Kon, O. L., & Spelsberg, T. C. (1982) *Endocrinology (Baltimore) 111*, 1925–1936.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Liarakos, C. D., Rosen, J. M., & O'Malley, B. W. (1973) *Biochemistry 12*, 2809–2816.
McKnight, G. S., Pennequin, P., & Schimke, R. T. (1975) *J. Biol. Chem. 250*, 8105–8110.
Means, A. R., Comstock, J. P., Rosenfeld, G. C., & O'Malley, B. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1146–1150.
Ogle, T. F. (1987) *Endocrinology (Baltimore) 121*, 28–35.
Oka, T., & Schimke, R. T. (1969) *J. Cell Biol. 41*, 816–831.
O'Malley, B. W., McGuire, W. L., Kohler, P. O., & Korenman, S. G. (1969) *Recent Prog. Horm. Res. 25*, 105–160.
Palmiter, R. D., Cattin, G. H., & Cox, R. F. (1973) *Cell Differ. 2*, 163–170.
Palmiter, R. D., Moore, P. B., Mulvihill, E. R., & Emtage, S. (1976) *Cell (Cambridge, Mass.) 8*, 557–572.
Palmiter, R. D., Mulvihill, E. R., Shepherd, J. H., & McKnight, G. S. (1981) *J. Biol. Chem. 257*, 7910–7916.
Perry, B. N., & Lopez, A. (1978) *Biochem. J. 176*, 873–883.
Pikler, G. M., Webster, R. A., & Spelsberg, T. C. (1976) *Biochem. J. 156*, 399–408.
Ross, P., & Ruh, T. S. (1984) *Biochim. Biophys. Acta 782*, 18–25.
Ruh, M. F., Singh, R. K., Mak, P., & Callard, G. V. (1986) *Endocrinology (Baltimore) 118*, 811–818.
Shyamala, G., Singh, R. K., Ruh, M. F., & Ruh, T. S. (1986) *Endocrinology (Baltimore) 119*, 819–826.
Singh, R. K., Ruh, M. F., Butter, W. B., & Ruh, T. S. (1986) *Endocrinology (Baltimore) 118*, 1087–1095.
Spelsberg, T. C. (1983) *Biochemistry 22*, 13–21.
Spelsberg, T. C., Steggles, A. W., & O'Malley, B. W. (1971) *J. Biol. Chem. 246*, 4188–4197.
Spelsberg, T. C., Webster, R. A., & Pikler, G. M. (1976) *Nature (London) 262*, 65–67.
Spelsberg, T. C., Knowler, J., Boyd, P. A., Thrall, C. L., & Martin-Dani, G. (1979) *J. Steroid Biochem. 11*, 373–388.
Spelsberg, T. C., Littlefield, B. A., Seelke, R., Martin-Dani, G., Toyoda, H., Boyd-Leinen, P., Thrall, C., & Kon, O. L. (1983) *Recent Prog. Horm. Res. 39*, 463–517.
Spelsberg, T. C., Gosse, B., Littlefield, B., Toyoda, H., & Seelke, R. (1984) *Biochemistry 23*, 5103–5112.
Tokarz, R. R., Harrison, R. W., & Seaver, S. S. (1979) *J. Biol. Chem. 254*, 9178–9184.
Towbin, H., Staehlin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Toyoda, H., Seelke, R. W., Littlefield, B. A., & Spelsberg, T. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4722–4726.
Tsai, Y-H., Sanborn, B. M., Steinberger, A., & Steinberger, E. (1980) *J. Steroid Biochem. 13*, 711–718.
Tuohimaa, P. J., Jussila, M., & Haapala, O. (1976) *J. Steroid Biochem. 7*, 1103–1108.

Deposition of Newly Synthesized Histones: Hybrid Nucleosomes Are Not Tandemly Arranged on Daughter DNA Strands†

Vaughn Jackson

Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226

Received May 15, 1987; Revised Manuscript Received October 1, 1987

ABSTRACT: Density labeling procedures have been utilized to study the dynamics of histone–histone interactions in vivo. Cells were labeled for 60 min with dense amino acids, and the label was chased for up to 22 h (two replication events for these cells). Nuclei were isolated and treated with formaldehyde to stabilize the histone–histone interactions with a covalent cross-link that produces an octameric complex of two each of H3, H2B, H2A, and H4. This complex was then extracted from the DNA and analyzed on density gradients. The results indicate that new H3,H4 deposits as a tetramer and does not dissociate in the subsequent chases. New H2A,H2B deposited as a dimer and also does not dissociate in subsequent chases. These new histones form hybrid octamers with old histones. On the basis of the new:old ratio in the hybrid octamers, we propose that additional old H2A,H2B from elsewhere in the genome interacts with tetramers of new H3,H4 to form the newly synthesized nucleosomes. It is also observed that 5% of the cross-linked complexes produced by formaldehyde are octamer–octamer (dioctamer). Upon analysis of the density of the dioctamer, the hybrid octamers were found adjacent to octamers that were homogeneous with respect to containing normal density histones. Control experiments are presented to demonstrate that the octamer–octamer cross-links are a product of intrastrand and not interstrand interactions between nucleosomes. These same control experiments also indicate that these procedures do not induce histone exchange during the preparative procedure prior to density gradient analysis. The significance of these results with regard to the dynamics of histone–histone interactions at the replication fork and the potential role in the maintenance of differentiation is discussed.

The basic repeat unit in chromatin is the nucleosome. Each nucleosome is composed of two each of histones H3, H2A, H2B, and H4 [see review of McGhee and Felsenfeld (1980)] organized in an octameric structure upon which two supercoils of DNA [approximately 200 base pairs (bp)] are associated. Histone H1 is thought to bridge one nucleosome to another in the repeat structure. There is increasing evidence that active genes have a more diffuse periodic structure than repressed genes. The mechanisms involved in establishing this more open

† This work was supported by National Institutes of Health Grant CA35829.

0006-2960/88/0427-2109$01.50/0

structure and in the maintenance of that conformation after DNA replication are largely unknown and an area of extensive investigation. We have focused our attention in this report to the mechanisms involved in histone deposition at the replication fork. Regulation of this deposition may actively define whether a gene is to be maintained in an active or inactive state.

A controversy exists as to whether the deposition of histones is regulated with regard to the newly replicated daughter strands. Seidman et al. (1979) reported data which suggested that during replication, parental histones segregated to the daughter strand where DNA was synthesized continuously (leading strand synthesis). The hypothesis is that during replication these parental histones do not release from the DNA and therefore maintain the same open or closed conformation as the prereplicative gene. Therefore, if replication origins were to exist within genes (presumably in introns), the bidirectional DNA synthesis from these sites would produce daughter strands each of which would contain DNA synthesized continuously and the associated parental histones. These parental histones would serve as a template to define the postreplicative distribution of newly synthesized histones on that portion of the genes synthesized discontinuously (lagging strand). This is an extremely popular concept in textbooks.

In contrast, a number of investigators have suggested, using varying procedures, that histones deposit randomly at the replication fork (Jackson et al., 1975; Freedlander et al., 1977; Russev & Hancock, 1982; Annunziato & Seale, 1984; Cusick et al., 1984; Jackson & Chalkley, 1985b; Pospelov et al., 1982; Sogo et al., 1986). The implication from these latter studies is that parental histones do not provide a template upon which the postreplicative distribution of new histones is determined. This distribution would be established by a yet undefined mechanism (see Discussion).

A common feature of all these previous studies is that the experimental protocols involved isolation of replicative DNA and analysis of the associated histones. Both mechanisms (conservative or random) can also be tested on the basis of experimental procedures which analyze histone–histone interactions. Conservative deposition predicts that all newly synthesized histones would be deposited as a single group in a nucleosome and that adjacent nucleosomes would also consist of exclusively new histones. Therefore, if newly synthesized histones were density labeled and the histones within the nucleosome cross-linked together into an octameric group, the density of the group would remain the same as the density of the individual newly synthesized histones. Also, cross-linking of the octamer in one nucleosome to the octamer of the adjacent nucleosome on the same daughter strand would produce a product (dioctamer) which would maintain the same density as the octamer alone. In a previous report (Jackson, 1987a), such procedures were used to determine whether the newly synthesized octamers at the replication fork are homogeneous with respect to newly synthesized histones. Those experiments indicated that they were not. Newly synthesized H3,H4 (tetramer) was found in an octameric complex with two each of parental H2A,H2B. Newly synthesized H2A,H2B (dimer) was found in an octameric complex with one each of parental H2A,H2B and two each of parental H3,H4. In this paper, the stability of these newly synthesized octamers during the cell cycle is described. The H3,H4 tetramer and H2A,H2B dimer were found to be remarkably stable with no detectable dissociation for up to 3 generations of cell growth. In addition, the density of the hybrid octamers was determined when cross-linked to adjacent octamers (referred to as dioctamer). The results show that the density of the dioctamers decreases by a factor of 2 relative to the density of the octamer alone. These results suggest that the hybrid octamers are primarily adjacent to octamers consisting entirely of parental histones. The significance of these results with regard to chromatin assembly and stability is discussed.

