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Specific Sites of Interaction Between Histones and DNA in Chromatin

(nuclease/DNA-electrophoresis)

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ABSTRACT **Staphylococcal nuclease digestion of purified chromatin from duck reticulocytes or calf thymus results in the production of a series of double-stranded DNA fragments of discrete molecular size, ranging from about 130 to 45 base pairs, which can be detected by polyacrylamide gel electrophoresis. Similar patterns of protected DNA fragments are obtained from limit digests of chromatin "reconstituted" from purified DNA and chromatin proteins. The results obtained with reconstituted material do not depend upon the origin of the DNA, which may be derived from a bacterial, viral, or homologous source. The specificity of the protective mechanism, therefore, resides in the structure of the bound histones, and probably not in any special nucleotide sequences present in the DNA. Removal of lysine-rich histones from chromatin before digestion results principally in disappearance from the digest of a DNA fragment about 130 base pairs long. Our preliminary results suggest that other elements of the digest pattern can be assigned uniquely to the remaining histone components. These results indicate that the binding of histones to DNA in chromatin involves a limited number of specific and very well defined contacts between protein and nucleic acid, which arise from structural properties of the histones.**

Many studies of the binding of histones to the DNA of chromatin have suggested that the histones interact with DNA through well defined sites on the proteins (1). Other evidence indicates that the histones may be bound to DNA in the form of specific complexes involving more than one histone species (2–4). It has been proposed (4) that such complexes are arranged in a regular repeating sequence along the DNA, giving rise to the tertiary DNA structure present in chromatin. Evidence suggesting that histone complexes may cover the DNA in a regular fashion is provided by studies of the products of nuclear autodigestion (5, 6). These studies reveal that autodigestion of nuclei results in release of DNA segments that are multiples of a subunit about 200 base pairs in length. Incubation of nuclei with added nuclease results in release of fragments of similar size (7).

In our laboratory we have made use of the enzyme staphylococcal nuclease as a probe of the structure of purified chromatin, and we have isolated those regions of the DNA that are sufficiently tightly covered by protein to be protected from digestion (8, 9). About half the DNA is susceptible to digestion. The rest is protected, and is reduced to double-stranded segments with a weight average length of about 110 to 130 base pairs regardless of the amount of enzyme used (9).

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In this paper we examine in more detail the nature of the reaction product of this digestion, and show that it consists of a well-defined set of DNA segments. We believe that these segments correspond to the points of intimate contact between histones and DNA, and reveal a regular and highly specific mode of interaction between them.

MATERIALS AND METHODS

Chromatin was prepared from washed nuclei isolated from calf thymus and duck reticulocytes, by methods described elsewhere (10). DNA was prepared by standard methods (11). Staphylococcal nuclease (specific activity 6000 U/mg) was obtained from Worthington Biochemical Corp.

Digestions were typically carried out at DNA concentrations of 300 μg/ml, in a solvent containing 1 mM Tris·HCl buffer at pH 8, 0.1 mM $CaCl_2$, and 4 to 10 μg/ml of nuclease. Reactions were stopped by addition of NaEDTA at pH 7. Insoluble limit digests were sometimes concentrated by centrifugation without any effect on the results. In all cases, the products were then incubated (37°, 1 hr) with 100 μg/ml of Proteinase K (E. Merck) after the solvent had been made 0.5 M or 1 M in NaCl. The solutions were extracted with buffered phenol, and the DNA was precipitated with two volumes of 95% ethanol (overnight, −20°), isolated by centrifugation, and redissolved in electrophoresis buffer at concentrations of about 5 mg/ml.

Electrophoresis was carried out in 6% polyacrylamide gels, with an acrylamide:methylene-bisacrylamide ratio of 19:1. The buffer system was that described by Peacock and Dingman (12). A sample volume of 20 μl was used, to which was added 5 μl of 40% sucrose containing brom phenol blue marker dye. Gels were run at 200 V on a 17-cm slab gel for about 2 hr. Gels were stained in "Stains-all" (Eastman) (0.005% in 1:1 formamide-water) overnight, destained in water for 3 hrs, and photographed. Color transparencies or monochrome negatives were scanned in a Joyce-Loebl microdensitometer. The measured density is linear with DNA concentration.

