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## The F3-F2a1 Complex as a Unit in the Self-Assembly of Nucleoproteins<sup>†</sup>

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**ABSTRACT:** A specific and stable interaction between histones f3 and f2a1 was demonstrated to take place in the absence of DNA. When a mixture of these histones was subjected to velocity sedimentation under conditions in which the separate histones are aggregated and precipitate, the mixture of f3 and f2a1 remained soluble and these histones appeared to cotransport through the gradient, indicating the establishment of an isolatable, stable f3-f2a1 complex. This isolated complex subsequently binds to DNA quantitatively to form nucleohistone. Stoichiometry data strongly suggest that histones f3 and f2a1 bind to DNA as a unit; this is the only type of f2a1 binding to DNA that can take place under mild conditions. Histone f1 can act as a modifier of the f3-f2a1-DNA interactions by augmenting

the formation of the f3-f2a1 complex and consequently enhancing the overall binding of these histones to DNA. No significant interactions of histones f2b and f2a2 with other histones could be demonstrated. Because of the findings reported here and the known affinity characteristics of the arginine-rich histones to DNA in native chromatin (in particular their simultaneous extraction from chromatin by salt), we suggest that the (f3 + f2a1)-DNA complex is a structural component of native chromatin. We would also like to propose that, in vivo, histones may possess a considerable amount of quaternary structure, which would greatly increase the specificity of their role as potential regulators of the structure and function of the eucaryotic chromosomes.

The role of histones in the structure and function of chromatin continues to be the subject of intensive investigation. The difficulties involved in unraveling the complexities of chromatin have been by-passed by exploiting various model systems; these studies have resulted in a considerable body of information on the characteristics of complexes between histones and DNA. A generalization applicable to most of this work is that complexes between DNA and each class of histones have significant structural features specific to that histone, although functional differences among the various histone-DNA complexes have not been as pronounced.

The significance of these findings, however, is difficult to assess. One problem is the inability to demonstrate specific native chromatin structures which are comparable to those observed for DNA-histone complexes. A less technical but more serious shortcoming of model systems is that the types

of structures formed by individual histone-DNA complexes may be markedly altered by the presence of other histones (or non-histone proteins) normally associated with chromatin. There may not be enough physical-chemical information in individual histone-DNA complexes to model the natural nucleoprotein.

It is to the latter problem that the present paper is directed. In a previous publication (Rubin and Moudrianakis, 1972) we demonstrated that the histones do interact during binding to DNA. The present paper contains evidence for a specific interaction between histones f3 and f2a1, on the role of the f3-f2a1 complex in histone binding to DNA, and evidence that f1 can enhance the formation of the f3-f2a1 complex. The mild procedures used here to demonstrate the binding of this f3-f2a1 complex to DNA, and the pattern of extraction of histones from chromatin by salt (Ohlenbusch et al., 1967), which suggest that these histones interact in chromatin, support our conclusion that *the complex between f3 and f2a1 may be a native structural component of chromatin.*

Since the completion of this work (Rubin, 1973), similar inferences have been made in studies in which histone interactions were monitored indirectly after chemical cross-linking (Kornberg and Thomas, 1974). More direct support for histone-histone interactions has been provided by D'Anna and Isenberg's studies of changes in the optical properties of

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histone mixtures (1973, 1974). In a preliminary report, Geoghegan et al. (1974) have claimed the existence of an f3-f2a1 dimer and tetramer. In all of these studies, however, histone interactions were monitored in the absence of DNA. The distinguishing feature of the present communication, we believe, is that the interactions reported here were monitored directly (a stable complex was isolated) without the use of external chemical modifiers (fixatives, etc.), and in the presence of the natural companion of histones in chromosomes, DNA.

#### Materials and Methods

**Preparation of Histones.** Calf thymus chromatin was prepared as described previously (Rubin and Moudrianakis, 1972) except that the nuclei were washed in a solution consisting of 0.085 *M* NaCl–0.05 *M* NaHSO<sub>3</sub>–3.0 *mM* MgCl<sub>2</sub> (pH 7.5). Approximately 1.5 g of chromatin was obtained from each preparation of 80 g (wet weight) of calf thymus.

**F1.** Crude f1 was extracted from sheared chromatin in a solution consisting of 0.05 *M* NaHSO<sub>3</sub> (Panyim and Chalkley, 1969), pH 7.5, by the addition of solid NaCl to a concentration of 0.55 *M* (Ohlenbusch et al., 1967). DNA was removed by centrifugation in a Beckman 30 rotor at 27,000 rpm for 16 hr. After dialysis against 0.05 *M* acetic acid and lyophilization to dryness, the f1 preparation was applied to an Amberlite CG 50 column. Non-histone proteins were eluted with 6% guanidinium hydrochloride; f1 was eluted with 11% guanidinium hydrochloride (Bonner et al., 1968).

**F2b–F2a2.** The nucleohistone pellet obtained after centrifuging chromatin through 0.55 *M* NaCl–0.05 *M* NaHSO<sub>3</sub> (pH 7.5) was suspended (by brief homogenization with a Teflon homogenizer) in a solution consisting of 4 *M* urea–0.05 *M* NaHSO<sub>3</sub> (pH 7.8) and carefully adjusted to 0.2 *M* NaCl–4 *M* urea–0.05 *M* NaHSO<sub>3</sub> (pH 7.8) (conductance equal to 14.5 mmho, 0°, using a Radiometer conductivity meter Type CDM 2d and electrode Type CDC 104), after the method of Kleiman and Huang (1972). The dispersed suspension was centrifuged in a 30 rotor at 27,000 rpm for 16 hr, and the contents of the top three-fourths of each centrifuge tube were used as purified f2b–f2a2.

**F3 and F2a1.** Nonsheared chromatin was used for the preparation of an f3 + f2a1 mixture. To the chromatin suspension in 2 *mM* EDTA were added solid urea, 1.0 *M* NaHSO<sub>3</sub> solution at pH 7.5, and solid NaCl so that the final solute concentrations were 6.0 *M* urea, 0.04 *M* NaHSO<sub>3</sub>, and 0.33 *M* NaCl (pH 7.8). After achieving homogeneity by mild homogenization in a Waring Blendor, the suspension was centrifuged in a 30 rotor at 25,000 rpm for 12–18 hr. The supernate, containing mostly f1, f2b, f2a2, and non-histone proteins (Kleinman and Huang, 1972), was discarded and the pellet was reextracted with the same solvent. After another centrifugation the pellet was suspended in a solution of 2 *M* urea–3 *M* NaCl–0.01 *M* NaHSO<sub>3</sub> and centrifuged in the 30 rotor at 25,000 rpm for 30 hr. The bottom third of the contents of each centrifugation tube (excluding the DNA pellet) was recentrifuged in a Ti50 rotor at 45,000 rpm for 12 hr and the supernate from this centrifugation was combined with the top two-thirds of the supernate from the previous centrifugation. This solution was then dialyzed extensively against 0.05 *M* acetic acid and lyophilized to dryness. Approximately 100 mg of protein containing mostly f3 and f2a1 was obtained from 600 mg of chromatin histone.