Materials and Methods

Cell Growth and Density Labeling. Chicken leukemic cell line transformed by Marek's virus (MSB)[1] cells were grown in 10% fetal calf serum with medium of 1:1 Dulbecco's MEM–RPMI-1640 and supplemented with 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (growth medium). Cells were grown in suspension culture without a CO_2 atmosphere. Exponential growth was (0.5–1.5) $\times 10^6$/mL with a 9–10-h cell cycle. One liter of cells was concentrated into 200 mL of Dulbecco's medium (lacking all amino acids) and incubated 20 min at 37 °C. This process depleted internal amino acid pools to increase the specific activity of density label in the subsequent pulse. Cells were then pelleted and resuspended into 10 mL of Dulbecco's medium (containing carrier-free ^{15}N-, ^{13}C-, and ^{2}H-labeled amino acids; Merck MB1808) and incubated at 37 °C for 15 min. Also included in the 10 mL of Dulbecco's medium were the amino acids tryptophan, cysteine, 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 [^{3}H]lysine (70 Ci/mmol, Amersham) and [^{3}H]arginine (50 Ci/mmol, Amersham) was added and incubation continued for 45 min. An aliquot of the cells was pelleted and quick-frozen (zero chase). The remaining cells were washed twice with growth medium and resuspended in the same medium at a density of 0.5×10^6 mL. At chase points of 4, 12, and 22 h, aliquots of cells were pelleted and quick-frozen.

Uniformly density-labeled cells were prepared by incubating cells for 3 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 and 50 μCi each of [^{3}H]lysine and [^{3}H]arginine per 10 mL of labeling medium. After incubation for 30 h, cells were harvested and frozen for later isolation of nuclei.

Preparation of Nuclei and Histone Octamer for Density Centrifugation. Nuclei were isolated from the frozen cells by washing 3 times in 1% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0, and then washed twice in the same buffer in the absence of detergent. Ten percent of the nuclei were set aside for acid extraction to isolate monomer histones (density markers). These nuclei were adjusted to a final concentration of 0.4 N H_2SO_4 and sonicated for 1 min on a Branson sonifier. This sample was centrifuged at 27000*g* 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 nuclei were suspended into 12 mM triethanolamine (TEA)/12 mM Na_3PO_4, pH 9.1 (TEA/PO_4), at 100 μg/mL DNA concentration and made 1% in formaldehyde. After incubation for 8 h at 4 °C, the chromatin was centrifuged to

[1] Abbreviations: MSB, chicken leukemic cell line transformed by Marek's virus; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEA, triethanolamine; Gdn-HCl, guanidine hydrochloride; CsFo, cesium formate; SDS, sodium dodecyl sulfate; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

remove the excess formaldehyde. The cross-linked histone complexes (histone octamer) are selectively removed from non-histone protein by acid extraction of the chromatin pellet. This pellet was adjusted to a final concentration of 0.4 N H_2SO_4 from a 4 N stock and immediately sonicated twice for 30 s at 4 °C. The solution was centrifuged at 20000*g* for 10 min, and the supernatant, containing the octamer, was dialyzed against two changes of 0.4 N H_2SO_4 and one change of 0.02 N H_2SO_4 over a 36-h period at 4 °C. The treatment with acid terminates the cross-linking, and the extensive dialysis removes the unreactive formaldehyde. This solution was then combined with the previously acid-extracted monomer histones and added to 2.4 g of guanidine hydrochloride (Gdn-HCl), 0.82 g of cesium formate (CsFo), 100 μL of 3 M Tris, pH 8.0, and the volume was adjusted to 4.0 mL. Centrifugation was at 54000 rpm for 96 h at 11 °C on an SW60Ti rotor; 150-μL fractions were collected, made to a final concentration of 0.4 N H_2SO_4, and directly dialyzed against 0.2 N H_2SO_4 and 5 mM 2-mercaptoethanol on a BRL (Bethesda Research Labs) 28-slot dialysis apparatus. Samples were then precipitated in 7 volumes of acetone at −20 °C overnight and redissolved by sonication into 0.5% sodium dodecyl sulfate (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 blue, 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 (1979). When reversal of cross-link (Jackson, 1978) was required for the proteins in the gel, the stained gel was cut into a strip containing the octamer or dioctamer region and incubated in two changes of 100 mL each of 1% SDS and 100 mM Tris, pH 6.8 (reversal solution), over a 90-min period. The strip was then heated at 100 °C for 30 min in fresh reversal solution containing 0.5 M 2-mercaptoethanol and reincubated for 60 min in three changes of reversal solution to remove mercaptoethanol. 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.

Preparation of Nuclease-Solubilized Chromatin. Nuclei were isolated as previously described and suspended in 10 mM $MgCl_2$, 10 mM Tris, pH 8.0, and 1 mM $CaCl_2$ at 1 mg/mL DNA. Staphylococcal nuclease (P-L Biochemicals) was added at 40 units/mL, and the nuclei were incubated for 10 min at 37 °C. The reaction was terminated by addition of ethylenediaminetetraacetic acid (EDTA) to a concentration of 20 mM; the nuclei were dialyzed against 1 mM EDTA–TEA/PO_4, pH 9.1, for 12 h and centrifuged at 5000 rpm for 5 min. The supernatant (nuclease-solubilized chromatin) could then be directly cross-linked with 1% formaldehyde for 8 h at 4 °C, acid-extracted, and dialyzed as previously described. To prepare mononucleosomes, the nuclease-solubilized chromatin was adjusted to 0.65 M NaCl and incubated with preequilibrated Bio-Rad AG50W-X4 resin for 60 min at 4 °C (Leffak, 1983). This procedure removes the majority of non-histone protein and histone H1. The resin was removed by centrifugation at 5000*g* for 10 min, and the supernatant (depleted chromatin) was dialyzed against 10 mM Tris and 0.2 mM EDTA, pH 8.0, for 12 h at 4 °C. The chromatin was adjusted to 3 mM $CaCl_2$ and staphylococcal nuclease added for redigestion to mononucleosomes. Reactions were terminated by addition of EDTA to 10 mM, and the samples were dialyzed against 1 mM EDTA–TEA/PO_4, pH 9.1, for 12 h. These samples were then treated with 1% formaldehyde; the cross-linked histones were isolated and electrophoresed as previously described.

RESULTS

Density Labeling and Isolation of Cross-Linked Histone Complexes. MSB cells were labeled for 60 min with dense amino acids and [^{3}H]lysine and [^{3}H]arginine. A portion of the cells was frozen for storage purposes (zero chase) and the remainder resuspended into fresh medium and chased for 4, 12, and 22 h. At each time point, a portion of these cells was frozen for later analysis. Since MSB cells have a cell cycle of 9–10 h, the 4-h time points represent cells that are in the growth phase and have not cycled through to the next replication event. At the 12- and 22-h time points, the cells have completed one and two replication events, respectively. Nuclei were isolated from the cells at each time point. Ten percent of the nuclei were acid-extracted to obtain density-labeled, radiolabeled monomer histones (fraction B of Figure 1). These histones are used as standards in the density gradients. The remaining nuclei were cross-linked with 1% formaldehyde at pH 9 for 8 h (Jackson, 1987a; also see Materials and Methods). These nuclei were then acid-extracted to isolate the cross-linked complex of histones (octamer) within the nucleosome. This octameric complex is fraction A of Figure 1 and is applied to the density gradients. The acid-insoluble nuclear material was resuspended in 1% SDS and incubated at 37 °C for 2 days to reverse protein–DNA cross-links (Jackson, 1978). These samples are fraction C of Figure 1. Fraction C is shown to illustrate that in this cross-linking procedure with formaldehyde, not only histone–histone (octamer) but also histone–DNA cross-links are produced. These latter cross-links dramatically decrease the formation of octamers by competing for the N-terminal regions of the histones. Approximately half of these regions cross-link to DNA and the remainder to each other to form the octameric complex (unpublished observations). In Figure 1 are also shown molecular weight markers which establish that the cross-linked product of these histones in the nucleosome has a molecular weight consistent with an octameric complex (110K). On the basis of the cell count and the decrease in specific activity of radiolabel with increasing chase times (Figure 1), we estimate the cell cycle to be 9 h for this experiment.

Density Gradient Analysis of Histone Complexes (Octamer). To determine the distribution of newly synthesized histones in the nucleosomes relative to parental histones, the density of the octamers was determined on cesium formate density gradients. Monomer histones were also added to these gradients to serve as markers for both normal density and fully dense positions. The normal density is reflected in the distribution of the bulk of the protein (stained gel of isolated fractions) and the fully dense position as the radiolabeled, density-labeled histones (as seen in the fluorogram). Variability in this fully dense position can potentially occur as it is dependent on the level of isotope incorporated in the initial pulse and the subsequent turnover and reutilization of label in the subsequent chases. Therefore, by mixing fraction A (octamer) and fraction B (monomer) from nuclei of the same time point, the markers automatically correct for these vari-

