Histone Composition of Chromatin Subunits Studied by Immunosedimentation†

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ABSTRACT: Chromatin subunits were prepared from HeLa cells by in situ digestion of nuclear DNA with micrococcal nuclease followed by sucrose gradient sedimentation. These 11S chromosomal particles (nucleosomes) contain a DNA fragment 140–180 base pairs long and an equal mass of histones, H2A, H2B, H3, and H4. Nucleosomes were incubated with purified antibodies to histones H2A and H2B and to hemoglobin A, and the resulting complexes were analyzed by ultracentrifugation. Of these, only anti-H2B bound specifically to nucleosomes. When sufficient antibody was present, all (>98%) the nucleosomes sedimented with increased velocities, indicating that all chromosomal particles contain H2B, as suggested by previous electron microscopic studies (Bustin, M., Goldblatt, D., and Sperling, R. (1976), *Cell 7*, 297). The amount of antibody reacting with H2B in the nucleosome was quantitated by densitometric scanning of gel electrophoresis patterns of the proteins in various nucleosome-anti-H2B complexes separated by sedimentation on isokinetic sucrose gradients. Under conditions where all particles had increased sedimentation velocities, from 1 to 3 IgG molecules are bound to each nucleosome, the ratio increasing from top to bottom of the sedimenting peak. When nucleosomes are thus dispersed on the basis of reaction with anti-H2B, the ratios of H2A to H4 and of (H2B + H3) to H4 are identical (±8%) for all fractions, suggesting that each nucleosome has an identical histone complement, two each of histones H2A, H2B, H3, and H4. Confidence limits for exclusion of other possible octamers are presented. The variation in ratio of bound antibody to nucleosome probably reflects a normal distribution during the titration, although differential exposure of H2B antigenic determinants in several populations of nucleosomes cannot be excluded as an explanation. The method used should be generally applicable to further studies of the composition and function of nucleosomes.

Eukaryotic chromatin is thought to be composed of repeating subunits each containing a 180–205 base pair segment of DNA associated with a core of histones to form a ν body or nucleosome (for review, see Felsenfeld (1975)). The histone core is likely an octamer, thought to contain two each of histones H2A, H2B, H3, and H4 (Thomas and Kornberg, 1975). The question of whether all nucleosomes have an identical histone composition remains unresolved.

The approach most used to study the organization of histones in chromatin has been cross-linking chromatin-bound proteins by various reagents (Kornberg and Thomas, 1974; Hyde and Walker, 1975; Chalkley, 1975; Chalkley and Hunter, 1975; Martinson and McCarthy, 1975; Van Lente et al., 1975; Bonner and Pollard, 1975; Thomas and Kornberg, 1975; Hardison et al., 1975), thereby localizing neighboring protein molecules. Specific histone–histone interactions are suggested by such studies on both chromatin and histones free in solution. Spectroscopic and hydrodynamic studies on histone–histone interactions have indicated that each of the histones H2A, H2B, H3, and H4 can interact with each other, albeit with differing association constants (D'Anna and Isenberg, 1974). When the assembly of histones alone and in combination was investigated, various histone combinations all generated the same basic structure (Sperling and Bustin, 1975). Analysis of the histone composition of nuclei of various organisms reveals that in some cases the four smaller histones are not found in equimolar ratios, even though the chromatin appears to possess subunit structure (Gorovsky and Keevert, 1975). It seems, therefore, that the uniformity of histone content of the chromatin subunits is still an open question.

We have used immunochemical techniques to assess the possible heterogeneity of the histone composition of nucleosomes. Previously we have shown that specific antibodies elicited by purified histones bind to chromatin (Bustin, 1973; Goldblatt and Bustin, 1975) and to metaphase chromosomes (Bustin et al., 1976b), and that such antisera can be used to evaluate the arrangement of histones in chromatin (Zick et al., 1975). Direct visualization of histone content in chromatin subunits by immunoelectron microscopy suggested that at least 95% of nucleosomes contain H2B (Bustin et al. 1976a). In this report, antibodies purified by affinity chromatography are used, for the first time, to probe the composition of nucleosomes by immunosedimentation. We have allowed purified histone antibodies to react with nucleosomes and have analyzed the resulting complexes by centrifugation. The binding of a single 7S IgG antibody molecule, 150 000 daltons, to a nucleosome, about 240 000 daltons, should create a heavier complex separable from unreacted chromatin particles. Analysis of the distribution of antibody bound vs. unreacted nucleosomes as a function of antibody added, as well as analysis of proteins present in antibody bound nucleosomes, allows determination of the distribution of histones in the population of nucleosomes.

Experimental Procedures

Purification of Antibodies. Calf thymus histones were purified as previously described (Bustin, 1973), and antibodies to them elicited in rabbits (Stollar and Ward, 1970; Goldblatt and Bustin, 1975). Immune sera were assayed by the microcomplement fixation technique (Wasserman and Levine, 1961). The IgG fraction from immune and control sera was