RESULTS

Chromatin prepared from duck reticulocyte nuclei was treated with staphylococcal nuclease, and the digestion stopped by addition of EDTA at various points in the course of the reaction. The DNA was purified and analyzed by electrophoresis on 6% polyacrylamide gels. The results of this experiment are shown in Fig. 1. From the earliest times of digestion, a complex but quite reproducible pattern of bands is observed. As digestion proceeds toward 50%, the band pattern

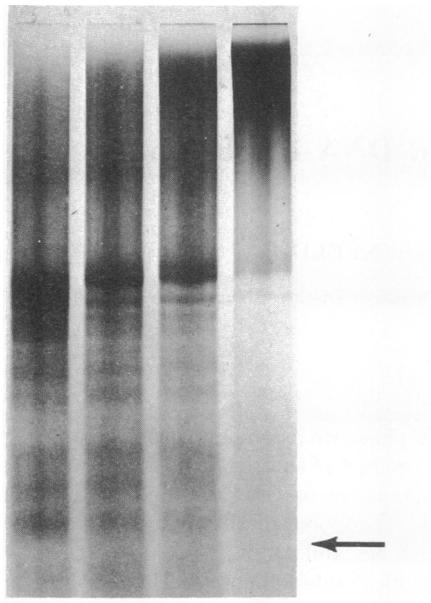

FIG. 1. Gel electrophoresis patterns of staphylococcal nuclease digestion products of duck reticulocyte chromatin. From left to right are 50, 20, 15, and 7% of total DNA digested as determined by solubility in 0.5 M perchloric acid, 0.5 M NaCl. Direction of migration is downward. Dye marker is indicated by arrow.

becomes more intense, while the polydisperse higher molecular weight DNA becomes less abundant. As this limit of digestion is approached, the bands reach their maximum intensity. At this point, the material between the slowest moving sharply defined band (R_F about 0.47) and the dye marker comprises about 75% of the DNA on the gel. The rest of the DNA is of slightly higher molecular weight and remains polydisperse. Digestion of protein-free DNA to the point of 50% acid solubility results only in the appearance of very heterogenous low-molecular-weight material.

The size of the DNA in three of the bands was determined by cutting the unstained bands out of the gel. The DNA was eluted and purified by hydroxyapatite chromatography. Its weight average molecular weight was measured both by short column equilibrium and sedimentation velocity centrifugation in the analytical ultracentrifuge (13). The fractions were also reanalyzed by gel electrophoresis to verify that they had not undergone degradation. We find that the slowest moving band is comprised of DNA, 128 base pairs in size. The other two bands correspond to DNAs that are 76 and 48 base pairs long. These data permit calibration of the gel and assignment of chain lengths to the other bands in the pattern (Fig. 2). Analysis of the sedimentation coefficient distribution of the limit digest (13) reveals that 80% of the DNA lies in the size range between 40 and 190 base pairs, so that the gel pattern of the limit digest reflects the properties of the bulk of the material. All of the protected DNA in the limit digest is double-stranded.

The pattern (Fig. 2) reveals a group of higher molecular weight fragments about 130 to 80 base pairs long, and another group about 60 to 45 base pairs long. Do any of the smaller DNA fragments arise from degradation of the higher molecular weight group? To answer this question we have examined the mass distribution of DNA in the banded region of the gel

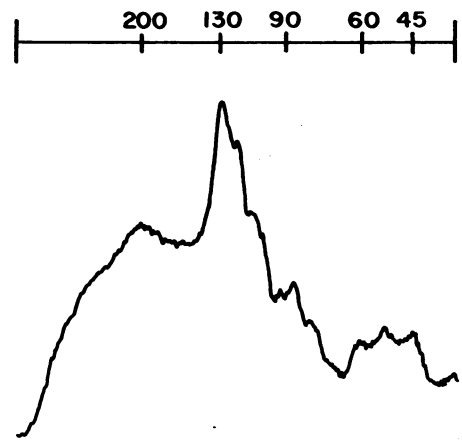

FIG. 2. Densitometer tracings of stained gel. 50% of reticulocyte chromatin DNA is digested. Migration from left to right in this and all other tracings. The line at top shows distance of migration for various DNA chain lengths (base pairs), obtained by calibration as described in *text*. Other points were obtained by interpolation using the relationship $\log_{10} Z = 2.6\text{–}1.05\ R_F$, where Z is the number of base pairs, and the parameters define the line through a graph of log Z versus R_F for the three calibration points.

