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Nonrandom Assembly of Chromatin during Hydroxyurea Inhibition of DNA Synthesis†

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ABSTRACT: Incubation of MSB-1 chicken lymphoblastoid cells with hydroxyurea leads to a rapid 25-fold decrease in the incorporation of [^{3}H]thymidine into DNA and a 5-fold decrease of [^{3}H]lysine into the nucleosome core histones. I have investigated whether the distortion in the normal proportion of histone–DNA synthesis results in alterations in the nucleosome assembly process and find that neither the stoichiometry of new histone synthesis nor the deposition is appreciably changed during hydroxyurea incubation. Protein cross-linking and micrococcal nuclease digestion show that the histones synthesized during hydroxyurea treatment form octamer structures and are assembled into typical nucleosome particles. Minor nucleosome subpopulations are found which exhibit altered sensitivity to nuclease digestion and which are depleted in new histones H3 and H4. When MSB-1 cells incubated in hydroxyurea are pulsed briefly with density-labeled amino acids and [^{3}H]lysine, the radiolabeled core histone octamers formed are as dense as individual monomer histones. These results suggest that the newly synthesized histone octamers are uniformly dense and do not contain mixtures of new and old histones. Thus, histones synthesized during hydroxyurea incubation are deposited nonrandomly and do not exchange with preexisting histones.

The assembly of chromatin in vivo reflects the interplay of several regulated processes, including the coordinated synthesis of histones and DNA during S phase and the synthesis of S-phase histone subtypes (and basal histone variants) in nucleosomal stoichiometry (DePamphilis & Wassarman, 1980; Wu & Bonner, 1981; Delegeane & Lee, 1982; Wu et al., 1982, 1983; Sariban et al., 1985). The ordered nature of chromatin replication is evident also in the sequential deposition of histones H3/H4 prior to H2A/H2B during nucleosome formation (Worcel et al., 1978; Senshu et al., 1978) and the nonrandom assembly and segregation of nucleosomes (Seale, 1976; Weintraub, 1976; Leffak et al., 1977; Weintraub et al., 1978; Prior et al., 1980; Roufa & Marchionni, 1982; Leffak 1983b, 1984; Trempe & Leffak, 1985; Kumar & Leffak, 1986).

The synthesis of histones can be partially uncoupled from that of DNA by using chemicals which specifically inhibit either protein or DNA synthesis [reviewed in DePamphilis and Wassarman (1980) and in Sariban et al. (1985)]. In HeLa and Ehrlich ascites tumor cells, the inhibition of histone synthesis by cycloheximide is correlated with a 60–80% decrease in DNA synthesis. Under these conditions, the segregation of preexisting histones occurs normally at the level of mono- and oligonucleosomes (Pospelov et al., 1982; Seale & Simpson, 1975), and new histones are deposited nonrandomly immediately after removal of the cycloheximide block (Leffak, 1983b).

Both cytosine arabinoside (ara-C)[1] and hydroxyurea (HU) are able to cause a rapid decrease of greater than 95% in [^{3}H]thymidine incorporation into DNA with a concomitant, selective decrease of [^{3}H]lysine incorporation into the core histones by 60–80% (Leffak, 1983a; Louters & Chalkely, 1985). Here again, several features of normal chromatin replication persist. During ara-C incubation, the molar stoichiometry of new core histones is maintained, and bulk

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[1] Abbreviations: ara-C, cytosine arabinoside; Na_2EDTA, disodium ethylenediaminetetraacetate; HU, hydroxyurea; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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nucleosomes are conservatively assembled (Leffak, 1983a,b). The clearest effect of the 100–200-fold decrease in the ratio of newly synthesized DNA to histone during ara-C treatment is a reversible close-packing of nucleosomes near the replication fork. The stoichiometry of new histone synthesis during hydroxyurea inhibition also appears normal; however, it has recently been reported that histones H2A/H2B synthesized during HU incubation readily exchanged with parental histones (Louters & Chalkley, 1985). As this conclusion appeared to be at odds with previous observations, I have reexamined the assembly of chromatin during HU incubation using nuclease digestion, protein density labeling, and protein cross-linking. I find that the core histones are assembled nonrandomly as octamers into chromatin, without exchange into preexisting nucleosomes.

