In Vivo Studies on the Dynamics of Histone–DNA Interaction: Evidence for Nucleosome Dissolution during Replication and Transcription and a Low Level of Dissolution Independent of Both†

Vaughn Jackson

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

Received June 19, 1989; Revised Manuscript Received September 5, 1989

ABSTRACT: Histones have been both radiolabeled and density-labeled with amino acids in vivo to determine the dynamics of histone–DNA and histone–histone interactions at the replication fork and on active genes. Proteins were uniformly labeled and subsequently chased for three cell generations. During the chase period, H3,H4 tetramers dissociated from the H2A,H2B dimers to re-form nucleosomes with the corresponding nondense histones synthesized during the chase period. These data suggest that the prereplicative nucleosomes are dissolved during advancement of the replication fork with release of associated histones in the form of the H3,H4 tetramers and H2A,H2B dimers. Experiments that involve density labeling of cells in the presence of actinomycin D indicate that the dynamic exchange of H2A,H2B that has been previously described [Jackson, V. (1987) *Biochemistry 26*, 2315–2324] is partially dependent on RNA polymerase movement. These results provide indirect evidence that nucleosome dissolution occurs during transcription. When deposition during replication and transcription is inhibited by simultaneous treatment of cells with cytosine arabinoside and actinomycin D, the majority of the newly synthesized histones are unable to deposit into nucleosome structure. The low level of deposition that is observed has characteristics similar to the deposition of uH2A and uH2B, and it is proposed that conjugation of H2A and H2B by ubiquitin occurs when these proteins are in a free pool within the nucleus. The new H3,H4 tetramers and new H2A,H2B dimers when prevented from depositing are not stable. New and old H3 and H4 intermix to form hybrid tetramers, and a similar intermixing is observed for the H2A,H2B dimers. A model is presented to describe the dynamics of histone–DNA interactions during replication and transcription.

The basic repeating unit of chromatin structure is the nucleosome. Each nucleosome consists of two each of histones H2A, H2B, H3, and H4, upon which two coils of DNA interact (145 bp[1] of DNA). The proposed function for these histone–DNA interactions is to provide compaction of the DNA while regulating gene expression. Since both DNA transcription and replication require the interaction of a polymerase with the nucleosome, it is of interest whether nucleosome dissolution occurs to facilitate these processes. This question is addressed in this paper by analyzing the dynamics of histone–DNA interactions in vivo. A model is proposed (Figure 1) that describes a dynamic process of histone interaction with DNA that is dependent on both transcription and replication. This model is based on previous in vivo studies that examined these interactions and on the data of this paper. These previous studies will be discussed briefly to provide background and to introduce the protocols that will be used in this paper.

Several investigators have indicated that the deposition of newly synthesized histone at the replication fork is not equivalent for each histone type (Senshu et al., 1978, 1985; Jackson & Chalkley, 1981a,b, 1985a; Jackson et al., 1981; Annuziato et al., 1982; Yamada & Senshu, 1987). While newly synthesized H3 and H4 were found to selectively deposit on newly replicated chromatin, the majority (70%) of new histones H2A and H2B did not deposit on new DNA. This result suggested that a dynamic state exists within the nucleus in which old histones H2A and H2B from elsewhere in the genome participate in deposition at the replication fork. This dynamic state, however, is restricted. When the new H2A and H2B that did deposit at the replication fork were chased through the cell cycle, no detectable release of these histones from the DNA was observed (Jackson & Chalkley, 1981a). Therefore, a limited region of the genome must be the site for the H2A and H2B exchange; otherwise, the chase should have caused the release of the new H2A and H2B. It has been proposed that the site of exchange is restricted to genes that are involved in active transcription (Jackson & Chalkley, 1981a). The model of Figure 1 suggests that transcription dissolves nucleosome structure and releases H2A and H2B. These histones enter a pool of free histones in which on a random basis these histones may redeposit either at the replication fork or at active genes. The model also indicates that H3 and H4 do not undergo this exchange process, but remain associated with the DNA during transcription. This aspect of the model is based on the observations that new H3 and H4 deposit specifically at the replication fork. If these histones exchanged similarly to H2A and H2B, only 30% would be found associated with replicated DNA. The data of this paper will indicate that inhibiting RNA synthesis with actinomycin D can also prevent the exchange of H2A and H2B. The majority of exchange for H2A and H2B is dependent on the active movement of RNA polymerase.

The model of Figure 1 illustrates that new histones H3 and H4 deposit onto newly replicated DNA as a tetramer asso-

† This work was supported by NCI 35829 and Wisconsin Heart Association 86-GA-72.

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

0006-2960/90/0429-719$02.50/0

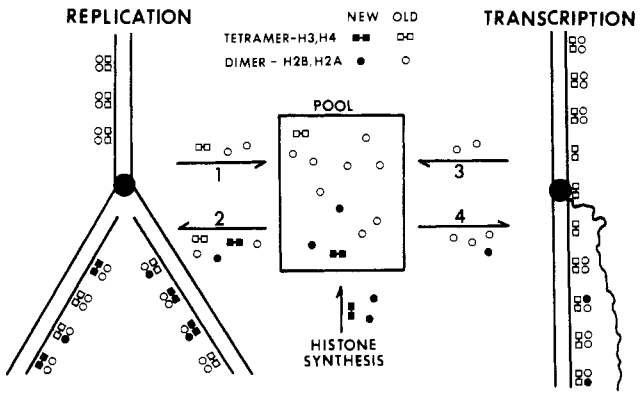

FIGURE 1: Model for the dynamics of histone–DNA interactions as a function of replication and transcription. The solid squares (H3,H4 tetramer) and solid circles (H2A,H2B dimer) indicate the newly synthesized density histones, and the open squares and circles indicate the old histones. The model is based on previously published data and the data of this paper (see introduction). Additional data of this paper will indicate that the model is incomplete in that a basal level of nucleosome dissolution occurs independent of replication and transcription.

ciated primarily with old H2A and H2B. New histones H2A and H2B deposit as a dimer with primarily old H2A, H2B, H3, and H4. The data in support of this specific aspect of the model are based on experiments in which cells were labeled with a short pulse of density-labeled amino acids and the histones within the nucleosome cross-linked with formaldehyde to form an octameric complex (two each of H3, H2B, H2A, and H4). Determining the density of these complexes revealed that new H3,H4 tetramers and new H2A,H2B dimers were not deposited in the same nucleosomes (Jackson, 1987). For this type of deposition to occur substantial release of old H2A,H2B dimers from preexisting nucleosomes would be required. On the basis of our earlier studies, this substantial release would be limited to a minor component of the genome (transcriptionally active genes) as the majority of new H2A and H2B when deposited on newly replicated DNA remain associated with that same DNA throughout the cell cycle (Jackson & Chalkley, 1981a). A much greater quantity of free old H2A,H2B dimers would be available, however, if prereplicative nucleosomes also dissolved (step 1 of Figure 1). The data of this paper provide evidence that nucleosome dissolution occurs during replication.

Several investigators have shown that histones continue to be synthesized at a basal level in the absence of replication and that the excess histones appear to be incorporated into nucleosomal structure (Gurley & Hardin, 1969; Tarnowka et al., 1978; Groppi & Coffino, 1980; Sheinin & Lewis, 1980; Wu & Bonner, 1981; Russev & Hancock, 1981; Waithe et al., 1983; Wu et al., 1981, 1982; Louters & Chalkley, 1985; Jackson & Chalkley, 1985a; Bonner et al., 1988). This incorporation of excess histone is observed when DNA synthesis is inhibited by treatment with drugs or during phases G1 and G2 of the cell cycle. Thus, in the model of Figure 1, it is possible to analyze deposition on nonreplicative DNA by preventing steps 1 and 2 through inhibition of DNA synthesis. Moreover, by simultaneous inhibition of RNA synthesis, it is possible to analyze deposition in which steps 1–4 are inhibited. Data presented here will indicate that a basal level of exchange continues to occur which is independent of both replication and transcription.

Two of the four core histones have been shown to participate in a posttranslational modification that involves conjugation to an 8.6-kDa protein, ubiquitin (Goldknopf & Busch, 1977; West & Bonner, 1980; Thorne et al., 1987). This modification has been strongly implicated as the first step in a process that involves an energy-dependent degradation pathway for protein turnover (Hershko et al., 1982). Histones H2A (5–15%) and H2B (2–5%) are found covalently attached to ubiquitin, and these histones are in turn found within nucleosomes (Matsui et al., 1979; Levinger & Varshavsky, 1980; Wu et al., 1981). In our analysis of the dynamics of histone–DNA interactions in the cell, the dependence of uH2A and uH2B deposition on replication and transcription was analyzed. Our data suggest that conjugation of H2A and H2B with ubiquitin occurs while these histones are in the free histone pool and that subsequent deposition of the conjugates into nucleosomes is independent of replication and transcription.