as a function of time. Fig. 3 shows the integral distribution of mass for four DNA samples obtained from chromatin at various stages of digestion. The distributions are identical, showing that all of the DNA species are generated at identical rates from the earliest times until the limit of digestion has been reached. The larger (130–80 base pair) segments are therefore not precursors of the smaller segments; all of the

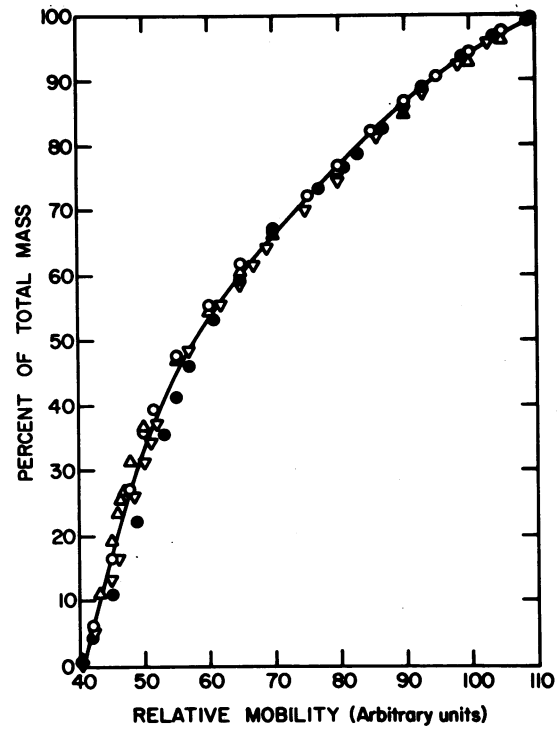

FIG. 3. The integral distribution function of the patterns show in Fig. 1, for the region between $R_F = 0.47$ and $R_F = 1$. The fraction of material of mobility less than X is plotted against X. △, ○, ▽, ● represent duck reticulocyte chromatin that has been 7, 15, 20, and 50% digested, respectively.

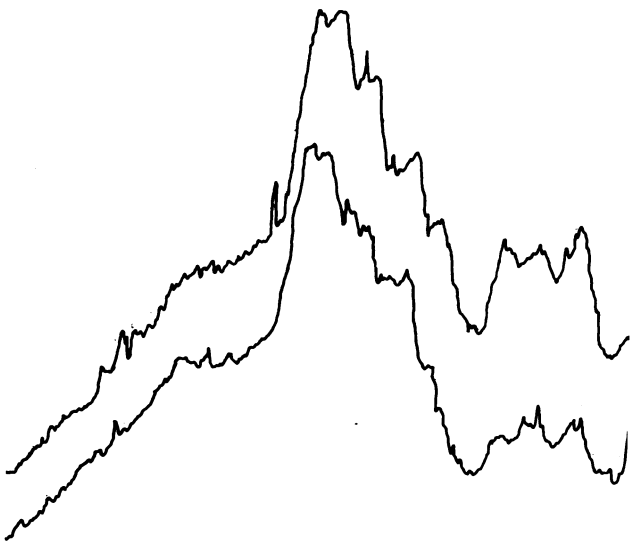

FIG. 4. Comparison of duck reticulocyte (upper) and calf thymus (lower) chromatin digests. About 50% of DNA was digested in each case.

segments are generated simultaneously by the same process, or by independent processes occurring at the same rate. The precursors of all of these bands are the polydisperse DNA segments spread through the gel at R_F values less than 0.47, which decrease in abundance as digestion proceeds. It should be noted that the polydisperse material, of size greater than 130 base pairs, often exhibits a peak or shoulder corresponding to sizes in the neighborhood of 200 base pairs, and some of the polydisperse material appears to persist in the limit digest. A relatively small variation in the amount and size of this material will be detected as a change in measured weight average molecular weight, and probably accounts for the variation in this average that can be observed if the chromatin sample or digestion conditions are varied (9).

