Specific Binding of Phosphorylated Non-Histone Chromatin Proteins to Deoxyribonucleic Acid*

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SUMMARY

The binding of phosphorylated non-histone proteins to DNA has been studied via the technique of DNA-cellulose chromatography. At an NaCl concentration of 0.14 M, approximately 1% of this protein fraction prepared from rat liver binds to rat DNA. Both the percentage of binding observed and the binding constant for the protein-DNA interaction are dependent on ionic strength. The phosphorylated proteins bind less efficiently to foreign DNAs than to host DNA, thereby demonstrating the specificity of the binding reaction. Treatment with various enzymes demonstrates the protein nature of the material involved in conferring the specificity of binding of the non-histone proteins. At physiological ionic strength, binding sites on the DNA are saturated at a value of approximately 1 µg of phosphorylated protein per 100 µg of DNA. The material which selectively binds to the DNA is still a heterogeneous family of phosphorylated proteins as demonstrated via sodium dodecyl sulfate acrylamide gel electrophoresis. The majority of these DNA-binding proteins fall in a molecular weight range of 30,000 to 70,000, and amino acid analyses confirm that these phosphorylated proteins are non-histones. The results are consistent with a proposed role of the phosphorylated non-histone proteins in specific gene control.

Nuclear proteins have received a great deal of attention in recent years because of their possible involvement in gene control mechanisms in higher organisms. Due to their limited heterogeneity and general lack of tissue specificity, it has been widely accepted that histones are not responsible for specific gene control (1-3). In contrast, the non-histone (or "acidic") chromatin proteins are both highly heterogeneous and tissue-specific (4-8) and exhibit many properties expected of genetic regulatory molecules (for review see Ref. 9).

Since specific genetic regulatory molecules which have been purified from microbial systems have turned out to be allosteric proteins which recognize and bind to specific sites on DNA, it might be expected that specific gene regulators in eukaryotic cells would also be able to bind specifically to DNA. In earlier experiments in our laboratory employing the technique of DNA-cellulose chromatography, it was demonstrated that a small portion of the non-histone chromatin proteins from rat liver would bind to rat DNA, but not to salmon or bacterial DNA (10). Subsequently Teng et al. (4), employing sucrose density gradient analysis, also found that non-histone proteins specifically bind to DNA and that the binding proteins are heavily phosphorylated.

The present studies describe in further detail the properties of nuclear protein binding to DNA in our DNA-cellulose chromatography system and provide additional evidence that the proteins which recognize and bind to specific gene sequences in eukaryotic cells are phosphorylated, non-histone proteins.

EXPERIMENTAL PROCEDURE

Isotope Administration and Isolation of Nuclei—Male Sprague-Dawley rats, weighing 150 to 250 g, were injected with [32P]orthophosphate in 0.9% NaCl at a dose of 3 mCi/100 g body weight. After 2 hours, the animals were killed and their livers were removed and quickly chilled.

Nuclei were isolated by a dense sucrose procedure adapted from Pogo et al. (11). All operations were performed at 4°. Thirty grams of liver were homogenized with scissors and dispersed in 300 ml of 0.3 M sucrose-3 mM MgCl2. The tissue was homogenized for 2 min at 6,000 rpm in a Sorvall Omni-Mixer with the small-bladed chamber. The resulting homogenate was filtered through double-napped flannelette, and centrifuged for 7 min at 1,000 × g. The resulting pellet was resuspended in 225 ml of 2.4 M sucrose-1 mM MgCl2 by homogenizing 2 min at 2,000 rpm in the Sorvall Omni-Mixer using the large-bladed chamber. The resulting suspension was centrifuged for 60 min at 70,000 × g. The nuclear pellets were collected and washed twice by resuspending in 0.01 M Tris-HCl (pH 7.5)-0.25 M sucrose-4 mM MgCl2 and centrifuging for 7 min at 1,000 × g.

Purification of Phosphorylated Non-Histone Proteins—The soluble proteins of the nuclear sap were removed from the purified nuclei by suspending them in 0.01 M Tris-HCl, pH 7.5, for 20 min. The nuclei were collected by centrifugation at 10,000 × g for 10 min and were then suspended in 1.0 M NaCl-0.02 M Tris-HCl, pH 7.5, to a final protein concentration of 2 mg per ml. The suspension was dispersed using a Polytron homogenizer (Brinkmann) for 30 s at setting 3. The resulting viscous solution was mixed with 1.5 volumes of 0.02 M Tris-HCl, pH 7.5, and the precipitated nucleohistone was removed by centrifugation at 200,000 × g for 2 hours. Bio-Rex 70 (Na+), which

