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 [^{3}H]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 (^{15}N, ^{13}C, ^{2}H) in the presence of [^{3}H]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-

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[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; $MgCl_2$, magnesium chloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethanolamine; PO_4, phosphate; H_2SO_4, 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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cleosome).] This radiolabeled complex was shown to have a density on CsFo gradients that suggested that these labeled octamers were homogeneous with respect to containing all newly synthesized histones. By inference, these octamers were deposited at the replication fork. How is it possible to have two such extremely opposite results? The resolution of this discrepancy is pivotal to our understanding of whether histones interact with each other in a static and/or dynamic manner as they package the DNA into a nucleosome and perhaps regulate the expression of that DNA in subsequent generations.

In 1981, a series of three papers were published describing four separate techniques for isolating replicative DNA that indicated that newly synthesized histones deposited on newly replicated DNA. However, the level of deposition was variable with histone type (Jackson & Chalkley, 1981a,b; Jackson et al., 1981). Whereas the majority of new H2A and H2B (70%) deposited on nonreplicated DNA, all newly synthesized H3 and H4 deposited on newly replicated DNA. The implications from these latter studies were that new H3 and H4 do not always deposit in the same nucleosome with new H2A and H2B. These results were in contrast to those reported in 1976 by the same group. In retrospect, two mistakes were made in the initial study. First, by use of [^{3}H]lysine ([^{3}H]arginine at high specific activity was not available at that time) as the radiolabel, histones H1, H2A, and H2B were selectively labeled. The mole percents of lysine in the histones are as follows: H3, 10%; H4, 9%; H2B, 16%; H2A, 11%; H1, 28% (Isenberg, 1979). Seventy-five percent of the lysine is in H1, H2A, and H2B. Second, newly synthesized histones H3 and H4 when first deposited are weakly associated with replicative DNA (Jackson et al., 1981) such that washes with hypotonic buffer containing a nonionic detergent remove the vast majority of these histones from the replicative DNA. Such a washing procedure was used in the 1976 experiments. In addition, procedures were not available at that time to reverse formaldehyde cross-links in order to analyze the proteins in the gradient by gel electrophoresis. Therefore, interpretations were made on the basis of the distribution of total radioactivity. If such procedures were available, gel electrophoresis would have detected the lower percentage of H3 and H4 and the specific deposition of those histones on replicated DNA. In 1978 (Jackson, 1978), procedures were developed to reverse formaldehyde cross-links and were used in the later studies. We emphasize the importance of analyzing the individual histones in whatever fractionation procedure is used.

These results of nonuniform deposition of each histone type at the replication fork remain in conflict, however, with the original results obtained from experiments that utilized dense amino acids (Leffak et al., 1977). Additional data from the same group (Leffak, 1983, 1984) have been reported and again interpreted as indicating that newly synthesized H2A and H2B as well as H3 and H4 deposit as a group in an octameric complex. In these latter reports, it was shown that density-labeled oligomeric complexes are produced which, after sedimentation to equilibrium on cesium formate gradients, can be resolved on polyacrylamide–SDS gels. In this way it was demonstrated that these complexes were as dense as the original density-labeled monomer histones (prior to cross-linking).

As a result of our earlier experiences (Jackson et al., 1976) in which we observed the importance of establishing the individual histone composition of cross-linked complexes, we felt it important to analyze the histone composition of complexes cross-linked by Lomant's reagent. Such an analysis has not been reported. Lomant's reagent [dithiobis(succinimidyl propionate), DSP] contains a disulfide bridge that allows reversal of the cross-link and analysis of the monomer histones. We have, therefore, repeated the procedures described by Leffak (1983, 1984) to determine whether newly synthesized histones are cross-linked as a 100% dense octamer. We find that, upon reversal of these oligomeric complexes, newly synthesized histones are present in hybrid octamers. These results are consistent with the results in the previous paper (Jackson, 1987) in which hybrid octamers were shown to exist by use of the cross-linking agent formaldehyde and are also consistent with our observations that specific histone types deposit at the replication fork to differing extents. In this paper, the radiolabeled dense oligomers produced by Lomant's reagent, which have been previously interpreted to be 100% new histones in octamers, are found to be non-histone proteins, which because of their molecular weight have been misinterpreted to be cross-linked histones.

Materials and Methods

Preparation of Chromatin and Density Gradient Centrifugation. MSB cells were grown in 10% fetal calf with medium of 1:1 Dulbecco's MEM–RPMI-1640 and supplemented with 50 mM HEPES. Exponential growth was maintained at a cell density between 1×10^6/mL and 1.5×10^6/mL with a 10-h cell cycle. Procedures for labeling of cells with [^{3}H]lysine, [^{3}H]arginine, and dense amino acids were as previously described (Jackson, 1987).

Labeled nuclei were isolated by being washed 3 times in 1% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM Tris, pH 8.0, and then washed twice in the same buffer in the absence of detergent. Nuclei were suspended at 1 mg/mL (DNA concentration), adjusted to 1 mM $CaCl_2$, and digested (to 5–10% DNA acid solubility) with 40 units of micrococcal nuclease (Pharmacia)/mL for 10 min at 37 °C. The digestion was terminated by addition of 20 mM EDTA, and the nuclei were dialyzed against 20 mM borate, pH 9.0, and 1.0 mM EDTA for 12 h at 4 °C. The solution was centrifuged at 5000*g* for 10 min, and the solubilized chromatin was either directly cross-linked with Lomant's reagent (DSP, Pierce) or further purified. Ten percent of the chromatin was set aside for acid extraction to isolate monomer histones (density markers). This was done by adjusting the chromatin to 0.4 N H_2SO_4, and after sonification on a Branson sonifier for 1 min, the sample was centrifuged at 27000*g* for 10 min. Histones were precipitated from the supernatant in 7 volumes of acetone at −20 °C overnight. The precipitate was dissolved in water and added to the density gradient.

Further purification of solubilized chromatin, which involved removing the majority of the non-histone protein and histone H1, was performed by adjusting the chromatin to 0.65 M NaCl and incubating with preequilibrated Bio-Rad AG 50W-X4 resin for 60 min at 4 °C (Leffak, 1983). The depleted chromatin was removed and either directly cross-linked with Lomant's reagent (cross-linking in 0.65 M NaCl) or dialyzed against 20 mM borate, pH 9, for 10 h before cross-linking. The cross-linking condition involved treatment of 2 mL of the chromatin (0.5 mg/mL DNA concentration) with 0.5 mg/mL Lomant's reagent for either 1 or 30 min at 20 °C. Reactions were terminated by addition of 2.4 g of Gdn-HCl followed by 0.85 g of CsFo. The volume was adjusted to 4.0 mL, and the sample was centrifuged to equilibrium at 54 000 rpm for 96 h at 11 °C in a SW60 Ti rotor. Twenty seven fractions were collected, adjusted to 0.4 N H_2SO_4, and dialyzed against the same at 4 °C for 18 h. The presence of acid is to ensure that no further cross-linking will occur after the Gdn-HCl is dialyzed away and before the SDS is added. Each fraction was then precipitated in 7 volumes of acetone at −20 °C and re-

