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Molecular Homogeneity of the Histone Content of HeLa Chromatin Subunits†

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ABSTRACT: Interaction of affinity chromatographically purified antihistone H3 and antihistone H4 with isolated HeLa core particles, followed by separation of unreacted and reacted particles by sedimentation, demonstrates that every core particle contains these histones. Taken together with our previous data indicating the presence of H2B in every nucleosome (Simpson, R. T., and Bustin, M. (1976), *Biochemistry 15,* 4305), these data lead to the conclusion that each core particle contains two each of the four smaller histones. In contrast to the lack of interference in binding of more than one molecule of antibody to a single species of histone to the core particle, steric hindrance exists when attempts are made to bind both anti-H3 and anti-H4 to core particles.

Ample experimental data support the notions that (1) there are nearly equal amounts of DNA and histones in most eukaryotic chromatin; (2) that the four histones H2A, H2B, H3, and H4 are present in nearly equimolar proportions; and (3) that chromatin is organized into a well-defined repeating unit composed of DNA and these four smaller histones. A question which is not fully resolved is whether each nucleosome present in a given tissue is identical in histone content. Recent evidence suggests that some type of heterogeneity in the structure of chromosomal subunits should be expected. Such data include the differential susceptibility of active genes to digestion by DNase I (Weintraub and Groudine, 1976; Garel and Axel, 1976), the presence of posttranscriptionally modified histones (for a review, see Hnilica, 1972), the presence of several species of histone H2A (Laine et al., 1976) and histone H3 (Marzluff et al., 1972; Garrard, 1976) in a single tissue type, changes in the species of histone present during development of a tissue (Cohen et al., 1975; Blankstein and Levy, 1976), and the lack of equimolar ratios of histones in some tissues (Gorovsky and Keevert, 1975).

It seems important, therefore, to devise approaches which will potentially enable us to distinguish between different species present in a mixture of chromatin subunits. Two levels of histone heterogeneity could potentially occur within the core particle: (1) the composition of the protein core could be variable, that is, different ratios of the four core histones might be present in different particles, or (2) all particles might contain two each of the four smaller histones, but there might be sequence variations among these histones in different particles, due, for example, to genetic polymorphism or post-transcriptional modification.

The availability of antibodies specific to purified histone fractions (Stollar and Ward, 1970; Bustin, 1973) which specifically interact with both isolated histones and chromatin-bound histones (Bustin, 1976) allows examination of possible heterogeneity in core particle histone composition at the molecular level. It is possible to examine the histone content of individual nucleosomes by spreading chromatin preparations on electron microscope grids and reacting the spread chromatin with antihistone sera (Bustin et al., 1976). Alternatively, purified nucleosomes can be reacted with purified antihistones and the reaction mixture subjected to sedimentation to separate those particles which have bound the antibody from those which have not (Simpson and Bustin, 1976). Using both these approaches, we have shown that each nucleosome in a tissue contains histone H2B. However, electron micrographs of rat liver nucleosomes reacted with antibodies to the other histones did not confirm the expectation that every particle would react with each specific antihistone antibody. Thus, 27, 54, and 43% of a population of rat liver nucleosomes failed to react with anti-H3, anti-H2A, and anti-H4, respectively (Goldblatt et al., 1977). In view of these results and because of the data of others suggesting possible heterogeneity in nucleosome composition, we have studied in further detail the histone composition of individual chromosomal subunit particles. We have purified core particles from HeLa cells, reacted them with

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purified antibodies to H3 and H4, and examined the binding of antibody to nucleoprotein by sedimentation. The results presented here demonstrate that each particle in the population contains each of the arginine-rich histones, making it likely that heterogeneity at the molecular level does not exist for the core particle histones in HeLa cells.

Experimental Section

Preparation of Histones and Antihistone Sera. Histones were extracted from washed calf-thymus nucleoprotein by extraction with 0.4 N sulfuric acid. The acid-extracted histones were dissolved in 6 M urea, 0.1 M mercaptoethanol, 1 mM Tris-Cl (pH 7.5), 1 mM phenylmethanesulfonyl fluoride, and incubated at room temperature for 2 h. The histones were then applied to a Bio-Gel P60 column equilibrated and eluted with 0.02 M HCl, 0.1 M NaCl, 0.02% sodium azide (Bohm et al., 1973). This chromatography resolves H1, H2B, H4, and a mixture of H2A and H3. The latter mixture was separated into its two components by gel filtration on Sephadex G-100 (Bohm et al., 1973). The purity of the preparations, judged by electrophoresis in polyacrylamide gels, was greater than 95%. Antisera against histone fractions were prepared from rabbits immunized with histone–RNA complexes (3:1) as recommended by Stollar and Ward (1970). Antisera were assayed by the microcomplement fixation technique (Wasserman and Levine, 1961). Details of methods for eliciting antibodies and titers of sera are presented elsewhere (Goldblatt and Bustin, 1975).

