Inhibition of Transcription in Vitro by a Non-histone Protein Isolated from Ehrlich Ascites Tumor Chromatin

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NINA C. KOISTRABA AND TUNG YUE WANG
From the Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14214

A non-histone protein has been isolated from Ehrlich ascites tumor chromatin. The minimum molecular weight of this non-histone protein, estimated by sodium dodecyl sulfate gel electrophoresis and amino acid analysis, is approximately 10 to 11,000. This non-histone protein is acidic, contains 9.7% alkali-labile phosphorus, binds to DNA; and inhibits transcription of DNA in vitro by the homologous RNA polymerase. The per cent inhibition of RNA synthesis is not affected by increasing amounts of RNA polymerase, but is reduced by addition of excess DNA. In the presence of the non-histone protein, incorporation of [γ-32P]GTP and [γ-32P]ATP into RNA in the in vitro RNA synthesizing system is inhibited, with no apparent change in the average chain length of the RNA product. Inhibition of RNA synthesis is completely eliminated if the DNA template is allowed to interact with ATP prior to the addition of the non-histone protein. These results indicate that the observed repression of in vitro RNA synthesis is due to the effect of the non-histone protein on the DNA, inhibiting the initiation of RNA chain formation.

In differentiated cells, regions of the DNA are obstructed such that only certain genome sequences are transcribed, resulting in the expression of cellular individuality (1-5). This is manifested in vitro by the reduced template capacity of eukaryotic chromatin, as compared with the template activity of naked DNA. Chromosomal proteins appear to be the biochemical determinants responsible for this gene restriction, and hence, the differentiated cellular state. Histones, which lack diversity and specificity, and whose synthesis is coupled to DNA replication, probably contribute to the permanent inactivation of certain regions of DNA (2). In contrast, the more transient repressions of gene expression appear to derive from the heterogenous, tissue-specific non-histone chromosomal proteins. For example, it has been shown that dehistonized chromatin has less template activity than naked DNA (6). Also, in mitotic cells, RNA synthesis is reduced as compared with S-phase cells. This reduction appears to be caused by non-histone proteins (7, 8). These observations suggest a repressing action by non-histone proteins participating in the overall control of differential gene expression.

In this paper, we report the isolation of a non-histone protein from a DNA-protein complex of Ehrlich ascites tumor chromatin that inhibits the initiation of RNA synthesis in an in vitro homologous RNA polymerase B system.

EXPERIMENTAL PROCEDURE

Isolation of Non-histone Protein—Male Albany Swiss mice (1200) weighing approximately 30 g each were injected intraperitoneally with 10⁶ Ehrlich ascites tumor cells per mouse and killed 14 days post-inoculation. Chromatin was isolated from these cells as described previously (9). Briefly, the ascites fluid was harvested, filtered through cheesecloth, and whole cells were collected by centrifugation.

The tumor cells were osmotically disrupted and the crude nuclei were sedimented by centrifugation at 100 x g for 10 min. The nuclei were purified by treatment with 1% Triton X-100 in 0.14 M NaCl containing 0.01 M Tris-HCl, pH 7.5, and 0.001 M MgCl₂, and were pelleted by centrifugation. In all subsequent solutions, phenylmethylsulfonylfluoride was present at a concentration of 1.6 mM.

The tumor nuclei were immediately washed three times with 0.14 M NaCl/0.01 M Tris-HCl, pH 7.5, followed by two washes with 0.05 M EDTA/0.05 M Tris-HCl, pH 8.0, and finally by three washes with 0.05 M Tris-HCl, pH 8.0. Chromatin was then isolated from the washed nuclei according to the procedure of Seligy and Miyagi (10). The chromatin, after washing with 0.05 M Tris-HCl, pH 8.0, was homogenized with 2.0 M NaCl containing 0.02 M Tris-HCl, pH 8.0, and the suspension was stirred for 4 to 6 hours. This mixture was clarified by centrifugation at 78,000 x g for 1 hour and the supernatant was dialyzed against 13 volumes of 0.4 M NaCl/0.01 M Tris-HCl, pH 8.0, to precipitate the DNA-protein complex. The latter was collected by centrifugation at 78,000 x g for 1 hour. The histones of the DNA-protein complex were removed by three extractions with a total of 2000 ml of 0.4 M H₂SO₄. The acid-extracted DNA-protein complex was treated with phenol according to the procedure of Teng et al., (11). The phenol phase was reduced to 1/10 its volume by dialysis against 0.1 M acetic acid/0.14 M β-mercaptoethanol, and then dialyzed against 0.05 M acetic acid/0.0 M urea/0.14 M β-mercaptoethanol for 24 hours. This was followed by a 2-hour dialysis against 0.1 M Tris-HCl, pH 8.4/0.06 M NaCl/0.01 M EDTA/0.14 M β-mercaptoethanol and then against 0.4 M NaCl/0.02 M Tris-HCl, pH 7.0, for 12 hours. The dialyzed protein was passed through a Bio-Rex 70 (Na⁺) column. The run-off protein was collected, dialyzed against several changes of 0.01 M Tris-HCl, pH 8.0, and concentrated by negative pressure. The yield was 100 to 120 μg of protein.