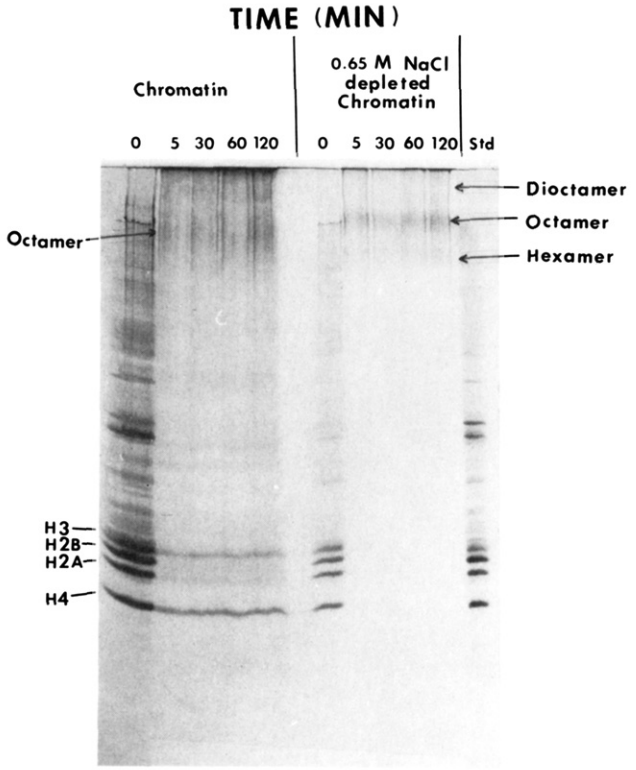

FIGURE 1: Time courses for reaction of DSP with chromatin. Reactions were completed on two types of chromatin, depleted and nondepleted. Nuclease-solubilized chromatin was isolated as described under Materials and Methods and either directly cross-linked with DSP in 20 mM borate, pH 9, or first depleted of the majority of non-histone protein by adjusting the chromatin to 0.65 M CaCl–20 mM borate, pH 9, and treatment with ion-exchange resin. This depleted chromatin was then dialyzed against 20 mM borate, pH 9, prior to treatment with DSP. At increasing times reactions were stopped by addition of SDS stop buffer and immediately electrophoresed on 18% polyacrylamide–SDS gels. Similar results are observed when Lomant's reagent is directly added to the depleted chromatin in the presence of 0.65 M NaCl (data not shown). The presence of this ionic strength has no effect on the distribution of oligomers formed.

dissolved by sonication into 0.5% SDS–10% glycerol–100 mM Tris, pH 6.8.

Electrophoresis of Proteins and Reversal of Lomant's Cross-Link. The electrophoresis conditions were as previously described (Jackson, 1987). To examine the histone composition of the cross-linked complexes, the stained gel was cut into strips encompassing the area containing the hexamer or octamer region and incubated according to condition C (see legend to Figure 2). The strip was then polymerized as previously described (Jackson, 1987) in a stacking gel, and the proteins within the strip were electrophoresed a second time.

RESULTS

Reaction Conditions for Production of a Histone Octamer by Lomant's Reagent (DSP) and Reversal of the Cross-Link. Solubilized chromatin was prepared by digestion of nuclei as described under Materials and Methods. The chromatin was treated with 0.5 mg/mL DSP, and at increasing times, samples were removed and added to SDS electrophoresis buffer to stop the reaction. Samples were immediately electrophoresed on 18% polyacrylamide–SDS gels to separate the cross-linked products. As shown in Figure 1, the reaction with this reagent is very rapid. After 5 min, no appreciable change is observed in the distribution of multimers formed. In addition, the octamer is extremely broad and diffuse in molecular weight. As suggested by Leffak (1983) in his studies with this reagent, treatment of the chromatin with Bio-Rad AG 50W-X4 in the presence of 0.65 M NaCl removes most non-histone protein. By including these procedures in our studies (Figure 1), the octamer is more well defined, and indeed, it is now possible to observe a second oligomeric complex. We have tentatively defined this second oligomer as a hexamer on the basis of its apparent molecular weight. We interpret these observations as indicating that in the absence of this depletion step non-histone proteins are cross-linked to histones, thereby causing the diffuse molecular weight. That such cross-linking occurs can be further demonstrated in the observation that DSP-cross-linked complexes of histones are acid insoluble (data not shown). This is also true of the octamer and hexamer produced by cross-linking of the depleted chromatin. As previously shown (Jackson, 1987), a characteristic of histones is their acid solubility, even after being cross-linked by formaldehyde into an octameric complex. The depletion step is therefore necessary to limit the non-histone–histone cross-linking by DSP. Indeed, if nuclei are cross-linked directly with DSP at pH 9, all material is cross-linked within 30 s of addition into a massive complex, which will not enter the acrylamide gel (data not shown). This extensive cross-linking can be observed even with the depleted chromatin (see top of gel in Figure 1).

DSP [dithiobis(succinimidyl propionate)] contains a disulfide bridge. We have established conditions for reversal of the disulfide bond by running several duplicate samples of the DSP octamer on an SDS gel. The octamer region of each lane was removed and subjected to different reversal conditions. The slices were then reelectrophoresed a second time to examine the proteins present in the octameric complex. These results are shown in Figure 2. Condition A is not sufficient to reverse the cross-link for all the oligomeric complexes. However, condition C results in complete reversal. More rigorous conditions result in unacceptable loss of protein by diffusion from the gel slice. We therefore used condition C for all subsequent reversals of the disulfide bond in DSP.

Analysis of Protein Composition in the Oligomeric Complexes Produced by DSP. As shown in Figure 1, several oligomeric complexes (dioctamer, octamer, and hexamer as suggested by their apparent molecular weights) are produced when depleted chromatin is cross-linked with Lomant's reagent. We wish to establish that histones are present in each of these complexes and to quantitate any difference in the level of H3, H2B, H2A, and H4. Using the 2-h time point of Figure 1 (depleted chromatin), we electrophoresed the sample in the first dimension (see gel strip of Figure 3) and using condition C reversed the cross-link to electrophorese the monomer proteins in a second dimension. As shown in Figure 3, these oligomeric complexes do not contain equimolar quantities of the histones. This can be seen by comparing the reversed complexes and the histone standard in the fluorogram. Histone H2B is much depleted, and to a lesser extent H2A is also depleted. This is true for all three oligomers. It is not that the reversal conditions are insufficient to reverse cross-links, for we have repeated this same experiment with condition E (see Figure 2) and have observed the same result (data not shown). A significant percentage of these histones are complexed in a nonreversible complex that remains at the top of the first-dimension gels. As will be shown later, much of this higher molecular weight protein is non-histone protein. However, if we concern ourselves specifically with those reversible histones that are in these complexes, it is possible to analyze the distribution of newly synthesized histones in the complexes by using density-labeled amino acids.

