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Chromatin Structural Changes in Synchronized Cells Blocked in Early S Phase by Sequential Use of Isoleucine Deprivation and Hydroxyurea Blockade†

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ABSTRACT: We have investigated the loss of histone H1 from chromatin [D'Anna, J. A., Gurley, L. R., & Tobey, R. A. (1982) *Biochemistry 21*, 3991–4001] and the structure of chromatin from Chinese hamster (line CHO) cells blocked in early S phase by sequential use of isoleucine deprivation G_1 block and 1 mM hydroxyurea (HU) blockade. Measurements of H1 content in the cell and histone turnover indicate that H1 is lost from the cell and that there is negligible replacement synthesis of H1 during the period of the S-phase block. As H1 is lost, chromatin appears to undergo structural change. After 10 h of HU block, the new deoxyribonucleic acid (DNA) and a portion of the old DNA have measured nucleosome repeat lengths (37 °C digestion) which are less than those of controls and similar to those observed by Annunziato and Seale [Annunziato, A. T., & Seale, R. L. (1982) *Biochemistry 21*, 5431–5438] for new immature chromatin in the absence of HU. By 24 h of HU block, nearly all of the chromatin has assumed a pseudoimmature conformation in which the nucleosome cores appear to be more closely packed along the DNA chain, but the new DNA is slightly more resistant than old DNA to attack by micrococcal nuclease. Electrophoretic analysis of nucleoprotein particles produced by micrococcal nuclease digestion of nuclei indicates that (1) the distribution of mononucleosome species changes during HU block and (2) some mononucleosome species appear to be enriched in normally minor proteins which may determine the electrophoretic mobility of the nucleoprotein particles in agarose–acrylamide gels. The results raise the possibility that (1) during the early stages of replication (or prior to the passage of the replication fork), H1 is dissociated from initiated replicons and (2) H1 does not reassociate in a concerted fashion with the H1-depleted chromatin until the replication fork has passed and, perhaps, a substantial portion of the replicon has been replicated.

Release of synchronized G_1 cells into hydroxyurea (HU)[1] inhibits the accumulation of dATP pools (Walters et al., 1973) and greatly reduces the rate of DNA synthesis (Tobey & Crissman, 1972), but it does not prevent cells from entering S phase (Walters et al., 1976a). Autoradiography employing high concentrations of high specific activity radioactive thymidine shows that G_1 cells enter S phase at the same rate in the presence of HU as in its absence (Walters et al., 1976a). Furthermore, during the first 10 h of HU block, there is an accumulation of new small DNA (Walters et al., 1976a; Cress

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[1] Abbreviations: bp, base pairs of DNA; DNA, deoxyribonucleic acid; FCM, flow cytometry; HU, hydroxyurea; $NaDodSO_4$, sodium dodecyl sulfate; NP-40, Nonidet P-40 nonionic detergent; PCA, perchloric acid; Cl_3CCOOH, trichloroacetic acid; TCB, 15 mM Tris-HCl and 3 mM $CaCl_2$, pH 7.2; TCMB, 15 mM Tris-HCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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& Bowden, 1981) whose molecular weight is $\leq 10^7$. Hence, it appears that HU allows cells to initiate replication, but it retards DNA elongation and the ligation of small to replicon-sized DNA fragments into bulk DNA (Walters et al., 1976b; Johnston, 1980).

Recently, this laboratory reported measurements of relative histone contents in the chromatin of blended (in isotonic saline) nuclei isolated from cells synchronized in S phase by sequential use of isoleucine-deprivation G_1 block and 1 m*M* HU blockade (D'Anna et al., 1982). After 10 h in HU, the ratio of histone H1 to core histones was reduced to ~70% of its value in exponentially growing cultures, and after 24 h, it was reduced to ~30%. These results suggested that (1) histone H1 was being lost from the cell (e.g., degradation), (2) H1 was located in a cytoplasmic or nuclear pool and was being lost during nuclear isolation, or (3) there was an accumulation of extraordinary quantities of core histones in the HU-blocked cells.

In addition to the questions about the reduction in histone H1 in isolated chromatin, we wondered whether the sequential use of isoleucine deprivation and HU blockade also was causing an accumulation of structurally immature chromatin. This question was prompted by the following considerations: (1) the release of synchronized G1 cells into HU-containing medium allows cells to initiate DNA synthesis and leads to an accumulation of small DNA fragments (Walters et al., 1976a; Cress & Bowden, 1981), and (2) treatment of exponentially growing cells with HU retards the maturation of chromatin (Jackson & Chalkley, 1981a).

Here, we report the results of investigations which address the questions pertaining to the fate of histone H1 and the structure of chromatin in HU-blocked S-phase cells. We also discuss the potential implications of the results to chromatin structural changes which occur during replication in the absence of HU.

Experimental Procedures

Cellular Growth, Synchronization, and Cell Cycle Analysis. Suspension cultures of Chinese hamster (line CHO) cells (D'Anna et al., 1982) were synchronized in G_1 by maintenance in isoleucine-deficient medium for 36 h (Tobey & Ley, 1971). Cultures were synchronized in S phase by sequential use of isoleucine deprivation (31–33 h) and resuspension in complete F-10 containing 1 mM hydroxyurea (Tobey & Crissman, 1972) where they were maintained for 10–24 h. During 10 h in HU, the G_1 cells enter S phase in a synchronous fashion, producing an average increase in the DNA content of about 3–5% per cell; after 24 h in HU, there is an average increase in the DNA content of about 15% relative to G_1 cells. The distribution of cellular DNA content in culture was determined by flow cytometry (FCM), as previously described (D'Anna et al., 1980).

Isotopic Labeling. For measurement of H1 and H4 turnover during HU block, 2 L of exponentially growing cells was grown (3.3–3.9 generations) and synchronized in G_1 in the presence of [^{3}H]lysine (60 Ci mM^{-1}). The cells were washed once with 10 mL of [^{3}H]lysine-free F-10 medium containing 1 mM HU before being released into 2.0 L of the same medium.

To distinguish new DNA from old DNA in HU-blocked S-phase cells, exponentially growing cultures were prelabeled for 24 h and synchronized in G_1 in the presence of 5 μCi L^{-1} [^{14}C]thymidine (53 mCi mM^{-1} in H_2O) to radiolabel "old DNA". The cells were then released into complete medium without radioactive thymidine; 1.5 h later, [^{3}H]thymidine (83 Ci mM^{-1} in H_2O) was added to radiolabel "new DNA". [^{3}H]Thymidine was added at a concentration of 9.4 mCi L^{-1} if cells were to be harvested after 10 h in HU or at a concentration of 0.6 mCi L^{-1} if cells were to be harvested after 24 h in HU.

Isolation of Nuclei and Extraction of Whole Histones. Nuclei were isolated by the use of (1) a nonionic detergent (NP-40) procedure (method 1) employing TCB (15 mM Tris-HCl–3 mM $CaCl_2$, pH 7.2) and homogenization in a Dounce homogenizer as previously described (D'Anna et al., 1982) and (2) an NP-40 procedure (method 2) employing RSB (10 mM NaCl, 3 mM $MgCl_2$, and 10 mM Tris, pH 7.4) and no homogenization (Gurley et al., 1983).