Preparation of DNA and DNA-cellulose Chromatography—DNA was prepared from nuclei isolated from Ehrlich ascites tumor cells by a modified procedure (9) of Marmur (12). Chromatography on DNA-cellulose was carried out according to the method of Alberts and Herrick (13), using native Ehrlich ascites tumor DNA.

Preparation and Assay of RNA Polymerase—RNA polymerase B
The specific activity of the RNA polymerase B was 120 to 170 units/mg of enzyme. One unit of RNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of radioactive label from 1 nmol of [3H]GTP into acid-insoluble material under the conditions described below.

RNA polymerase activity was assayed in a reaction mixture of 0.025 mol of Tris-HCl, pH 7.9, 0.75 mM of MgCl₂, 12.5 mM of (NH₄)₂SO₄, 0.17 mM of EDTA, pH 7.9, 1.0 mM of β-mercaptoethanol, 0.0625 mM each of ATP, CTP, and UTP, and 0.00625 mM of [3H]GTP (1 µCi), 5 µg of Ehrlich ascites tumor DNA, the enzyme, and, where indicated, other additions. The reaction mixture was incubated at 37°C for 1 hour. Precipitation of the sample by 5% trichloroacetic acid and preparation of the acid-precipitated sample for counting were as described previously (9).

Incorporation of [γ-32P]ATP and [γ-32P]GTP into RNA—RNA chain initiation was studied by incorporation of [γ-32P]ATP and [γ-32P]GTP into RNA, employing the RNA polymerase assay mixture and conditions as described above, except that all the ribonucleoside triphosphates, labeled or unlabeled, were 0.0625 mM each and DNA, 10 µg. Separate mixtures contained either [γ-32P]ATP (1360 cpn/pmol) or [γ-32P]GTP (8600 cpn/pmol) and [γ-32P]GTP (61 cpn/pmol). The reaction mixtures were incubated at 37°C for the respective time periods as indicated in the figures. At the end of the reaction mixtures were chilled and 1 µg of carrier yeast RNA was added. To each mixture 5.15 ml of 0.01 M sodium pyrophosphate, pH 8.0, in 0.5% sodium dodecyl sulfite, and 2 ml of water-saturated, redistilled phenol were added. This mixture was shaken immediately at 37°C for 3 min and the aqueous phase was collected. The interphase was re-extracted with 2 ml of sodium pyrophosphate and sodium dodecyl sulfite as before, from which the aqueous phase was collected and pooled with the first aqueous phase. This aqueous solution was subjected to phenol extraction. The resulting aqueous phase was brought to 20% potassium acetate, pH 4.5. RNA was precipitated by addition of 2 volumes of cold ethanol. Upon standing at 20°C for 12 hours, the precipitate was collected by centrifugation for 15 min at 12,000 × g. The pellet was dissolved in 0.05 M Tris-HCl, pH 8.0. Precipitation of the sample by 5% trichloroacetic acid and preparation of the acid precipitate for counting were as described previously (9).