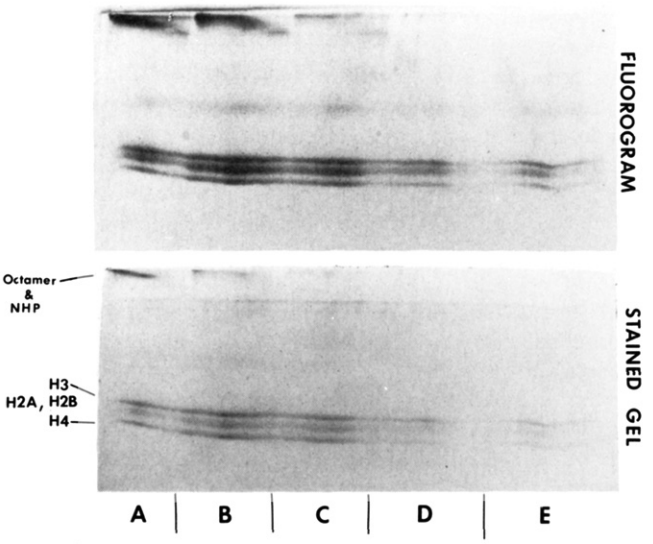

FIGURE 2: Establishment of conditions for reversal of DSP cross-links. Cells were labeled with [^{3}H]lysine and [^{3}H]arginine for 60 min. Nuclei were isolated, and depleted chromatin was prepared and cross-linked with DSP as described under Materials and Methods. After electrophoresis, stained gel slices containing equivalent quantities of octamer were incubated under varying conditions. Condition A is an incubation for 30 min in 1% SDS, 100 mM Tris, and 0.5 M 2-mercaptoethanol (buffer A); condition B is an incubation for 2 h in buffer A; condition C is an incubation for 60 min in buffer A followed by heating in the same at 100 °C for 5 min; condition D is an incubation for 60 min in buffer A followed by heating in the same at 100 °C for 30 min; condition E is an incubation for 60 min in buffer A followed by heating in the same at 100 °C for 60 min. After these treatments, gel slices were incubated at 20 °C for 60 min in two changes of buffer A (minus the 2-mercaptoethanol) and directly polymerized into an acrylamide stacking gel (Jackson, 1987).

Density Gradient Analysis of the Radiolabeled, Density-Labeled DSP Oligomers Produced from Depleted Chromatin. MSB cells were labeled for 60 min with ^{15}N, ^{13}C, and ^{2}H-labeled amino acids and [^{3}H]lysine and [^{3}H]arginine. Nuclei were isolated and treated with micrococcal nuclease, and the soluble chromatin was isolated. Chromatin was depleted of non-histone protein and H1 histone by adjusting to 0.65 M NaCl and incubating for 60 min at 4 °C with AG 50W-X4 resin. Ten percent of this depleted chromatin was acid extracted to isolate monomer histones, which serve as internal density markers in the CsFo gradients (Jackson, 1987). The remaining chromatin was treated with DSP (0.5 mg/mL) for 30 min at 20 °C. The reaction was stopped by addition of guanidine hydrochloride (Gdn-HCl). Gdn-HCl is an effective agent for terminating the reaction (reacts with the *N*-hydroxysuccinimide ester of DSP forming a covalent linkage with the propionate) and is needed in the gradients to prevent adventitious binding between proteins. Cesium formate (CsFo) was added along with acid-extracted monomer histones (density markers) and centrifuged to equilibrium at 53 000 rpm for 4 days. Fractions were collected and electrophoresed on 18% polyacrylamide–SDS gels to determine the distribution of radiolabeled, density-labeled proteins (fluorogram) and nonlabeled proteins (stained gel). The results of the analysis are shown in Figure 4. Microdensitometric analysis of these data (Figure 5A) shows that the normal density (ND) of the stained proteins for both hexamer and monomer centers around fraction 15. The density of the radiolabeled proteins (labeled with dense amino acids) is fraction 8 for octamer, hexamer, and monomer histones (see also Figure 4, HD). Because the density of the octamer and hexamer appears to be similar to that of the monomer, density-labeled histones, one may interpret these data as indicating that newly synthesized histones

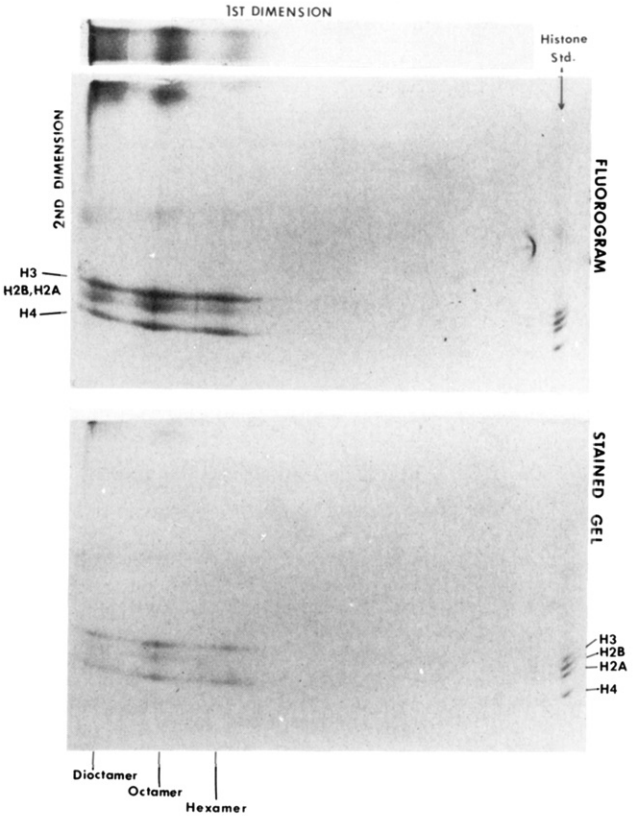

FIGURE 3: Second-dimension analysis of protein content present in the cross-linked products of DSP. Depleted chromatin was cross-linked for 2 h with 0.5 mg/mL DSP at 20 °C. Samples were directly electrophoresed on an 18% polyacrylamide–SDS gel. After electrophoresis, the gel was stained with Coomassie and then destained, and a gel slice was taken of the lane containing the cross-linked complexes. This gel strip is shown in the upper panel. The strip was treated by condition C (see legend to Figure 2) and polymerized into a stacking gel for electrophoresis in the second dimension on an 18% polyacrylamide–SDS gel. The histone standard was isolated from the same depleted chromatin by acid extraction (see Materials and Methods).

form oligomeric complexes, exclusive of old histones, and deposit as a homogeneous complex on DNA, presumably newly replicated DNA. Such a conclusion has been made on the basis of these procedures (Leffak, 1984). However, it is important to establish that, indeed, these complexes are derived from the histones.

As we have established reversal conditions for this reagent, we have cut gel strips of the octamer and hexamer regions as indicated by the horizontal dotted lines in Figure 4 (stained gel). These were reversed under condition C of Figure 2 and then electrophoresed a second time on 18% polyacrylamide–SDS gels to determine the protein component in each fraction. The results are shown in Figure 6. A large percentage of the radiolabeled protein present in the various regions of the gel in Figure 4 do not consist of oligomers of histones but rather of what we tentatively describe as non-histone protein (NHP). These monomer non-histone proteins are located in the dense region of the gradient (fraction 8), which was initially described as the location for the radiolabeled, density-labeled octamer and hexamer (Figure 4). From the data of Figure 6, it can be seen that the histones of the reversed octamer and hexamer are actually located in fractions 11–16. The presence of these non-histone proteins provides a false impression that cross-linked histone complexes are located in the same region of the gradient where the monomer, density-labeled histones are located. They are not. From the data of Figure 4, it is therefore possible to grossly misinterpret the distribution of

