Assembly of Active Chromatin†

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ABSTRACT: In eukaryotic cells, transcriptionally active chromatin and inactive chromatin exist in dissimilar configurations which correlate with their functional states. The present study asks whether the differences in structure and function of active and inactive chromatin are also reflected in the process of replication. We have used mild micrococcal nuclease digestion [Bloom, K. S., & Anderson, J. N. (1978) *Cell (Cambridge, Mass.) 15*, 141–150] to release selectively an active chromatin fraction, S1, from MSB cell nuclei. The S1 fraction is greater than 6-fold enriched in sequences complementary to polyadenylated RNA and thus consists of virtually pure active chromatin. As isolated, the S1 chromatin comprises mononucleosomes which contain approximately 160 base pairs of DNA bound by core histones but which are enriched in non-histones and free of histone H1. Chemical cross-linking of S1 chromatin yields octamers containing the normal stoichiometry of core histones. When MSB cells are pulse labeled with isotopically dense amino acids and the recovered S1 chromatin is isolated and cross-linked, the labeled histone octamers are found in a single hybrid density octamer band on isopycnic gradients. These results indicate that, in contrast to the conservative assembly of transcriptionally inactive nucleosomes, the deposition of histones during the replication of active chromatin follows a nonrandom, semiconservative pathway.

The bulk of eukaryotic chromatin is organized into nucleosomes whose core complexes contain two each of histones H2A, H2B, H3, and H4 bound to approximately 145 base pairs of DNA. While a nucleosome repeat can be obtained on micrococcal nuclease digestion of transcribed chromatin (Lacy & Axel, 1975; Davis et al., 1983), several structural properties attributed to active chromatin distinguish it from bulk chromatin [reviewed in Weisbrod (1982)]. Among these are an increased susceptibility to nuclease digestion (Weintraub & Groudine, 1976; Bloom & Anderson, 1978), the presence of non-histones HMG 14/17 (Weisbrod & Weintraub, 1979; Levy-Wilson et al., 1979; Stalder et al., 1980), the absence of histone H1 (Levy-Wilson et al., 1979), a decreased level of DNA methylation (Weintraub et al., 1981; Jones, 1985), and association with the nuclear matrix (Ciejek et al., 1983; Davis et al., 1983; Rose & Garrard, 1984). Analysis of nucleosomal proteins indicates that the same species of histones are present in bulk and transcribed cores (Levy-Wilson et al., 1979; Micolas et al., 1983), although the data of Baer and Rhodes (1983) suggest that active cores which are bound to RNA polymerase II undergo a structural perturbation accompanying the release of one histone H2A/H2B dimer per histone octamer. Structurally disrupted nucleosomes have been reported to occur on transcriptionally active *Drosophila* heat-shock genes (Wu et al., 1979), murine ribosomal and immunoglobulin genes (Davis et al., 1983; Rose & Garrard, 1984), and chicken ovalbumin genes (Bellard et al., 1982; Bloom & Anderson, 1982), and Ryogi and Worcel (1985) have argued that this distorted chromatin corresponds to the "dynamic" nucleosomes which are assembled on plasmid DNAs injected into *Xenopus* oocytes. Under torsional stress, these transcriptionally active nucleosomes are proposed to undergo a transition to a symmetrically opened, "half-nucleosome" conformation (Weintraub et al., 1976; Oudet et al., 1977).

During the replication of bulk chromatin, temporal and structural constraints are imposed on nucleosome assembly such that there is a defined order of histone deposition (Senshu et al., 1978; Worcel et al., 1978) and a partitioning of new histones from old in individual nucleosomes (Leffak et al., 1977; Weintraub et al., 1978; Leffak, 1983a, 1984; Prior et al., 1980; Trempe & Leffak, 1985). In an attempt to determine whether the structural (and functional) differences between active and inactive chromatin were associated with differences in their behavior at replication, we have isolated and characterized an active chromatin fraction from chicken lymphoblastoid MSB cells. The active chromatin fraction consists of mononucleosomes with about 160 base pairs of DNA complexed with core histones and non-histones. After in vivo labeling of MSB cells with isotopically dense amino acids, histone cross-linking, and isopycnic centrifugation, we find that the newly replicated core octamers of active chromatin have a density intermediate between uniformly dense and light particles, indicating that new and old histones have been incorporated into the same nucleosome core. These data are consistent with a nonrandom, semiconservative mechanism for the assembly of active nucleosomes.