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had previously been equilibrated with 0.4 M NaCl-0.02 M Tris-
HCl, pH 7.5, and was then added to the supernatant at a ratio of
20 mg of Bio-Rex per mg of protein. After stirring slowly for
10 min, the suspension was centrifuged for 10 min at 6,000 × g
and the supernatant was withdrawn. The resin was washed by
suspending it in 10 to 15 ml of 0.4 M NaCl-0.02 M Tris-HCl, pH
7.5 and centrifuging again for 10 min at 6,000 × g. The two
supernatants were then combined and calcium phosphate gel
(12) was added at a ratio of 0.46 mg of gel per mg of protein.
After slowly stirring for 20 min, the suspension was centrifuged for
5 min at 6,000 × g and the supernatant was discarded. The
gel was washed by resuspension in 10 to 15 ml of 1.0 M (NH₄)₂-
SO₄-0.05 M Tris-HCl, pH 7.5, using a motor-driven stirrer at
1,000 rpm, followed by centrifugation at 6,000 × g for 5 min.
The supernatant was again discarded and the gel was then dis-
solved by gentle homogenization with a Teflon Potter-Elvehjem
tissue grinder in 0.3 M EDTA (pH 7.5)-0.33 M (NH₄)₂SO₄ in a
ratio of 0.2 ml of solution per mg of gel. The suspension was
allowed to stand for 1 hour in the cold with occasional rehomog-
emization and the insoluble residue was then removed by cen-
trifugation for 15 min at 33,000 × g. The supernatant was then
diaлизed overnight against 0.05 M Tris-HCl, pH 7.5.

The final protein product contains about 1.3% phosphorus by
weight and will generally be referred to as the phosphorylated
non-histone protein fraction (previously referred to as the “nu-
clear phosphoprotein” fraction).

**DNA-Cellulose Chromatography—**DNA was extracted from
rat and mouse tissues according to the phenol procedure of
Kirby and Cook (13). DNA from salmon sperm, *Escherichia
coli* and *Clostridium perfringens* was obtained commercially, but
each preparation was subsequently run through the Kirby pro-
cedure so that all DNAs would receive equivalent treatment.
DNA-cellulose was prepared by mixing 6 ml of DNA solution
(1 mg per ml in 0.01 M Tris-Cl (pH 7.2)-0.001 M Na₂EDTA)
with 2 g of Munktell 410 cellulose (Bio-Rad) which had previ-
ously been washed with ethanol. The material was allowed to
dry overnight in an open Petri dish and was then put in a vac-
uum desiccator for 1 week. It was then washed and stored
frozen as described elsewhere (14). The final product contained
about 1 mg of DNA bound per g of cellulose.

**Binding of phosphorylated non-histone proteins to DNA-
cellulose** was studied via the technique of gradient dialysis (15).
The ³²P labeled protein preparation was first incubated for 15
min at 25° with deoxyribonuclease (1 μg per ml) in the presence
of 1 mm Mg²⁺ and was then dialyzed against several changes of
cold 0.6 M NaCl, 0.001 M Na₂EDTA, 0.001 M β-mercaptoetha-
ol, 10% glycerol, and 0.02 M Tris-HCl, pH 7.5 ("dialysis
buffer"). An aliquot of the protein solution containing 50 to
400 μg of protein in 0.5 to 1.0 ml was mixed with 250 mg of
DNA-cellulose which had been previously washed with the same
dialysis buffer. The mixture was then dialyzed for 2 hours
against dialysis buffer containing 5 μ U urea and then against
successive 2-hour changes of dialysis buffer-5 μ U urea in which
the NaCl concentration was sequentially lowered to 0.4, 0.2, and
0.14 M. The mixture was finally dialyzed overnight against
0.14 M NaCl dialysis buffer without urea. All dialyses were
performed at 4°. The DNA-cellulose was then placed in a
0.5-cm diameter glass column and washed successively with
dialysis buffer made up with 0.14, 0.06, and 2.0 M NaCl.
Fractions (0.5 ml) were collected and counted in Bray’s solution (16)
in a Beckman liquid scintillation counter.

**Acrylamide Gel Electrophoresis—**Protein fractions were con-
centrated in the cold by dehydrating in dialysis tubing covered

**RESULTS**

**Binding of Rat Phosphorylated Proteins to Rat DNA—**All bind-
ing studies were performed with phosphorylated proteins which
had been prerun with DNA-cellulose made from salmon sperm
DNA. Only those proteins which did not bind to the salmon
sperm DNA-cellulose column were used in subsequent studies,
thereby largely (although not entirely) eliminating the problems
of nonspecific binding. Fig. 1 shows that, when such pretreated
proteins are chromatographed on rat DNA-cellulose, about 1% of
the radioactivity binds to the column and is eluted at 0.6 M
NaCl. A small, but reproducible amount of binding occurs in
the absence of DNA.