Materials and Methods

Cell Growth and Labeling. Chicken leukemic cell line transformed by Marek's virus (MSB) cells were grown in 10% fetal calf serum with medium of 1:1 Dulbecco's MEM–RPMI-1640 and supplemented with 50 mM HEPES (growth medium). Cells were grown in suspension culture without a CO_2 atmosphere. Exponential growth was (0.5–1.5) × 10^6/mL with a 9–10-h cell cycle. One liter of cells was concentrated into 200 mL of Dulbecco's medium (lacking all amino acids) and incubated 20 min at 37 °C. This process depleted internal amino acid pools to increase the specific activity of label in the subsequent pulse. Cells were then pelleted and resuspended into 10 mL of dense labeling media which consisted of Dulbecco's medium and 10 mg of carrier-free ^{15}N-, ^{13}C-, and ^{2}H-labeled amino acids (Merck MB1808). Also included in the 10 mL of Dulbecco's medium were the amino acids tryptophan, cysteine, glutamine, and asparagine, as these were not present in the mixture of dense amino acids. After a 10-min incubation, 0.5 mCi each of [^{3}H]lysine (70 Ci/mmol, Amersham) and [^{3}H]arginine (50 Ci/mmol, Amersham) was added and the incubation continued for 20 min. Cells were pelleted and quick-frozen. For experiments in which a chase of the density label was done, an aliquot of the cells was resuspended in fresh medium at 0.5 × 10^6 mL and incubated 4 h, after which time the cells were pelleted and quick-frozen. When DNA or RNA synthesis was inhibited, cells were incubated with 20 μg/mL cytosine arabinoside or 10 μg/mL actinomycin D.

Uniformly density labeled cells were prepared by incubating cells for three generations in dense labeling medium containing 10% fetal calf serum and 50 μCi each of [^{3}H]lysine and [^{3}H]arginine per 10 mL of labeling medium. After an incubation of 40 h, cells were harvested and frozen for later isolation of nuclei. Because undialyzed serum was present during the labeling to maintain appropriate growth, the specific activity of density in the histones is less than observed in the 30 min pulse experiments. Fraction 9 of the gradients is the density observed for the long-term labeling (Figure 3) and fraction 7 for the 30-min pulse (Figure 9).

Preparation of Nuclei and Histone Octamer for Density Centrifugation. Nuclei were isolated from frozen cells by washing three times in 1% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, and 10 mM Tris, pH 8.0, and then washed twice in the same buffer without detergent. Ten percent of the nuclei were set aside for acid extraction to isolate monomer histones. These nuclei were adjusted to a final concentration of 0.5 N H_2SO_4 and sonicated for 1 min on a Branson sonifier. The sample was centrifuged at 27000*g* for 10 min, and the supernatant, containing the histones, was precipitated in 7 volumes of acetone at −20 °C overnight. The precipitate was dissolved in water for analysis by gel electrophoresis. In the density-labeling experiments, these monomer histones served

as density markers in the gradient. The remaining 90% of nuclei were suspended in 12 mM TEA/12 mM Na_3PO_4, pH 9.1 (TEA/PO_4), at 100 μg/mL DNA concentration and made 1% in formaldehyde. After incubation for 8 h at 4 °C, the chromatin was pelleted at 10000*g* to remove the excess formaldehyde. This pellet was adjusted to a final concentration of 0.4 N H_2SO_4 from a 4 N stock and immediately sonicated twice for 30 s at 4 °C. The cross-linked histone complexes (histone octamer) are selectively removed from non-histone protein by acid extraction of the chromatin pellet. The solution was centrifuged at 20000*g* for 10 min, and the supernatant, containing the octamer, was dialyzed against two changes of 0.4 N H_2SO_4 and one change of 0.02 N H_2SO_4 over a 36-h period at 4 °C. The treatment with acid terminates the cross-linking, and the extensive dialysis removes the unreactive formaldehyde. Direct electrophoretic analysis was then completed on samples that were only radiolabeled. For experiments with density-labeled octamers this solution was then combined with the previously acid-extracted monomer histones from the same preparation and added to 2.4 g of Gdn-HCl, 0.82 g of CsFo, and 100 μL of 3 M Tris, pH 8.0, and the volume was adjusted to 4.0 mL. Centrifugation was at 54000 rpm for 96 h at 11 °C on an SW60Ti rotor; 150-μL fractions were collected, made to a final concentration of 0.4 N H_2SO_4, and directly dialyzed against 0.2 N H_2SO_4 and 5 mM 2-mercaptoethanol on a BRL (Bethesda Research Labs) 28-slot dialysis apparatus. Samples were then precipitated in 7 volumes of acetone at −20 °C overnight and redissolved by sonication into 0.5% SDS, 10% glycerol, and 100 mM Tris, pH 6.8.

Electrophoresis of Proteins and Reversal of Formaldehyde Cross-Link. The electrophoresis conditions were a modification of the Laemmli (1970) procedure. The electrophoresis buffer was 0.1% SDS, 25 mM Tris, and 200 mM glycine, pH 8.3, and the separating gel was 18% acrylamide, 0.09% methylenebis(acrylamide), 0.1% SDS, and 0.75 M Tris, pH 8.8. Electrophoresis was at 150 V for 18 h at 4 °C, after which time the gels were stained with 0.5% Coomassie blue, 40% methanol, and 10% acetic acid for 12 h and destained in the same (without stain). Gels were scanned with a modified Gilford densitometer and fluorographed by the procedure of Laskey and Mills (1979). When reversal of cross-link (Jackson, 1978) was required for the proteins in the gel, the stained gel was cut into a strip containing the octamer region and incubated in two changes of 100 mL each of 1% SDS and 100 mM Tris, pH 6.8 (reversal solution) over a 90-min period. The strip was then heated at 100 °C for 30 min in fresh reversal solution containing 0.5 M 2-mercaptoethanol and reincubated for 60 min in three changes of reversal solution to remove mercaptoethanol. This gel strip was directly polymerized into a stacking gel consisting of 2.5% acrylamide, 0.13% methylenebis(acrylamide), 125 mM Tris, pH 6.8, and 0.1% SDS. The separating gel was the same as previously described except the acrylamide to bis(acrylamide) ratio was changed from 200:1 to 100:1.

Results

Evidence for Nucleosome Dissolution during Replication. Figure 2 illustrates an experimental protocol designed to determine whether the tetramer and dimer dissociate from an octameric complex during nucleosome replication. Following a modified version of the classic Meselson and Stahl (1958) experiments, cells were labeled for several generations with density-labeled and radio-labeled amino acids. Subsequently, half the cells were quick-frozen and the remainder suspended in fresh medium containing nondense and nonradioactive

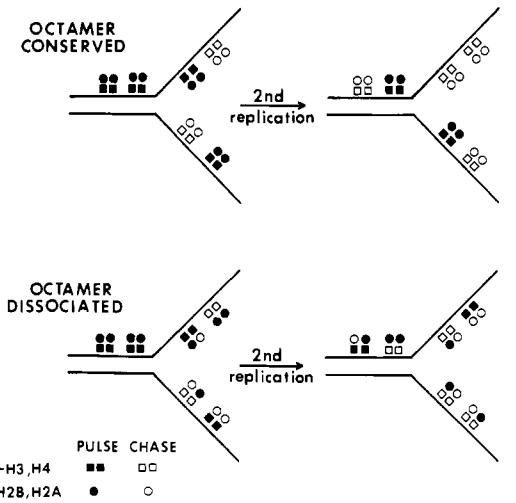

FIGURE 2: Illustration of a protocol designed to determine whether nucleosomes dissolved as a function of replication. The solid squares (H3,H4 tetramer) and solid circles (H2A,H2B dimer) indicate the density-labeled histones produced by incubating cells for three generations in heavy medium (pulse). The open squares and circles indicate the histones synthesized during a chase period when the labeling medium has been replaced with normal medium (chase). If the octamer is conserved, the density of the octameric complex produced in the initial pulse will not change in the subsequent chase. Octameric dissociation will be reflected in the formation of octameric complexes during the chase that have decreased their content of density-labeled histones.

amino acids. After a chase of 48 h (three cell divisions), these cells were harvested and nuclei were isolated. The cells that were harvested after the initial labeling period were mixed with a 10-fold excess of unlabeled cells (mix control) and nuclei were isolated. Solubilized chromatin was prepared and treated with formaldehyde at pH 9 for 8 h to allow formation of the octameric complex of histones within the nucleosomes. The octamers were isolated by acid extraction and centrifuged to equilibrium on CsFo gradients in the presence of un-cross-linked (monomer) histones which served as density markers in the gradients. After fractionation, samples were electrophoresed on SDS–PAGE, the gels were stained to localize the proteins (both monomer and octamer), and the octamer region encompassing fractions 1–28 was sliced out. These gel strips were heated at 100 °C for 30 min to reverse the formaldehyde cross-link. The histone composition of octamers of different densities was then determined by fusing the gel strip to a second SDS–PAGE and reelectrophoresing in the same dimension. The results of this analysis are shown in Figure 3 and densitometrically analyzed for quantitation in Figure 4. The fluorogram for the 48-h chase shows that the labeled H3 and H4 are distributed at a greater density relative to labeled histones H2A and H2B. The H3 and H4 distribution is halfway between the high-density position and the normal-density position in the gradient. Normal density (ND) is based on the location of the unlabeled histones in the stained gel. High density (HD) is based on the location of the labeled monomer histones from the same nuclear preparation which were present in the gradient. These were quantitated from the first SDS–PAGE in which the octamer region was sliced out (data not shown). This intermediate density indicates that H3 and H4 are present in a nucleosome in which half of the histones are labeled and half are not: hence, H3 and H4 are present in nucleosomes as tetramers associated with dimers of nonlabeled H2A and H2B. As shown in Figure 2, if the octameric complex of histones within the nucleosome did not dissociate, the fully dense octameric complex of the initial labeling should have maintained that same density in the