Materials and Methods

Preparation of Nucleic Acids. DNA was isolated from MSB cell nuclei or isolated chromatin fractions after treatment with RNase A (100 μg/mL, 1 h, 37 °C) followed by proteinase K (100 μg/mL, 4 h, 37 °C, phenol/chloroform extraction, and ethanol precipitation (Maniatis et al., 1982). Cytoplasmic RNA was prepared and polyadenylated RNA selected by two cycles of oligo(dT)–cellulose (Collaborative Research) chromatography with the inclusion of 1 unit/μL RNasin (Promega) in the binding and elution buffers (Maniatis et al., 1982) and the storage buffer [10 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris),[1] (pH 8.0), and 1 mM Na_2EDTA]. For cDNA synthesis, 6 μg of poly(A) RNA was

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[1] Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Na_2EDTA, disodium ethylenediaminetetraacetate; SSC, standard saline citrate; bp, base pair(s); SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTSP, dithiobis(succinimidyl propionate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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incubated in a total 30-μL reaction volume containing 50 mM Tris (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 2 mM dithiothreitol, 100 μg/mL bovine serum albumin, 1 unit/μL RNasin, 1 mM dGTP, 1 mM dCTP, 1 mM TTP, 0.1 mM dATP, 20 μM deoxyadenosine 5′-(α-[^{35}S]thiotriphosphate) (500 Ci/mmol), 100 μg/mL $(dT)_{12-18}$, and 30 units of reverse transcriptase (Promega) for 75 min at 37 °C. The reaction was stopped by adding Na_2EDTA to 10 mM and the RNA template hydrolyzed by the addition of NaOH to 25 mM and incubation at 37 °C for 8 h. cDNA was separated from unincorporated nucleotides by spun column chromatography (Maniatis et al., 1982).

Hybridization. DNA was glyoxalated according to Thomas (1981) and spotted onto nitrocellulose (Schleicher & Schuell, BA85) by using a dot blot apparatus (BRL). Total genomic DNA was sonicated to an average length of 400 base pairs (judged by agarose gel electrophoresis) prior to spotting. Filters were baked at 80 °C for 2 h in a vacuum oven, prehybridized in 50% formamide, 5× Denhardt's solution [0.1% bovine serum albumin, 0.1% Ficoll, and 0.1% poly(vinylpyrrolidone)], 5× SSC [0.75 M NaCl and 0.075 M sodium citrate (pH 7)], 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA at 42 °C, and hybridized to an approximate 3-fold sequence excess of ^{35}S-cDNA in 50% formamide, 5× SSC, 50 mM sodium pyrophosphate, 250 μg/mL salmon sperm DNA, 100 μg/mL poly(adenylic acid) (Collaborative Research), and 1× Denhardt's solution at 42 °C for 24 h. Filters were rinsed at room temperature in 2× SSC, and 0.5% SDS and at 50 °C in 0.5× SSC and 0.1% SDS for a total of 4 h. Filters were air-dried, sprayed with ENHANCE (NEN), and exposed to preflashed film at −80 °C for 5 days.

Cell Culture and Chromatin Preparation. MSB cells, a line of chicken lymphocytes transformed by Marek's virus (Akiyama & Kato, 1974), were grown and labeled with dense amino acids (Merck MB1808) and [^{3}H]lysine (NEN) essentially as described (Leffak, 1983a). Chromatin was fractionated according to Bloom and Anderson (1978) using sterile solutions and equipment, at 4 °C. Phenylmethanesulfonyl fluoride and iodoacetamide were included in all isolation and digestion buffers at 1 mM. MSB cells were spun out of culture at low speed and washed with cold phospate-buffered saline. The cells were lysed in STKM buffer [350 mM sucrose, 10 mM Tris (pH 7.4), 25 mM KCl, and 5 mM $MgCl_2$] containing 0.5% Nonidet-P40 and pelleted in a table-top centrifuge. The resulting pellet of nuclei was washed twice with STKM and finally suspended in STKM at 5×10^8 nuclei/mL (A_{260} ~100) and warmed to 37 °C for 3 min. $CaCl_2$ was added to 0.5 mM, and the nuclei were mildly digested (to a maximum of 2–4% DNA acid solubility) with micrococcal nuclease (200 units/mL; Boehringer) at 37 °C for 5 min. The nuclei were chilled on ice, and EGTA was added to 1 mM. The nuclei appeared intact by light microscopy and were pelleted in a microcentrifuge (15000g) for 5–10 s. The supernatant of this first centrifugation was designated S1. The pellet was resuspended in 10 mM Na_2EDTA and incubated on ice for 10 min. The suspension was microcentrifuged (10 s) again and the supernatant designated S2. Chromatin was cross-linked with DTSP [dithiobis(succinimidyl propionate), Pierce] as described (Leffak, 1983a). DNA concentration was estimated by assuming that 1 A_{260} unit (in 0.1% SDS) equals 50 μg of chromatin DNA.