Materials and Methods

MSB-1 cells (Akiyama & Kato, 1974) were cultured in RPMI-1640 containing 5% fetal calf serum, 1% chicken serum, and 50 μg/mL gentamycin (Schering), in a humidified, 5% CO_2 atmosphere. The effect of hydroxyurea on DNA synthesis was examined by incubating half of a logarithmically growing culture of cells with hydroxyurea at zero time and pulsing aliquots of drug-treated and control cells (duplicate aliquots of 2×10^6 cells each) for 10 min with 20 μCi/mL [^{3}H]thymidine at 0, 5, 10, 20, 40, and 60 min. Comparable results were obtained over a range of hydroxyurea doses from 3 to 20 mM. Incorporation was ended by lysis of the cells in 0.5% sodium dodecyl sulfate (SDS), digestion with proteinase K, and quantitation of 10% trichloroacetic acid precipitable radioactivity (Leffak, 1983a).

Protein radiolabeling was accomplished by resuspending approximately 10^7 control cells or cells incubated with 20 mM HU for 20 min in 2 mL of their respective conditioned media and incubating for an additional 20 min with 200 μCi/mL [^{3}H]lysine. Cells were broken by suspension in lysis buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P40] and nuclei pelleted by low-speed centrifugation. Nuclei were washed twice in lysis buffer and digested with micrococcal nuclease to ca. 5% acid solubility. Total nuclear proteins were resolved by electrophoresis of an aliquot of the reaction mixture on a 15% polyacrylamide–SDS gel. Soluble chromatin was isolated after low-speed centrifugation of the nuclease digestion reaction mixture, lysis of the pelleted nuclei in 10 mM Na_2-EDTA (pH 7.4), and recentrifugation. Chromatin was cross-linked in 50 mM $Na_2B_4O_7$, pH 9.0, and 0.4–0.6 M NaCl using dithiobis(succinimidyl propionate) (Pierce) at 0.5–1 mg/20 A_{260} units (Leffak, 1983b). Stock solutions of 500 mM $Na_2B_4O_7$, 1.2 M NaCl, and 20 mg/mL dithiobis(succinimidyl propionate) were added rapidly to the chromatin while vortexing. Chromatin samples were centrifuged on denaturing cesium formate–guanidinium chloride gradients.

SDS–PAGE was performed according to Laemmli (1970), as modified by Weintraub et al. (1975), except that mercaptoethanol was omitted to permit visualization of the cross-linked histone octamer. Low ionic strength PAGE of nucleoprotein fragments was carried out at 4 °C according to Albanese and Weintraub (1980), with buffer recirculation. The positions of nucleosome monomers and oligomers were ascertained by second-dimension DNA gel electrophoresis (Albright et al., 1980). For second-dimension 15% polyacrylamide–SDS gel electrophoresis of cross-linked proteins, gel slices were soaked in 1% 2-mercaptoethanol and 100 mM Tris-HCl (pH 6.8) for 15–30 min and in 0.5% SDS and 50 mM Tris-HCl (pH 6.8) for 15–30 min. Nucleoprotein gel slices were soaked in 0.5% SDS and 50 mM Tris-HCl (pH 6.8) for 15–30 min before reelectrophoresis. Gels were fluorographed either according to the procedure of Laskey and Mills (1975) or using a commercial enhancer (New England Nuclear); Kodak XR-5 film was sensitized to linear response by preflash. Quantitative densitometry of fluorograms was carried out by using an Apple IIE assisted LKB Ultroscan 2202 soft laser densitometer equipped with the LKB 2190 Gelscan program. Multiple fluorographic exposures of each gel were taken to ensure that the scanned film signal was linearly proportional to the band radioactivity.