The pattern we observe is not unique to duck reticulocyte chromatin. We obtain essentially identical patterns with digests from calf thymus chromatin (Fig. 4) and from rat liver chromatin (data not shown). It is known that many of the structural and biological properties of chromatin can be regenerated from DNA and purified chromatin proteins if these are recombined under controlled conditions of gradually decreasing salt and urea concentration. Staphylococcal nuclease digestion of reconstituted duck reticulocyte chromatin reveals a DNA band pattern qualitatively similar to that observed with native chromatin (Fig. 5). The reconstituted chromatin digest has a more clearly defined peak in the neighborhood of 200 base pairs than that given by native chromatin. The 130 base pair peak is slightly decreased in height, and all of the bands, including those in the range 65–45 base pairs, are broadened. Nonetheless, almost all of the peaks of native chromatin digests are retained. The source of the DNA used in these reconstitution experiments appears to have no effect upon the result: if DNA from a bacterial source, *Micrococcus luteus*, (Fig. 5) or from the bacteriophage λ (data not shown), is substituted for duck DNA and recombined with reticulocyte chromatin proteins, the limit digest of the resulting complex behaves like that obtained by reconstitution with the homologous DNA (Fig. 5).

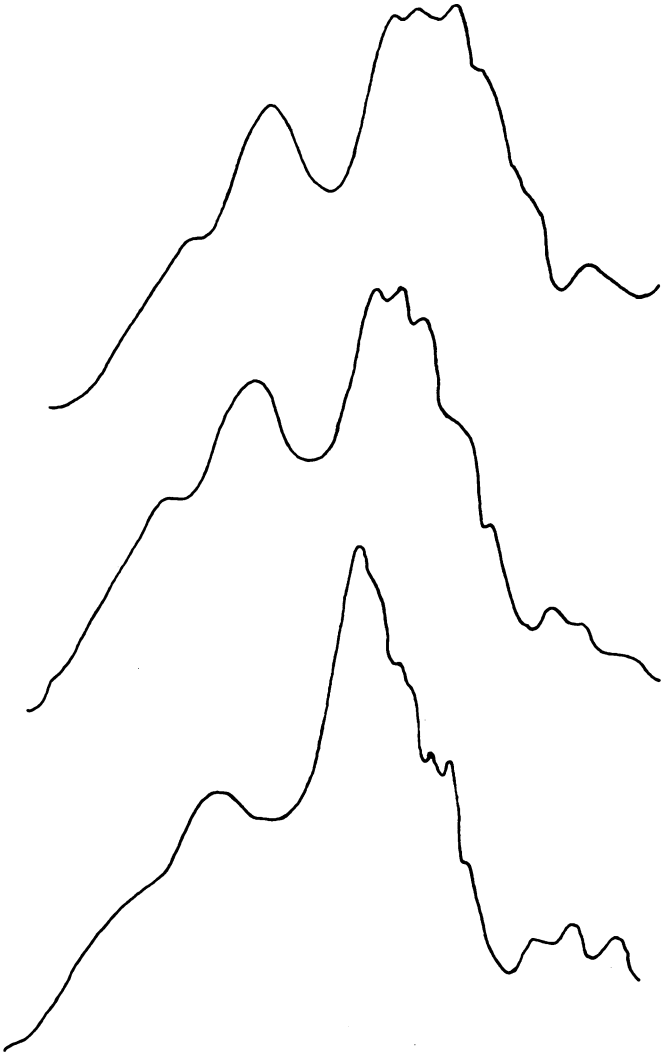

FIG. 5. Digests of reconstituted complexes. Duck reticulocyte chromatin proteins were prepared by centrifugation of chromatin in 3 M NaCl, 5 M urea, 1 mM Tris·HCl buffer at pH 8. The supernate was mixed with either duck DNA or *Micrococcus luteus* DNA and reconstituted by salt-urea gradient dialysis, beginning with 2 M NaCl, 5 M urea, and followed at 80-min intervals by 1.2 M, 1.0 M, 0.8 M, and 0.6 M NaCl (all in 5 M urea), then 0.6 M NaCl without urea, and finally 0.1 mM EDTA at pH 7. Top curve, reconstitution with *M. luteus* DNA, 50% digest; middle curve, reconstitution with duck DNA, 48% digest; lower curve, native duck reticulocyte chromatin, 50% digest. Curves are normalized to the same total amounts of DNA in the gel.