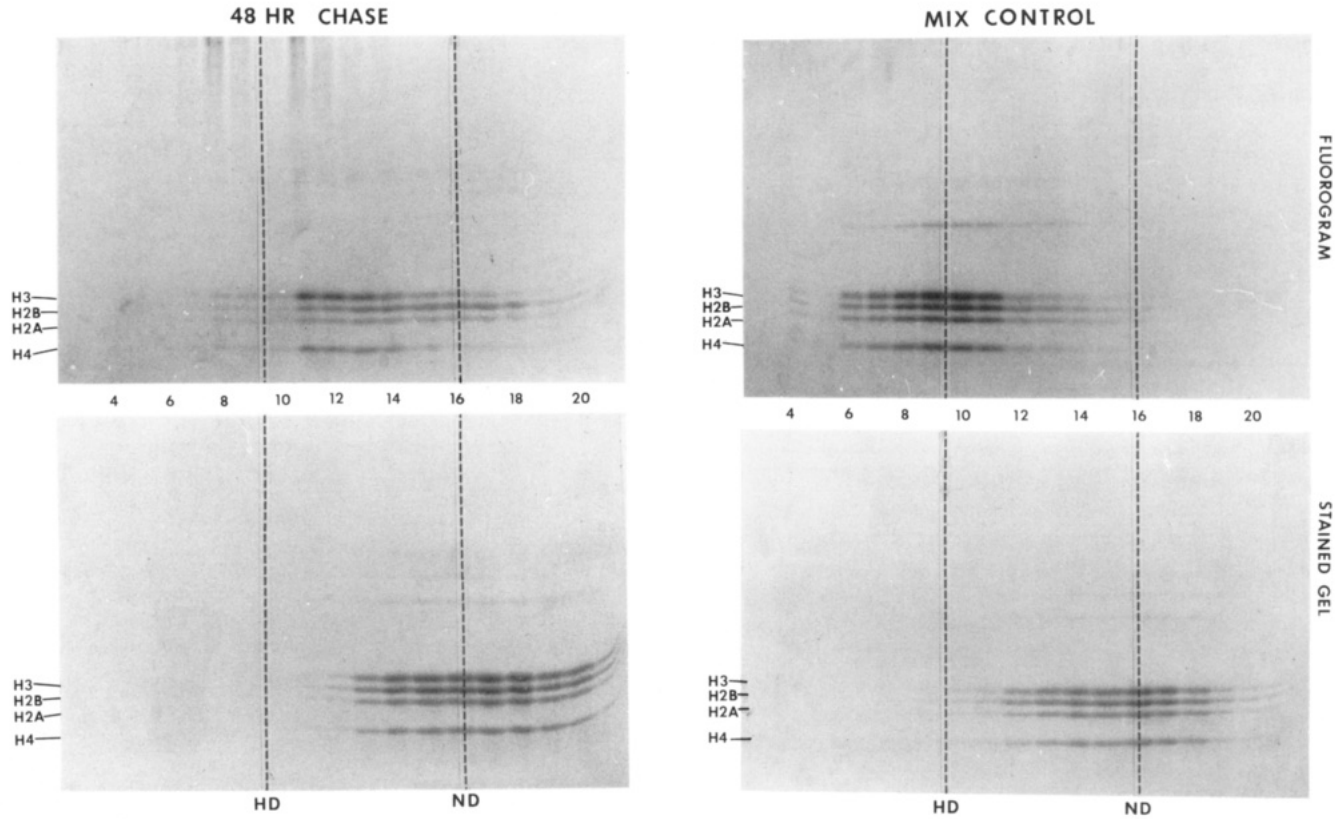

FIGURE 3: Gel electrophoretic analyses of the histones in the octamers for the experiment of Figure 2. After fractionation of the formaldehyde-cross-linked octameric complexes on density gradients, the individual fractions were electrophoresed on SDS–PAGE so that the octamer region of the gel from fraction 1 to fraction 28 could be sliced out and heated at 100 °C for 30 min to reverse the cross-link of the histones in the complexes. Subsequently, the proteins were reelectrophoresed in the same dimension to resolve the individual histones as illustrated in this figure. The vertical dotted lines mark the location in the density gradient in which the monomer normal-density histones (ND) and high-density histones (HD) were distributed. These locations were defined by the first gel electrophoreses when the monomer histones were resolved from the octamer.

subsequent chase. All four histones, H3, H2B, H2A, and H4, should have been distributed at the HD position of the gradient. The data of Figure 3 require that nucleosomes dissociate such that labeled tetramers could form new octameric complexes with nonlabeled dimers. The fluorogram of the mix control in Figure 3 shows that these results are not due to any artifactual effect such as an induced randomization brought about by the preparative procedure or the formaldehyde cross-linking. All four labeled histones, H3, H2B, H2A, and H4, are distributed at the HD region of the gradient. We conclude that the dissociation of the H3,H4 tetramers from the H2A,H2B dimers occurs during the replication event. This conclusion is also based on earlier studies which have indicated that within a cell cycle the exchange of H2A and H2B is very limited for the majority of the genome (Jackson & Chalkley, 1981a). Such an extensive breakdown of nucleosomes as observed in the data in Figure 3 must be a consequence of replication.

These results provide the bases for step 1 of Figure 1 in which prereplicative nucleosomes are shown to dissolve such that the H3,H4 tetramers and H2A,H2B dimers are free to interact with the incoming newly synthesized histones. This mixture in turn redeposits on the daughter strands (step 2) with no specific requirement for deposition other than that an H3,H4 tetramer (new or old) interacts with two H2A,H2B dimers (new or old) to form a nucleosome.

Evidence for Histone Excess in the Nuclei of Cells in Which DNA Synthesis Has Been Inhibited. The model of Figure 1 proposes that nucleosome dissolution occurs as a function of RNA polymerase movement and causes the release of H2A,H2B dimers (step 3) which interact with the pool of histones generated from replication and protein synthesis. During S phase (Cox, 1976), transcription (nucleotides incorporated) occurs at a 3–5-fold greater rate than replication (nucleotides replicated); therefore, in this model the pool of old H2A,H2B dimers is shown to be 3 times greater relative to the newly synthesized H2A and H2B which enter the pool. Because the ratio of new versus old histone in this intranuclear pool is dependent on steps 1–4, any procedure that will affect these steps should changes this ratio. Therefore, in the absence of DNA synthesis, steps 1 and 2 are prevented. Inhibiting RNA synthesis prevents steps 3 and 4, providing that the movement of the polymerase is a prerequisite for nucleosome dissolution.

As shown in Figure 5A, cytosine arabinoside (20 μg/mL) rapidly inhibits DNA synthesis. The rate of inhibition is so rapid for MSB cells that even the simultaneous addition of drug with [^{3}H]thymidine (0 time sample) to an aliquot of cells results in no uptake of [^{3}H]thymidine. In contrast, RNA synthesis is unaffected by the cytosine arabinoside. Similarly, as shown in Figure 5B, RNA synthesis can be rapidly inhibited by actinomycin D (10 μg/mL), whereas DNA synthesis continues to occur with a $t_{1/2}$ of 30 min. Thus, steps 1 and 2 can be inhibited separately from steps 3 and 4. All steps are prevented when both inhibitors are present.

As shown in Figure 6 (isolated nuclei), and graphically illustrated in Figure 5C, the rapid effect on DNA and RNA synthesis by these inhibitors does not cause a rapid decrease in histone synthesis. At specific points after treatment with the inhibitor, cells were incubated for 10 min with [^{3}H]lysine and [^{3}H]arginine, and the proteins of the isolated nuclei were analyzed by SDS–PAGE. As shown in the fluorogram, when

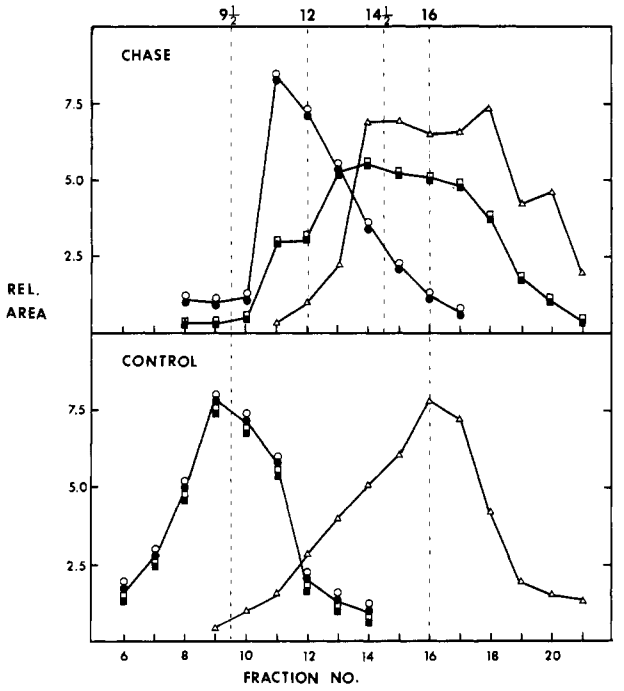

FIGURE 4: Quantitation of the histone distribution from the data of Figure 3. Densitometric analyses of the data in Figure 3 is plotted to determine the distribution of the histones. In this way the relative distribution of the individual labeled histones (fluorogram) in the density gradient can be determined: H3 (○); H4 (●); H2A (■); H2B (□). The distribution of the normal-density histones (△) was quantitated from the stained gel. For the chase experiment the average distribution in the gradient for H3 and H4 was fraction 12 and for H2A and H2B was fraction 14-$^1/_2$. The relative distribution for these labeled histones, between fraction 16 (normal density) and fraction 9-$^1/_2$ (high density) was 35% dense for H2A and H2B and 61% dense for H3 and H4. For the mix control experiment the relative density for all four labeled histones was 100%. The 48-h chase was sufficient to complete three rounds of replication on the nucleosomes.

