Histone Synthesis and Deposition in the G1 and S Phases of Hepatoma Tissue Culture Cells†

Vaughn Jackson*
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
Roger Chalkley
Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242
Received February 28, 1985

ABSTRACT: Hepatoma tissue culture cells were synchronized in G1 and in S phase in order to examine the level of synthesis of different histone types and to determine the rate, timing, and location of their deposition onto DNA. We observe a basal level of synthesis in G1 (5% of that seen in S phase) for H2A.1, H2A.2, H3.2, H2B, and H4. The minor histone variants X and Z are synthesized at 30% of the rate observed in S cells. The rate of synthesis of the ubiquinated histones uH2A.1,2 is not as depressed in G1 cells as seen for H2A.1 and H2A.2. Histones synthesized in G1 are not deposited on the DNA of these cells at equivalent rates. Thus, histones H3.2 and H4 are not deposited significantly until S phase begins, at which time deposition occurs selectively on newly synthesized DNA. The deposition of H2A.1, H2A.2, H2B, X, and Z proceeds in G1; however, it occurs to a 2–4-fold lower extent than seen for the deposition of H1, HMG 14, and HMG 17. The deposition of all histones synthesized in S phase occurs rapidly, but there are variations in the sites of deposition. Thus, newly synthesized H3.1, H3.2, and H4 deposit primarily on newly replicated DNA whereas H2A.1, H2A.2, uH2A.1, 2, and H2B deposit only partially on new DNA (30%) and mostly on old. H1, HMG 14, and HMG 17 are deposited in an apparently fully random manner over the chromatin. To interpret these observations, we propose a model which includes a measure of histone exchange on the chromatin fiber. The model emphasizes the dynamics of histone–histone and histone–DNA interactions in regions of active genes and at replication forks.

Evidence has accumulated over a number of years that histone synthesis is tightly coupled with DNA synthesis (Hohmann, 1981; Robbins & Borun, 1967; Butler & Mueller, 1973). However, more recent evidence has indicated that there is also a basal level of synthesis, both in nonproliferating cells and in exponentially growing cells when they are not in S phase, which appears to be independent of DNA synthesis (Brown, 1980; Zlatanova & Swetly, 1978; Groppi & Coffino, 1980; Wu & Bonner, 1981; Wu et al., 1982, 1983). In nondividing systems, all the major histones including the variant types are synthesized and assumed to be deposited on the DNA (Wu et al., 1982, 1983). This observation presupposes that a concomitant turnover of histone in the nucleus also occurs to accommodate the influx of histone if the net histone content is to remain unchanged. Such turnover of histone has been reported (Commerford et al., 1982). Grove & Zweidler (1984) have recently shown that a significant turnover of H2A and H2B occurs in both proliferating and nonproliferating cells. In addition, they have shown changes in the histone variant proportions during terminal differentiation of erythroleukemic cells. On the other hand, it has also been reported that turnover of histone may not be necessary to accommodate the additional histone within the nucleus. Thus, during the development of neuronal perikaryons, the observed decrease in spacer length between nucleosomes is correlated with a continued synthesis of histone in the absence of DNA synthesis (Brown, 1980).

For exponentially growing cells, the basal level of histone synthesis (G1 synthesis) appears to differ both qualitatively and quantitatively from that seen in S phase. Groppi & Coffino (1980) have reported that in the G1 phase of S49 cells histone synthesis occurs at an equivalent rate in S-phase synthesis (an extensive uncoupling of histone and DNA synthesis). Wu & Bonner (1981) and Marashi et al. (1982) have reported that in several cell lines, CHO, Reuber, L1210, HeLa, and IMR90, the level of synthesis for histones in G1 is no larger than 10% of that observed in S-phase cells. The reported distribution of histone mRNA between G1- and S-phase cells is also consistent with these latter observations (Marashi et al., 1982; Stein et al., 1978). Qualitatively, Wu and Bonner (1981) have reported that in contrast to nonproliferating cells, exponential cells in the G1 phase synthesize primarily the histone variants X, Z, and H3.3 and only in S phase is there a 10-fold increase in synthesis of the major histone types H2A.1 and H2A.2, H3.1 and H3.2, H2B, and H4. In this latter work as well as in the earlier studies with nonproliferating cells, the actual deposition of these histones on the DNA was not analyzed in detail. However, if deposition and integration into chromosomal structures are occurring (particularly in G1 phase), these observations reflect a rather dynamic interaction between histones themselves as well as with DNA.

In our earlier studies, we analyzed the deposition of newly synthesized histones onto DNA and observed that in a randomly growing population of hepatoma tissue culture (HTC) cells, the site of deposition varies according to the specific histone. Using four separate techniques (Jackson & Chalkley, 1981a,b; Jackson et al., 1981), we demonstrated that newly synthesized H3 and H4 are deposited specifically on newly replicated DNA, that H2A and H2B are deposited on both new and old DNA, and that new H1 is deposited on old DNA. The implication of these observations is that a dynamic exchange of some histones is occurring, particularly with H1 and less so for H2A and H2B, which results in the observation that

† This investigation was supported by U.S. Public Health Service Grants CA35829 (to V.J.) and GM47224 (to R.C.).

0006-2960/85/0424-6921$01.50/0

these histones do not deposit selectively on newly replicated DNA. However, because of the need to distinguish between synthesis and deposition of histones for S-phase cells as opposed to the G1 cells in a randomly growing population of cells, we have reanalyzed deposition in G1- and in S-phase cells separately. We will be able to discuss the dynamics of basal histone synthesis and deposition in both G1 and S phases as well as the deposition of the major histones in S-phase cells.

Experimental Procedures

Synchronization and Labeling of Cells. HTC (hepatoma tissue culture) cells were grow in suspension culture at (2–4) $\times 10^5$/mL in Swims S77 medium (5% newborn/5% calf serum) with a cell cycle of 18 h. The cells were synchronized in the following way. They were first converted into a monolayer culture by growing in the presence of 2.5 mM $CaCl_2$. After 24 h, the flasks were vigorously shaken to remove loosely attached cells. Fresh medium containing 0.25 mM colcemid (Moore et al., 1979) was then added. After 8 h, mitotic cells were removed by gentle rotation, collected by centrifugation at 2000*g* for 10 min, washed, and resuspended at 2×10^5 cells/mL in fresh medium. Within 90 min, the detached cells doubled in number. For labeling with [^{3}H]lysine and iododeoxyuridine (IdUrd) in combination, aliquots of cells were incubated for 30 min in the presence of 1×10^{-4} M iododeoxyuridine. The cells were then centrifuged at 2000*g* for 5 min and resuspended in modified Swims S77 medium (minus lysine, +IdUrd) for incubation at 37 °C for 15 min. The cells were then centrifuged at 2000*g* for 5 min, resuspended in 10 mL of [^{3}H]lysine (0.2 mCi/mL) in the modified Swims S77 medium, and incubated at 37 °C for 10 min. Cells were centrifuged at 2000*g* for 5 min, resuspended in fresh medium containing 1×10^{-4} M IdUrd and incubated for 60 min. These cells were centrifuged at 2000*g* for 5 min and then resuspended in Dulbecco's medium containing 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and 1% formaldehyde. After incubation at 4 °C for 2 h the fixation was removed, and the cells were resuspended into fresh medium for incubation overnight at 4 °C (Jackson & Chalkley, 1981b). The overnight incubation ensures removal of unreacted formaldehyde prior to freezing the cells for storage or isolation of nuclei. For experiments which involve determining the relative rates of histone synthesis in G1 and S phases, the procedures are the same as described except that the IdUrd pretreatment was omitted and upon completion of the [^{3}H]lysine pulse the cells were immediately frozen. The preparative procedure for preparing acid-soluble proteins from whole cell extracts is as described in the legend to Figure 2.