Total histones were obtained in two ways. In the first procedure, nuclei were blended in isotonic saline containing 0.05 M sodium bisulfite. Histones were extracted from the sedimented chromatin of the blended nuclei with 0.20 M H_2SO_4, and histones were precipitated with acetone (D'Anna et al., 1982). In the second procedure, pelleted nuclei from ~1.4×10^8 cells were transferred to an Omnimixer blending cup with three aliquots of 0.75 mL of 0.20 M H_2SO_4. The nuclei were blended on ice at half-speed for 30 s and then at full speed for another 1.5 min. The suspension of blended chromatin in H_2SO_4 was transferred to a centrifuge tube where it was left for 45 min before centrifugation. The H_2SO_4 supernate and the supernate from subsequent 0.8- and 0.6-mL H_2SO_4 extractions (45 min) were clarified by centrifugation for 30 min at 7700*g*. After dialysis (molecular weight cutoff 3500; Arthur H. Thomas Co.) against 1 L of 0.10 M acetic acid for 24 h and 1 L of 10 mM HCl for 24 h, the supernates were lyophilized and weighed in preparation for electrophoresis.

PCA Extraction of H1 for Quantification of H1 Content. To determine whether H1 was being lost from the cell during prolonged S-phase block in HU, histone H1 was extracted from known numbers of cells whose average DNA contents were determined by FCM. One portion of cells [(2–3) $\times 10^8$] was used for extraction of total histones and determination of the H1:H4 ratio; the other portion was used to quantify the total amount of H1 in the cells.

Cells to be used for quantification of H1 were harvested and transferred to a graduated centrifuge tube in a total of 10.0 mL of isotonic saline. Duplicate aliquots (100 μL) of the suspended cells were taken to measure the cell concentration. Pelleted cells were transferred directly to an Omnimixer microblender cup with 3 × 0.80 mL of the 0.83 M PCA. They were blended on ice for 1 min at half-speed and for 1.5 min at full speed. The blended chromatin was transferred to a glass tube where it was left to stand for 15 min to extract H1. After centrifugation, the supernate and the supernate from two subsequent 0.40-mL extractions with 0.83 M PCA (which first were used to wash the blender cup) were combined and cleared by centrifugation in the Sorvall centrifuge for 30 min at 7700 *g*. The cleared supernate was made 20% in trichloroacetic acid (Cl_3CCOOH) by the addition of 100% Cl_3CCOOH; it was left overnight at 3 °C to precipitate H1 and other proteins. The next day, the precipitated proteins were recovered by centrifugation at 7700*g* for 20 min. The precipitate was washed first with acidified acetone and then with acetone. Finally, it was dissolved in water and lyophilized. The sample was dissolved in a measured volume of electrophoresis buffer and subjected to electrophoresis in acid–urea gels. H1 was quantified from its amido black absorbance profile (D'Anna et al., 1982).

Digestion of Nuclei with Micrococcal Nuclease. To determine the percent of acid-soluble DNA generated during digestion with micrococcal nuclease (Worthington, EC 3.1.4.7), cells (~1.5×10^8) were grown in the presence of radioactive

thymidine, and nuclei were isolated by the NP-40, TCB procedure (method 1) described above, except that TCMB (10 mM Tris, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) was substituted for TCB in the last nondetergent wash. The nuclei were suspended in 950 μL of TCMB and dispensed in 95-μL aliquots. After preincubation for 2 min, prewarmed micrococcal nuclease was added to make the solution ~65 absorbance activity units mL^{-1} in nuclease, and digestion was performed at 37 °C. Digestion was stopped by adding 4.5% (w/v) PCA to make the samples 3.6% in PCA and by placing the tubes in ice. After centrifugation for 10 min (full speed) in a clinical centrifuge at 3 °C, duplicate aliquots of 100 mL were counted in 12 mL of New England Nuclear NEN-963 scintillation cocktail. For determination of the total radioactivity associated with complete digestion of radiolabeled DNA, at least two aliquots (treated with nuclease and PCA) were incubated at 70 °C for 35 min, and 100-mL aliquots were counted in 12 mL of NEN-963.

For determination of the percent acid-soluble DNA and isolation of DNA for electrophoretic analysis, nuclei from 3 × 10^8 cells were isolated and suspended in ~1.90 mL of TCMB. Aliquots were dispensed for determination of the percent acid-soluble DNA as a function of the time of digestion as described above, and the rest of the sample was measured and made 65 absorbance activity units mL^{-1} in nuclease (37 °C digestion). Aliquots were withdrawn periodically and added to 100 mM EDTA, pH 8.0 (to 10 mM), to stop the digestion. The digested aliquots were made 0.67 M in sodium acetate, 0.2% in $NaDodSO_4$, and 50 μg mL^{-1} in proteinase K. They were left at room temperature for 20–24 h and extracted with phenol and chloroform (Britten et al., 1974). DNA was precipitated by adding 2.75 volumes of 95% ethanol and placing the sample in a freezer (−15 °C) for at least 16 h.

Nuclei also were digested with micrococcal nuclease for the electrophoretic analysis of the resulting nucleoprotein particles. Nuclei from ~1.5 × 10^8 cells were suspended in 0.57 mL of TCMB, and 90 absorbance units of micrococcal nuclease were added. Digestion was stopped by the addition of 20 mM EDTA (pH 7.2) to 2 mM, and the nuclear suspension was dialyzed for ~12 h against 0.5 L of 1.5 mM EDTA, pH 7.2 at 3 °C. After dialysis, the nuclear suspension was centrifuged 10 min at 9000*g*. An aliquot of the supernate was taken to determine the DNA concentration (A_{260nm}), and the rest of the sample was made 5% in glycerol by the addition of one-third volume of 20% glycerol–2 mM EDTA buffer containing bromphenol blue (Jackson et al., 1981).

Electrophoresis. Histones were separated on 0.5 × 25 cm 2.5 M urea, 15% acrylamide, and 5.2% acetic acid cylindrical gels (Panyim & Chalkley, 1969), and histones were quantified as previously described (D'Anna et al., 1982). DNA fragments were subjected to electrophoresis in 3 mm thick, 10 cm long 0.5% agarose–2.5% acrylamide gels in Loening (1967) buffer. *Hae*III fragments of ϕX174 DNA (Bethesda Research Laboratories, Inc.) were loaded on two lanes of each gel as DNA mobility markers. After electrophoresis, the gel was stained for 1 h with ethidium bromide (1 μg mL^{-1}) and photographed with transillumination through an orange filter with Polaroid type 55 film. When radiolabeled DNA was used, each lane was sliced into 2.2-mm pieces and dissolved as described for histones, except the H_2O_2 contained 1% (v/v) concentrated ammonium hydroxide (Albanese & Goodman, 1977).

Nucleoprotein particles were separated by subjecting the equivalent of 25 μg of DNA to electrophoresis in 3 mm thick, 13.5 cm long 0.5% agarose–3.5% acrylamide gels without glycerol (Todd & Garrard, 1977; Annunziato et al., 1981). Electrophoresis was performed at 4 °C (150 V) in low ionic strength buffer (4.8 mM Tris, 9.6 mM sodium acetate, and 0.32 mM EDTA, pH 8.0) with buffer recirculation. After electrophoresis, the gels were stained with ethidium bromide and processed as described above for DNA.

The proteins associated with the nucleoprotein particles were analyzed by two-dimensional nucleoprotein–sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis employing the general procedures of Albright et al. (1980) and Annunziato et al. (1981).