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obtained by precipitation with 40% ammonium sulfate at 4 °C. Purified histone fractions were coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Corp.) for use as affinity adsorbents. Activated Sepharose (1.5 g) was washed with HCl (1 mM) as recommended by Pharmacia Corp. The swollen Sepharose was added to about 40 mg of H2B or H2A in 4.0 ml of 0.1 M $NaHCO_3$, 0.2 M NaCl, pH 8.1, and incubated at 25 °C with shaking for 4 h. The adsorbent was poured into a small column and uncoupled histone was removed by washing with 0.01 M HCl and 0.5 M NaCl, until the absorbance of the eluate at 230 nm was less than 0.05. About 85% of the input histone was coupled to the Sepharose. Remaining active groups on the adsorbent were blocked by incubation for 2 h with 1 M ethanolamine, 0.01 M Tris-Cl, pH 8.5, and then the adsorbent was washed exhaustively with PBS.[1] The washed adsorbent was added to 20 ml of antiserum and incubated with vigorous shaking for 1–2 h at 20 °C and overnight at 4 °C. The reaction mixture was poured into a column and washed with PBS containing 0.5 M NaCl with or without 0.06% (v/v) Triton X-100 until absorbance at 230 nm was less than 0.1. After rinsing the column with PBS, bound antibody was eluted with 0.5 M ammonia, 0.5 M NaCl, pH 11.2. The eluate was collected into tubes containing 0.5 M Tris-Cl, pH 8.0, and dialyzed into PBS. After concentration approximately fourfold by placing the bags in dry Sephadex G-150, the antibody preparations were redialyzed against PBS and passed through an 0.45-μm Millipore filter. The yield of purified antibody from 20 ml of antiserum was 5–9 mg for anti-H2B and 8 mg for anti-H2A, based on $A_{280}^{1\%} = 14$. The purified antibodies were stored at −20 °C. The control antibody, elicited against hemoglobin A_{oxy} in sheep and similarly purified by affinity chromatography but using elution with 4 M guanidine hydrochloride, was a generous gift from Dr. Alan Schechter.

Goat anti-rabbit sera and goat anti-rabbit IgG were obtained from Cappel Laboratory. Immunodiffusion was performed in 1% agarose, phosphate buffer, pH 8, using Ouchterlony plates obtained from Meloy Laboratories. The activity of purified antibodies towards nucleosomes was tested by immunoadsorbance, as described before (Bustin, 1973; Goldblatt and Bustin, 1975), except that particles were precipitated with 10 mM $CaCl_2$.

Preparation of Labeled Nucleosomes. HeLa cells, clone S3, were maintained in logarithmic growth at 2–4 × 10^5 cells/ml in Eagle's spinner medium No. 2 supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 5% horse serum. DNA was labeled by addition of 0.5–1.0 mCi/l. of [^{3}H]thymidine (New England Nuclear Corp.) for 24 h prior to harvesting the cells. Cells were harvested by centrifugation, washed once with Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution, and suspended in 0.25 M sucrose, 3 mM $CaCl_2$, 10 mM Tris-Cl, pH 8.0. Triton X-100 was added to a final concentration of 1% (v/v) and the mixture was homogenized with ten strokes of a Dounce homogenizer equipped with a loose-fitting pestle. Nuclei were collected by centrifugation at 1600g for 1 min and washed by resuspension in and sedimentation from the above buffer lacking the detergent. The nuclei were then washed twice in 0.25 M sucrose, 0.1 mM $CaCl_2$, 1 mM Tris-Cl, pH 8.0, and suspended in this buffer at 20 A_{260} units/ml. This suspension was incubated with 250 units/ml of micrococcal nuclease (Worthington Biochemical Corp.) at 0 °C for 1 h with stirring. After digestion, the nuclei were pelleted by centrifugation at room temperature for 2 min at 3000g. The pellet was suspended in 0.25 mM EDTA, pH 7.0, by passage through a 25-gauge needle ten times at 0–4 °C and layered over preparative sucrose gradients.

All centrifugations, both preparative and analytical, were done using sucrose gradients in 0.25 mM EDTA, pH 7.0, with a meniscus concentration of 5% (w/w) sucrose. The gradients were isokinetic for a particle with density 1.51 at 4 °C and were formed using parameters described by McCarty et al. (1974). Preparative centrifugations were generally carried out in a Spinco SW41 rotor at 4 °C using sample volumes of 1.0 ml and centrifugation to $\omega^2 t = 5.5 \times 10^{11}$ radians^2 s^{-1}. Most analytical centrifuge runs were also carried out in the SW41 rotor but with a sample volume of 0.1 ml. Some analyses used the Spinco SW60 rotor with a sample volume of 25–50 μl.

Preparative gradients were emptied by inserting a tube to the bottom of the centrifuge tube and pumping out through a DB-G spectrophotometer equipped with a flow cell and log recorder. The 11S monomer nucleosome peak was collected and dialyzed against two changes of 0.25 mM EDTA, pH 7.0, at 4 °C for 24 h.

Antibody–Nucleosome Interactions. Incubations of 11S chromosomal subunits (usually 0.4–1.0 μg of DNA) and varying concentrations of antibodies were carried out in various mixtures of 0.25 mM EDTA, pH 7.0, and PBS—the storage buffers for the two reactants. Incubation with shaking was carried out for 1.5 h at 37 °C and 2–4 h at 4 °C. Samples were then applied to sucrose gradients for analytical ultracentrifugation as described above.

Analytical sucrose gradients were emptied as described above but, rather than monitoring the absorbancy of the eluate, it was collected directly into scintillation vials for radioactivity measurements. Generally, 30 fractions of about 0.44 ml each were collected from each SW41 tube. Water, 0.5 ml, was added to each vial followed by 8 ml of Aquasol (New England Nuclear Corp.) and the samples were counted in a Beckman LS250 scintillation counter. When sucrose gradients used for analysis of the proteins in various fractions were emptied, the eluate was collected in test tubes, several gradients were pooled, and an aliquot was utilized for radioactivity measurements. Appropriate fractions were dialyzed to water at 4 °C, lyophilized, and used for polyacrylamide gel electrophoretic analysis.

Electrophoresis in Polyacrylamide Gels. Samples for polyacrylamide gel electrophoresis were dissolved in 1% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.064 M Tris, 0.032 M H_3PO_4, and heated to 100 °C for 2 min. Gels, containing 15% acrylamide, 0.13% bis(acrylamide) in the buffer described by LeStourgeon and Rusch (1973), were formed in a slab gel apparatus. Stacking gels contained 3% acrylamide, 0.08% bis(acrylamide). Samples, usually 20 μl, were applied and electrophoresis was carried out at a constant current of 20 mA until the bromphenol blue tracking dye had migrated to within 5 mm of the bottom of the gel. Gels were stained in 0.2% Coomassie blue (Sigma Chemical Co.) in 50% methanol, 7% acetic acid for 30–60 min, and destained overnight in 40% methanol, 7% acetic acid with a small quantity of Dowex 1X2 resin. Gels were photographed using Polaroid Type 55 P/N film. Negatives were scanned with an E-C Apparatus Corp. Densitometer or a Beckman Acta III spectrophotometer equipped with a linear transport device.