Preparation and Fractionation of Chromatin. The formaldehyde-cross-linked cells were washed 3 times in 1% Triton X-100, 0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane (Tris), and 10 mM $MgCl_2$ and suspended into 4 M guanidine hydrochloride (Gdn·HCl) and 10 mM ethylenediaminetetraacetic acid (EDTA). The nuclei were sonicated at a concentration of 2 mg/mL (DNA) for 60 s at setting 3 in a Branson sonifier. The samples were centrifuged at 27000*g* for 10 min to remove membranes. The supernatant containing the solubilized chromatin was added to 1.2 g of CsCl and the volume adjusted to 4.6 mL with 4.0 M Gdn·HCl and 10 mM EDTA. Centrifugation to equilibrium was at 35 000 rpm for 72 h at 4 °C in a Beckman SW56 rotor.

Reversal of the Formaldehyde Cross-Links and Analysis by Gel Electrophoresis. The fractions from the CsCl gradient were treated with 20 μL of 2-mercaptoethanol and heated at 100 °C for 30 min (Jackson & Chalkley, 1981b; Jackson, 1978). Samples were adjusted to a final concentration of 0.4 N H_2SO_4 and dialyzed against two changes of 2 L each of 0.4 N H_2SO_4 and 50 mM 2-mercaptoethanol at 4 °C. Samples were sonicated at setting 3 for 60 s and centrifuged at 10000*g* for 20 min, and acid-souble proteins in the supernatant were precipitated in 7 volumes of acetone at −20 °C overnight. The proteins were dissolved into 8 M urea, 0.9 HOAc, 0.5 M 2-mercaptoethanol, and 0.25% methyl green and then electrophoresed on 12% acrylamide gels containing 0.08% bis(acrylamide), 0.9 N acetic acid, 8 M urea, and 0.45% Triton X-100 at 25 °C (250 V for 24 h) (Zweidler, 1978). The gels were stained in 0.1% Coomassie R, 40% methanol, and 10% acetic acid for 12 h, destained in the same system lacking dye, and scanned on an RFT-II scanning densitometer. Either gels were fluorographed directly (Laskey & Mills, 1979) or gel strips were cut for electrophoresis in the second dimension. The strips were soaked in 1% sodium dodecyl sulfate (SDS) and 100 mM Tris, pH 6.8, for 60 min and polymerized into a stacking gel consisting of 2.5% acrylamide, 0.12% bis(acrylamide), and 0.125 M Tris, pH 6.8. The running gel was 18% acrylamide, 0.35% bis(acrylamide), 0.1% SDS, and 0.75 M Tris, pH 8.8. The electrophoresis buffer was 0.1% SDS, 0.025 M Tris, and 0.2 M glycine, pH 8.3, and electrophoresis was at 150 V for 18 h at 4 °C. Staining and fluorography were as previously described (Jackson & Chalkley, 1981c).

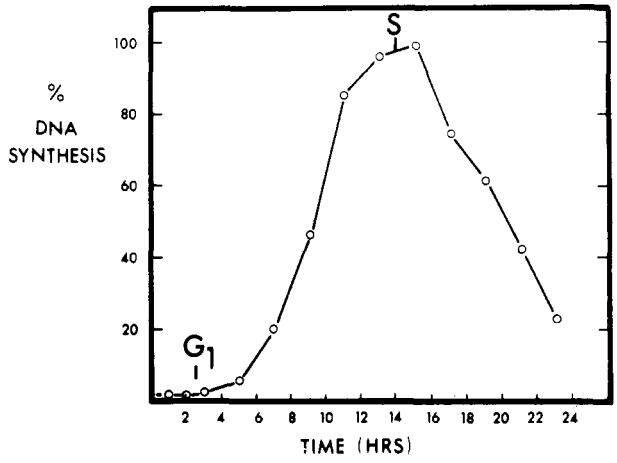

FIGURE 1: Rates of synthesis of DNA after colcemid block in HTC cells. After release of the 8-h block, 3-mL aliquots of cells (3×10^5 cells/mL) were incubated with 20 Ci/mL [^{3}H]thymidine for 10 min. Cells were centrifuged at 10000*g* for 1 min, and the pellet was immediately sonicated into 5 mL of 0.5 M NaCl and 0.5 M perchloric acid and recentrifuged. The pellets were rewashed by repeated sonication 3 more times and finally suspended into scintillation fluid for counting.

Results

Rates of Histone Synthesis in G1- vs. S-Phase Cells. HTC cells were grown as subconfluent monolayers for 2 days and subsequently treated with colcemid (10 μg/mL) for 8 h. Mitotic cells were detached by gentle rotation and cells harvested and resuspended in fresh medium. The extent of DNA synthesis in these synchronized cells was measured at various times after selective detachment. As shown in Figure 1, significant DNA synthesis begins approximately 5 h following release from the colcemid block, and it reaches a maximum 8 h later. At 2 h after release, the extent of DNA synthesis is only 1% of that seen after 12 h. For comparison purposes between G1- and S-phase cells in the following experiments, we have labeled cells with [^{3}H]lysine for 10 min at either the 2-h (G1 cells) or the 12-h (S phase) time points. Acid-soluble extracts for these cells were prepared and equal amounts analyzed by electrophoresis on two-dimensional gels. The results

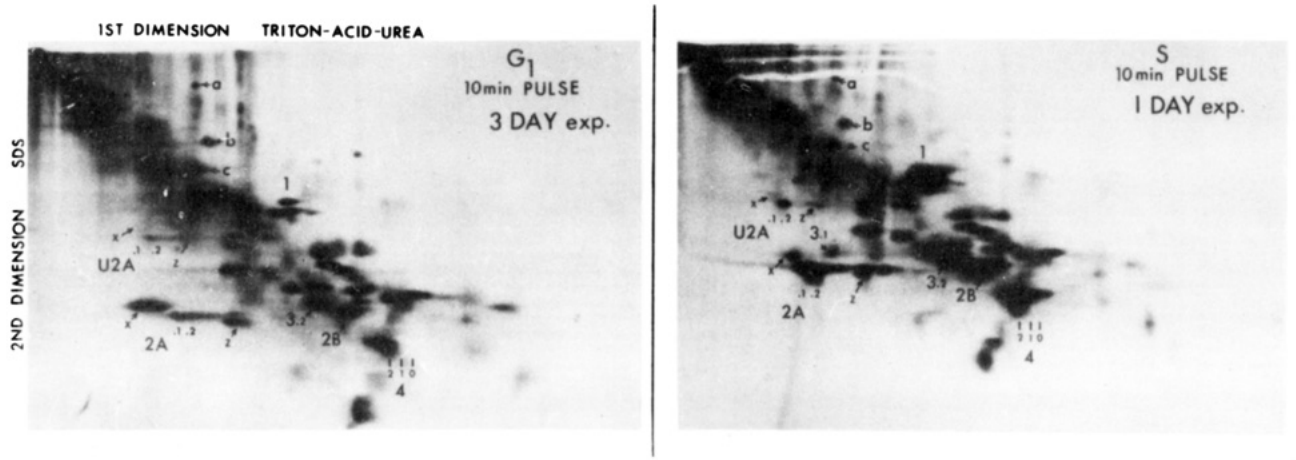

FIGURE 2: Two-dimensional gel analysis of histone synthesis in G1- and S-phase cells. HTC cells were labeled with [^{3}H]lysine as described in the text, collected by centrifugation, and sonicated into 0.4 N H_2SO_4. After centrifugation at 27000g for 20 min, the acid-soluble supernatant was precipitated with 7 volumes of acetone and redissolved such that the protein from 4×10^6 cells was electrophoresed on Triton–acetic acid–urea gels. After electrophoresis, gel strips were soaked in SDS buffer and electrophoresed in a second dimension in SDS. Proteins labeled a, b, and c in the figure are used as markers to determine equivalent exposure lengths for non-histone proteins. To achieve this exposure length, the gel from the G1 cells was exposed for 3 days, and that from the S-phase cells underwent a 1-day exposure. (The rate of protein synthesis in G1 cells is 3 times slower.) Gels were scanned densitometrically, and the ratio of S/G1 synthesis was determined for each histone. These data are in Table I. Different fluorograph exposure times with a G1/S ratio of 3/1 were used in order to maintain linearity in the scans for both minor and major histones. The photographs utilized in this figure are overexposed for the major histones in order to illustrate that the quantity of minor ubiquinated components can be determined. Identification of protein components on these two-dimensional gels is based on the data of West & Bonner (1980b).