Other Methods. Cesium formate/guanidinium chloride isopycnic centrifugation, gradient fractionation, gel electrophoresis, and fluorography were performed as described previously (Leffak, 1983a,b). Silver staining of gels followed the method of Wray et al. (1981).

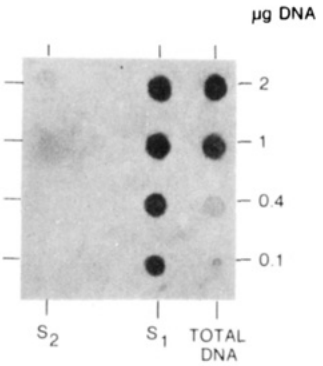

FIGURE 1: Dot blot hybridization of chromatin fractions. S1DNA was isolated from the supernatant chromatin which leaked out of MSB nuclei during mild micrococcal nuclease digestion. S2 DNA was isolated from the S1 pellet by solubilization in 10 mM Na_2EDTA. The S2 pellet was discarded. Increasing amounts of S1, S2, and total DNA (sonicated to ca. 400 base pairs) were spotted onto nitrocellulose. The filter was hybridized to a sequence excess of ^{35}S-cDNA complementary to MSB cell total cytoplasmic polyadenylated RNA and fluorographed (see Materials and Methods).

RESULTS

S1 Chromatin Is Enriched in Transcribed DNA. Under conditions of low ionic strength (Bloom & Anderson, 1978), mild micrococcal nuclease digestion of nuclei from a variety of cell types preferentially releases mononucleosomes containing transcribed DNA (Bloom & Anderson, 1978; Nicolas et al., 1983; Seale et al., 1983). Similar digestion of MSB cell nuclei releases approximately 5% of the total nuclear DNA (beyond that which becomes acid soluble) into the first supernatant, S1, upon centrifugation. DNA dot hybridization to ^{35}S-labeled cDNA synthesized from total polyadenylated cytoplasmic RNA was used to estimate the relative concentration of transcribed DNA sequences in S1 vs. total genomic DNA. Under these conditions, DNA from the S1 fraction shows a minimum 6-fold increase in hybridization over unfractionated DNA, while the S2 chromatin, which is retained in the nuclei after digestion, is virtually devoid of cDNA-hybridizable sequences (Figure 1). Additional hybridizations using a probe complementary to the transcriptionally inactive chicken α-globin genes indicated that these sequences were approximately equally represented in the S2 and total DNA preparations and strongly depleted in the S1 chromatin fraction (not shown). A 6-fold enrichment for transcribed sequences may be an underestimate of the true enrichment for active chromatin in S1 due to the presence of nontranscribed sequences in total DNA complementary to mRNA; however, this result is quantitatively similar to those obtained with avian oviduct (Bloom & Anderson, 1978; Nicolas et al., 1983) and HeLa cells (Seale et al., 1983). On the basis of solution hybridization studies indicating that approximately 18% of MSB chromatin is transcribed (Seidman et al., 1978), we conclude that the S1 fraction comprises greater than 90–95% active chromatin.

Composition of S1 Chromatin. The DNA in the S1 fraction is contained primarily in nucleoprotein species which have electrophoretic mobilities similar to those of nucleosome core and chromatosome (Simpson, 1978) particles (Figure 2A). In contrast, the S2 fraction is depleted in the low-mobility mononucleosomes and displays a series of higher molecular weight oligomers. On agarose gel electrophoresis, DNA isolated from S2 displayed the canonical nucleosome repeat ladder, with the monomer band depleted relative to unfractionated DNA, while DNA purified from S1 chromatin contained a single pre-

