Study of the Loosely Bound Non-Histone Chromatin Proteins

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

NINA C. KOISTRABA, RICHARD A. MONTAGNA, AND TUNG YUE WANG

From the Division of Cell and Molecular Biology, Faculty of Natural Science and Mathematics, The State University of New York at Buffalo, Buffalo, New York 14214

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 enhanced 25- to 30-fold in phosphorus which were capable of stimulating DNA-templated RNA synthesis in vitro. The stimulation of transcription from DNA was template-specific, the stimulation being manifested with Ehrlich ascites tumor RNA polymerase.

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Another fraction, rich in phosphoproteins, binds specifically to DNA of the tissue of origin (5-9) 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 10⁶ Ehrlich ascites tumor cells. The mice were killed 15 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 x g for 10 min in a Sorvall refrigerated centrifuge. The packed cells were washed two times in 10 volumes 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 x 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) 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 x 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), and collected by centrifugation. The washed nuclei were resuspended in 10 volumes of 0.14 M NaCl-0.01 M Tris-HCl, pH 8.0, and centrifuged at 1400 X 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 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).

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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 method of Alberts and Herrick (18). Briefly, 24 mi of DNA solution (5 mg per ml of native DNA in 0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA, and 0.14 M sodium citrate) was added dropwise into the suspension and stirring was continued for 30 min. The DNA concentration of the chromatin suspension was adjusted to 100 pg of DNA per ml with 0.6 M NaCl-0.02 M Tris-HCl, pH 8.0. An additional 1.2 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 X g for 48 hours (15), and the resulting pellet was extracted with 0.35 M NaCl-0.02 M Tris-HCl, pH 8.0 in the same manner. The extracted chromatin pellet was collected and further centrifuged at 78,000 X g for 5 hours. The resulting supernatant was dialyzed against 20 volumes of 0.05 M Tris-HCl, pH 7.0, and centrifuged in the same manner. The 0.35 M NaCl-extracted chromatin was further extracted sequentially with 1.0 M NaCl and 2.0 M NaCl, dialyzed against 0.4 M NaCl-0.01 M Tris-HCl, pH 7.0, and centrifuged in the same manner. The 0.6 M, 1.0 M, and 2.0 M NaCl chromatin extracts thus obtained were separately treated with Bio-Rex 70. The unadsorbed proteins were dialyzed against 20 volumes of 0.05 M NaCl-0.01 M Tris-HCl, pH 7.0, and lyophilized. The lyophilized material was dissolved in and dialyzed against appropriate buffer for SDS-PAGE gel electrophoresis.

Preparation 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-0.02 M Tris-HCl. The above treatment was repeated three times. The dry DNA-cellulose powder was then suspended in and dialyzed against 0.1 M Tris-HCl, pH 8.4, containing 0.01 M EDTA and 0.14 M β-mercaptoethanol. 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 X g for 10 min. The aqueous phase was re-extracted with an equal volume of buffer-saturated phenol and washed free of unbound DNA by suspending the resulting precipitate in cold 95% ethanol. The crude DNA was collected on a stirring rod and dissolved in 0.1 M Tris-HCl, pH 8.4, by addition of 0.5% SDS and extracted alternately with autodigested pronase (1 mg per ml) for 4 to 6 hours. The DNA solution was made to 0.5% SDS and extracted twice with a chloroform-isopropyl alcohol (24:1) mixture. After centrifugation, the nucleic acid was collected on a stirring rod and redissolved in 0.1 M Tris-HCl (pH 8.4)-8.6 M urea-0.01 M EDTA and collected by centrifugation. This was repeated three more times. The DNA concentration of each reconstituted chromatin was adjusted to 50 µg per ml. The following combinations of Ehrlich ascites chromosomal components were used in preparing the reconstituted chromatin: (a) histone and non-histone in a ratio of 1:12.1; (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 1.12:1.34:1. In each case, the final DNA concentration of each complex used was 1 mg per ml.

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 procedure of Marushige and Bonner (13) modified by Seligy (14). The isolated chromatin was washed free 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 1.0 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 Dounce homogenizer and stirring for 20 min. The extract was collected by centrifugation at 105,000 X g for 2 hours. The resulting pellets were cut into small pieces and re-extracted three more times with 1.0 parts (w/w) of Buffer B (0.35 M NaCl-0.02 M Tris-HCl, pH 7.5). The extracted chromatin was saved, whereas supernatants 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 4 hours, and lyophilized until use.

