Localization of the Sites Along Nucleosome DNA Which Interact with NH₂-terminal Histone Regions*

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Trypsin digestion of HeLa nucleosomes produces the same series of discrete histone breakdown products observed previously by others during digestion of chromatin, thus, trypsin excises the NH₂-terminal ends of the histones from the chromatin core particle. The resulting nucleoprotein complex sediments at 9 S, has an increased molecular ellipticity at 280 nm, and has DNase I-susceptible sites at 10 nucleotide intervals. Nucleosomes containing a ³²P label at the 5'-DNA termini were digested sequentially with trypsin and DNase I. Following trypsin digestion, the segments of nucleosome DNA 20 to 35 and 60 to 80 nucleotides from the 5' end became more susceptible to DNase I, suggesting that these segments interact with the trypsin-sensitive regions of the histones.

The concept that eukaryotic chromatin is composed of nucleoprotein subunits, called "nucleosomes" (2) seems well established, and a variety of models has been proposed for the structure of the chromatin subunit (1, 3-10). However, the details of nucleosome structure are not well understood; more complete knowledge of the protein-DNA interactions within the chromatin subunit is necessary in order to fully understand the mechanisms leading both to the packaging of DNA within the nucleus and to the regulation of the expression of genetic information. For example, there may be only subtle differences between nucleosomes containing transcribed DNA sequences and those containing sequences which are repressed (11-13).

Therefore, we have developed methods for preparing homogeneous populations of intact nucleosomes (14) and for studying nucleosome structure in more detail than was previously possible (15-18). Here, we have examined the contribution to nucleosome structure of the trypsin-sensitive NH₂-terminal ends of the histones. The results indicate that the NH₂ ends of the histones interact with the segments of nucleosome DNA which are 20 to 35 and 60 to 80 nucleotides from the 5'-DNA termini. However, the NH₂ ends do not, by themselves, determine the distribution of DNase I-susceptible sites at 10-nucleotide intervals along nucleosome DNA, and they are not required for the maintenance of the nucleoprotein complex in a nucleosome-like conformation.

MATERIALS AND METHODS

Cell culture, preparation of nucleosomes, labeling of nucleosome DNA with ³²P, digestion with DNase I, analysis of DNA and protein, and measurement of radioactivity were as previously described (14, 15, 19-21).

Sedimentation velocity was determined in the Beckman model E ultracentrifuge at 48,000 rpm and 20° in 0.1 M NaCl, 10 mM Tris/Cl, pH 8, using the automatic photoelectric scanner system. Circular dichroism measurements were made in the same buffer, as previously described (22).

Trypsin-digested nucleosomes were hydrolyzed for 20 h in 6 N HCl at 110°. Amino acid analysis was performed using conventional automated equipment. Amino acids were detected fluorometrically as o-phthalaldihydde condensation products (23).

Trypsin, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, was from Worthington Biochemical Corp. Digestions were at 20° in 5 mM Tris/Cl, pH 8, at a trypsin concentration of 5 μg/ml and a nucleosome concentration of 10 A260/μl units/ml. Digestions were terminated by the addition of a 20-fold (w/w) excess of lima bean trypsin inhibitor (Worthington Biochemical Corp.); preliminary experiments indicated that this amount of trypsin inhibitor was required to halt the reaction rapidly. Zero time controls received trypsin inhibitor prior to exposure to trypsin. In all experiments, the extent of proteolysis was monitored by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis of nucleosomal proteins, as previously described (14). Under the conditions described, no detectable intact histones remained after 128 min of digestion. Trypsin was stored as a stock solution (1 mg/ml) at 4° in 1 mM HCl; lima bean trypsin inhibitor was stored as a stock solution (5 mg/ml) at 4° in 5 mM Tris/Cl, pH 8. Both solutions were stable for months.