Approximately 50 mg of the f3–f2a1 extract was chromatographed on a Bio-Gel P60 column (2.6 cm i.d. × 150

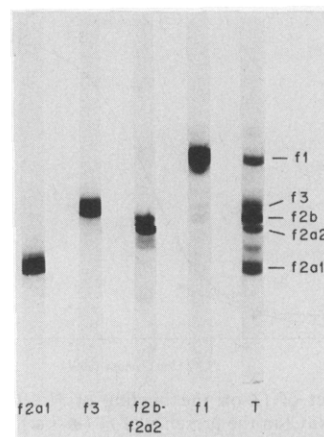


FIGURE 1: Polyacrylamide gel electrophoresis of the purified histone preparations used in these studies. Histones migrated to the cathode, which was at the bottom.

cm) by elution with 0.01 *M* HCl at 20 ml/hr (Fambrough and Bonner, 1969). Approximately 12 mg of f2a1 and 18 mg of f3 were recovered.

The purity of the histone fractions was analyzed by polyacrylamide gel electrophoresis according to the method of Panyim and Chalkley (1969). As shown in Figure 1 no contamination by any other proteins was detectable in the f3 preparation or the f2a1 preparation. The f2a2–f2b preparation contained approximately 5% contamination with a few faster moving proteins. The f1 preparation was contaminated with a small percentage of non-histone proteins.

**Radiolabeling of Histones.** Part of each histone preparation was radiolabeled by reaction with [<sup>14</sup>C]cyanamide (I.C.N.). Details of this procedure, along with evidence that a high degree of native conformation and biological activity is maintained in these slightly modified proteins, will be published elsewhere (manuscript in preparation). Specific radioactivity varied among the different histones from approximately 10,000 to 100,000 cpm/mg.

**Preparation of DNA.** Calf thymus DNA was prepared from pellets taken from the histone preparations. The crude DNA was digested with RNase (Worthington, "RASE") at 50 µg/ml for 2 hr and with Pronase and was then subjected to three phenol extractions as described previously (Rubin and Moudrianakis, 1972). The extent of protein contamination was determined from concentrated solutions of the purified DNA. No protein was detected in such DNA preparations, setting the maximum limit of possible protein contamination at less than 1% by weight. The DNA had a hyperchromicity of 36% and an average molecular weight of approximately  $25 \times 10^6$  (determined by electron microscopy).

**DNA–Histone Complex Formation and Purification.** Each preparation of histone was dissolved in 0.5 *mM* EDTA (pH 7.5) at a concentration of 1–2 mg/ml. For making mixtures of histones, individual histone solutions were mixed together and an equal volume of 4.0 *M* NaCl–4.0 *M* urea–0.5 *mM* EDTA (pH 7.5) was added. After incubation at 0° for 30 min, each solution of histone(s) was added dropwise to 150 µg of DNA while being stirred constantly on ice in a solvent consisting of 2.0 *M* NaCl–2.0 *M* urea–0.5 *mM* EDTA (pH 7.5). The final volume of each histone–DNA mixture was 1.5 ml. The histone–DNA mixtures were placed in dialysis bags 0.25 in. in diameter (A. H. Thomas, Inc., preboiled) and dialyzed against 1 l. of 2.0 *M* NaCl–0.5 *mM* EDTA (pH 7.5) at 0° for at least 1 hr to remove most

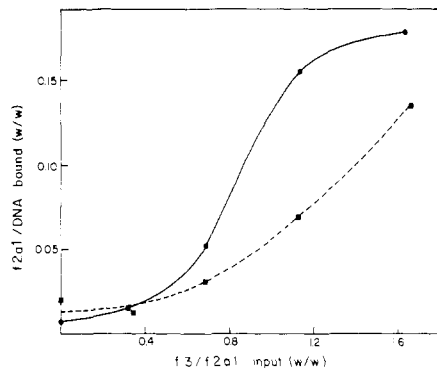


FIGURE 2: Effect of f3 on the binding of [ $^{14}\text{C}$ ]cyanamide-f2a1 to DNA in 0.9 M NaCl in the presence of f1 (●—●) or in the absence of f1 (□—□). The amount of histone bound to DNA was determined by the radioactivity of the histone-DNA complex after removal of unbound histone through a Bio-Gel A50 column. F2a1/DNA input ratio = 0.64. F1/DNA input ratio = 1.0. The apparent leveling-off of the solid line does not represent saturation of DNA by f2a1 (compare to Figures 5 and 6), but probably reflects some triviality of this experimental design, perhaps related to the presence of f1.

of the urea (see Results). The bags were then placed in 100–200 times the volume of the appropriate NaCl concentration (in 0.5 mM EDTA, pH 7.5) and dialyzed overnight at 0°.

Nonbound histone was removed by passing the DNA-histone complexes through Bio-Gel A50 columns (28 cm  $\times$  1.8 cm) eluted with the appropriate solvent at room temperature. Fractions were pooled and concentrated by vacuum dialysis against 0.5 mM EDTA (pH 7.5). For all histone-DNA mixtures more than 90% of the input DNA was recovered in the void volume, and approximately two-thirds of this histone-DNA complex was used for subsequent analysis.

**Analytical Determinations.** DNA concentration was determined either by absorbance at 260 nm using  $E_{260\text{nm}}$  (1 mg/ml) 21.4 at all histone/DNA ratios or by the diphenylamine method of Burton (1968). The extinction coefficient of DNA did not change upon complexing with histones unless the solutions became turbid.

Protein concentration was determined by the procedure of Lowry et al. (1951), modified for dilute solutions as described previously (Rubin and Moudrianakis, 1972).