If the peak of bound material is collected, concentrated, and
rerun with a fresh batch of rat DNA-cellulose, a much larger
percentage of the radioactivity is now found to bind to the column
(Fig. 2). This experiment demonstrates that a small fraction of
the phosphorylated non-histone proteins has been selectively
purified on the basis of its affinity for DNA under these condi-
tions.

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Effects of various enzymatic treatments on binding of phosphorylated non-histone proteins to DNA

The purified phosphorylated non-histone protein fraction labeled with $^{32}$P was incubated for 30 min at 25° in the presence of one of the enzymes listed in the table. The incubation with ribonuclease was performed in the presence of 2 M NaCl-5 M urea (15). Binding of the treated protein preparations to DNA-cellulose was performed as described in the text and total radioactivity present in the 0.6 M NaCl elution step was determined. Activity of the various enzyme preparations was checked by measuring the rate of hydrolysis of known standards of yeast RNA, soluble starch (Merck), lecithin, p-toluenesulfonyl-L-arginine methyl ester, or benzoyl arginine ethyl ester as described elsewhere (22).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Enzyme concentration</th>
<th>Radioactivity bound to DNA</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>20 mg/ml</td>
<td>745 pm</td>
<td>61%</td>
</tr>
<tr>
<td>a-Amylase</td>
<td>20 mg/ml</td>
<td>772 pm</td>
<td>27%</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>30 mg/ml</td>
<td>702 pm</td>
<td>11.5%</td>
</tr>
<tr>
<td>Trypsin</td>
<td>30 mg/ml</td>
<td>321 pm</td>
<td>59.6%</td>
</tr>
<tr>
<td>Papain</td>
<td>50 mg/ml</td>
<td>118 pm</td>
<td>85.1%</td>
</tr>
<tr>
<td>Pronase</td>
<td>100 mg/ml</td>
<td>56 pm</td>
<td>92.9%</td>
</tr>
</tbody>
</table>

Inhibition increases. The majority of subsequent experiments were performed at 0.14 M NaCl, even though maximal binding was not observed at this ionic strength, because this ionic strength is the best available approximation of the situation in vivo.

Effects of Enzymatic Treatments on DNA-Protein Binding—In order to determine the chemical nature of the material responsible for the observed binding of our protein fraction to DNA, the effects of various types of enzymatic pretreatments were determined. As can be seen from the results summarized in Table I, ribonuclease, a-amylase, and phospholipase C have no significant effect on the binding of the phosphorylated protein fraction to DNA, thus suggesting that RNA, polysaccharides, and phospholipids do not play critical roles in this process. Since the phosphorylated protein fraction is also routinely pretreated with deoxyribonuclease, it is also unlikely that any DNA in our protein samples is involved in the binding reaction.

The protein nature of the binding material is substantiated by its sensitivity to proteolytic enzymes such as papain and pronase. The decreased effectiveness of trypsin in inhibiting the binding reaction is consistent with our previous observation that this phosphoprotein fraction is relatively resistant to tryptic digestion. The conclusion that the radioactivity bound to DNA is protein in nature is further substantiated by amino acid analyses which showed that 90% of the $^{32}$P in our protein fraction is present as phosphoserine, while the remaining 10% is accounted for as phosphothreonine.

Amino Acid Compositions of Protein Fractions—Amino acid analyses were performed to confirm the non-histone nature of these phosphorylated proteins and to determine whether the DNA-binding fraction could be differentiated on the basis of its amino acid content. Table II compares the amino acid composition of the total phosphorylated non-histone chromatin protein fraction with the fraction which binds to DNA in 0.14 M NaCl. As can be seen, the over-all amino acid compositions are quite similar, but the bound fraction is reproducibly richer in serine and lower in total charged (acidic and basic) residues. These data also show that both the total and bound protein fractions
are acidic (non-histone) proteins, with the total fraction being slightly less acidic (acidic to basic ratio of 1.24) than the bound fraction (acidic to basic ratio of 1.36). Table II also includes data which allow comparison of these phosphorylated non-histone proteins to the total non-histone fraction as well as to a typical histone fraction. It can be seen that these phosphorylated non-histone protein fractions are clearly distinguishable from both histones and the total non-histones, being more acidic than the histones but less acidic than the average non-histone.