Determination of Nucleosome Repeat Lengths. The distance of DNA band migration in the electrophoretic gels was measured directly from photographic negatives of the ethidium bromide fluorescence. DNA size was determined from a calibration curve of log M_r vs. the distance of migration constructed from the number of base pairs (bp) per fragment (Sanger et al., 1977) and the electrophoretic mobilities of *Hae*III restriction fragments from ϕX174 DNA. The nucleosome repeat length of total DNA was calculated by the linear regression method of Thomas & Thompson (1977).

To determine the nucleosome repeat length of new DNA, semilog plots were constructed from the sizes of the total DNAs (approximately the same as old DNA), determined from ethidium bromide fluorescence and the positions of the old DNA band maxima in the DNA radiolabel profiles. These straight lines and the positions of the new DNA bands in the radiolabel profiles were used to calculate the sizes of the new DNA fragments. The nucleosome repeat length of the new DNA was then calculated by the linear regression method.

Results

Reduction of H1 Content in Early S-Phase, HU-Blocked Cells. Two sets of experiments were performed to investigate whether H1 was being lost during the isolation of chromatin or whether it was truly depleted in vivo in early S-phase cells blocked with HU.

To determine whether H1 was being lost during nuclear isolation, (1) histones were extracted from the isolated nuclei by blending the isolated nuclei directly in H_2SO_4, and (2) cytoplasmic fractions (obtained with detergent) and all subsequent nuclear washes were acidified (individually) with PCA, cleared, and treated with Cl_3CCOOH to precipitate H1, H1°, and HMG (along with other) proteins. The precipitated proteins from all fractions were then subjected to analysis by electrophoresis in long, acid–urea–polyacrylamide gels. In addition to our usual nuclear isolation procedure (method 1), which employs homogenization with a Dounce homogenizer, we employed a second procedure which does not (method 2). The second method was used as a control in case H1 might be mechanically released by nuclear rupture during homogenization.

Figure 1a shows gels of the precipitated cytoplasmic proteins, precipitated nuclear wash proteins, and nuclear histones from exponentially growing cells (method 2, lanes 1–3) and from cells synchronized in S phase by sequential use of isoleucine deprivation and 24 h of HU blockade (method 2, lanes 4–6; method 1, lanes 7–11). Figure 1a (lanes 12 and 13) also shows the total PCA-soluble, Cl_3CCOOH-precipitable proteins from the cells of similar exponentially growing and 24-h HU-blocked cultures. Bands in the HMG1 and HMG2 regions are clearly seen in all of the cytoplasmic and nuclear wash fractions, but only in lanes 9 and 10 can traces of bands be seen in the H1 region. Based on the total amount of recovered H1 (computed from the absorbance of H1 on the gels and the total material in each fraction), we estimate that less than 5% of the total H1 is recovered from the cytoplasmic

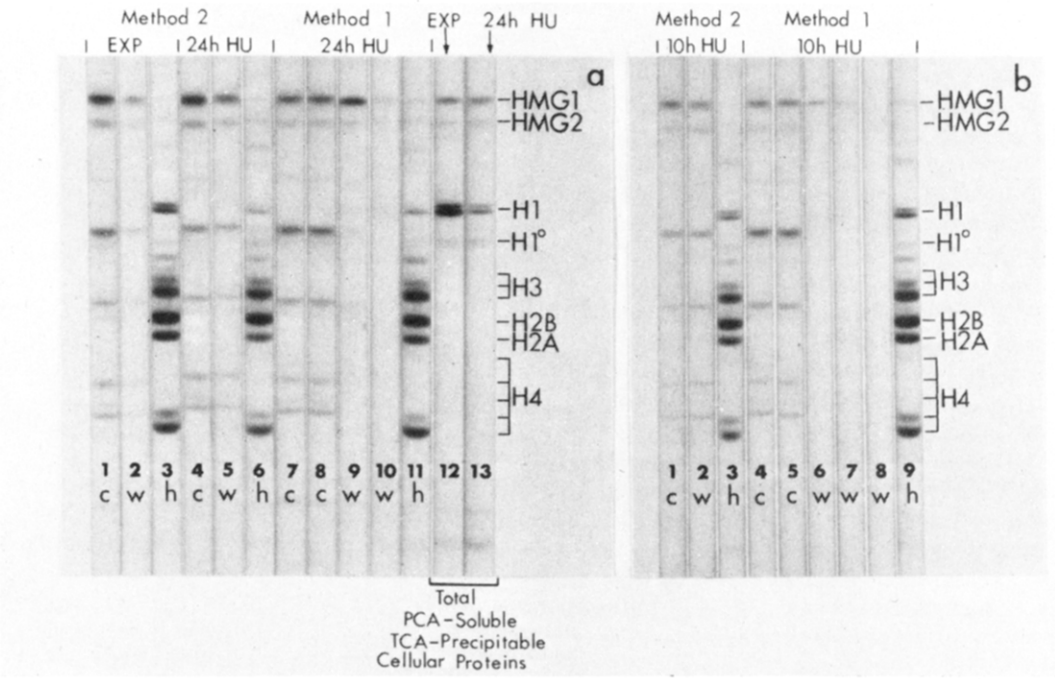

FIGURE 1: Acid–urea–polyacrylamide gels of H_2SO_4-soluble nuclear histones (h) and the PCA-soluble, Cl_3CCOOH-precipitable proteins from the cytoplasm (c) and nuclear washes (w) obtained during nuclear isolation. Nuclei were isolated from exponentially growing (EXP), 10-h HU-blocked (10h HU), and 24-h HU-blocked (24h HU) cells by methods 1 and 2 as indicated in the figure. The total PCA-soluble, Cl_3CCOOH-precipitable proteins from exponentially growing cells and 24-h HU-blocked cells are given by gels 12 and 13 of panel a. About 10–15% of the total PCA-soluble, Cl_3CCOOH-precipitable cytoplasmic proteins, 50–100% of the total PCA-soluble, Cl_3CCOOH-precipitable wash proteins, and 3–4% of the total nuclear histones were loaded on their respective gels.

fraction of nuclear washes. Since control experiments indicate that NP-40 does not interfere with the recovery of H1, we conclude that the "lost" H1 is not relegated to a cytoplasmic pool, nor is more than 5% lost during nuclear isolation. Data from S-phase cells blocked with HU for 10 h (Figure 1b) lead to the same conclusion.

We note that the same H1:H4 absorbance ratio is obtained whether (1) nuclei are blended directly in H_2SO_4 or (2) nuclei are first blended in isotonic saline and then extracted with H_2SO_4 (D'Anna et al., 1982). Further, we find that less than 1% of any histone is recovered from the isotonic saline supernate from blended nuclei. Thus, it does not appear that any substantial portion of the histones is loosely bound in the HU-blocked cells.

To confirm our previous notion that histone H1 was being depleted in HU-blocked cells (as opposed to some extraordinary accumulation of core histones) (D'Anna et al., 1982), we compared both the H1 content per unit DNA (as outlined under Experimental Procedures) and the H1:H4 absorbance ratio between exponentially growing cells and cells blocked in early S phase for 24 h (where the H1:H4 ratio is minimal). In one experiment, we found the H1 content per unit DNA in the HU-blocked culture was 37 ± 1% of the value in exponentially growing cells, and the H1:H4 ratio was reduced to 35 ± 1% of the value in the exponentially growing culture; a second experiment gave respective values of 34 ± 2% and 39 ± 1%. We conclude, therefore, that there is a true loss of H1 per unit DNA in the HU-blocked cells and, thus, no need to postulate the existence of extraordinary quantities of core histone in the nucleosome.