Results

Preparation and Characterization of Nucleosomes. Digestion of HeLa cell nuclear DNA in situ with micrococcal

[1] Abbreviations used are: PBS, 0.1 M NaCl, 0.02 M sodium phosphate, pH 7.0; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

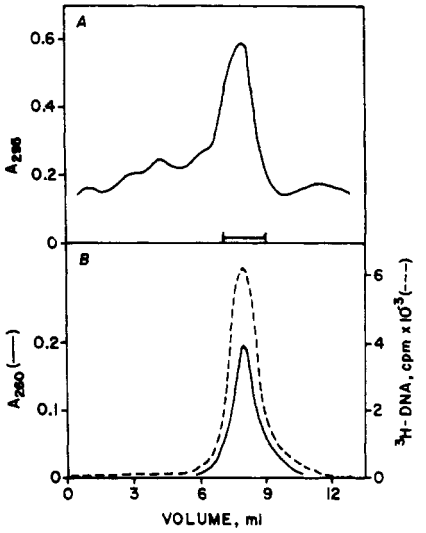

FIGURE 1: (A) Sucrose gradient sedimentation of micrococcal nuclease digestion products of Hela cell nuclei. Hela cell nuclei were digested with micrococcal nuclease as described under Experimental Procedures. The nuclear lysate was centrifuged on an isokinetic sucrose gradient with a meniscus sucrose concentration of 5% (w/w) at 4 °C and 32 000 rpm for 15 h using the Spinco SW41 rotor. The direction of sedimentation was from right to left. The bar indicates the fraction of the gradient pooled as 115 particles or nucleosomes. (B) Resedimentation of nucleosomes isolated from a preparative gradient as illustrated in (A). Pooled, dialyzed nucleosomes were sedimented under exactly the same conditions as in (A). Optical density at 260 nm (—) and tritium labeled DNA (- - -) are plotted. The direction of sedimentation was from right to left.

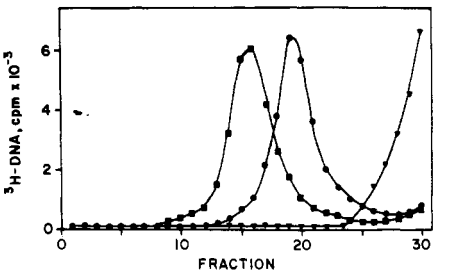

FIGURE 2: Inactivation of serum nuclease by lyophilization. Sedimentation of nucleosomes alone (●—●) or incubated with fresh rabbit serum (▼—▼) or lyophilized, reconstituted rabbit serum (■—■). Nucleosomes isolated as in Figure 1 were incubated in 0.25 mM EDTA, pH 7.0, either alone or with 5 μl of fresh or lyophilized rabbit serum per 0.8 μg of DNA for 1.5 h at 37 °C and 4 h at 4 °C and then centrifuged on isokinetic sucrose gradients in the SW41 rotor for 10 h at 40 000 rpm and 5 °C. The direction of sedimentation was from right to left.

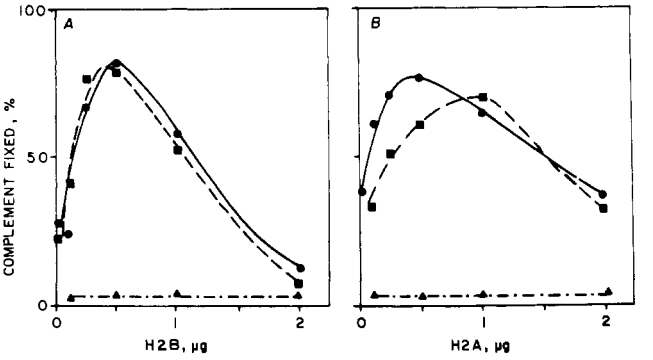

FIGURE 3: Purification of active antibodies by affinity chromatography. Immunological activity was measured by micro-complement fixation using purified histone fractions as antigens. Panel A: anti-H2B serum, 1:3000 (●—●); anti-H2B antibodies, 1:1000 (■- -■) after elution from affinity column; material not bound to the histone–Sepharose column, 1:1000 (▲- - -). Panel B analogous to panel A except with anti-H2A serum.

nuclease generates multimers of the chromosomal subunit and, with time, converts much of the nuclear DNA into monomeric DNA fragments of about 160 base pairs. Under the conditions used in this study, 45–50% of the DNA present in lysed nuclei after digestion sediments in sucrose gradients as the major peak with a sedimentation coefficient of about 11 S (Figure 1A). The fraction indicated by the bar was collected and analyzed. When resedimented at an analytical level, the 11S particles migrate as an homogenous population (Figure 1B). DNA from these particles was purified by phenol extraction and analyzed on 3 and 6% acrylamide gels after phenol extraction, using as standards the DNA fragments generated from SV-40 DNA by digestion with *Haemophilus aegyptus* restriction endonuclease III. More than 85% of the DNA in these particles is double stranded, with a length of 140–180 base pairs. Smaller amounts of material are larger (330–380 base pairs) and smaller (<140 base pairs) fragments. As evaluated by electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels, the particles contain histones H2A, H2B, H3, and H4 in apparently similar amounts, and trace amounts of H1 and nonhistone proteins. By generally accepted criteria, these 11S particles are purified chromatin subunits or nucleosomes as previously obtained and characterized by others (Senior et al., 1975; Finch et al., 1975).