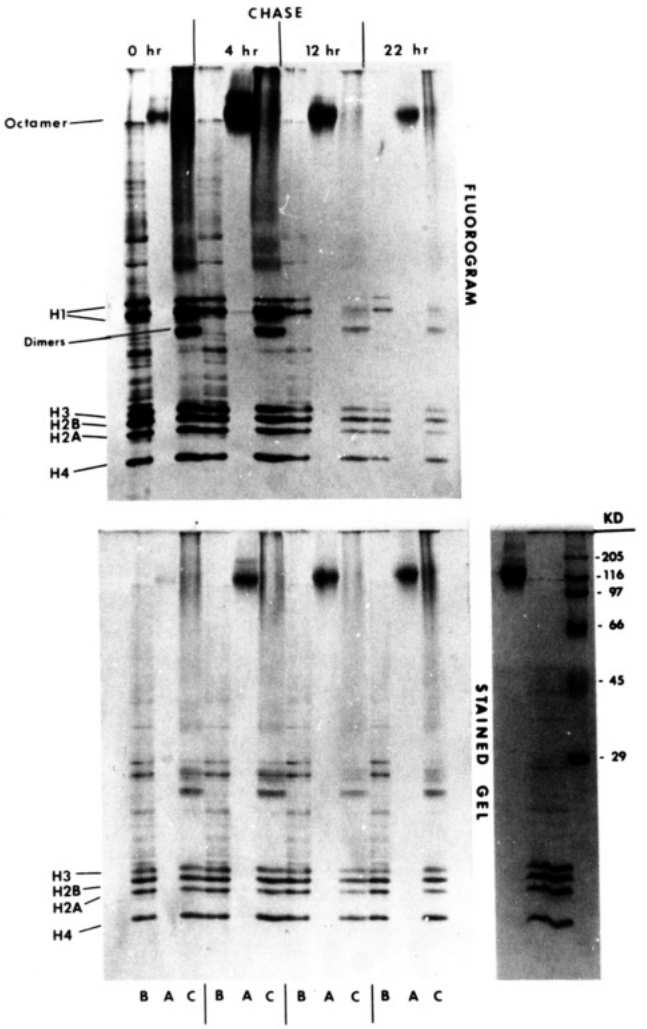

FIGURE 1: Fractions of histones used for density gradient analysis of the stability of histone–histone interactions. MSB cells were pulsed for 60 min with dense amino acids and chased for 4, 12, and 22 h. Nuclei were isolated and 10% acid-extracted to isolate monomer histone (fraction B), and the remainder was treated with 1% formaldehyde for 8 h. This sample was also acid-extracted to isolate the cross-linked octamer complex (fraction A). The acid-insoluble pellet of the cross-linked nuclei was incubated at 37 °C for 2 days in 1% SDS and 100 mM Tris, pH 8, to reverse the cross-link between histone and DNA so that these histone complexes (fraction C) can be analyzed on 18% SDS–PAGE gels. The fluorogram indicates the radiolabel present in the isolated histones during the pulses and chases. A 5-fold less amount of the 0-h octamer (fraction A) was applied to the gel to provide a direct comparison of radioactive intensity with the octamer in the 22-h chase (fraction A). The decrease in specific activity of the histone octamer and monomer histones during the chases is consistent with the cell count and establishes the cell cycle to be 9 h. Sigma molecular weight size markers (MW-SDS-200) are shown to establish the apparent molecular weight of the cross-linked histone complex of fraction A. See also Figure 5 for molecular weight analysis.

abilities and allow for a direct determination of relative amounts of new and old histone in these octamers. These procedures have been used to analyze the distribution of new vs old histone in the octamer during a 60-min pulse (Jackson, 1987a). Those experiments indicated that new H3,H4 deposited as a tetramer and was associated primarily with old H2A,H2B in the octamer. New H2A,H2B deposited as a dimer in an octamer containing another dimer of old H2A,-H2B and two each of old H3,H4. In Figure 2, the stability of these octamers after a 4-h chase was determined. The stained gel ("1st gel") illustrates the distribution of the bulk of the protein from the density gradient and therefore the normal density position for proteins. As shown in Figure 2,

Table I: Percent Density Shift[a]

	octamer		dioctamer	
chase time (h)	H2A,H2B	H3,H4	H2A,H2B	H3,H4
0	24[b]	45[b]	11	22
4	32	55	17	27
12	29	47	11	21
22	24	42	11	21

[a] Example calculation for 4-h chase of Figure 3: for octamer, (16.5 − 11.25)/(16.5 − 7) = 55% for H3,H4 and (16.5 − 13.5)/(16.5 − 7) = 32% for H2A,H2B; for dioctamer, (16 − 13.5)/(16 − 7) = 27% for H3,H4 and (16 − 14.5)/(16 − 7) = 17% for H2A,H2B. [b] Data taken from Jackson (1987a).

fraction 16.5 is the midpoint of the Gaussian distribution for the unlabeled monomer histones and octamer. Fraction 7 is the midpoint of the Gaussian distribution for radiolabeled, density-labeled monomer histones H3, H2B, H2A, and H4 (fluorogram of Figure 2). To identify the individual histone types in the octamer, the region indicated by the dotted line was removed and after reversal of the cross-link (Jackson, 1978) reelectrophoresed in the same dimension. As shown in the stained gel of Figure 2 ("OCTAMER REVERSED"), histones H3, H2B, H2A, and H4 are distributed equally in each fraction with the midpoint for the Guassian distribution at 16.5 (vertical dotted line). However, the distribution for the newly synthesized, density-labeled histones is not equivalent in each fraction. Histones H3 and H4 are distributed asymmetrically more dense relative to new H2A and H2B. These data have been analyzed densitometrically, and as shown in Figure 3, two separate Gaussian distributions are observed for H3,H4 as compared to H2A,H2B. There is little indication of overlap of the type where new H3, H4, H2B, and H2A are in the same octamer. Such an overlap would be indicated by shoulders on these Gaussian curves. From the data of Figure 3, the midpoint for the Gaussian distribution of H3,H4 is found to be fraction 11.25. As shown in the calculations of Table I, the density shift for H3,H4 is therefore 55% and indicates that half the histones in these octamers are new histones. As H3 and H4 are the new histones in these octamers, we conclude that a tetramer of new H3,H4 has deposited with two each of old H2A,H2B. For the octamers containing new H2A,H2B, the density shift is 32%, or approximately two of eight histones are present as new. Therefore, a dimer of new H2A,H2B has deposited into an octamer where the remaining six histones were old. As these density distributions for both sets of histones are similar to the 60-min pulse (Jackson, 1987a), we conclude that the new H3,H4 tetramers and new H2A,H2B dimer do not dissociate during the 4-h chase or reassociate in a new complex where new H3, H4, H2A, and H2B are in the same octameric complexes.

Stability of H3,H4 Tetramer and H2A,H2B Dimer after Second and Third DNA Replication Cycles. To determine whether dissociation of these histone complexes occurs during the replication of associated DNA, the labeled cells which were chased for 12 and 22 h were prepared and the histone fractions of Figure 1 analyzed on density gradients. As the cell cycle was 9 h for this experiment, these time points represent conditions where one (12 h) and two (22 h) replications have been completed. As shown in Figure 4 ("1st gel"), no detectable change is observed in the specific activity of density label in the monomer histones when compared to Figure 3 ("1st gel"). In all three chase conditions, there is a 9.5 fraction difference in density between the unlabeled histones and labeled histones. These data indicate that turnover and reutilization of label are minimal in these chases. Therefore, it is possible to readily determine the density for the octamers in these chases and

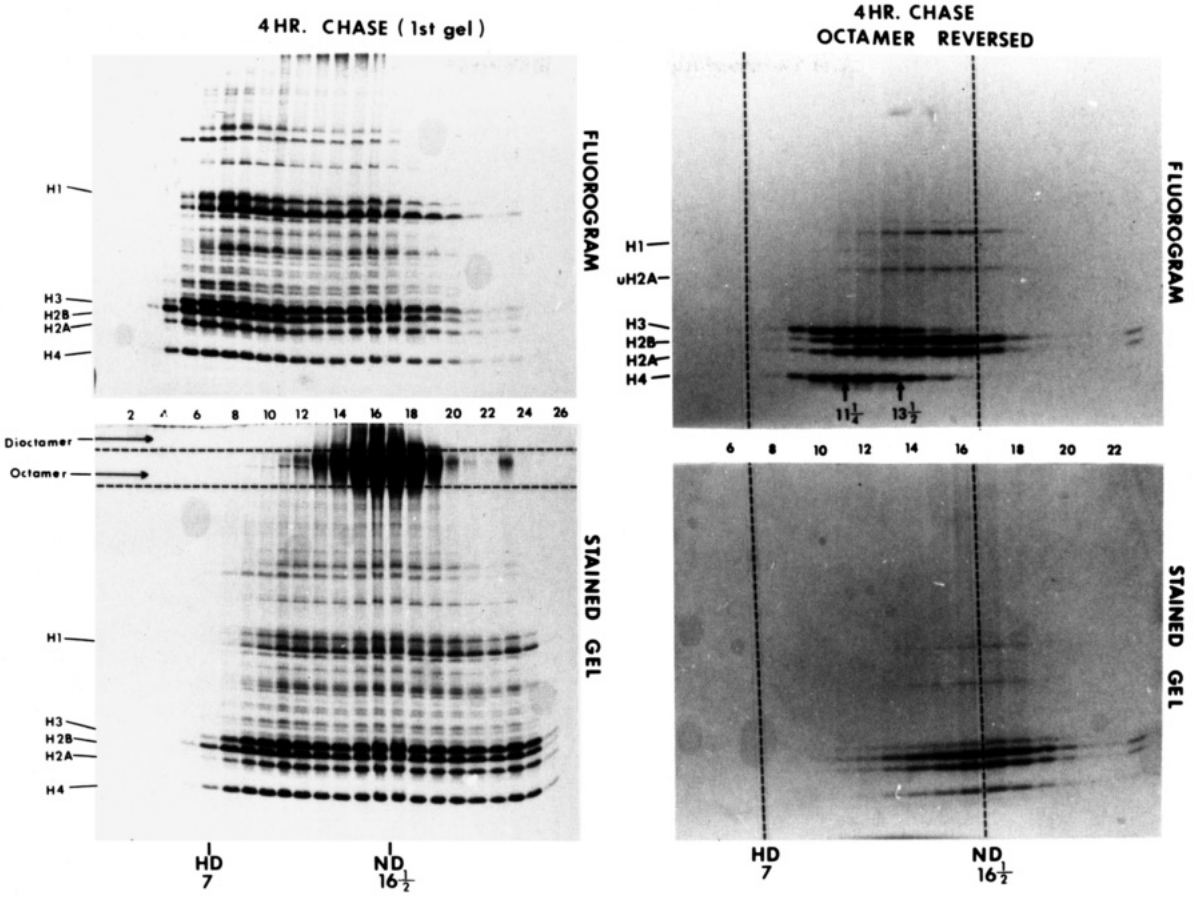

FIGURE 2: Density gradient analysis of the octamer produced from a 60-min pulse with radiolabeled and density-labeled amino acids followed by a 4-h chase. Octamer (fraction A) and monomer histones (fraction B) are combined and sedimented to equilibrium on a CsFo–Gdn-HCl density gradient. Fractions were electrophoresed on an 18% SDS–PAGE gel (left-hand panel), and before fluorography of the stained gel, the octamer region was removed at the position of the horizontal dotted line and the gel strip heated at 100 °C for 30 min (see Materials and Methods). The proteins in the gel strip were electrophoresed in the same dimension, and that gel was stained and fluorographed (right-hand panel of this figure). The vertical dotted line (ND) refers to the normal density of histones. It is the fraction number corresponding to the midpoint of the Gaussian distribution (scanned densitometrically) for the stained proteins in the right panel. The vertical dotted line indicating high density (HD) is determined from the first SDS gel in the left panel. It is derived from densitometric analysis of the radiolabeled monomer histones H3, H2B, H2A, and H4. The total area from all four histones is combined as a single value. This was done even though the individual densities of the monomer histones are different. This can be seen in the fluorogram of Figure 2 (left-hand panel) 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, the total area of these histones is used to give a single average value for each fraction number. The vertical arrows and corresponding fraction numbers indicate the midpoint for the Gaussian distribution of the two sets of histones as determined densitometrically. These are the same fraction numbers shown in Figure 3.

