

Deposition of Newly Synthesized Histones: New Histones H2A and H2B Do Not Deposit in the Same Nucleosome with New Histones H3 and H4[†]

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ABSTRACT: We have developed procedures to study histone-histone interactions during the deposition of histones in replicating cells. Cells are labeled for 60 min with dense amino acids, and subsequently, the histones within the nucleosomes are cross-linked into an octameric complex with formaldehyde. These complexes are sedimented to equilibrium in density gradients and octamer and diocamer complexes separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With reversal of the cross-link, the distribution of the individual density-labeled histones in the octamer is determined. Newly synthesized H3 and H4 deposit as a tetramer and are associated with old H2A and H2B. Newly synthesized H2A and H2B deposit as a dimer associated with old H2A, H2B, H3, and H4. The significance of these results with respect to the dynamics of histone interactions in the nucleus is discussed. Control experiments are presented to test for artifactual formation of these complexes during preparative procedures. In addition, reconstitution experiments were performed to demonstrate that the composition of these octameric complexes can be determined from their distribution on density gradients.

The mechanism for deposition of chromosomal proteins on newly replicated DNA has been an area of considerable interest. This is because the organization of these proteins on newly replicated chromatin may be responsible for the maintenance of that chromatin in a transcriptionally active or repressed state. The proteins primarily responsible for the packaging of DNA into a condensed state in the nucleus of a cell are the histones H1, H3, H2B, H2A, and H4 [see review by McGhee and Felsenfeld (1980)]. In the primary structure of the chromosome, histones H3, H2B, H2A, and H4 are organized in an octameric structure (two each of the four) upon which two supercoils of DNA [200 base pairs (bp)] are associated. This complex, termed a nucleosome, is repeated along the DNA strand with histone H1, thought to be the protein associated with the DNA bridging one nucleosome to another. There is increasing evidence that this primary structure is different between active and repressed chromatin (McGhee & Felsenfeld, 1980). In active genes, the DNA is in a more open conformation and the histones are distributed in a less precisely periodic structure along the DNA strand. How this organization is maintained during DNA replication for both daughter strands is not known.

One of the more fundamental questions that has been addressed with regard to histone deposition is "Do newly synthesized histones deposit on newly replicated DNA?" The answer to the question is of importance, because it addresses the dynamics of histone-DNA interactions and the role each histone type plays in the transmission of this epigenetic information from parent to daughter cells. Experimental procedures designed to answer these questions, as well as the interpretations of the data, have varied widely. Leffak (Leffak et al., 1977; Leffak, 1983, 1984) has described experiments in which histones were labeled with dense amino acids and subsequently cross-linked into octameric complexes after deposition within the nucleosome (analysis based on histone-histone interactions). His data indicated that newly synthesized dense histones segregate together. Therefore, the

conclusion has been drawn that all new histones deposit as new octamers on newly replicated DNA. A corollary to this conclusion is that a static state exists between the histones and DNA; i.e., once a histone deposits on DNA, it remains associated with that DNA throughout the life of that cell. In contrast, a number of laboratories reported an opposite conclusion based on studies designed to isolate replicative chromatin (Senshu et al., 1978, 1985; Jackson & Chalkley, 1981a,b, 1985; Jackson et al., 1981; Annunziato et al., 1982). In these cases, analyses are based on histone-DNA interactions. For example, Jackson and Chalkley utilized four separate procedures for isolating replicative chromatin from nonreplicative chromatin. By labeling cells with [³H]lysine and subsequently applying the fractionation procedures, it was observed that not all the newly synthesized histones were associated with replicative chromatin. While newly synthesized histones H3 and H4 were selectively associated with replicative chromatin, the majority of new histones H2A and H2B (70%) did not deposit on new DNA. No detectable quantity of H1 was associated with new DNA. These results suggested that both static and dynamic interactions of histone with DNA exist and these interactions were dependent on histone type. On the basis of these latter observations, a hypothesis has been made that new histones H3 and H4 do not deposit selectively in the same nucleosome with new H2A and H2B.

The apparent inconsistencies between the experiments that analyze histone-histone interactions (density-labeled amino acids) and those that analyze histone-DNA interactions led us to redesign experimental procedures to test histone-histone interactions. In this paper, we describe experiments that involve density labeling histones in MSB cells, isolating the nuclei, and cross-linking the histones with formaldehyde into an octameric structure. We have determined the density of the labeled octamer and found it to be of a hybrid density. Reversal of the cross-link and analysis of the histones within these octamers indicate that newly synthesized H3 and H4 deposit as a tetramer associated with old H2A and H2B. New H2A and H2B appear to deposit as dimers in nucleosomes separate and distinct from those containing new H3 and H4. We discuss the relevance of these observations with respect

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to the dynamics of histone-histone and histone-DNA interactions.

MATERIALS AND METHODS

Procedures for Labeling of Cells. MSB cells were grown in medium consisting of 1:1 Dulbecco's MEM-RPMI-1640 (Gibco, Grand Island, NY) supplemented with final 50 mM HEPES¹ and 10% fetal calf serum. These cells grow between 1×10^6 /mL and 1.5×10^6 /mL with a 10-h cell cycle in suspension culture without a CO₂ atmosphere. Cells from 1 L were pelleted and resuspended in 200 mL of Dulbecco's medium (lacking amino acids) and incubated 20 min at 37 °C. This procedure depletes the internal pool of amino acids and at the same time does not affect the DNA replication rate (V. Jackson, unpublished observations). Cells were then pelleted and resuspended into 10 mL of Dulbecco's medium (containing carrier-free ¹⁵N-, ¹³C-, and ²H-labeled amino acids, Merck MB1808) and incubated 37 °C for 15 min. Also included in the 10 mL of Dulbecco's medium (dense labeling medium) were the amino acids tryptophan, cysteine, hydroxyproline, glutamine, and asparagine as these are not present in the mixture of dense amino acids. After the 15-min incubation, 0.5 mCi each of [³H]lysine (70 Ci/mmol, Amersham) and [³H]arginine (50 Ci/mmol, Amersham) was added and incubation continued for 45 min. Cells were then sedimented, collected by centrifugation, and prepared as described below.

To label cells for three generations in dense labeling medium, MSB cells were suspended at a density of 0.5×10^6 /mL in the dense labeling medium, which included 10 mg of dense amino acids—500 µCi each of [³H]lysine and [³H]arginine per 10 mL of labeling medium. After incubation for 30 h, cells were harvested and frozen for later isolation of nuclei.

Preparation of Chromatin and Density Gradient Centrifugation. Nuclei were isolated by washing 3 times in 1% Triton X-100, 0.25 M sucrose, 10 mM MgCl₂, and 10 mM Tris, pH 8.0, and then washed twice in 10 mM MgCl₂ and 10 mM TEA, pH 7.5. Ten percent of the nuclei was set aside for acid extraction to isolate monomer histones (density markers). The nuclei solution was adjusted to a final concentration of 0.4 N H₂SO₄ and sonicated with a Branson sonifier for 1 min. The sample was centrifuged at 27000g for 10 min and the supernatant, containing the histones, precipitated in 7 volumes of acetone at -20 °C overnight. The precipitate was dissolved in water to be added to the density gradient. The remaining 90% of the nuclei was suspended into 12 mM TEA and 12 mM PO₄³⁻, pH 9.1 (TEA/PO₄), at 100 µg/mL DNA concentration and made up to 1% in formaldehyde. After incubation for 8 h at 4 °C, the chromatin was centrifuged; the pellet made to a final concentration of 0.4 N H₂SO₄ from a 4 N stock and immediately sonicated twice by a Branson sonifier at setting 4 for 30 s each at 4 °C. The solution was centrifuged at 20000g for 10 min, and the supernatant, containing the octamer, was dialyzed against two changes of 0.4 N H₂SO₄ and one change of 20 mM H₂SO₄ over a 36-h period at 4 °C. The treatment with acid prevented further cross-linking, and the extensive dialysis removed the unreacted formaldehyde (Jackson, 1978). This solution was then combined with the previously acid-extracted monomer histones and added to 2.4

g of guanidine hydrochloride, 0.82 g of cesium formate, and 100 µL of 3 M Tris, pH 8.0, and volume adjusted to 4.0 mL. Centrifugation was performed at 54000g for 96 h at 11 °C in an SW60 Ti rotor. Fractions of 150 µL were collected, adjusted to a final concentration of 0.4 N H₂SO₄, and directly dialyzed against 0.4 N H₂SO₄ and 5 mM 2-mercaptoethanol on a BRL (Bethesda Research Laboratories) 28-slot dialysis apparatus. Samples were then precipitated in 7 volumes of acetone at -20 °C overnight and redissolved by sonication into 0.5% SDS, 10% glycerol, and 100 mM Tris, pH 6.8.

