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Interaction of Histone f2a1 with T7 Deoxyribonucleic Acid. Cooperativity of Histone Binding†

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ABSTRACT: Hydrodynamic studies are performed on histone f2a1 and reconstituted histone f2a1–T7 DNA complexes. The results of cesium chloride banding of intact and sheared nucleohistone complex indicate that histone f2a1 molecules anneal to DNA in a cooperative fashion starting at the two ends of the DNA molecule. Histone f2a1 is modified by the removal of 18 amino acids from its carboxyl-terminal end by cleavage with cyanogen bromide. The CNBr-modified histone f2a1 molecule no longer binds cooperatively to the DNA. Histone f2a1 aggregates in 0.1 M NaCl but not in 0.005 M NaCl. On the other hand, the CNBr-modified histone f2a1 does not aggregate in 0.1 M NaCl. Sedimentation velocity and viscosity studies indicate that histone binding to DNA causes folding of the DNA only under conditions where the histone molecules themselves aggregate. We suggest that the basic halves of the histone molecules bind electrostatically to the DNA and the carboxyl ends aggregate through the interactions of hydrophobic amino acid residues. A "roof-shingling" mechanism is proposed to illustrate the cooperative binding of histone molecules to DNA. Both the cooperativity of histone binding and the folding of the DNA are observed only with the intact histone f2a1 molecules.

Histones may play roles in the maintenance of the structural integrity of the nucleoprotein (Pardon and Wilkins, 1972), the control of chromosome conformational changes (Sadgopal and Bonner, 1970) and as modifiers of template activity in gene regulation (Johns, 1972). An extreme biological restriction on structural variation is demonstrated by the fact that in over one billion years since the divergence of plants and animals, very little change in the amino acid sequences of the histones has occurred (DeLange and Smith, 1972).

We report here that histone f2a1 molecules appear to anneal to DNA in a cooperative fashion starting at the two ends of the DNA molecule. This artificial nucleohistone may be cross-linked with glutaraldehyde and banded in CsCl density gradients.

Histone f2a1 may be modified by the removal of 18 amino acid residues from the carboxyl-terminal end by cleavage with cyanogen bromide. The residual protein which remains after removal of the peptide will also bind to DNA. But it is not stabilized by glutaraldehyde and dissociates from DNA in concentrated CsCl. Furthermore, we find that the interactions induced by 0.1 M NaCl, which occur with this histone both when free in solution and when bound to DNA, are lost when the peptide is removed from the C-terminal end.

These observations may be taken to imply the existence of a "roof-shingle" fit between successive histone molecules, in analogy with shingling a roof where it is necessary to start at the eaves. The hydrophobic end of each histone may overlap the basic portion of the previous molecule, locking it into place where it winds around the DNA groove.

Materials and Methods

Histone f2a, a mixture of f2a1 and f2a2, and histone f2a1 were isolated from calf thymus as previously described (Ziccardi and Schumaker, 1972).

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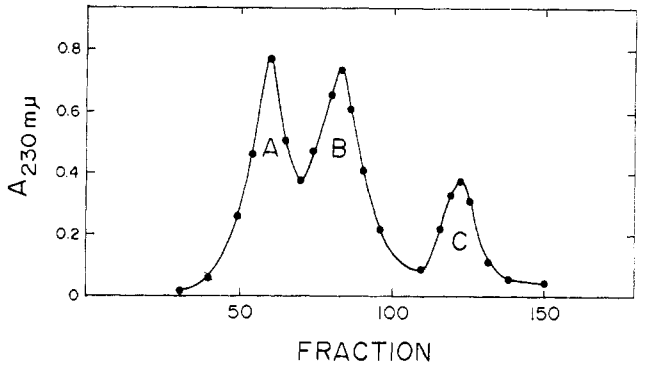

FIGURE 1: Chromatographic separation of CNBr-treated histone f2a into three components: (A) histone f2a2, (B) CNBr-modified histone f2a1, and (C) the 18 amino acid peptide released from the carboxyl-terminal end of histone f2a1. A Bio-Gel P-60 column (150 × 4 cm) was used and was eluted with 0.01 M HCl. The fractions concontain 14 ml, and the flow rate was 80 ml/hr.