compare these results with those for the pulse and 4-h chase. As shown in Figure 4 ("OCTAMER REVERSED"), the distribution of labeled H3,H4 and H2A,H2B remains unchanged. Even after a 22-h chase, the separate Gaussian distribution of H3,H4 relative to H2A,H2B remains the same as the 4-h chase of Figure 2. Table I tabulates the percent density shifts in these hybrid octamers for the pulse and chases. These data indicate that the H3,H4 tetramer and H2A,H2B dimer appear to be remarkably stable and at first glance would suggest that these complexes remain tightly associated to DNA (as a nucleosome), even during replication.

Density Gradient Analysis of Octamer–Octamer Complexes (Dioctamer). If these complexes remain associated with the DNA being replicated, then potentially these histones could serve as a template for defining an open or closed structure for that gene in the daughter strands. For example, if the parental histone segregates to the strand where leading strand synthesis occurs (conservative deposition), then under circumstances where the replication origin is located within a gene, both daughter strands will receive a tandem copy of parental histones. This model therefore predicts that parental histones segregate with one strand and new histones deposit on the other strand. The observation that octamers of hybrid density are produced during histone deposition is not inconsistent with this model. These octamers may be produced by an interaction with old histones extracted from other regions of the genome other than at the replication fork. The parental histones of the prereplicative nucleosomes may still segregate according to conservative deposition. Therefore, this model would predict that hybrid octamers are tandemly arranged on the other daughter strand.

When nuclei are cross-linked with formaldehyde at pH 9, the major product is the octamer. However, a small percentage (5%) is a cross-linked product of octamer to octamer (see Coomassie-stained gel of left panel in Figure 5). As shown later (Figures 7 and 8), this product does not result from interstrand cross-links but is a result of intrastrand cross-link of the tandemly arranged nucleosomes. Therefore, it is possible to test this model of conservative deposition.

Nuclei were isolated from cells labeled 60 min with dense amino acids and [^{3}H]lysine and [^{3}H]arginine. The nuclei were treated with formaldehyde and the cross-linked complexes isolated by acid extraction. This sample was centrifuged to equilibrium on density gradients and resolved on 12% SDS–

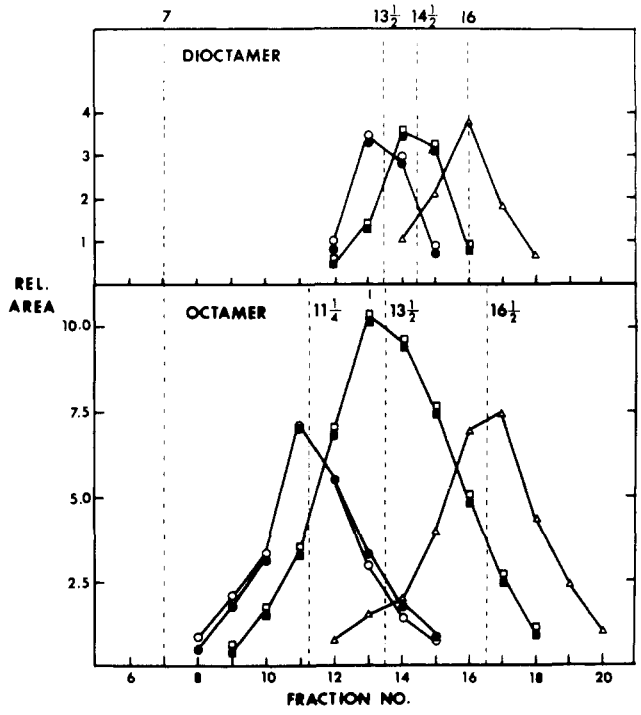

FIGURE 3: Densitometric analysis of the histone distribution of the reversed octamer (Figure 2) and reversed dioctamer (Figure 5) from the 4-h chase. (△) Relative area (REL./AREA) of the four monomer histones in the stained gel; (■) relative area of H2A in the fluorogram; (□) relative area of H2B in the fluorogram; (○) relative area of H3 in the fluorogram; (●) relative area of H4 in the fluorogram. The relative area is calculated by densitometrically determining the area for each histone in each fraction. All integrated values for that histone type are divided by the lowest significant value. When the data are plotted as shown in this figure, the total area under the curve is determined. The vertical dotted line indicates the average value (midpoint) for the density of the proteins in that curve. These density positions are indicated as fraction numbers. Percentage density shifts for the histones are calculated from these values (see Table I).

PAGE gels. The gel was stained with both Coomassie and silver and is shown in Figure 5A. The octamer–octamer product, dioctamer, is clearly resolved from the octamer and therefore can be easily isolated by cutting the gel at the place indicated by the dotted line. As shown in Figure 5B ("DIOCTAMER REVERSED"), the density shift of the labeled histones is significantly less than what was observed for the octamer (Figures 2 and 3). This decrease in density suggests that the hybrid octamers are not tandemly arranged on a replicated daughter strand.

As it is potentially possible that a conservative deposition is established by a rearrangement of the deposited histones in a time frame where the replicated DNA has matured, the dioctamer from a 4-h chase was analyzed. A similar analysis was also done for the 12- and 22-h chases. These latter chases define the distribution of labeled histones in the dioctamer after the associated DNA has been replicated. As shown in Figure 6, the distribution of H3,H4 and H2A,H2B remains significantly less dense than for the octamers (compare Figure 6 with Figures 2 and 4). A densitometric analysis of the dioctamer from the 4-h chase is shown in Figure 3 so that it can be compared with a similar analysis of the octamer from the same 4-h chase. There is a substantially significant decrease in density of the dioctamer relative to the octamer. Table I tabulates the percent density shifts for the dioctamers in the pulses and chases. These numbers are then used to compute the relative change in density between octamer and dioctamer. These data are shown in Table II. In all cases, the relative density shift is approximately 2, indicating that these hybrid

Table II: Relative Density Shift between Octamer and Dioctamer[a]

chase time (h)	H2A,H2B	H3,H4
0	2.2	2.0
4	1.9	2.0
12	2.6	2.2
22	2.2	2.0

[a] Example calculation of data from Table I for 4-h chase: octamer/dioctamer = 55%/27% = 2.0 for H3,H4.

octamers are not tandemly arranged but rather interspersed with octamers consisting of old histones (normal density) on the DNA strands. These data do not support a conservative deposition. The data of Table II are consistent with an ordered mechanism of the type where every other octamer is deposited as a hybrid followed by a normal density octamer. The data are also consistent with a random deposition. Statistically, in a random deposition one would predict that of six dioctamers produced, five of them are of the type hybrid-normal. Only one would be produced which would consist of a hybrid–hybrid complex.

In the cross-linking of octamer to octamer, potentially histone H1 could be involved in the cross-linking. This histone is thought to bridge from one nucleosome to the other, and as there are data suggesting that histone H1 is extensively mobile (Carin & Thomas, 1981; Jackson & Chalkley, 1981a,b), the cross-linking of old H1 to the dioctamer may influence the ultimate density of the complex. However, as shown in Figures 5 and 6, histone H1 is barely detectable in the dioctamer as is the case of the octamer (Figures 2 and 4). Therefore, the predominant cross-linking is between H3,H2B,H2A and H4 in producing the dioctamer. The change in density is a result of the cross-linking of hybrid octamers to octamers with a normal density.

In these experiments, the octamer and dioctamer complexes that have been analyzed are those which were acid soluble and therefore not cross-linked to DNA (fraction A). As shown in Figure 1, approximately 50% of the histones cross-link to DNA (fraction C). Potentially, these histones could represent a subfraction in which the deposition of newly synthesized histones follows a different pathway. Therefore, fraction C for each of the chases has been analyzed on density gradients, and the histones in the octamer and dioctamer region have been characterized. The distribution of density-labeled histones remains the same as described for the acid-soluble histone complexes (data not shown).

Because the cross-linking with formaldehyde was done with nuclei, it is possible that the hybrid-normal dioctamer is caused by interstrand cross-links, due to the compact nature of nuclei. We have repeated the 60-min pulse experiments using nuclease-solubilized chromatin and have observed the same decrease in density for the dioctamer (data not shown). In addition, control experiments will be described which demonstrate that these observations are not due to interstrand cross-links.