Preparation and Characterization of Purified Antibodies. Our initial studies of the interaction of anti-histone sera with nucleosomes revealed several problems that deserve consideration. Most fresh rabbit sera contain a nuclease activity that degrades nucleosome DNA to nonsedimenting material (Figure 2). This nuclease activity is inhibited in part by EDTA and is completely absent in serum samples reconstituted after lyophilization (Figure 2).

Figure 2 also shows that lyophilized nonimmune serum binds to nucleosomes in 0.25 mM EDTA, 1 mM Tris-Cl, pH 7.0, and increases the sedimentation coefficient of the particles. Further, we have found that both crystalline bovine serum albumin and IgG fractions prepared by precipitation by 40% $(NH_4)_2SO_4$ bind to nucleosomes, particularly in solutions of low ionic strength. In PBS or solutions of higher ionic strength, this nonspecific binding is reduced but not eliminated. In addition, we have noted that both rabbit sera and IgG fractions contain a protease activity that degrades histones, likely the same protease noted by Weintraub et al (1975). Therefore, in addition to using lyophilized serum to eliminate nuclease activity, we have used purified antibodies to minimize nonspecific binding of protein to nucleosomes and have taken further measures to inhibit protease activity (see below).

Antibodies were purified by affinity chromatography as described under Experimental Procedures. Anti-histone activity present in anti-H2A and anti-H2B sera was absorbed onto the homologous histone–Sepharose column material (Figure 3). The material that passed through the column was devoid of immunological activity when assayed by complement fixation using purified histones as antigen. Good recovery of antibody activity was obtained in the material eluted with 0.5 M ammonia, 0.5 M NaCl, pH 11.2 (Figure 3).

When analyzed by Ouchterlony diffusion, the purified antibodies are seen to belong to the IgG class (Figure 4). A single sharp precipitin line is obtained by immunodiffusion for both antibodies with goat anti-rabbit sera and goat anti-rabbit IgG. Immunoelectrophoresis of anti-H1 antibody prepared by this same technique verified this conclusion (Bustin and Kupfer, 1976). The purified anti-H2B antibodies can recognize H2B

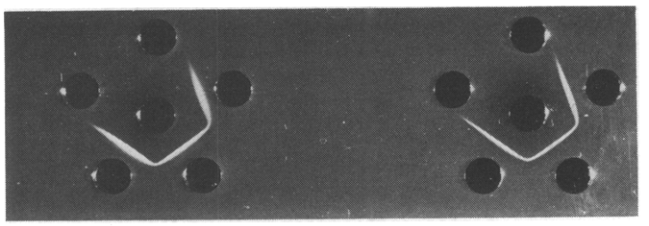

FIGURE 4: Anti-histone activity resides in the IgG fraction. Ouchterlony immunodiffusion proceeded for 20 h at 25 °C with antibody preparations at about 0.9 mg/ml in PBS. Left center well, goat anti-rabbit serum; right center well, goat anti-rabbit IgG. Clockwise from the top well: anti-H1 antibody, anti-H2A antibody, and anti-H2B antibody.

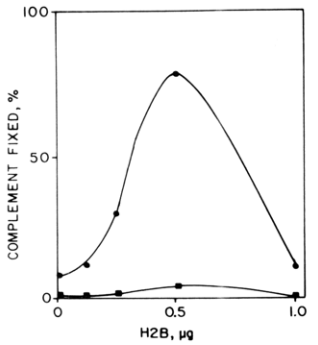

FIGURE 5: Adsorbance of anti-H2B antibody on nucleosomes. Anti-H2B (10 μg) was added to 50 μg of nucleosome (as DNA) in 0.4 ml of 0.25 mM EDTA, 1 mM Tris-Cl, pH 6.9. The mixture was incubated with shaking for 90 min at 25 °C and 4 h at 4 °C. At the conclusion of the incubation, nucleosomes were precipitated by making the solutions 10 mM in $CaCl_2$ and centrifugation for 30 min at 5000g and 4 °C. Supernatant (185 μl) was assayed by complement fixation. Control antibody incubated in the absence of nucleosomes, 1:2500 (●—●). Antibody incubated in the presence of nucleosomes, 1:2500 (■—■).

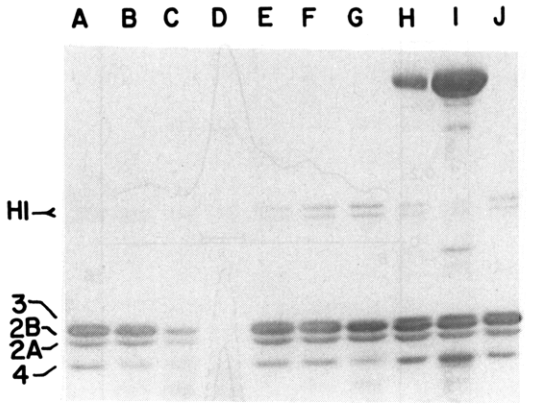

FIGURE 6: Proteolytic activity in antibody preparations. Histones at 1.0 mg/ml in 0.2 M Tris-Cl, pH 7.45, were incubated at 37 °C for 4 h with various amounts of antibody. (A and J) Histones alone; (B–D) histones plus 40, 80, and 160 μg of anti-H2B antibody; (E–G) histones plus 40, 80, and 160 μg of anti-H1 antibody; (H–I) histones plus 5 and 20 μl of lyophilized normal rabbit serum.