RESULTS

Effect of Trypsin on Nucleosome Histones and DNA - Digestion of HeLa nucleosomes with trypsin results in the gradual disappearance of intact histones, accompanied by the simultaneous appearance of several discrete smaller products. The kinetics of digestion (not shown) indicates that the histones within the nucleosome are not all equally sensitive to the protease; H3 is degraded most rapidly, followed by H2A, H4, and finally H2B. Similar observations have been made previously (24-26). Fig. 1 shows that trypsin digestion of HeLa nucleosomes generates a characteristic population of cleavage fragments in a pattern which is virtually identical with those previously described (24, 26, 27). Analysis of such fragments by others has suggested that trypsin digestion results primar-
Histones und Nucleosome Structure

FIG. 1. Proteins in native and trypsin-digested nucleosomes. Nucleosomes were digested for 0 min (left slot) or 128 min (right slot) and the reaction stopped with trypsin inhibitor. The digest was made 1% in sodium dodecyl sulfate and 1% in β-mercaptoethanol and was electrophoresed in a discontinuous 18% polyacrylamide gel. Migration was from top to bottom. The positions of migration of the four smaller histones are indicated.

illy in the removal of 20 to 30 amino acid residues from the NH₂ end of each histone (24).

We have isolated the trypsin-resistant nucleoprotein complex by gel filtration, using nucleosomes prepared from cells labeled in vivo with [3H]lysine and [3H]arginine. Fig. 2 shows that during gel filtration approximately half of the radioactivity in the digest passes through the column unretarded. This fraction contains all the input material absorbing at 260 nm and the proteins in this first peak have an electrophoretic pattern identical with that shown in Fig. 1. The protein/DNA ratio of these nucleoprotein complexes is 0.7 to 0.8 g/g, and they have an amino acid composition which is consistent with the idea that trypsin excises the NH₂-terminal histone regions (Table I). Thus, these results, taken together, indicate that the organization of HeLa nucleoprotein, as measured by its digestion with trypsin, is indistinguishable from that of other tissues. Further, it seems that trypsin digestion of HeLa nucleosomes removes the lysine- and arginine-rich NH₂ ends of the histones, as first shown for chromatin by Weintraub and Van Lente (24).

Trypsin contains no detectable endonuclease or exonuclease contamination, as measured both by polyacrylamide gel electrophoresis of DNA purified from trypsin-digested nucleosomes and by measurement of acid-soluble radioactivity generated during trypsin digestion of nucleosomes labeled in vivo with [3H]thymidine (data not shown).

Physical Properties of Trypsin-digested Nucleosomes—Both native and trypsin-digested nucleosomes sediment as homogeneous boundaries during analytical ultracentrifugation. The $\bar{s}_{20w}$ of native nucleosomes is 10.7 S; that of the trypsin-digested particles is 9.0 S. No material sedimented with the velocity of protein-free DNA.

The results of analytic ultracentrifugation implied that trypsin digestion does not release DNA from the nucleosome. This was confirmed by analyzing the trypsin-digested nucleoprotein complex by polyacrylamide gel electrophoresis. Fig. 3 shows that, as digestion proceeds, the mobility of the remain-

![Fig. 2. Gel filtration of trypsin-digested nucleosomes. Nucleosomes labeled in vivo with [3H]lysine and [3H]arginine were digested with trypsin for 128 min and the reaction stopped with trypsin inhibitor. The mixture was made 10 mM in MgCl₂ and layered over a column (1 x 5 cm) of Bio-Gel P-150 equilibrated with 10 mM MgCl₂, 5 mM Tris/Cl, pH 8. Fractions were eluted with the same buffer and analyzed for radioactivity by liquid scintillation counting in Aquasol (New England Nuclear Corp.).](http://www.jbc.org/)