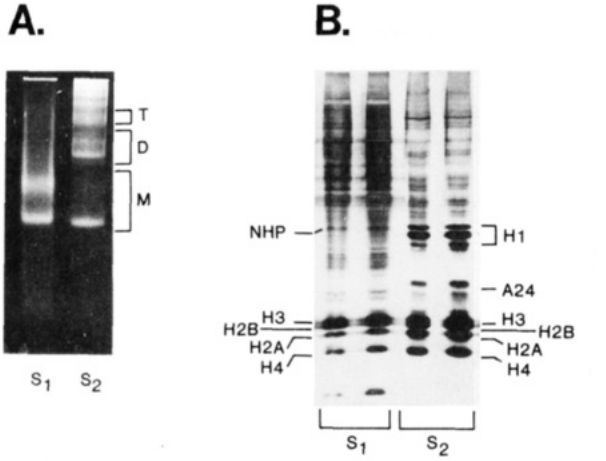

FIGURE 2: Electrophoretic pattern of protein and nucleoprotein particles from fractionated chromatin. (A) Aliquots taken directly from the S1 and S2 chromatin fractions were electrophoresed on a nondenaturing 5% polyacrylamide gel at 4 °C with buffer recirculation and stained with ethidium bromide; M, nucleosome monomer; D, dinucleosomes; T trinucleosomes. Aliquots were adjusted to yield approximately equal intensities of the fastest migrating monomer particle in each lane. (B) Aliquots of S1 and S2 chromatin (containing approximately 0.005–0.01 A_{260} unit) were mixed with sample buffer and subjected to SDS–PAGE on a 0.5 mm thick, 15% acrylamide minigel. NHP, non-histone protein. Proteins were visualized by silver staining (Wray et al., 1981).

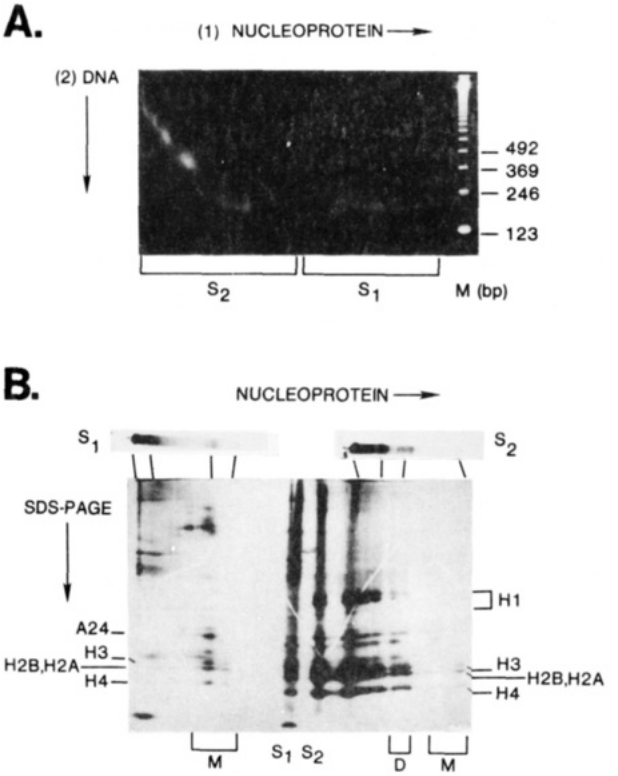

FIGURE 3: Second-dimension analysis of protein composition and DNA fragment lengths of isolated nucleoprotein particles. (A) S1 chromatin and S2 chromatin were electrophoresed in a first-dimension nucleoprotein gel as shown in Figure 2A. After visualization with ethidium bromide, individual lanes were excised, placed horizontally across a 6% polyacrylamide gel containing 0.5% SDS, and sealed in place with 0.5% agarose in running buffer for second-dimension electrophoresis. Directions of first-dimension (nucleoprotein) and second-dimension (DNA) electrophoresis are indicated by arrows. M, 123 base pair ladder size marker DNA. (B) S1 chromatin and S2 chromatin were prepared from MSB cells labeled for ca. 2 h with [^{3}H]lysine (200 μCi/mL, in RPMI 1640 containing one-tenth the normal amount of cold lysine) and electrophoresed in the first dimension on a 5% polyacrylamide gel, as in Figure 2A. After visualization with ethidium bromide, individual lanes were excised, equilibrated with 125 mM Tris-HCl (pH 6.8) and 0.1% SDS, and placed horizontally across the stacking gel of a 15% SDS–PAGE gel. Arrows indicate the relative directions of first- and second-dimension electrophoresis. The horizontal insets at the top of panel B are fluorograms of first-dimension S1 (left) and S2 (right) nucleoprotein gel lanes from the same gel as the lanes used for second-dimension SDS–PAGE. Total proteins from the S1 and S2 fractions were loaded in between the nucleoprotein gel slices for comparison. The positions of nucleosome monomers (M) and dimers (D) in the first dimension and core histones, histone H1, and semi-histone A24 in the second-dimension run are indicated. After electrophoresis, gels were fixed in methanol/acetic acid and fluorographed.