We note that estimates of relative H1 and H4 contents from [^{3}H]lysine incorporation and the number of lysines per molecule (62 and 11, respectively) indicate that there is only 0.25 ± 0.01 H1 molecule for each molecule of H4 in exponentially growing cells (H1:H4 absorbance ratio of 0.50). Consequently, an average of only one nucleosome in two contains H1 in

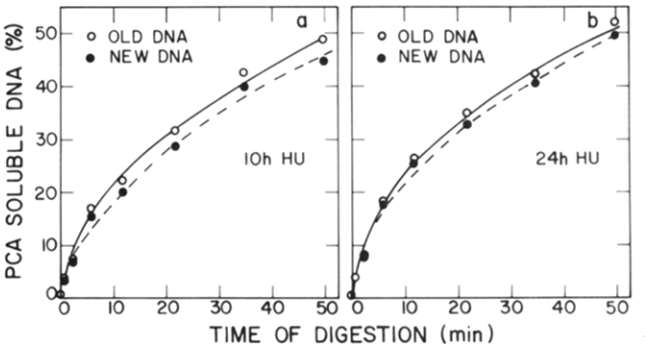

FIGURE 2: Percentages of acid-soluble new and old DNAs released by micrococcal nuclease digestion of nuclei as functions of time. (a) Cells were synchronized in G_1 in the presence of [^{14}C]thymidine to label old DNA and then released into complete medium containing 1 mM HU (for 10 h) and [^{3}H]thymidine to radiolabel new DNA. (b) Cells were synchronized and radiolabeled as in (a), except cells were blocked 24 h in HU.

exponentially growing cells, and this value is substantially reduced in the HU-blocked cells.

Sensitivity of New DNA to Digestion with Micrococcal Nuclease. To determine if there was an accumulation of immature chromatin, we investigated whether (1) the DNA from chromatin replicated during S-phase block (new DNA) was more sensitive than that of mature chromatin to digestion (to acid-soluble size) by nucleases (Seale, 1975; Hildebrand & Walters, 1976; Levy & Jakob, 1978) and (2) the measured nucleosome repeat lengths of new chromatin (from micrococcal nuclease digests of nuclei at 37 °C) were shorter than those of bulk chromatin (Levy & Jakob, 1978; Murphy et al., 1978; Seale, 1978; Jackson et al., 1981; Annunziato & Seale, 1982).

Measurement of the percent acid-soluble DNA as a function of the time of digestion of nuclei from cells blocked for 10 and 24 h in HU (Figure 2) shows that new DNA is not degraded more rapidly than old DNA by micrococcal nuclease. Re-

producibly, we find (1) the new DNA is slightly more resistant to digestion to acid-soluble size than old DNA, and (2) the difference in the digestion of new and old DNA is somewhat greater after 10 h in HU than after 24 h in HU. Thus, prolonged S-phase block for 10 or 24 h does not prevent new chromatin from maturing to a structural form that is equally or more resistant than old DNA to attack by micrococcal nuclease.

Since histone H1 is lost from chromatin in the HU-blocked cells, we investigated whether the loss of H1 would substantially increase the exposure of DNA to attack by micrococcal nuclease, compared with exponentially growing cells. However, digestion of a mixture of isolated nuclei from exponentially growing cells labeled with [^{3}H]thymidine and 24-h HU-blocked cells labeled with [^{14}C]thymidine showed no difference in the rate of digestion of their DNA (results not shown).

Nucleosome Repeat Lengths in HU-Blocked Cells. To determine whether there were differences in the electrophoretic patterns and the nucleosome repeat lengths of the new and old DNAs from HU-blocked cells, nuclei from radiolabeled cells were digested wtih micrococcal nuclease, and their DNA fragments were subjected to electrophoresis in agarose–polyacrylamide gels.

Figure 3a–c shows electrophoretic patterns (isotope incorporation profiles) of the DNA fragments produced by different extents of digestion of nuclei from cells blocked 10 h with HU. In each of the digests, the maxima of the new and old DNAs coincide in the mononucleosome bands, but (1) the new DNA migrates from half to one slice faster than the old DNA in the dinucleosome and other resolved multimers, and (2) the new DNA exhibits a higher background between bands. Also, there are changes in the relative prominence of the old and new DNAs during the course of digestion (Figure 3a–c). At the early times of digestion, the dinucleosome–tetranucleosome bands of old DNA are relatively more prominent than those of new DNA, but as digestion continues, they are eclipsed by the new DNA. In contrast, the mononucleosome-sized new DNA is always relatively less prominent than the old DNA. During digestion from 1.4 to 24% acid solubility, there is a 9% increase in the ^{3}H:^{14}C ratio for DNA on the gel. Thus, these results, like those in Figure 2, indicate that, after 10 h in HU, new chromatin is more resistant than old chromatin to attack by micrococcal nuclease. In addition, the results suggest that the conformation of the new chromatin is such that it is relatively more resistant than old DNA to cleavage to the mononuclease size or that the new DNA mononucleosome is quickly degraded.

Similar results are obtained when the micrococcal nuclease fragments from 24-h HU-blocked cells are analyzed in agarose–polyacrylamide gels (Figure 3d–f); however, (1) the new DNA is shifted only 0–0.5 gel slice ahead of DNA in the profiles, and (2) there is only a 3% change in the ^{3}H:^{14}C ratio of the DNA counts on the gel in going from 1.3 to 21.3% acid-soluble DNA.

Measurement of nucleosome repeat lengths of new and total (similar to old) DNA from HU-blocked cells and total DNA from G_1 and exponentially growing cells (Figure 4) shows that the measured nucleosome repeat lengths change as a function of the extent of digestion as observed by others (Lohr et al., 1977; Jackson et al., 1981; Annunziato & Seale, 1982). More interestingly, however, the changes are different between the new DNA and old DNA of the HU-blocked cells and between the total DNA of HU-blocked cells and the exponentially growing or G_1 controls.