Electrophoresis of Proteins and Reversal of Formaldehyde Cross-Link. The electrophoresis conditions were a modification of the Laemmli (1970) procedure. The electrophoresis buffer was 0.1% SDS, 25 mM Tris, and 200 mM glycine, pH 8.3, and the separating gel was 18% acrylamide, 0.09% methylenebis(acrylamide), 0.1% SDS, and 0.75 M Tris, pH 8.8. Electrophoresis was at 150 V for 18 h at 4 °C after which the gels were stained with 0.1% Coomassie, 40% methanol, and 10% acetic acid for 12 h and destained in the same (without stain). Gels were scanned with a modified Gilford densitometer and fluorographed by the procedure of Laskey and Mills (1975). When reversal of cross-link was required for the proteins in the gel, the stained gel was cut into a strip containing the octamer region and incubated in two changes of 100 mL each of 1% SDS and 100 mM Tris, pH 6.8 (reversal solution). The strip was then heated at 100 °C for 30 min in fresh reversal solution, containing 0.5 M 2-mercaptoethanol. The strip was then reincubated for 60 min in three changes of reversal solution to remove mercaptoethanol followed by a 30-min incubation in 20% methanol, 1% SDS, and 100 mM Tris, pH 6.8, to decrease the size of the swollen gel. This gel strip was directly polymerized into a stacking gel consisting of 2.5% acrylamide, 0.13% methylenebis(acrylamide), 125 mM Tris, pH 6.8, and 0.1% SDS. The separating gel was the same as previously described except the acrylamide to bis(acrylamide) ratio was changed from 200:1 to 100:1.

RESULTS

Methods for Analysis of the Composition of Histones in Newly Synthesized Nucleosomes. In order to study histone-histone interactions, it was necessary to develop procedures to isolate covalently cross-linked octamers. The cross-linking agent, formaldehyde, has been chosen because it requires close proximity between proteins to effect a cross-link and also because of the high yield of histone octamer formed. Figure 1 illustrates a time course in which nuclei were cross-linked with 1% formaldehyde at pH 9.0. It is emphasized that this cross-linking was done at pH 9 and not pH 7, as the latter condition does not result in cross-linking to the octamer level but rather only to the dimer and trimer levels. Figure 1A shows the acid-soluble complexes formed. Figure 1B shows those histones that were acid insoluble or, in other words, those histones that were cross-linked to the DNA as a result of the formaldehyde treatment. In order to observe these latter cross-linked histones, however, it was necessary to reverse the cross-link between the histones and DNA. This was accomplished by solubilizing the acid-insoluble material in 1% SDS and 100 mM Tris, pH 8, and incubating at 37 °C for 2 days. The samples were then directly electrophoresed on 18% polyacrylamide-SDS gels. This reversal procedure results in a very limited reversal of protein-protein cross-links (Jackson, 1978). The streaking effect on the gel in Figure 1B is due to the presence of free DNA in the samples.

The kinetics of cross-linking of histones to each other and/or to DNA is a pH-dependent process. At pH 7, the N-terminal tails of the histones are cross-linked efficiently to the DNA,

¹ Abbreviations: MSB, chicken leukemic cell line transformed by Marek's virus; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; MgCl₂, magnesium chloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethanolamine; PO₄, phosphate; H₂SO₄, sulfuric acid; Gdn-HCl, guanidine hydrochloride; CsFo, cesium formate; SDS, sodium dodecyl sulfate; NaCl, sodium chloride; PAGE, polyacrylamide gel electrophoresis.

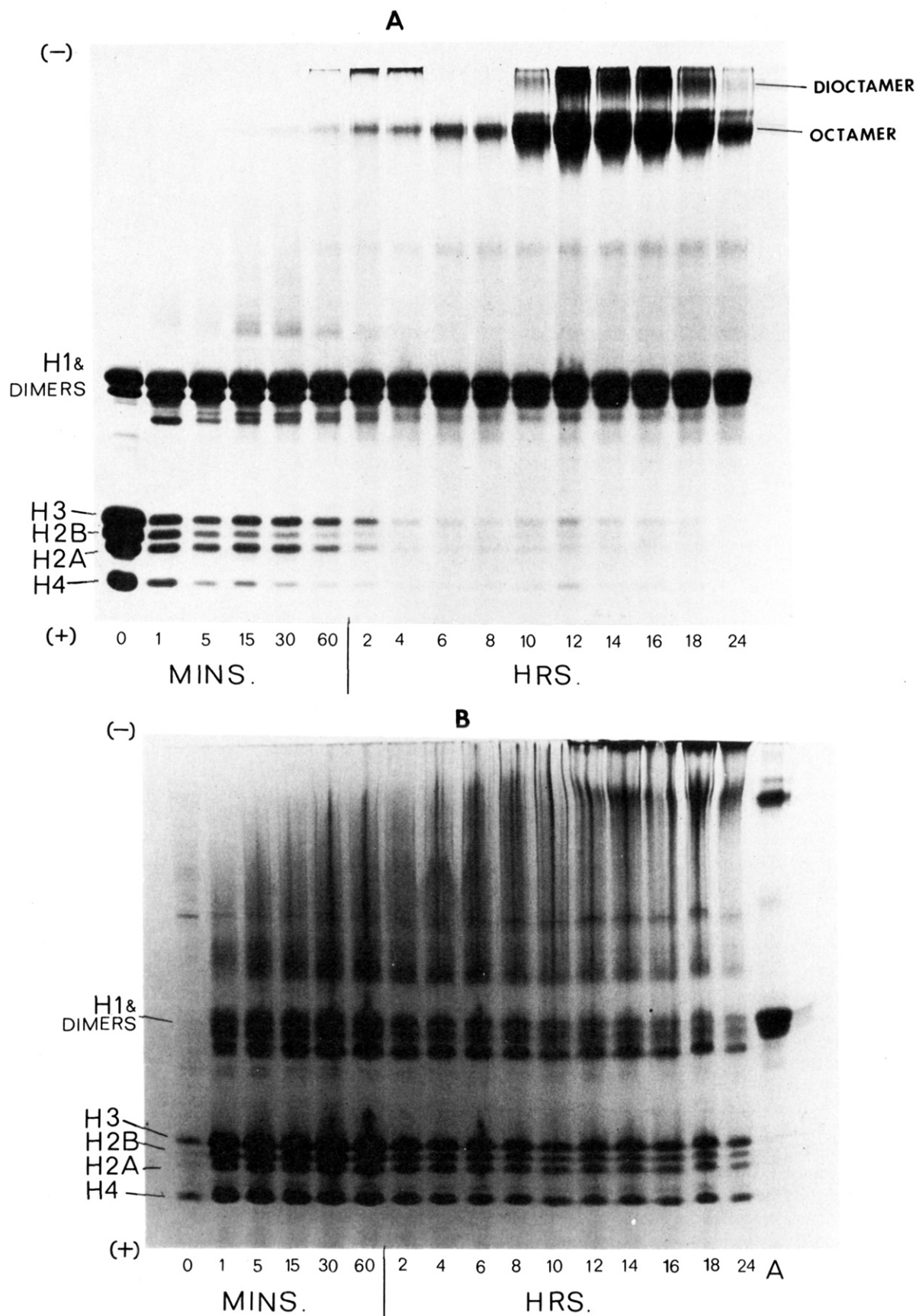


FIGURE 1: Time course analysis of the oligomeric products produced by formaldehyde treatment of nuclei at pH 9. Nuclei at 2 mg/mL of DNA were treated with 1% formaldehyde in 100 mM borate, pH 9, at 4 °C. At each time point fractions were made to a final concentration of 0.4 N H₂SO₄, sonicated, and centrifuged at 20000g for 10 min. (A) The supernatant was dialyzed against 0.4 N H₂SO₄ to remove excess formaldehyde followed by dialysis against 20 mM HCl. Samples were made 0.5% SDS and 100 mM Tris, pH 6.8, and electrophoresed on an 18% polyacrylamide-SDS gel. (B) The pellet of acid-insoluble material was suspended by sonication in 1% SDS and 100 mM Tris, pH 8.0, and incubated at 37 °C for 2 days. Samples were directly electrophoresed on an 18% polyacrylamide-SDS gel. The sample marked A in (B) is the 8-h sample of (A) reelectrophoresed as a marker for this gel.