Radioactivity was counted in a TriCarb liquid scintillation spectrometer (Packard, Model 2002) using 2,5-diphenyloxazole + 1,4-bis[2-(5-phenyloxazolyl)]benzene fluor in a Triton-toluene solvent. After correcting for quenching by water, the counts per 10 min of each complex containing a known amount of DNA were converted to micrograms of protein according to a previously determined value of specific radioactivity of the radiolabeled histone used to form the complex.

**Sucrose Gradient Sedimentation.** Each histone preparation was dissolved in 0.5 mM Tris (pH 7.5), mixed with one or more other histone solutions, and made to 1.2 M NaCl–0.5 mM Tris (pH 7.5). A volume of 0.2 ml was layered on a 5–20% sucrose gradient (containing 1.2 M NaCl–0.5 mM Tris, pH 7.5) and centrifuged in a Spinco SW 50.1 rotor at 45,000 rpm for 34 hr at 22°. Each gradient was collected from the bottom into 24 fractions, each 0.2 ml in volume, and analyzed for protein by absorbance at 230 nm.

## Results

### Enhancement of Binding of F2a1 to DNA by F3 and by

Table I: F2a1 Binding to DNA in  $2 \times 10^{-3}$  M NaCl.

Other Histone(s) Present <sup>a</sup>	f2a1 Bound (% of Input) <sup>b</sup>	f2a1 Bound (% of Input) <sup>c</sup>
None	7	3
f2b + f2a2	9	6
f3	26	25
f1	16	14
f3 + f1	54	48

<sup>a</sup> Histone/DNA input ratio = 0.60. <sup>b</sup> F2a1/DNA input ratio = 0.40; [ $^{14}\text{C}$ ]cyanamide f2a1 was used. <sup>c</sup> F2a1/DNA input ratio = 0.40; values obtained from densitometer tracings of gels in which the bound, nonradiolabeled histones were electrophoresed and the amount of f2a1 was estimated relative to a control gel containing a known amount of f2a1. Experiment c serves as a control to experiment b and shows that the results obtained from the use of [ $^{14}\text{C}$ ]cyanamide histone are valid, i.e., if any conformational change is introduced to the histone molecule by this labeling procedure, such a hypothetical change must be small since, in the binding experiments reported here, modified histone behaved indistinguishably from unmodified, “native” histone.

**F1.** If a mixture of f2a1 and DNA is dialyzed from 2.0 M NaCl to 0.9 M NaCl, very little f2a1 forms a stable complex with DNA. F3, however, greatly enhances the ability of radiolabeled f2a1 to bind to DNA in 0.9 M NaCl, as shown in Figure 2. As the f3/f2a1 input ratio is increased above 0.4, the amount of f2a1 which is excluded from a Bio-Gel A50 column along with DNA increases nonlinearly. It is obvious, then, that f3 causes an increase in the f2a1/DNA ratio even when it is the *only* other histone added to a mixture of f2a1 and DNA. Contrary to that, *f1 alone does not enhance* the binding of f2a1 to DNA in 0.9 M NaCl (Figure 2), but the addition of f1 to a mixture of f3–f2a1 histones further increases the bound f2a1/DNA ratio by another two- to threefold. The combined f3–f1-dependent enhancement of the binding of f2a1 to DNA appears to be as much as 15-fold.

These enhancements can also be demonstrated if DNA-histone mixtures are dialyzed down to very low ionic strengths before nonbound histone is removed. Mixtures of radiolabeled f2a1 and DNA were prepared in 2.0 M NaCl at an f2a1/DNA input ratio of 0.40 in the presence of a 1.5-fold excess (by weight) of another histone. After dialysis down to  $2 \times 10^{-3}$  M NaCl and removal of unbound histone, the amount of radioactivity associated with DNA was determined. As shown in Table I, the amount of f2a1 bound to DNA in  $2 \times 10^{-3}$  M NaCl in the absence of other histones was approximately three times that which bound in 0.9 M NaCl. Histones f2b + f2a2 had no significant effect on the binding. However, as in 0.9 M NaCl, f3 stimulated the binding, bringing the amount of f2a1 to 26% of input, and had a *synergistic* effect with f1, increasing the binding of f2a1 to more than half of the input amount. Except for the higher binding of f2a1 to DNA in the absence of other histones and the twofold stimulation by f1 in the absence of f3, the specificity and extent of enhancement of f2a1 binding to DNA in  $2 \times 10^{-3}$  M NaCl were very similar to that in 0.9 M NaCl. Since in a similar experiment in which non-radiolabeled histones were used, comparable results were obtained (Table I), it is certain that the data obtained with the radiolabeled histones are not artifacts of some hypothetical modification of histone structure by the process of radiolabeling.

Table II: Effect of Urea on Binding of Histones f3 + f2a1 to DNA.

Urea Molarity <sup>a</sup>	Histone Bound in 1.0 M NaCl (% of Input f3 + f2a1) <sup>b</sup>
0	3
0.5	3
1.0	7
1.3	29
2.6	32

<sup>a</sup> Initial urea concentration in mixture of histones and DNA in 2.0 M NaCl. Urea was then removed by dialysis against 2.0 M NaCl. <sup>b</sup> F3/DNA input ratio = 0.7; f2a1/DNA input ratio = 0.7.

**Urea Effect.** As described under Materials and Methods, the present binding studies were done (except where noted otherwise) with 2.0 M urea in the initial histone-DNA mixture followed by removal of the urea by dialysis against 2.0 M NaCl, a solvent in which no stable interactions between DNA and histones take place (Ohlenbusch et al., 1967). The histones were then allowed to bind to DNA by dialysis to lower ionic strengths. However, if f3 and f2a1 were mixed with DNA in 2.0 M NaCl in the *absence* of urea, very little histone was bound to DNA after dialysis to 1.0 M NaCl. Most of the input histone was aggregated, forming a turbid suspension that was completely separable from free DNA. The effect of adding urea to a mixture of DNA and histones f3 and f2a1 in 2.0 M NaCl is shown in Table II. These data were obtained using the normal method for forming and purifying histone-DNA complexes; that is, the histone-DNA mixtures in 2.0 M NaCl containing increasing concentrations of urea were dialyzed for 1 hr against 2.0 M NaCl before the histones and DNA were allowed to interact by dialysis to 1.0 M NaCl. The 1 hr dialysis against 2.0 M NaCl removed more than 90% of the urea, so that complex formation took place in the presence of approximately 0.02–0.01 M urea. The results shown in Table II indicate that below 1.0 M urea in the initial histone-DNA mixture, very little histone is bound to DNA, but the level of binding rapidly increases as the urea concentration is raised above 1.0 M. It appears, therefore, that the presence of 2.0 M urea in the initial histone-DNA mixture enhances the binding of f3 and f2a1 up to tenfold. However, since no significant amount of urea is present during the removal of the salt when DNA-histone complexes are forming, urea is not required during the binding of histones to DNA.

