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$$\text{API gravity} = \frac{141.5}{\text{specific gravity of the liquid at 15.5 C}} - 131.5$$

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Chromatin Structure: Oligomers of the Histones

The histones comprise an $(F2A1)_2(F3)_2$ tetramer, a different oligomer of F2A2 and F2B, and monomer of F1.

Roger D. Kornberg and Jean O. Thomas

Biochemical and x-ray diffraction results concerning the oligomeric structure of the histones are presented. The results show pairwise associations in solution, two types of histone forming a tetramer and two other types of histone forming a different oligomer. The same pairwise associations appear to occur in chromatin.

Introduction to the Histones

There are five main types of histone in the chromatin of eukaryotes, known as F1 (or I) with a mass of 21,000 daltons; F2A1 (or IV), of 11,300 daltons; F2A2 (or IIb1), of 14,500 daltons; F2B (or IIb2), of 13,700 daltons; and F3 (or III), of 15,300 daltons. There are usually nearly equimolar amounts of all the histones except for F1, of which there is about half as much (*1*).

The arrangement of histones and DNA in chromatin has been studied by x-ray diffraction (*2*, *3*), leading to the proposal (*4*) of a "super-coil" model. The x-ray data have not been sufficient, however, to prove the validity of the model. Various studies have been undertaken to supplement the x-ray data, such as studies of the association behavior of the histones (*5*–*7*) and studies of complexes of one or another type of histone with DNA (*6*, *8*). In most cases, histones purified to homogeneity from calf thymus were used. Four of the histones, F2A1, F2A2, F2B, and F3, were observed to form large self-aggregates. For example, F2A1 and F3, at *p*H 7 and ionic strength 0.1, form self-aggregates of sedimentation coefficient 19*S* and molecular weight about 10^6. Such aggregates may be the cause of anomalous stoichiometries in complexes of the histones with DNA. For example, five to ten times as much F2A1 will bind to a given weight of DNA as is bound to the same weight of DNA in chromatin.

Oligomers of the Histones in Solution

The tendency of histones to form large aggregates may be a consequence of the denaturing conditions under which they are prepared. The histones are usually extracted from chromatin in acid and fractionated by ethanol and acetone precipitation, followed by gel filtration in acid or by ion-exchange chromatography in guanidine hydrochloride. We decided to try milder methods and, in particular, the procedure of van der Westhuyzen and von Holt (*9*), which involves extracting the histones from chromatin in 2*M* sodium chloride–50 m*M* sodium acetate (*p*H 5) and fractionating the extract by Sephadex G-100 gel filtration in 50 m*M* sodium acetate at *p*H 5. The Sephadex G-100 elution profile consists of two peaks, both coming after the excluded volume and thus of molecular weight less than about 10^5. The first (higher molecular weight) peak contains F1, F2A1, and F3; and the second (lower molecular weight) peak contains F2A2

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and F2B. In other words, F2A1 and F3, which are almost the same as F2A2 and F2B in molecular weight, behave like F1, which has nearly twice their molecular weight. It seemed to us that this procedure might yield a dimer of F2A1 and F3 and monomers of F2A2 and F2B. In view of indirect evidence (see below) for pairwise associations of the histones in chromatin, F2A1 with F3, and F2A2 with F2B, it seemed that a dimer of F2A1 and F3 could be significant. So we followed the procedure of van der Westhuyzen and von Holt (*9*), including the final step of precipitating F2A1 and F3 with ammonium sulfate to separate them from F1, and then looked for dimers. Instead we found an $(F2A1)_2(F3)_2$ tetramer.