dominant size class of fragments, approximately 160 bp in length (data removed). Analysis of the S1 and S2 proteins by SDS–PAGE revealed that the S1 supernatant contains the normal complement of core histones, is virtually devoid of histone H1, and is enriched in non-histone proteins relative to S2 (Figure 2B). Second-dimension electrophoresis of the DNA from a nucleoprotein gel similar to that of Figure 2A demonstrated that both the slow and fast mononucleosomes of S1 (and S2) chromatin contain DNA of approximately 160 bp (Figure 3A). Evidence exists indicating that the slightly greater length of DNA in the slow monomers can contribute to a high-affinity binding site for non-histones (Stein & Townsend, 1983; Swerdlow & Varshavsky, 1983), accentuating the difference in electrophoretic mobility of these nucleoproteins. Protein analysis by second-dimension SDS–PAGE showed that the fast S1 monomers contain stoichiometric amounts of the core histones while the slow monomers additionally contain non-histones and ubiquitinated histones (Figure 3B). It is unlikely that the high molecular weight non-histones which comigrate with mononucleosomes in the nucleoprotein dimension are actually associated with these particles, rather than ribonucleoprotein or protein oligomers. As shown below, these large non-histones do not become cross-linked to the core histones. Ubiquitinated H2A (semi-histone A24) is not consistently observed in the S1 fraction, despite the use of protease inhibitors in all buffers [see also Baer & Rhodes (1983)], but its absence is correlated with the appearance of a low molecular weight peptide which is the major species running faster than histone H4 on the gels of Figures 2B and 3B. On the basis of its size and inverse correlation with A24, this spot may represent free ubiquitin (Matsui et al., 1982). The appearance of A24 in both S1 (active) and S2 (inactive) chromatin fractions is consistent with the previously reported uniform ubiquitinization of H2A molecules (Trempe & Leffak, 1982).

Assembly of Active Chromatin. We have previously demonstrated the nonrandom deposition and inheritance of the core histones, non-histones HMG 14/17, and histone H1 in total chromatin after pulse labeling of cells with isotopically dense amino acids and [^{3}H]lysine, protein cross-linking, and isopycnic centrifugation (Leffak et al., 1977; Weintraub et al., 1978; Leffak, 1983a, 1984). In order to analyze the assembly of active chromatin by this procedure, it was necessary to characterize the products of cross-linking the S1 chromatin fraction. When S1 chromatin which had been cross-linked with DTSP was resolved by 5–22% acrylamide gradient SDS–PAGE, the primary cross-linked product had the electrophoretic mobility of a histone octamer derived particle with M_r ~130 000 (Leffak, 1983a) (Figure 4). Only relatively small amounts of higher molecular weight material were observed. However, S2 chromatin cross-linked under similar conditions produced a family of cross-linked particles representing integral multiples of the core octamer.

Our earlier results had shown that nucleosomes assembled during an in vivo pulse with density-labeled amino acids

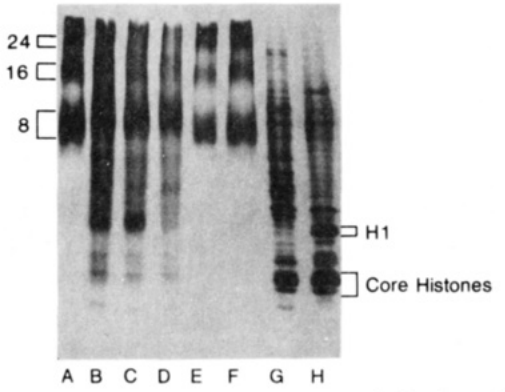

FIGURE 4: SDS–PAGE of cross-linked chromatin fractions. S1 and S2 chromatins were isolated from MSB cells radiolabeled with [^{3}H]lysine as in Figure 3B, dialyzed vs. 10 mM EDTA, and cross-linked with DTSP at 1:1 (1 mg of DTSP/mg of DNA) or 3:1 (3 mg of DTSP/mg of DNA) weight ratios (see Materials and Methods). Aliquots of cross-linked chromatin were mixed with an equal volume of SDS–PAGE sample buffer (minus 2-mercaptoethanol) and electrophoresed on 5–22% polyacrylamide gradient SDS gels. After electrophoresis, gels were fixed and fluorographed. A, mixture of equal amounts of S2 chromatin cross-linked at 1:1 and 3:1 ratios; B, mixture of equal amounts of S1 chromatin cross-linked at 1:1 and 3:1 ratios; C, S1 cross-linked at a 1:1 ratio; D, S1 cross-linked at a 3:1 ratio; E, S2 cross-linked at a 3:1 ratio; F, S2 cross-linked at a 1:1 ratio; G, un-cross-linked S1; H, un-cross-linked S2. 8, 16, and 24 denote cross-lined histone octamers, 16-mers, and 24-mers, respectively.