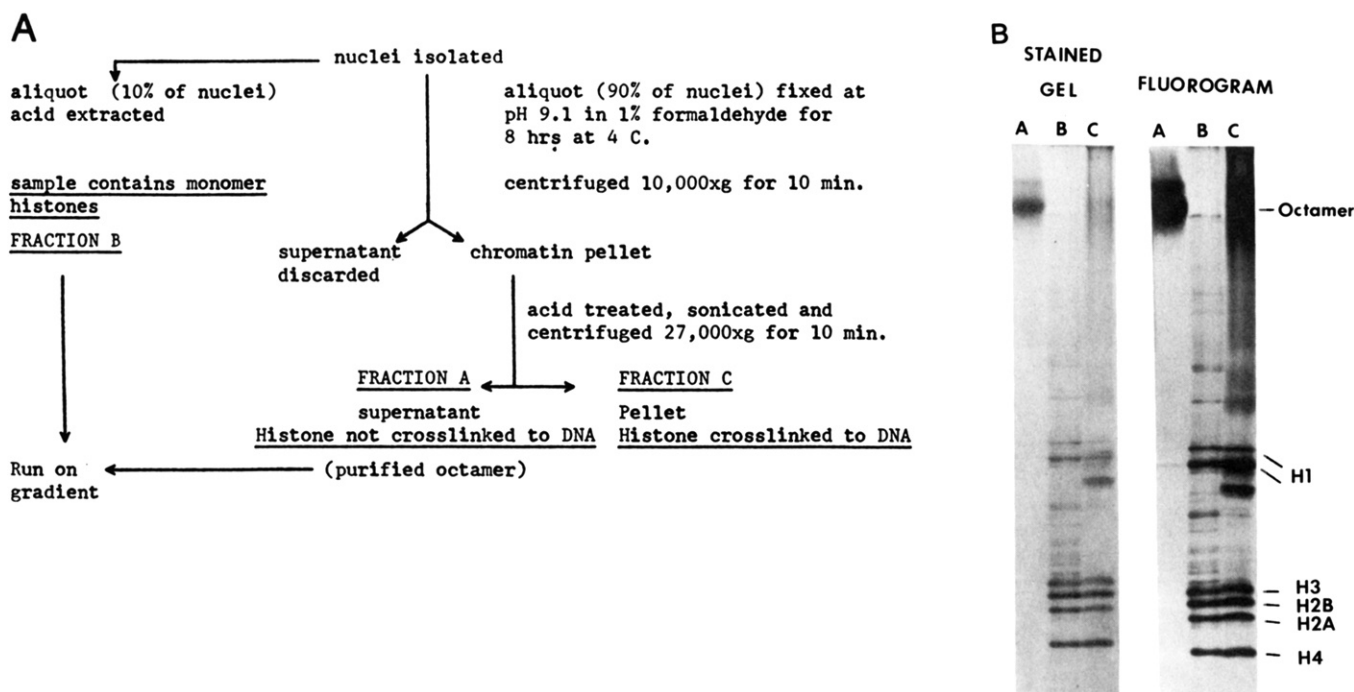


FIGURE 2: (A) Protocol for analysis of the distribution of new histone in newly synthesized nucleosomes. The MSB cells were labeled with ^{15}N -, ^{13}C -, and ^2H -labeled amino acids and [^3H]arginine and [^3H]lysine. Fractions A and B were mixed and placed on CsFo-Gnd-HCl gradients at 53 000 rpm for 96 h at 11 °C. The tubes were fractionated, and the samples were dialyzed and electrophoresed (see Figure 3). (B) The histone complexes present in fractions A–C were analyzed by electrophoresis in an 18% polyacrylamide–SDS gel. The stained gel is fluorographed to illustrate the distribution of radiolabeled complexes. This gel indicates what is in these fractions before centrifugation to equilibrium.

presumably through the major groove. Cross-linking to DNA in the core regions of the histones also occurs (Jackson, 1978). At this same pH, the core regions of the histones cross-link in the nucleosome to form dimers and trimers, but no higher oligomers (Jackson, 1978) are formed. In order for higher oligomers to form, the N-terminal tails must cross-link to each other. This happens at pH 9 where the interaction of the N-terminal tails with the DNA is weakened. There is less cross-linking to DNA and greater cross-linking to other N-terminal tails. The involvement of N-terminal tails in this process is suggested by the result that trypsin digestion of the octamer produces histone cores cross-linked as dimers and trimers lacking the N-terminal tails (unpublished information). Therefore, approximately 50% of the histones form octameric complexes (Figure 1A) and the remainder are cross-linked to DNA at various stages of oligomeric formation (Figure 1B). A further illustration of this effect is the observation that a large percentage (~90%) of histone H1 is released from the DNA during formaldehyde treatment at pH 9 (see Figure 7). Although H1 is not a part of the octameric complex within the nucleosome (McGhee & Felsenfeld, 1980; see also Figure 5B), the release of H1 from DNA due to formaldehyde treatment is of great concern, as it suggests that the core histones within the nucleosome may slide or be released from the DNA, providing uninterpretable information. Data are shown later which demonstrate that the histones of the octamer are not released from DNA by formaldehyde treatment (vide infra).

The protocol for investigating the deposition of new and old histones in nucleosomes is illustrated in Figure 2. MSB cells were labeled 60 min in medium containing dense amino acids and tritiated arginine and lysine. Nuclei were isolated, and histones (fraction B) were directly acid extracted (10% of nuclei). The remaining nuclei (90%) were treated with formaldehyde at pH 9 for 8 h at 4 °C. The chromatin was sedimented at 10000g for 10 min, and the supernatant was discarded (containing excess formaldehyde and released histone

H1). The chromatin pellet was directly acid extracted and the acid-soluble protein (histones) dialyzed exhaustively against 0.4 N H_2SO_4 . The treatment with acid is necessary to remove non-histone protein (acid insoluble). Without this step, a considerable ambiguity can occur as to what is or is not an octamer on the subsequent density gradients (Jackson, 1987). As shown in Figure 2B, this fraction, denoted as fraction A, consists only of the cross-linked octamer product. Fraction C represents the cross-linked protein products that were cross-linked to the DNA (acid-insoluble pellet) and were freed from the DNA by the reversal procedure described for Figure 1B. In the procedure of Figure 2, fraction B (monomer histones) and fraction A (octamer) were combined and placed on a CsFo-Gdn-HCl gradient for centrifugation to equilibrium. The monomer histones are included as density markers to establish the normal density region (unlabeled histones) and the dense region (radiolabeled histones) of the gradient. Fractions were taken, dialyzed to remove the salts, and electrophoresed on 18% polyacrylamide–SDS gels. These results are shown in Figure 3. As shown in the fluorogram, the radiolabeled, density-labeled proteins primarily distribute between fractions 6 and 8 (HD). The peak of the stained monomer histones is at fraction 16, as is also true for the stained octamer, and indicates the location in the gradient for normal density histones (ND). The distribution of the radiolabeled, density-labeled octamer is between normal density and fully dense proteins (fractions 11–14). As shown in Figure 4, microdensitometric analysis of these data indicates that these octamers have a density that is less than half the density of the density-labeled monomer histones. Such a distribution indicates that the newly synthesized histones have formed hybrid octamers with old, normal density histones.