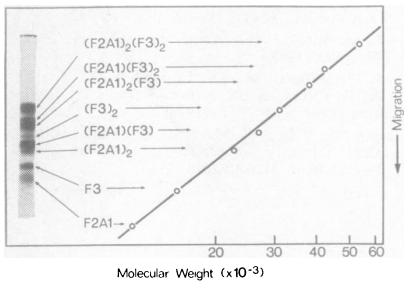

Fig. 1. Cross-linking of F2A1 and F3. An ammonium sulfate precipitate of F2A1 and F3 was prepared by the method of van der Westhuyzen and von Holt (*9*) from calf thymus chromatin [prepared by the method of Zubay and Doty (*25*), including 50 m*M* sodium bisulfite as a protease inhibitor (*26*)]. The precipitate was dissolved at a protein concentration [determined by the method of Lowry *et al.* (*27*)] of about 2 mg/ml in 50 m*M* sodium acetate–50 m*M* sodium bisulfite, *p*H 5.0, and dialyzed against 70 m*M* sodium phosphate–10 m*M* sodium bisulfite, *p*H 8.0 (ionic strength, 0.25). The protein concentration was adjusted to 0.5 mg/ml by dilution with the dialysis buffer, and dimethyl suberimidate (freshly dissolved at 20 mg/ml in the same buffer) was added to a final concentration of 1 mg/ml. The mixture was kept for 3 hours at room temperature, then dialyzed against 0.25 m*M* phenylmethyl sulfonyl fluoride (PMSF) at 4°C, and freeze-dried. The residue was dissolved in SDS sample buffer, containing 0.1 m*M* PMSF and already boiling to prevent proteolysis (*28*), and analyzed in 7.5 percent SDS–polyacrylamide gels according to Weber and Osborn (*29*). The gels were fixed, stained with Coomassie brilliant blue, and destained as described (*30*). The positions and relative intensities of bands were the same in experiments at lower ionic strength (40 m*M* triethanolamine hydrochloride–10 m*M* sodium bisulfite, *p*H 8.0; ionic strength, 0.05) and when the shorter cross-linking reagent, dimethyl pimelimidate [prepared by the method of McElvain and Schroeder (*31*)] was used. The positions of the bands were the same in experiments at *p*H 7 (90 m*M* sodium phosphate, *p*H 7.0; ionic strength, 0.2) and at *p*H 9 (0.15*M* NaCl–0.1*M* sodium borate–10 m*M* sodium bisulfite, *p*H 9.0; ionic strength, 0.25); but the relative intensities were different, the lower molecular weight bands being relatively more intense at *p*H 7 and the higher molecular weight bands more intense at *p*H 9, reflecting the *p*H dependence of the reactivity of ε-amino groups.

The evidence for this tetramer comes from cross-linking and sedimentation experiments. Cross-linking was carried out by the method of Davies and Stark (*10*) which involves treating the protein with dimethyl suberimidate, a bifunctional amino group reagent, and determining the molecular weights of the products by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. A tetramer of identical subunits gives four bands on the gel, corresponding to monomer, dimer, trimer, and tetramer. In a case of nonidentical subunits a more complex pattern of bands would be expected. The mixture of F2A1 and F3 prepared according to van der Westhuyzen and von Holt gives eight bands (Fig. 1). The positions and relative intensities of the bands were the same in experiments performed at protein concentrations of 0.1, 1, and 2 mg/ml, showing that all the bands derive from intramolecular reaction. The monomer bands were identified by comparison with a control in which dimethyl suberimidate was omitted. The other bands were identified by finding combinations of F2A1 and F3 whose molecular weights, when plotted against the positions of the bands in the gel, gave a straight line [Fig. 1; the molecular weight scale was not calibrated with other proteins because the mobilities of histones in SDS gels are anomalous (*11*)]. All the bands expected for an $(F2A1)_2(F3)_2$ tetramer are present, but none of the additional bands that would arise from tetramers of other compositions. The relative intensities of the bands are roughly as expected for an $(F2A1)_2(F3)_2$ tetramer (1 : 2 : 1 for the dimer bands and 1 : 1 for the trimers), but not exactly as expected, presumably because probabilities of forming cross-links depend on proximities and reactivities of amino groups.