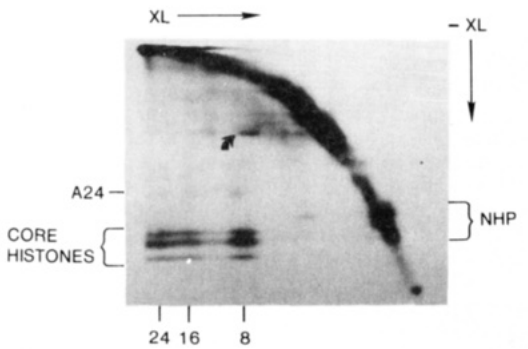

FIGURE 5: Two-dimensional analysis of protein particles obtained by cross-linking S1 chromatin at 0.4 M NaCl. S1 chromatin was isolated from MSB cells labeled with [^{3}H]lysine as above and mixed with an equal volume of 0.8 M NaCl while vortexing. The chromatin was cross-linked with 2 mg of DTSP/mg of DNA and electrophoresed in the first dimension on a 5–22% polyacrylamide gradient SDS gel as in Figure 4. The first-dimension slice was excised, equilibrated with SDS–PAGE sample buffer containing 1% 2-mercaptoethanol, and placed horizontally across the stacker of a 15% polyacrylamide–SDS gel for second-dimension electrophoresis. The resulting gel was fixed and fluorographed. Arrow, see text.

comprised uniformly dense core octamers associated with light histone H1 and non-histones (Leffak, 1983a; Trempe & Leffak, 1985). In order to examine exclusively the assembly of the core octamer of active chromatin, we attempted to cross-link the S1 chromatin at elevated ionic strength (0.4 M NaCl) to promote dissociation of the non-histones. Previous work had demonstrated that cross-linking at 0.6–0.7 M NaCl leads to the dissociation of H1 and non-histone proteins in the absence of inner histone rearrangement (Leffak, 1983a, 1984). Consequently, S1 chromatin was cross-linked with DTSP in 0.4 M NaCl and submitted to SDS–PAGE as in Figure 4. Second-dimension electrophoresis after cleavage of the cross-links with 2-mercaptoethanol demonstrated that the major product of S1 cross-linking in 0.4 M NaCl is an octamer containing stoichiometric amounts of the core histones (Figure 5). A single high molecular weight non-histone protein (arrow in Figure 5) is incorporated into a particle which comigrates

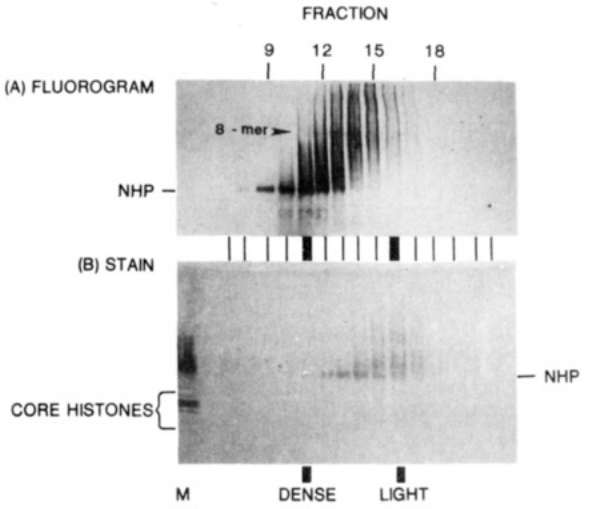

FIGURE 6: Isopycnic banding of density-labeled, cross-linked S1 chromatin. MSB cells were labeled with dense amino acids in the presence of trace [^{3}H]lysine for 45 min and mixed with a 4-fold excess of unlabeled cells. S1 chromatin was prepared and cross-linked in 0.4 M NaCl with 2 mg of DTSP/mg of DNA. The cross-linked chromatin was centrifuged to equilibrium on a denaturing cesium formate/guanidinium chloride gradient. The gradient was fractionated (into 28 fractions) from below and desalted by microdialysis against water (6 h) and then SDS–PAGE sample buffer (omitting 2-mercaptoethanol) overnight. Fractions 6–22 were electrophoresed on a 5–22% polyacrylamide gradient SDS gel which was stained (lower panel) and fluorographed (upper panel). The cross-linked histone octamer is only faintly visible on the stained gel due to its decreased binding of Coomassie Blue (Leffak, 1983b). DENSE and LIGHT refer, respectively, to the peak positions of un-cross-linked, ^{3}H-labeled, dense core histones (fractions 10–13) and unlabeled, light core histones (fractions 14–18) run together on a parallel gradient. (An internal marker of un-cross-linked chromatin was not included to avoid overlap of the 8-mer and non-histone protein signals.) For internal comparison, note that the tritiated, high molecular weight protein aggregates band near the light marker, while the tritiated, un-cross-linked proteins smaller than the octamer band near the dense marker. Arrow indicates the cross-linked histone octamer of S1 chromatin. M, marker chromatin, NHP, non-histone protein.

with the leading edge of the histone octamer population in first-dimension electrophoresis. This protein is not cross-linked to the core octamer, however, based on molecular weight and variability of its position relative to the core octamer at higher levels of DTSP cross-linking and on independent electrophoretic runs (not shown).