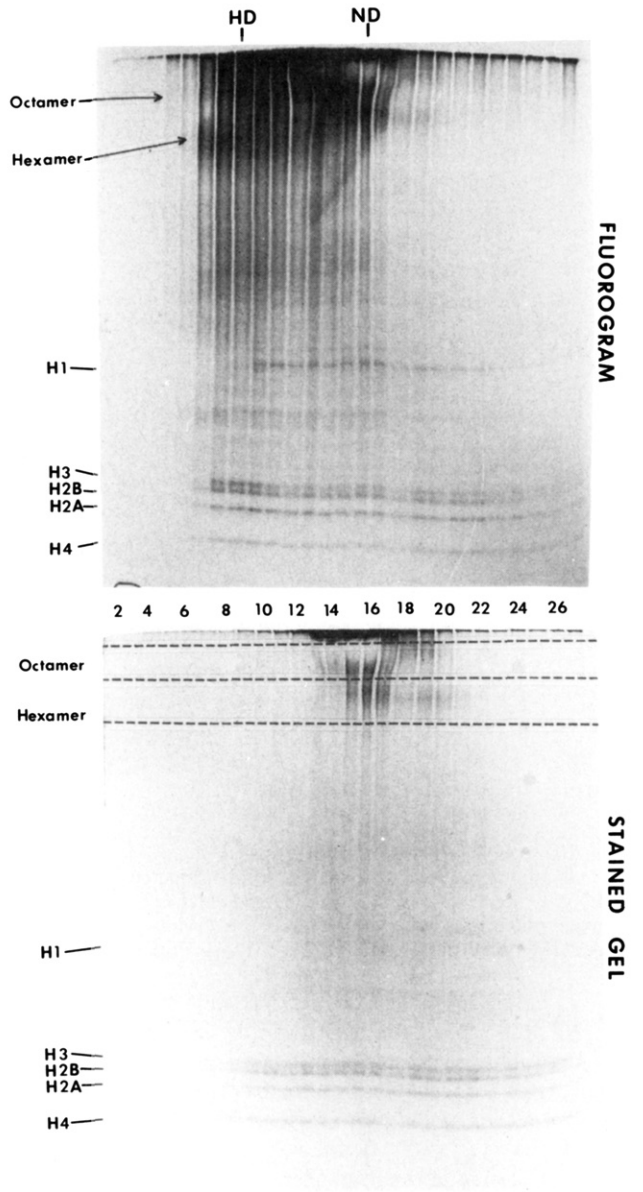

FIGURE 4: Density gradient analysis of the distribution of density-labeled DSP oligomers and monomer histones. Nuclei were isolated from cells labeled with ^{15}N, ^{13}C, and ^{2}H-labeled amino acids and [^{3}H]lysine and [^{3}H]arginine for 60 min. Nuclei were digested with micrococcal nuclease, and after depletion of non-histone protein by treatment with AG 50W-X4 resin in 0.65 M NaCl–20 mM borate, pH 9, 10% of this chromatin was directly acid extracted (see Materials and Methods). The remainder was cross-linked with 0.5 mg/mL DSP for 30 min at 20 °C. The reaction was terminated by adding Gdn-HCl. CsFo and the acid-extracted histones (monomer histones as density markers) were then added, and the solution was centrifuged to equilibrium. Fractions were collected, dialyzed, and electrophoresed on 18% polyacrylamide–SDS gels. The dotted horizontal lines on the stained gel indicate where a duplicate stained gel was cut to produce two gel strips each of which was electrophoresed a second time to produce the data of Figure 6. High density (HD) and normal density (ND) indicate the central location where density-labeled and non-labeled histones, respectively, have focused in the density gradient.

newly synthesized histones on the gradient.

Microdensitometric analysis has been done on the data of Figure 6 to determine the distribution of the radiolabeled, density-labeled histones. As shown in Figure 5B for the analysis of the octamer, hybrid density is a characteristic of this complex. The focused position for labeled H3 and H4 is 13 and for H2A and H2B is fraction 14.5. For the stained, nondense protein the focused position is fraction 16. Since fraction 8 represents the 100% dense position (Figure 5, HD),

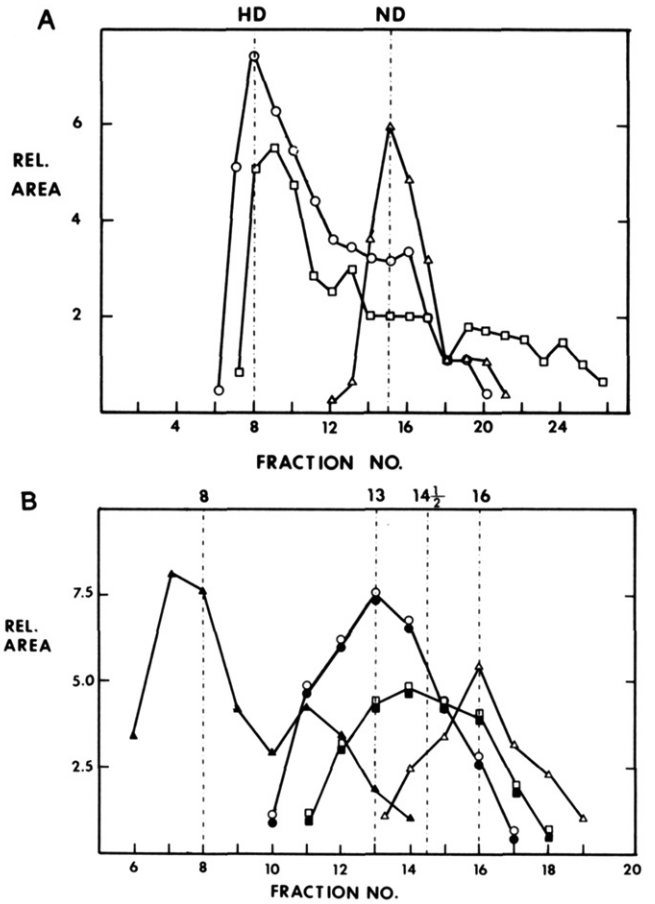

FIGURE 5: Densitometric analysis of the polyacrylamide gels of Figure 4 and Figure 6. (A) In Figure 4 the hexamer region of both the fluorogram (○) and the stained gel (△) and the monomer histone (H3, H2B, H2A, and H4) region of the fluorogram (□) were densitometrically scanned and the areas integrated. The relative area was determined by dividing each integrated value by the lowest significant value, which was then defined as 1.0. Values less than 1 are also plotted; however, the error in these values is significant due to the small measured areas. The octamer region has also been analyzed in this way, but the data are not included in this figure as the octamer region is not as clearly resolved compared to the hexamer region. The distribution of radiolabeled octamer, however, remains the same as that of the hexamer. (B) In Figure 6 the gel containing the uncross-linked (reversed) proteins from the reelectrophoresed gel strip (octamer region from Figure 4) was scanned densitometrically: NHP (non-histone protein) region (▲) of the fluorogram; H3 (○), H4 (●), H2B (□), and H2A (■) region of the fluorogram; H3, H2B, H2A, and H4 region (△) of the stained gel.

we estimate the following for the octamer: (16 − 13)/(16 − 8) = 3/8 = 38% or three of eight histones consist of new H3 and H4. For H2A and H2B, the values are (16 − 14.5)/(16 − 8) = 1.5/8 = 19% or 1.5 of 8 histones in the octamer are new H2A and H2B. These values for the distribution of labeled histone in the octamer are less than those reported in the previous study with the formaldehyde-cross-linked octamer (Jackson, 1987). This effect may be due to the presence of a small percentage of non-histone protein (normal density) that was cross-linked to the histone complexes, reducing the overall density. However, the ratio of deposition of new H3 and H4 as compared to new H2A and H2B (3/8 ÷ 1.5/8 = 2) is identical with the value observed in the hybrid octamers from the previous study (Jackson, 1987) and, therefore, further supports the conclusion that newly synthesized histones H3 and H4 primarily deposit in new nucleosomes separate from new H2A and H2B.