### Table 1

Amino acid composition of trypsin-digested nucleosomes

<table>
<thead>
<tr>
<th>Amino acid*</th>
<th>Theoreticalb</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid + asparagine</td>
<td>6.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Serine</td>
<td>4.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Glutamic acid + glutamine</td>
<td>10.3</td>
<td>10.1</td>
</tr>
<tr>
<td>Glycine</td>
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<td>16.2</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Cysteine</td>
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<td>0</td>
</tr>
<tr>
<td>Valine</td>
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<td>7.7</td>
</tr>
<tr>
<td>Methionine</td>
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<td>1.4</td>
</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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<td>2.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>9.4</td>
<td>9.1</td>
</tr>
</tbody>
</table>

* Proline is not detected in this system.

b The following assumptions were made: (a) HeLa histones have the identical amino acid composition as calf thymus histones; (b) the composition of the histones was as described in Ref. 28; (c) trypsin treatment resulted in the removal of the NH₂-terminal amino acids as follows: H₂A, 1-42; H₂B, 1-34; H₃, 1-27; H₄, 1-23.
Histones and Nucleosome Structure

FIG. 3 (left). Polyacrylamide gel electrophoresis of trypsin-digested nucleosomes. Nucleosomes were digested for increasing times with trypsin; the reaction was stopped with trypsin inhibitor, made 0.1 \( \times \) in electrophoresis buffer, and electrophoresed in a 5% polyacrylamide gel using the Tris/borate/EDTA buffer system (20). Migration was from top to bottom. Digestion times, in minutes, were: A, 0; B, 1; C, 2; D, 4; E, 8; F, 16; G, 32; H, 64; I, 128. Each slot contained 5 \( \mu \)g of DNA in the nucleoprotein complex. Slot J contained 5 \( \mu \)g of purified nucleosome DNA. The gel was stained with ethidium bromide and photographed using ultraviolet transillumination.

FIG. 4 (right). Circular dichroism of trypsin-digested nucleosomes. Circular dichroism spectra of native (---) and trypsin-digested (----) nucleosomes was measured in both 10 mM Tris/Cl, pH 8, and 0.1 M NaCl, 10 mM Tris/Cl, pH 8 with identical results. The DNA spectrum (----) was obtained after the addition of solid sodium dodecyl sulfate to a final concentration of 1%.

FIG. 5. DNase I digestion of native (○) and trypsin-digested (●) nucleosomes. \( ^{2}P \)-labeled native nucleosomes were digested with trypsin for 128 min. The reaction was stopped with trypsin inhibitor, made 10 \( \mu \)M in MgCl\(_{2}\), and digested for the indicated times with DNase I (100 units/ml at 37°). Trichloroacetic acid-precipitable material was collected on glass fiber filters and measured by liquid scintillation counting.

Stained gel (left) and autoradiogram (right) of single-stranded DNA fragments generated during DNase I digestion of trypsin-digested nucleosomes. Nucleosomes were digested with trypsin for 128 min. The reaction was stopped with trypsin inhibitor, made 10 \( \mu \)M in MgCl\(_{2}\) and digested with DNase I (100 units/ml at 37°). Digestion times (in minutes) were: A, 0; B, 1; C, 2; D, 4; E, 8; F, 16.

solution after trypsin digestion. The results also reveal that trypsin digestion produces no increase in the heterogeneity of the nucleosome population (as measured by the breadth of bands on the gel), confirming the analysis of boundaries in the ultracentrifuge.

Fig. 4 shows that the molecular ellipticity at 280 nm of trypsin-digested nucleosomes is about twice that of native particles, but is still less than half that of protein-free DNA. These results suggest that at least some of the DNA in trypsin-digested nucleosomes has assumed a conformation more like that of protein-free DNA. We attempted to determine whether this increase in molecular ellipticity reflects an overall change in DNA conformation or whether certain regions of nucleosome DNA undergo a greater change than other regions; for example, following trypsin digestion, those DNA segments which interact in the native particle with the trypsin-sensitive regions of the nucleosomal histones might undergo a greater
Our previous observations indicated that the intact nucleosome contains a DNase I-susceptible site at each 10-nucleotide interval, just as in intact particles. This finding indicates that the trypsin-sensitive NH\(_2\) ends of the histones are not required for the maintenance of the DNA in a nucleosome-like conformation, having a DNase I-susceptible site each 10 nucleotides. Thus, the trypsin-resistant histone regions must also play an important role in determining the general conformation of nucleosome DNA.