DNA synthesis is inhibited (-DNA, +RNA), histone synthesis decreases, but this decrease is not sufficiently rapid to prevent an accumulation of new histone in the nucleus. As shown in Figure 6, the rate of decrease in histone synthesis is equivalent for all five histones and, therefore, all five histones accumulate within the histone pool in the nuclei of MSB cells. These histones are unable to deposit at the replication fork due to the lack of DNA. This rate of decrease in histone synthesis is expected on the basis of previous studies demonstrating an accelerated rate of histone mRNA turnover when DNA synthesis is inhibited (Baumbach et al., 1984; Morris et al., 1986; Bandyopadhyay et al., 1987). DNA and histone syntheses are tightly coupled. Therefore, as expected, when RNA synthesis is inhibited and DNA synthesis continues (+DNA, -RNA), the decrease in histone synthesis is significantly reduced, with the reduction being parallel to the rate of DNA synthesis (Figure 5). This tight coupling of histone and DNA synthesis maintains a condition in which excess free histones are kept to a minimum.

Evidence for a Limited Number of Deposition Sites for the Excess Histones. The excess histones produced when DNA synthesis is inhibited were assayed for deposition into nucleosome structures by treatment of the isolated nuclei at each time point (Figure 6) with formaldehyde at pH 9 to form the octameric complex. By in vitro analyses, Louter and Chalkley (1984) have shown that the formation of the octameric complex by formaldehyde requires that the histones be in a nucleosome structure. DNA must be wrapped around the core histones to produce the short distances needed for formaldehyde cross-links between histones. This method is very useful for assaying nucleosome formation. Subsequently, the cross-linked octamers were extracted and electrophoresed on SDS-PAGE as shown in Figure 7A. A gel slice encompassing the octamer region, as indicated by the dotted line, was taken and heated at 100 °C for 30 min to reverse the cross-link, and the proteins within the gel slice were reelectrophoresed in the same dimension (Figure 7B). As shown in the fluorogram of Figure 7B, the quantity of newly synthesized histones present in the octamer decreases rapidly when DNA synthesis is inhibited. Figure 5D graphically illustrates that this decrease is substantially more rapid than the rate of inhibition of histone synthesis. Therefore, at the 10-min time point during which 80% of synthesis occurs, only 10% of the histones can deposit into a nucleosome. In the case of the control (c, no inhibitor treatment), all newly synthesized histones are deposited into octameric complexes. These data indicate that a subpopulation of the excess histones is actively deposited into nucleosome structure. A significant pool of histones is retained within the nucleus which is not nucleosomal and is presumably in equilibrium with old histones that were released during the deposition of the new ones. By definition, this deposition is occurring on nonreplicative DNA as DNA synthesis is inhibited. The more rapid decrease in deposition relative to histone synthesis implies that the sites of exchange are limited and become rapidly saturated. If the only factor limiting histone exchange in the nucleus was the quantity of excess histones present, then the percentage of deposition relative to histone synthesis should have remained constant. This conclusion was further tested by determining octamer formation in cells in which RNA synthesis was inhibited and DNA synthesis continued (Figure 7, +DNA, -RNA). The percentage deposition (octamer formation) closely parallels the rate of histone synthesis throughout the time course (compare Figure 5, parts C and D). This result would be predicted if the size of the histone pool in the nucleus remained constant throughout the treatment with actinomycin D due to the continual deposition of newly synthesized histones on newly replicated DNA. All of the histones that are synthesized are deposited.

Evidence That Not All Deposition Sites Are Dependent on Active Transcription. The saturation of deposition sites under conditions in which DNA synthesis is inhibited suggests that there are limited sites for histone exchange. The sites for this limited exchange may be sites of active transcription and, as such, inhibition of both transcription and replication would be expected to terminate any formation of labeled cross-linked octameric complexes. As shown in Figure 6 (-DNA, -RNA) and graphically illustrated in Figure 5D, inhibition of both DNA and RNA synthesis further decreases octamer formation (from 10% to 5% in 10-min time point, Figure 5D). However, as the process is not totally inhibited, a limited exchange must still occur independent of active transcription.

An additional feature of the data in Figure 7B is the differential decrease in deposition that occurs for the various histones. When DNA synthesis is inhibited, the rate of decrease in deposition for histones H3 and H4 is 3-fold more rapid compared to that of H2A and H2B. As shown in Figure 6, this effect is not due to a difference in histone synthesis. The saturation of deposition sites for H3 and H4 is more rapid than for H2A and H2B, suggesting that there may be differences in sites of deposition for these sets of histones under conditions during which deposition is prevented at the replication fork. To further evaluate this differential deposition, cells were incubated with the inhibitors for 30 min by using the conditions of Figures 6 and 7. Subsequently, the cells were

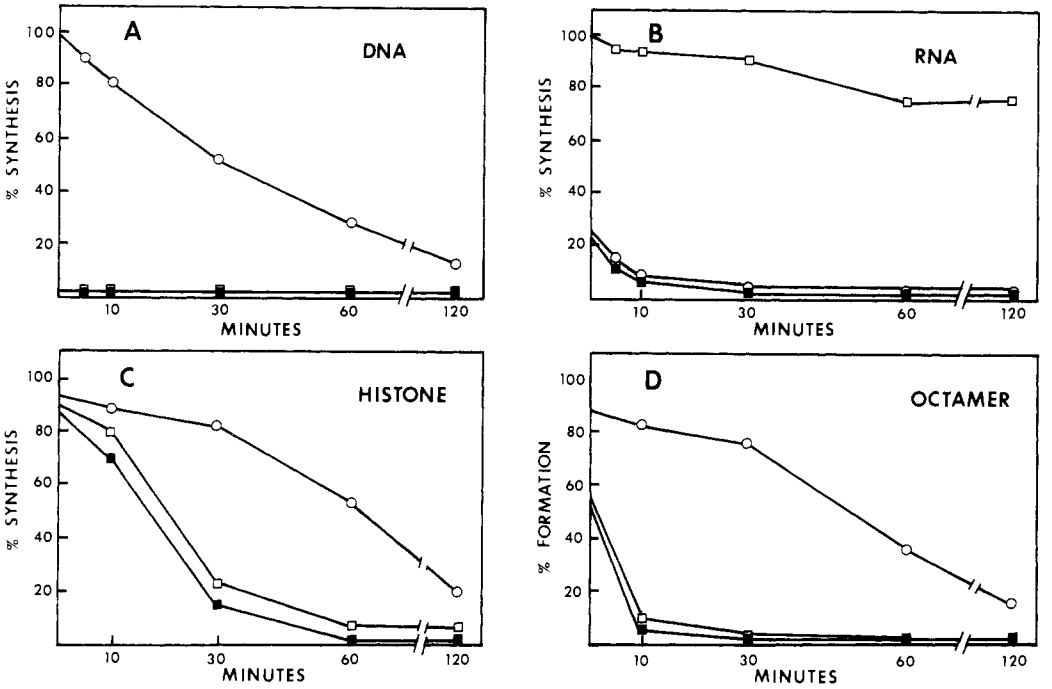

FIGURE 5: Rates of DNA, RNA, histone, and octamer synthesis that occurs during inhibition of DNA and/or RNA synthesis: (A) rates of DNA synthesis; (B) rates of RNA synthesis; (C) rates of histone synthesis; (D) quantitation of octamer that can be formed from the synthesized histone by formaldehyde treatment. Rates were determined when cells were treated with 20 μg/mL cytosine arabinoside (□) or 10 μg/mL actinomycin D (○) or when both inhibitors were added simultaneously (■). For (A) and (B) aliquots of cells were taken during treatment with the inhibitors and added to either [^{3}H]thymidine or [^{3}H]uridine and incubated for 5 min. Cells were lysed and precipitated with TCA to determine uptake of nucleotide. For (C), data taken from Figure 6 were densitometrically quantitated for the core histones, H2A, H2B, H3, and H4, and plotted to determine the rate of synthesis. For (D) data of Figure 7, in which the formaldehyde-cross-linked octamer was analyzed, were quantitated for the four histones and plotted to determine the percentage of synthesized histones that can be cross-linked into an octameric complex. Quantitation of both (C) and (D) requires determination of specific activity of labeled histone (fluorogram) vs total histone (stained gel) for each time point for both Figures 6 and 7.

incubated for 10 min with [^{3}H]lysine and [^{3}H]arginine and then incubated in the absence of label for 4 h. The 4-h chase was done both in the presence and in the absence of the inhibitors. Following the same protocol of isolation and electrophoresis as described for Figure 7, the histones in the cross-linked octameric complexes were then electrophoresed and fluorographed. As shown in Figure 8, additional deposition does occur for histones H3 and H4. After a 4-h incubation, H3 and H4 are deposited at a level similar to that of histones H2A and H2B. This deposition is independent of whether the inhibitors were present during the subsequent incubations; the deposition is independent of replication and transcription. The model of Figure 1 proposes that deposition of H3 and H4 occurs specifically at the replication fork and the deposition of H2A and H2B may occur either at the replication fork or active genes. As deposition of H3 and H4 is occurring in the absence of replication and transcription, albeit at a much reduced rate, these data indicate that this model must be modified to account for this nonreplicative deposition.