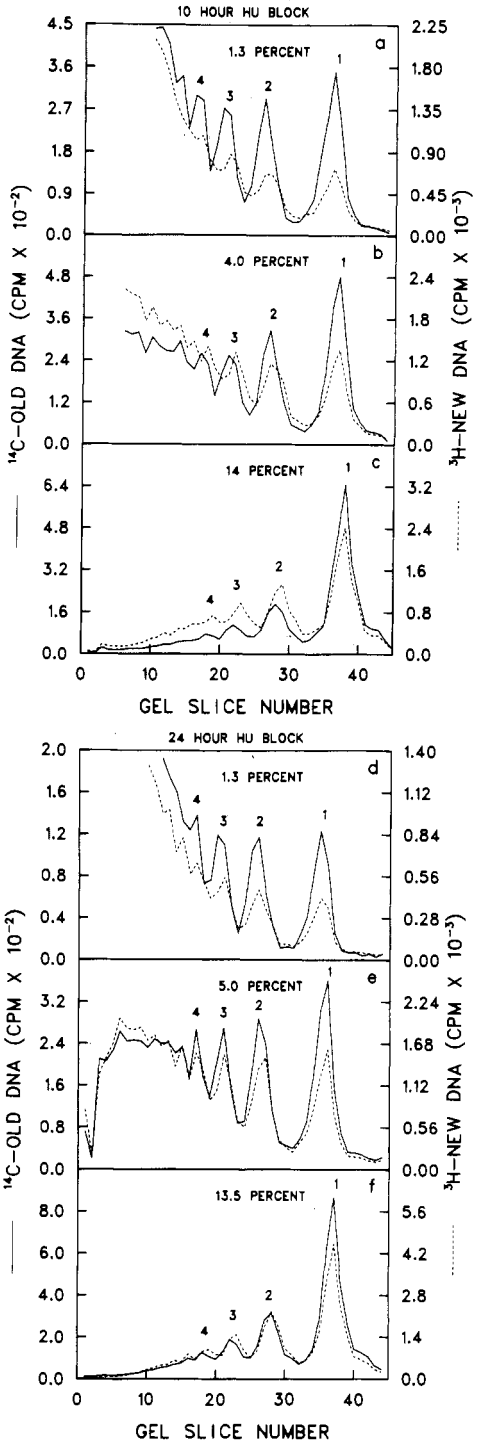

FIGURE 3: Electrophoretic radiolabel profiles of old (—) and new (---) DNA fragments produced by micrococcal digestion of nuclei from S-phase cells blocked 10 (a–c) and 24 h (d–f) with HU. Cells were radiolabeled, as described in Figure 2. DNA fragments were separated in 0.5% agarose–2.5% polyacrylamide gels; the direction of migration is from left to right. The percent value in each panel indicates the extent of digestion of the old DNA to acid-soluble size. Numbers above the bands indicate the number of nucleosome units in the DNA fragment.

After 10 h of HU block (when there is a 5% increase in DNA mass and an ~30% loss of H1 per nucleosome), the measured nucleosome repeat lengths of new DNA are between 7 and 10 bp less than those of total DNA (similar to old DNA) throughout the digestion period; thus, in the HU-blocked S-phase cells, the measured nucleosome repeat lengths of new DNA are less than those of old DNA, just as they are in experiments employing very brief periods of pulse labeling in

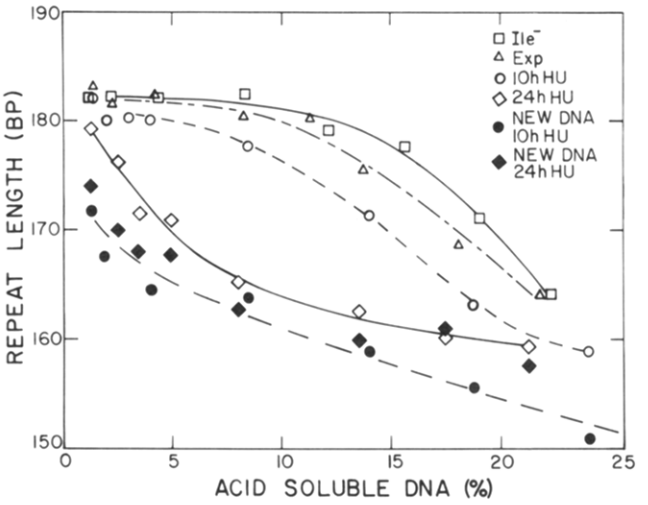

FIGURE 4: Nucleosome repeat length as a function of the percent acid-soluble DNA: total DNA from exponentially growing cells (△); G_1-blocked (isoleucine deprivation) cells (□); cells blocked in S phase by sequential use of isoleucine deprivation and 10 (○) or 24 h (◇) of HU blockade; new DNA from the cultures blocked in S phase by sequential use of isoleucine deprivation and 10 (●) or 24 h (◆) of HU blockade.

the absence of drugs (Levy & Jakob, 1978; Murphy et al., 1978; Seale, 1978). Not only is the nucleosome repeat length of new DNA shorter than that of old DNA but also the nucleosome repeat length of the old DNA decreases as a function of acid-soluble DNA more rapidly than the repeat length of exponentially growing or G_1-blocked cells.

After 24 h in the HU (when there is approximately a 15% increase in the DNA content and a 70–80% loss of H1), the nucleosome repeat lengths of new DNA are 2–3 bp less than those of old DNA (Figure 4), but the repeat lengths of the new and old DNAs are substantially less than those measured for the control G_1 cells and much more like those measured for the new DNA from cells blocked 10 h in S phase with HU. It appears, therefore, that as the time of S-phase block is increased and the content of H1 in chromatin is reduced, the measured nucleosome repeat lengths of old chromatin are reduced so that they are similar to those measured for newly replicated, immature chromatin (Murphy et al., 1978; Jackson et al., 1981; Annunziato & Seale, 1982).

Resolution of Nucleosome Species Containing Old and New DNAs. The similar shapes and positions of the mononucleosome DNA bands (band 1) in Figure 3 suggested that the distributions of new and old mononucleosome species [see Todd & Garrard (1979) and Albright et al. (1980)] in the respective 10- and 24-h HU-blocked cells might be the same. To determine whether this was so or whether mononucleosome species containing H1 might be partially depleted in new chromatin [as expected from the loss of H1 and as suggested for newly replicated DNA in the absence of drugs (Schlaeger, 1982)], we digested the isolated nuclei of radiolabeled cells to ~5% DNA acid solubility and subjected the resulting nucleoprotein particles to electrophoresis in 0.5% agarose–3.5% acrylamide gels (Todd & Garrard, 1979) without glycerol (Annunziato et al., 1981).

Examination of the gels (Figure 5a) and the DNA isotope incorporation profiles (Figure 5b) from exponentially growing and HU-blocked cells indicates that the major mononucleosome (M) bands (MI–MIII) and the minor bands (MIV and MV) (Todd & Garrard, 1979) are present in all of the samples, but (1) there are changes in the distribution of mononucleosome species MI–MV in going from exponentially growing to 10-h HU-blocked to 24-h HU-blocked cells, (2)

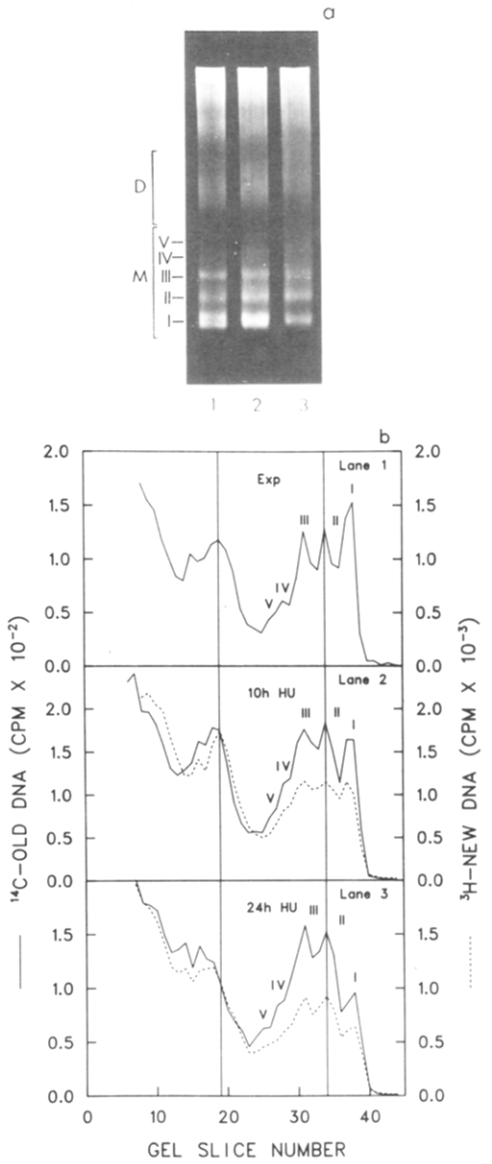

FIGURE 5: Electrophoretic gels (a) and DNA radiolabel profiles (b) of separated nucleoprotein particles produced by digestion of nuclei (37 °C, 5% acid-soluble DNA) from exponentially growing cells (lanes 1) and cells blocked in S phase by 10 (lanes 2) or 24 h (lanes 3) of exposure to HU. DNA was radiolabeled as described in Figure 2 to distinguish old (^{14}C) and new (^{3}H) DNA. The direction of migration is from top to bottom in (a) and from left to right in (b). Mononucleosome particles (M) and dinucleosome particles (D) are designated in accordance with the nomenclature of Albright et al. (1980).

there is relatively less new than old chromatin in the mononucleosome regions of the HU-blocked cells, compared with the respective dinucleosome and higher multimer regions, and (3) the bands in the dinucleosome region of the 24-h HU-blocked cells are shifted to positions of reduced electrophoretic mobility.