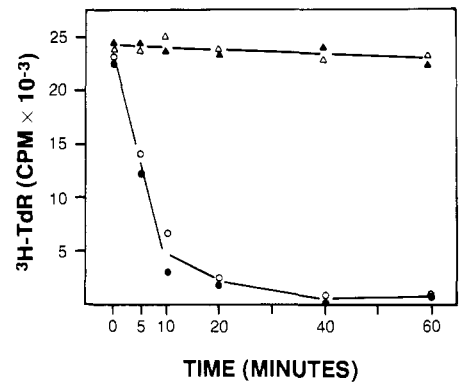

FIGURE 1: Inhibition of DNA precursor incorporation by HU. HU was added to half of a logarithmically growing culture of MSB-1 cells (time zero), and duplicate aliquots were removed from the drug-treated and control cells after 0, 5, 10, 20, 40, and 60 min for pulse-labeling with [^{3}H]thymidine and measurement of trichloroacetic acid precipitable incorporation. Circles, HU-treated cells; triangles, control (no HU) cells.

Results

Hydroxyurea Inhibition of [^{3}H]Thymidine Incorporation in MSB-1 Cells. Hydroxyurea (HU) was added to a logarithmically growing population of MSB-1 cells, and aliquots were removed at timed intervals for pulsed labeling with [^{3}H]thymidine. As shown in Figure 1, incubation of MSB-1 cells with HU decreases the rate of incorporation of the DNA precursor by more than 90% within 20 min, with further inhibition to more than 95% during the following 40 min. The MSB-1 cells remain viable during prolonged exposure to HU as demonstrated by the recovery of DNA synthesis after removal of the drug (not shown). A curve similar to that shown in Figure 1 is obtained when HU and [^{3}H]thymidine are added concurrently to MSB-1 cells, and the increment of [^{3}H]thymidine incorporation is plotted against time. Although not corrected for changes in precursor pool sizes, these data suggest a significant decrease in the extent of DNA synthesis resulting from HU treatment, consistent with previous investigations using other cell types.

HU Inhibition of Histone Synthesis in MSB-1 Cells. Treatment of MSB-1 cells with 20 mM HU leads to a selective decrease in the synthesis of core and H1 histones, in parallel with the inhibition of DNA synthesis. MSB-1 cells were incubated with [^{3}H]lysine for the final 20 min of a 40-min HU incubation, and nuclei were isolated. Nuclei were also isolated from an equal number of control cells incubated identically with [^{3}H]lysine but without drug. Both samples were digested mildly with micrococcal nuclease and loaded directly on 15% polyacrylamide–SDS gels. While HU incubation does not detectably alter the pattern of Coomassie blue staining of the bulk nuclear proteins (Figure 2A, lanes 1 and 2), fluorography of the gel (Figure 2A, lanes 3–5) and densitometric quantitation (Figure 2B) indicate an approximate 80% decrease in [^{3}H]lysine incorporation into the histones. The effect does not

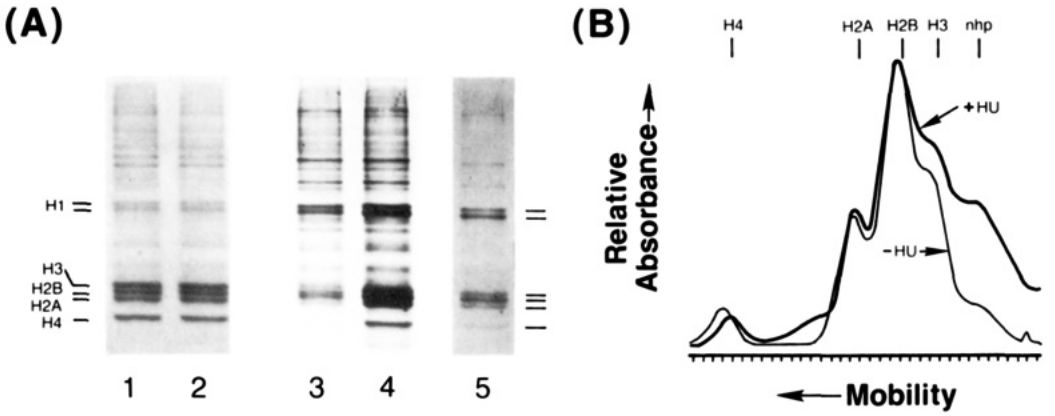

FIGURE 2: (A) Inhibition of histone synthesis by HU. Cells were labeled with [^{3}H]lysine in the presence or absence of HU and the total nuclear proteins resolved on a 15% polyacrylamide–SDS gel. Lanes 1 and 2, Coomassie stain; lanes 3 and 4, fluorogram of lanes 1 and 2 (48-h exposure); lanes 1 and 3, HU-treated nuclei; lanes 2 and 4, control nuclei; lane 5, 8-h reexposure of lane 4. (B) Densitometric scan of the core histone region of lanes 3 (thick line) and 5 (thin line). nhp, non-histone protein.