Purification of the Immunoglobulin (Ig) Fraction by Chromatography on DE-52. DE-52 (150 g) (Whatman) was prepared for chromatography as recommended by the manufacturer. The washed, degassed, and defined slurry was suspended in 0.015 M sodium phosphate (pH 8.0) and packed at 5 psi into a 2.5 × 40 cm column. Sera, 25–35 mL previously dialyzed against 0.015 M sodium phosphate (pH 8.0), was applied to the column and elution performed at room temperature with a linear gradient consisting of 400 mL of the starting buffer and 400 mL of 0.3 M sodium phosphate at pH 8.0. Flow rate was 32 mL/h and fractions were collected on a time basis.

Affinity Chromatography. Cyanogen bromide activated Sepharose 4B (Pharmacia) was washed as detailed by the manufacturer. To 1.5 g of dry powder, swollen in water to about 6 mL, 20 mg of H3 or H4 was added in 0.1 M sodium bicarbonate. After shaking at room temperature for 4 h, the slurry was poured into a 0.9 cm wide column and washed with 0.01 M HCl, 0.4 M NaCl until the absorbance of the eluate at 230 nm was less than 0.05. Any remaining active groups were blocked by incubating the column material for 20 min at room temperature with 1.0 M ethanolamine, 0.1 M Tris-Cl (pH 8.5). The adsorbent was then washed extensively with 0.01 M sodium phosphate (pH 7.0), 0.3 M NaCl, and stored at 4 °C. Fifty-four percent of the input H3 and 87% of the H4 were bound to the matrix. For purification of antihistone antibodies, the histone Sepharose was incubated with 45–50 mL Ig fractions in a flask with constant shaking for 60 min at 37 °C and for 18 h at 4 °C. The slurry was poured into a column and the liquid recycled twice through the adsorbent. The columns were washed in the cold with 0.01 M sodium phosphate (pH 7.0), 0.3 M NaCl, 0.05% Triton X-100, until the absorbance of the eluate at 230 nm was less than 0.05. Bound antibodies were eluted with 0.1 M ammonia (pH 11.3), 0.4 M NaCl, immediately adjusted to pH 9.5, and dialyzed against 0.01 M Tris-Cl (pH 7.0), 0.1 M NaCl, 0.25 mM EDTA. Antibody concentrations were estimated using a value of 1.4 for the absorbance at 280 nm of a 1 mg/mL solution of rabbit Ig.

Preparation of Chromatin Subunits. HeLa cell culture, labeling of DNA with tritiated thymidine, isolation of nuclei, and preparation of core particles were carried out exactly as previously described (Whitlock and Simpson, 1976; Simpson and Bustin, 1976).

Interaction of Core Particles with Antibody. HeLa core particles were incubated with various concentrations of antibodies in 0.25 mM EDTA, 1 mM Tris-Cl (pH 7.0), for 60 min at room temperature and 18 h in the cold with constant shaking (Simpson and Bustin, 1976). The incubation mixture was applied to a sucrose gradient made in 0.25 mM EDTA (pH 7.0) with a meniscus sucrose concentration of 5% (w/w), isokinetic for a particle with a density of 1.51 (McCarty et al., 1974). Samples were routinely centrifuged in a Spinco SW41 rotor for 16 h at 38 000 rpm and 4 °C. Gradients were emptied by inserting a tube to the bottom of the centrifuge tube and pumping out. Fractions, usually 0.4 mL, were collected and counted in a Beckman LS250 scintillation counter after addition of 0.4 mL of water and 9.0 mL of Aquasol (New England Nuclear Corp.).

Results

Rabbit antisera cannot be used directly to study the organization of histones in core particles by immunosedimentation (Simpson and Bustin, 1976). This method requires that the antibody population be present in sufficient excess so that every antigen will react with at least one antibody. Because the antigen–antibody reaction has a finite stability constant and because low concentrations of core particles are used, it is expected that high concentrations of antibody will be necessary in this method. At these high concentrations, when antiserum is used, albumin and other unidentified components bind nonspecifically to chromatin subunits. Further, rabbit sera contain nucleases and proteases which degrade subunits during the incubation periods. Ig fractions prepared by ammonium sulfate precipitation were previously shown to be insufficiently pure for immunosedimentation studies (Simpson and Bustin, 1976); here we have explored the possibility that Ig preparations obtained by chromatography of serum on DEAE-cellulose would be suitable reagents for such studies.