Analysis for Artifacts Due to Interstrand versus Intrastrand Cross-Links. In the formation of the dioctamer, if interstrand cross-links were to occur, then naturally the hybrid octamer will be cross-linked to a normal density octamer, as the DNA in these cells would be associated primarily with normal density histones. It is therefore extremely important to establish whether this process occurs. We have chosen two procedures to test for such interstrand cross-links.

In the first case, mononucleosomes were cross-linked with formaldehyde. Theoretically, dioctamers would be produced only by intermononucleosomal cross-links of octamers. Nuclei were isolated from MSB cells that had been labeled for 30 min

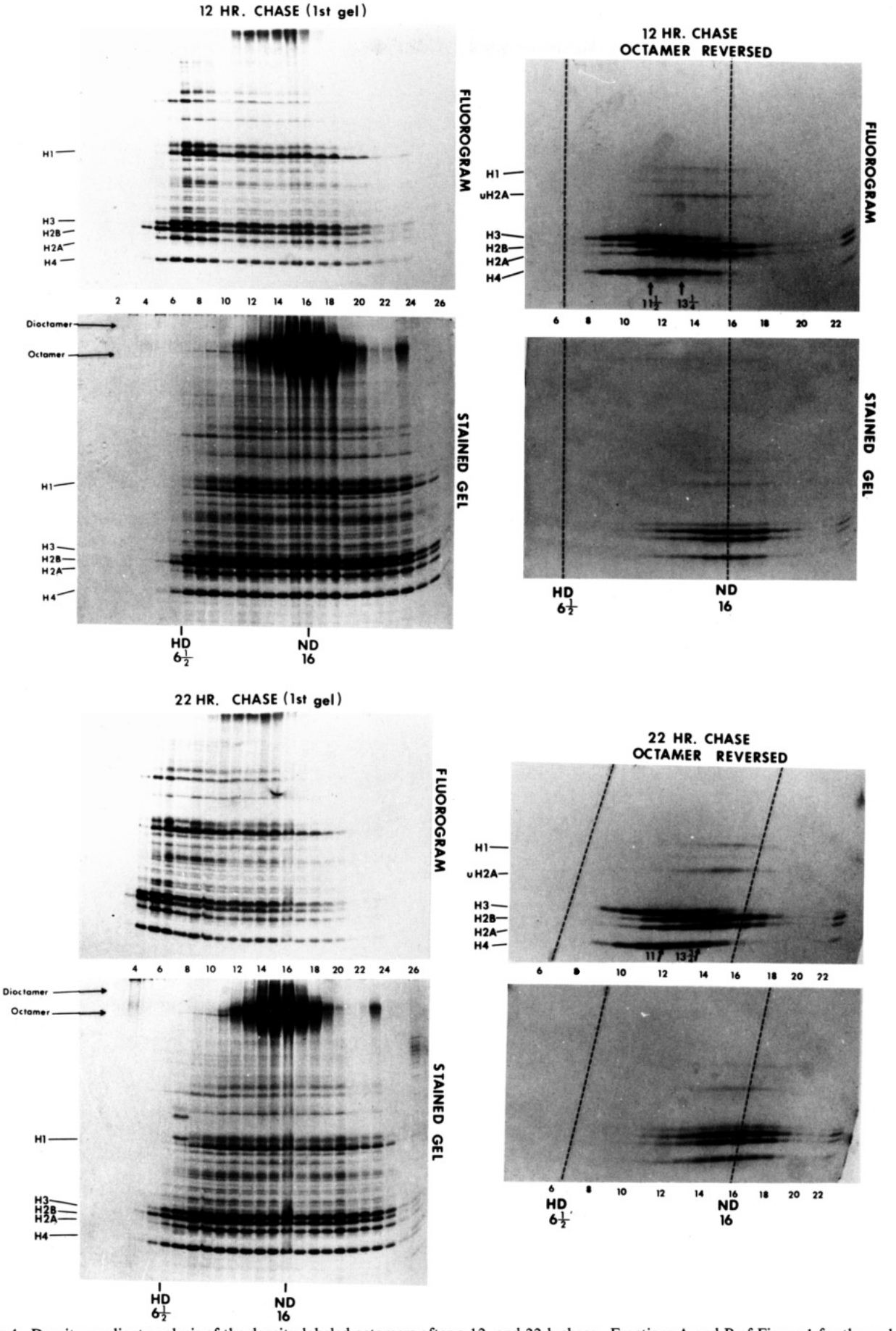

FIGURE 4: Density gradient analysis of the density-labeled octamers after a 12- and 22-h chase. Fractions A and B of Figure 1 for these chases are combined and centrifuged to equilibrium. The 18% SDS–PAGE gel in the left panel ("1st gel") from each chase shows the distribution of the proteins after fractionation and electrophoresis. Gel strips are obtained as described in the legend to Figure 2, the cross-link is reversed, and proteins are reelectrophoresed in the same dimension. These results are in the right panel. To maintain fluorographic intensity with increasing chase times, the gels for the 12-h chase were fluorographed twice as long as the gels for the 4-h chase. The gels for the 22-h chase were fluorographed 4 times longer.

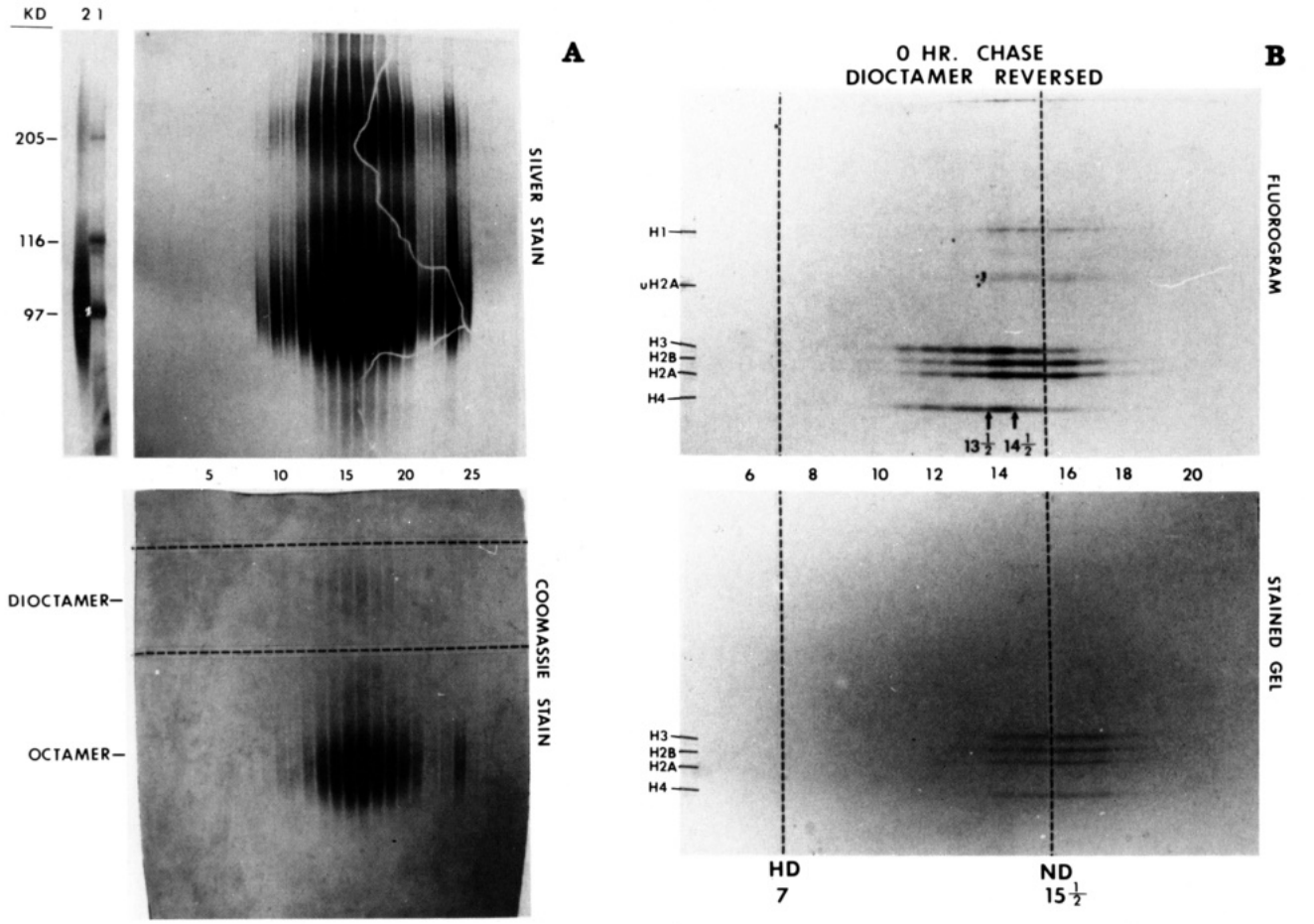

FIGURE 5: Density gradient analysis of the density-labeled dioctamer after a 60-min pulse with dense amino acids. (A) Fractions from the density gradient were electrophoresed on 12% SDS–PAGE to clearly resolve the octamer from dioctamer. Because of the low percentage of dioctamer produced (5%) as seen in the Coomassie-stained gel, this same gel was stained with silver (Wray et al., 1981) to increase sensitivity. (B) A duplicate gel was made, the dioctamer region excised, and the cross-link reversed as previously described. The histones in the gel strip were electrophoresed in the same dimension with the results shown in the right panel. (1) Sigma size markers are shown in the left panel (MW-SDS-200) with the (2) octamer, dioctamer sample. Sizes are measured in kilodaltons ("KD").