Histone f2a1 was cleaved by cyanogen bromide (Eastman Kodak) following the procedure of DeLange *et al.* (1968). Histone f2a (40 mg/ml) in 70% formic acid was added to an equal volume of 21 mg/ml of CNBr in 70% formic acid, and was allowed to react for 24 hr at room temperature in the dark. The solution was diluted tenfold with distilled water and lyophilized over NaOH pellets. Histone f2a1 is cleaved by CNBr at amino acid residue 84, the only methionine residue in the molecule. A peptide consisting of 18 amino acids is freed from the carboxyl end. Histone f2a2 is the other component of histone f2a and contains no methionine residues. The CNBr digest of histone f2a is separated by exclusion chromatography (Bio-Gel P-60; 150 × 4 cm; eluted with 0.01 M HCl) into three components which include histone f2a2; the amino-terminal portion of histone f2a1 (84 amino acid residues), subsequently referred to as CNBr-modified histone f2a1; and the 18 amino acid peptide (elution profile is shown in Figure 1). Polyacrylamide disc gel electrophoresis indicated that the CNBr-modified histone f2a1 was contaminated by some uncleaved histone f2a1. The contaminant was removed by exclusion chromatography on a Bio-Gel P-60 column (22 × 2 cm) eluted wth 0.1 M NaCl. Separation is possible since native uncleaved histone f2a1 aggregates in 0.1 M NaCl and elutes in the void volume while CNBr-modified histone f2a1 does not aggregate and remains monomeric. Cyanogen bromide modified histone f2a1 is now electrophoretically pure, and its amino acid composition is consistent with that predicted from the amino acid sequence of histone f2a1 (DeLange *et al.*, 1969).

DNA was prepared from T7 bacteriphage by phenol extraction. Phage in 1 M NaCl and phosphate–EDTA buffer was extracted five times with solvent-saturated phenol at a temperature of 50°. Freshly distilled phenol was used. Phenol was removed by exhaustive dialysis and an ultraviolet absorption spectrum was characteristic of protein free DNA. (Absorbance at 260 mμ/absorbance at 230 mμ = 2.34.)

Histone–DNA complexes were prepared at 4° by the following procedure: DNA (200–250 μg/ml in 5 M urea–4 M NaCl–0.001 M EDTA) was mixed with an equal volume of histone (80–100 μg/ml) in 5 M urea and then dialyzed for a minimum of 3 hr each against five solutions of decreasing ionic strength, 1.0, 0.4, 0.3, 0.1, and 0.001 M NaCl; all in the presence of 5 M urea and 0.0025 M EDTA. The urea is required to solubilize the histones in the presence of high concentrations of salt. As the salt is removed by dialysis, the electrostatic attractive forces between the histone and DNA allow the formation of a nucleohistone complex. Urea was then removed by dialysis against 0.001 M NaCl, 3.3×10^{-4} M sodium citrate, and 10^{-4} M EDTA (pH 7.0). Finally, the histone–DNA complex was dialyzed against the desired solvent. To prevent the loss of histone during dialysis, a small pore size cellulose casing (Nojax-Union Carbide) was used in the place of normal dialysis tubing. Urea was recrystallized in 95% ethanol. For these histone–DNA complexes, (+/−) = 0.3, where (+/−) is the ratio of the sum of basic amino acid residues to DNA phosphates in the complex (this corresponds to 20 μg of histone/50 μg of DNA).