It is of obvious interest to determine whether specific protein components give rise to specific portions of the DNA band pattern. Histones are the predominant protein components in our preparations of chromatin from reticulocytes and calf thymus. Methods have been described (14) for selectively removing the lysine rich histone from chromatin without rearranging the other histone species. These methods remove histone f1 and, in the case of the erythroid chromatin, histone f2c. If this partially stripped chromatin is digested to the limit, the gel band pattern shown in Fig. 6 is obtained. A graph of the difference between this pattern and the one obtained

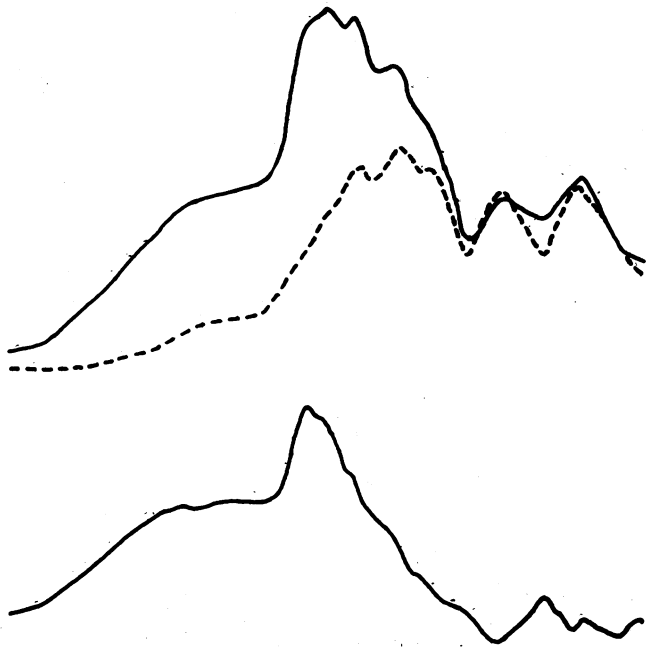

FIG. 6. Digestion of reticulocyte chromatin from which lysine rich histones f1 and f2c have been removed. Solvent used in digestion contained, in addition to Ca^{++}, about 1 mM Mg^{++} that remained from this procedure. DNA digestion products (broken line) are superimposed on the pattern obtained from native reticulocyte chromatin digested in identical solvent (solid line). The curves are normalized to the same starting quantities of chromatin DNA, taking into account that 50% of the native chromatin is resistant to digestion, while only 32% of the DNA from the partly stripped chromatin is resistant. The bottom curve is the difference pattern between these curves.

with whole chromatin is also shown. It is evident that the major change accompanying removal of lysine rich histones is the disappearance of the largest DNA species, 130 base pairs in length, from the limit digest, as well as the disappearance of most of the polydisperse material of larger size. Other DNA segments, including those in the range 120–80 base pairs, remain, though there may be a reduction in their relative abundance. Most of the complex band pattern must therefore be attributed to the remaining arginine rich (f2a1 and f3) and slightly lysine rich (f2a2 and f2b) histones. We have begun experiments involving reconstitution of individual histone fractions with DNA. Our preliminary results (W. Melchior *et al.*, in preparation) show that some of the higher molecular weight bands are generated by histones f2a2 and f2b, while at least one other band appears to arise from histones f2a1 and f3.

Although the electrophoretic pattern shown in Fig. 1 is reproducible and can be obtained easily from a variety of natural and reconstituted chromatin preparations, this pattern is not obtained under all conditions. For example, if chromatin is exposed to staphylococcal nuclease at higher ionic strength (50 mM KCl, 13 mM NaCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM Tris·HCl at pH 8), similar to that used in the nuclear autolysis experiments of Hewish and Burgoyne (6), the characteristic band pattern shown in Fig. 1 is almost completely abolished. If purified duck DNA and reticulocyte chromatin proteins are mixed together in this same solvent, and the precipitated complex is resuspended in 1 mM Tris·HCl buffer at pH 8, before nuclease digestion, the predominant product of digestion conducted for short periods of time appears on 6% acrylamide gels as a band with R_F about 0.05; this R_F corresponds to molecular sizes greater than 300 base pairs, which are poorly resolved in this system. More extensive digestion results in disappearance of most of the intensity in this band, and appearance of DNA of smaller size. However, the characteristic band pattern obtained with chromatin appears only faintly. We conclude that digestion or reassembly of chromatin at high ionic strength does not lead to the same pattern of protection observed at lower ionic strength. This may be related to our earlier observation that protein exchange among sites on DNA can occur under such conditions (8).