**Evidence for Complex Formation between F3 and F2a1.** There are two general ways in which f3 might stimulate f2a1 binding to DNA: either by interacting first with the DNA, thus increasing the affinity of this complex for f2a1; or by modifying f2a1, thus increasing the affinity of f2a1 for DNA. The latter mechanism implies a direct interaction of f3 with f2a1 in the absence of DNA, an interaction which might result in the formation of a stable f3-f2a1 complex.

Sucrose gradient ultracentrifugation was used to test this hypothesis. Histone solutions were centrifuged through sucrose gradients which contained 1.2 M NaCl, an ionic strength in which the binding of f2a1 to DNA takes place (unpublished observation). In the absence of other histones all the f2a1 sedimented through the sucrose gradient as shown in Figure 3. This rapid sedimentation is due to the well-known self-aggregation of f2a1 in salt solutions (Ed-

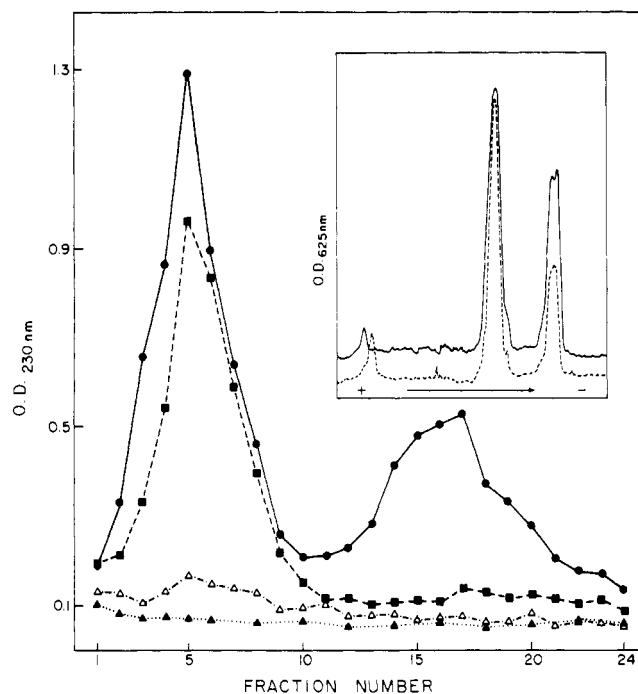


FIGURE 3: Sucrose gradient centrifugation profiles of histone preparations and their mixtures. The amount of each histone applied to a single gradient was as follows: 270  $\mu$ g of f2a1 ( $\Delta$ — $\Delta$ ); 290  $\mu$ g of f3 ( $\square$ — $\square$ ); 231  $\mu$ g of f2a1 + 553  $\mu$ g of f3 ( $\square$ — $\square$ ); 231  $\mu$ g of f2a1 + 553  $\mu$ g of f3 + 297  $\mu$ g of f1 ( $\bullet$ — $\bullet$ ). Inset: Densitometer tracings of polyacrylamide gel electrophoresis. Fractions 2–9 from each sucrose gradient were pooled, concentrated, and analyzed by gel electrophoresis. The tracings show the analyses from the gradient in which both f3 and f2a1 were applied (—) and the gradient in which f3, f2a1, and f1 were applied (—). Direction of protein migration is indicated by the arrow. Viewing the inset from right to left, the two main peaks correspond to histones f2a1 and f3, and the small peak on the far left corresponds to a polymerized form of f3.

wards and Shooter, 1969). In 0.1 M NaCl, pH 2.0, Diggle and Peacocke (1971) identified a 25S aggregate of f2a1 containing approximately 200 monomers. Similarly, little f3 was found within the sucrose gradient, in agreement with the results of Diggle and Peacocke (1971), who showed that in salt solutions at neutral pH, f3 forms aggregates of up to approximately 50 monomers.

In contrast to the fast sedimentation of the individual histones, a protein band having a much lower *s* value was found if a mixture of f3 and f2a1 was sedimented through a sucrose gradient. At an f3/f2a1 input ratio of 2.4, 20% of the protein was found in a symmetrical band having an *s*<sub>20,w</sub> of 4, as shown in Figure 3. This material was composed of approximately equal amounts of f2a1 and f3 (but see Table V) as determined by the quantitative gel electrophoresis analysis shown in the inset of Figure 3. When the ultracentrifugation was done for one-third of the time, still only the 4S, f3-f2a1 band was found in the gradient, indicating that the rest of the protein was present in aggregates of greater than 13S value.

The cotransport of f3 and f2a1 indicates that these histones can form a stable complex. To determine if this complex has unique binding properties, the fractions from the sucrose gradient were pooled, dialyzed down to water, and lyophilized to dryness. The histones were dissolved in water and the solutions were made to 2 M NaCl and mixed with DNA in 2 M NaCl in the absence of urea. Table III shows that more than two-thirds of this histone complex will bind to DNA after dialysis down to 0.9 M NaCl; on the con-

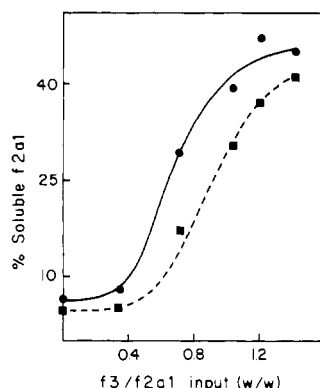


FIGURE 4: Effect of f3 on the solubility of [ $^{14}\text{C}$ ]cyanamide-f2a1 in the presence of f1 (●—●) and in the absence of f1 (■—■). The f2a1 concentration was 50  $\mu\text{g}/\text{ml}$  and the f1 concentration was 59  $\mu\text{g}/\text{ml}$ .