Histone and DNA concentrations were measured by Lowry *et al.* (1951) and diphenylamine (Burton, 1956) tests, respectively. There is no loss of histone during the extensive dialysis. The same histone to DNA ratio was found in the final histone–DNA complex as was originally mixed together. Preparative centrifugation showed that all histone was bound to the DNA in the complexes. The complex was centrifuged for 4 hr at 40,000 rpm in a SW50L rotor in 0.005 M NaCl for native histone f2a1 and in 0.1 M NaCl for CNBr modified histone f2a1. Control experiments showed that all DNA would pellet while DNA-free histone would remain in the supernatant under these conditions. Lowry tests on the supernatant solutions resulting from the centrifugation of histone–DNA complexes reveal no histone in the supernatants, indicating that at least 95% of the histone in the original solution was bound to the DNA.

Ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanning system. Sedimentation velocity experiments were performed at 20° at 15,000 rpm (histone–DNA complexes), 30,000 rpm (T7 DNA) and 59,780 rpm (histones). In the latter case a synthetic boundary cell (capillary type) was used. Sedimentation equilibrium in a CsCl density gradient was used to determine the buoyant densities of T7 DNA and the histone–DNA complexes. Samples were run for 24 hr at 44,000 rpm and 25°. Prior to sedimentation equilibrium centrifugation of the histone–DNA complexes, they were fixed with glutaraldehyde to prevent the dissociation of protein from the DNA in CsCl. Histone–DNA complex (20 μg/ml in 0.005 M NaCl–3.3×10^{-4} M sodium citrate, pH 7.0) was mixed with an equal volume of 6% glutaraldehyde (Sigma Chemical Co.) in the same buffer. The solution was allowed to sit in the dark at room temperature for 4 hr, then the excess glutaraldehyde was removed by dialysis.

Viscosity measurements were performed using a 3-bulb Ubbelohde capillary viscometer. The histone–DNA complex was sheared to lower molecule weights in a Virtis mixer (Arthur H. Thomas Co.). For shearing, the DNA was at a concentration of 5.9 μg/ml in 0.005 M NaCl, 3.3×10^{-4} M sodium citrate, and 10^{-4} M EDTA (pH 7.0).

Results

Sedimentation of Histone f2a1 and Cyanogen Bromide Modified Histone f2a1. In 0.005 M NaCl histone f2a1 (mol wt 11,280) sedimented as a monomer with a sedimentation coefficient of 0.6 S. However when the ionic strength was raised to 0.1 M NaCl, the molecules aggregated and had a sedimentation coefficient of 19 S. Cyanogen bromide modified histone f2a1 did not aggregate in 0.1 M NaCl but had a sedimentation coefficient of 0.8 S at this high ionic strength. The data are described in Table I. Thus, with a loss of 18 amino acids from its carboxyl end, histone f2a1 no longer aggregated in 0.1 M NaCl.

TABLE I: Sedimentation Velocity Data on Histone f2a1 and Cyanogen Bromide Modified Histone f2a1.

Sample[a]	$s_{20,w}$ (S)[b]
Native histone f2a1 in 0.005 M NaCl	0.6
Native histone f2a1 in 0.1 M NaCl	19.2
CNBr-modified histone f2a1 in 0.1 M NaCl	0.8

[a] Samples are at a concentration of 4 mg/ml and in 3.3×10^{-4} M sodium citrate–10^{-4} M EDTA (pH 7.0) as well as the respective sodium chloride concentrations. [b] Values of $s_{20,w}$ were calculated from the observed sedimentation coefficients, $s_{20,s}$ by the expression $s_{20,w} = s_{20,s}\,\eta_{20,s}\,(1 - \bar{V}\rho_{20,w})/(1 - \bar{V}\rho_{20,s})$, where $\eta_{20,s}$ and $\rho_{20,s}$ are the relative viscosities and densities of the respective solvents.