The multiplicity of bands does not appear to arise from partial breakdown of histones to smaller discrete fragments by proteases endogenous to chromatin or present in the nuclease preparation. The proteins present in the limit digest have been examined by sodium dodecyl sulfate gel electrophoresis; no significant change in amount of histones was observed, and no smaller molecular weight fragments were detected. The method of chromatin preparation we use appears to remove endogenous proteases (10). We have also carried out nuclease digestion in the presence of 0.01 M sodium bisulfite to inhibit proteolytic activity; the DNA band pattern is not affected by this treatment.

DISCUSSION

We find that the digestion of chromatin by staphylococcal nuclease results in the production of a complex but reproducible pattern of DNA fragments in the size range 130–45 base pairs. The precision with which the enzymic process produces these discrete DNA fragments suggests that there is a highly specific arrangement of the histones on the surface of the DNA, in which certain well-defined portions of the polypeptide chains are in intimate contact with the DNA, or have folded the DNA in such a way that precisely determined lengths of nucleic acid are protected from digestion. The sizes of these segments are probably not determined by the presence of any long special sequences of nucleotides; similar patterns are obtained when nucleoprotein complexes are reconstructed using eukaryotic, bacterial, or viral DNA, which do not share such sequences to any significant extent. These results are consistent with our observation that the majority of chromatin proteins bound to DNA are randomly distributed with respect to base sequence (15). The determining factor in the production of the DNA fragments apparently lies in the structure of the histones themselves, and the way in which the histones attach themselves to DNA in the native chromatin structure.

The limit digest we have examined contains about half the DNA of the original chromatin. The remainder is digested to acid soluble oligomers that are free of protein. We have argued elsewhere (8, 9) that this is not the result of displacement of some histones into solution, followed by binding of these histones to the remaining nucleoprotein. Any analysis of structure that depends upon partial digestion of one of the components is open to the criticism that the procedure perturbs the structure. In the present case, we do not exclude the possibility that local sliding or folding back of segments of the polypeptide chains occurs during digestion. Nonetheless, the reproducible and precisely defined nature of the digestion

products strongly suggests that we are dealing with an aspect of the interaction between nucleic acids and histones which is of importance in the native chromatin structure.

Given the size of the protected DNA segments, it is unlikely that a single histone molecule could provide the necessary protection; it is much more likely that the histones are bound in clusters on the DNA surface. Kornberg and Thomas (4) have shown that the arginine rich histones (f2a1 and f3) occur in solution as a tetramer that contains two of each molecule. They have proposed that the basic chromatin structure involves one such tetramer, and a second tetramer that contains the slightly lysine rich histones (f2a2 and f2b), thus comprising a repeating unit of eight histone molecules which occurs once for each 200 base pairs. Our experiments suggest that there may be a discrete 200 base pair segment in the polydisperse DNA appearing in the gel at $R_F < 0.47$. Comparison of our results with those of Hewish and Burgoyne (6) is not straightforward, since in their case the digestion was carried out in nuclei, at much higher ionic strength, and with an endogenous enzyme. The results of Noll reported by Kornberg (7) were also obtained using nuclei.

These 200 base pair subunits may comprise a stable reaction intermediate in digestion within nuclei, but not in digestion of purified chromatin, which results in a set of still smaller "subunits." These subunits are all produced at identical rates throughout the digestion process. The 130 base pair subunit, as well as all of the polydisperse material of size greater than 130 base pairs, is related to the presence of lysine rich histones. It is not yet clear whether the protective action of lysine rich histones requires the presence of other histones as well. The 130 base pair band may arise from an indirect action of lysine rich histone on the other histones.

The other subunits observed in the gel must be generated by the binding of histones f2a1, f3, f2a2, and f2b. If the subunits are in fact created by specific interactions between histones and DNA, it is difficult to interpret the size and number of the bands as arising from a single kind of repeating unit that is 200 base pairs long. It seems more likely that there are two or more different kinds of repeating clusters of histone segments bound to the DNA, perhaps in the way proposed by Weintraub and Van Lente (16).

We have shown that the specific subunit pattern of protected DNA can be generated by reconstitution of the nucleoprotein from its components, and that portions of the total pattern disappear when lysine rich histone is removed. Our preliminary results suggest that the other components of the band pattern are identifiable with specific groups of histones. The interaction of histones and DNA must involve protein complexes tightly bound over very well defined lengths of DNA. The definition appears to be determined principally by the histones, not the DNA, and indicates that the histone structures involved must be specific, and very limited in variety.

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