Table III: Effect of Fractionation on Binding of f3 + f2a1 to DNA in 0.9 M NaCl.

f3 + f2a1 Input	Amount Bound (% of Input)
Unfractionated	6
Fractionated	68

trary, less than one-tenth of a mixture of f3 and f2a1 which has not previously been fractionated on a sucrose gradient will bind to DNA. Only if 2 M urea is present during the initial mixing of histone with DNA in 2 M NaCl will significant binding of unfractionated f3-f2a1 occur (Figure 2). It appears, therefore, that (a) when histones f3 and f2a1 are made to interact under the appropriate conditions they can form a stable and isolatable complex, (b) the purified complex formed between f3 and f2a1 has a very high affinity for DNA, and (c) the stoichiometry of this complex is (f2a1)<sub>1</sub>(f3)<sub>1</sub>.

**The F3-F2a1 Complex as the Unit of Binding.** Since the proportion of the input f3 + f2a1 that forms a f3-f2a1 complex is approximately equal to the proportion that binds to DNA, it is likely that this f3-f2a1 complex binds to DNA as a unit. In order to determine how well this relationship is maintained, a comparison was made between the extent of f3-f2a1 complex formation and the binding of these histones to DNA. For convenience, the extent of complex formation between f3 and f2a1 was assessed by incubating the histones for 4 hr at 0° in a 1.2 M NaCl solution and then determining the amount of radiolabeled f2a1 which remained in the supernate after centrifuging at 5000 rpm for 15 min. The amount of f2a1 solubilized by f3 is directly related to the formation of an f3-f2a1 complex, although a proportion of the soluble f2a1 may have an *s* value greater than that of the 4S complex.

The results of this study are shown in Figure 4. In the absence of other histones, only approximately 5% of the input f2a1 was soluble in 1.2 M NaCl. However, f3 specifically solubilized f2a1. Although f1 alone had little effect, the f3-dependent solubility of f2a1 was further enhanced as much as tenfold by the simultaneous presence of f1. Histones f2b + f2a2 had no effect (data not shown). The f3-stimulated and f3-f1-stimulated enhancement of f2a1 solubility had a stoichiometry very similar to that of their enhancement of f2a1 binding to DNA (Figure 2), except that the solubility

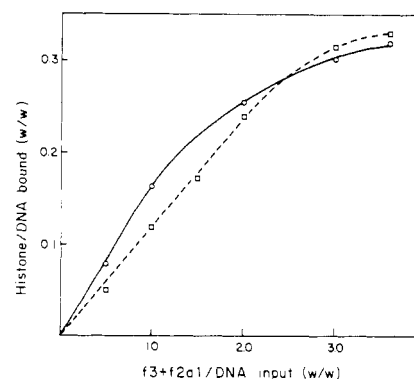


FIGURE 5: Effect of the input ratio of histone to DNA on the binding of f3 and f2a1 to DNA in 0.9 M NaCl. In one experiment f3 binding to DNA was monitored by the use of [ $^{14}\text{C}$ ]cyanamide-f3 (○—○); in a parallel experiment f2a1 binding to DNA was monitored by the use of [ $^{14}\text{C}$ ]cyanamide-f2a1 (□—□). The f3/f2a1 ratio was 2.50 at all input ratios of histone to DNA.

enhancement required a lower f3/f2a1 ratio and reached a higher maximum than the binding enhancement. These comparisons suggest that f2a1 must first become soluble before it can bind to DNA, although not all soluble f2a1 forms a stable complex with DNA, presumably due to the presence of aggregates of f2a1 which cannot bind to DNA. At all f3/f2a1 ratios, therefore, the ability of f3 to enhance the binding of f2a1 to DNA can be accounted for by its ability to solubilize f2a1—that is, by the formation of a f3-f2a1 complex.

If the f3-f2a1 complex binds to DNA as a unit, these histones should bind simultaneously to DNA. To test this, increasing amounts of a mixture of f3 and f2a1 in which the ratio of f3 to f2a1 was held constant were allowed to complex with DNA in the usual manner. In one experiment radiolabeled f3 was used; in a parallel experiment only f2a1 was radiolabeled. Figure 5 shows the results after unbound protein was removed by Bio-Gel chromatography in 0.9 M NaCl. The binding of f3 and f2a1 is coordinate, which supports the view that these histones bind as a unit to DNA. However, although the ratio of f3 to f2a1 was constant at all input concentrations of histone, this ratio (i.e., f3/f2a1) of histones bound to DNA decreased slightly as the concentration of input histones increased. It appears, therefore, that the binding of these histones to DNA cannot be entirely accounted for by an absolutely invariant stoichiometry or size of the f3-f2a1 complex.

**Binding of F3 to DNA.** The previous figures suggest that f3 can form a complex with f2a1 and that this complex binds to DNA. Although the formation of this complex is required for the binding of f2a1 to DNA, it may not necessarily be required for the binding of f3 to DNA. In fact, if only f3 is allowed to complex with DNA, 13% of the input will bind in 0.9 M NaCl and 30–40% will bind in low ionic strength (Table IV). Therefore, the binding of f3 to DNA in the presence of f2a1, as shown in Figure 5, may not necessarily be explained by the binding of a simple f3-f2a1 complex. This complex might vary in the ratio of f3 to f2a1, or f3 might bind to DNA itself, even in the presence of f2a1.

In an attempt to distinguish between these alternatives, sucrose gradient centrifugation was used to determine the stoichiometry of the f3-f2a1 complex. Mixtures of f3 and f2a1 at a constant ratio but at different total protein concentrations were centrifuged through sucrose gradients as

Table IV: Binding to DNA.

A. In $2 \times 10^{-3}$ M NaCl		B. In 0.9 M NaCl	
Other Histone(s) Present <sup>a</sup>	f3 Bound (% of Input) <sup>b</sup>	f2a1/f3 Input Ratio	f3 Bound (% of Input) <sup>b</sup>
None	32	0	13
f1	34	0.5	9.5
f2b + f2a2	28	1.5	3.3
f2a1	12	3.0	3.1
f2a1 + f1 + f2b + f2a2	13		

<sup>a</sup> Histone/DNA input ratio = 0.64. <sup>b</sup> F3/DNA input ratio = 0.42; [<sup>14</sup>C] cyanamide f3 was used.

shown in Figure 3. At all concentrations of histone applied, one band formed which peaked at fraction 5 or 6, where the 4S f3-f2a1 complex sedimented; the composition of these bands is shown in Table V. It can be seen that the f3/f2a1 ratio of banded material decreases as the concentration of histone applied to the gradient increases. At low concentrations of protein much of the f2a1 aggregates, leaving f3 complexed with a relatively small amount of f2a1. As the concentration of protein increases, the ratio of f3 to f2a1 in the complex decreases, approaching unity at an average complex concentration of approximately 260  $\mu$ g/ml. This variation in the composition of the histone complex parallels the decrease in the ratio of f3 to f2a1 bound to DNA as the histone concentration increases (Figure 5). It appears, therefore, that the preferential binding of f3 to DNA at low protein concentrations might be explained by the binding of an f3-f2a1 complex enriched in f3.