Preparation of Nuclear Sap Proteins—Ehrlich ascites tumor nuclei 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 X g for 15 min. The supernatant was collected and further centrifuged at 78,000 X g for 5 hours. The resulting supernatant was dialyzed against 1,000 volumes of 0.02 M Tris-HCl, pH 8.0, for 6 hours and lyophilized. The lyophilized powder was dissolved in the appropriate buffer and dialyzed for at least 6 hours before use.

Preparation of Cytoplasmic Soluble Proteins—Washed Ehrlich ascites tumor nuclei 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 X g for 15 min. The supernatant was collected and further centrifuged at 78,000 X g for 5 hours. The resulting supernatant was dialyzed against 1,000 volumes of 0.02 M Tris-HCl, pH 8.0, for 6 hours and lyophilized. The lyophilized powder was dissolved in the appropriate buffer and dialyzed for at least 6 hours before use.

Preparation of Nucleoplasmic Soluble Proteins—Ehrlich ascites tumor nuclei isolated as described previously were extracted for 1 hour with 100 volumes of 0.14 M NaCl-0.01 M Tris-HCl, pH 8.0. A total of three such extractions were performed and the extracts were collected by centrifugation at 20,000 X g for 15 min. The three extracts were pooled, dialyzed against 0.05 M NaCl-0.01 M Tris-HCl, pH 7.0, and lyophilized. The lyophilized material was dissolved in and dialyzed against appropriate buffer for SDS-PAGE gel electrophoresis.

The abbreviations used are: SDS, sodium dodecyl sulfate; SSC, 0.15 M sodium chloride-0.015 M sodium citrate.
To determine the amount of DNA bound to the cellulose, an aliquot of the DNA cellulose in Buffer D (0.05 M NaCl-0.001 M 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 dialyzed against a buffer blank.

EDTA-0.01 M aliquot of the DNA cellulose in Buffer D (0.05 M NaCl-0.065 M Tris-HCl, pH 6.8)-0.1% 2-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, 0.27% bis-acrylamide, 0.125 M Tris-HCl, pH 8.0, 0.1% SDS, 0.065 M NaCl, N',N',N'-tetramethylethylenediamine, 0.5 μg urea, 0.15% ammonium persulfate to the gel tubes. The acrylamide solution was overlaid with 30 ml of isobutyl alcohol and allowed to polymerize for 18 hours. A stacking gel of 200 μl of a final solution of 2.5% acrylamide, 0.07% bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.065 M NaCl, N',N',N'-tetramethylethylenediamine, 0.5 μg urea, 0.15% ammonium persulfate, was added about 0.5 cm above the separating gels and again 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 glycine, 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 removed from the electrophoresis apparatus and stained for 2 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 Gilford linear transport. Molecular weight markers used consisted of thyroglobulin (molecular weight 67,000), ovalbumin (molecular weight 43,500), and trypsin (molecular weight 23,090).

The incubation was at 37° for 60 min. At the end of the incubation, the reaction mixture was boiled for 20 min in a boiling water bath, and added with 10 ml of toluene cocktail. The radioactivity was counted in a Packard liquid scintillation spectrometer.

**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 from chromatin of 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 percent 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 0.55 M Proteins**—Fig. 1 shows SDS polyacrylamide gel electrophoretic patterns of the 0.30 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.
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. Belzer 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 with either 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
Ehrlich ascites tumor reconstituted in a ratio of 1:0.94; DNA
0.35 ascites tumor chromatin components. Reconstitution was per-
chromatin and nucleoproteins reconstituted from various Ehrlich
ascites tumor DNA and whole histone reconstituted in a ratio of
various templates used were: DNA-histone (O--O), Ehrlich
0.94; DNA-O.35 ascites tumor native DNA.
RNA polymerase as described under "Experimental Procedure." The
tivities were assayed with Ehrlich ascites tumor nucleoplasmic
amount of DNA present in each template. Template ac-
data are consistent with the results obtained with chromatin
reconstitution, suggesting that the 0.35 DNA-cellulose. This fraction is eluted with
DNA (Ehrlich ascites 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 re-
action. PS-0.35 m NHP from Ehrlich ascites tumor chromatin was
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 (A-A); 1:1 (O--O).
The absorbance at 280 nm.