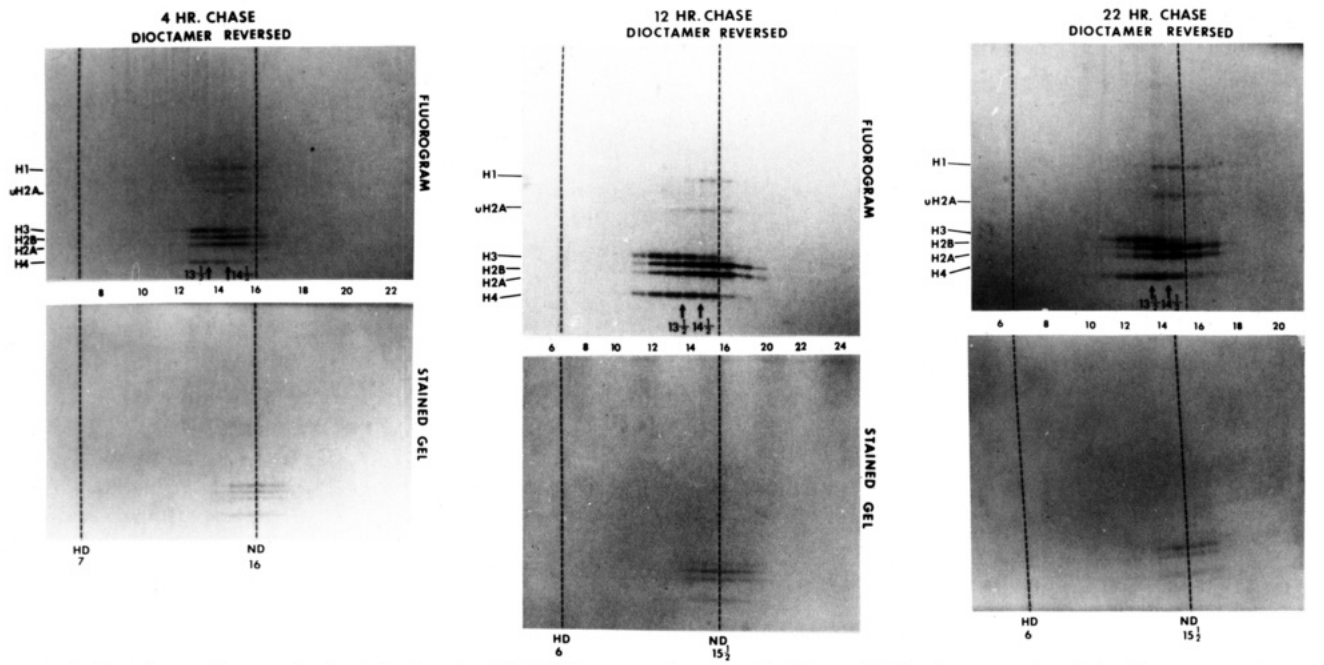

FIGURE 6: Density gradient analysis of the density-labeled dioctamer from the 4-, 12-, and 22-h chase samples. The dioctamer regions of Coomassie-stained gels, similar to the gel in the left panel of Figure 5, were excised and, after reversal of the cross-link, electrophoresed in the same dimension to determine the histones in the dioctamer. The vertical dotted lines (HD and ND) of this figure were determined as described in Figure 2. After densitometric analysis of these histones, the total area of the Gaussian distribution was determined (see Figure 3 for the dioctamer of the 4-h chase). The fraction number at the midpoint of the curve (vertical arrows) defines the average density shift for the two sets of histones. The percent density shift can then be calculated as shown in Table I.

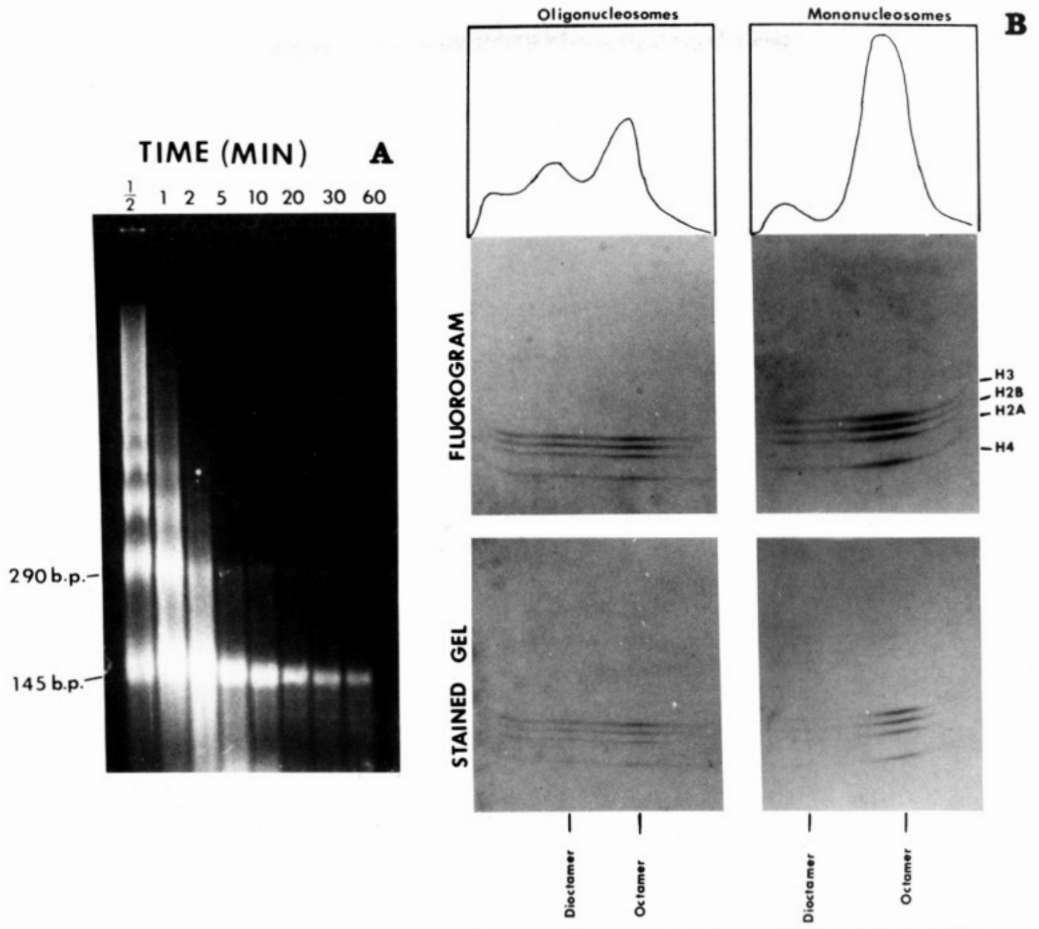

FIGURE 7: Analysis of dioctamer produced in oligonucleosomes and mononucleosomes. Nuclease-solubilized (depleted) chromatin was prepared as described under Materials and Methods. (A) Time course of digestion with staphylococcal nuclease. (B) Two-dimensional analysis of dioctamer produced by formaldehyde cross-linking. The 30-s and 5-min time points were dialyzed against TEA/PO_4, pH 9.1, and then cross-linked with formaldehyde. The cross-linked histone complexes were isolated by acid extraction and electrophoresed in the first dimension. Gel strips of the lanes containing the histones were heated at 100 °C for 30 min as previously described to reverse the cross-link, turned 90°, and electrophoresed in a second dimension. Gels were stained and fluorographed. Densitometric analysis was done on histone H3 of the fluorogram to quantitate the relative distribution of dioctamer to octamer.

with [^{3}H]lysine and [^{3}H]arginine. As shown in Figure 7A, radiolabeled, solubilized chromatin (see Materials and Methods) was redigested with nuclease to produce the 145 bp mononucleosome used for this analysis. Samples were taken at 30 s (oligonucleosomes) and 5 min (mononucleosomes) and treated with 1% formaldehyde for 8 h. These samples were electrophoresed on 18% SDS–PAGE gels. A slice of the stained gel, encompassing the top of the gel through the octamer region, was taken for both samples, and the cross-link was reversed and reelectrophoresed in a second dimension. As shown in Figure 7B, the higher oligomers of histones are much in evidence in the oligonucleosome sample. The higher percentage of dioctamer (20%) seen in this sample, as compared to when cross-linking is done with nuclei (5%; see Figure 5), is due to the formation of close-packed nucleosomes when H1 is removed in 0.65 M NaCl (McGhee & Felsenfeld, 1983). In the mononucleosome sample, the production of dioctamer is barely detectable relative to the octamer. That amount of dioctamer is consistent with the quantity of close-packed dinucleosome which remains in the 5-min time point (Figure 7A).