DEAE-Cellulose Chromatography. A typical elution profile of rabbit serum chromatographed on DE-52 under the conditions described in the Experimental Section is presented in Figure 1. Although the Ig component usually elutes earliest in this chromatographic system (Fahey and Terry, 1967), antibody to H4 trailed at least as far as fraction 40, determined by Ouchterlony immunodiffusion. Various fractions, indicated in Figure 1, were diluted to $A_{280} = 1.4$ and tested for antibody content by complement fixation. The maximal complement fixed is indicated by the histogram displayed on the elution profile in Figure 1. About half of the anti-H4 activity applied to the column was recovered; this activity was split into two major fractions. Somewhat less than half the anti-H4 antibody was recovered in the early eluted peak, the usual elution position of rabbit Ig components; the remainder of the activity eluted later. Others have documented the inverse relationship between the charge of an antigen and the charge of the antibodies it elicits (Sela and Mozes, 1966). It might be expected, therefore, that the basic histones would elicit relatively acidic Ig, consistent with a large fraction of the Ig population eluting later from the basic cellulosic adsorbent. Essentially identical results with these were obtained with anti-H3 sera.

Pooled fractions containing the anti-H4 activity contained about 15% of the protein applied to the column. This partially purified antihistone was tested as a reagent for immunosedimentation. At a molar excess of Ig to histone of about 400.

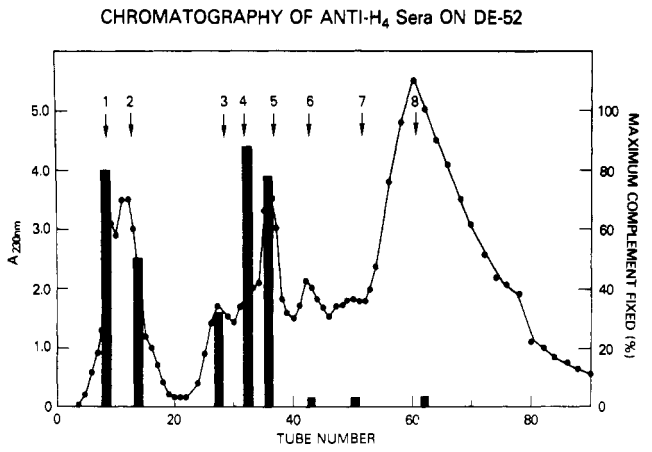

FIGURE 1: Chromatography of anti-H4 on DEAE-cellulose. Thirty-five milliliters of anti-H4 serum was applied to a DE-52 column, prepared and eluted as described in the Experimental Section. Selected fractions, indicated by arabic numerals and arrows, were adjusted to contain 1 mg of protein per mL, diluted 400-fold, and tested for complement fixing activity with H4. The maximum complement fixed is indicated by the solid bars.

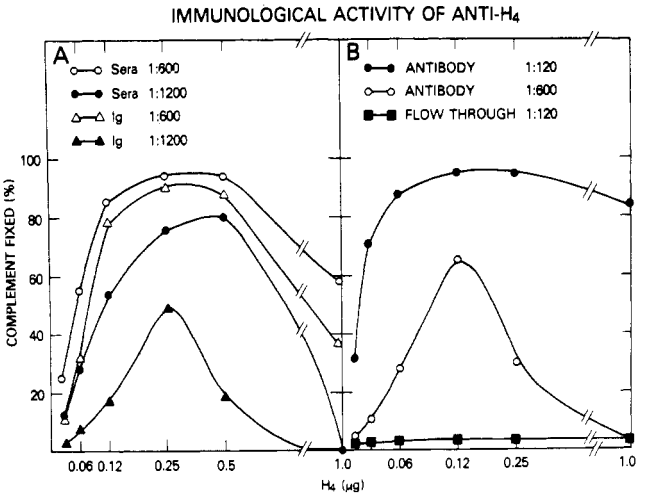

FIGURE 2: Immunological activity of anti-H4 at various stages of purification: (A) comparison of the pooled DE-52 fraction to the original antiserum; (B) activity recovered after affinity chromatography. In both cases, the dilutions are normalized to the original volume of antiserum applied to the DE-52 column.

about 40% of the core particles sedimented more rapidly than the original particle population. However, at a similar excess of Ig to histone, about 30% of core particles bound protein and sedimented more rapidly when control Ig from nonimmunized rabbits was used. Similar results were obtained for anti-H3 sera. Antibodies of higher specific activity were therefore purified by affinity chromatography on histone–Sepharose.