The changes in the distribution of mononucleosome species in going from lane 1 to lane 3 in Figure 5 suggest that during the HU block there occur (1) a relative increase in mononucleosome species MII–MV at the expense of MI (best seen in Figure 5b) and (2) a depletion in the faster migrating band of the MI doublet in Figure 5a. [Also notice that, reproducibility, the new DNA MI peak (Figure 5b, lane 2) migrates slightly slower than the MI peak of the old DNA.] Since (1) Albright et al. (1980) have shown that the major noncore histone proteins in the MIII–MV bands of untreated cells are H1 and HMG proteins and (2) certain multinucleosome

species are directly digested to MI species (Todd & Garrard, 1979), it is tempting to assume that (1) there are relatively more mononucleosomes containing H1 (or H1°) and HMG 14/17 proteins in the HU-blocked cells, and (2) MI is reduced because those species giving rise to the faster MI are depleted in the rearranged chromatin. While the second assumption seems reasonable, the first does not. Although there appears to be an increase in the quantities of HMG 14/17 class proteins in the H_2SO_4-extracted proteins from HU-blocked cells (unpublished results), there is also the enormous loss of H1.

To determine whether there was a change in the proteins in the MIII–MV regions of HU-blocked cells, we separated the nucleoprotein particles in a first-dimension agarose–polyacrylamide gel and then analyzed their proteins in a second-dimension $NaDodSO_4$ gel. Comparison of the two-dimensional gels from the digested nuclei of exponentially growing cells (Figure 6a) and 24-h HU-blocked cells (Figure 6b) indicates a clear loss of H1 in the region of MIII nucleosomes and a general increase in non-histone proteins in the HU-blocked cells. Several of the non-histone proteins (designated 1a, 1b, 2a, 2b, 3a, 3b, and 4) are of special interest, because they (1) are visible in both gels, (2) are enhanced in the HU-blocked cells, and (3) give prominent spots at positions which correspond to those of the mononucleosome species in the first-dimension gel. Since there are so many bands, we can only make a semiquantitative comparison of the proteins at this time. Relative to core histones, the differences between the proteins from the 24-h HU-blocked cells and the exponentially growing cells in each of the mononucleosome bands are as follows: (1) ~2–3 times as much protein 1a is found in MI (perhaps associated with the slower MI species); (2) approximately twice as much protein 2a is found in MII; (3) ~25% as much H1 and 4–6 times as much protein 3a (really two bands of similar electrophoretic mobility) are found in MIII; (4) ~65% as much H1 and 2–4 times as much of proteins 3b and 4 are found in the MIV–MV region. (Protein 3b also appears to be enhanced between MIII and MII.) In addition, proteins 1b and 2b are severalfold enhanced in the MI and MII regions, respectively. Thus, these data indicate that (1) H1 is especially depleted in the region of MIII nucleosomes, and (2) other proteins, especially proteins 3a, 3b, and 4, are enhanced in the HU-blocked cells. The results also (1) suggest that proteins 3a, 3b, and 4, like H1 and HMG proteins (Albright et al., 1980), determine the characteristic electrophoretic mobilities of the MIII–MV species, (2) emphasize the danger of characterizing mononucleosome species solely by their electrophoretic mobilities, and (3) raise the possibility that the loss of H1 is related to the enhancement in non-histone proteins.

These data do not indicate to us why the dinucleosomes from the 24-h HU-blocked cells have a reduced electrophoretic mobility compared with those from exponentially growing control cells.

Turnover of H1 and H4 during HU Block. Since (1) some H1 is still found in the mononucleosome region of chromatin from HU-blocked cells, (2) histones are known to turn over in isoleucine-deprivation G_1-blocked cells (Gurley et al., 1972; Zlatanova, 1981) and in HU-blocked S-phase cells (Gurley et al., 1974; Nadeau et al., 1978; Russev et al., 1980), and (3) nucleosome cores appear to be more closely packed after S-phase block (this report), we investigated whether there was extensive turnover of H1 prior to its loss from chromatin and extensive turnover of H4, which might account, mechanistically, for the rearrangement of nucleosomes in the HU-blocked cells. Cells were grown and synchronized in G_1 in the presence

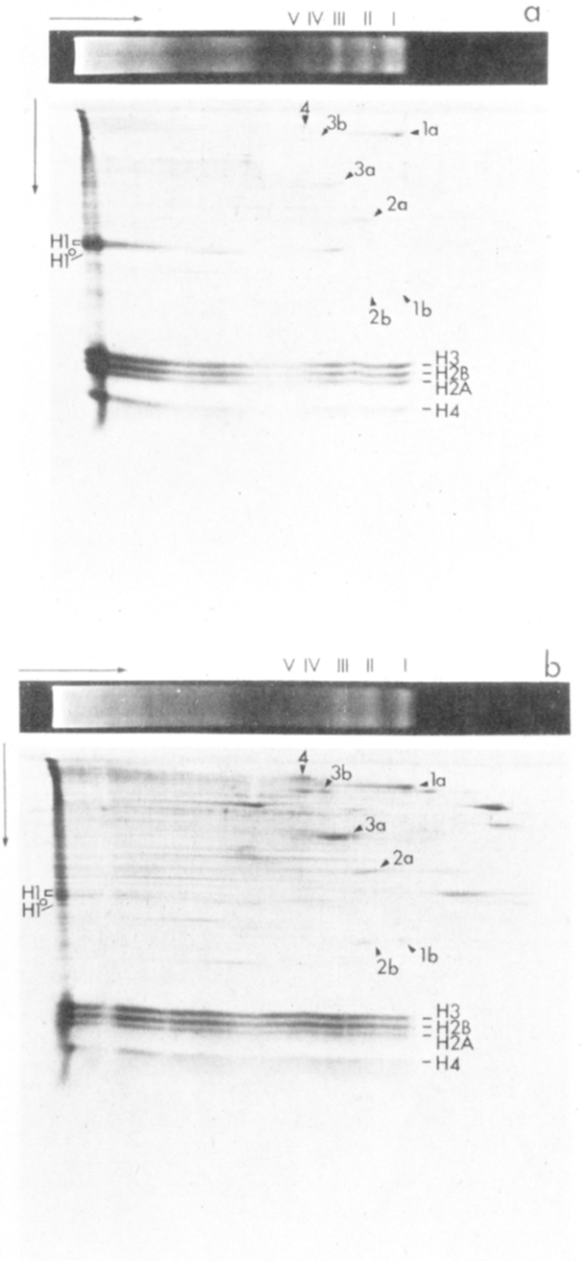

FIGURE 6: Two-dimensional electrophoretic analysis of the proteins associated with the nucleoprotein particles produced by micrococcal nuclease digestion (37 °C) of nuclei from exponentially growing cells (a) and cells blocked in S phase by sequential use of isoleucine deprivation and 24 h of HU blockade (b). The first-dimension gel (migration from left to right) is described in the legend to Figure 5; the second dimension is a 15% $NaDodSO_4$ slab gel (migration from top to bottom). The first-dimension gels shown are lighter loads than those actually used so that the nucleoprotein bands may be identified.