Evidence for Nucleosome Dissolution during Transcription. With the preliminary indications that a limited exchange (nucleosome dissolution) occurs which is partially dependent on active transcription (Figure 5D), an additional experiment was done to test for nucleosome dissolution. This experiment involved the incorporation of density-labeled amino acids into cells in which RNA synthesis was inhibited and a subsequent analysis of the cross-linked octamer. As described previously (Jackson, 1987; see also Table I), when density-labeled newly synthesized H2A and H2B deposit into a nucleosome, an octamer complex is formed with one old H2A,H2B dimer and one old H3,H4 tetramer (two of eight are dense). The distribution of this octameric complex on density gradients is 24% of the distance between the HD (high density as determined by the monomer density-labeled histones) region and the ND (normal density as determined by the Coomassie stained protein) region. When newly synthesized density-labeled H3 and H4 deposit into a nucleosome, they deposit as a new tetramer primarily with two old H2A,H2B dimers and their distribution is 45% of the distance between HD and ND region. In the model of Figure 1, the active movement of RNA polymerase is shown to cause nucleosome dissolution and the

Table I: Percent Density Shift[a]

synthesis	H3,H4 (%)	H2A,H2B (%)	uH2B,H2A or uH2A,H2B (%)
+DNA[b] (pulse) +RNA	45	24	18
+DNA (pulse) -RNA	63	24, 63	18
-DNA (pulse) -RNA	53	29	18
-DNA (pulse) +RNA	53	24	18
-DNA (4-h chase) +RNA	35	12	12

[a] Example calculation for -DNA, +RNA (pulse). For H3 and H4 the center of the peak is fraction 11, (15.5 - 11)/(15.5 - 7) = 53%. For H2A and H2B the center of the peak is fraction 13.5, (15.5 - 13.5)/(15.5 - 7) = 24%. For uH2A and uH2B the center of the peak is fraction 14, (15.5 - 14)/(15.5 - 7) = 18%. Fraction 15.5 is the center of the peak for nonlabeled histones (stained gel). Fraction 7 is the center of the peak for the labeled monomer histones and therefore marks the location in which labeled histones in the octameric complex would be distributed if all were labeled (Jackson, 1987). [b] Data taken from Jackson (1987).

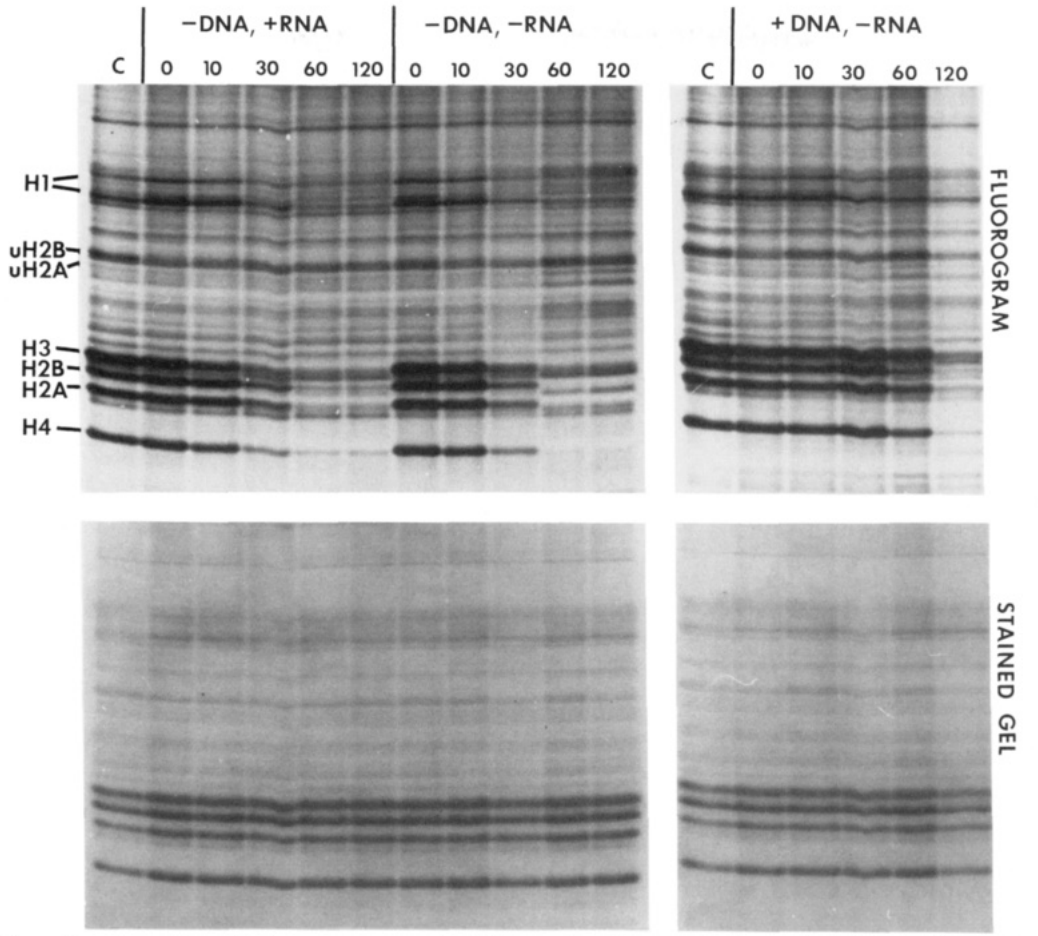

FIGURE 6: Gel electrophoretic analyses of histone synthesis when DNA and/or RNA synthesis was inhibited. Cells were incubated with the inhibitors for increasing lengths of time at which point aliquots were taken for 10-min incubations with [^{3}H]lysine and [^{3}H]arginine. The cells were then rapidly frozen for isolation of nuclei as described under Materials and Methods. The '0' time point represents conditions during which the inhibitors and radiolabel were added simultaneously; 'c' is the control to indicate the histone synthesized in absence of the inhibitors. Conditions for treatment were as follows: (−DNA, +RNA) cells are incubated with 20 μg/mL cytosine arabinoside; (+DNA, −RNA) cells are incubated with 10 μg/mL actinomycin D; (−DNA, −RNA) cells are incubated with both inhibitors simultaneously.

release of old H2A,H2B dimers (step 3), which are then available to form an octameric complex with new H3,H4 tetramers at the replication fork. After RNA polymerase passage, the nucleosomes are shown to re-form by the addition of either new or old H2A,H2B dimers (step 4). If RNA synthesis was inhibited and steps 3 and 4 were prevented, the quantity of new H2A,H2B dimer in the nuclear pool would increase significantly such that the quantity of new histones within each newly deposited octameric complex would be greatly increased. However, the complexes would not be absolutely homogeneous for new histone since both old H2A,H2B dimers and old H3,H4 tetramers would continue to be released as part of the replication process (step 1). The quantity of density-labeled new H3, H4, H2B, and H2A together in an octameric complex should significantly increase when RNA synthesis is inhibited.

Cells were labeled with dense amino acids for 30 min in the presence of actinomycin D. Nuclei were isolated and treated with formaldehyde to cross-link the histones within the nucleosomes into octameric complexes. The isolated complexes were then centrifuged to equilibrium on CsFo–GuCl gradients and the fractions electrophoresed on SDS–PAGE gels. The octamer region of the gel was heated at 100 °C for 30 min and reelectrophoresed as described for the experiment in Figure 3. As shown in Figure 9D and densitometrically quantitated in Figure 10, new H2A and H2B can be resolved into two approximately equal peaks. The first peak, which is located at fraction 13.5, represents octameric complexes in which 24% of the histones are new (fraction 13.5 is 24% relative to ND–HD). The interpretation of this observation is that the new H2A and H2B of this fraction deposit into a nucleosome as a dimer with six old histones, one old H2A,H2B dimer, and one old H3,H4 tetramer. If RNA synthesis had not been inhibited, all the new H2A and H2B would have been distributed in such an octameric complex (Jackson, 1987; see Table I). The unique effect of the inhibition of RNA synthesis is that a second peak has been generated in which the other half of the new H2A and H2B are now located at fraction 10. New H3 and H4 are also distributed at fraction 10 (fraction 10 is 63% relative to ND–HD). The significant shift in density for all four histones (Table I) indicates that these two sets of new histones are interacting with a much higher frequency to form nucleosome structure. Because this increased frequency is due to the inhibition of RNA synthesis, these data suggest (according to the model of Figure 1) that a major part of the exchange process is dependent on the movement of RNA polymerase.

Evidence for Octamer Instability When in the Free Histone Pool. As shown previously, histone deposition continues to occur under conditions in which DNA synthesis is inhibited. This deposition on nonreplicative DNA is rapidly saturable. To study the dynamics of deposition for the excess histones, MSB cells were incubated with density-labeled amino acids in the presence of cytosine arabinoside for 30 min. An aliquot of cells was harvested and the remainder resuspended in fresh medium containing cytosine arabinoside for a chase of 4 h.