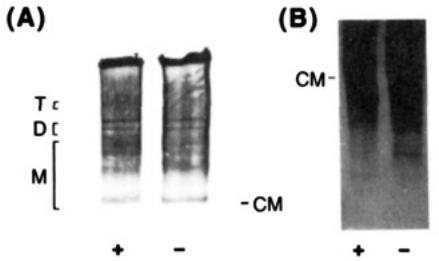

FIGURE 3: Nucleoprotein fragment pattern of HU chromatin. Soluble chromatin was isolated from HU-treated and control cells labeled with [^{3}H]lysine as in Figure 2 and resolved on a 4.5% polyacrylamide gel. A fluorogram of the gel is shown. (A) Mononucleosome–oligonucleosome region. (B) Subnucleosome region. (+) HU-treated chromatin; (–) control chromatin. CM, core mononucleosome; M, mononucleosome; D, dinucleosome; T, trinucleosome.

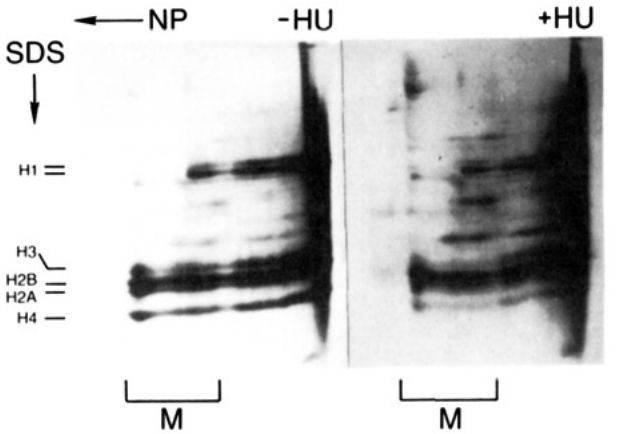

FIGURE 4: Second-dimension gel electrophoresis of nucleoprotein fragment histones. Samples run in parallel on the first-dimension gel shown in Figure 3 were submitted to second-dimension SDS–PAGE. A fluorogram of the second-dimension gel is shown. The directions of the first-dimension (NP) electrophoresis and second-dimension (SDS) electrophoresis are indicated by arrows. (+HU) Chromatin labeled in HU; (–HU) control chromatin; M, mononucleosome region of the first-dimension gel.

result in a universal decrease in the labeling of nuclear proteins and therefore implies a more specific inhibition of histone protein synthesis. As seen in Figure 2B, the fluorographic signal from each of the four core histones (and H1, not shown) is decreased proportionately, suggesting that the synthesis of any one histone is not differentially inhibited to an appreciable extent and that there is no preferential loss of histones during preparation of the nuclei. Similar results are obtained when chromatin solubilized by micrococcal nuclease digestion is examined (see below).

Effects of HU on Nucleosome Structure. To investigate whether the inhibition of DNA and histone synthesis resulted in a change in the structure of newly assembled nucleosomes, the nucleoprotein gel electrophoretic patterns of chromatin isolated from cells labeled with [^{3}H]lysine in the presence or absence of HU were compared. As shown in Figure 3A, when approximately equal numbers of counts of radiolabeled chromatin from control and HU-treated cells are run on nondenaturing PAGE, the nucleosome particle profiles are virtually indistinguishable. This result contrasts with that obtained when DNA synthesis in MSB-1 cells is inhibited with ara-C. Under those conditions, synthesis of the core histones is coordinately depressed by approximately 80%, but histone H1 synthesis is decreased by approximately 65–70%, and a quantitatively different pattern of [^{3}H]lysine-labeled nucleosome monomers and oligomers is obtained. The generation of an altered nucleoprotein pattern after ara-C inhibition may therefore be due to the differential inhibition of core vs H1 (or non-histone) protein synthesis (Stein & Bina, 1984) or the difference in the mechanism of ara-C inhibition compared to that of HU (Cozzarelli, 1977; Fridland, 1977; Streifel & Howell, 1981).