Affinity Chromatography. The bulk of the protein present in the pooled DE-52 fractions does not bind to H4–Sepharose. After extensive washing of the column at neutral pH, elution of bound antibody with ammonia removes about 2% of the original protein. As shown by the complement fixation data in Figure 2, this material represents nearly 85% of the anti-H4 activity present in the DE-52 pooled sample. As expected, the material which did not bind to H4–Sepharose is totally devoid of antibody activity against this histone. The purification obtained by this procedure is about 50-fold; overall, the purification of anti-H4 from serum was about 500-fold.

Similar results were obtained for anti-H3. The two peaks on the DE-52 elution profile containing the antibody activity contained 18% of the total protein and 70% of the antibody activity. Figure 3 shows that nearly 90% of the anti-H3 activity applied to the affinity column was recovered in association with the 15% of the protein that bound to the adsorbent. The nonbound material did contain a low level of anti-H3 activity, corresponding to about 5% of the original. Compared to the original antiserum, anti-H3 antibodies were purified 320-fold.

Interaction of Purified Antibodies with Chromatin Subunits. To determine whether every nucleosome contains H4, core particles from HeLa cells were incubated with anti-H4 antibodies purified on H4–Sepharose prior to sedimentation. The Ig fraction which did not bind to the affinity adsorbent was used as a control. Figure 4 shows that increasing the amount of anti-H4 added to the core particle preparation leads to a progressive shift toward higher sedimentation coefficients. At a molar ratio of anti-H4 to H4 of about 260, all the core particles have moved from the sedimentation position of the original population. In agreement with previous results with anti-H2B (Simpson and Bustin, 1976), the sedimenting peak at high ratios of antibody to particle is broad compared to the original distribution. This probably reflects dispersion of the core particle population due to variations in the amount of

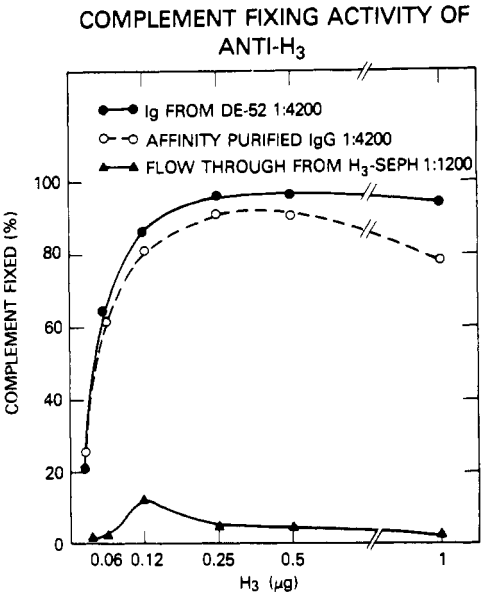

FIGURE 3: Immunological activity of the anti-H3 fraction purified by affinity chromatography. Complement fixation by the Ig fraction from DE-52 chromatography and the absorbed and nonabsorbed fractions from affinity chromatography, as indicated. All dilutions are normalized to the original volume of antiserum applied to the DE-52 column.

antibody bound to the nucleosomes. The data presented in Figure 4 show that an identical amount of control Ig does not alter either the position or the breadth of the sedimentation envelope of core particles. Thus, the anti-H4 antibodies bind specifically to core particles under these conditions and it can be concluded that every core particle contains at least one molecule of histone H4.