Having demonstrated that the histones of active nucleosomes could be cross-linked into canonical octamers, it was possible to test whether new and old histones became assembled into individual active nucleosome cores during replication. MSB cells were pulsed with RPMI 1640 growth medium containing isotopically dense (^{15}N, ^{13}C, ^{2}H) amino acids for 45 min in the presence of tracer [^{3}H]lysine, and nuclei were isolated. The nuclei were digested mildly with micrococcal nulcease to release the transcriptionally active S1 chromatin fraction, which was cross-linked with DTSP in the presence of 0.4 M NaCl. The resulting cross-linked chromatin was centrifuged to equilibrium on a denaturing cesium formate/guanidinium chloride density gradient. Under these centrifugation conditions, protein is dissociated from DNA, and proteins not cross-linked to one another band independently [see Trempe & Leffak (1985) and references cited therein]. The gradient was fractionated, and individual fractions were desalted by microdialysis prior to SDS–PAGE on a 5–22% acrylamide gradient SDS gel. The gel was stained to visualize the bulk proteins and fluorographed to allow detection of the banding pattern of the dense, ^{3}H-labeled histones. Figure 6 shows that the octamers (identified by second-dimension SDS–PAGE)

which contain density-labeled histones band with hybrid density (fractions 12–14), midway between the marker peaks for completely dense (fraction 11) and light (fraction 16) protein. Inasmuch as this gradient system is capable of resolving particles which are 25% density labeled (one dense protein molecule in four) from those which are 50% density labeled (Leffak et al., 1977; Leffak, 1983a), this result implies that the newly synthesized octamer cores of active chromatin contain on the average 50% dense and 50% light histones, as if these octamers were assembled from one newly synthesized and one parental histone tetramer. Because the pulse-labeled histones represent less than 10% of the histone in the S-phase cell population (and less than 5% of the total histone in the entire culture), this mixing of new and old protein in individual core octamers cannot have occurred by a process of randomization in vivo or dissociation and equilibration in vitro since no signal is detected in octamers in the light region of the gradient. Instead, these results argue that newly synthesized and parental histones are incorporated during the replication of transcriptionally active chromatin by a nonrandom mechanism. This result is in contrast to the assembly of bulk chromatin, where we have shown that newly synthesized and parental histones are not assembled into the same nucleosomes (Leffak et al., 1977; Weintraub et al., 1978; Leffak, 1983a, 1984; Trempe & Leffak, 1985).

Discussion

Mild micrococcal nuclease digestion of MSB cell nuclei leads to the selective solubilization of nucleosomes containing transcribed DNA sequences. In accord with results obtained by using other cell types, the active S1 fraction contains the normal complement of core histones but is depleted of histone H1 and enriched in non-histones (see the introduction). Two species of S1 mononucleosome are resolved on nondenaturing PAGE. The fast monomers contain core histones bound to ~160 bp DNA. The slower moving form contains DNA slightly larger than 160 bp and additional non-histones. When S1 chromatin is cross-linked under ionic conditions which dissociate non-histones, second-dimension SDS–PAGE indicates that each active nucleosome core contains the inner histones in approximately equimolar stoichiometry.

When MSB cells are pulse labeled for 45 min with isotopically dense amino acid medium (plus tracer amounts of [^{3}H]lysine), less than 10–20% of the histones in an S-phase cell become labeled. Density-labeled nuclei were digested mildly with micrococcal nuclease and the resulting S1 mononucleosomes reacted with DTSP to yield covalently cross-linked core octamers. These octamers formed a single hybrid density band on a denaturing cesium formate/guanidinium chloride isopycnic gradient, demonstrating that they contain approximately equal amounts of dense (newly synthesized) and light (preexisting) histones. Although symmetry arguments make such a semiconservative model attractive, in view of the inequality of histone molecular weights and the sensitivity of these gradients, it is not possible to be certain that each labeled octamer does not contain three or five dense histones. Nevertheless, because light histones are in large excess, had the density-labeled ^{3}H-histones randomized with the remaining histones in the cell during in vivo labeling or subsequent chromatin isolation, these ^{3}H-histones would have appeared in the light region of the gradient. We conclude, therefore, that the hydrid density S1 octamers were assembled by a nonrandom mechanism which incorporated both new and old histones into the same nucleosomes.