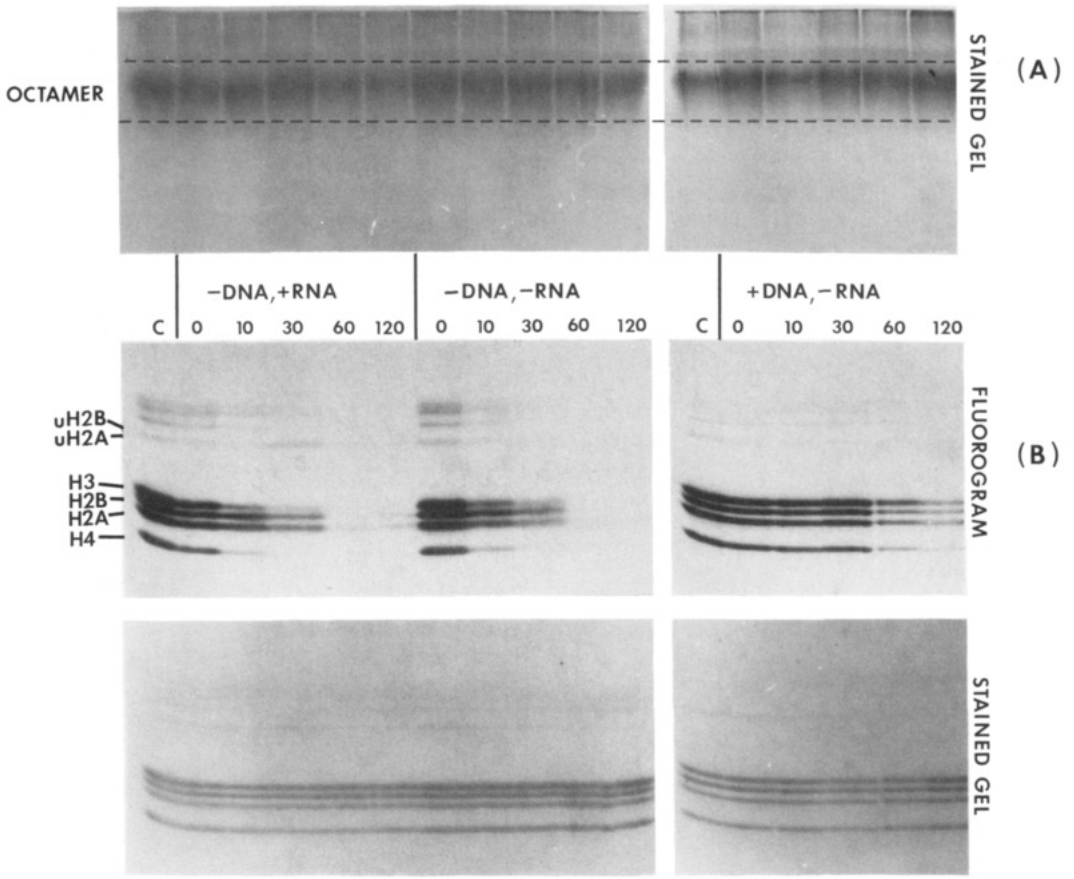

FIGURE 7: Gel electrophoretic analyses of the cross-linked octameric complexes isolated from cells that have been treated with inhibitors by the protocol of Figure 6. An aliquot of the isolated nuclei for each time point of Figure 6 was treated with formaldehyde for 8 h at 4 °C in 12.5 mM TEA/12.5 mM PO_4, pH 9.1. The octameric complexes were then acid-extracted from the DNA and electrophoresed on SDS–PAGE (panel A). Subsequently, the octamer region of the gel bounded by the horizontal dotted lines of this figure was sliced out and heated at 100 °C for 30 min to reverse the cross-link, and the histones within the gel were reelectrophoresed to quantitate the individual histones in the fractions (panel B).

Subsequently, the nuclei were isolated from the cells, octameric complexes were formed by formaldehyde treatment, and these complexes were then analyzed by density gradient fractionation and SDS–PAGE. As shown in Figure 9B and plotted in Figure 10, the octameric complexes produced in the 30-min pulse contain both new H3 and H4 and new H2A and H2B. The two sets of histones are distributed in different regions of the gradient. The peak of new H3 and H4 is at fraction 11, and the peak of new H2A and H2B is at fraction 13.5. This distribution indicates that H3 and H4 are deposited on the nonreplicative DNA as a new tetramer with old H2A and H2B (53%, Table I) and new H2A,H2B are deposited as a dimer with six old histones (24%, Table I). Such a result is predicted on the basis of the model of Figure 1, in which even if steps 1 and 2 are terminated due to the lack of DNA synthesis, steps 3 and 4 will continue to occur. Thus, the continual release and redeposition of old H2A,H2B dimers during transcription keeps the histone pool diluted with old histones. As was shown in Figure 7, Figure 9B also indicates that when DNA synthesis is inhibited, H3,H4 tetramers continue to deposit into an octameric complex, although much reduced compared to H2A,H2B. The model of Figure 1 requires modification to account for this low-level deposition of H3 and H4 on nonreplicative DNA.

As shown in Figure 9A and plotted in Figure 10, when these labeled cells were chased for 4 h, the distribution of newly synthesized histone changed dramatically. A much greater quantity of new H3 and H4 is deposited, and these additional histones are distributed in a broad peak of decreasing density that centers at fraction 12.5 (35%, Table I). The distribution of new H2A and H2B is also a broad peak of decreasing density that centers at fraction 14.5 (12%, Table I). These data indicate that the excess labeled histones which did not deposit in the initial pulse are intermingling with nonlabeled histones within the pool such that the labeled H3,H4 tetramers have become new-old tetramers. A similar effect is observed for the H2A,H2B dimers in which only one dense H2A or H2B eventually deposits in a nucleosome to give the 12% density of the octamer (one of eight dense). This instability of both tetramers and dimers is not normally observed when DNA synthesis continues. Under these conditions chases of greater than 20 h do not show any detectable change in the density of the deposited octameric complexes (Jackson, 1988). Thus, when left for an extended period in an undeposited form within the nucleus, the H3,H4 tetramers and H2A,H2B dimers will not maintain stable interactions but will intermix with old histones.

Further Evidence for a Basal Level of Histone Exchange in the Absence of Replication and Transcription. To further evaluate this exchange that occurs in the absence of DNA synthesis, density-labeling experiments were done in the presence of both cytosine arabinoside and actinomycin D. As was previously shown in the data of Figure 6, the additional inhibition of RNA synthesis caused an approximately 2-fold decrease in deposition for all four core histones. Theoretically, if the only source of old histones for intermixing with new ones is from the dissolution of nucleosomes at the replication fork or from RNA polymerase movement during transcription, the

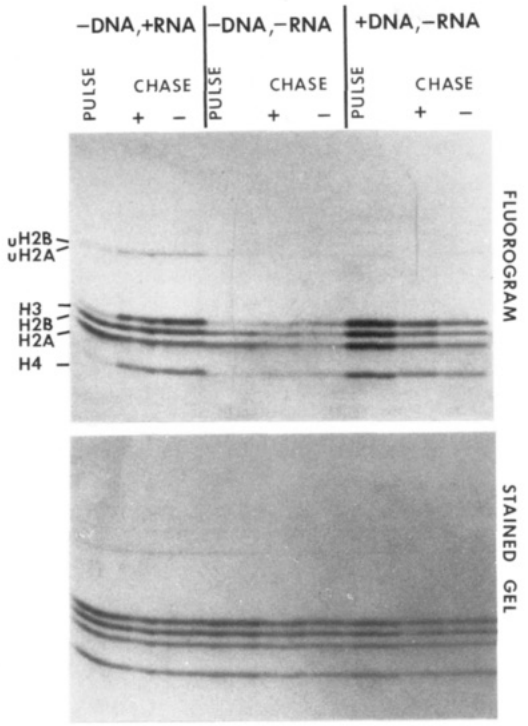

FIGURE 8: Gel electrophoretic analyses of histones deposited into nucleosomes after a 4-h chase in the presence and/or absence of inhibitors. After incubation of cells with the inhibitors for 30 min, the cells were labeled for 10 min with [^{3}H]lysine and [^{3}H]arginine (30-min time point of Figure 7). An aliquot was immediately frozen (pulse) and the remainder rinsed and resuspended in fresh medium in the presence (+) and absence (−) of the inhibitors (chase). After a 4-h chase, cells were harvested and nuclei isolated from all samples. After formaldehyde treatment of the nuclei to produce the octameric complexes, these complexes were resoved by gel electrophoreses using the protocol described in Figure 7. After reversal of cross-link, the individual histones were resolved by SDS–PAGE.

inhibition of these activities should create a condition where only new histone would be present in the excess pool (steps 1–4 terminated). Any subsequent deposition into nucleosome structures would create an octameric complex more homogeneous with respect to density-labeled histones. The predicted distribution would be similar to the data of Figure 3 (mix control) in which density-labeled and nonlabeled histones do not intermix. When this experiment was done (Figure 9C), no such homogeneity was observed. The distribution of the labeled histones indicates that new H3,H4 tetramers are depositing into nucleosome structures with primarily old H2A,H2B dimers (53%, Table I) and new H2A,H2B dimers are depositing with an old H2A,H2B dimer and an old H3,H4 tetramer (29%, Table I). The free histone pool is obtaining a source of old histones that is independent of replication and transcription.