**Dependence of DNA Binding on Protein Concentration**—The total amount of phosphorylated protein which binds to DNA-cellulose columns was found to vary as a function of the concentration of phosphoprotein employed during the gradient dialysis. As can be seen from Fig. 4, the amount of protein bound to DNA increases as a function of protein concentration until a maximum is reached, at which point the binding levels off. The level at which this plateau occurs depends both on the amount of DNA present and the ionic strength of the binding medium. When binding is carried out at a final salt concentration of 0.14 M NaCl, the observed level of protein-DNA binding is 2- to 3-fold higher.

Experiments of this type also allow a rough estimate of the binding constant of these phosphorylated proteins for DNA, which is defined as the protein concentration at which one-half maximal binding occurs. As can be seen by comparing Curves A and B, the value of the binding constant is reduced at the lower ionic strength.

**Species Specificity of DNA-Protein Binding**—If the DNA binding of phosphorylated non-histone proteins observed in this system is related to the control of specific gene activity, then one would expect the binding to be relatively specific for host DNA. Therefore, binding of rat liver phosphorylated proteins to DNAs from a variety of sources has been compared. As can be seen from the data in Table III, binding of 32P-labeled proteins to DNA-cellulose was performed by gradient dialysis to a final salt concentration of 0.14 M NaCl as described in the text and total radioactivity present in the 0.6 M NaCl elution step was determined. All columns were normalized to contain 200 μg of DNA adsorbed to 200 mg of cellulose.

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Radioactivity bound to DNA (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>626</td>
</tr>
<tr>
<td>Rat testes</td>
<td>671</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>578</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>210</td>
</tr>
<tr>
<td>Calf liver</td>
<td>175</td>
</tr>
<tr>
<td>Salmon sperm</td>
<td>120</td>
</tr>
<tr>
<td>E. coli</td>
<td>110</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>84</td>
</tr>
</tbody>
</table>

**Table II**

**Amino acid compositions of nuclear protein fractions**

Amino acid compositions are expressed in terms of moles per 100 moles of amino acids recovered. For comparative purposes, data for total non-histone protein (26) and F2b histone (24) are included. ND, not determined.

<table>
<thead>
<tr>
<th>Phosphorylated non-histone proteins</th>
<th>Total moles</th>
<th>Bound to DNA moles</th>
<th>Total non-histone moles</th>
<th>Histone (F2b) moles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.2</td>
<td>7.5</td>
<td>6.3</td>
<td>14.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0</td>
<td>2.3</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.1</td>
<td>6.3</td>
<td>5.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.8</td>
<td>9.1</td>
<td>9.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>14.1</td>
<td>12.8</td>
<td>12.4</td>
<td>9.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.5</td>
<td>4.5</td>
<td>5.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Serine</td>
<td>10.2</td>
<td>12.8</td>
<td>7.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Proline</td>
<td>6.6</td>
<td>8.1</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.0</td>
<td>8.5</td>
<td>7.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.2</td>
<td>7.8</td>
<td>7.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.4</td>
<td>trace</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>5.0</td>
<td>6.1</td>
<td>5.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.4</td>
<td>1.0</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.0</td>
<td>2.5</td>
<td>4.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.4</td>
<td>6.2</td>
<td>9.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.9</td>
<td>1.3</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Phelyalanine</td>
<td>2.9</td>
<td>3.2</td>
<td>3.9</td>
<td>2.0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>ND</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Total moles</td>
<td>23.9</td>
<td>21.9</td>
<td>21.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Total non-histone moles</td>
<td>19.3</td>
<td>16.1</td>
<td>13.4</td>
<td>24.5</td>
</tr>
<tr>
<td>Acidic to basic moles</td>
<td>1.24</td>
<td>1.38</td>
<td>1.63</td>
<td>0.59</td>
</tr>
</tbody>
</table>
In our earlier studies on the binding of nuclear proteins to DNA-cellulose columns (10) the total non-histone protein fraction labeled with \(^{32}\)P was employed. The current experiments, using a purified \(^{32}\)P-labeled phosphoprotein subfraction of the non-histone proteins, provide more direct evidence that the specific binding molecules are phosphorylated proteins. Due to the increased purification of our protein fraction, the amount of binding observed in the current studies is severalfold higher than the 0.1% reported previously. The present results are qualitatively consistent with those of Teng et al. (4) who found that the phenol-soluble non-histone phosphoproteins specifically bind to host DNA. However, they found that up to 13% of their phosphoprotein fraction bound to DNA, a value considerably higher than found in the present studies. Aside from the obvious differences in the methods of protein isolation and assay procedures, a major factor involved in this apparent discrepancy is probably the difference in ionic strength employed during the binding reaction. Since both the overall level of the binding as well as the binding constant have been shown by the present experiments to be greatly affected by ionic strength, care is needed in comparing the results from different experiments. Indeed, many variables which have not yet been systematically studied most likely also affect the degree of binding observed, so that one must be generally cautious in interpretations of the significance of the absolute quantity of material bound.