The observation that the octamer produced by DSP is a hybrid octamer consisting of both new and old histones could

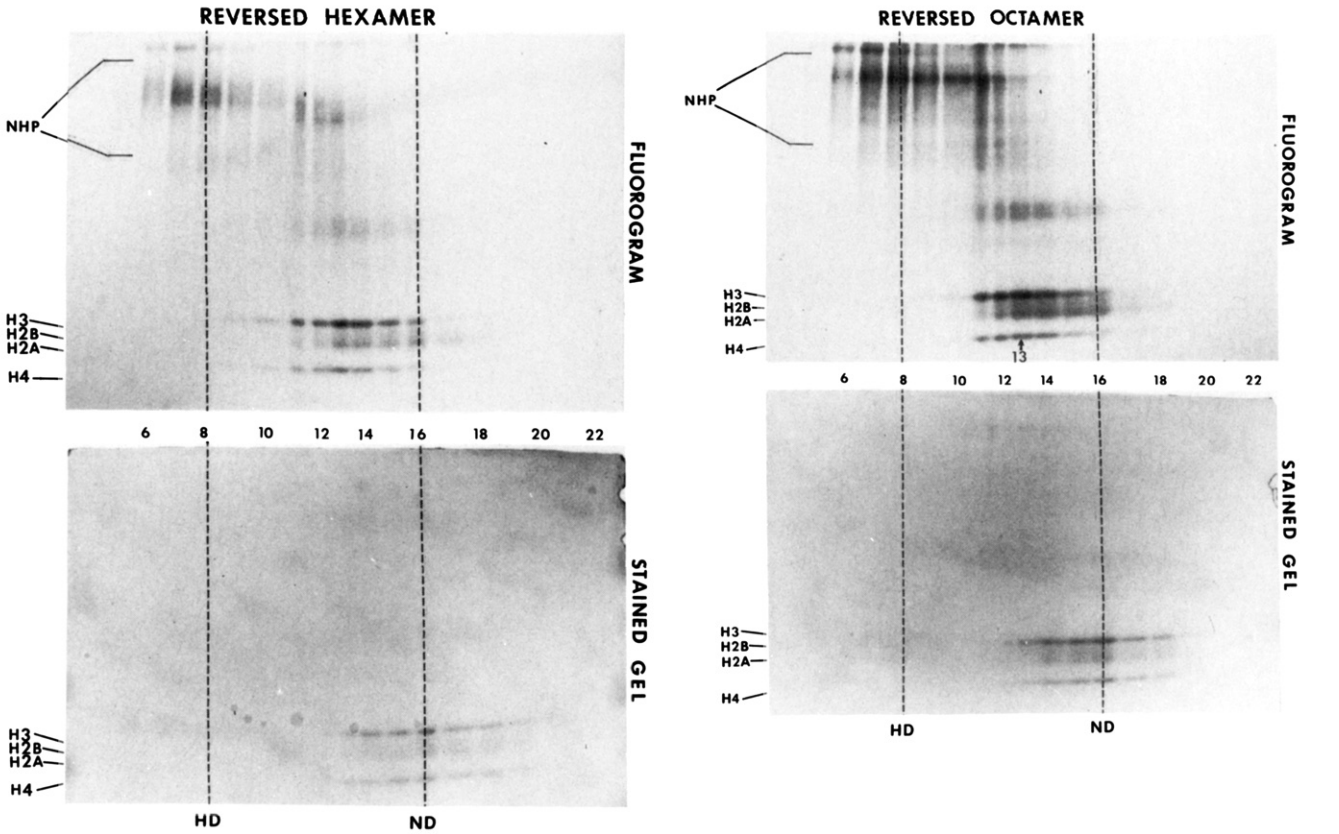

FIGURE 6: Reelectrophoresis of the gel strips containing hexamer and octamer from Figure 4. These strips contain cross-linked and non-cross-linked proteins of the hexamer and octamer region from fractions 3–23. All proteins in the gel strips were un-cross-linked (disulfide bridge reduced) by treatment according to condition C (see Figure 2). Strips were then directly polymerized into a stacking gel and electrophoresed on 18% polyacrylamide–SDS gels. NHP (non-histone protein) refers to the un-cross-linked proteins at the top of the gels. The vertical arrow (fraction 13) is the focused density for the octamer containing new H3 and H4 (see also densitometric analysis of these data in Figure 5B).

be the result of a number of potential artifacts. The utilization of chromatin, depleted of protein by ion-exchange resins in the presence of 0.65 M NaCl, may accelerate histone movement or loss of H2A and H2B on DNA. In addition, cross-linking with this reagent at this ionic strength (0.65 M NaCl) may magnify the release of histones and create the hybrid octamer. Also, the extended length of time for cross-linking (30 min) may significantly increase the quantity of non-histone protein complexed with the histone oligomers. In the following experiment these concerns are addressed.

Density Gradient Analysis of the DSP Octamer Produced from Nondepleted Chromatin. Nuclei were isolated from MSB cells that were previously labeled with [^{3}H]lysine, [^{3}H]arginine, and dense amino acids for 60 min. The nuclei were digested with micrococcal nuclease, and the solubilized chromatin was directly cross-linked with DSP for 1 min without the depletion step. The reaction was terminated by addition of Gdn-HCl, and the sample was centrifuged to equilibrium. Fractions were collected and electrophoresed on 18% polyacrylamide–SDS gels. The diffuse octamer region (see Figure 1) was removed as described in Figure 4, and the strips were treated according to condition C to reverse the disulfide bridge of the DSP. The proteins in the strips were electrophoresed a second time to determine the distribution of the proteins. As shown in Figure 7A, the results are similar to those results shown in Figure 6. Non-histone proteins with molecular weights similar to that of the octamer are again distributed in fractions 7–9. No radiolabeled histones from the reversed octamer are located in those same fractions. These results indicate that the procedures used to deplete chromatin of non-histone protein are not a causative factor for producing these hybrid octamers. The hybrid character (new and old histones in same nucleosomes) of these octamers is a characteristic of the deposition process in the cell.

Is it possible that the radiolabeled protein, described as NHP protein, is in fact high-density histone octamer, which for some unknown reason would not reverse under the conditions used for the normal density octamer? This would result in a misinterpretation of the level of hybrid octamer. We believe this is not the case. If one examines Figures 6 and 7A, the radiolabel in the fraction 8 region represents several bands rather than one broad band. A broad band is characteristic of the histone octamer. If unreversed octamer were still present, the bands would remain diffuse. We interpret these several bands to represent specific monomer proteins, which initially electrophorese rather diffusely as in Figure 4. This diffuse characteristic is probably due to the presence of a guanidine moiety covalently linked to one end of the propionate (DSP) and the protein linked to the other. Guanidine hydrochloride (Gdn-HCl) was used to terminate the cross-linking reaction. By applying the reversal procedure to cleave the disulfide bond, that portion of the DSP covalently attached to the guanidine group is removed from the proteins, and these proteins now electrophorese more sharply in the reelectrophoresis with the same molecular weight as in the first electrophoresis.

Additional experiments have been done to determine whether the high molecular weight proteins in the dense region of the gradients are non-histone proteins. These experiments emphasize the importance of extracting the formaldehyde-cross-linked histones with acid prior to analysis on density gradients.

Analysis on Density Gradients of the Formaldehyde-Cross-Linked Octamer without Selective Acid Extraction.