The second method used to test for interstrand cross-links was to mix density-labeled chromatin (all histones density-labeled) with unlabeled chromatin and subsequently cross-link with formaldehyde. If interstrand cross-links were generated, the density of the dioctamer would be half the density of the octamer. MSB cells were labeled for 3 generations in the presence of dense amino acids and [^{3}H]lysine and [^{3}H]arginine. Nuclei were isolated from these uniformly density-labeled cells and digested with staphylococcal nuclease to produce nuclease-solubilized chromatin (see Materials and Methods). Nuclease-solubilized chromatin was also made from unlabeled cells. These two solubilized chromatins were either mixed (1:10) before treatment with formaldehyde ("MIX BEFORE FIX") or mixed after treatment with formaldehyde ("MIX AFTER FIX") to produce cross-linked histones (octamer and dioctamer). These samples were then centrifuged to equilibrium on cesium formate density gradients. Included in the gradients were monomer histones (mixed in a 1:10 labeled:unlabeled ratio) to serve as density markers on these gradients. After electrophoresis on SDS gels, the octamer and dioctamer regions were sliced out, and the cross-link was reversed. These strips were then reelectrophoresed in the same dimension with the results shown in Figure 8. The "MIX AFTER FIX" gradient indicates that the density of the octamer was the same as the dioctamer. This is shown by the vertical dotted line which passes through fraction 9. This same fraction is the midpoint position in the gradient where the radiolabeled monomer histones are distributed, as seen in the original SDS gel (data not shown). This experiment is an important control for it demonstrates that increases in molecular weights from monomer histone to octamer to dioctamer do not alter the inherent density of these histone complexes as seen on the density gradients. We have previously done

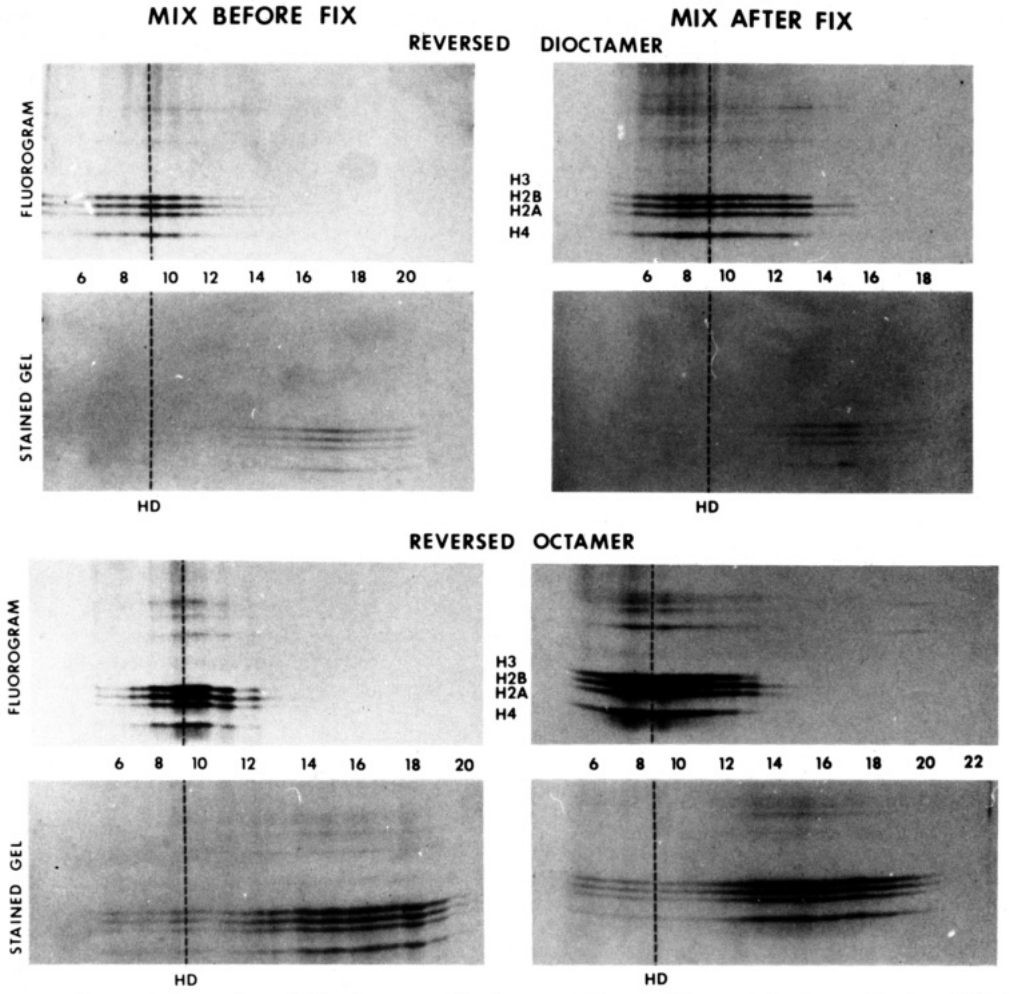

FIGURE 8: Mixing experiments to determine whether interstrand or intrastrand cross-links occur in the production of the dioctamer. When density labeling cells for 3 generations, it was necessary to use undialyzed fetal calf serum to maintain appropriate growth. As a result, the specific activity of density in the histones is less than what is observed when pulse-labeling experiments are done where serum is omitted. In this instance, the high density (HD) position is fraction 9 rather than fraction 7.

reconstitution experiments to further establish this point (Jackson, 1987a). Therefore, the decrease in density seen in the earlier experiments for the dioctamer as compared to the octamer (Tables I and II) must be due to cross-links of density-labeled octamer to normal density octamer. The "MIX BEFORE FIX" data of Figure 8 also indicate that both octamer and dioctamer have the same density. However, in this instance, because the mixing of chromatins occurs prior to fixation, these data indicate that intrastrand cross-links produced the dioctamers. There is no indication that the octamers from 1 part of dense chromatin were cross-linked to octamers in the 10 parts of nondense chromatin. If interstrand cross-links were to occur, they would have produced dioctamers whose density would have focused at fractions 13 and 14. This is clearly not seen. These data also indicate that histone exchange during treatment with formaldehyde does not occur. As seen in Figure 8 ("MIX BEFORE FIX"), there is no asymmetry of density distribution between histones H3,H4 and H2A,H2B, which would be the case if H2A,H2B was able to exchange between nucleosomes during the treatment. As the conditions for fixation used in these experiments are the same as used for the previous analysis (Figures 1–6), the decrease in density of the dioctamer relative to octamer in those experiments must be due to the positioning of hybrid octamers next to normal density octamers on the same DNA strand.

Discussion

It has been previously shown (Jackson, 1986a) that newly synthesized histones form octameric complexes with old parental histones (hybrid octamers). Two principal classes were observed in those studies. One class consisted of newly synthesized H3,H4 (tetramer) associated with parental H2A,H2B. The second class consisted of newly synthesized H2A,H2B (dimer) associated with a parental H2A,H2B (dimer) and two each of parental H3,H4 (probably as a tetramer). Because of the Gaussian distribution of octamers containing new H3,H4 and octamers containing new H2A,H2B as seen on the density gradients (Figure 3), there was no apparent formation of complexes where both new H3,H4 and new H2A,H2B are present in the same octamer. The experiments of the present report indicate that this separate distribution remains unchanged for up to 22 h. The experiment with the 4-h chase of the density-labeled histones shows that the H3,H4 tetramer and H2A,H2B dimer do not dissociate within the cell cycle. These complexes also do not dissociate when associated with DNA that is replicated once (12-h chase) or twice (22-h chase). These observations are consistent with those reported by Prior et al. (1980), where it was observed by fluorescence analysis that H3–H3 interactions are maintained for several generations when these histones are injected into physarum. Therefore, in order to maintain such stable complexes, these histones would appear to be in an octameric complex that remains tightly associated to DNA through many generations. Such a conclusion has been made by Manser et al. (1980) in their studies with fused cells and radiolabeling with [^{3}H]arginine. However, these studies do not preclude possible

transient release of these complexes from DNA. The stability of the octamer in the absence of DNA and more specifically of the H3,H4 tetramer and H2A,H2B dimer in in vitro studies is well established (Isenberg, 1979). It has also been suggested that the octamer is sufficiently stable in vivo to slide along a chromatin fiber prior to deposition on newly replicated DNA (Stein et al., 1985). Thus, if there were a transient release of an octamer at the replication fork, it may not be of sufficient duration to allow dissociation of the complexes prior to redeposition. As discussed below, a number of previous studies indicate that parental histones distribute randomly to the two daughter strands at the replication fork, which therefore suggests that they may be transiently released during replication.

There is extensive data which suggest that histones deposit on DNA randomly (Jackson et al., 1975; Freedlander et al., 1977; Russev & Hancock, 1982; Annunziato & Seale, 1984; Cusick et al., 1984; Jackson & Chalkley, 1985b; Pospelov et al., 1972; Fowler et al., 1982; Sogo et al., 1986). By using a variety of procedures designed to isolate newly replicated DNA and associated proteins, these investigators have concluded that neither old nor new histones selectively segregate to either of the two daughter strands. However, experiments which have analyzed histone–histone interactions using density-labeled amino acids have been interpreted as indicating a conservative deposition where newly synthesized histones selectively segregate to one daughter strand (Leffak et al., 1977; Leffak, 1983, 1974). In these studies, density-labeled new histones were cross-linked with Lomant's reagent. We have repeated those procedures with this reagent and have observed that a misinterpretation exists as to what is truly a histone octamer due to the effect Lomant's reagent has on non-histone proteins [see Jackson (1987b)]. When formaldehyde is used as a cross-linking agent, these problems are avoided, and as observed in this report, tandem arrays of newly synthesized histones on one of the two daughter strands are not observed. Additional support for conservative deposition has come from experiments which involved nuclease treatment of SV40 minichromosomes after synthesis in the presence of cycloheximide (Seidman et al., 1979). However, other investigators have repeated these same experiments and made the opposite conclusion of a random deposition (Cusick et al., 1984). We have also repeated these same procedures and see no indication of a conservative deposition (unpublished observations).