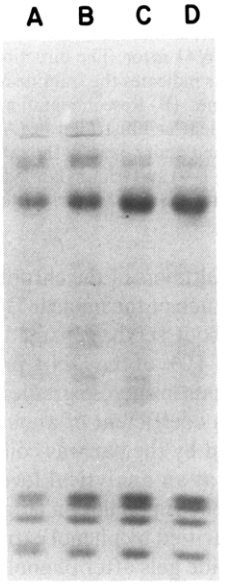

FIGURE 7: Inactivation of histone protease activity by phenylmethanesulfonyl fluoride. Two preparations of anti-H2B were studied. (A,B) Anti-H2B prepared as under Experimental Procedures with column washing with 0.1 M NaCl in PBS; (C,D) anti-H2B prepared as under Experimental Procedures with column washing with 0.5 M NaCl, 0.06% Triton X-100, in PBS; (A,C) antibody controls; (B,D) antibody treated with 1 mM phenylmethanesulfonyl fluoride for 1 h at 20 °C in PBS. After incubation, with or without the inhibitor, 34 μg of antibody was incubated with 5 μg of nucleosome (as DNA) for 1.5 h at 37 °C and 2 h at 4 °C. Samples were dialyzed to water, lyophilized, and analyzed on sodium dodecyl sulfate containing polyacrylamide gels.

antigenic determinants in nucleosomes. Incubation of the antibody with nucleosomes results in adsorption of immunological activity on the particles (Figure 5) in a way analogous to the loss of activity upon incubation of antisera with whole chromatin (Bustin, 1973; Goldblatt and Bustin, 1975). As will be shown below, these preparations lacked nuclease activity and, at the concentrations necessary for immunologic studies, did not bind nonspecifically to nucleosomes. Some of the antibody preparations did, however, contain protease activity.

Weintraub et al. (1975) reported that IgG preparations contain a protease that preferentially degrades histone H2B in a mixture of histones at high ionic strength. A protease specific for H2B conceivably could bind to H2B–Sepharose columns. The results presented in Figure 6 indicate that an anti-H2B antibody preparation is enriched in histone protease as compared with an anti-H1 antibody preparation or whole serum. In these experiments, histones were incubated at low ionic strength at 37 °C with various amounts of antibodies or lyophilized normal rabbit serum. In each case, histone degradation was observed but was most severe with the purified anti-H2B antibody. There was no apparent preferential degradation of any one histone. The protease is also active against histone in purified nucleosomes and, in this case, histone H2B is degraded somewhat more rapidly than the others (Figure 7). The protease activity could be inhibited by reacting the antibody preparations with 1 mM phenylmethane-sulfonyl fluoride in PBS for 1 h at 20 °C (Figure 7). Therefore, all subsequent operations were carried out with antibody preparations that had been treated in this manner. We have additionally found that when the H2B–Sepharose columns, to which the serum was added, are washed with PBS, containing 0.5 M NaCl and 0.06% Triton X-100, the antibody recovered is devoid of protease activity (Figure 7).

Interaction of Antibodies with Nucleosomes. Isolated nucleosomes sediment on sucrose gradients in 0.25 mM EDTA, pH 7.0, as a sharp zone with a sedimentation coefficient of 11 S (Figure 8). Addition of anti-H2B to nucleosomes shifts this sharp band to higher sedimentation coefficients and broadens the zone considerably (Figure 8). Addition of greater amounts of antibody leads to a progressive shift to the faster sedimenting species until almost no nucleosome DNA (<1%) sediments at the peak fraction of the control nucleosomes. The breadth of

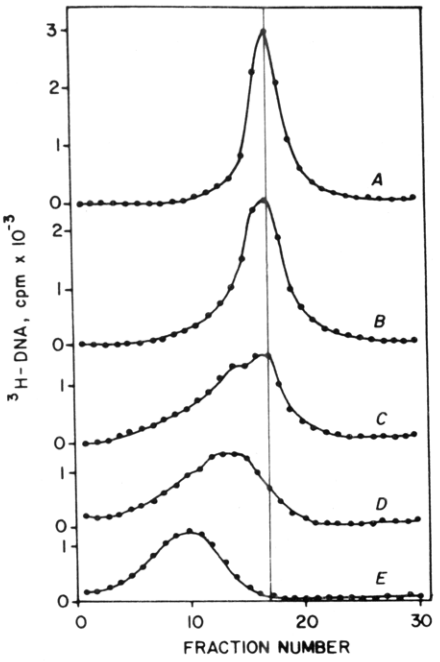

FIGURE 8: Altered sedimentation behavior of nucleosomes incubated with anti-H2B antibody. Incubation and sedimentation carried out as in the legend to Figure 2. Samples are: (A) nucleosomes alone; (B–E) 0.8 μg of nucleosomes (as DNA) plus (B) 5, (C) 15, (D) 30, and (E) 75 μg of anti-H2B antibody. At the highest concentration of antibody about 25% of the nucleosomes sedimented to the bottom of the gradient.

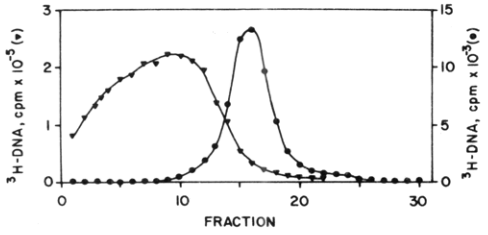

FIGURE 9: Quantitation of the interaction of anti-H2B antibody with nucleosomes. Above: 1200 μg of anti-H2B and 30 μg of nucleosomes (as DNA) were sedimented on duplicate isokinetic gradients and the collected gradients were pooled. A 1% aliquot was counted (▼). A control sample of nucleosomes was centrifuged in parallel (●—●). Samples were selected, dialyzed to water, lyophilized, and analyzed on 15% polyacrylamide gels, as shown in Figure 10.