Using similar density labeling procedures, we have shown previously that the histone octamer core of bulk chromatin is conservatively assembled from completely new or completely old histones when chromatin replicates under normal cell culture conditions or under drug-induced conditions of new histone or new DNA excess (Leffak et al., 1977; Weintraub et al., 1978; Leffak, 1983a; Trempe & Leffak, 1985). Given the conservative assembly [and segregation (Leffak, 1984)] of bulk histone octamers, it was proposed that the active conformation of chromatin could be propagated laterally along the chromatin fiber [Weintraub et al., 1978; see also Kmiec & Worcel (1985)]; however, no simple model emerged as to how the active conformation of chromatin could be transmitted during replication at the level of the single nucleosome. Consistent with the present demonstration that the replication of active nucleosomes nonrandomly combines approximately equal amounts of new and parental histones, one conceivable mechanism for the propagation of an active nucleosome conformation could involve the semiconservative segregation of parental histones. Recently, Ryogi and Worcel (1985) proposed that active nucleosomes exist in a torsionally driven, "dynamic", half-nucleosome state. If, by analogy to allosteric enzyme systems, both tetramers of a single core octamer are constrained to exist in the same state, either "static" or "dynamic", the presence of a dynamic parental tetramer could induce a similar conformation in an incoming tetramer. Among several possible mediators of this postulated allosteric effect are direct histone–histone contact, the binding of nonhistone proteins, and torsional strain on the nucleosomal DNA. Thus, the dynamic conformation of chromatin may play a role in replication as well as transcription.

Baer and Rhodes (1983) have shown that, at the moment they are bound by RNA polymerase II, active nucleosomes are deficient in histones H2A and H2B. Because the density-labeling pulse in our experiments lasted for less than 1 h, it is unlikely that the newly replicated nucleosomes would have encountered RNA polymerase molecules before they were harvested. Therefore, our demonstration that newly replicated S1 histones did not randomize during the course of the labeling pulse is not inconsistent with the observations of Baer and Rhodes. In fact, we have reported that over a 4–6 generation chase period ~10% of pulse-labeled histones do appear in light nucleosomes (Leffak, 1984) and suggested that these may represent a particular subpopulation of total histones.

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High Molecular Weight Forms of the Insulin Receptor†

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ABSTRACT: The insulin receptor of liver, adipose, and placental plasma membranes was photoaffinity labeled with radioiodinated $N^{\epsilon B29}$-(monoazidobenzoyl)insulin. Three specifically labeled bands of 450, 360, and 260 kilodaltons (kDa) were identified in each tissue by polyacrylamide gel electrophoresis of the membranes solubilized in sodium dodecyl sulfate (SDS). The 360- and 260-kDa bands corresponded to partially reduced forms of the 450-kDa band. The distribution of radioactivity between the three insulin receptor bands was dependent on the tissue, the purity of the receptor preparation, and the conditions of solubilization in SDS. The 360- and 260-kDa bands became more prominent in each tissue with an increasing time of solubilization in SDS. However, with a short solubilization time in SDS, the 450-, 360-, and 260-kDa bands of the receptor were distributed approximately in a ratio of 85:15:0 in all three tissues. Inclusion of sulfhydryl alkylating reagents during solubilization in SDS altered this ratio to about 95:5:0. We conclude that the 450-kDa band represents the predominant form of the photolabeled insulin receptor and that the 260-kDa and probably the 360-kDa form as well were generated during the experimental manipulations preceding identification of the receptor. However, the appearance of the 360- and 260-kDa bands was not due to reductant present in SDS or buffer solutions and could not be accounted for by proteolytic degradation of the receptor. Furthermore, purification of the receptor over 2000-fold did not prevent the appearance of the 360- and 260-kDa bands. The effect of sulfhydryl alkylating reagents [*p*-(chloromercuri)benzenesulfonate, iodoacetamide, and *N*-ethylmaleimide] on the appearance of the receptor suggests that a sulfhydryl component of the membranes, which copurifies with the insulin receptor, can reduce specific disulfide bonds of the insulin receptor during solubilization in SDS.

The insulin receptor is minimally composed of a 130-kilodalton (kDa)[1] (α) insulin binding subunit and a 90-kDa (β) protein kinase subunit. cDNA cloning of the human insulin receptor has recently demonstrated that α and β subunits of the receptor are the products of a single gene (Ullrich et al., 1985). However, a number of high molecular weight forms of the plasma membrane receptor are observed on nonreducing SDS–polyacrylamide gels. To account for these high molec-

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[1] Abbreviations: SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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