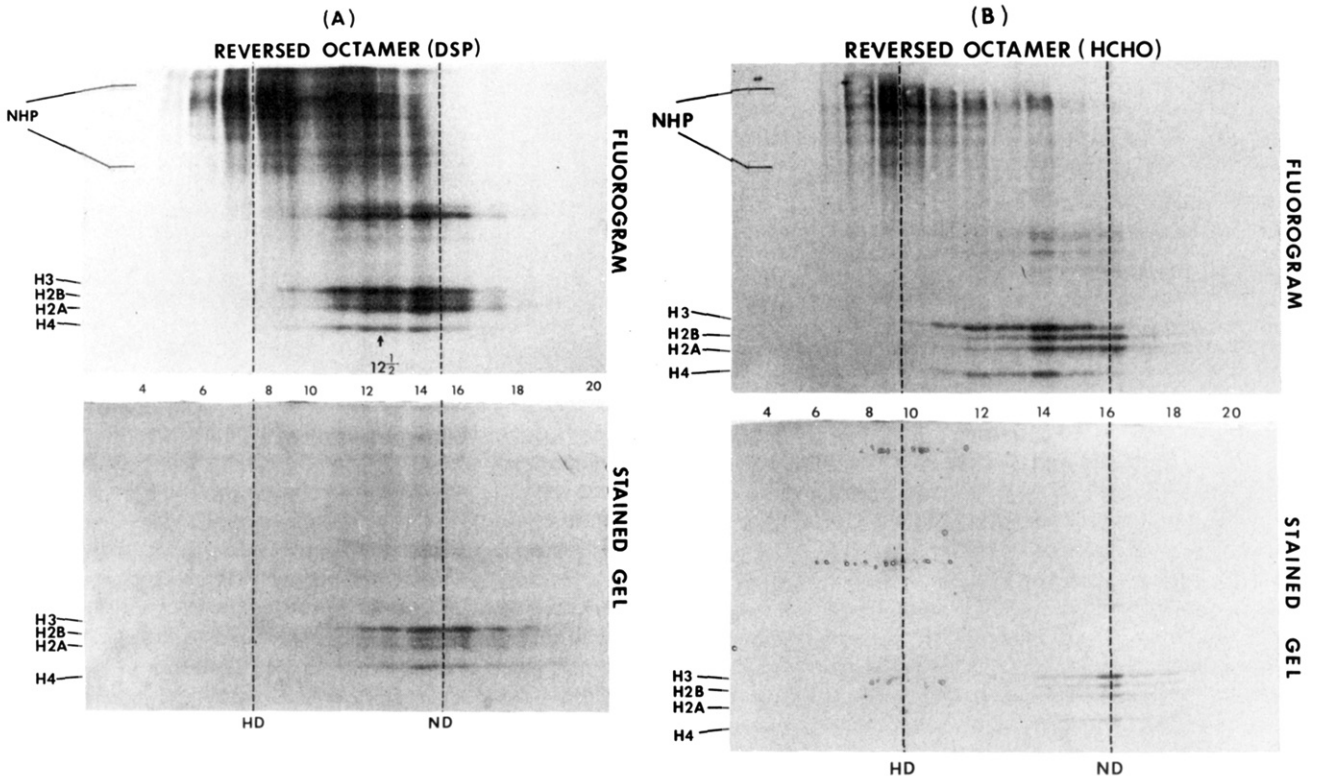

FIGURE 7: Gel electrophoretic analysis of octamers produced by DSP (A) and formaldehyde (B) cross-linking after separation of the density-labeled oligomers on density gradients. MSB cells were labeled for 60 min with [^{3}H]lysine, [^{3}H]arginine, and dense amino acids. (A) Nuclease-solubilized chromatin was prepared (see Materials and Methods) and directly cross-linked with 0.5 mg/mL DSP at 20 °C for 1 min. This chromatin had not previously been treated with resin at 0.65 M NaCl concentration to remove the majority of non-histone protein. The short term of cross-linking (1 min) is designed to minimize non-histone protein cross-linking to histones. The reaction was terminated by addition of Gdn-HCl, and after addition of CsFo and the acid-extracted monomer histones (density standards), the sample was centrifuged to equilibrium on density gradients. Fractions were electrophoresed on 18% polyacrylamide–SDS gels as described for Figure 4. Subsequently, the octamer region was removed from the gel from fraction numbers 3–23 and treated according to condition C (see Figure 2) to reduce the disulfide bond of the DSP. Proteins in the gel strip were electrophoresed a second time to determine the location of the histones in the fractions. The vertical arrows (fraction 12.5) is the focused density for the octamer containing new H3 and H4. (B) The nuclease-solubilized chromatin was depleted of non-histone protein by treatment with resin in 0.65 M NaCl. The chromatin was subsequently dialyzed into 20 mM borate, pH 9, and cross-linked with formaldehyde (HCHO) for 8 h at 4 °C. Gdn-HCl and CsFo were directly added, and the sample was centrifuged to equilibrium. Fractionation and gel electrophoreses were as described for (A). The gel strip containing the octamer region was heated at 100 °C for 30 min in the presence of 2-mercaptoethanol to destroy the formaldehyde cross-link (Jackson, 1987), and the proteins were reelectrophoresed a second time to determine the location of histones in the fractions.

Nuclease-solubilized chromatin was isolated from cells that had been labeled with dense amino acids for 60 min. Depleted chromatin was prepared as described under Materials and Methods and then immediately dialyzed against 20 mM borate, pH 9. This chromatin was fixed with formaldehyde for 8 h at 4 °C, and the fixation was terminated by addition of Gdn-HCl in a similar manner to the termination of fixation for DSP. In the previous paper (Jackson, 1987) the fixation with formaldehyde was terminated by treatment with acid, and subsequently the acid-soluble histones were isolated. This step has been omitted in this experiment. CsFo and the acid-extracted monomer histones (density markers) were then added, and the sample was centrifuged to equilibrium. Fractions were collected and electrophoresed on 18% polyacrylamide–SDS gels. The octamer region from fractions 1–27 was excised and heated at 100 °C for 30 min to reverse the formaldehyde cross-link (Jackson, 1987). The proteins within the gel strip were then reelectrophoresed on 18% polyacrylamide–SDS gels. As shown in Figure 7B, an extensive number of proteins (NHP) are located in fraction 9 of this density gradient. This distribution of proteins is very similar to the data of Figure 6, where depleted chromatin was cross-linked with DSP prior to equilibrium density centrifugation. When these data are compared with the results of the previous paper (Jackson, 1987; Figure 5B), it is evident that these proteins are insoluble in acid and can be separated from histones by acid treatment. The proteins in fraction 9 of Figure 7B are non-histone protein. The proteins in fraction 9 of Figure 5B are likely to also be non-histone protein, not cross-linked histones. Why then not acid extract the DSP octamer, as is done in the isolation of the formaldehyde octamer (Jackson, 1987)? Such acid extraction will remove the vast majority of this non-histone protein. However, as indicated previously, the DSP octamer is almost totally acid insoluble, and therefore, it is necessary to use the Gdn-HCl treatment to terminate the reaction. It is unavoidable to sediment the sample to equilibrium in the presence of RNA, DNA, and the nonhistone proteins.

Analysis on Density Gradients of [^{3}H]Tryptophan-Radiolabeled, Density-Labeled DSP Octamer. A characteristic of the histone sequences is that in all cases the amino acid tryptophan is absent (Isenberg, 1979). It is, therefore, possible to radiolabel cells with [^{3}H]tryptophan and determine whether radiolabeled high molecular weight proteins are found in the dense region of the gradient. If the radiolabeling remains, non-histone proteins must be the source, not cross-linked histones. MSB cells were radiolabeled with [^{3}H]tryptophan in the presence of dense amino acids for 60 min. Nuclei were isolated and digested with micrococcal nuclease, and the solubilized chromatin was depleted of the majority of its non-histone protein by treatment with AG 50W-X4 resin in 0.65 M NaCl, as was done for the data of Figures 4 and 6.