This conclusion is further supported by the following experiment. Histone-DNA complexes were made by the addition of increasing concentrations of unlabeled f3 + f2a1 to DNA, followed by dialysis down to 0.9 M NaCl as described under Materials and Methods. After removing unbound histone by Bio-Gel chromatography and determining the histone/DNA ratio of the complexes, additional f3 that was radiolabeled was added directly to the (f3 + f2a1)-DNA complexes in 0.9 M NaCl. Unbound histone was again removed and the amount of additional f3 that was able to bind to the preformed complexes was determined by radioactivity counting. Table VI shows that as the (f3 + f2a1)/DNA ratio increases, the ability of additional f3 to bind decreases. This result suggests that the f3-f2a1 complex binds to sites on DNA that are the same as many of the sites that f3 alone occupies, although a small amount of f3 will bind to DNA heavily loaded with f3-f2a1 complex. The apparent competition between f3 and the f3-f2a1 complex suggests that little if any f3 not associated with f2a1 will bind to DNA if f2a1 is also present, although the binding of a 4S or aggregate form of f3 completely lacking in f2a1 cannot be completely ruled out.

The previous data show that f2a1 has a significant effect on the form in which f3 binds to DNA. Histone specificity and the extent of modification by f2a1 are shown in Table IV. In the specificity study the affinity of f3 for DNA was determined after dialysis to 0.9 M NaCl in the presence of a 1.5-fold excess of another histone. It is clear that *only f2a1 affects the binding of f3*, but, as shown in Table IVB, as the amount of f2a1 increases, the ability of f3 to bind to DNA actually decreases. The reason for this repression of binding is that when excess f2a1 is present, a highly aggre-

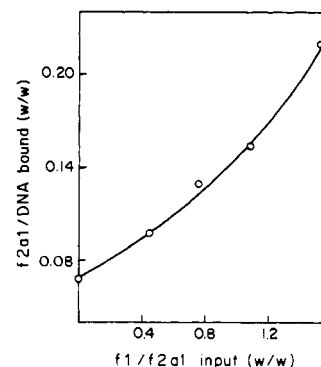


FIGURE 6: Effect of f1 on the binding of f2a1 to DNA in 0.9 M NaCl in the presence of a constant amount of f3. F2a1/DNA input ratio = 0.64. F3/DNA input ratio = 0.56.

gated f3-f2a1 complex forms. By complexing with excess f2a1, f3 becomes unavailable for binding—it is found coprecipitated with f2a1 after low-speed centrifugation. This effect of excess f2a1 is also manifested in the binding of f2a1 to DNA in the presence of f3; Figure 2 shows that no increase in f2a1 binding occurs at an f3/f2a1 ratio of 0.5 or less because f2a1 precipitates (Figure 4) together with f3. It is likely that the aggregation of the f3-f2a1 complex is a direct consequence of the aggregation of f2a1, which occurs in the absence of f3 (Figure 3 and Edwards and Shooter, 1969).

**The Role of F1.** It can be seen from Figure 2 that f1 stimulates the binding of f2a1 to DNA in 0.9 M NaCl *only in the presence of f3*. Figure 6 shows that at a constant f3/f2a1 ratio, f1 causes a nonlinear enhancement of f2a1 of up to 300%. The f3 requirement for this f1 stimulation demonstrates that f1 is acting on the complex between f3 and f2a1. This effect might be brought about by f1 (a) forming a DNA-f1 complex with enhanced affinity for the f3-f2a1 complex, (b) modifying the f3-f2a1 complex and increasing its affinity for DNA, or (c) changing the stoichiometry of the components of the f3-f2a1 complex. In order for f1 to affect binding sites on DNA for the f3-f2a1 complex, it would most likely interact directly with DNA before the f3-f2a1 complex binds to DNA. To test this, radiolabeled f1 of high specific radioactivity was prepared, and its ability to bind to DNA in the presence or absence of f3 and f2a1 was determined in different ionic strengths. The results, shown in Table VII, indicate that f1 alone does not form a stable complex with DNA at ionic strengths of 0.9 M or higher. In the presence of f3 and f2a1, some f1 binding occurs, but this is still only 0.5% or less of the input. It is unlikely, therefore, that f1 increases the affinity of DNA for the f3-f2a1 complex since no stable interaction takes place between f1 and DNA at the ionic strengths in which f2a1 binds.

Figure 4 shows that f1 can enhance the solubility of f2a1 by twofold in the presence of f3. This might be brought about by increasing the amount of f3-f2a1 complex formed or by increasing the f2a1/f3 ratio in the complex. Sucrose gradient centrifugation was used to distinguish between these possible mechanisms. When f1 was added to a mixture of f3 and f2a1 in a 1.2 M NaCl solution, two peaks were observed after centrifugation, as shown in Figure 3. One band migrated to the same position as the f3-f2a1 complex formed in the absence of f1, and this protein consisted entirely of f3 and f2a1, as shown in the inset of Figure 3. The other band contained only f1 of 1S value, similar

Table V: Stoichiometry of the f3-f2a1 Complex Isolated by Sucrose Gradient Centrifugation.

Histone Mixture Layered on Gradient (Solvent = 0.2 ml of 1.2 M NaCl)		Histone Composition of 4S Complex (Fractions 2-9) <sup>b</sup>			
Amount f3 + f2a1 <sup>a</sup> (μg)	Amount f1 (μg)	Amount 4S Complex at Half-Height (μg/ml)	% of Input		f3/f2a1 Ratio
			f2a1	f3	
392	0	32	4	14	8.8
784	0	95	17	21	2.8
1,173	0	144	25	19	1.8
784	297	134	38	24	1.5

<sup>a</sup> F3/f2a1 input ratio = 2.4. <sup>b</sup> Determined by quantitative gel electrophoresis.

Table VI: Binding of Additional f3 to (f3 + f2a1)-DNA Complexes.