Strictly speaking, the pattern of cross-linked products is consistent with either some mixture of monomers, dimers, or a homogeneous solution of $(F2A1)_2(F3)_2$ tetramer. The possibility of a mixture was ruled out and the tetramer shown to be homogeneous by sedimentation experiments. We observed (Fig. 2) a single sharp sedimentation boundary (sedimentation coefficient 3*S*) and the behavior expected of a homogeneous species at sedimentation equilibrium (a linear plot of log concentration as a function of the square of the distance from the axis of rotation). The sedimentation equilibrium results give a value of 53,900 for the molecular weight (assuming a partial specific volume of 0.72 $cm^3\ g^{-1}$). This is in good agreement with the value of 53,200 expected for the molecular weight of an $(F2A1)_2(F3)_2$ tetramer.

When F2A1 and F3 prepared by acid and solvent extraction (*12*) were mixed and analyzed in cross-linking and sedimentation experiments as described above, only a small amount of tetramer was observed. Most of the material was in the form of high molecular weight aggregates which were excluded from SDS gels after cross-linking and which sedimented rapidly (sedimentation coefficient about 13*S*).

Since F2A1, F3, and F1 are eluted together from Sephadex G-100 in the van der Westhuyzen and von Holt procedure, and since F2A1 and F3 form a tetramer, it seemed that F1 might form a dimer (*13*). However, previous work (*5*, *14*) on F1 prepared by acid extraction has shown only monomers.

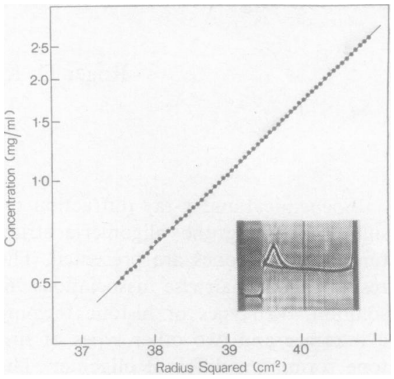

Fig. 2. Sedimentation experiments on F2A1 and F3. A mixture of F2A1 and F3 was prepared as described in Fig. 1 and dialyzed against 0.15*M* NaCl–25 m*M* sodium phosphate–10 m*M* sodium bisulfite (*p*H 7.0; ionic strength, 0.25). Sedimentation velocity measurements were made with a protein concentration of 4 mg/ml, in a Beckman Spinco model E centrifuge at a speed of 60,000 rev/min, with a double sector cell, and with the schlieren phase plate at 60°. The photograph shown was taken 32 minutes after full speed was reached. Sedimentation equilibrium measurements were made with the model E at a speed of 12,000 rev/min and methods described by Durham (*32*). The temperature was 20°C throughout.

In fact, F1 prepared by the van der Westhuyzen and von Holt procedure is also a monomer, as shown by cross-linking experiments (in which only a monomer band was observed on the gel) and sedimentation equilibrium experiments (which showed a homogeneous species with a molecular weight of 21,500 under the conditions given in Fig. 2, in good agreement with the expected molecular weight of about 21,000). This leaves open the question of why F2A1, F3, and F1 are eluted together from Sephadex G-100. A possible explanation is that the tetramer of F2A1 and F3 is relatively compact and the monomer of F1 extended [the sedimentation coefficient of F1 is known (*5*, *15*) to be anomalously low].

The association behavior of F2A2 and F2B in the mixture that arises from the van der Westhuyzen and von Holt procedure has been difficult to analyze. Cross-linking experiments give the pattern of bands shown in Fig. 3. The most rapidly migrating species was found, by comparison with a control in which dimethyl suberimidate was omitted, to comprise both monomers. The other species were identified as dimers, trimers, tetramers, pentamers, and hexamers by the linearity of a semilog plot of the sort shown in Fig. 1. (The plot was constructed using the average of the molecular weights of F2A2 and F2B. Plots constructed in this way and for the extreme cases of all the bands arising from F2A2 or all from F2B gave the same straight line. In other words, the resolution of the gel was not sufficient to distinguish the various possible combinations of F2A2 and F2B.)