However, even without attaching too much significance to the absolute value of percent binding, one can come to some general conclusions based on the general level of binding which has been observed. Under ionic strength conditions which approximate physiological conditions, a relatively small number of sites on DNA are bound specifically by phosphorylated, non-

**FIG. 6.** Comparison of the heterogeneity of DNA-binding proteins obtained from experiments where binding is carried out at different ionic strengths. \(^{32}\)P-Labeled phosphorylated non-histone proteins from rat liver were bound to rat DNA-cellulose via gradient dialysis to a final salt concentration of either 0.14 or 0.01 M NaCl. The protein peaks which elute at 0.6 M NaCl were collected, concentrated, and analyzed via SDS-acrylamide gel electrophoresis. The radioactivity in the gels is plotted as a function of distance of migration. Note the somewhat increased heterogeneity of the material bound at the lower ionic strength, especially in the region of the arrows.
histone proteins. Since a relatively small percentage of the genome is active in differentiated cells of multicellular organisms, this observation is consistent with what might be expected for genetic regulatory molecules. In addition, based on available data for the DNA and non-histone protein content of rat liver nuclei, one can calculate that the average liver cell contains about 10^5 molecules of phosphorylated non-histone proteins which specifically bind to DNA. Although this value is relatively large, this protein fraction is still highly heterogeneous and may represent most or all of the DNA-associated proteins involved in specific regulation.

The current studies provide strong support for the idea that specific recognition and interaction occurs between phosphorylated, non-histone proteins and DNA. Enzymatic treatments of this protein fraction suggest that non-protein components are not responsible for the specific binding observed. Likewise, all of the DNA samples tested in this system have gone through the same phenol purification procedure which lowers protein contamination to extremely low levels (12). Thus, it is unlikely that protein contaminants in the DNA preparations are responsible for the differences in binding observed when DNAs from different sources are employed.

The phosphorylated proteins which specifically bind to DNA are still highly heterogenous as judged by SDS-acrylamide gel electrophoresis. Indeed, although there were some detectable differences between the bound and unbound fractions, it was somewhat surprising to find that the degree of heterogeneity of the DNA-binding fraction is almost as great as that of the total phosphoprotein fraction. This observation emphasizes an inherent limitation in employing SDS-acrylamide gel electrophoresis for the differences in binding observed when DSAs from different sources are employed.

The phosphorylated proteins which specifically bind to DNA are still highly heterogenous as judged by SDS-acrylamide gel electrophoresis. Indeed, although there were some detectable differences between the bound and unbound fractions, it was somewhat surprising to find that the degree of heterogeneity of the DNA-binding fraction is almost as great as that of the total phosphoprotein fraction. This observation emphasizes an inherent limitation in employing SDS-acrylamide gel electrophoresis for the analysis of extremely heterogeneous mixtures, since it is obvious that a protein “band” of defined molecular weight may still consist of a family of proteins with different functional properties. Some differences between the total non-histone phosphoproteins and the specific DNA-binding fraction are also evident from amino acid analyses, which in addition also confirm that the DNA-binding proteins are truly non-histone in nature.

The present experiments demonstrate, then, that the phosphorylated non-histone proteins exhibit one property expected of genetic regulatory molecules, that is, the ability to specifically recognize and bind to host DNA sequences. But specific DNA binding is only one property expected of genetic regulatory molecules and in itself does not prove the hypothesis that these proteins are involved in specific gene control. However, these phosphorylated non-histone proteins also have been shown to exhibit other properties expected of genetic regulatory molecules. Changes in their phosphorylation rate have been shown to correlate with changes in gene activity in a variety of systems (25–29), they have been shown to be highly heterogeneous and tissue-specific (4, 6), and addition of these phosphorylated proteins has been shown to alter the rate of RNA synthesis in vitro (4, 30, 31). In addition, their rapid phosphorylation and dephosphorylation provide a possible mechanism for the continual modulation and control of their structural and functional properties (32–34). Thus, evidence obtained from a variety of independent experimental approaches strongly points to a role of the phosphorylated non-histone proteins in gene regulation. The current studies on the specificity of their binding to DNA lends further support to this conclusion.

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