are shown in Figure 2. We observe from the fluorogram that a measure of synthesis of the major histones does occur in G1 cells; however, the rate differs substantially between S phase and G1 phase as is evidenced by the 1-day exposure for the S-phase material. We have quantitated these differences by microdensitometric analysis of the fluorograms and have collected this information in Table I (column 2). We observe that the major histones are synthesized at an approximately 20-fold greater rate in S phase relative to G1. In contrast, the histone variants X and Z and their ubiquinated conjugates are synthesized at only a 2-fold greater rate in S phase. This relatively tight coupling of histone synthesis to DNA replication for H2A, H2B, H3, and H4 has been previously reported (Wu & Bonner, 1981) as well as the lack of coupling with DNA synthesis for the variants X and Z. What has not been previously described is the tight coupling of the synthesis of histone H1 with DNA replication as observed in these cells. The data of Table I also confirm that in HTC cells we are observing a basal level of histone synthesis in G1 cells which is about 5-fold greater than that needed to match the rate of DNA synthesis.

Deposition of Histones in S Phase. Since we observe that the synthesis of the major histones is significantly coupled with DNA replication in HTC cells, we wondered if these histones would selectively deposit on the replicated DNA. In addition, since the synthesis of X and Z variants occurs in both G1- and S-phase cells, we have asked whether the deposition of these histones is excluded from replicative DNA, a result which would indicate that these two depositional events (basal vs. replicative) might well operate independently of one another. To do these studies, it is necessary to use a procedure which can define whether the newly synthesized histones are deposited on newly synthesized DNA. We have previously shown that after incorporation of the density-labeled iododeoxyuridine (IdUrd) into newly replicated DNA, we can fractionate this DNA and its associated histones from unlabeled chromatin (Jackson & Chalkley, 1981b). This is achieved by using CsCl density gradients containing chromatin isolated from cells previously treated with the cross-linking agent formaldehyde. Since we have developed procedures to reverse such cross-links,

Table I[a]

histone	S-phase/G1-phase synthesis ratio	rel deposition on new DNA in S phase	extent of deposition in G1 phase
uX	2	1	2
uZ	2	1	2
u2A.1,2 (A24)	5	3	2
X	2	1	3
Z	2	1	4
2A.1	18	5	2
2A.2	19	5	2
1	20	1	1
3.1	*b*	22	
3.2	21	21	11
2B	20	4	5
4	22	23	12
HMG 14	nd[c]	1	1
HMG 17	nd	1	1

[a] The ratio of the amount of synthesis of a given histone in S phase relative to that in G1 phase was determined directly from the data shown in Figure 2. The relative deposition onto new DNA in S-phase cells was calculated from the data of Figure 4 and is reported in terms of the specific activity of histones in fraction 7 divided by that in fraction 10. We have experimentally determined by labeling cells with [^{3}H]thymidine in the presence of IdUrd that these gradients provide a 22-fold purification of newly replicated DNA (Jackson & Chalkley, 1981b). Thus, a value of 22 reflects selectivity for histone deposition on only replicative DNA. The extent of deposition of a given histone onto DNA in G1 cells is calculated from the data of Figure 6 and is reported in terms of the ratio of the specific activity of histone in fraction 22 divided by that in fraction 10. Clearly, the larger the number, the less effectively is it deposited onto DNA. [b] Assume 100% S (not detected in G1). [c] Not determined.

the proteins associated with the IdUrd containing DNA can be analyzed by gel electrophoresis after fractionation. This procedure is illustrated in the following experiment to test for the site of deposition of histones newly synthesized in S phase.

Synchronized, S-phase HTC cells were obtained by collecting cells 12 h after release from mitotic block. These cells were treated with IdUrd for 30 min prior to addition of [^{3}H]lysine for 10 min. Subsequently, the radiolabeled material was chased for an additional 60 min in the continued presence

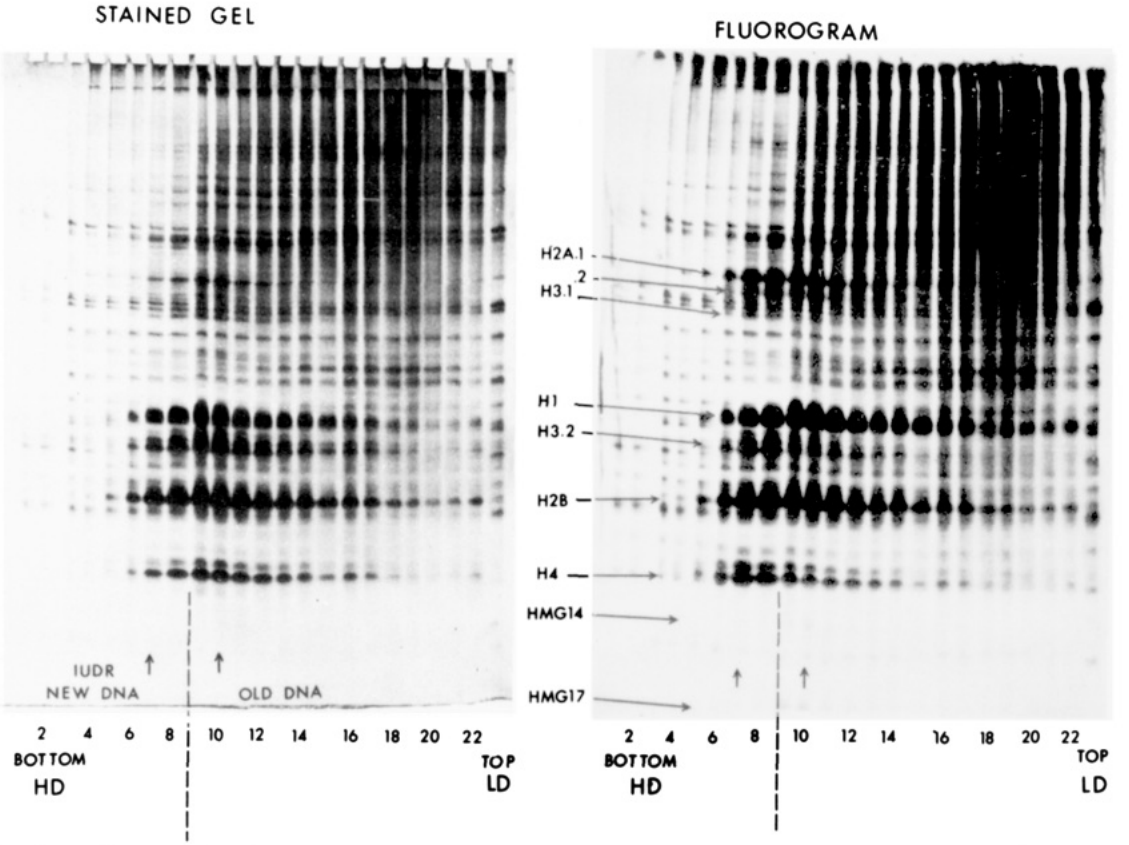

FIGURE 3: Density gradient analysis of histone synthesis and deposition in S-phase cells. HTC cells were labeled with [^{3}H]lysine and IdUrd as described in the text. Chromatin was isolated from the fixed cells and fractionated by centrifugation to equilibrium on CsCl gradients. The cross-links between protein and DNA were reversed as described under Experimental Procedures, and the protein was analyzed by gel electrophoresis on Triton–acetic acid–urea gels. HD (high density) and LD (low density) refer to the orientation of the CsCl gradients. The dashed line approximates the separation between IdUrd-containing chromatin and normal density chromatin. The arrows indicate the two fractions, fractions 7 and 10, that are electrophoresed in a second dimension for Figure 4.

