are similar to the tightly bound non-histone chromosomal proteins (12) and are different from the soluble cytoplasmic proteins (37). Although the 0.35 M proteins have been reported to be similar to nucleoplasmic proteins (35), the latter are notably distinct from the cytoplasmic proteins (36).

Non-histone chromosomal proteins have been shown to contain factors (38, 39) which stimulate RNA synthesis in vitro. The factors bind to RNA polymerase but not to DNA (39). In this respect, the non-histone protein stimulating factors differ from the fraction described in the present work. Our studies of the 0.35 M NaCl-soluble chromatin proteins have revealed that both native DNA binding and phenol extraction yielded fractions that are enriched in phosphoprotein. It is the enriched phosphoprotein fraction that binds specifically to homologous DNA and exhibits template-specific stimulation of RNA synthesis. The results support the original findings by Teng et al. (5, 6), Shea and Kleinsmith (10), and Kleinsmith et al. (7) and indicate a functional role of the 0.35 M NHP in gene activation. Whether phosphoproteins, rather than unphosphorylated proteins participate directly in the stimulation of RNA synthesis cannot be assessed from the present study. Other studies, however, have indicated a correlation of gene activity with protein phosphorylation (10, 40–44).

The requirement of homologous RNA polymerase (or eukaryotic RNA polymerase) but not M. luteus (or a prokaryotic) RNA polymerase for stimulation of DNA-templated RNA synthesis by the specific DNA-binding protein fraction also suggests the possibility of a prior binding of the proteins to RNA polymerase. This could lead to a specific recognition binding to DNA. Such an interpretation has indeed been advanced by Teng et al. (6) who suggested that the active phosphoprotein fraction functions like the sigma factor in E. coli RNA polymerase. If this reasoning is correct, direct demonstration of RNA polymerase in association with the specific DNA-binding protein and of an effect of the fraction on initiation of RNA synthesis are necessary. A highly purified RNA polymerase and knowledge of its subunits are prerequisites to our understanding of the mechanism of this activating process.

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