Although the data of Figure 4 indicate that hybrid octamers were formed, they do not indicate which histone types are old, normal density histones and which are new, high-density histones. However, procedures have been developed to reverse the formaldehyde cross-link within the octamer so that this

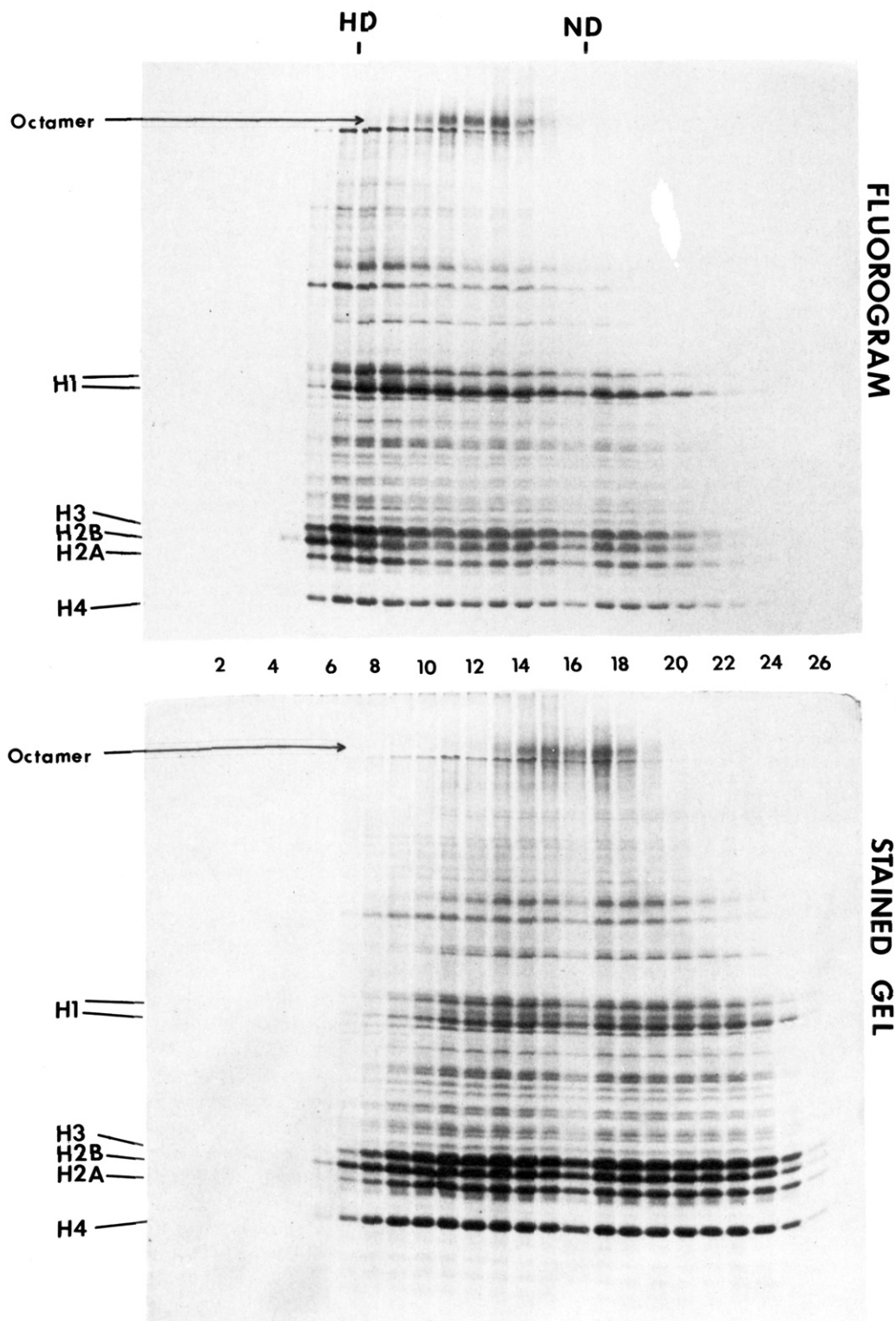


FIGURE 3: Density gradient analysis of the density-labeled octamer produced during a 60-min pulse with radiolabeled and density-labeled amino acids. Octamer (fraction A) and monomer histones (fraction B) were combined and sedimented to equilibrium in a CsFo-Gdn-HCl density gradient. Fractions were collected, dialyzed to remove salts, and electrophoresed on SDS gels. HD (high density) indicates the midpoint position of density-labeled monomer histones. ND (normal density) indicates the position of normal density histones. Note that the focusing of proteins in these gradients is directly proportional to molecular weight. Therefore, monomer histones are more diffuse throughout the gradient (fractions 8–22) as compared to the cross-linked octamer (fractions 13–18). This more diffuse Gaussian distribution does not mean that equilibrium has not been established. Centrifugation at 53 000 rpm for 4 days is more than sufficient to establish equilibrium. Note also that the density-labeled monomer histones (fractions 5–10) are focused more sharply than the monomer normal density histones (fractions 7–24). This is due to the greater centrifugal force in the lower region of the gradient relative to the center of the gradient in the SW60 rotor. Note that fraction 16 is depleted in over all protein. This depletion serves as a marker for the orientation of the stained gel with the fluorograms.

determination can be made. Shown in Figure 5A is a stained gel of a CsFo-Gdn-HCl gradient similar to the one described in Figure 3. In this case, however, before the gel was fluorographed, the octamer region of the gel containing fractions

1–26 (area between horizontal dotted lines) was sliced out. (It should be noted in the fluorogram shown in Figure 5A that this region of the gel is missing.) This gel strip was heated at 100 °C for 30 min in 1% SDS, 100 mM Tris, pH 6.8, and

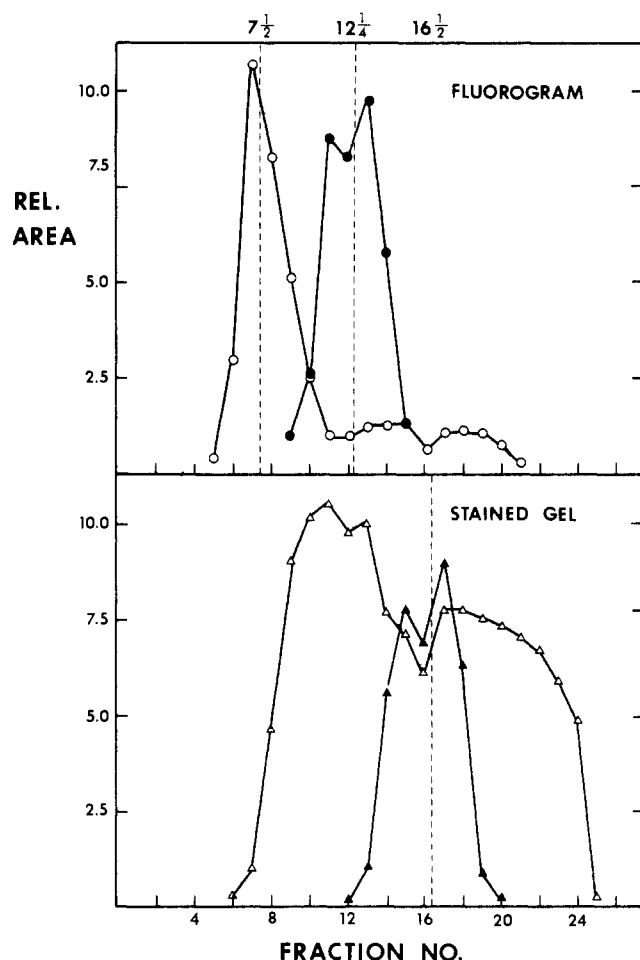


FIGURE 4: Microdensitometric analysis of the stained gel and fluorogram of Figure 3: (Δ) relative area of monomer histones in stained gel; (\blacktriangle) relative area of octamer in stained gel; (\circ) relative area of radiolabeled, density-labeled monomer histones; (\bullet) relative area of radiolabeled, density-labeled octamer. The relative area was calculated by determining the lowest significant integrated value and dividing all other values in the set by it. In the case of the monomer histones, the area from all four histones was combined as a single value. This was done even though the individual densities of the monomer histones are different. This point can be most clearly seen in the fluorogram of Figure 3 where H2B is seen to have a one-fraction greater density than H3, H2A, or H4. Since the octamer is composed equally of all four histones, (two each of H3, H2B, H2A, and H4), the total area of these histones is used to give a single average value for each fraction number. The positions of the vertical dotted lines were determined from the total area under each curve. The average value for the fraction number was then determined and taken to indicate the average density position for the proteins in that curve. These density positions are indicated as fraction numbers.