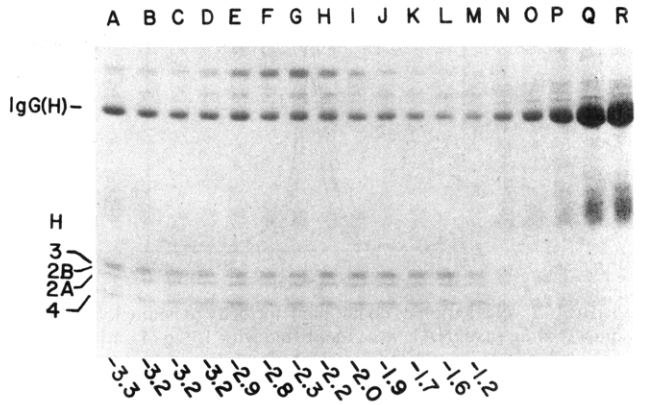

FIGURE 10: Electrophoresis of proteins from nucleosome fractions shown in Figure 9. One-third of each sample was applied to the gel except for two-thirds of samples 1 and 2. From left to right the slots represent fractions 1–13, 15, and 17–20. The positions of migration of the four smaller histones are indicated as is the migration position of the heavy chain of IgG, molecular weight 50 000. The peak in slots C–I of protein with a subunit molecular weight of about 70 000 likely represents a small contamination of the IgG anti-H2B with IgM, having a sedimentation coefficient of about 20 S. The numbers at the bottom of the figure are the molar ratio of IgG to nucleosome for those fractions which contained labeled DNA.

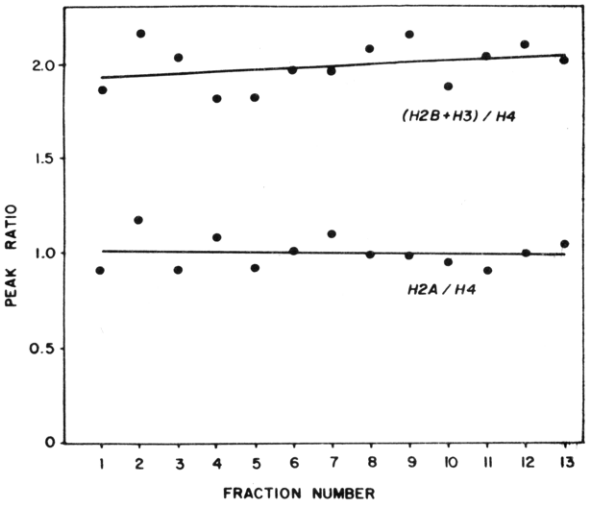

FIGURE 11: Ratios of histones in nucleosomes fractions shown in Figure 10. The negative of a photograph of the gel shown in Figure 10 was scanned and peak areas corresponding to histones H4, H2A, and H2B + H3 was determined. The ratios of areas for H2A/H4 (lower curve) and (H2B + H3)/H4 (upper curve) are plotted for fractions 1–13 of the original gradient (lanes A–M of Figure 10). The lines drawn are linear least-squares regression lines for the data.

the distribution is unaltered when antibody is treated or not treated with phenylmethane-sulfonyl fluoride and, therefore, does not arise from partial proteolytic breakdown of H2B. Further, the shift in sedimentation pattern is observed with either treated or untreated antibody and, therefore, does not arise from a change in particle shape consequent to cleavage of H2B. Similarly, the sedimentation of the nucleosomes is not affected by their application to the gradient in PBS and the antigen–antibody reaction appears to be independent of whether incubation was in PBS or dilutions of PBS. Therefore, we conclude that the altered sedimentation pattern of particles incubated with anti-H2B results from the binding of the antibody to H2B determinants exposed in the nucleosome. These data demonstrate directly that all nucleosomes contain at least one H2B molecule.

The increased breadth of the particle distribution when reacted with anti-H2B compared to particles alone could arise from heterogeneity in the histone content of the particles or other features of interactions of antibody preparations with nucleosomes. This question was evaluated by scaling up the analytical experiment (Figure 9), isolating the various fractions, and determining their protein composition by polyacrylamide gel electrophoresis (Figure 10). The ratio of antibody to nucleosome chosen was that which shifted >90% of the particles from their original sedimentation position.

By visual inspection, all the DNA-containing fractions of the sucrose gradient appear to contain equal amounts of the four smaller histones (Figure 10). This conclusion was examined further by densitometric scanning and determination of the peak areas corresponding to histones H4, H2A, and (H3 + H2B); H3 and H2B are not resolved in the scans. The ratios of content of H2A/H4 and of (H2B + H3)/H4 are shown in Figure 11. To compensate for differences in staining intensity, the data have been normalized so that the average is 1 and 2 for the two distributions, respectively. It is apparent that there

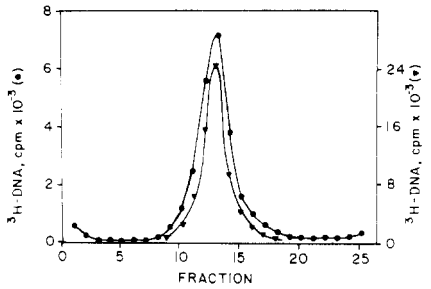

FIGURE 12: Nucleosomes do not bind antibody to hemoglobin. Nucleosomes, 0.4 μg (as DNA), were incubated with 15 μg of anti-Hb A_{oxy} as in the legend to Figure 2 and sedimented on isokinetic sucrose gradients. The direction of sedimentation was, right to left, (▼) +antibody, (●) −antibody.

is essentially no slope to either distribution—that is, no trend for accumulation of one histone relative to another at either end of the sedimentation profile. Rather, the data seem to reflect constant ratios of the contents of these three histone groups across the entire peak, with scatter due to experimental error. The standard deviations of the ratios of (H2B + H3) to H4 and of H2A to H4 are 6 and 8%, respectively.