Evidence That uH2A and uH2B Exchange Is Independent of Replication and Transcription. As shown in Figure 6, newly synthesized H2A and H2B are conjugated with ubiquitin and are subsequently deposited into nucleosome structure, as reflected by their presence in the cross-linked octameric complex (Figures 7–10). Of interest is the observation that the level of conjugation increases as the quantity of excess histone increases when DNA synthesis is inhibited. Thus, after 30 min, that quantity of H2A synthesized in the 10-min pulse is modified by 30% as compared to the control in which 5% is modified (Figure 6). This pool of excess histone is vulnerable to extensive modification by ubiquitin. As shown in the density-labeling experiments of Figure 9, these conjugated histones are incorporated into octameric complexes whose densities provide information with regard to the sites of deposition for these conjugates. Under normal conditions (+DNA, +RNA), uH2A and uH2B have a density shift of 18%. The decrease in density from 24 to 18% is not indicative of a lack of dimer formation. Rather, it reflects the conjugation of a nonlabeled ubiquitin to the dimer, which decreases the relative percentage of density-labeled histones within the octameric complex (Seale, 1981; Wu et al., 1981). The nonlabeled ubiquitin pool is substantially greater than the labeled ubiquitin which can be synthesized in the pulse (Haas, 1988). Of interest in the data of Figure 9D is the distribution of the conjugated H2A,H2B dimers when RNA synthesis is inhibited. Whereas half of the H2A,H2B dimers take on a new density of 63% (Table I), uH2A and uH2B remain at 18%. We interpret these data to indicate that deposition of these conjugated histones occurs at sites in which the exchange process occurs independent of RNA polymerase movement. Otherwise, half of the uH2A and uH2B should have shifted from 18% to approximately a 50% density shift. These sites would appear to be similar to the sites of deposition for the four core histones when DNA and RNA synthesis is inhibited. Further evidence in support of this point is that uH2A and uH2B are also susceptible to the same intermingling with old histones that occurs for the other histones when DNA synthesis is inhibited and the histone excess is chased for 4 h (density shift from 18 to 12%, Table I). Thus, the deposition of the conjugated forms of H2A and H2B may be a marker for the basal level of deposition that occurs independent of replication and transcription.

Discussion

Evidence has been presented which indicates that nucleosome dissolution occurs during replication. This evidence is based on the observed dissociation of uniformly density labeled octameric complexes into H3,H4 tetramers and H2A,H2B dimers. Theoretically, the breakdown may occur at any time in the cell cycle and simply reflect an extensive dynamic interchange of histones on all the DNA. However, such extensive interchange has not been seen. Rather, previous studies using density-labeled DNA and radiolabeled newly synthesized histones have shown that for the majority of the genome once histones are deposited, they remain associated with that same DNA, providing the chase does not continue through the next replication event for that region of DNA (Jackson & Chalkley, 1981a, 1985b). Therefore, the data of Figure 3 suggest that the release of the H3,H4 tetramer and/or H2A,H2B dimer occurs as a consequence of replication.

The temporary synthesis of histones in the presence of DNA synthesis inhibitors provides a means for analysis of histone deposition at nonreplicative sites. Our data indicate that only a small percentage of these excess histones, primarily H2A and H2B (Figure 7), are able to deposit into nucleosomal structure. Louters and Chalkley (1985) described similar selective deposition of H2A and H2B in cells in which DNA synthesis was inhibited; however, our data indicate that H3 and H4 also deposit, but only during subsequent chases of the label (Figure 8). This deposition of H2A and H2B is partially dependent on transcription as shown by the decrease in deposition when both transcription and replication are inhibited (Figure 5D). The same dependence on transcription was also observed in the density-labeling experiments of Figure 9D. The increased percentage of density-labeled histones within individual octamers was a direct reflection of the inhibition of transcription, such that H2A,H2B dimers were no longer capable of exchanging (steps 3 and 4 of model 1 inhibited). The occurrence of this exchange and its dependence on active

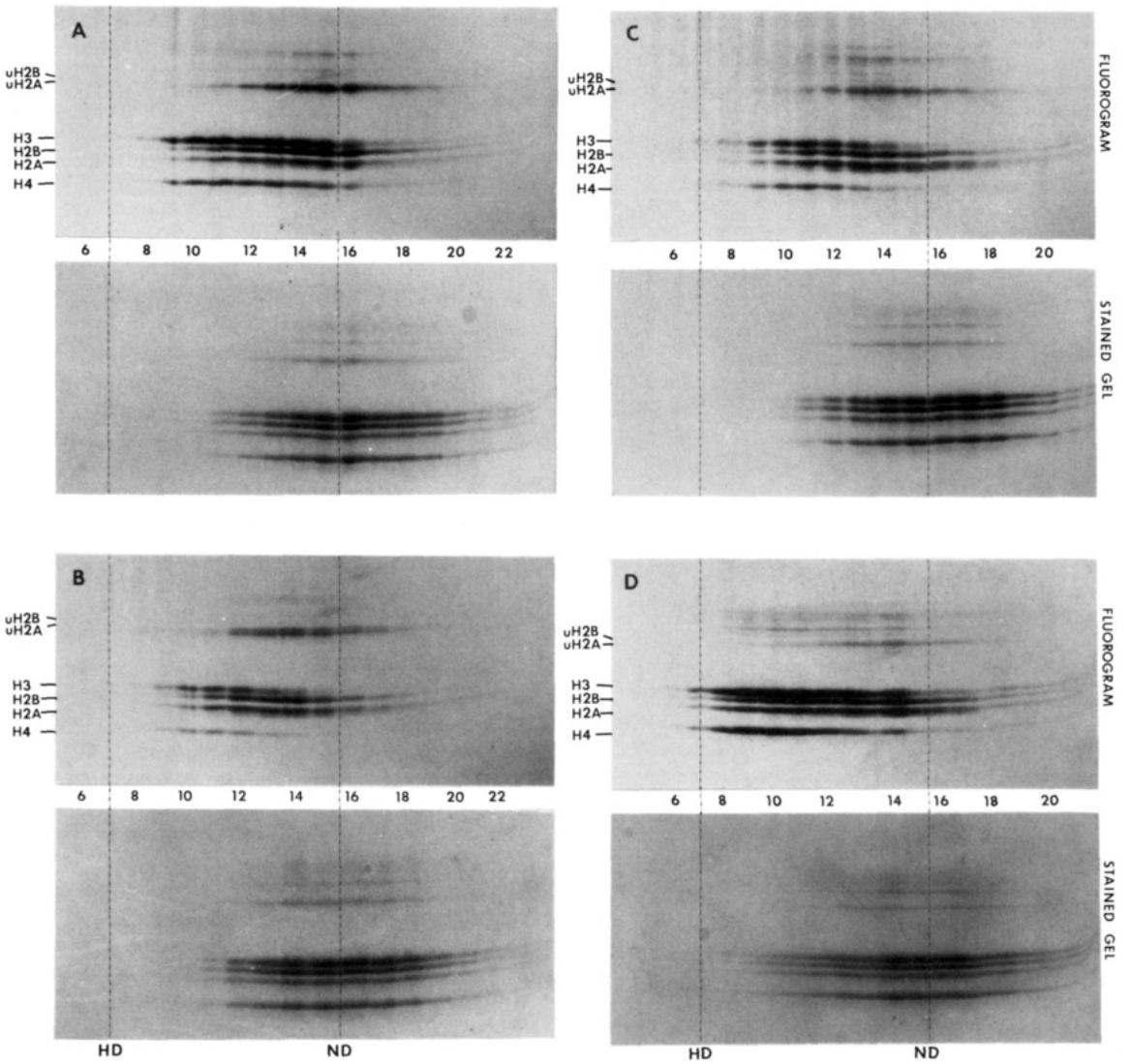

FIGURE 9: Density gradient fractionation of octameric complexes isolated from cells that have been density-labeled when DNA and/or RNA synthesis is inhibited. Cells were preincubated with the inhibitors for 10 min prior to an incubation of 30 min with ^{15}N-, ^{13}C-, and ^{2}H-labeled amino acids and [^{3}H]lysine and [^{3}H]arginine. Nuclei were isolated, an aliquot of which was directly acid-extracted to isolate monomer histones that served as density markers for the gradients (HD and ND). The remaining nuclei were treated with formaldehyde to form the cross-linked complexes which were then acid-extracted and centrifuged to equilibrium on CsFo–GuCl gradients along with the marker monomer histones within the same gradient. Following the protocol described in Figure 3, the reversed octameric complexes from the gradients were resolved on SDS–PAGE as shown in this figure. (B) Cells labeled for 30 min in the presence of cytosine arabinoside (–DNA, +RNA, pulse). (A) Same as (B) except an aliquot of the cells was resuspended in fresh normal medium and chased for 4 h in the presence of inhibitor (–DNA, +RNA, chase). (C) Cells labeled for 30 min in the presence of both cytosine arabinoside and actinomycin D (–DNA, –RNA). (D) Cells labeled for 30 min in the presence of actinomycin D (+DNA, –RNA). The lengths of exposures for fluorography were as follows: panel A and B, 20 days; panel C, 30 days; panel D, 10 days. The fluorography of (D) is overexposed with respect to the core histones so that the distribution of uH2A and uH2B can be observed. In this way the data of Figure 10 and Table I can be obtained.

transcription suggest that RNA polymerase movement facilitates the dissolution of the nucleosome.

The low level of exchange occurring independent of replication and transcription is rapidly saturated. This effect does not imply that there are a limited number of specific deposition sites in which this process occurs. If that were the case, the chase of Figure 8 would not have produced any further deposition. Rather, we interpret these data as indicating that deposition occurs throughout the genome and is only limited with respect to the number of exchange sites available at any given time. Thus, when excess histones are present in a nucleus, only a small percentage can deposit during a 10-min pulse (compare 10-min time points of parts C and D of Figure 5). As additional sites become available during subsequent chases, further deposition of the radiolabeled histones occurs, particularly for H3 and H4 (Figure 8). On the basis of the pulse–chase data of Figure 8, less than 5% of the new histones deposit in the absence of replication and transcription. Under normal conditions, this large excess of histones would have directly deposited on DNA of the replication fork or transcriptionally active genes. The data indicate that histones remain associated with the DNA in a nucleosome structure except for one of the following conditions: (1) during active transcription; (2) for the rare occasion when basal exchange occurs at that specific nucleosome; (3) when replication occurs on that nucleosome.