0.5 M 2-mercaptoethanol. This procedure destroys the formaldehyde cross-link leaving an intact, chemically unmodified protein (Jackson, 1978). The strip was then polymerized into an 18% polyacrylamide-SDS gel and electrophoresed in the same dimension to determine which radiolabeled, density-labeled histones were present in the hybrid octamers of each fraction of the gradient. These results are shown in Figure 5B. The stained gel of this figure shows that the four histones of the normal density octamer are present in equimolar concentrations in each fraction. In contrast, newly synthesized H3 and H4 are distributed in fractions of the gradient where newly synthesized H2A and H2B are noticeably deficient. Likewise, newly synthesized H2A and H2B are distributed in fractions where newly synthesized H3 and H4 are deficient. Figure 6 shows the densitometric analysis of these data. Two separate Gaussian curves are observed for the two sets of

histones. The midpoint for the curve of new H3 and H4 is fraction 13.5 and for new H2A and H2B is fraction 15. The interpretation of these data is that new H3 and H4 are found in octamers that contain a larger percentage of density-labeled histones than are new H2A and H2B. As this density gradient has been calibrated for density on the basis of the distribution of labeled and unlabeled monomer histones (Figure 5A), the percentage density shift can be calculated. The density shift for new H3 and H4 is 45% and for new H2A and H2B is 24% (Figure 6). The interpretation for these observations is that a tetramer of new H3 and H4 is deposited with two each of old H2A and H2B within a nucleosome and a dimer of new H2A and H2B deposited with one each of old H2A and H2B and two each of old H3 and H4. Deposition of new H3 and H4 and new H2A and H2B in the same nucleosome would appear to be very limited. This conclusion is based on the lack of any shoulder on the dense side of the Gaussian distribution of the labeled H2A and H2B. Such a shoulder would have suggested that one each of new H3, H4, H2A, and H2B was in the same octameric complex. The intermingling of new histones with old histones to form nucleosomes suggests that a dynamic state of histone-histone interactions exists within the nucleus. These points will be more extensively addressed under Discussion.

Analysis for Histone Rearrangement Caused by Formaldehyde Treatment. The hybrid octamers described in the previous figures could have been produced as a result of the movement of histones H2A and H2B due to formaldehyde treatment. We have chosen two experimental procedures to test for this possibility.

In the first instance, we have assayed for the release of histone by treatment of nuclei with formaldehyde followed by immediate sedimentation on 5–20% sucrose-formaldehyde, pH 9, gradients. As shown in Figure 7A, histone H1 is released into the supernatant whereas the core histones are not. The core histones (H3, H2B, H2A, and H4) remain in the pellet associated with DNA. To determine whether formaldehyde modification of lysine groups on these histones might loosen the structure sufficiently to produce sliding, the following experiment was performed. Nuclei at pH 9 were treated with NaCl to a final concentration of 0.5 M and made 1% formaldehyde. This sample was layered on a 5–20% sucrose gradient, containing 0.5 M NaCl and 1% formaldehyde, pH 9, and immediately centrifuged as before. As shown in Figure 7B, the presence of the formaldehyde at this ionic strength has no greater effect on releasing histones H2A and H2B than in the absence of salt (Figure 7A). Thus the electrostatic and hydrophobic interactions that exist between the core histones and DNA are not significantly affected by the presence of formaldehyde. We have also included an analysis of the salt-dependent release of histones from DNA in the absence of formaldehyde in order to demonstrate the sensitivity of this assay. In Figure 7C, a sucrose gradient is shown in which the nuclei were treated with 0.7 M NaCl prior to sedimentation through sucrose containing 0.7 M NaCl. The pellet fraction of Figure 7C is significantly depleted of H2A and H2B. These histones are found on the top of the gradient. This depletion is seen even more significantly when the NaCl concentration is increased to 1.0 M (Figure 7D). For the formaldehyde treatment used in the previous studies to artifactually produce the hybrid octamers, the data of Figure 7A should have resembled the data of Figure 7D. This is clearly not the case. These experiments also demonstrate that a small shift of salt concentration from 0.5 M to 0.7 M NaCl (Figure 7C) results in a small, but significant, release of H2A and

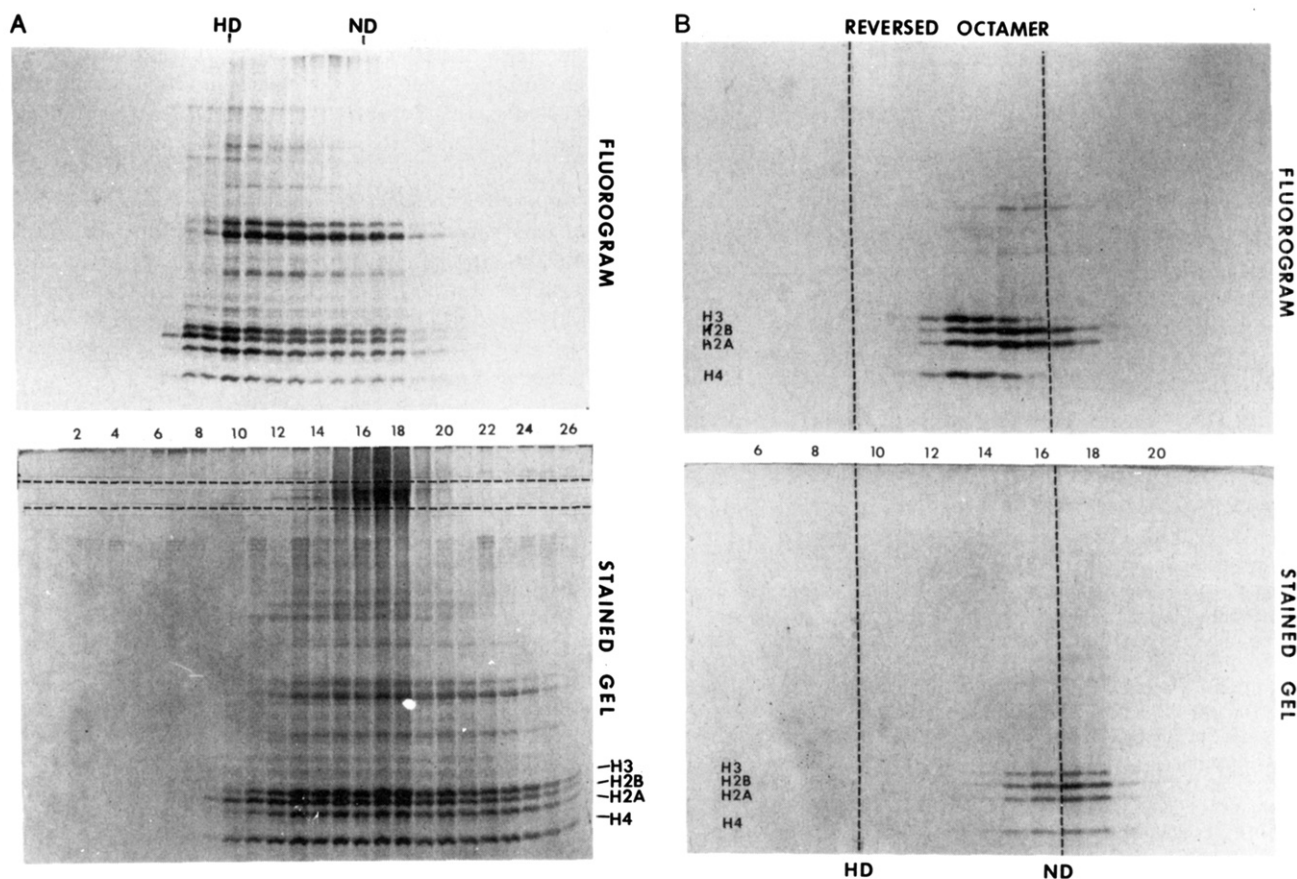


FIGURE 5: Analysis of the newly synthesized and old histone composition of the hybrid octamers. (A) An experiment similar to that described in Figure 3 was done. In this instance, the gel slice of the stained gel, as indicated by the horizontal dotted line, was cut away and the formaldehyde cross-link reversed as described in (B). The remaining gel was fluorographed to determine the distribution of density-labeled monomer histones. Since this density position can vary for each labeling experiment due to variation in the specific activity of density label in the cell, it is necessary to establish this fully dense position on each gradient with monomer histones from the same cells used to produce the cross-linked octamer. In this instance, fraction 9.5 represents the fully dense position and was the value used for the analysis in (B) and the microdensitometric analysis of Figure 6. (B) The gel strip of (A) was heated at 100 °C for 30 min in the presence of 1% SDS, 0.5 M mercaptoethanol, and 100 mM Tris, pH 6.8, and reelectrophoresed in the same dimension on 18% polyacrylamide-SDS gels. The vertical dotted line HD indicates the fraction where density-labeled histones distribute and was obtained from the fluorogram of (A). The vertical dotted line ND was taken directly from the stained gel of (B).