of [^{3}H]lysine and then released into complete medium containing 1 mM HU but lacking [^{3}H]lysine. Measurements of H1-specific activity as a function of time (Figure 7a) are somewhat noisy, but independent experiments indicate there is little change following release from G_1 block. Since there is substantial loss of H1 and no evidence of cytoplasmic H1 pools, we conclude that H1 is degraded from chromatin, or it is dissociated from chromatin and degraded in the cell with very little replacement synthesis ($\lesssim$10% of H1 is newly synthesized when ~75% of H1 is depleted from the cell).

In contrast to the behavior of H1, the measured specific activity of H4 decreases following release from G_1 block (Figure 7b). The largest decrease occurs between 0 and 2 h

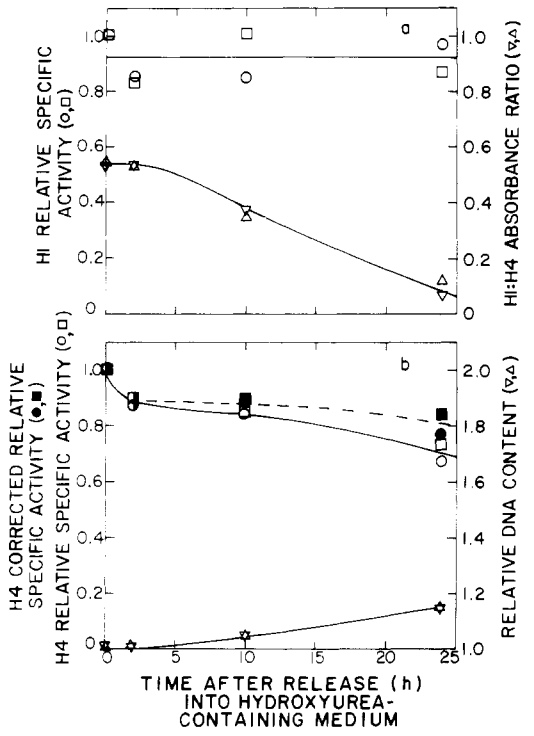

FIGURE 7: Relative specific activities of histones H1 (a) and H4 (b) following the release of synchronized G_1 cells into 1 mM HU blockade. The H4 corrected relative specific activity is the relative specific activity adjusted for a presumed increase of new H4 to accompany the increase in cellular DNA mass. H1:H4 absorbance ratios and increases in DNA content are also plotted in (a) and (b), respectively.

when there is no DNA synthesis; thus, it is clear that H4 turns over during this period. Between 2 and 10 h, there is a 3–5% increase in DNA mass and a 10–14% decrease in specific activity (Figure 7b), and between 10 and 24 h, there is a 10% increase in DNA mass and a 11–15% decrease in specific activity.

Since we have seen no evidence for rapidly degraded DNA in nuclear digests of chromatin which might suggest naked DNA, we assume that the new DNA in the HU-blocked cells is associated, at least, with core histones. If we correct the specific activities of H4 for a presumed increase in H4 mass associated with synthesis of new DNA (this is probably an underestimate, because it does not allow for additional H4 and other core histones that may be synthesized to occupy DNA as nucleosome cores become more closely packed), we find a 10–12% decrease in H4 specific activity between 0 and 2 h, negligible change (and negligible turnover) between 2 and 10 h, and a 5–11% decrease in specific activity between 10 and 24 h. Thus, between 2 and 24 h (cells enter S phase at ~4 h), 5–12% of the H4 molecules turn over, and at 24 h, a total of ~18–25% of the total H4 molecules are new, because of turnover and the presumed synthesis of additional H4 mass. While we do not know what percentage of chromatin has rearranged during 24 h of S-phase block with HU, 5–12% turnover of H4 (removal and replacement of H4 on chromatin) is probably not enough to account for the apparent massive rearrangement of chromatin observed in the 24-h HU-blocked cells.

Discussion

Loss of H1 and Changes in Chromatin Structure. These results (1) confirm our previous notion that histone H1 is depleted from the chromatin of the synchronized S-phase cells (D'Anna et al., 1982) and (2) show that chromatin undergoes substantial structural changes during early S-phase block.

Investigation of H1 content in HU-blocked S-phase cells indicates that H1 is lost from both chromatin and the cell and that there is minimal replacement synthesis of H1. Since histone H1 is easily degraded by proteases and there is no evidence for cytoplasmic pools of H1, it seems most likely that H1 is degraded in the cell, as opposed to more complicated means of disposal.

During the time that H1 is lost, chromatin appears to undergo structural reorganization. After 10 h of HU block (3–5% increase in DNA content; ~30% decrease in H1 content), the new chromatin synthesized during the block yields a distribution of nucleosome repeat lengths whose values are, for the most part, less than those of bulk chromatin at the same extent of digestion and similar to those observed for new chromatin within ~10 min of synthesis in the absence of drugs (Jackson et al., 1981; Annunziato & Seale, 1982). In addition, a portion of the old chromatin undergoes structural reorganization so that its nucleosome cores are more closely spaced along the DNA chain. After 24 h of HU block (when there is an ~15% increase in DNA content and a 70–80% loss of H1), the measured nucleosome repeat lengths of *both* new and old chromatins are reduced and similar to those measured for new DNA after 10 h of HU block.

Although the "newly replicated chromatin" of HU-blocked cells has a smaller repeat length than bulk chromatin, it is slightly more resistant, rather than more sensitive, than old chromatin to attack by micrococcal nuclease. Therefore, HU block allows new chromatin to mature until it is equally resistant as bulk chromatin to attack by nuclease, but HU prevents chromatin from achieving the proper nucleosome repeat lengths associated with true structural maturity. Furthermore, HU block appears to cause old chromatin to assume a "pseudoimmature" conformation in which the nucleosome cores are in closer than normal proximity (or extremely mobile; see below) along the DNA.

Since the old chromatin in HU-blocked cells undergoes structural change as H1 is lost from the chromatin, the two events appear to be directly related. Estimates of H1 content indicate that there is an average of only one H1 per two nucleosomes in exponentially growing cells. [This is even lower than the value of 0.64 molecule of H1 per nucleosome in mouse mastocytoma cells (Albright et al., 1979).] This relatively low value in total chromatin is reduced to 1 molecule of H1 per 2.8 nucleosomes after 10 h in HU and only 1 molecule of H1 per 7–10 nucleosomes after 24 h in HU. In addition, other indirect evidence suggests that H1 is lost first from those regions containing new DNA (so that the new chromatin contains $\lesssim$1 H1 molecule for every 7–10 nucleosomes): (1) the nucleosome repeat length of new chromatin is much less than that of bulk chromatin after 10 h in HU, but as H1 is lost, nearly all of the chromatin is reorganized to a smaller repeat length; (2) the average nucleosome repeat length of the new chromatin in the 10-h HU-blocked cells (after digestion to 4.5% acid solubility at 37 °C) is less than or equal to the 166–168 bp required for the stability of the platysome (McGhee & Felsenfeld, 1980; Simpson, 1978; Nelson et al., 1979) which contains H1 and core histones; (3) new DNA appears to occur preferentially in the slower migrating MI mononucleosome band, rather than in the faster one, which becomes depleted as H1 is lost from the total chromatin during the 24 h of HU block (Figure 5).