Polyacrylamide Gel Electrophoresis—Nondenaturing 5% polyacrylamide gel electrophoresis was performed according to the procedure described by Krakow (14) and 20% sodium dodecyl sulfite polyacrylamide gel electrophoresis was performed according to that of Laemmli (15). Two-dimensional gel electrophoresis was performed essentially as described by Suria and Liu (16). The first dimension (top gel) consisted of isoelectric focusing in 8 M urea with ampholine, pH range 3.1 to 10.0, at 100 volts for 6 hours. The second dimension consisted of electrophoresis of the isoelectrically focused gel onto a sodium dodecyl sulfate gel slab as described by Laemmli (15). The value indicates molecular weight × 10³. Sodium dodecyl sulfate buffer front (B.F.) is seen below the protein band. (C) two-dimensional gel electrophoresis of 30 µg of non-histone protein was performed as described by Suria and Liu (16). The first dimension (top gel) consisted of isoelectric focusing in 8 M urea with ampholine, pH range 3.1 to 10, at 100 volts for 6 hours. The second dimension consisted of electrophoresis of the isoelectrically focused gel onto a sodium dodecyl sulfate gel slab for 3.75 hours at 30 mA per slab. The gels of A, B, and the slab gel were stained for 12 hours in 45% methanol/10% acetic acid/0.1% Coomasie brilliant blue and destained with 5% methanol/10% acetic acid. The isoelectrically focused gel of C was stained for 4 hours in 60% ethanol/10% acetic acid/0.1% Coomasie brilliant blue and destained with 30% methanol/10% acetic acid.

RESULTS Polyacrylamide Gel Electrophoresis of the Non-histone Protein

The non-histone protein isolated from the DNA-protein complex of Ehrlich ascites tumor chromatin appeared as one major protein band when subjected to electrophoresis in a nondenaturing polyacrylamide gel, as shown in Fig. 1A. Under denaturing conditions (Fig. 1B), the non-histone protein migrated as a single protein band in 20% sodium dodecyl sulfate polyacrylamide gel, having an estimated molecular weight of approximately 11,000, migrating slightly faster than monomeric cytochrome c. Fig. 1C shows a two-dimensional gel electrophoretic pattern of the non-histone protein. In the first dimension, the protein was isoelectrically focused at pH 5.3 as a single band. When the isoelectrically focused gel was subjected to 15% sodium dodecyl sulfite gel electrophoresis in the second dimension, the protein migrated with the sodium dodecyl sulfate buffer front, indicating subunit molecular weight of 12,000 or less.

To determine whether trace amounts of other proteins were present, the non-histone protein was radioiodinated with 125I and subjected to electrophoresis in a nondenaturing polyacrylamide gel system. As shown in Fig. 2A, only one major radioactive peak was detected. Sodium dodecyl sulfite gel electrophoresis of the 125I-non-histone protein also showed 1 radioactive band (Fig. 2B). However, in both cases, there was a small amount of radioactive material at the front shoulder, indicating some contamination.

Amino Acid Composition of Non-histone Protein—Table I shows the amino acid composition of the non-histone protein. Conspicuously absent were cysteine and methionine. The ratio of acidic to basic amino acid residues was 1.42. Since the non-histone protein had an isoelectric pH of 5.3 by isoelectric focusing, the non-histone protein is acidic. From the amino acid

1 J. Chan, R. M. Loor, and T. Y. Wang, unpublished data.
GEL SLICE

Fig. 2. (A) Electrophoresis of $^{131}$I-non-histone protein (0.05 mg) on nondenaturing 5% polyacrylamide gel as described in Fig. 1. The gel was sliced into 1-mm sections and each slice was counted in a Beckman gamma 300 system counter. (B) Electrophoresis of $^{131}$I-non-histone protein (0.2 mg) (---) and DNA-binding $^{131}$I-non-histone protein (approximately 0.05 mg) (-----) on 10% sodium dodecyl sulfate polyacrylamide gel as described in Fig. 1. Samples were subjected to electrophoresis for 24 hours at 0.5 mA/tube. The gels were sliced into 1-mm sections and each slice was counted as in A.

Table I

Amino acid composition of non-histone protein

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar %</th>
<th>Minimal no. of residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.7</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.6</td>
<td>6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.1</td>
<td>8</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.9</td>
<td>4</td>
</tr>
<tr>
<td>Serine</td>
<td>7.5</td>
<td>6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.9</td>
<td>11</td>
</tr>
<tr>
<td>Proline</td>
<td>7.3</td>
<td>6</td>
</tr>
<tr>
<td>Cystine</td>
<td>11.6</td>
<td>10</td>
</tr>
<tr>
<td>Alanine</td>
<td>10.8</td>
<td>9</td>
</tr>
<tr>
<td>Valine</td>
<td>7.7</td>
<td>6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.8</td>
<td>2</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.1</td>
<td>4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.5</td>
<td>3</td>
</tr>
</tbody>
</table>

(Glutamic acid + aspartic acid)/(lysine + histidine + arginine)

1.42

analysis, the minimum molecular weight of the non-histone protein was calculated to be 10,273. Analysis of the non-histone protein for alkali-labile phosphorus showed that it contained 2.7% phosphorus, thus being a phosphoprotein.