of IdUrd to ensure complete deposition of all histones labeled during the pulse. Cells were treated with formaldehyde for 2 h and chromatin was subsequently isolated and fractionated on CsCl gradients. The cross-links were reversed and the proteins analyzed on Triton-acetic acid–urea gels with the results shown in Figure 3. Comparing the fluorogram with the stained gel, we observe that the majority of the newly synthesized histones H3 and H4 are localized in fractions 6–8; histones H2A and H2B are much more broadly distributed throughout the density gradient, and significant amounts are found in the range of fractions 7–16. New histones H1 shows a distribution very closely resembling that of the bulk, non-density-labeled chromatin as seen in the stained gel. The asymmetry of distribution of new histones reflects the various associations (or lack of) with IdUrd-containing DNA. Because of the number of proteins associated with the DNA by these procedures, it is difficult to resolve the individual components by electrophoresis in only one dimension (particularly in the top right-hand corner of the fluorogram). Therefore, we have taken samples from fraction 7 (proteins associated primarily with IdUrd-containing DNA) and fraction 10 (proteins associated primarily with old, unlabeled DNA) and subjected these proteins to electrophoresis in a second dimension in SDS. These results are shown in Figure 4 for both the fluorogram and stained gels. With these gels, it is more readily apparent which and to what extent newly synthesized histones are associated with replicative DNA. This is seen dramatically for histones H3 and H4 which show a strong fluorographic signal when extracted from fraction 7, but a very weak signal when extracted from fraction 10 in the control density region of the gradient. This asymmetry is not seen for the other histones. These data have been quantitated in terms of the ratio of fluorographic intensity/Coomassie stain intensity, and the results are summarized in Table I (column 3). These results confirm our previous observations that the deposition of new histones on replicative DNA is not the same for all histones. Histones H3.1, H3.2, and H4 are 20-fold enriched for IdUrd-containing DNA relative to old DNA, a value indicative of complete selectivity for deposition on replicative DNA [see Jackson & Chalkley (1981b)]. These results are in contrast to histones H1, HMG 14 and 17, X, Z, and their ubiquinated adducts where a specific activity of 1 indicates that no selectivity for deposition on replicative DNA is observed. New histones H2A.1, H2A.2, H2B are distributed on both old and new DNA. We calculate that new H2A and H2B (with a specific activity of 5) are deposited on replicative DNA to the extent of approximately 30%.

Histone Deposition in G1 Cells. Since we have observed that a low but significant level of histone synthesis occurs in G1-phase cells, we asked whether these histones are deposited onto the DNA of these cells. To do this, it is necessary to utilize procedures which prevent adventitious binding of histones to DNA during the isolation procedures. We have previously reported that fixation of whole cells with formaldehyde prior to isolation of chromatin at high ionic strengths can avoid this problem. Subsequent fractionation of the chromatin on CsCl gradients results in a separation into three general groups (see Figure 5): component "a" contains mature chromatin (DNA and associated proteins), component "b" contains replicating, immature chromatin, and component "c" contains proteins not as yet associated with DNA [see Jackson & Chalkley (1981a)]. We have therefore applied these pro-

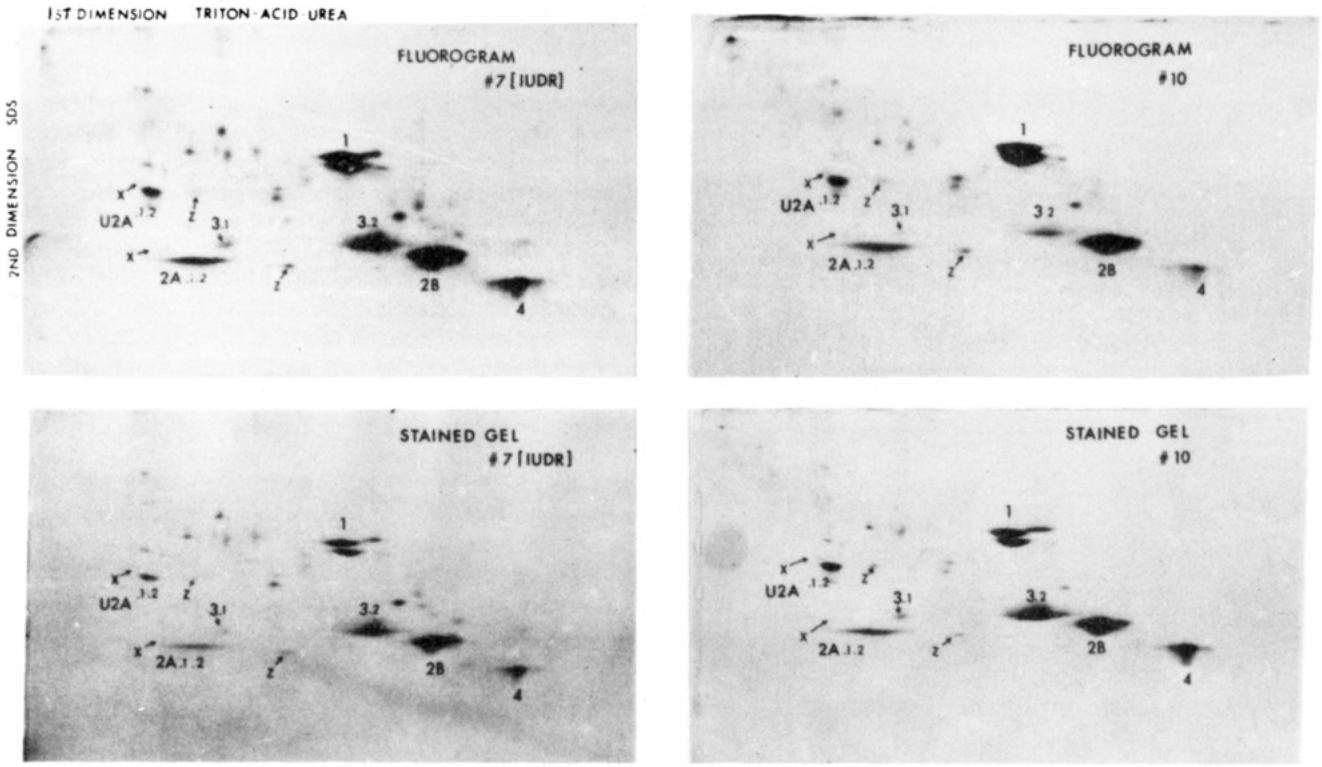

FIGURE 4: Two-dimensional gel analysis of histone deposition in S-phase cells. Gel slices of fractions 7 and 10 from the experiment of Figure 3 were soaked in SDS buffer and electrophoresed in a second dimension in SDS. Labeled histones in fraction 7 are associated primarily with IdUrd-containing DNA and in fraction 10 with normal density DNA. Microdensitometric scans of both the fluorogram and the stained gel for each histone are used to compute the relative specific activity. This value is shown in Table I (column 3).

cedures to study the extent of deposition of histones in G1 cells.