In cross-linking experiments at a tenfold lower protein concentration, the positions of the bands were the same, but the intensities of the dimer and the higher molecular weight bands relative to the monomer were greatly diminished. This suggests that F2A2 and F2B form oligomers in a reversible way. The simplest possibility is that they form short chains, such as $(\text{-F2A2-F2B-})_n$ or $(\text{-F2A2-F2A2-F2B-F2B-})_n$, by reversible polymerization. We have not so far established this or excluded other possibilities, such as association behavior like that of a mixture of F2A2 and F2B prepared by acid extraction, which Kelley has shown (*15*) to comprise dimers of the two histones and higher aggregates of only F2B.

We have also carried out a cross-linking experiment on a mixture of the tetramer of F2A1 and F3 and oligomers of F2A2 and F2B. Equal weights of the pairs of histones described in Fig. 1 (F2A1 and F3) and Fig. 3 (F2A2 and F2B) were combined, treated at a total protein concentration of 1 mg/ml with dimethyl suberimidate, and analyzed in SDS gels, as for the experiment shown in Fig. 1. The pattern of bands appeared to be the sum of the patterns shown in Figs. 1 and 3, but many of the bands overlapped. The dimers could be completely resolved in a 17.5 percent SDS–polyacrylamide gel run according to Laemmli (*16*); there were four bands, corresponding exactly in position and intensity to the three dimer bands given by the tetramer and the one dimer band of the F2A2-F2B oligomers. Evidently no additional oligomers are formed.

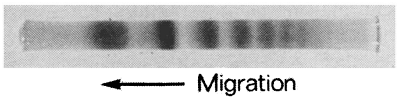

Fig. 3. Cross-linking of F2A2 and F2B. The mixture of F2A2 and F2B resulting from the van der Westhuyzen and von Holt (*9*) procedure was treated at a protein concentration of 0.5 mg/ml with dimethyl suberimidate and analyzed in SDS gels as for the experiment shown in Fig. 1.

Oligomers of the Histones in Chromatin

The histones thus appear to be associated in pairs in solution, F2A1 with F3 and F2A2 with F2B. Many lines of evidence seem to us to suggest that the same pairs of histones are associated in chromatin. The strongest evidence comes from the work of Ilyin *et al.* (*17*) on the exchange of histones between chromatin and free polynucleotides. In 5 m*M* tris-HCl, *p*H 8 at 0°C, there is no exchange of F2A1 and F3 (no detectable exchange in 24 hours), slow exchange of F2A2 and F2B (a half-time for exchange of about 24 hours), and rapid exchange of F1 (complete exchange in 15 minutes). An example of very different evidence which seems to us to suggest the same pairing of histones comes from amino acid sequence work. F2A1 and F3 have been conserved in sequence during evolution [there are two differences in F2A1 (*18*) between pea seedling and calf thymus and four differences in F3 (*19*)], F2A2 and F2B appear less conserved [eight differences in F2A2 (*20*) between trout testis and calf thymus and three differences in the first 22 residues of F2B (*21*)], and F1 appears variable [eight differences in the first 34 residues between rabbit thymus and calf thymus (*22*)].