Fig. 7 shows that the DNA in trypsin-treated nucleosomes is more rapidly degraded by DNase I than the DNA in native particles. This finding is consistent with the circular dichroism measurements, which suggested that trypsin digestion allows at least some regions of nucleosome DNA to assume a conformation more similar to that of protein-free DNA.

DISCUSSION

A detailed understanding of nucleosome structure is relevant to several fundamental problems. One involves the nature of the protein-DNA interactions required both for the
initial packing of the DNA into a nucleoprotein complex and for the subsequent maintenance of this complex as an ordered structure. A second involves the subtle alterations in this basic structure which may lead to the specific expression of genetic information.

The results of these experiments indicate that the complete removal of the trypsin-sensitive N\(_H\)\(_T\) ends of the histones leads to a change in the conformation of at least some of the DNA within the nucleosome (its increased molecular ellipticity and increased nuclease susceptibility) and yet does not destroy its structural feature of having nuclease-susceptible sites at 10- nucleotide intervals. These observations imply (a) that the N\(_H\)\(_T\) ends of the histones are not required in the initial folding of the DNA into a nucleoprotein complex) and (b) that the N\(_H\)\(_T\) ends do not, by themselves, determine the distribution of potential nuclease-susceptible sites along nucleosome DNA (although they do affect the relative susceptibilities of particular sites). These studies indicate that the nucleosome is a quite stable structure, since the rather drastic alterations in composition produced by trypsin digestion do not dramatically alter its structural features. Thus, the trypsin-resistant regions of the different histones must not only interact with each other to form the protein core of the nucleosome but must also have important interactions with nucleosome DNA.

Although removal of the N\(_H\)\(_T\) regions of the histones produces relatively minor changes in overall nucleosome structure, the nuclease susceptibility of certain sites along nucleosome DNA is substantially increased following excision of these regions. Following trypsin digestion, the sites 20 to 35, 60 to 80, and, likely, 105 to 120 nucleotides from the 5' end of the DNA, protected against DNase I digestion in the native structure, the nuclease susceptibility of certain sites along nucleosome DNA as loci which interact with the N\(_H\)\(_T\)-terminal regions of the four smaller histones.

Trypsin digestion leads to a reduction in the sedimentation coefficient of the nucleosome from 10.1 to 9.0 S. In contrast, in the presence of 6 M urea, undigested core particles sediment at about 6 S (14). This suggests that the decrease in the sedimentation coefficient of trypsin-digested nucleosomes is primarily related to a loss of mass, rather than to any substantial change in overall conformation. This implies that the N\(_H\)\(_T\) ends of the histones are not required to maintain the nucleosome in its native compact shape. Thus, if the nucleosome is composed of two "half-nucleosomes" (10), the N\(_H\)\(_T\) ends of the histones do not serve to bind the two halves together.

These experiments also show that the decrease in the interaction between nucleosome DNA and the N\(_H\)\(_T\) ends of the histones is accompanied by an increase in the interaction between the DNA and a different protein (in this case, a nuclease). This suggests the possibility that more subtle alterations in the interactions between nucleosome DNA and the N\(_H\)\(_T\) ends of the histones, such as those which might result from histone modification (28–33), might also increase the relative accessibility of certain sites along DNA to other proteins, such as RNA polymerase, hormone-receptor complexes, or other regulatory macromolecules. Such decreases in the strength of interactions between DNA and the N\(_H\)\(_T\) ends of the histones might also play a role in the increased DNase I susceptibility of the transcribed regions of the genome (12, 13).

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