H2B. The presence of formaldehyde in 0.5 M NaCl (Figure 7B) does not mimic this 0.2 M increase in NaCl concentration. Thus, in the absence of salt, which is the condition for the cross-linking in the density experiments, any change in the electrostatic environment due to the formaldehyde treatment cannot be greater than if these nuclei were suspended in 0.2 M NaCl, or approximately physiological ionic strength.

In the second procedure to test for histone release within the nucleosome due to formaldehyde treatment, mixing experiments were done. MSB cells were labeled for three generations in dense amino acids. Nuclei from these cells were isolated and mixed at a ratio of 1:10 with nondense, unlabeled nuclei. This mixing was done before the formaldehyde treatment (mix before fix) or after the formaldehyde treatment (mix after fix). This latter mixing condition serves as an important control for these studies, as it establishes that when cross-linked octamers are produced from nuclei containing a uniform population of density labeled histones (three generations of labeling), these octamers maintain the same density as the monomer histones from which they were derived. As shown in the fluorogram of Figure 8 (mix after fix), the octamer does indeed maintain the same density as the original monomer histones. The mix before fix experiment is designed to test whether the hybrid octamers observed in the experiments of Figures 2–5 are produced by the formaldehyde treatment. However, in this latter experiment, it is important to additionally demonstrate that all four radiolabeled, den-

sity-labeled histones are equally distributed in each fraction. Therefore, in this experiment after the fractions from the density gradient (mix before fix) are electrophoresed on an 18% polyacrylamide-SDS gel, the octamer region was sliced out with procedures previously shown (see Figure 5). The cross-link was reversed, and the gel slice was reelectrophoresed in the same dimension. As shown in Figure 8, histones H2A and H2B are distributed in the fully dense position (fraction 8) as are H3 and H4. There is no indication of intermixing of density-labeled and normal density histone within the same octamer. If this intermixing had occurred, there would be not only a decrease in density of the labeled octamer but also a potential shift in distribution of labeled H2A and H2B as compared to H3 and H4. This latter shift would be exhibited under circumstances where H2A and H2B were exchangeable within a nucleosome during formaldehyde treatment. These data indicate that formaldehyde treatment does not cause the exchange of the core histones within the nucleosome.

From the hybrid density observed for H2A and H2B in the data of Figures 5 and 6, the conclusion has been made that these octamers consist of two dense-labeled and six nondense histones, or 25% dense histones per octamer. To test whether it is valid to assume that a density shift of 25% on these gradients is attributable to two of the histones being new H2A and H2B, we have performed reconstitution experiments. One part dense nuclei was mixed with 5 parts of nondense nuclei and adjusted to 2.0 M NaCl. The sample was dialyzed 18

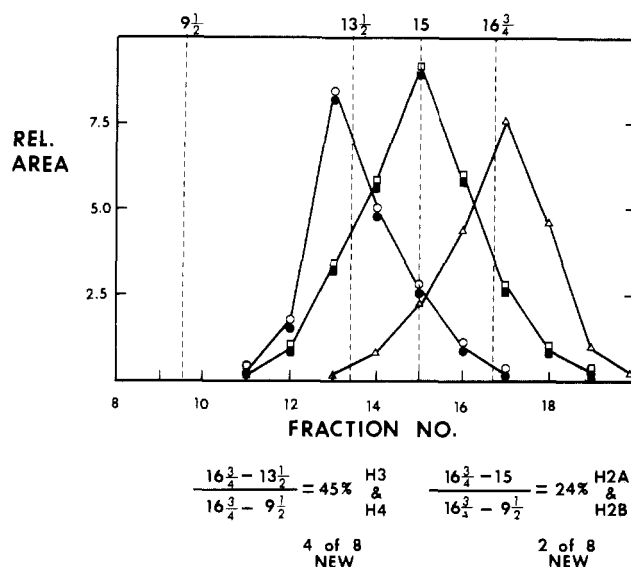


FIGURE 6: Microdensitometric analysis of the fluorogram and stained gel of Figure 5B: (Δ) relative area of the four monomer histones in the stained gel; (\blacksquare) relative area of H2A in the fluorogram; (\square) relative area of H2B in the fluorogram; (\circ) relative area of H3 in the fluorogram; (\bullet) relative area of H4 in the fluorogram. The total area under the curve was determined, and the dotted line indicates the average value for the density of the proteins in that curve. These density positions are indicated as fraction numbers. Percent density shift for the histones was calculated from these values and indicates that the hybrid octamers containing new H3 and H4 consist of 45% dense histones and the hybrid octamers containing new H2A and H2B consist of 24% dense histone.

h at 4 °C against the same salt concentration followed by stepwise dialysis to low ionic strength (Tatchell & Van Holde, 1977). This procedure results in dissociation of the octameric complex of histones within nucleosomes (Godfrey et al., 1980) such that upon reconstitution onto DNA the density-labeled histones are distributed 1 part in 5 parts in the nucleosomes. The sample was then treated with formaldehyde; the octamer was isolated and sedimented to equilibrium on the density gradients with the added monomer histones as controls. The octamer region of the subsequent SDS gel was removed, the cross-link was reversed, and the proteins in the slice were reelectrophoresed. As shown in Figure 8 (reconstitution), the density shift observed is $(16.5 - 15)/(16.5 - 8) = 17.6\%$. On a theoretical basis, a 1:5 mix is expected to produce a 16.7% density shift. From the similarity in these values, it is concluded that the number of density-labeled histones in these octamers is directly proportional to the distance (measured in fractions) between normal density histones and the fully dense ones.

DISCUSSION

Procedures have been described to study the deposition of histones into the newly synthesized nucleosome. These procedures involve cross-linking the histones to each other within the nucleosome to produce an octameric complex of protein. By radiolabeling and density labeling of the newly synthesized histones in MSB cells, the density of the octameric complexes containing these histones was found to be of a hybrid density. It has been shown that this hybrid density is a result of newly synthesized histones being associated with old histones in the same nucleosome. We have described two experimental procedures to test for artifactual formation of these hybrid densities and found that our experimental protocols do not produce them.

These observations are in conflict with earlier data (Leffak et al., 1977; Leffak, 1983, 1984), which indicate that the

density of such octameric complexes was reflective of a homogeneous population of all newly synthesized histones. The basic difference between the earlier studies and the studies described in this paper is the reagent used to produce the covalent cross-link. In previous work, Lomant's reagent was used whereas formaldehyde was used in these studies. We have addressed these differences in a separate paper and have been able to resolve the source of the discrepancy. There is significant ambiguity as to what is or is not an octameric structure of histones, due to the presence of non-histone proteins. These proteins can be mistakenly interpreted to be cross-linked histone complexes when Lomant's reagent is used as the cross-linking agent (Jackson, 1987).

We have described procedures to reverse the formaldehyde cross-link that were used to produce the octameric complexes and with these procedures have been able to characterize the newly synthesized histones in the octamer. Two separate classes of newly synthesized octamer appear to occur. One class consists of a tetramer of new H3 and H4 associated with two each of old H2A and H2B. The other class contains one dimer of new H2A and H2B, associated with six old histones (one each of H2A and H2B and two each of H3 and H4). These conclusions are based on the observation that new H3 and H4 are in hybrid octamers exhibiting a density consistent with four of eight histones being newly synthesized. In nucleosomes containing new H2A and H2B, the density is consistent with a content of two dense histones of the eight histones. Since the density in the latter case is reflective of a two of eight organization, it is also conceivable that homodimers of new H2A and homodimers of new H2B could deposit in separate nucleosomes. Since heterodimers of these two histones have been observed in a number of *in vitro* studies (Isenberg, 1979), the data obtained in this study have been interpreted on this basis.