(f3 + f2a1)/ DNA Ratio of Complex Used	Additional f3 Bound (f3/DNA Ratio) <sup>a</sup>
0	0.143
0.28	0.054
0.48	0.044

<sup>a</sup> [<sup>14</sup>C]Cyanamide f3 was used; f3/DNA input ratio = 1.5.

to that obtained by others (Haydon and Peacocke, 1968; Edwards and Shooter, 1969). There was, however, a 40% increase in the net amount of protein which banded at the 4S region, and Table V shows that this f1-induced increase can be accounted for by a greater than twofold increase in the net amount of f2a1 without a significant change in the net amount of f3 in that region of the gradient. Therefore, the ability of f1 to increase the amount of f2a1 found bound onto DNA can be explained by an *f1-dependent change in the stoichiometry of the f3-f2a1 complex*—that is, f1 can cause a twofold enhancement in the ratio of f2a1 to f3 in the ca. 4S f3-f2a1 complex.

Since f1 was not detectable in the f3-f2a1 complex, the mechanism of the f1-induced increase in the f2a1/f3 ratio is not clear. However, the asymmetry of the f1 band shown in Figure 3 and the fact that a small amount of f1 was found in the 4S f3-f2a1 complex after centrifugation for a one-third shorter duration (unpublished observations) suggest that a reversible complex may form between f1 and some component of the f3-f2a1 complex. Table I shows that f1 alone can enhance the binding of f2a1 to DNA in 2 mM NaCl, while it has no effect on the binding of f3 to DNA (Table IV). It appears, therefore, that f1 might interact directly with f2a1, perhaps preventing its aggregation so that more f2a1 is available for complexing with f3.

## Discussion

Figure 7 illustrates some of the interactions among the histones which are inferred from this study. *F2a1 alone does not bind to DNA under mild conditions because it is present as a massive aggregate.* But if f3 also is present, at least some of this aggregation is prevented by the formation of an *array* of f3-f2a1 complexes, whose *stoichiometry* depends in part on the *concentrations* of these histones. The stoichiometry is also *modified* by f1, which presumably forms an easily dissociable complex with f2a1, thereby making more f2a1 available for complexing with f3. The f3-f2a1 complex represents the *only means by which f2a1 can bind to DNA under mild conditions*. The f3-f2a1 com-

Table VII: F1 Binding to DNA.

Other Histone(s) Present	F1 Bound	
	[NaCl] (M)	(% of Input) <sup>a</sup>
(f3 + f2a1) <sup>b</sup>	2.0	0.24
(f3 + f2a1) <sup>b</sup>	1.4	0.25
None	1.4	0.05
(f3 + f2a1) <sup>b</sup>	0.9	0.53
None	0.9	0.03

<sup>a</sup> [<sup>14</sup>C]Cyanamide f1 was used; f1/DNA input ratio = 1.3.

<sup>b</sup> F3/DNA input ratio = 0.81; f2a1/DNA input ratio = 0.58.

plex, once formed, is stable, can be isolated as such, and can subsequently bind to DNA as a unit. F3 alone also aggregates, but a significant quantity can bind to DNA in the absence of other histones. However, as more f2a1 is present, more f3 binds to DNA in the form of an f3-f2a1 complex. The f3-f2a1 complex also aggregates, particularly at high f2a1/f3 ratios, thereby diminishing the amount of f3 that would otherwise have bound to DNA in the absence of f2a1.

Some of these observations for histone-histone interactions have recently been reported by others, but to our knowledge the present study is the first on the interactions of preformed histone complexes with DNA. Kelly (1973) has isolated an f2a2-f2b complex from acid-extracted calf thymus chromatin. D'Anna and Isenberg (1973, 1974), by the use of circular dichroism and fluorescence anisotropy, have thoroughly monitored changes in the secondary and tertiary structure of histones in mixtures and have concluded that histone f2b forms strong complexes with histone f2a2 as well as with f2a1, while histones f2a2 and f2a1 interact only weakly with each other. The nature of these experiments necessitates extensive interpretation of the experimental measurements, thus making some of the conclusions rather speculative. Also, since these studies were carried out in the absence of DNA, it is not clear if any of the above-mentioned interactions have any role in regulating the binding of histones to DNA. The results we report here suggest that an association of histone f2a1 with either histone f2b or histone f2a2 is of negligible significance (see Table I), particularly if histone f3 also is present during the formation of the nucleohistone complex (Figure 5). On the basis of electrophoretic separation in acrylamide gels of mixtures of the histones f2a1 and f3, which were previously fixed with a protein cross-linking reagent, Kornberg and Thomas (1974) have proposed that these histones interact to form an (f2a1)<sub>2</sub>(f3)<sub>2</sub> tetramer. The proposed stoichiometry was based on the relative electrophoretic migration,



rather than on direct chemical analysis, of the resolved complexes. The authors point out that technical difficulties prevented them from calibrating the molecular weight scale; thus assignments of the type and number of histones present in each band were made arbitrarily on a best-fit basis. The validity of such treatment is questionable. For example, it has not been demonstrated that a histone complex with the composition  $(f2a1)_3(f3)_1$  (mol wt 49,200) can be resolved from that of  $(f2a1)_2(f3)_2$  (mol wt 53,200) under the conditions employed in their experiments, especially since "the mobilities of histones in SDS gels are anomalous" (Kornberg and Thomas, 1974, p 866). Furthermore, the molecular weight value for the f2a1-f3 complex estimated by these authors from sedimentation equilibrium results, a value which supports the  $(f2a1)_2(f3)_2$  stoichiometry, may not be correct since it was obtained by "assuming a partial specific volume of  $0.72 \text{ cm}^3 \cdot \text{g}^{-1}$ " (Kornberg and Thomas, 1974, p 866). This value may be significantly inaccurate since the migrating complex is an artificially cross-linked structure, presumably more hydrated than an "average" native protein. Small errors in this number could introduce large errors in the estimation of the protein mass present in this complex.

It is clear, then, that although different laboratories have independently arrived at a general agreement that histones f2a1 and f3 interact in a 1:1 molar ratio, the precise number of subunits in such a complex, as well as its geometry, has not yet been established conclusively. Therefore, any attempt to attribute the significance of this complex to the fragments of nuclease digestion of chromatin (Sahasrabudhe and Van Holde, 1974) or to the nodules (Anderson and Moudrianakis, 1969) or  $\nu$  bodies (Olins and Olins, 1974) observed in electron microscopic preparations of chromatin could be needlessly speculative and may lead to erroneous conclusions at this time.