Recently, Schlaeger (1982) has suggested that H1 is depleted or loosely bound in newly replicated chromatin in the absence of drugs. Those results appear to be supported by our interpretation of the data of Galili et al. (1981) and Jackson

et al. (1981). In contrast, Annunziato & Seale (1982) have found new DNA in MIII nucleosomes within 30 s of synthesis of H1. Although their conclusion was based on the electrophoretic mobilities of mononucleosome particles containing new DNA, the new DNA MIII bands were very sharp and probably do contain H1 as opposed to, or in addition to, other proteins such as those we observed in Figure 7.

Are Nucleosomes Sliding during Digestion? Our interpretation of structural rearrangements in chromatin is predicated on the assumption that chromatin is not rearranging during digestion. At least three observations support that interpretation: (1) preincubation of nuclei of HU-blocked cells at 37 °C [a condition which retards sliding (Spadafora et al., 1979)] does not alter the electrophoretic pattern of DNA fragments produced by nuclease digestion (results not shown); (2) digestion of chromatin at 2 °C [a condition which retards sliding (Spadafora et al., 1979; Weischet, 1979] is complex (Greil et al., 1976), but it does not stop the nucleosome repeats from changing during digestion (results not shown); and (3) there is no rapidly digested new DNA to suggest the presence of naked DNA during digestion of chromatin from HU-blocked cells. In this regard, Annunziato & Seale (1982) have presented similar arguments in discussing the reduced nucleosome repeat length of newly replicated chromatin in the absence of drugs.

While the aforementioned observations provide a basis for our interpretation, they are not proof. Indeed, while this paper was submitted, Smith & Chalkley (1983) reported that formaldehyde fixation of cells abolishes the shortened repeat length of new chromatin in the absence of drugs, providing support for sliding during digestion. We note, however, that removal of H1 in vitro is insufficient to cause sliding during digestion (Lawson & Cole, 1979; Allan et al., 1980). Consequently, if chromatin rearrangement occurs during digestion, our results indicate that the rearranged chromatin is structurally different from the bulk mature chromatin and that it is especially sensitive to rearrangement. The possible involvement of the apparently enhanced proteins, 3a, 3b, and 4, in nucleosomal sliding is intriguing but unknown.

Implications for Chromatin Structural Changes during Replication. Previously, this laboratory suggested that the release of G_1 cells into S-phase HU block may accentuate the loss of H1 from chromatin (during replication) which normally goes unnoticed (D'Anna et al., 1982). A major question was not addressed: "Why does the loss of H1 in the synchronized HU-blocked cells greatly exceed the synthesis of new DNA?" To answer this question and to account for a number of other observations regarding replication, we suggest the following: (1) during the early stages of the initiation of replication (or prior to passage of the replication fork), H1 is dissociated (by whatever mechanism) from the initiated replicon-sized units of chromatin, and (2) H1 does not reassociate with the newly replicated chromatin in a concerted fashion until the replication fork has passed and, perhaps, the equivalent of 40–80 nucleosomes or the whole replicon has been replicated [see Jackson & Chalkley (1981a) and Worcel et al. (1978)]. The implications of these hypotheses are outlined below.

Replication of eukaryotic cells is accomplished by the bidirectional initiation of clusters of replicating units called replicons (Prescott, 1976; Hand, 1978). It has been estimated (Prescott, 1976) that at least 25 clusters of replicons initiated in sequence are needed to maintain a continuous rate of DNA synthesis during the S phase in mammalian cells. If H1 were lost from chromatin during the initiation of a replicon cluster, one would observe an initial 4% loss of H1, which would increase to ~8% in perfectly synchronized cells were H1 not to reassociate with DNA until replication of the replicon was completed. [Following release of G_1 cells into complete medium to resume cell-cycle traverse, we observe a decrease in the H1:core histone ratio of ~10% when 70% of the cells are in S phase (D'Anna et al., 1983).] If another cluster of replicons were to fire at approximately the time that synthesis of the first one was completed, its dissociated H1 and newly synthesized H1 would be available to reassociate with the completed replicon. Such an occurrence would be consistent with (1) the reports that newly synthesized H1 has little tendency to associate with newly synthesized DNA (Jackson & Chalkley, 1981a,b; Annunziato et al., 1982) and (2) the suggestion of Worcel et al. (1978) that H1 associates with newly replicated DNA after the core histones. With regard to the association of H1 with newly replicated chromatin, Annunziato & Seale (1982) have observed that pulse-labeled chromatin does not fully mature until ~10 min after the removal of the radioactive isotope. On the basis of a fork progression rate of 0.5 μm/min (Prescott, 1976), ~85 nucleosome lengths of DNA would have been synthesized before the new chromatin was fully mature. Allowing for residual radioactive DNA pools and a lower rate of fork movement, we estimate that a minimum unit size of 40–85 nucleosomes is required for chromatin maturation and, perhaps, concerted reassociation of H1.

In HU-blocked cells, replication is initiated, but elongation is greatly retarded (Walters et al., 1976a,b). If H1 dissociates from initiated replicons and does not reassociate in a concerted fashion until ≥40 nucleosomes have been synthesized (or until the H1-depleted region is replicated), one would expect a continued loss of H1 just as we have observed. Alternatively, it is equally plausible that H1 does not dissociate, in total, from the replicon at the time of initiation, but a "displacing protein" precedes the replication fork to dislodge H1. In either case, the loss of H1 would be expected to relax the higher orders of chromatin structure and greatly facilitate the processing of chromatin—especially if replication involves the reeling of chromatin through a replication complex on the nuclear matrix (Pardoll et al., 1980; Berezney & Buchholtz, 1981).

While this simplified model of replication is attractive, we do not know whether H1 loss is an artifact; e.g., H1 synthesis might be specifically inhibited by HU during S phase, or proteolysis of H1 might be switched on by the accumulation of deoxyribonucleotide triphosphate pools during S-phase block [see Surowy & Berger (1983)]. (In this regard, treatment of G_1 cells with HU for 10 h does not alter the H1 content, but after 24 h, there is an approximate 12% reduction in H1. Thus, while HU appears capable of altering H1 content in G_1 cells, the effects are substantially less than during S phase.) In support of our suggestions, Schlaeger (1982) has mentioned in passing that (1) the altered structure of chromatin (~165 bp repeat length) is associated with a small fraction of chromatin "not confined to the replicating chromatin at the growing point alone" (in the absence of drugs) and (2) the maturation of newly replicated chromatin "depends strongly on continued DNA synthesis (E. J. Schlaeger and W. Pülm, unpublished results)". If our suggestions are correct, other inhibitors besides HU and selected temperature-sensitive mutants should enhance the loss of H1 and inhibit the full maturation (this report; Jackson & Chalkley, 1981a) of newly replicated chromatin. These ideas are experimentally testable.

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