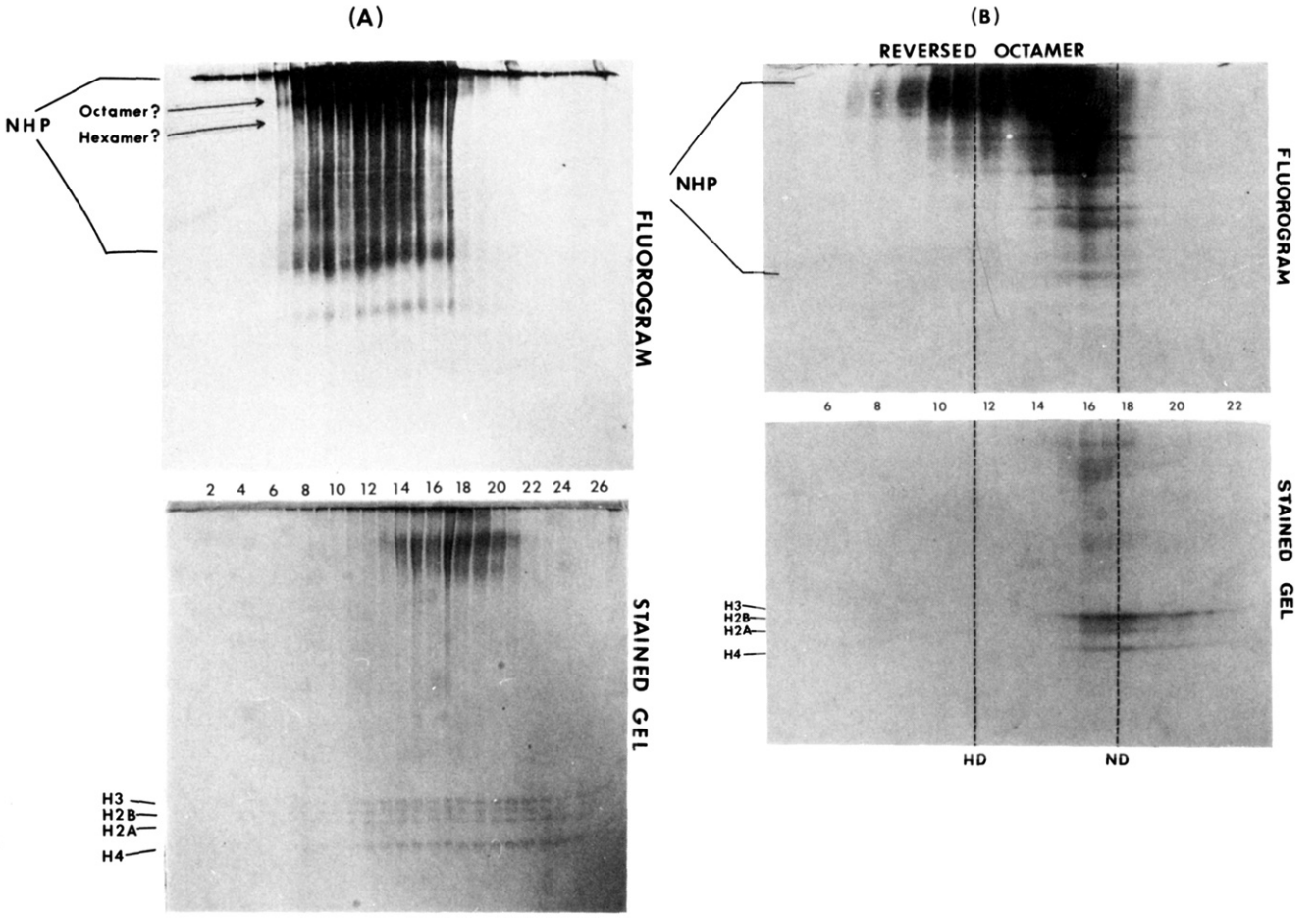

FIGURE 8: Gel electrophoretic analysis of [^{3}H]tryptophan-labeled, density-labeled octamers produced by DSP and fractionated on density gradients. (A) Depleted chromatin was prepared and cross-linked as previously described in the legend to Figure 4 and centrifuged to equilibrium. Fractions were collected, dialyzed, and electrophoresed on 18% polyacrylamide–SDS gels. (B) The octamer region of a duplicate stained gel of (A) was sliced out from fractions 2–23 and the gel slice treated according to condition C. Proteins in the strips were reelectrophoresed a second time on 18% polyacrylamide–SDS gels.

Subsequently, the chromatin was treated with DSP for 30 min, and the reaction was terminated by addition of Gdn-HCl. CsFo and acid-extracted monomer histones from the same nuclei were added, and the sample was centrifuged to equilibrium. Fractions were collected, dialyzed, and electrophoresed on 18% polyacrylamide–SDS gels. As shown in Figure 8A, the distribution of the [^{3}H]tryptophan-labeled proteins in the fluorogram is very similar to the distribution of [^{3}H]lysine-labeled proteins in the fluorogram of Figure 4. Diffuse protein bands are observed with molecular weights of an octamer and hexamer, under conditions where histones would not be radiolabeled. When a reelectrophoretic analysis was performed on the octamer region to determine the distribution of monomer proteins (cross-link destroyed by reduction of disulfide bridge), as shown in Figure 8B, these proteins were similar in molecular weight and location on these gradients as those of the [^{3}H]lysine-labeled proteins of Figures 6 and 7A. These additional data further indicate that non-histone proteins are the source of the dense high molecular weight protein that has previously been misinterpreted as histone octamers.

Discussion

We have described studies utilizing DSP to cross-link radiolabeled density-labeled histones into oligomeric complexes. We have shown that these complexes are of hybrid density and contain both new and old histones. However, it is also observed that non-histone proteins are cross-linked to these complexes, producing a decreased density for the complexes. Such cross-linking is manifested to a large extent in the acid insolubility of these complexes. It therefore is difficult to assign the number of new vs. old histones present in these complexes. Nevertheless, because an asymmetry of distribution in density exists for new histones H2A and H2B vs. new histones H3 and H4 on these gradients, new H3 and H4 must be organized in a more dense complex than new H2A and H2B. These results are consistent with the observations described in the previous paper (Jackson, 1987) that new H3 and H4 deposit as a tetramer and a new H2A and H2B deposit as a dimer. The tetramer and dimer primarily deposit in separate nucleosomes.

We have shown that because of the inability to acid extract and purify the cross-linked products produced by DSP prior to centrifugation on the density gradients, a significant quantity of non-histone protein remains present in the chromatin. This is true even after depletion of the majority of these proteins by a cation-exchange resin. The presence of these proteins produces an effect that can cause one to interpret mistakenly that cross-linked octameric complexes exist in the region of the gradient where density-labeled proteins distribute (fractions 7–9 on these gradients). Density-labeled proteins do indeed distribute in those regions of the gradient. They do so because of the inherent density of the monomeric non-histone proteins synthesized in the presence of the dense amino acids, the inherent density being the same as that of the histones synthesized at the same time in the same cells.