The findings of the present study on the role of urea in histone interactions are of considerable interest. The presence of 2.0 *M* urea in the initial histone-DNA mixture containing 2.0 *M* NaCl is essential for the subsequent binding of f2a1 to DNA; 1.0 *M* urea is not sufficient (Table II). This observation is consistent with that made by Bekhor et al. (1969) and Huang and Huang (1969) concerning the importance of urea during the "reconstitution" of chromatin, and it is similar to that made by Shih and Fasman (1971) concerning the binding to DNA of f2a1 in particular. However, in most studies of DNA-histone complexes, a high concentration of urea is invariably maintained during dialysis to low ionic strength as the nucleohistone complexes are forming. Our data show that significant binding of f2a1 to DNA occurs in the absence of urea as long as urea and f3 are present in the initial histone-DNA mixture. If 2.0 *M* urea (but not f3) is present during the dialysis of an f2a1 + DNA mixture to 0.9 *M* NaCl, a considerable amount of f2a1 does bind to DNA, but no more than that which binds to DNA if f3 is present and urea is absent. We suggest that urea is necessary to prevent the aggregation of f2a1 (and possibly f3) but that *once the f3-f2a1 complex forms, urea is no longer necessary* for the binding of these histones to DNA. One disadvantage of having urea present during histone-DNA complex formation is the possibility that it disrupts chemical interactions involved in the natural binding of histones to DNA. That urea does, in fact, disrupt relevant interactions is suggested by our (unpublished) observation that the binding of f2a1 to DNA in the presence of 2.0 *M* urea is no longer enhanced by f3, and by the finding of

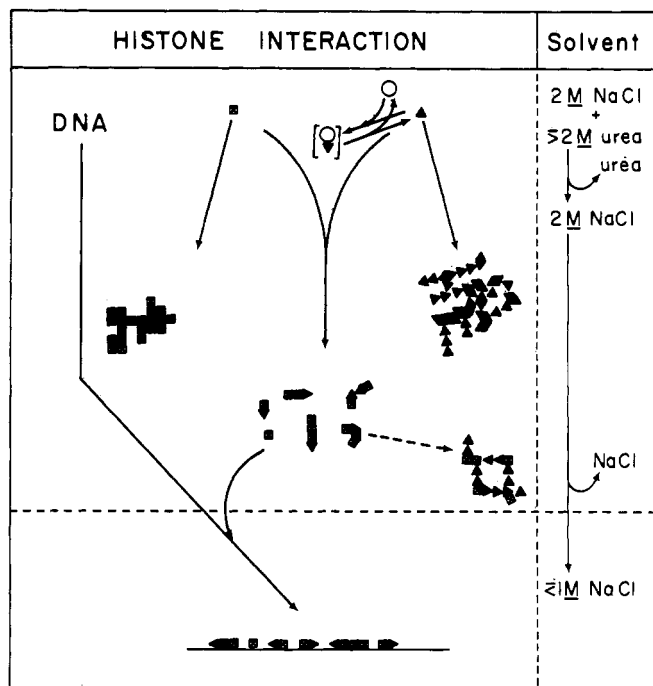


FIGURE 7: Working model which proposes the histone interactions involved in the binding of f3 and f2a1 to DNA. The monomers are represented as follows: f3 ( $\boxtimes$ ), f2a1 ( $\blacktriangle$ ), f1 ( $\circ$ ). In the presence of 2 *M* NaCl and 2 *M* urea no stable interactions may exist between histones or between histones and DNA. After removal of urea, but still in high salt, most of histone f3 aggregates in complexes of varying size, as does all of histone f2a1 separately. As the salt concentration is decreased, another type of interaction begins to take place, i.e., the formation of f3-f2a1 complexes whose stoichiometry is dependent on the relative concentration of the proteins. In low ionic strength these complexes, as well as some free f3, can bind to DNA.

Kleiman and Huang (1972) that 6.0 *M* urea reduces the affinity of all the histones for DNA except that of f1.

Whether the f3-f2a1 complex is an integral part of the structure of chromatin is the most important consideration of the present study. The observation that this complex can be formed and isolated in the absence of DNA from one reaction mixture and then added to DNA and bind almost quantitatively is certainly novel, and perhaps most relevant to the structure and synthesis of eucaryotic chromosomes. A variety of qualitative data exists on the relative binding strengths of these two histones in chromatin as measured by their ability to be dissociated by various agents. One relevant fact applies to most of these studies: the dissociation of f3 from chromatin is invariably concomitant with the dissociation of f2a1, regardless of the agent employed. This phenomenon is observed in 1.0–2.0 *M* NaCl (pH 7.8) (Ohlenbusch et al., 1967; Wagner and Spelsberg, 1971; Kleiman and Huang, 1972); in ethanol containing 1.25 *M* HCl (Johns, 1964); in  $\text{H}_2\text{SO}_4$  between pH 1.8 and 1.2 (Murray, 1966); in 0.01–0.05 *M* sodium deoxycholate (pH 8.0) (Smart and Bonner, 1971); and in 0.3–0.6 *M* NaCl–0.04 *M*  $\text{NaHSO}_3$ –6.0 *M* urea (pH 7.8) (Kleiman and Huang, 1972). Although other explanations are possible, we suggest that the reason f3 is dissociated from chromatin simultaneously with f2a1 is that these histones are present in chromatin in the form of an f3-f2a1 complex. This conclusion is also supported by the data of Varshavsky and Georgiev (1972), who showed that extraction of chromatin with a 20-fold excess of yeast tRNA in very low ionic strength removed all histones except f3 and f2a1; after formaldehyde



fixation and shearing, the residual (f3 + f2a1)-DNA complex could be separated from essentially naked DNA, which constituted one-third of the chromatin DNA. This finding suggests that f3 and f2a1 are distributed nonrandomly in chromatin, and we predict that they are actually *bound together as a unit*.

Any model of chromatin structure should be consistent with the available data on how the components of chromatin interact with one another under mild conditions. Our demonstration of a specific f3-f2a1 complex, modified by f1, supports the view that these interactions play an integral role in the structure and perhaps the biosynthesis of chromatin.

#### Added in Proof

While this paper was in press, a communication by Hyde and Walker (1975) appeared which agrees with our conclusions here but not with those of Kornberg and Thomas (1974); that is, histones f2a1 and f3 first interact to form a dimer, which is the "basic polymerising unit," and this complex can be induced to "undergo extensive polymerisation" by salt or when in high concentration.

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