Binding of Non-histone Protein to DNA—To examine DNA affinity of the non-histone protein, the $^{131}$I-non-histone protein was charged onto an Ehrlich ascites tumor DNA-cellulose column previously equilibrated with 0.14 M NaCl/0.01 M Tris-HCl, pH 7.4. After washing the DNA-cellulose column successively with 0.14 M NaCl/0.01 M Tris-HCl, pH 7.4, and 0.6 M NaCl/0.01 M Tris-HCl, pH 7.4, the radioactive protein was eluted with 1.0 M NaCl/0.01 M Tris-HCl, pH 7.4. Electrophoresis of a aliquot of the eluted $^{131}$I-protein was then performed in sodium dodecyl sulfate polyacrylamide gel. As shown in Fig. 2B, a single radioactive band migrated identically with that observed prior to DNA-affinity chromatography. Nuclease and protease activities, determined as described elsewhere (9) using radioactively labeled DNA, RNA, and protein substrates, were not detectable in the non-histone protein. We conclude that the non-histone protein is DNA-binding, and consists mainly of one polypeptide or multiple polypeptide of molecular weight approximately 10,273.

Inhibition of RNA Synthesis in Vitro by the Non-histone Protein

The non-histone protein markedly inhibited the DNA-dependent RNA polymerase B reaction in the presence of homologous template (Fig. 3). This inhibitory activity co-migrated with the major stainable protein band when subjected to electrophoresis under nondenaturing conditions (Fig. 1A). The inhibitory effect of the non-histone protein was not significantly alleviated by increasing the concentration of Ehrlich ascites tumor RNA polymerase but was proportionately reduced by increasing amounts of the template (Table II). The data indicate that the inhibition of RNA synthesis in vitro by the non-histone protein results from interaction of the non-histone protein with the template rather than with the enzyme.

In order to determine whether this inhibitory action of the non-histone protein on RNA synthesis is on chain initiation or on chain elongation, RNA chain initiation was assayed by measuring the incorporation of $[\gamma^{-32}P]ATP$ and $[\gamma^{-32}P]CTP$ into RNA. The results are shown in Fig. 4. In the presence

Fig. 3. Inhibition of DNA-dependent RNA synthesis in vitro by the non-histone protein. Ehrlich ascites tumor RNA polymerase B, 0.04 unit, and Ehrlich ascites tumor DNA, 5 µg, were used in the in vitro RNA synthesizing system. Abscissa indicates the amounts of non-histone protein added and the ordinate represents counts per min of $[\gamma^{-32}P]GMP$ incorporated in 60 min using the standard RNA synthesizing system as described in the text.

Table II

Effect of template and enzyme concentration on RNA synthesis inhibited by non-histone protein

Assay conditions were as described in the text with modifications of the amounts of DNA and RNA polymerase as indicated. In addition each reaction tube contained 3.7 µg of the non-histone protein.

<table>
<thead>
<tr>
<th>DNA</th>
<th>RNA polymerase</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>unit</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>0.05</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>21</td>
</tr>
<tr>
<td>3.7</td>
<td>0.01</td>
<td>39</td>
</tr>
<tr>
<td>3.7</td>
<td>0.02</td>
<td>35</td>
</tr>
<tr>
<td>3.7</td>
<td>0.05</td>
<td>39</td>
</tr>
</tbody>
</table>
of the non-histone protein. RNA synthesis, as determined by the incorporation of \[^{3}H\]UMP into RNA, was inhibited by 50% at the end of a 60-min incubation period. Similarly, incorporation of \[^{3}P\]ATP and of \[^{3}P\]GTP into RNA was reduced to 49 and 53%, respectively. The average chain length of the RNA product (Fig. 5), computed from the initiation data, was virtually identical for the control and the non-histone protein-inhibited RNA synthesis. The data indicate that the non-histone protein inhibits initiation of RNA chains and has no effect on the rate of RNA synthesis in vitro.