Although the patterns of control and HU nucleosome monomers and oligomers are comparable, overexposure of these gels reveals that there are subtle quantitative differences in the patterns of subnucleosome fragments derived by micrococcal nuclease digestion (Figure 3B). This suggests that the slowing of replication fork movement by HU may lead to enrichment for a normally minor subpopulation of nucleosomes with differential sensitivity to micrococcal nuclease.

Chromatin which had been separated on a first-dimension nondenaturing nucleoprotein gel was resolved on a second-dimension SDS–polyacrylamide gel to examine its histone composition. In agreement with the report of Louters and Chalkley (1985), a depletion of labeled histones H3 and H4 is observed in the nucleosome monomer region of the gel (Figure 4, +HU panel); however, densitometric quantitation of the +HU chromatin sample shows a 2–3-fold increase in the relative intensity of the H3 or H4 signal relative to the signal from the H2 histones in dinucleosomes (or oligonucleosomes) compared to mononucleosomes (Figure 5); this effect is significantly less evident in the –HU chromatin. Since the fluorograms of Figure 4 reveal only newly synthesized, labeled histones, if these histones were exchanging at random into bulk chromatin, the stoichiometry of labeled histones should be the same in mono- and oligonucleosomes. That this is not the case suggests that a fraction of newly synthesized

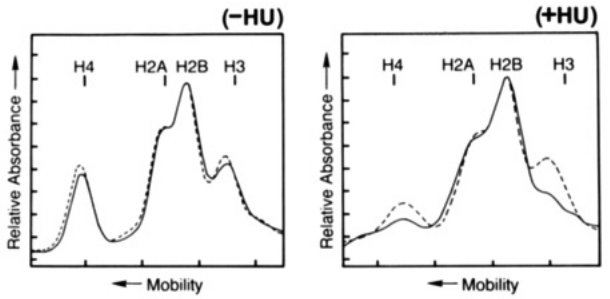

FIGURE 5: Stoichiometry of labeled histones in HU and control nucleosomes. Representative densitometric scans across the monomer (solid line) and dimer (dashed line) regions of the second-dimension gel fluorograms of Figure 4. (+HU) Chromatin labeled in HU; (-HU) control chromatin.

H2A and H2B is being assembled into a subpopulation of nucleosomes which are preferentially cleaved to mononucleosomes by micrococcal nuclease; this nucleosome subpopulation becomes enriched by HU inhibition of replication.

Cross-Linking of Histones Synthesized during HU Inhibition of DNA Synthesis. Reaction of solubilized chromatin with the reversible cross-linker dithiobis(succinimidyl propionate) [Lomont's reagent (Lomont & Fairbanks, 1976)] results in the formation of cross-linked core histone octamers from both nucleosome monomers and oligomers (Leffak et al., 1977); the cross-linking of nucleosome oligomers in solutions of moderate salt concentration (0.35–0.6 M NaCl) increases the proportion of core octamers relative to 16-mers and larger particles (Leffak, 1983b). Because histone octamers can be generated from chromatin which has not been digested to mononucleosomes, chemical cross-linking is likely to be less influenced by chromatin superstructure than is nuclease digestion. I therefore chose to use Lomont's reagent cross-linking to examine further the assembly of nucleosomes during HU inhibition. Chromatin was isolated by mild micrococcal nuclease digestion from cells pulse-labeled with [^{3}H]lysine in the presence or absence of HU and cross-linked in the presence of 0.4 M NaCl. The data of Figure 6A show that the core histones are completely cross-linked to octamers and larger particles by this treatment. Although qualitatively similar patterns are obtained upon cross-linking, compared to the control histones a greater proportion of histones labeled in HU are found in the lower molecular weight octamer-based particles than in larger particles containing a core octamer plus histone H1 or residual non-histones. Second-dimension SDS–PAGE after cleavage of the cross-links with 2-mercaptoethanol suggests that the HU histones are cross-linked into a repeating, octamer-derived unit in the nucleosomal stoichiometry (Figure 6B). The proteins which are eluted in the 0.4 M NaCl buffer and appear on the arc of un-cross-linked material are non-histone proteins, based on their solubility in trichloroacetic acid and perchloric acid (not shown).