HTC cells were synthesized into G1 (2 h after release from mitotic block) and labeled with [^{3}H]lysine for 10 min in the presence of IdUrd in a protocol identical with that reported for the labeling of S-phase cells (Figure 3). This protocol involves the use of IdUrd which also indicates the extent and nature of any S-phase histone synthesis and deposition, since placement of histones on IdUrd-containing DNA (S-phase cells) can be distinguished from an association with normal density DNA (G1 cells) (see Figure 3). We have previously shown that histone deposition in S-phase cells is a rapid process occurring within 1 min after histone synthesis (Jackson & Chalkley, 1981a). Therefore, the 60-min chase of the labeled histones in G1 should provide ample opportunity for deposition to occur. After such a labeling procedure, the cells were then treated with formaldehyde for 2 h, and chromatin was then isolated and fractioned on CsCl gradients. The cross-links were reversed, and proteins associated with the various regions on the gradient were analyzed on Triton–acetic acid–urea gels as shown in Figure 5A. An important conclusion derived from Figure 5A is that the deposition of histone observed in this experiment is exclusively that characteristic of G1 cells. If contamination of our preparation with S-phase cells had occurred, the newly synthesized H4 and H3 would be found in the IdUrd region of the gradient. The complete absence of both histones in the IdUrd region is evidence that we are observing events which are restricted to G1 cells. The histone distribution results shown in Figure 5A reveal that whereas in the stained gel where the bulk of the unlabeled histones are in region a (mature chromatin), the fluorogram indicates that the labeled histones do not selectively distribute to this region. The results vary with the histone class. Thus, H1 is primarily associated with mature chromatin (region a) and H3/H4 are primarily found in the region of nondeposited material. Histones H2A/H2B span all regions where chromatin is found in the gradient. In order to be able to quantitate the amounts of the various histone fractions, especially the minor classes, we have used a two-dimensional analysis to study the material associated with fraction 10 (banding with mature chromatin and presumably deposited) and with fraction 22 (which lacks mature chromatin and reflects undeposited material).

The data of Figure 6 confirm that the distribution of the labeled histones in fractions 22 and 10 varies with histone type. New histones H3 and H4 are found primarily in fraction 22 and are not deposited on DNA, whereas most of histone H1 is found in fraction 10, which indicates that histone H1 is deposited on G1 DNA. We have determined the specific activity for all the histones and their variants. These data are shown in the fourth column of Table I. A value of unity indicates (see legend to Table I) that the deposition of newly synthesized material is complete after the 60-min chase. As this value increases, the extent of deposition is less complete. A value of 2 indicates that deposition of that histone is a factor of 2 lower than that for histone H1. Thus, the observed value of 11–12 for new histones H3 and H4 is indicative of nearly total inhibition of deposition.

The 60-min chase utilized in this experimental procedure ensures that the cells are still in the G1 phase of the cycle. Therefore, the deposition of the major histones H2A and H2B must result from an ongoing exchange of these histones with H2A and H2B molecules already associated with the DNA, an exchange perhaps not dissimilar with that observed for these same two histones when synthesized in S-phase cells. This experiment also indicates that the variants X and Z and the ubiquinated adducts behave quite similarly to H2A/H2B with respect to deposition.

Analysis of the data of Figures 5A and 6 reveals that even 60 min after the conclusion of the [^{3}H]lysine pulse, most of the H4 synthesized during the pulse is diacetylated. In S-phase cells, H4 is found in the diacetylated form immediately fol-

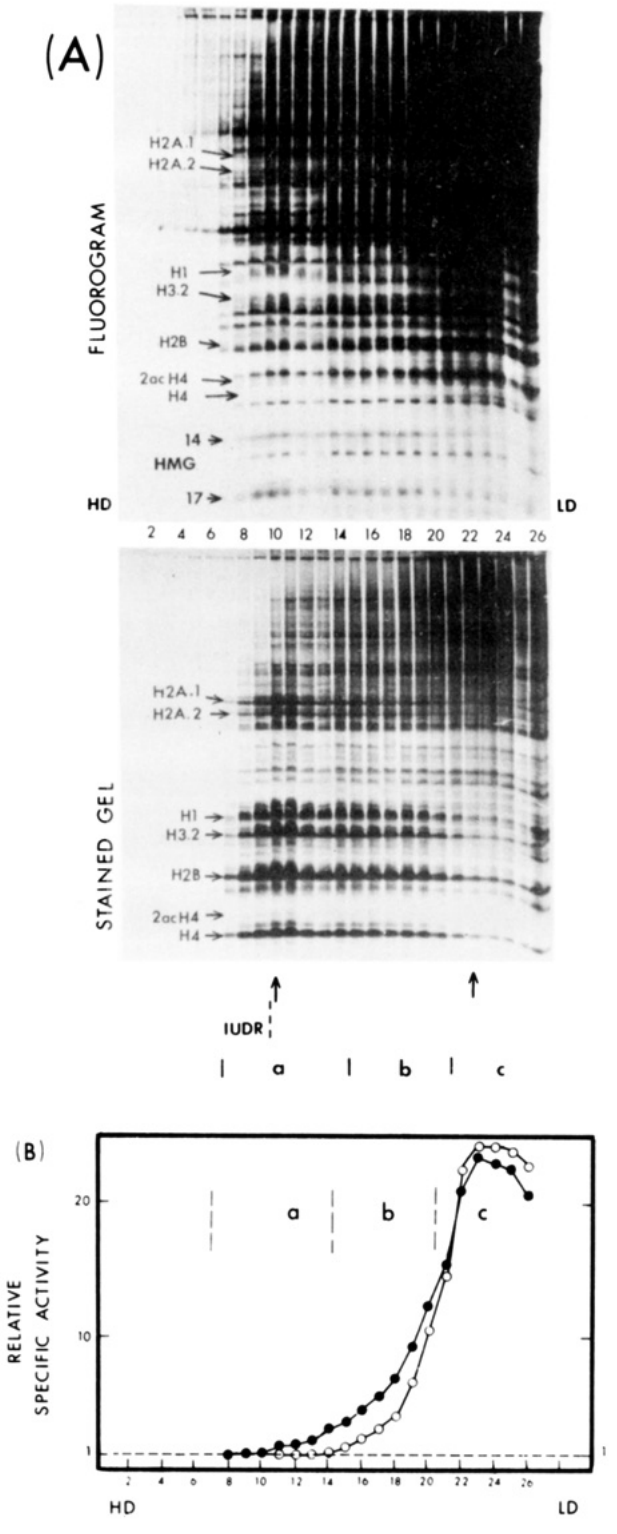

FIGURE 5: Analysis of deposition of newly synthesized histone in G1 cells. (A) HTC cells were labeled with both [^{3}H]lysine and IdUrd as described in the text. Preparation of cross-linked chromatin, fractionation on CsCl gradients, and electrophoresis on Triton–acetic acid–urea gels were as previously described. Regions a, b, and c refer to sections within the gradients where the heterogeneity of the protein/DNA ratio is such that the chromatin can be fractionated into three regions: region a, mature chromatin; region b, immature chromatin ($t_{1/2}$ ~5 min); region c, undeposited proteins [see Jackson & Chalkley (1981a)]. Within region a, if IdUrd is present in the DNA, the density of the IdUrd-containing chromatin is increased and a partial separation achieved within region a. (B) Specific activity of newly synthesized H4 within these gradients is shown for a 10-min pulse with [^{3}H]lysine (○) and for a 10-min pulse with a 60-min chase (from gel in panel A) (●).

lowing synthesis (Ruiz-Carrillo et al., 1975; Jackson et al., 1976), but it is rapidly deacetylated to the parental and monoacetylated form ($t_{1/2}$ ~ 5 min). Thus, it appears that the nondeposited state of H4 is characterized not only by its location in the CsCl density gradients but also by the lack of turnover of the acetylation modification. This will be further substantiated when we analyze the S-phase deposition of H4 synthesized (and not deposited) in G1 (see below).