Sedimentation Velocity and Viscosity Studies on the Histone–DNA Complex. Sedimentation velocity and viscosity data are described in Table II. In 0.005 M NaCl the sedimentation coefficient of the native histone f2a1–T7 DNA complex was close to that of T7 DNA. However, in 0.1 M NaCl the sedimentation coefficient nearly doubled. Combination of sedimentation and viscosity data indicated that little aggregation occurred. Thus to account for the large increase in sedimentation coefficient, the nucleohistone apparently folded into a faster sedimenting form in 0.1 M NaCl, with a decrease in hydrodynamic volume of over 50%. Unlike histone f2a1–T7 DNA complex, CNBr modified histone f2a1–T7 DNA complex did not sediment much faster than T7 DNA in 0.1 M NaCl (see Table II). Although there was a small amount of faster moving complex observed in the sedimentation profile, most of the complex moved with the same velocity as free DNA. It should be again mentioned that all complexes have the same amount of histone bound to DNA. Thus, the removal of 18 amino acids from the carboxyl end of histone f2a1 had markedly diminished its ability to cause the folding of T7 DNA in 0.1 M NaCl.

Sedimentation Equilibrium in CsCl Density Gradients of Intact and Sheared Nucleohistone Complexes. Histone–DNA complexes were fixed with glutaraldehyde prior to banding in order to prevent dissociation of protein from DNA in the CsCl. When studied in these gradients, native histone f2a1–T7 complex was found as a heterogeneous band at 1.63 g/cm^3. Assuming additivity of densities, the buoyant density of the nucleoprotein complex indicates that it consists of 20% histones by weight. Since the complex was 30% histone before CsCl banding, approximately one-third of the original histone may have been dissociated from the DNA in CsCl. But the remaining two-thirds are held in place by glutaraldehyde treatment. On the other hand, the CNBr-modified histone f2a1–T7 DNA complex banded sharply at 1.70 g/cm^3. Since 1.70 g/cm^3 is the buoyant density of pure T7 DNA, it was obvious that all of the CNBr-modified histone f2a1 became dissociated from the DNA in CsCl, even after glutaraldehyde treatment.

The glutaraldehyde-fixed native histone f2a1–T7 DNA complex was then sheared in a Virtis mixer. Table III correlates the degree of shearing with its corresponding sedimentation coefficient and molecular weight. For the three different samples, the glutaraldehyde-fixed complex was broken in approximately one, two, and three places, respectively. Figure 2 illustrates the CsCl banding profiles for the unsheared and sheared samples. Unsheared complex

TABLE II: Sedimentation Velocity and Viscosity Data on T7 DNA and Histone–DNA Complexes.

Sample[a]	$s^0_{20,w}$[b] (S)	$[\eta]$ (dl/g)	Mol Wt[c]
0.005 M NaCl			
T7 DNA	27.9	119	22×10^6
Histone f2a1–T7 DNA complex	29.1		
	27.9	102	25
0.1 M NaCl			
T7 DNA	31.8	96	22
Histone f2a1–T7 DNA complex	57.8		
	66.2	33	39
	55.5		
CNBr-modified histone f2a1–T7	33.0		
DNA complex	34.3	81	26
	34.7		

[a] Samples were in 3.3×10^{-4} M sodium citrate–10^{-4} M EDTA (pH 7.0) as well as above NaCl concentrations. [b] Duplicate and triplicate sedimentation coefficients are from independently prepared complexes. [c] Calculated from the Flory–Mandelkern expression: $NS\eta_0[\eta]/M^{2/3}(1 - \bar{v}\rho) = \beta$, where $\beta = 2.50 \times 10^6$ for 0.1 M NaCl and 2.36×10^6 for 0.005 M NaCl. The expected molecular weight for the histone–DNA complex is 31×10^6 daltons.

banded exclusively at 1.63 g/cm^3. On breaking the complex in half we found that most (97%) of the complex still banded at 1.63 g/cm^3. However, a second break in the complex released 21% of the DNA as free DNA banding at 1.70 g/cm^3. After three breaks, 43% of the DNA was released from the complex while the remaining half was found in a broad band between 1.56 and 1.62 g per cm^3. From the distribution of densities it may be shown that shearing has not "stripped

TABLE III: Correlation of Extent of Shearing of Native Histone f2a1–T7 DNA Complex with Molecular Weight.