Histones Synthesized in HU Are Assembled Nonrandomly into Nucleosomes. Using a protein density labeling method, we have previously shown that nucleosome core histones are deposited nonrandomly under normal cell culture conditions, when DNA replication is inhibited by ara-C, and when cells recover from cycloheximide inhibition of protein synthesis. I adopted a similar approach to examine directly whether histones synthesized in HU exchanged in vivo with histones preexisting in chromatin. MSB-1 cells were preincubated in medium containing ^{2}H,^{13}C,^{15}N-density-labeled amino acids and HU for 30 min, following which tracer [^{3}H]lysine was added for an additional 20 min. On the basis of the rate of histone synthesis during control and HU labeling, it is estimated that less than 1–2% of the total histones in any cell are labeled by this procedure. In this protocol, it is intentional that the dense histones are a very small fraction of the total histone mass, since the smaller the pool of dense histones the greater its sensitivity to mixing with preexisting histones.

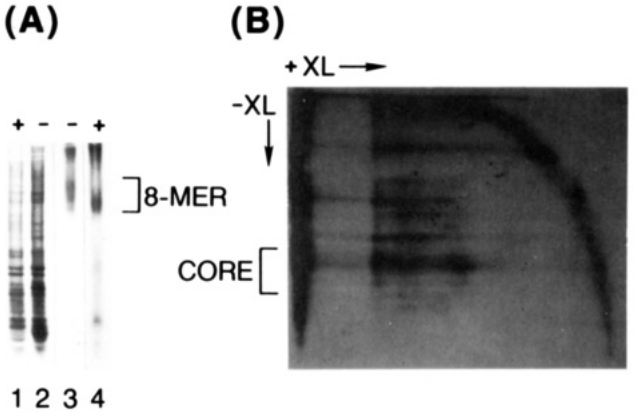

FIGURE 6: Cross-linking of histones synthesized during HU incubation. (A) Chromatin was isolated as in the experiment of Figure 2, reacted with the cross-linker dithiobis(succinimidyl propionate), and separated on a 5–22% gradient polyacrylamide–SDS gel. Lanes 1 and 2, un-cross-linked chromatin; lanes 3 and 4, cross-linked chromatin; lanes 1 and 4, HU chromatin; lanes 2 and 3, control chromatin. The histone octamer region of the gel is bracketed. (B) A lane of the first-dimension gel shown in (A) containing cross-linked chromatin labeled with [^{3}H]lysine in HU was incubated with 2-mercaptoethanol to cleave the cross-links and analyzed by second-dimension SDS–PAGE. The directions of first-dimension (+XL) and second-dimension (-XL) SDS–PAGE are indicated by arrows. Fluorograms are shown in (A) and (B).

Solubilized chromatin was cross-linked with Lomont's reagent in buffer containing 0.6 M NaCl, so as to remove non-histones and histone H1 without disrupting the nucleosome core (Leffak, 1983b). The cross-linked chromatin was dissolved in a cesium formate–guanidinium chloride solution and centrifuged to equilibrium. On these dissociating isopycnic gradients, the DNA pellets, while the histones which are not cross-linked to one another band independently. Gradients containing un-cross-linked light and dense histones were run in parallel to provide density markers. Fractions from the gradients were desalted by microdialysis and electrophoresed on 5–22% gradient polyacrylamide–SDS gels. Fluorography of the gels reveals the banding patterns of the [^{3}H]lysine-labeled histones and the density of the cross-linked particles which contain histones deposited during HU incubation. The fluorograms of Figure 7 show that the octamers which contain dense histones are as dense as individual dense histones. Moreover, no appreciable signal is evident from octamers in the light or hybrid density regions of the gradient, as would result from a mixture of newly synthesized dense histones and preexisting light histones. These data indicate that the new histones are assembled into chromatin in the normal stoichiometry and argue strongly that the majority of new histone does not mix or exchange with old histones during chromatin replication in the presence of hydroxyurea.