Since other evidence suggests that nucleosomes contain an octamer of histones (Thomas and Kornberg, 1975), the constancy of ratios of histones observed here provides rather good evidence that the octamer is, indeed, two each of histones H2A, H2B, H3, and H4, for all nucleosomes. We cannot, of course, from the scans rule out the presence of nucleosomes all containing two molecules each of histones H2A and H4 but having varying ratios of H2B and H3 making up the remaining four histone molecules. However, visually, we can detect easily variations of 20% in ratio between purified H2B and H3 (i.e., 0.8 or 1.2:1 vs. 1:1) and the absence of such variations on inspection of the gel makes us feel that even this potential heterogeneity is unlikely.

We can calculate confidence limits for the conclusion that the histone ratios indicate compositional homogeneity for all nucleosomes. Suppose that in the presence of mainly ($H2A_2H2B_2H3_2H4_2$) there was a fraction, X, of nucleosomes with the composition ($H2A_3H2B_3H3H4$). We purposely alter H2A and H2B as one pair and H3 and H4 as another pair on the basis of studies indicating their strong interactions (Kornberg and Thomas, 1974; Roark et al. 1974; D'Anna and Isenberg, 1974). It can be easily shown that the following ratios are obtained for the histones as a function of the fraction, X, of the ($H2A_3H2B_3H3H4$) species:

$$\frac{(H3 + H2B)}{(H4)} = \frac{4}{2 - X}$$

$$\frac{(H2A)}{(H4)} = \frac{X + 2}{2 - X}$$

Such an octamer, richer in H2B, would tend to accumulate near the bottom of the sedimenting peak after interaction with anti-H2B. Since the bottom-most fractions do not differ from the average, and the mean and standard deviations of the two plotted ratios are 2 ± 0.12 and 1 ± 0.08, we can calculate the value of X which would make these ratios exceed the 90% confidence limits of the distribution to be 0.21 or 0.16 for the (H3 + H2B)/H4 and H2A/H4 data, respectively. Thus, the bottom fraction must contain less than 20% of a putative ($H2A_3H2B_3H3H4$) nucleosome, and the proportion of this possible octamer in the total would obviously be much less. Similar considerations apply to a potential octamer containing only one molecule each of H2A and H2B near the top of the sedimentation profile. The magnitude of the changes in ratio accompanying a larger population of a different histone octamer is really quite large—for example, if the bottom of the gradient contained equal ratios of $H2A_3H2B_3H3H4$ and the equimolar octamer, and the top contained equal ratios of the equimolar octamer and ($H2AH2BH3_3H4_3$), the ratio of (H2B + H3)/H4 would vary from 2.67 at the bottom to 1.60 at the top and the ratio of H2A/H4 would vary from 1.67 to 0.60. This level of variance in histone ratios markedly exceeds the variance in the experimental data.

In spite of the apparent constancy in histone content across the sedimentation profile, the amount of IgG bound to each nucleosome varies across the sedimentation profile (Figure 10). It seems likely that the broadening of the peak associated with antibody binding to nucleosomes is attributable to the differing mass of antibody bound to various particles. The molar ratio of antibody to nucleosome, measured by quantitative densitometry of IgG heavy chains using human IgG as a standard, varies from about 1 at the top of the sedimenting peak to over 3 at the bottom (Figure 10). The bulk of the nucleosomes bind about two IgG molecules per particle. We see differing amounts of antibody bound to particle subpopulations and yet an apparently constant content of the antigenic histone H2B in all particles (Figures 10 and 11). The conditions for this experiment were selected as the point where nearly all particles had moved from their original sedimentation position; that is, nearly all particles appeared to have bound at least one IgG. Thus, it is apparent that we have not reached antibody saturation (cf. Figures 8 and 9). Since the antibody population must be heterogeneous, directed against a variety of antigenic determinants, it is not surprising that some particles will have bound more antibody and others less at any given point in the titration, thereby generating a normal distribution. While this seems the simplest explanation, we cannot exclude differential exposure of H2B antigenic determinants in several populations of nucleosomes as the basis for differential binding of anti-H2B.

Studies with an antibody to hemoglobin, a protein absent from nucleosomes, demonstrate that the shift in sedimentation of the 11S particles consequent to binding of anti-H2B is not due to nonspecific binding of antibody. When incubated with nucleosomes at antibody to DNA mass ratios of 50, anti-Hb A_{oxy} did not alter the sedimentation profile of the particles in any way (Figure 12). Hence, in contrast to crude IgG fractions, albumin or whole serum, purified antibodies against determinants not present in the nucleosome do not alter the sedimentation properties of the nucleoprotein particle. We conclude that the shift in sedimentation coefficient observed after interaction of anti-H2B with nucleosomes reflects the specific binding of the anti-histone antibody to its antigenic determinant in the chromosomal particles.

In contrast to the availability of antigenic determinants of H2B in the nucleosome to antibody elicited against pure H2B, the determinants of H2A appear to be shielded when this histone is complexed with its partners and DNA in the nucleosome. Titration of nucleosomes with anti-H2A up to levels of 50 μg of antibody per μg of DNA caused only a slight shift in sedimentation profile of the nucleosomes (data not shown). The conclusion that anti-H2A does not bind to nucleosomes is reinforced by a preparative level experiment for this system analogous to that shown for anti-H2B. Again, all the DNA-containing fractions contain apparently identical complements of the four smaller histones. However, in this case, practically no IgG heavy chains are associated with the nucleosome peak.

Rather, all the IgG is above the nucleosomes in the gradient, sedimenting with a velocity expected for a 7S protein. Previously, it has been reported that antigenic determinants of H2A are the least exposed in chromatin (Goldblatt and Bustin, 1975).

Discussion

A fundamental unresolved question concerning the structure of eukaryotic chromatin and chromosomes is whether all nucleosomes contain identical complements of histones. It is now well established that most of the interphase chromatin is organized into subunits which are roughly spherical in shape, sediment at about 11 S, and are composed of approximately equal masses of proteins and DNA (see Felsenfeld, 1975). These particles have been observed in the electron microscope in preparations obtained from ruptured nuclei (Olins and Olins, 1974), from virus (Griffith, 1975), from sonicated and native chromatin (Senior et al., 1975; Oudet et al., 1975; Langmor and Wooley, 1975), and from reconstituted chromatin (Germond et al., 1975). Studies on isolated nucleosomes have suggested that, on the average, each particle is composed of about 200 base pairs of DNA complexed with 140 000 daltons of protein.