To further examine the reaction of the interaction between the non-histone protein and the transcription apparatus, a series of experiments was conducted in which the order of addition of the non-histone protein and labeled ATP in the RNA synthesizing system was varied. The results are summarized in Table III. It is seen that when the non-histone protein was preincubated with DNA (Reaction 2) or was added at the onset of the reaction (Reaction 9) and then RNA synthesis was allowed to proceed, RNA synthesis was markedly inhibited. However, if the non-histone protein was added after ATP had interacted with the DNA-RNA polymerase complex (Reaction 3) or after the first phosphodiester bond was formed (Reactions 4–6), subsequent addition of the non-histone protein and the remaining ribonucleoside triphosphates resulted in no significant change in RNA synthesis as compared with the 50-min RNA synthesis control (Reaction 1). The non-histone protein was also ineffective when added after RNA synthesis had commenced (Reactions 7 and 8). \[^{3}H\]ATP alone, in the absence of other three ribonucleoside triphosphates, gave insignificant incorporation (Experiment 2). These results, taken together, indicate that the non-histone protein inhibits transcription in vitro by interacting with DNA and preventing the initiation of the RNA chain.

**DISCUSSION**

In the present work, we have described the isolation of a non-histone protein from Ehrlich ascites tumor DNA-protein complex by phenol extraction. Data have been presented to show that the non-histone protein inhibits the DNA-dependent RNA polymerase reaction by inhibiting the initiation of RNA chains. The inhibition of RNA synthesis by the non-histone protein is alleviated by increasing concentrations of DNA, but not by increasing amounts of RNA polymerase. This, together with the data from RNA initiation experiments and the DNA-binding characteristic of the non-histone protein, indicate that the non-histone protein inhibits transcription by acting on the DNA. The non-histone protein is apparently tightly bound in the DNA-protein complex, since, after binding to DNA-cellulose, the non-histone protein is eluted by 1 M NaCl.

**TABLE III**

Inhibition of RNA synthesis by non-histone protein

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>[^{3}H]ATP incorpo- rated</th>
<th>pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DNA</td>
<td>[^{3}H]ATP + (C,G,U)TP</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>2. DNA + NHP</td>
<td>[^{3}H]ATP + (C,G,U)TP</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3. DNA + [^{3}H]ATP</td>
<td>NHP + (C,G,U)TP</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>4. DNA + [^{3}H]ATP + CTP</td>
<td>NHP + (C,G,U)TP</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>5. DNA + [^{3}H]ATP + GTP</td>
<td>NHP + (C,G,U)TP</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>6. DNA + [^{3}H]ATP + UTP</td>
<td>NHP + (C,G,U)TP</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>7. DNA + [^{3}H]ATP + (C,G,U)TP</td>
<td>NHP</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>8. DNA + [^{3}H]ATP + (C,G,U)TP</td>
<td>NHP</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>9. DNA + NHP + [^{3}H]ATP + (C,G,U)TP</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers indicate reactions as cited in the text.
*Total incubation time, 10 min.
In eukaryotes, most of the transcriptive restriction of DNA is undoubtedly due to histones. The apparent lack of species specificity of histones has, however, made it difficult to assign a specific regulatory role to the histones. Non-histone proteins, on the other hand, are complex, manifest tissue variations (1, 3, 21-23) and interact with DNA (9, 24-27). The non-histone proteins have been shown to contain fractions that activate transcription in vitro (9, 11, 28-31), indicating a potential role of non-histone proteins in gene activation. The data reported in this paper suggest, moreover, that non-histone proteins also contain component(s) which participate in negative control of gene activity. These observations indicate, therefore, that non-histone chromosomal proteins are involved in the differential regulation of gene expression.

If the non-histone protein described in this report indeed operates at the gene level, it should bind to specific recognition sites of DNA for selective transcription of genes. The non-histone protein could also be tissue-specific. Study of the non-histone protein-inhibited transcripts, an examination of such inhibitory non-histone protein from other tissue systems, and of the specific DNA sequences to which the non-histone protein binds would establish its regulatory role in gene control. These are problems for further investigations.

*Note Added in Proof—After submission of this paper the work of Natori et al. (32, 33) came to our attention. These investigators isolated a protein (R protein) from crude extract of whole Ehrlich ascites tumor cells which inhibited transcription of native DNA in the homologous RNA polymerase B reaction. RNA synthesis in vitro was inhibited only when R protein was added at the onset of the RNA polymerase reaction, thus indicating a repression at the RNA initiation step. They further showed that the R protein was a ribonucleoprotein containing 70% CG-rich RNA and had a molecular weight of 38,000, determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. This would give an estimated molecular weight of 11,000 for the protein. Whether the R protein of Natori et al. is a non-histone chromosomal protein is unknown.*

REFERENCES

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Inhibition of transcription in vitro by a non-histone protein isolated from Ehrlich ascites tumor chromatin.
N C Kostraba and T Y Wang


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