Previous published data have indicated that the individual, newly synthesized histones do not deposit in an equivalent manner at the replication fork (Jackson & Chalkley, 1981a,b, 1985; Jackson et al., 1981). All of the newly synthesized H3 and H4 and 30% of new H2A and H2B were found to deposit specifically at the replication fork. The remaining 70% of new H2A and H2B deposited at sites other than at the replication fork. Preliminary evidence suggested that the site of deposition was a discrete location, and a model was proposed that active histone exchange occurred for H2A and H2B on genes during transcription. A corollary to this model was that old H2A and H2B were exchanging with new H2A and H2B during this process and that these old histones were in turn depositing at the replication fork in place of the new ones. The presence of these additional old H2A and H2B at the replication fork, from a source other than the parental nucleosomes which are being replicated, is very important in understanding the data described in this paper. If one were to assume that the only source of old H2A and H2B was dissociation of prereplicative nucleosomes during advancement of the replication fork, then reassociation of hybrid octameric complexes between new and old histones would produce a hybrid octamer of 50% new histones. Whereas such an octamer is observed for new H3 and H4, it is not observed for new H2A and H2B. Also, a significant and detectable percentage of new H3 and H4 as a tetramer (approximately 25%) is expected to be associated with a dimer of new H2A and H2B and therefore contain a hybrid density of 75%. This is clearly not seen. By assuming an additional source of old H2A and H2B to dilute the pool of new H2A and H2B at the replication fork, then a tetramer of new H3 and H4 would be expected to associate randomly

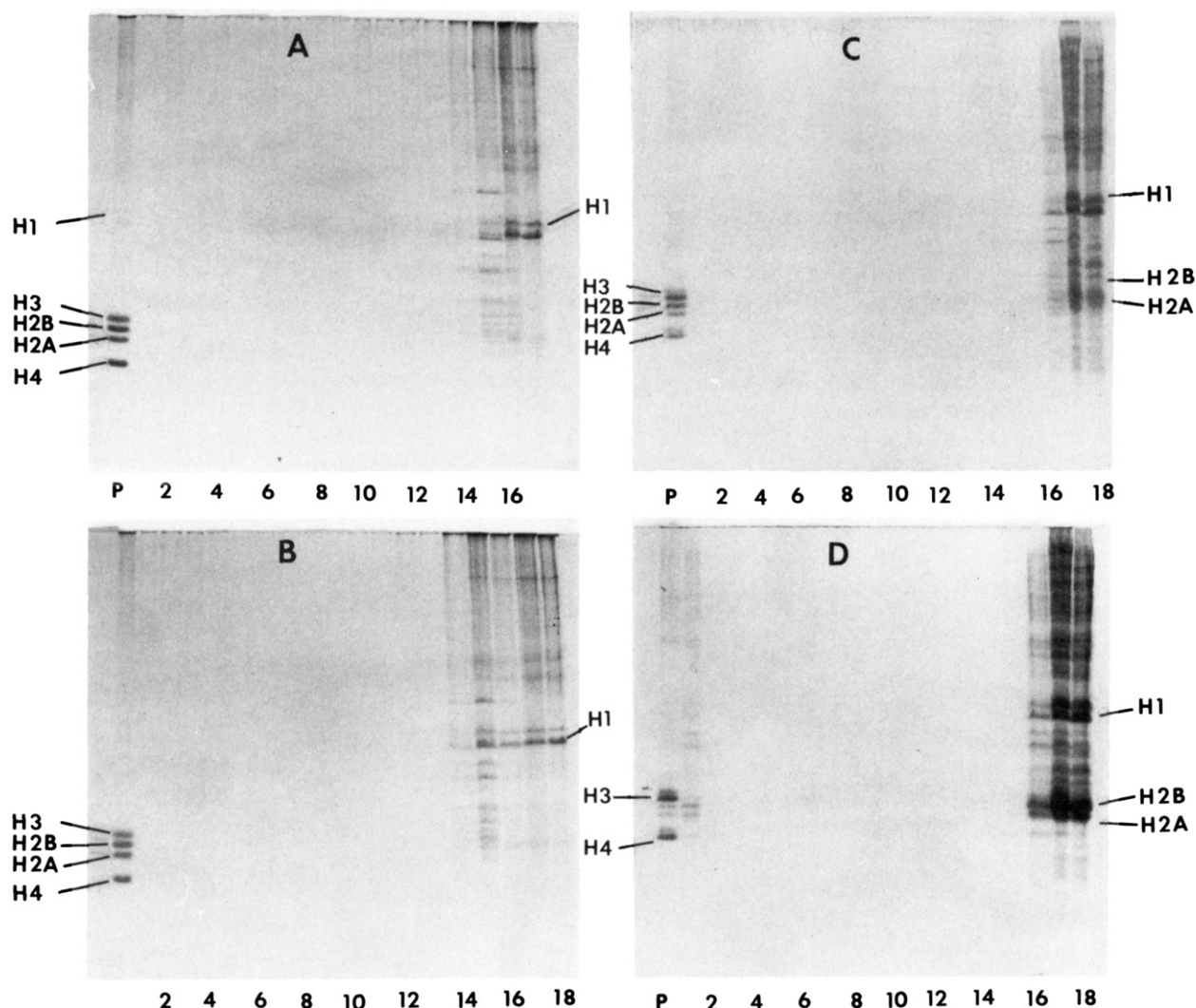


FIGURE 7: Sucrose gradient analysis of histones released as a result of formaldehyde treatment. (A) Cells were labeled for 30 min with [3 H]lysine and [3 H]arginine and nuclei isolated as described under Materials and Methods. Nuclei at 1 mg/mL were treated with 1% formaldehyde in TEA- PO_4 , pH 9.0, and immediately sedimented through a 5–20% sucrose–1% formaldehyde and TEA- PO_4 , pH 9, gradient at 40000 rpm for a total of 15 min. Fractions were made 4 M Gdn-HCl and 0.5 M mercaptoethanol and heated at 100 °C for 30 min. This procedure destroys all formaldehyde and any cross-links (Jackson, 1978). The samples were then dialyzed against 0.4 N H_2SO_4 followed by precipitation in 7 volumes of acetone. The precipitates were sonicated into loading buffer and electrophoresed on 18% PAGE-SDS gels. (B) Same as (A) except that the nuclei were pretreated with 0.5 M NaCl prior to formaldehyde treatment and immediately sedimented on a sucrose gradient containing 0.5 M NaCl. (C) Nuclei were made 0.7 M NaCl and sedimented through a sucrose gradient containing 0.7 M NaCl. In this instance, no formaldehyde treatment was done. Therefore, the fractions were directly dialyzed against 0.4 N H_2SO_4 and precipitated in acetone. (D) Same as (C) except nuclei were made 1.0 M NaCl and sedimented through a sucrose gradient containing 1.0 M NaCl. In all four gradients, P refers to the pellet of chromatin. For the pellets, after dialysis against 0.4 N H_2SO_4 , it was necessary to sonicate and remove the DNA by centrifugation at 20000g for 10 min. The supernatant was then precipitated in acetone. The quantity of protein applied to the polyacrylamide gel for the pellet fractions was $1/10$ of that compared to the other fractions.

with predominantly old H2A and H2B. It is even conceivable that the parental octameric histones within the prereplicative nucleosome do not dissociate from the DNA during replication or from each other. Providing the pool of old H2A and H2B is sufficiently large, one would still observe the hybrid octamers observed in this paper. Experiments are in progress to test whether the prereplicative parental histones are conserved as octamers or dissociated during replication. In earlier studies, it was observed that 70% of new H2A and H2B deposited on DNA at sites other than the replication fork (Jackson & Chalkley, 1981a,b, 1985). Therefore, the new H2A–H2B dimer described in this paper would appear to be deposited as two of eight histones in nucleosomes that are primarily not at the replication fork, but possibly on transcriptionally active genes.

The data indicate that new H3 and H4 deposit in the nucleosome as a H3–H4 tetramer. A number of *in vitro* studies have indicated that H3 and H4 form tetramers under ap-

propriate conditions (Isenberg, 1979). Therefore, it is not surprising that *in vivo* such an interaction represents a part of the mechanism for deposition of these two histones. What is not apparent is the time frame upon which these interactions are established. Presumably, to establish a homogeneous tetramer of new H3–H4, this interaction would be established soon after synthesis and before transport to the nucleus. Otherwise, hybrid tetramers would be formed with parental H3 and H4 at the replication fork. This assumes, however, that such parental tetramers would dissociate into monomeric H3 and H4, as a result of replication of the DNA upon which they are associated. Preliminary data involving chases of the density pulses indicate that not only is the H3,H4 tetramer maintained intact but also the H2A–H2B dimer (manuscript in preparation). Therefore, there would appear to be no spatial requirement for when the tetramer is formed since interchange of H3 and H4 within old tetramers appears to be excluded. *In vitro* studies have strongly suggested that core histones form