Deposition of G1-Synthesized Histones in S-Phase Cells. The lack of deposition of new H3 and H4 in G1 suggests that a mechanism exists which prevents it. In principle, this might involve a process which permits these new histones to be selectively degraded or might maintain them in a state such that deposition can only occur when DNA synthesis is initiated. The following experiment was designed to attempt to distinguish between these possibilities. Synchronized G1 cells were labeled for 10 min with [^{3}H]lysine before resuspension in fresh medium after which they were chased for 3 h. The total time elapsed since the mitotic block was 5 h. The onset of S phase occurs at this time (see Figure 1). Cells were then suspended in IdUrd for either 60 min or 5 h to density label newly synthesized DNA. The cells were fixed with formaldehyde for 2 h, and chromatin was isolated and fractionated on CsCl gradients. The nature of the proteins in the gradient was analyzed by electrophoresis as shown in Figure 7A. After 1 h in S phase when DNA synthesis is increasing (but clearly not occurring in all cells), a small percentage of labeled histones H3 and H4 synthesized in G1 is now deposited on newly replicated DNA (cf. Figure 5) as indicated by the distribution of histones into the IdUrd region of region a; however, much of labeled H3/H4 is still found in the lighter regions of the gradient, and H4 is still extensively diacetylated. However, when we extend the length of the IdUrd treatment to 5 h (mid S phase), all the labeled H3 and H4 histones are removed from region c and shifted to region a (Figure 7B). The H4 acetylation now resembles that seen in mature chromatin. These data indicate that histones synthesized in G1 are not selectively degraded but are eventually utilized for packaging of DNA, a depositional process which can occur at a significant rate for H1, H2A, and H2B histones on G1 DNA and for histones H3 and H4 only when replicated DNA is present.

Discussion

This work confirms the previous reports of Bonner and his co-workers that profound differences are seen between different histone fractions in the extent to which their synthesis is coupled to that of DNA synthesis. In addition, we have identified dramatic differences in the ways that histones are deposited on the chromatin both in the presence and in the absence of DNA synthesis.

Histones H1, H2A.1,2, H3.1,2, H2B, and H4 all show a tight, but not exclusive, coupling with DNA synthesis. Thus, they are all synthesized in S phase at a rate which corresponds directly with the amount of DNA which is made. In G1-phase cells in which DNA synthesis has been reduced to 1% of that occurring in S phase, the extent of synthesis of these histones is now 5% of that seen in S phase. In contrast, the minor histone fractions X and Z and the ubiquinated adducts X, Z, and H2A show much more modest decreases in synthetic activity in passing from S phase to G1 phase.

Thus, overall, the synthetic activity of all histone fractions in G1-phase cells when DNA synthesis has been reduced by 100-fold relative to S-phase cells is running at 8% of that seen in S-phase cells and as such constitutes the basal histone synthetic activity in HTC cells. Although the exact quantitation varies, our results are in general agreement with those

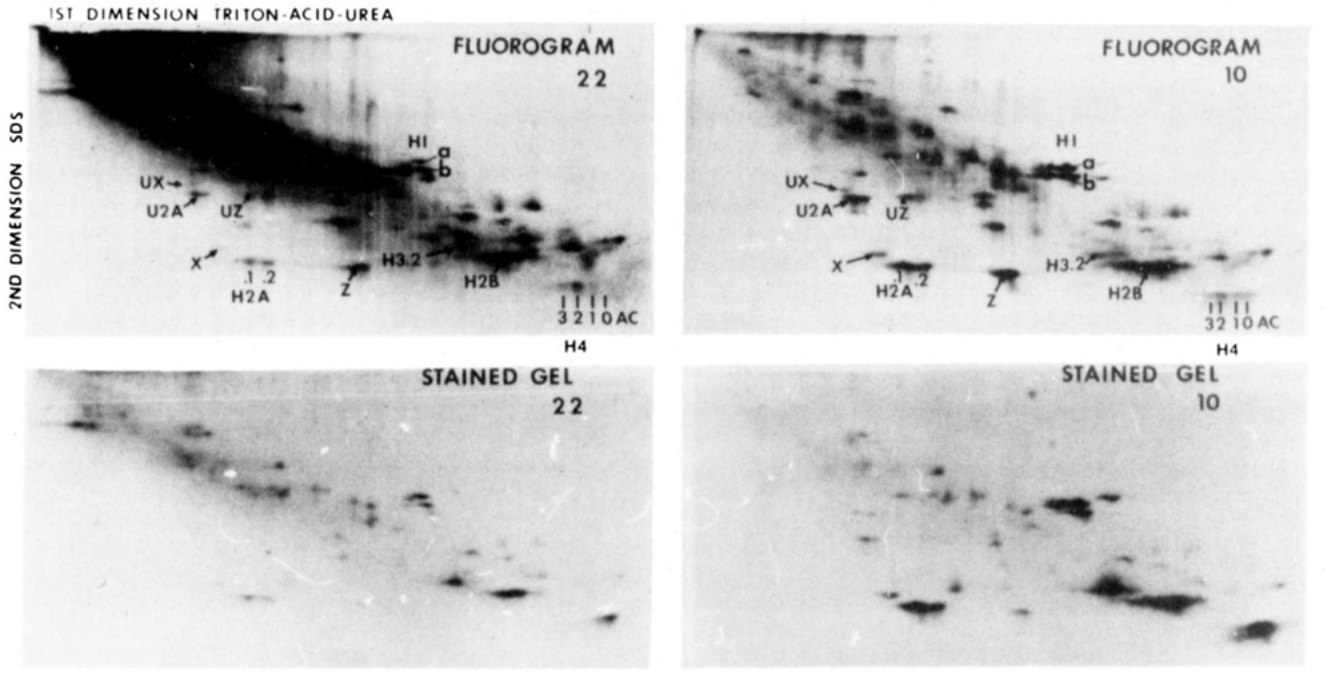

FIGURE 6: Two-dimensional gel analysis of histone deposition in G1 cells. Gel slices of fractions 10 and 22 from the experiment of Figure 5 were soaked in SDS buffer and electrophoresed in a second dimension in SDS. Labeled histones in fraction 10 are deposited on DNA, and those in fraction 22 are not deposited on DNA. Microdensitometric scans of each histone for both the fluorogram and the stained gel are used to compute the relative specific activity shown in Table I (column 4).

reported for other cell lines (Wu & Bonner, 1981; Tarnowka et al., 1978). They are also consistent with much earlier observations which indicated that essentially complete cessation of DNA synthesis by drugs such as hydroxyurea and arabinocytidine (*ara-C*) is not accompanied by a correspondingly complete inhibition of histone synthesis (Balhorn et al., 1973).

The presence of significant basal level histone synthesis in HTC cells in G1 phase led us to question whether some or all of these histones could become associated with chromatin and to compare these results with those obtained for deposition during S phase. The pattern of histone deposition in cells synchronized into S phase follows that previously observed for randomly growing populations of HTC cells (Table I). Histones H4/H3 (and related forms) are deposited with some considerable selectivity onto newly replicated DNA. Histones H2A/H2B (and related forms) seem to be distributed between deposition on either new (30% of incoming histone) or old (70%) DNA. So far as we can ascertain, H1 and the HMG proteins are deposited randomly on the chromosome. The pattern of histone deposition in G1 cells in the presence of a very small amount of DNA synthesis is consistent with that obtained in S-phase cells. Thus, in the absence of significant DNA synthesis, we do not observe deposition of those H3/H4 molecules which are made in the G1 phase, though these molecules can be subsequently deposited when DNA synthesis is renewed in the subsequent S phase. In contrast, a large fraction of histones H2A/H2B and the variant histone molecules are deposited onto the G1-phase chromatin. Since such materials are found in nucleosomes of unchanged electrophoretic mobility (Jackson et al., 1981), and since exchange of histones H2A/H2B has been demonstrated in vivo (Jackson + Chalkley, 1981a,b), it is likely that deposition is occurring by exchange in G1 phase-cells rather than by simple surface association.