Several laboratories have developed techniques for isolating an enriched fraction of replicative chromatin to determine whether all newly synthesized histones deposit on replicative

DNA. In all cases the conclusion has been made that only new H3 and H4 were selectively deposited on newly replicated DNA. The majority of new H2A and H2B deposited on nonreplicative DNA (Senshu et al., 1978, 1985; Jackson & Chalkley, 1981a,b, 1985; Jackson et al., 1981; Annunziato et al., 1982). Hybrid nucleosomes (both new and old histones in same nucleosome) have also been reported in experiments where cells were labeled with dense amino acids and the labeled nucleosomes fractionated on TEA/metrizamide gradients (Russev & Hancock, 1981). Although all these reports specifically examined histone–DNA interactions, by inference the data are strongly suggestive that new H3 and H4 are organized in nucleosomal structures where new H2A and H2B are much depleted. Other investigators have utilized additional techniques for isolating replicative chromatin and have reported data consistent with these conclusions. However, in those reports the interpretation of the data was that new H3 and H4 were first deposited on new DNA and as the DNA matured 10 min (Worcel et al., 1978) or 8 min (Cremisi & Yaniv, 1980) later into nonreplicated DNA (SV40 replication rate is ~15 min) new H2A and H2B were subsequently deposited in the same nucleosome. Due to the length of time for both pulses (2 or 8 min) and chases, it is not likely that a sequential deposition is the correct interpretation. In addition, these experimental procedures are unable to determine the age of the DNA upon which new H2A and H2B were deposited. It is noteworthy that in vitro histones H3 and H4 have been shown to produce a nucleosomal-type structure in the absence of H2A and H2B, and therefore suggestive of a sequential mechanism (Simon et al., 1978). However, it has also been reported in other in vitro experiments that histones, as cross-linked octameric complexes of H3, H2B, H2A, and H4, efficiently supercoil DNA into a nucleosomal structure (Stein et al., 1977, 1979). It is therefore possible that simultaneous deposition actually occurs. Pulse and chases measured in terms of seconds will be needed to establish whether sequential deposition truly exists in vivo.

These observations taken as a whole are supportive of two separate depositional mechanisms. The first mechanism would relate to deposition at the replication fork, a process that is selective for new H3 and H4 and to a much lesser extent for new H2A and H2B. Thus, a prediction would be that hybrid octamers are present at the replication fork. The data from the previous paper (Jackson, 1987) and this paper provide the first direct evidence that newly synthesized histones are, indeed, organized in hybrid octamers and that H3 and H4 are deposited as tetramers in the nucleosome. The second mechanism relates to deposition presumably away from the replication fork. The histones involved in this deposition are new histones H1, H2A, and H2B. The location in the genome for this deposition is yet to be established, although preliminary data suggest that the exchange of new H2A and H2B with old H2A and H2B in a nucleosome is not a generalized phenomenon but must be restricted to 5–10% of the genome. This conclusion is based on the observation that, once a specific subset of new H2A and H2B deposits on newly replicated DNA (~30% new H2A and H2B), these histones are not detectably released from that DNA throughout the cell cycle (Jackson & Chalkley, 1981b, 1985). If a generalized exchange of these histones were to occur, then these deposited histones should have been released from the DNA. However, if this exchange were restricted to 5–10% of the replicated genome, the procedures would not have detected this low percentage of release from DNA. We suggest that regions of the genome involved in transcriptional activity might be the source of this dynamic exchange of histones H2A and H2B.

The density labeling of histones and subsequent cross-linking with DSP into oligomeric complexes is a procedure that has been used to demonstrate that conservative deposition of histones occurs at the replication fork (Leffak et al., 1977; Leffak, 1983, 1984). In these procedures, the data were interpreted as indicating that, when molecular weight complexes of octamer–octamer (16-mer) were analyzed on density gradients, the density of these complexes was the same density as the putative 100% dense octamer. Therefore, the conclusion was made not only that all new histones formed 100% new octamers but that the new octamers tandemly repeated on the same daughter strand (conservative deposition). The data we have described in this paper suggest that this conclusion may also be a misinterpretation. Additional support for conservative deposition has come from experiments that utilized nuclease digestion of SV40 DNA, synthesized in the presence of cycloheximide (Seidman et al., 1979). However, these same experiments have been repeated by a separate group (Cusick et al., 1984), and the data obtained supported a random deposition. A number of additional investigators have also suggested, using varying procedures that fractionate chromatin on the basis of histone–DNA interactions, that histones deposit randomly at the replication fork (Jackson et al., 1975; Freedlander et al., 1977; Fowler et al., 1982; Annunziato & Seale, 1984; Jackson & Chalkley, 1985; Sogo et al., 1986). Experiments are in progress in our laboratory to test whether the octamer–octamer (16-mer) produced from density-labeled cells (formaldehyde cross-linked) actually maintains a density similar to that of the octamer alone.

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Thermodynamic Investigation of Monoclonal Antibody Alkylated Nucleoside Interaction as a Model for Epitope Recognition on Nucleic Acids†

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ABSTRACT: The objective of this investigation is examination of the dominant forces that govern complex formation between a series of monoclonal antibodies directed against O^6-ethyl-2′-deoxyguanosine. These monoclonal antibodies (coded as ER-6, ER-3, and EM-1) provide the basis for a thermodynamic comparative evaluation of the potentially different forces that stabilize the various monoclonal antibody (mAb) alkylated nucleoside complexes. The binding affinities of ER-6, ER-3, and EM-1 are measured in terms of specific (O^6-ethyl-2′-deoxyguanosine, or O^6-EtdGuo) and nonspecific (O^6-methyl-2′-deoxyguanosine, or O^6-MedGuo) antigens, under a variety of experimental conditions, including pH, sodium chloride addition, 1-propanol addition, and temperature, via a nitrocellulose affinity filter assay. The binding isotherms were analyzed via a least-squares routine fit to a two independent binding sites model. The temperature dependence of the van't Hoff enthalpies for the specific O^6-EtdGuo interaction ranges from −15.18 to −18.60 kcal mol^{-1}, while for O^6-MedGuo the range was extended from −2.72 to −20.66 kcal mol^{-1}. The standard and unitary entropies were negative for those mAb interactions with O^6-EtdGuo as well as for ER-6/O^6-MedGuo complex formation. However, it was found that the interactions between ER-3 and EM-1 with O^6-MedGuo led to decidedly positive entropic values. These results indicate two different dominant forces at work in complex stabilization. The interaction of the three mAb's with their specific antigen, as well as ER-6/O^6-MedGuo interaction (nonspecific), may well be controlled by van der Waals type forces, while ER-3 and EM-1 interactions with nonspecific antigen imply formal charge neutralization electrostatics as the dominant force.

The interactions between protein and DNA are recognized as important events in regulation of gene expression, DNA repair, and other biological processes. An understanding of the recognition mechanism that facilitates binding reactions between proteins and ligand(s) requires knowledge of the forces (hydrophobic, electrostatic, van der Waals, etc.) that govern the interactions. Hybridoma technology (Kohler & Milstein, 1976) has provided a means of obtaining specific monoclonal antibodies (mAb's) against alkylated nucleosides (Poirier, 1981; Baan et al., 1982; Adamkiewicz et al., 1982), thereby permitting thermodynamic and other biophysical studies on individual (nucleoside) epitope–mAb interactions. We have chosen a series of three monoclonal antibodies, each reactive against the alkylated nucleoside O^6-ethyl-2′-deoxyguanosine (Rajewsky et al., 1980; Muller & Rajewsky, 1981; Adamkiewicz et al., 1982), for a comparative thermodynamic evaluation of the forces that stabilize the mAb-alkylated nucleoside complex. Using this novel protein–DNA epitope interaction model, we are investigating whether structure–function relationships of this recognition phenomena can be

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