Similarly, the data presented in Figure 5 demonstrate that each of the chromatin subunits also contains histone H3. All the core particles moved from their original sedimentation position on the gradient when a 130-fold molar excess of anti-H3 to H3 was added to the chromatin preparation. In this case the control Ig, the material not bound to H3–Sepharose, led to some broadening and movement of the position of the core particle peak. This probably reflects some complexing of core particles by the small amount of anti-H3 activity which

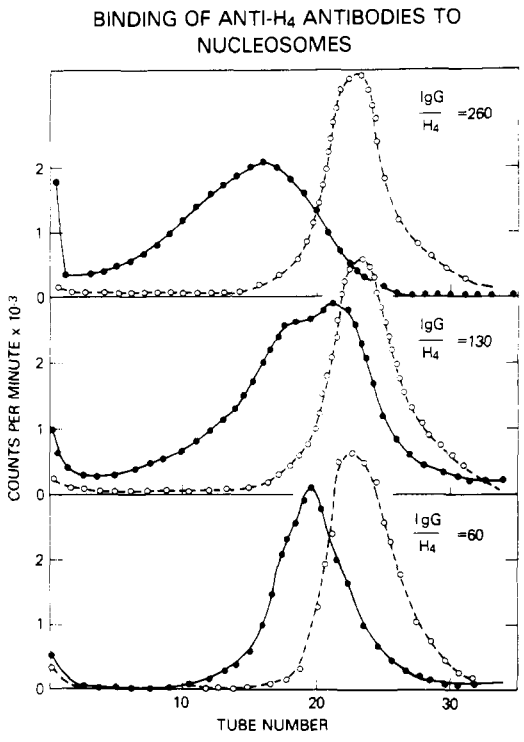

FIGURE 4: Binding of anti-H4 to HeLa core particles. Varying amounts of antibody, as indicated, were incubated with 1.3 μ-g of core particles in 0.2 mL of 0.25 mM EDTA, 1 mM Tris-Cl (pH 7.0), 1 mM phenylmethanesulfonyl fluoride. Samples were sedimented on isokinetic sucrose gradients and the distribution of core particles was assayed by scintillation counting. The direction of sedimentation was from right to left; (●) antibodies; (○) control, flow-through from the H4-Sepharose column.

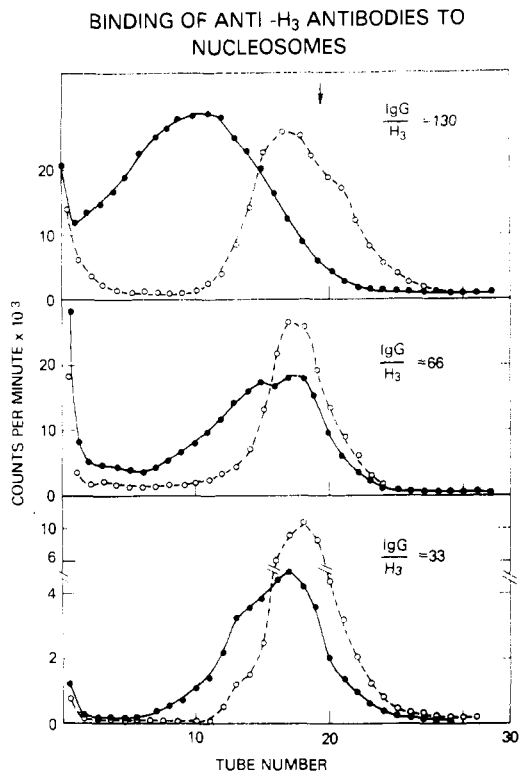

FIGURE 5: Binding of anti-H3 to HeLa core particles. Conditions as in Figure 4, except 2.2 μg of core particles was used. Arrow points to the migration position of core particles in the absence of added proteins.

did not bind to the affinity adsorbent (see Figure 3). Nevertheless, the antibody preparation led to much larger alterations in sedimentation behavior than did this control.

Steric Hindrance in Antibody Binding to Core Particles. Studies of antibody interaction with chromatin have suggested that the various antihistone antibodies could bind without interference from one another; thus, when antisera to various histones were sequentially added to chromatin, only a slight inhibition of the binding of the second antibody was observed (Bustin, 1973). Subsequently, it was noted that the antigenic determinants in chromatin-bound histones are much less available than those determinants in isolated histones (Goldblatt and Bustin, 1975). The availability of purified core particles allows more quantitative approaches to questions of availability of histone determinants and possible steric hindrance in binding of several antibodies. In isolated core particles, all histones have determinants exposed which can react with antibodies to H2B, H3, and H4. Furthermore, binding of one antibody to each core particle does not appear to preclude the binding of a second similar antibody, since the midpoint of the immunosedimentation envelope for all three antihistones corresponds to binding of two IgG molecules per particle (Figures 5 and 6; Simpson and Bustin, 1976). An experiment was performed to see if steric hindrance might exist in the binding of two different antibodies to core particles. Figure 6A shows a shift in sedimentation behavior for about 70% of the core particle population when a 100-fold molar excess of anti-H4 was added to nucleosomes. Figure 6B shows that a 200-fold molar excess of anti-H3 shifts the sedimentation envelope for all the core particles present. In Figure 6C, a 200-fold molar excess of anti-H3 plus a 70-fold molar excess of anti-H4 were added together to the core particles. The sedimentation behaviors of the resultant complexes are closely