Although we have reported that H2A and H2B are able to exchange with nonreplicating chromatin, it is important to recognize that these histones are not exchanging with all regions of the chromatin. This was previously shown by analyzing the behavior of that 30% of new H2A/H2B which deposited on new DNA. If they were fully available for exchange during the ensuing cell generation, we should have seen a shift of H2A/H2B from the density-labeled DNA with which it was initially associated during subsequent chases. This does not occur to any degree (~20%) (Jackson & Chalkley, 1981b) and leads us to the conclusion that 80% of the deposited histones H2A/H2B are not available for exchange in a given cell.

We therefore propose the following model to account for the depositional behavior of the various histones. It is based upon the following premises. (1) Newly synthesized histones H3/H4 do not exchange extensively and are primarily deposited on newly synthesized DNA—though whether deposition occurs as an initial tetramer or as an octamer containing new or old H2A/H2B is not specified. (2) H1 histone on the chromatin can exchange at most positions with new incoming H1. Whether exchange occurs in the absence of incoming H1 is not specified. (3) Histones H2A/H2B are dissociated from the chromatin regions of active transcription and are free to exchange with incoming newly synthesized H2A/H2B.

We have illustrated this mechanism in Figure 8. On the average, 5×10^7 nucleotides/min are transcribed by RNA polymerase while 1×10^7 nucleotides/min are replicated during an 8-h S phase (Cox, 1976). The movement of RNA polymerase along the DNA could then release sufficient H2A and H2B to produce a pool that would dilute the incoming newly synthesized H2A and H2B. Any one of these histones, new or old, could deposit on replicated DNA or redeposit on active genes. A transcription/replication ratio of 5/1 would predict that approximately 20% of newly synthesized H2A/H2B would be available for deposition on new DNA, the remainder on active genes. We further propose that in the transcriptional region H3 and H4 remain associated with the DNA to serve as nucleation sites for re-formation of the nucleosome. This conclusion is based on our previous experiments which indicate once new H3 and H4 are deposited on the newly replicated DNA, they remain associated with that DNA

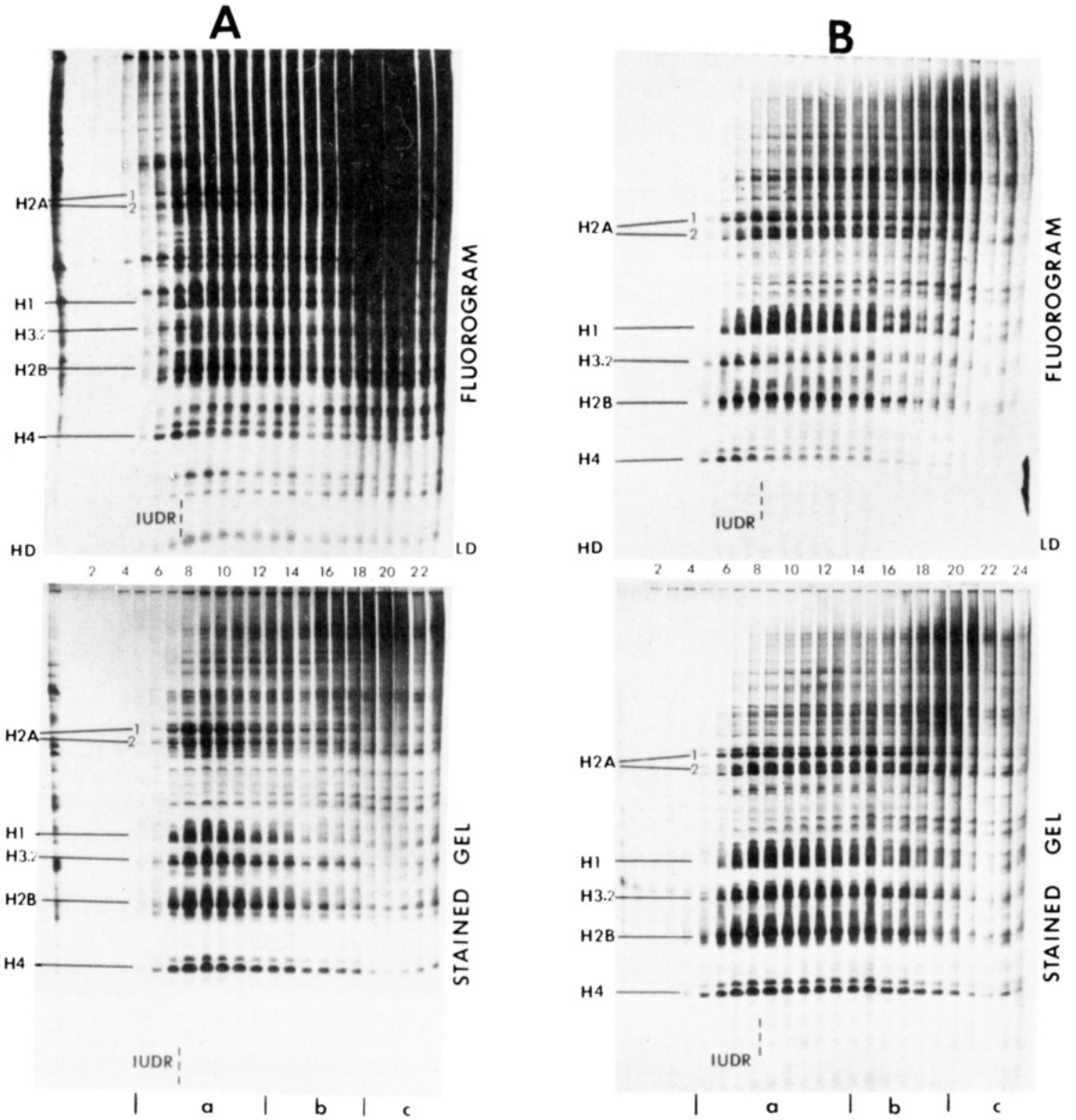

FIGURE 7: Analysis of the behavior of histones synthesized in G1 and their deposition on DNA in S phase. HTC cells were labeled as described in the text. The protocol for preparation and fractionation of the fixed chromatin is as previously described. After the 3-h chase of the [^{3}H]lysine pulse, cells were incubated with IdUrd for either 1 (A) or 5 h (B).