Extent of Shearing	$s_{20,w}$[a]	$M_w/(M_w)_0$[b]	Mol Wt (Complex)[c]	Approx No. of Breaks
Unsheared	29.2S	1	30.8×10^6	0
Low[d]	25.5	0.66	20.3	1
Medium	19.4	0.30	9.2	2
High	18.0	0.24	7.4	3

[a] All samples are at 5.9 μg/ml. [b] Since the nucleohistone has the same sedimentation coefficient as T7 DNA in 0.013 M NaCl, we have used the DNA data to estimate molecular weights. From the data of Eigner and Doty (1965) in 0.013 M Na^+, a plot of log $s_{20,w}$ *vs.* log (M_w) was constructed and from it the following equations were derived: $s_{20,w} = 0.092(M_w)^{0.83}$ or $M_w/(M_w)_0 = (s_{20,w}/(s_{20,w})_0)^3$; s_0 and $(M_w)_0$ correspond to unsheared complex. [c] $(M_w)_0$ = M_w of T7 DNA (22×10^6 daltons) + $0.4 \times (22 \times 10^6)$ for the added weight of histone in the complex. [d] Shearing was performed using a 5-ml spherical container and Virtis mixer for 1 min on the third line setting (low), for 5 min on the fifth line setting (medium), and 10 min on the seventh line setting (high)

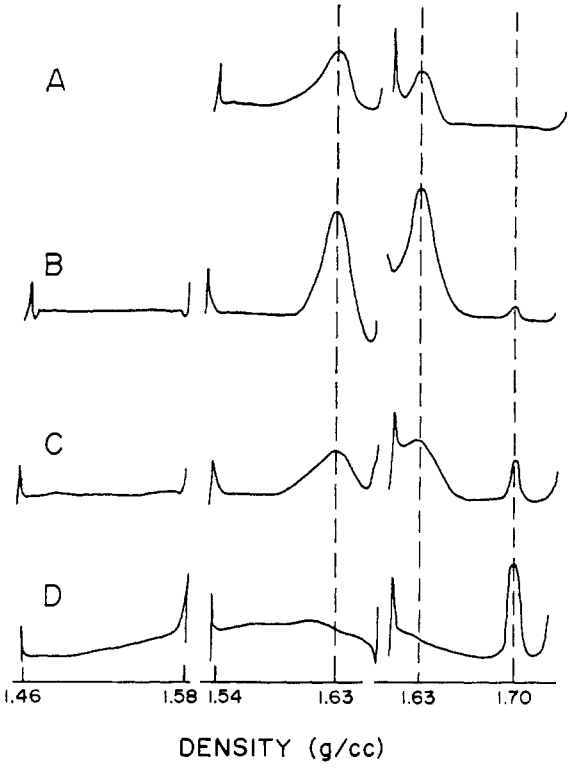

FIGURE 2: CsCl banding profiles for intact (A), half (B), halves and quarters (C), and quarter (D) complex molecules. The spike at the far left is the meniscus. Note the increase in free DNA (1.70 g/cm^3) as complex is sheared to smaller sizes. Each sample was also banded in CsCl gradients of lower densities as illustrated. Concentrations were estimated by measuring areas beneath the peaks.

off" protein from the DNA. These data are summarized in Table IV.