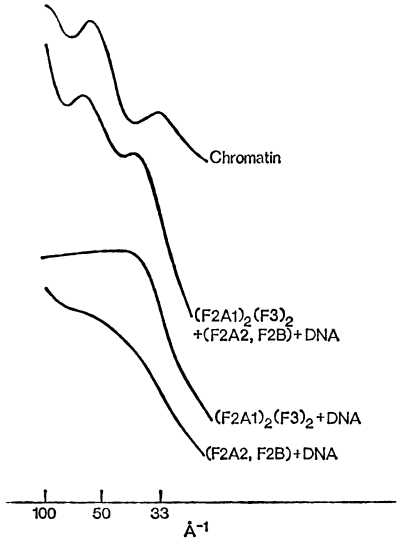

Fig. 4. X-ray patterns of chromatin and of complexes of oligomers of histones with DNA. Chromatin was isolated as described in Fig. 1, treated with ion-exchange resin in 0.65*M* NaCl–50 m*M* sodium phosphate–10 m*M* sodium bisulfite, *p*H 7.0, to remove F1 (*33*), dialyzed against 0.15*M* NaCl–25 m*M* sodium phosphate–10 m*M* sodium bisulfite (*p*H 7.0; ionic strength, 0.25) and centrifuged in a Spinco 60 Ti rotor at 50,000 rev/min for 20 hours, all at 4°C. Complexes of oligomers of histones with DNA were formed from 2 mg of one of the pairs of histones described in Figs. 1 and 3, or 2 mg of each of the pairs, and 5 mg of DNA [isolated from calf thymus (*34*) and sheared by passage through a French press (*35*) to pieces of about 2500 base pairs, as measured by analytical velocity sedimentation in alkali (*36*)]. The histones and DNA were mixed in 2*M* NaCl–0.1*M* sodium phosphate, *p*H 7.0, in a total volume of 25 ml, and dialyzed and centrifuged as described above for chromatin. The pellets of chromatin, $(F2A1)_2(F3)_2$ + (F2A2, F2B) + DNA, $(F2A1)_3(F3)_3$ + DNA, and (F2A2, F2B) + DNA contained 17, 19, 14, and 11 percent by weight of chromatin or histone + DNA. The pellets were sealed in 1-mm path length specimen holders and photographed with the use of a fine-focus rotating anode x-ray tube and mirror-monochromator focusing camera (*37*). Exposures were for about 20 hours at room temperature. Films were analyzed using an Optronics Photoscan model P-1000 digital microdensitometer interfaced to a PDP 11/10 computer, with a program devised by Dr. R. A. Crowther; the optical densities were radially integrated to obtain the traces shown above (*38*).

This evidence suggests pairwise associations of the histones in chromatin but says nothing of details, such as whether the F2A1 and F3 pair, which occurs as an $(F2A1)_2(F3)_2$ tetramer in solution, also occurs as a tetramer in chromatin. The most direct evidence for an $(F2A1)_2(F3)_2$ tetramer in chromatin is that a complex formed from tetramers, F2A2-F2B oligomers, and DNA gives the same x-ray pattern as chromatin (Fig. 4, upper two traces). Tetramers and F2A2-F2B oligomers are both required to give the x-ray pattern (Fig. 4, lower two traces), but F1 is not—in keeping with previous observations (*3*, *23*) that removing F1 from chromatin does not affect the x-ray pattern. Further implications of these results are discussed in the accompanying article (*24*).

We are currently studying associations of the histones in chromatin by cross-linking. There are two difficulties that do not arise in experiments on the histones in solution: the amino side chains are involved in salt linkages with the phosphate groups of DNA and are thus less available for chemical modification; and the presence of five rather than two histones complicates identification of products from molecular weights. Preliminary results do show less cross-linking of histones in chromatin than in solution, but cross-linked products up to pentamers are readily observed and call for further investigation.

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Chromatin Structure: A Repeating Unit of Histones and DNA

Chromatin structure is based on a repeating unit of eight histone molecules and about 200 DNA base pairs.

Roger D. Kornberg

Evidence is given in the preceding article (*1*) for oligomers of the histones, both in solution and in chromatin. Here I wish to discuss this and other evidence in relation to the arrangement of histones and DNA in chromatin. In particular, I propose that the structure of chromatin is based on a repeating unit of two each of the four main types of histone and about 200 base pairs of DNA. A chromatin fiber may consist of many such units forming a flexibly jointed chain.

Introduction to Chromatin Structure

Chromatin of eukaryotes contains nearly equal weights of histone and DNA. This corresponds, on the basis of the molecular weights and relative amounts of the five main types of histone, F1, F2A1, F2A2, F2B, and F3, to roughly one of each type of histone per 100 base pairs of DNA with the exception of F1, of which there is half as much. The arrangement of histones and DNA involves repeats of structure. The first evidence of this comes from the work of Wilkins and co-workers (*2*) who obtained x-ray diffraction patterns from whole nuclei of cells showing relatively sharp bands. Chromatin isolated from the nuclei as a nearly pure complex of histone and DNA gives x-ray patterns with the same bands. Further x-ray work (*3–5*) has shown that these bands correspond

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