Amount of the unadsorbed fraction from
Escherichia coli DNA-cellulose. The un-run off fraction
from E. coli DNA-cellulose column (top left peak) was chromo-
tographed 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>Bound to E. coli DNA</th>
<th>Bound to tumor DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Cytoplasmic soluble protein</td>
<td>13.7</td>
<td>0</td>
</tr>
<tr>
<td>Total non-histone proteins</td>
<td>18.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Total 0.35 m NHP</td>
<td>18.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Analysis of Ehrlich ascites DNA binding proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Weight</th>
<th>Alkali-labile phosphorus in protein (mg/g)</th>
</tr>
</thead>
<tbody>
<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>3.2</td>
</tr>
<tr>
<td>Total 0.35 m NHP</td>
<td>97.2</td>
<td>2.8</td>
</tr>
<tr>
<td>DNA-binding fraction</td>
<td>95.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

classified into three groups: heterologous and homologous DNA binding proteins, representing 18% 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_{480} 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 36,000 or less as compared with the unfractuated proteins and are also heterogeneous as seen in Fig. 5.

Stimulation of DNA-templated RNA Synthesis in Vitro by Specific DNA-binding Fraction of 0.35 M NHP—The detection of a 0.35 M NHP fraction enriched in phosphoprotein and binding selectively to homologous DNA raises the question of what effect it may have on transcription of DNA. To investigate this, the 0.35 M NHP fraction that selectively bound to tumor DNA was tested in an Ehrlich ascites tumor nucleoplasmic RNA polymerase system as described under "Experimental Procedure." As seen in Fig. 6, the specific DNA binding protein fraction stimulates RNA synthesis in vitro. At low protein concentrations, the stimulation is in proportion to the amount of added proteins. With further increase of the proteins relative to Ehrlich ascites tumor DNA template, the stimulated RNA synthesis reaches saturation equivalent to a 60% increase above a DNA control. RNA isolated from the specific DNA-binding fraction, when added to the RNA synthesizing system, had no effect on the template activity of DNA. This shows that RNA alone is not capable of stimulating transcription of DNA. When the tumor DNA was substituted by heterologous DNAs prepared from rat liver, calf thymus, and chicken erythrocytes, no stimulation of RNA synthesis by the specific DNA-binding protein fraction of tumor chromatin was evident. The stimulation of DNA-templated RNA synthesis by the specific DNA binding 0.35 M NHP fraction is, therefore, template-specific, effective only with homologous DNA. This result is consistent with the fact that the specific DNA-binding proteins bind selectively to tumor DNA.

![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.](http://www.jbc.org/)

![Fig. 6. Template specificity in activated RNA synthesis in vitro stimulated by the DNA binding 0.35 M NHP fraction. Each reaction tube contained 5 μg of DNA and amounts of the specific DNA binding protein fraction as indicated in Ehrlich ascites tumor nucleoplasmic RNA polymerase reaction. Control tube containing 5 μg of Ehrlich ascites tumor DNA without added protein gave an incorporation of 2350 cpm of [3H]GMP into acid-insoluble material and is considered to be 100% template activity. Assay conditions and preparation of sample for counting are described under "Experimental Procedure." The DNA templates used in this study were from Ehrlich ascites tumor (○), calf thymus (△), rat liver (□), and chicken erythrocytes (▼).](http://www.jbc.org/)
that the loosely bound proteins of Ehrlich ascites tumor chromatin cannot contain a phosphoprotein-rich fraction that is capable of stimulating template-specific, RNA polymerase-specific transcription of DNA from either RNA polymerase from Micrococcus luteus DNA and amounts of the specific DNA binding fraction as indicated in Ehrlich ascites tumor nucleoplasmonic RNA polymerase reaction (O—O) and in Micrococcus luteus RNA polymerase reaction (Δ—Δ). The template activities of controls containing 5 μg of DNA in either RNA polymerase system are considered to be 100%.

The preferential effect of the specific DNA binding 0.35 M NHP fraction on tumor DNA-templated RNA synthesis in vitro prompted us to examine the requirement of the enzyme source. Because the above stimulation was observed by using Ehrlich ascites tumor RNA polymerase II, a prokaryotic RNA polymerase from Micrococcus luteus was tested. As shown in Fig. 7, the specific DNA-binding protein fraction had no effect on RNA synthesis in the M. luteus RNA polymerase reaction templated by tumor DNA. These data, taken together, show that the loosely bound proteins of Ehrlich ascites tumor chromatin contain a phosphoprotein-rich fraction that is capable of stimulating template-specific, RNA polymerase-specific transcription from DNA.

DISCUSSION

In this work, the non-histone proteins loosely bound to 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 proteins (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 nucleoplasmonic 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- and RNA polymerase-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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