Discussion

The amino acid sequence of histone f2a1 reveals that the molecule can be divided into two halves (DeLange *et al.*, 1969). The amino-terminal half contains a large proportion of basic amino acids while the carboxy-terminal or "hydrophobic" half has an amino acid composition comparable to many globular proteins. From proton magnetic resonance studies Boublic *et al.* (1970a) concluded that when the ionic strength is increased, helix formation occurs within the carboxy-terminal half of the molecule, followed by aggregation from a specific interaction involving the carboxy-terminal half of the molecule. Similar results were found for histone f2b (Boublic *et al.*, 1970b). From gel filtration studies (Edwards and Shooter, 1970) it was concluded that histone f2b at high ionic strength formed an aggregate consisting of "a compact core of hydrophobic groups from which protrude the remaining basic parts of the molecule."

Our sedimentation results indicate that histone f2a1 forms aggregates in 0.1 M NaCl but not in 0.005 M NaCl where charge repulsion between the basic amino acid residues is strong enough to keep the molecules from aggregating. With the removal of 18 amino acids from its hydrophobic end, histone f2a1 no longer aggregates in 0.1 M NaCl. This is strong evidence to confirm the above conclusion that histones aggregate through interactions involving their hydrophobic ends.

Sedimentation velocity studies indicate that under conditions where histone f2a1 aggregates (*i.e.*, 0.1 M NaCl), the

TABLE IV: Data from Sedimentation Equilibrium of Intact and Sheared Native Histone f2a1–T7 DNA Complex in a CsCl Density Gradient.

Sample	% DNA Uncomplexed by Histone	Buoyant Density of Complex (g/cm^3)
Unsheared complex	0	1.630
Complex sheared in half	3	1.632
Complex sheared into halves and quarters	21	1.625
Complex sheared into quarters	43	1.56–1.62

binding of histone to DNA causes folding of the DNA. On the other hand, if conditions are such that the histone molecules do not aggregate (*i. e.*, 0.005 M NaCl or the use of CNBr-modified histone f2a1), no change in the tertiary structure of the DNA is observed on binding histone. Thus, we can conclude that nonspecific T7 DNA contraction due to charge reduction on histone binding plays a minor role in the folding of DNA. Our data support the following as the predominant mechanism: the basic halves of the histone molecules bind electrostatically to DNA and the carboxy ends of the protein molecules aggregate through the interactions of hydrophobic amino acid residues drawing regions of DNA covered with protein close together and thus folding the DNA. A similar model has been proposed to explain rosettes observed in the electron microscope after interacting histone f2a1 with T7 DNA (Olins and Olins, 1971).

An important question to be answered concerns the mechanisms by which histone molecules are placed on the DNA. Are they randomly distributed throughout the DNA, or do they bind in a cooperative manner where after the first molecule binds to a particular site others are quickly laid down next to another, covering a large region? The latter mechanism seems more likely if histones are to cover certain regions of DNA which are not to be expressed.

After glutaraldehyde fixation, native histone f2a1–T7 complex bands at a density lower than that of T7 DNA due to the presence of bound protein. However, CNBr-modified histone f2a1–T7 DNA complex bands at the density of T7 DNA, implying that no protein has been fixed to the DNA. Glutaraldehyde may form bridges between amino groups (*e.g.*, ϵ-amino of lysine) in adjacent protein molecules. Fixation to DNA would result when protein molecules physically wrapped around the DNA are connected by glutaraldehyde. On the other hand, if protein molecules were being covalently linked by glutaraldehyde directly to a DNA base, we would expect the cyanogen bromide modified histone also to be linked to the DNA. Discussions of glutaraldehyde and formaldehyde interactions with proteins and nucleoproteins are given by Habeen and Hiramoto (1968) and Brutlag *et al.* (1969).

The fact that native histone f2a1–DNA complex has two-thirds of its histones fixed by glutaraldehyde and that CNBr-modified histone f2a1 DNA complex does not implies that CNBr-modified histone molecules are randomly placed on the DNA, while much of the native histone f2a1 binds with positive cooperativity. An interesting question immediately arises: are these native histone f2a1 molecules bound in one region of the DNA, or are there numerous clusters of histones throughout the DNA molecule?