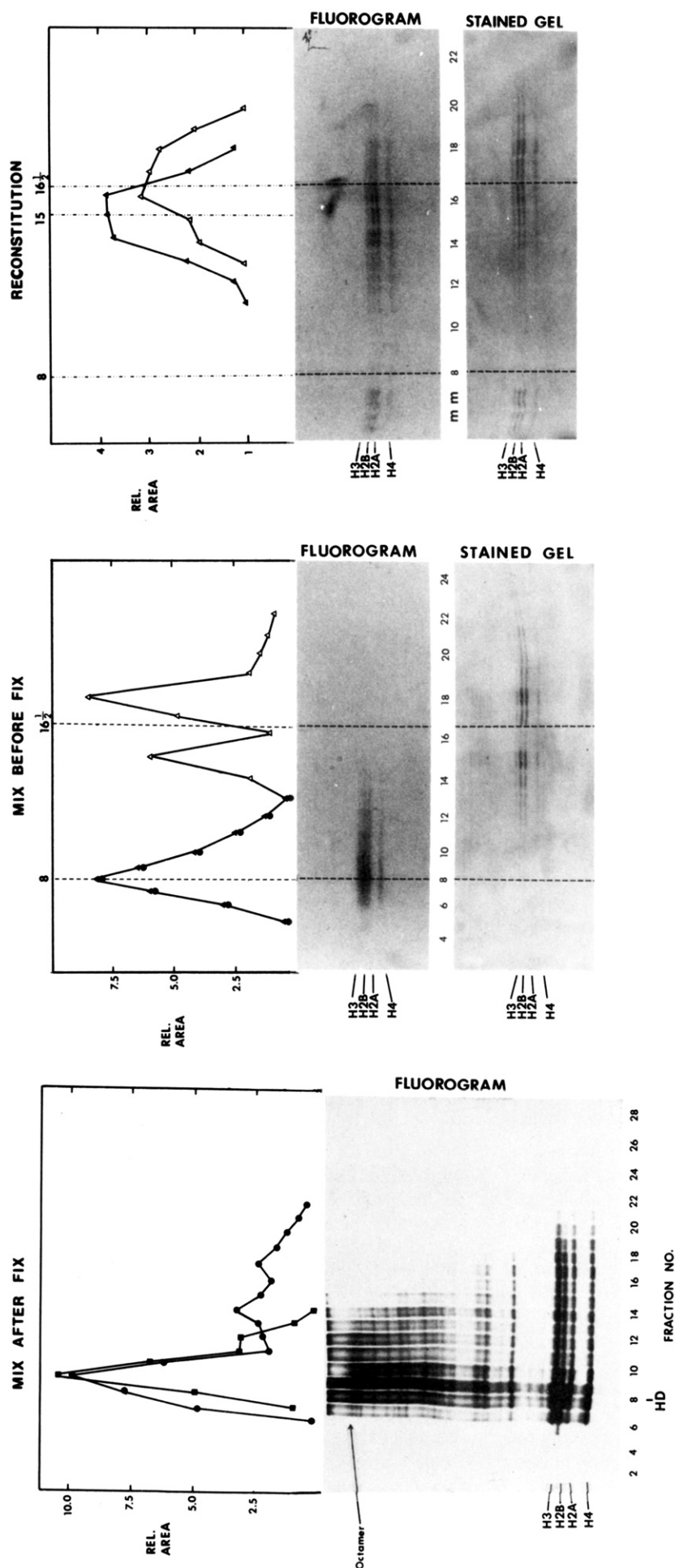


FIGURE 8: Mixing experiments designed to test for histone rearrangement caused by formaldehyde treatment and reconstitution experiments to establish the sensitivity of these gradients to density changes in the hybrid octamer. Nuclei were isolated from cells labeled for three generations with dense amino acids and mixed either before fixation with formaldehyde (mix before fix) or after fixation (mix after fix) with a 10-fold excess of nonlabeled nuclei. The chromatin was then acid extracted (acid-soluble octamer, see protocol of Figure 2) and mixed with monomer histones that had been previously extracted from nuclei (mixed 1:10). (Mix after fix) The fluorogram of the SDS gel for the fractionated gradient is shown: (●) relative area of the monomer histones, H3, H2B, H2A, and H4; (■) relative area of the octamer. (Mix before fix) After SDS gel electrophoresis of the fractions from the gradient (same as mix after fix), the octamer region was removed as described in Figure 5, the cross-link was reversed, and the monomer histones were reelectrophoresed from the gel slice in the same dimension: (▲) relative area of H3 and H4 in the fluorogram; (●) relative area of H2A and H2B in the fluorogram; (Δ) relative area of the histones in the stained gel. On loading the sample for fraction 16, less sample was applied for alignment of gels (same procedure

as for Figure 3). (Reconstitution) One part of dense nuclei was mixed with 5 parts of nondense nuclei. After adjustment to 2.0 M NaCl and dialysis against the same for 18 h, the sample was dialyzed stepwise from 2.0 to 1.2 to 0.6 M NaCl to 12 mM TEA-12 mM PO_4 , pH 9.1, over a 24-h period (Tatchell & Van Holde, 1977). The chromatin was treated with formaldehyde as previously described; octamer was isolated and sedimented to equilibrium with monomer histone present as a density marker. The octamer region was sliced out and the cross-link reversed and reelectrophoresed in the same dimension. (▲) Relative area of all four histones in the fluorogram; (Δ) relative area of all four histones in the stained gel. Marker histone standards (m m) were electrophoresed with the proteins in the gel slice to define orientation between the fluorogram and the stained gel. The vertical broken lines indicate the average density on the basis of the total area under the curves. The fully dense fraction on the gradients (fraction 8) is determined from the monomer histones of the first electrophoresis. Since the nuclei used in these three experiments were derived from the same three-generation dense labeling, fraction 8 (as seen in mix after fix data) represents that position for fully dense histones in all cases for this figure.

stable octameric complexes when free in solution and will deposit on DNA to produce nucleosomes when in this form (Stein et al., 1977, 1979). The data of this paper are consistent with such a deposition occurring in vivo.

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Deposition of Newly Synthesized Histones: Misinterpretations due to Cross-Linking Density-Labeled Proteins with Lomant's Reagent[†]

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ABSTRACT: We have reinvestigated studies using Lomant's reagent to cross-link newly synthesized density-labeled histones into octameric complexes to determine the nature of histone deposition. The analysis has additionally included procedures for reversal of the cross-link in order to analyze the individual histones in these complexes. These studies indicate that density-labeled, newly synthesized histones form hybrid octameric structures composed of both new and old histones. These studies also suggest that previous interpretations by other investigators for the production of homogeneous complexes (100% dense octamer containing 100% new histones) are misinterpretations due to the presence of non-histone protein that contaminates the preparation, even under conditions where much of this non-histone protein is removed by use of ion-exchange resins. These non-histone proteins can be fractionated on density gradients as un-cross-linked proteins with molecular weights that mimic those of cross-linked histone complexes.

The mechanism whereby histones package DNA during DNA replication has been extensively studied by a number of investigators. The interpretation of various experiments has in some instances resulted in a difference of opinion with regard to this mechanism. For example, in addressing the fundamental and apparently simple question whether all newly synthesized histones deposit on newly replicated DNA, a conflict exists. In 1976, it was reported (Jackson et al., 1976) that newly synthesized histones did not deposit on newly replicated DNA. By density labeling newly replicated DNA with iododeoxyuridine and labeling the histones with short pulses of [³H]lysine, it was observed on CsCl density gradients that the formaldehyde-cross-linked, iododeoxyuridine-containing chromatin did not associate with the newly synthesized

histones. In 1977, a separate group (Leffak et al., 1977) reported that all newly synthesized histones deposited on newly replicated DNA. This was shown experimentally by labeling newly synthesized histones with dense amino acids (¹⁵N, ¹³C, ²H) in the presence of [³H]lysine, isolating the nuclei, and cross-linking the histones into an octameric complex with Lomant's reagent (DSP).¹ [Octamer is a term for the complex of histones, two each of H3, H2B, H2A, and H4, that is associated with 200 base pairs of supercoiled DNA (a nu-

¹ Abbreviations: DSP, dithiobis(succinimidyl propionate) (Lomant's reagent); MSB, chicken leukemic cell line transformed by Marek's virus; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; MgCl₂, magnesium chloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethanolamine; PO₄, phosphate; H₂SO₄, sulfuric acid; Gdn-HCl, guanidine hydrochloride; CsFo, cesium formate; SDS, sodium dodecyl sulfate; NaCl, sodium chloride; HCHO, formaldehyde; EDTA, ethylenediaminetetraacetic acid.

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