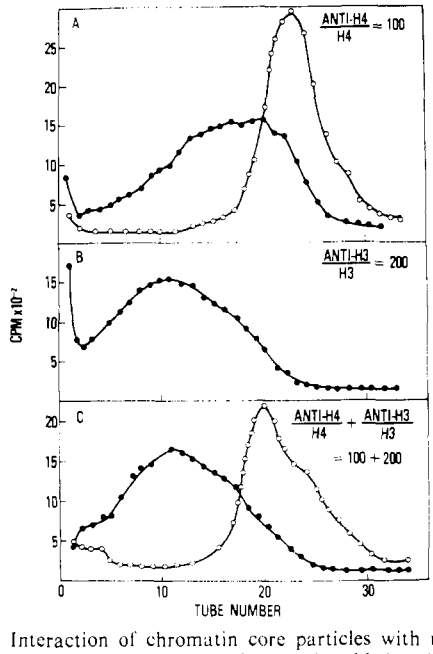

FIGURE 6: Interaction of chromatin core particles with mixtures of anti-H3 and anti-H4. The excesses of antibody added to 1 μg of core particles are indicated in each panel. Conditions and symbols are as in legend to Figure 4.

similar to those of the complexes obtained with anti-H3 alone. Importantly, no material is observed sedimentating in positions which would indicate the association of four or more Ig molecules with a particular particle. Such data are most simply interpreted as indicating steric hindrance in the binding of anti-H3 and anti-H4 to core particles. Extension of such studies to other pairs of histones will be of interest in mapping the

surface relationships of the components of the protein nucleus of the core particle.

Discussion

Our recent immunoelectron microscopic studies suggested that not all the nucleosomes present in a tissue could interact with antisera to all of the histone fractions. Thus, only 73% of rat liver nucleosomes reacted with anti-H3, 46% with anti-H2A, and 57% with anti-H4 (Goldblatt et al., 1977). These findings, together with data of others suggesting the possibility of heterogeneity in core particles, prompted us to study the molecular composition of histones using immunosedimentation. This method allows the detection of molecular heterogeneity, that is, nonequivalence of content of the major histone polypeptides; it does not relate to the possible occurrence of sequence heterogeneity among the histones in chromatin subunits.

The results presented here show that each of the chromatin subunits obtained from HeLa cells contains at least one copy of the histones H3 and H4. This finding, together with previous results indicating (1) that each core particle contains at least one molecule of histone H2B, (2) that when a preparation of core particles is dispersed by reaction with anti-H2B, all fractions contain equal ratios of the four major histones (Simpson and Bustin, 1976), and (3) that whole chromatin from most tissues contains equal ratios of the four smaller histones (see Elgin and Weintraub, 1975), demonstrate that each histone octamer must be composed of two each of histones H2A, H2B, H3, and H4. It is still likely that sequence heterogeneity exists within the population of core particles; that is, that different particles differ in their content of modified histones or histones differing in sequence due to genetic polymorphism.

The electron microscopic studies indicated that antihistone antisera detected an apparent heterogeneity in nucleosomes. Several options to explain the lack of reactivity of all chromatin subunits with anti-H2A, anti-H3, and anti-H4 were presented (Bustin et al., 1977). It is apparent that the options which relate to molecular compositional heterogeneity are not tenable, on the basis of this current investigation. Whether differential exposure of determinants due to orientation on the electron microscope grid, due to the presence of nonhistone proteins, or other unknown cause, is the case, remains to be determined.

An interesting extension of this approach to the composition and structure of the core particle will be the determination of steric hindrance in binding of antibodies to various pairs of histones in the core particle. Immunoelectron microscopic mapping of positions of proteins in the ribosome has shown great promise in determining the spatial array of proteins in this more complex nucleoprotein (Lake, 1976; Tischendorf et al., 1974). For the chromatin core particle lack of any definitive structural feature which allows orientation of the particle seems to make such studies more difficult. However, it should be possible to obtain some information about the proximity of various proteins to one another by probing the surface of the core particle and that of the octameric protein isolated from the core particle (Stein et al., 1977) with antibodies to specific histone fractions.

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