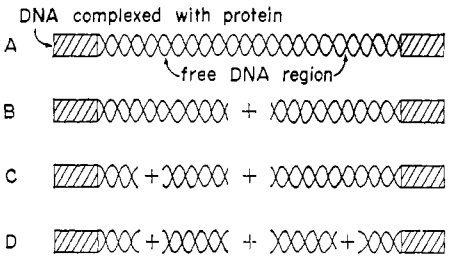

FIGURE 3: Model of native histone f2a1–T7 DNA complex with histones bound predominantly on ends of DNA molecule (A); if complex is sheared in half (B), the molecules retain the same buoyant density as in unsheared; when the complex is sheared into three pieces (C), approximately one-quarter of the free DNA is released; if complex is sheared into four pieces (D), approximately one-half of the free DNA is released.

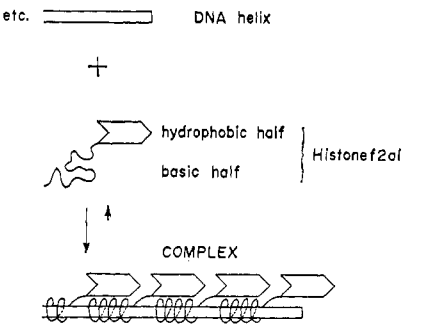

FIGURE 4: Cooperative interactions between DNA and several histone molecules are illustrated as a "roof-shingling" mechanism starting at the end of the DNA molecule. The hydrophobic half of each succeeding histone covers the basic half of the previous molecule, locking it into place around the DNA helix. The hydrophobic halves are shown to possess sites of interactions with each other. Furthermore, it is suggested that the cooperative binding of histone to DNA cannot initiate in the middle of the double helix because of unfavorable interactions between the DNA phosphates and the hydrophobic half of the histone.

The data from Table IV on the banding properties of sheared native complex give strong evidence for only two regions of histone binding. When the complex is broken in half, almost no free DNA is released, and the density of the complex is unchanged. Thus, the two halves contain equal amounts of histone. The second break releases one quarter of the molecules as free DNA, and the third break releases a second quarter. Thus, the cooperatively bound native histone f2a1 appears to bind to DNA only in specific areas and not randomly throughout the DNA.

A simple model can be devised to explain the actual figures in Table IV. It appears that histone binding initiates at the two ends of the DNA molecules and then histones are cooperatively added from these points. Thus, we would have histones predominantly on the ends of the DNA molecule. The model is illustrated in Figure 3 and correlated with the shearing and banding results.

Heterogeneity of the smaller sized complex is probably due to the greater influence of protein in the complex density. An alternative model can be imagined where a single symmetric, central portion of the DNA is covered with histone. If the first break occurs near the center, no free DNA would be released and the density would remain unchanged. The existence of such an initiation site would imply that the histone can recognize a particular DNA sequence. We think this rather unlikely and strongly prefer the model with initiation at the ends.

Although more work is necessary to substantiate the proposal concerning the initiation sites for histone binding, we can conclude that native histone f2a1 binds to DNA in a cooperative manner, while the CNBr-modified histone f2a1 binds randomly to DNA. To illustrate the cooperative binding of the native molecule, we suggest the existence of a "shingle-layering" mechanism as portrayed in Figure 4.

Our work shows that much of the histone f2a1 binds cooperatively to T7 DNA. Shih and Fasman have also presented evidence implying cooperative binding of histone f2a1 to DNA. Histone f2a1 molecules are also involved in the folding of the nucleoprotein upon raising the salt concentration to 0.1 M NaCl. These capabilities are lost upon the cleavage of 18 amino acids from the carboxy end of the histone molecule. Thus, the intact histone molecule is necessary for the performance of its functions. This is consistent with the fact that its amino acid sequence has remained essentially constant for over one billion years.

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