Close examination of the fluorogram of Figure 7A shows that a substantial fraction of the radiolabeled cross-linked particles larger than octamers band at a hybrid density. This effect has been observed previously and attributed to the conservative deposition of new nucleosomes in patches of 6–10 units and the cross-linking of dense and light octamers across the patch boundaries (Posepelov et al., 1982; Leffak, 1983b).

DISCUSSION

The inhibition of DNA synthesis by ara-C or hydroxyurea occurs through different mechanisms, yet each of these com-

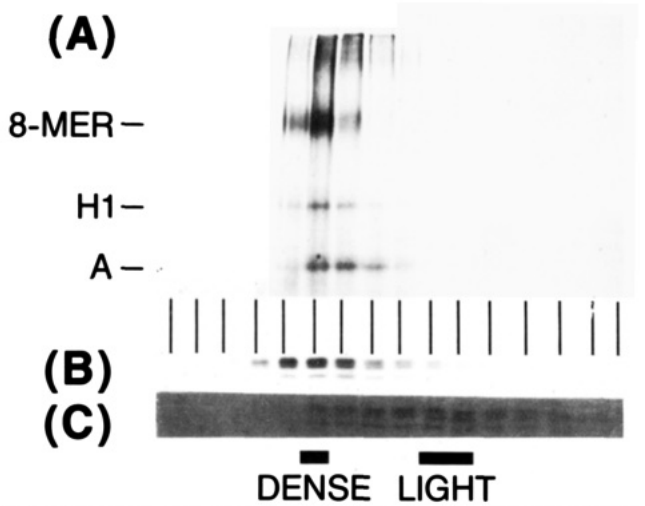

FIGURE 7: Conservative deposition of histones synthesized during HU incubation. Histones were labeled with [^{3}H]lysine and dense amino acids during hydroxyurea incubation (Materials and Methods), and chromatin was isolated. (A) The chromatin was cross-linked and banded on a cesium formate–guanidinium chloride gradient. Fractions 5 (density = 1.20 g/mL) through 23 (density = 1.37 g/mL) were desalted and analyzed by SDS–PAGE. A fluorogram of the gel is shown. 8-mer, histone octamer; H1, histone H1; A, an uncharacterized, hybrid density protein, possibly ubiquitinated (Trempe & Leffak, 1982). An aliquot of the same chromatin sample was run without cross-linking on a parallel gradient and fractionated identically. Fractions 5 (density = 1.20 g/mL) through 23 (density = 1.37 g/mL) were desalted and submitted to SDS–PAGE. Panel B is the fluorogram of this gel showing the banding pattern of the un-cross-linked, radioactive (dense) proteins. Panel C shows the Coomassie staining of the core histone region of the gel to reveal the banding pattern of the un-cross-linked, bulk (light) histone in the sample. LIGHT and DENSE labels beneath panel C refer to the peak band positions of cross-linked, ^{3}H-labeled, light and un-cross-linked, ^{14}C-labeled, density-substituted proteins from a mixture of control chromatins run on another parallel gradient.

pounds causes a decrease in the incorporation of labeled precursor into histones (Cozzarelli, 1977; Streifel & Howell, 1981; Wawra & Wintersberger, 1983), implying that the inhibition of replication leads to the decrease in histone synthesis. The core histones synthesized during ara-C incubation are deposited conservatively near the replication fork without mixing with preexisting histones, to form bona fide nucleosomes. These nucleosomes are qualitatively similar to control nucleosomes as judged by nondenaturing gel electrophoresis, but are more closely packed. On removal of the ara-C, the spacing of these nucleosomes returns to normal, in the absence of significant loss or randomization of the histones synthesized in the presence of the drug (Leffak, 1983a). The present results suggest that a similar situation may occur during the inhibition of DNA replication by hydroxyurea.