The exact organization of the histones in the nucleosome is not known. Cross-linking of histones in chromatin by a variety of bifunctional reagents demonstrates several types of close histone–histone interactions. Histone complexing and interactions also have been shown by cross-linking of isolated histones (Thomas and Kornberg, 1975; Sperling and Bustin, 1975; Weintraub et al., 1975). In the face of the variety of complexes observed, it is uncertain whether these represent different histone–histone interactions in a single type of chromatin subunit or whether they might arise from the presence in chromatin of several types of subunit histone cores. This concern about the possibility of differing types of nucleosome protein cores is reinforced by electron microscopic observations of formation of a common type of complex by various combinations of histones (Sperling and Bustin, 1975) and a report that the four smaller histones might not always occur in equimolar amounts. This latter observation was made in the micronucleus of *Tetrahymena pyriformis*, an organelle which apparently lacks H1 and H3 but appears to still possess a subunit organization for its chromatin DNA (Gorovsky and Keevert, 1975).

Immunochemical techniques allow identification of a specific histone while it is still complexed to DNA in chromatin. Specific binding of anti-histone sera to chromatin has been observed by us and used to evaluate the exposure of histone antigenic determinants in various types of chromatin preparations (Bustin, 1973; Goldblatt and Bustin, 1975) and to localize at a low resolution level the histones present in metaphase chromosomes (Bustin et al., 1976b). Using antibodies and electron microscopy, it was possible to show that most of the nucleosomes resolvable on a grid contained H2B (Bustin et al., 1976a). Here we have used a novel immunochemical approach to investigate potential heterogeneity in histone makeup of nucleosomes in a more quantitative way.

In developing the necessary methods for studying antibody nucleosome interaction, we had to overcome certain difficulties which might not be noted using more usual methods of assessment of antigen–antibody interactions. Two problems, a nuclease activity in the sera and nonspecific binding of both albumin and unidentified components present in ammonium sulfate prepared IgG fractions, could be overcome by using antibodies purified by affinity chromatography. The third problem, the presence in some affinity chromatographically purified anti-H2B of a protease activity, could be eliminated by treatment of antibody preparations with the serine protease inhibitor phenylmethane-sulfonyl fluoride.

The H2B antibody preparation obtained by affinity chromatography reacted with nucleosome-bound H2B as detected by immunoadsorbance studies. When incubated with nucleosomes, the particle mass was increased consequent to binding of antibody and hence the coated particles migrated on centrifugation as more rapidly sedimenting species. A direct correlation exists between the amount of antibody added to nucleosomes and the proportion of the total DNA which sediments as the heavier material. Essentially all the nucleosomes can be made to sediment more rapidly than the 11S parent particle by addition of sufficient anti-H2B, suggesting that all particles contain this histone. Quantitative analysis of fractions obtained by sedimentation of antibody reacted nucleosomes shows that each fraction contains a full complement of the four smaller histones and that these histones appear to occur in identical ratios in all fractions.

While the histones are found in apparently identical proportions across the sedimenting peak, the amount of anti-H2B bound to the particles does differ. We feel that this likely represents heterogeneity in the antibody population and lack of antibody saturation, but we can not exclude differing arrangements or conformations of H2B in different subpopulations of nucleosomes. The present data allow us to strongly suggest *compositional* homogeneity of the nucleosomes; further study is required to establish *structural* homogeneity of these particles.

In contrast to H2B, histone H2A apparently has its antigenic determinants shielded when it is present in intact nucleosomes. Thus, only a small shift in sedimentation behavior is observed for particles incubated with large excesses of anti-H2A and, when evaluated by gel electrophoresis, essentially no antibody was found bound to nucleosomes after such incubation and sedimentation. Experiments designed to loosen the structure of the nucleosome by heating and/or partial nuclease digestion may allow compositional analysis of the nucleosome with another antibody.

In considering the structure of the entire eukaryotic genome, it should be noted that we have examined only a portion (about 40%) of the DNA of the nucleus in these studies. A portion of the chromatin DNA is degraded during the nuclease digestion. While it now appears that both repressed and transcribed structural genes exist in a subunit conformation (Lacy and Axel, 1975), there remains the possibility that biologically important regions of the chromatin, e.g., DNA or RNA polymerase initiation sites, hormone receptor binding sites, etc., may exist in either a non-nucleosome conformation or alternatively contain nucleosomes of differing histone composition. Heterogeneity in nucleosomes has been suggested by Cohen et al. (1975). The present techniques would not be expected to detect nucleosome heterogeneity at levels of less than 5% of the total. Additionally, HeLa cells contain a full complement of histones. It would be of some interest to study by immunosedimentation the nucleosome composition of chromatin from a tissue that lacked one of the four small histones. Finally, while we demonstrate that each nucleosome is likely to have a similar composition of histones, it is not certain that in each nucleosome the various histones are in the same structure. Subtle differences in the organization of histones, for example, due to histone modification, which may be too minute to be detected by the techniques used here, may, nevertheless, have influences on the biological activity of chromatin.

The methodological approach used here appears novel and in principle should be applicable to other studies of composition and structure of nucleosomes. Binding of antibody to antigenic determinants exposed in the nucleosome increases the particle mass and so its sedimentation coefficient. Thus, particles that bind antibody are separable from those that do not. Nucleosomes that contain binding sites for RNA polymerase, hormone receptors, or specific nonhistone proteins should similarly be separable from the bulk of the genetic material by this technique, facilitating the study of segments of chromatin which differ functionally from the remainder of the genome.

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