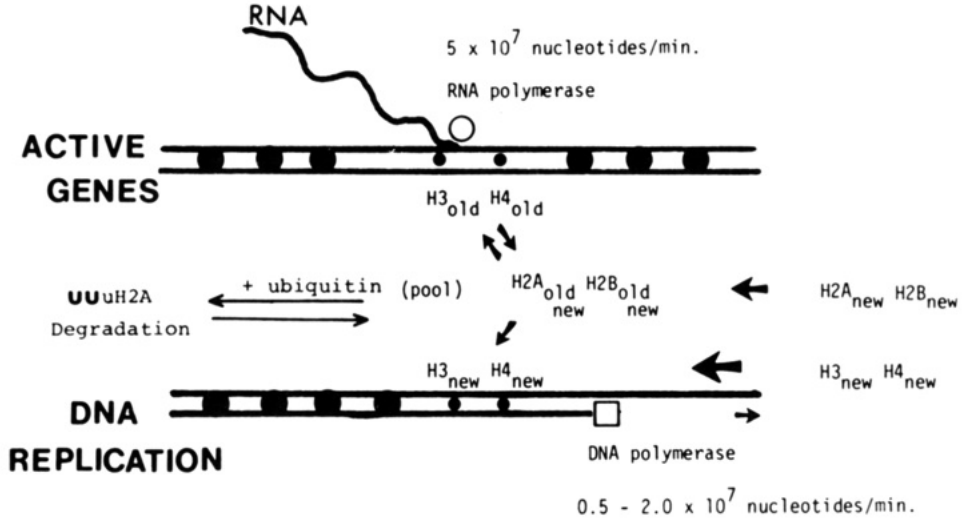

FIGURE 8: Model for histone exchange and deposition during transcription and replication.

region until the next replication event (Jackson & Chalkley, 1981b).

The model illustrated in Figure 8 predicts that the greater the polymerase activity, the more effectively will newly synthesized H2A.1,2 and H2B deposit on the transcribed DNA. Of course, if the gene upon which deposition has occurred is subject to repeated transcription, then new histone will be redistributed from the DNA to form a steady-state radiolabeled pool. One might expect that as the cell moves into S phase with the subsequent 20-fold increase in synthesis of H2A.1,2 and H2B, that these new histones would be the major histones in the pool and therefore the only ones available for deposition on newly replicated DNA. That would be true, however, only if the transcriptional activity between G1- and S-phase cells remained the same. Interestingly, for HTC cells the RNA polymerase activity increases 10-fold when going from G1 to S as seen by [^{3}H]uridine incorporation (data not shown). Thus, there is not only an increase of the flux of new histones into the nucleus at S phase but also a concomitant increase in the quantity of histones being released by RNA polymerase activity. The net result is that the ratio of old to new histone within the pool remains relatively the same for both G1 and S cells.

In the model of Figure 8, we illustrated that the histone pool may be subjected to degradation through energy-dependent turnover by way of ubiquitin conjugates. If the energy-dependent process reported by Hershko et al. (1980) and Haas et al. (1982) is occurring at equivalent rates for G1- and S-phase cells, then the observed relative increase in synthesis of uH2A.1,2 in G1 can be explained in terms of selective ubiquination and potential degradation of new H2A.1,2 that is synthesized in G1. This process need not reflect a lack of deposition of the new H2A.1,2 but only that its existence in the nuclear pool is extended relative to the rate at which it is deposited on the transcribed gene. Once the new H2A.1,2 is deposited on the DNA, it would no longer be available for ubiquination. Thus, when DNA synthesis begins in S phase and the deposition of new H2A increases, the level of H2A will proportionally decrease. The process of ubiquinating H2A does not of itself prevent deposition of this conjugated protein on DNA. Indeed, our data (Table I) indicate that uH2A is deposited onto G1 DNA to the same extent as H2A and also shows the same selectivity for deposition on IdUrd-containing DNA in S-phase cells. Thus, the process of ubiquination may of itself represent a mechanism not only for protein turnover but also for facilitating the exchange process at an active gene. Levinger & Varshavsky (1982) have similarly suggested that the ubiquination process of histones serves not only for energy-dependent degradation but also for destabilizing nucleosomes within active genes. Therefore, the ubiquination step might be seen as a mechanism to precondition a nucleosome so that release and exchange can occur readily for both uH2A and H2A if and when a RNA polymerase transcribes the associated DNA sequences.

In this regard, the observed rapid turnover of the ubiquitin moiety compared to the H2A sequences supports such a mechanism (Wu et al., 1981; Seale, 1981). In addition, the observed lack of uH2A in mitotic cells would then be expected for transcriptionally inactive and condensed chromatin (Matusi et al., 1979; Wu et al., 1981). Goldknopf et al. (1980) have reported the decreased content of uH2A during chicken erythropoiesis which again correlates with the presence of uH2A with transcriptionally active genomes. Since more than one ubiquitin molecule per protein is required before energy-dependent proteolysis occurs (Haas et al., 1982), we might expect, therefore, that the concentration of uH2A within the pool will determine whether the major activity is proteolysis or exchange with other H2A's on the DNA. In G1 cells, turnover of new H2A would be expected to be greater than that seen for new H2A in S-phase cells.

The data we have presented indicate that HTC cells carefully regulate the synthesis of histone such that the histone to DNA ratio is maintained. If histone synthesis exceeds the need for deposition, our data suggest that energy-dependent proteolysis by ubiquination of H2A and perhaps H2B (West & Bonner, 1980a) might occur. In contrast, when the synthesis of H3 and H4 exceeds the need, these histones are held in a transient state and will only deposit once DNA synthesis begins. This transient state is presumed to be localized to the nucleus as we have no evidence to suggest that the transport process from the cytoplasm is inhibited. A similar process has been observed in the *Xenopus* oocyte where histones are presynthesized prior to fertilization and are deposited on the DNA once replication begins (Adamson et al., 1977; Arceci et al., 1977). In this case, a depositional factor is implicated in this process (Laskey & Earnshaw, 1980; Kleinschmidt & Franke, 1982). A similar factor may be present in somatic cells as evidenced by the lack of H3 and H4 deposition prior to DNA replication.

ACKNOWLEDGMENTS

We thank our colleagues in the laboratory for their helpful suggestions and encouragement.

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Histone Segregation on Replicating Chromatin†

Vaughn Jackson*
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226
Roger Chalkley
Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242
Received February 28, 1985

ABSTRACT: We have reinvestigated the mode of segregation of preexisting histones onto replicating chromosomes. Since our previous data have indicated that only histones H3 and H4 do not appear to move from their association with the DNA strand with which they are bound until the next round of replication, we have concentrated our attention on these two histones. The strategy we have employed involved density labeling of DNA and radiolabeling of the histones of interest. Subsequently, we followed the association of histones and DNA during further rounds of DNA replication. One can make predictions concerning the nature of the association between specific histones and particular DNA strands depending on the mode of deposition. The results have confirmed our previous findings that histones segregate randomly. The possibility that such a result is a consequence of turnover of radiolabel in non-histone proteins and subsequent reutilization for histone synthesis has been tested directly. This process appears to be occurring to only a very limited extent. The implications of these conclusions for chromatin structure and gene control are discussed.

The mechanism whereby chromosomal proteins are deposited on replicating DNA molecules is of great interest. This is not only because of the intrinsic value of the description of a complex biological process but also because it appears that a specific arrangement of proteins is found in the region of transcriptionally active genes and the possibility exists that a part or all of this organization may be established during the replicational process.

The way in which old (preexisting) histones are distributed to daughter DNA strands can take one of three general pathways. One pathway involves unilateral distribution, in which all the preexisting histones are associated with only one of the two daughter strands. Obviously, several variations within this theme are possible; for instance, old histones could be associated exclusively with either the leading or the lagging side of the replication fork. These possibilities are documented in Table I. This general method of segregation is sometimes described as conservative segregation. Bilateral distribution, in which the preexisting histones ahead of the replication fork become associated with both daughter DNA molecules, constitutes the remaining two pathways since this may occur in two general ways, either in a random manner or in an ordered manner. In the latter case, the histones would show an affinity for a particular DNA strand.

We have attempted to address this question using an experimental protocol in which histones were labeled with [^{3}H]lysine and DNA was density labeled with iododeoxyuridine during generation-long pulse periods (Jackson et al.,

† This work was supported by National Institutes of Health Grants GM47224 (to R.C.) and CA35829 (to V.J.) and by the Diabetes and Endocrinology Research Center at The University of Iowa.

0006-2960/85/0424-6930$01.50/0