Incubation of MSB-1 cells with HU leads to a ca. 25-fold decrease in the incorporation of [^{3}H]thymidine and a 5-fold decrease in [^{3}H]lysine incorporation. The disproportionate inhibition of DNA and histone synthesis does not, however, appear to alter the stoichiometry of new core histones, their ability to form histone octamers or nucleoprotein particles, or their nonrandom assembly into chromatin. This is consistent with the conclusions of Russev et al. (1980), who found that the histones synthesized during HU inhibition of DNA synthesis in EAT cells were correctly assembled into nucleosomes. In contrast, the recent report by Louters and Chalkley (1985) found that histones H2A and H2B synthesized during HU incubation of hepatoma tissue culture cells can displace preexisting histones. In that work, the electrophoretic comigration of histone and non-histone proteins was not unequivocally excluded, and the densitometric comparison of widely disparate film exposures left open the possibility that the effects observed were largely due to the sequential incorporation of histones H3/H4 and H2A/H2B into nascent chromatin (Worcel et al., 1978; Senshu et al., 1978). This interpretation is consistent with the present observations that a fraction of chromatin is enriched during HU incubation which is preferentially cleaved to mononucleosomes by micrococcal nuclease, and which is depleted in newly synthesized H3/H4.

I have also utilized a sensitive biological assay to probe for the nonrandom deposition or exchange of histones synthesized during HU treatment. When MSB-1 cells are incubated with HU, density-labeled amino acids, and tracer [^{3}H]lysine, approximately 1–2% of the histones in a cell undergoing replication become labeled. I have shown previously that the cesium formate–guanidinium chloride gradients used here are able to resolve octamers containing exclusively dense histones from octamers which contain two or more light histones (Leffak, 1983b); thus, after cross-linking, mixing of the labeled histone pool with the large excess of unlabeled histones is readily detectable by this method. The absence of hybrid density, labeled histone octamers which could arise by the mixing of new and old histones at random or by semiconservative octamer assembly (Kumar & Leffak, 1986) demonstrates that the great majority of new histone does not mix with old histones during chromatin assembly in HU. In conjunction with the finding that the two-dimensional gel electrophoretic pattern of histones synthesized in HU most closely resembles that of the histones synthesized during the G_1 phase of the cell cycle (Wu & Bonner, 1981; Sariban et al., 1985), my data imply that histone deposition during G_1 may also occur by a nonrandom mechanism. As such, the slow turnover or replacement of S-phase histones (Commerford et al., 1982) would not imply random histone deposition a priori (Djondurov et al., 1983; Louters & Chalkley, 1985).

I previously used a similar density labeling approach to show that histones synthesized under normal culture conditions or during ara-C incubation are deposited and segregated nonrandomly (Leffak, 1983a,b). While it has been suggested that these results may be explained by the equilibration of new histone with a nonrandomly selected, limited pool of preexisting histone during chromatin assembly (Louters & Chalkley, 1985), this view overlooks the fact that randomization of the labeled histones was not detected over 5–6 subsequent cell generations and chromatin replications, during which time the labeled histones would have been exposed repeatedly to the putative randomizing environment.

My data disagree with a recent report by Jackson (1987) that newly synthesized dense histones mix with preexisting histones in individual octamer particles, in that I did not find octamers containing newly synthesized histones in the light or hybrid density regions of my gradients [Figure 7; see also Leffak (1983b)]. It appears that the chromatin isolation procedure and extensive exposure to high pH prior to cross-linking used in the experiments of Jackson (1987) may have led to histone exchange in vitro and the generation of particles of atypical histone stoichiometry. In the present work, I have closely followed the labeling protocol of Louters and Chalkley (1985), and my data show that most newly synthesized histone is not found in mixed octamers (Figure 7), yet an identifiable population of new nucleosomes exists which is depleted in labeled H3 and H4 (Figure 4). The absence of detectable amounts of hybrid density histone octamers argues against the possibility of an ongoing exchange of new histone H2A or H2B

with the total pool of preexisting histones during replication in HU nor can the present results be explained by postulating that all four subtypes of new, dense histones equilibrate with a pool of recently synthesized/deposited histones, in view of the clear depletion of new H3 and H4 in the most nuclease-sensitive nucleosomes. While I cannot exclude the possibility that these nucleosomes arise through a low-level, nonstoichiometric exchange of newly synthesized core histones with a limited pool of parental histones, the present observations are not consistent with the large-scale exchange of new and old histones but can be reconciled most simply by a model in which new histones are sequentially and nonrandomly incorporated into nucleosomes, with the H3 and H4 histones preceding H2A and H2B.

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