Data in support of a dynamic state for histone–DNA interactions are extensive. The observation that in the G1 growth phase of the cell H2A,H2B continues to be synthesized and deposited on DNA is indicative of such an interaction for these histones in the nucleus (Jackson & Chalkley, 1985a; Wu & Bonner, 1981; Wu et al., 1981, 1982; Waithe et al., 1983; Groppi & Coffino, 1980). In vitro experiments have demonstrated that H1 (Carin & Thomas, 1981) and H2A,H2B (Louters & Chalkley, 1984) are capable of exchanging in chromatin. In vivo studies have also indicated that these same three histones exchange in and out of nucleosomes (Senshu et al., 1978, 1985; Yamasu & Senshu, 1987; Russev & Hancock, 1982; Jackson & Chalkley, 1981a,b, 1985a; Jackson et al., 1981; Annunziato et al., 1982). In these latter studies, this conclusion is based on the observation that when replicated chromatin is isolated by a variety of procedures and the proteins analyzed, not all the newly synthesized histones are associated with the new DNA. Within the limits of detection, all new histone H3 and H4 are found associated with replicated DNA but, in contrast, only 30% of new H2A,H2B and no detectable new H1. Therefore, the majority of new H2A,-H2B,H1 is depositing elsewhere in the genome, thereby displacing old histones of the same type which in turn deposit at the replication fork. It is this interchange of old histone that brings about conditions where the new H3,H4 tetramer almost exclusively forms an octamer with old H2A,H2B. The new H2A,H2B dimer primarily deposits distant to the replication fork. It has been suggested that regions of the genome involved in transcriptional activity might be the source of this dynamic exchange of histones H2A and H2B (Jackson & Chalkley, 1981b, 1985a). It is worth noting that transcriptional chromatin is reported to be depleted in H2A,H2B (Baer & Rhodes, 1983) and that the H3 associated with active ribosomal DNA of physarum is more accessible to sulfhydryl reagents (Prior et al., 1984). A more open conformation at active genes may be reflected in a weakened interaction between H3,H4 and H2A,H2B such that the latter histones can readily deposit at the replication fork.

The question then arises as to how a gene maintains an active or inactive state after replication if histones are deposited randomly. It is perhaps time to more seriously consider the possibility that transcription factors in and of themselves can maintain active states simply through their apparent ability to inhibit histone binding to regulatory regions. Examples of this are seen in the in vitro studies with TFIIIA and 5S gene (Gottesfeld & Bloomer, 1982; Brown, 1984) and in the nucleosome-free regulatory region of SV40 (Varshavsky et al., 1979; Saragosti et al., 1982; Jackobovits et al., 1982). An additional mechanism may involve the maintenance of negative superhelical stress on daughter strands prior to histone deposition. The importance of negative superhelical stress in gene activity has recently been reported (Villeponteau et al., 1984; Ryoji et al., 1984). In our replication studies, we have preliminary data which suggest that a lack of negative superhelical stress on the daughter strands severely affects the ability for histones to deposit in vivo (unpublished results). Thus, one additional mechanism of defining an open conformation may relate to the continuity of the double-stranded DNA after replication.

ACKNOWLEDGMENTS

I thank Drs. Bettie Sue Masters, Tim Herman, and Kent Wilcox for advice and critical comments in preparation of the manuscript.

REFERENCES

Annunziato, A. T., & Seale, R. (1984) *Nucleic Acids Res. 12*, 6179–6196.

Baer, B. W., & Rhodes, D. (1983) *Nature (London) 301*, 482–488.

Brown, D. D. (1984) *Cell (Cambridge, Mass.) 37*, 359–365.

Carin, F., & Thomas, J. D. (1981) *J. Mol. Biol. 146*, 513–537.

Cusick, M. E., DePamphilis, M. L., & Wassarman, P. M. (1984) *J. Mol. Biol. 178*, 249–259.

Fowler, E., Farb, R., & El-Saidy, S. (1982) *Nucleic Acids Res. 10*, 735–748.

Freedlander, E., Taichman, L., & Smithies, O. (1977) *Biochemistry 16*, 1802–1812.

Gottesfeld, J., & Bloomer, L. S. (1982) *Cell (Cambridge, Mass.) 28*, 781–791.

Groppi, V. E., & Coffino, P. (1980) *Cell (Cambridge, Mass.) 21*, 195–205.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159–191.

Jackobovits, E. B., Bratosin, S., & Aloni, Y. (1982) *Virology 120*, 340–348.

Jackson, V. (1978) *Cell (Cambridge, Mass.) 15*, 945-954.
Jackson, V. (1987a) *Biochemistry 26*, 2315-2324.
Jackson, V. (1987b) *Biochemistry 26*, 2325-2333.
Jackson, V., & Chalkley, R. (1981a) *Cell (Cambridge, Mass.) 23*, 121-134.
Jackson, V., & Chalkley, R. (1981b) *J. Biol. Chem. 256*, 5095-5103.
Jackson, V., & Chalkley, R. (1985a) *Biochemistry 24*, 6921-6930.
Jackson, V., & Chalkley, R. (1985b) *Biochemistry 24*, 6930-6938.
Jackson, V., Granner, D. K., & Chalkley, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4440-4444.
Jackson, V., Marshall, S., & Chalkley, R. (1981) *Nucleic Acids Res. 9*, 4563-4580.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-683.
Laskey, R. A., & Mills, A. D. (1979) *Eur. J. Biochem. 56*, 335-342.
Leffak, I. M. (1983) *Nucleic Acids Res. 11*, 2712-2732.
Leffak, I. M. (1984) *Nature (London) 307*, 82-85.
Leffak, I. M., Grainger, R., & Weintraub, H. (1977) *Cell (Cambridge, Mass.) 12*, 837-846.
Louters, L., & Chalkley, R. (1984) *Biochemistry 23*, 547-552.
Manser, T., Thacher, T., & Rechsteiner, M. (1980) *Cell (Cambridge, Mass.) 19*, 993-1003.
McGhee, J. D., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115-1156.
McGhee, J. D., & Felsenfeld, G. (1983) *Cell (Cambridge, Mass.) 32*, 1205-1215.
Pospelov, V., Russev, G., Vassilov, L., & Tsanev, R. (1982) *J. Mol. Biol. 156*, 79-91.
Prior, C. P., Cantor, C. R., Johnson, E. M., & Allfrey, V. G. (1980) *Cell (Cambridge, Mass.) 20*, 597-608.
Prior, C. P., Cantor, C. R., Johnson, E. M., Littau, V. C., & Allfrey, V. G. (1984) *Cell (Cambridge, Mass.) 34*, 1033-1042.
Russev, G., & Hancock, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3143-3147.
Ryoji, M., & Worcel, A. (1984) *Cell (Cambridge, Mass.) 37*, 21-32.
Saragosti, S., Cereghini, S., & Yaniv, M. (1982) *J. Mol. Biol. 160*, 133-146.
Seidman, M. M., Levine, A. J., & Weintraub, H. (1979) *Cell (Cambridge, Mass.) 18*, 439-449.
Senshu, T., Fukuda, M., & Ohashi, M. (1978) *J. Biochem. (Tokyo) 84*, 985-988.
Senshu, T., Yamasu, K., & Ohsawa, T. (1985) *Eur. J. Biochem. 150*, 575-580.
Simon, R. H., Camerini-Otero, R. D., & Felsenfeld, G. (1978) *Nucleic Acids Res. 5*, 4805-4818.
Sogo, J. M., Stahl, H., Koller, T., & Knippers, R. (1986) *J. Mol. Biol. 189*, 189-204.
Stein, A., Bina-Stein, M., & Simpson, R. T. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2780-2784.
Stein, A., Holley, K., Zeliff, J., & Townsend, T. (1985) *Biochemistry 24*, 1783-1790.
Varshavsky, A. J., Sundin, O., & Bohn, M. (1979) *Cell (Cambridge, Mass.) 16*, 453-466.
Villeponteau, B., Lundell, M., & Martinson, H. (1984) *Cell (Cambridge, Mass.) 39*, 469-478.
Waithe, W. I., Renaud, J., Nadeau, P., & Pallotta, D. (1983) *Biochemistry 22*, 1778-1783.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197-203.
Wu, R. S., & Bonner, W. M. (1981) *Cell (Cambridge, Mass.) 27*, 321-330.
Wu, R. S., Kohn, K. W., & Bonner, W. M. (1981) *J. Biol. Chem. 256*, 5916-5920.
Wu, R. S., Tsai, S., & Bonner, W. M. (1982) *Cell (Cambridge, Mass.) 31*, 367-374.
Yamasu, K., & Senshu, T. (1987) *J. Biochem. (Tokyo) 101*, 1041-1049.

Optical Properties of Tb^{3+}-Phospholipid Complexes and Their Relation to Structure†

Helen N. Halladay and Matthew Petersheim*
Department of Chemistry, Seton Hall University, South Orange, New Jersey 07079
Received September 9, 1987; Revised Manuscript Received November 19, 1987

ABSTRACT: The excitation and emission properties of Tb^{3+}-phospholipid complexes are shown to provide structural and dynamic information about the general ligand field of the bound lanthanide. Most of the conclusions derive from the relative behavior of 4f to 5d and 4f to 4f electronic transitions of Tb^{3+}. These observations demonstrate the unique properties of Tb^{3+} as a probe of cation binding sites on phospholipid membranes and its potential in describing changes in the lipid phase state.

Among the several parameters which can alter the phase behavior of phospholipid membranes, cation binding can induce some of the most dramatic, e.g., lateral phase separation (Graham et al., 1985; Wilschut et al., 1985; Leventis et al., 1986), intramembrane inverted micelle formation (Bearer et al., 1982; Verkleij, 1984; Smaal et al., 1987a,b), and membrane fusion (Sundler & Papahadjopoulos, 1981; Sundler et al., 1981; Bearer et al., 1982; Duzgunes et al., 1984; Wilschut et al., 1985; Leventis et al., 1986). The objective of a wide range of studies involving nuclear magnetic resonance (Hauser et al., 1975; Grasdalen et al., 1977; Baruskov et al., 1980; Chrzeszczyk et al., 1981; McLaughlin, 1982; Altenbach & Seelig, 1984; Miner & Prestegard, 1984), infrared (Dluhy et

† This research was supported by a Cottrell Grant from the Research Corp.

0006-2960/88/0427-2120$01.50/0