The data of this paper suggest that a "free" histone pool exists in the nucleus from which exchange with nucleosomal histones can occur primarily, but not exclusively, during replication and transcription. The term free does not imply lack of association with the chromatin, for if that were the situation, the nuclei which are prepared under hypotonic conditions would lack these histones. Rather, these histones weakly interact (electrostatic interaction) with chromatin, as

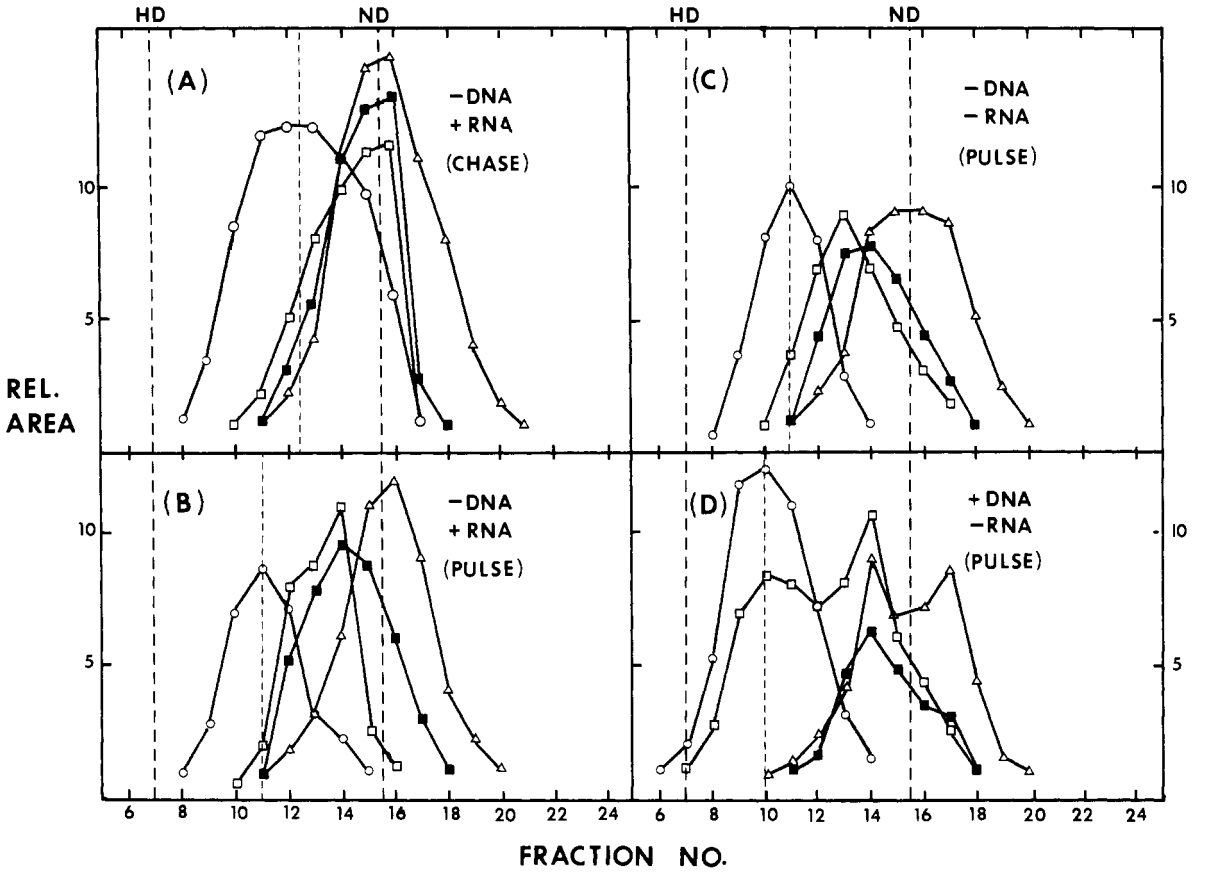

FIGURE 10: Quantitation of the histone distribution from the data of Figure 9. Because the distributions of labeled H3 and H4 are similar, the integrated values from the densitometry for both were quantitated together as a single point for each fraction (○). Similarly, the distributions of H2A and H2B (□) were quantitated together with uH2A and uH2B (■). The distribution of the normal-density histones is determined from all four histones in the stained gel (△). The average density for each set of histones was then determined (measured in fraction numbers) and used to quantitate the percentage of density-labeled histones present in the octameric complexes (see Table I).

reflected by extraction in 200 mM NaCl [data not shown; see also Louter and Chalkley (1984), Groppi and Coffino (1980), and Seale (1981b)]. Indeed, much of the past controversy regarding whether histones continue to be synthesized when DNA synthesis is inhibited or absent (G1 phase) can be explained either in the way nuclei were isolated or whether total cell extracts were analyzed. Such loose association may involve a transient formation of histone octamers prior to deposition (Voordoux & Eisenberg, 1978; Stein et al., 1979, 1985; Eisenberg & Felsenfeld, 1981). However, such complexes cannot be cross-linked as an octamer with formaldehyde unless within a nucleosome (Louter & Chalkley, 1984). As shown in Figure 9A, histone interactions within the H3,H4 tetramers and H2A,H2B dimers are not maintained when the histones are in this free pool for an extended time prior to deposition. This instability might be predicted on the basis of in vitro studies that show a similar instability at physiological ionic strength in the absence of DNA (D'Anna & Isenberg, 1974a,b; Eickbush & Moudrianakis, 1978). To maintain histone interactions within the tetramers and dimers, which is observed under normal conditions, the number of histones within the free pool would need to be kept to a minimum. In this way, the exchange reactions (steps 1–4 of Figure 1) would be sufficiently rapid to prevent the breakdown of these complexes. Thus, a particular histone complex, new or old, is only transiently present in this free pool of histones.

There is considerable evidence to indicate that histones are degraded in both proliferating and nonproliferating cells. The rate of turnover for the core histones follows the format of H2A $\approx$ H2B $\gg$ H3 > H4 (Djondjurov et al., 1983; Grove & Zweidler, 1984; Tsvetkov et al., 1989; Wunsch & Lough, 1989). The metabolic requirement of replacing general proteins which spontaneously denature due to usage and/or inherent instability does not exclude the histones. The low level of exchange that occurs in the absence of replication and transcription probably reflects this replacement process. This prompts the hypothesis that as a consequence of spontaneous denaturation and/or normal exchange during replication and transcription, the histones in the free pool are available for degradation. Those histones, especially H2A and H2B, which are more involved in exchange processes, may be more susceptible to spontaneous denaturation. As illustrated in Figure 8, the more rapid exchange of H2A and H2B relative to H3 and H4 may cause this increased vulnerability. Degradative pathways such as the energy-dependent pathway of ubiquitin conjugation may play an important role in this process (Hershko et al., 1982). The recent observation that multiubiquitin chains are found on specific lysines of short-lived proteins (Chau et al., 1989) is of interest, since similar chains are observed on histone H2A and H2B (Nickel et al., 1987, 1989). However, this conjugation cannot be limited to denatured histones because these modified histones are present in nucleosomes. Thus, ubiquitination must play an additional role in nuclear function.

Data of this paper and from other studies can be interpreted to indicate that ubiquitination occurs when the histones are in the free pool. According to the model of Figure 1, inhibition of DNA synthesis will produce excess histones in the nucleus. The data of Figure 6 show that as the concentration of free histones increases, the percentage of uH2A and uH2B increases. A similar effect has been observed in G1 phase cells when the basal level of histone synthesis produces an excess

of histones in the nucleus. For HTC cells this basal synthesis is 5% of that observed in S phase. As a result, the ratio of uH2A/H2A increases 4-fold compared to S-phase cells [Jackson & Chalkley, 1985a; see also Goldknopf et al. (1980) and Wu et al. (1981)]. Additional evidence comes from the study of metaphase cells. According to the model of Figure 1, any condition in which DNA, RNA, and histone synthesis is absent will virtually eliminate the presence of a free pool of histones, a condition that exists in metaphase cells. The total uH2A and uH2B content would be predicted to decrease substantially. This decrease may be very rapid if the uH2A and uH2B that are in nucleosomes continue to exchange. The data of Figure 9 suggest that such basal exchange does occur for uH2A and uH2B in the absence of replication or transcription. In this way the histones released from a nucleosome would be subjected to a new equilibrium of conjugation in which a lower percentage of H2A and H2B would contain ubiquitin. That conjugation and deconjugation is a rapid process has been reported (Wu et al., 1981; Seale, 1981). Redeposition of these unconjugated histones would result in a substantial decrease in the content of uH2A and uH2B in the chromosomes. Several investigators (Matsui et al., 1979; Wu et al., 1981; Mueller et al., 1985; Roboy et al., 1986) have reported that uH2A and uH2B are absent in metaphase chromatin. In the experiments of this paper in which both RNA and DNA syntheses are inhibited by drug treatment, a similar loss of uH2A and uH2B was not observed. This is because histone synthesis occurs temporarily, maintaining the free histone pool. Additional evidence has been provided from experiments in which RNA synthesis is inhibited and DNA synthesis continues. According to the model of Figure 1, a free pool of histones will be present which is primarily maintained by the synthesis of new histone, as transcription-dependent exchange (steps 3 and 4) is inhibited. Therefore, uH2A and uH2B would continue to be made and incorporated into nucleosome structure. Ericsson et al. (1986) have similarly observed by immunoanalysis that the content of uH2A within the nucleus does not change when RNA synthesis is inhibited by actinomycin D. These combined observations suggest that the presence of excess histones in the nucleus correlates with the observed level of uH2A and uH2B. Preliminary in vitro experiments in our laboratory also indicate that the conjugation process is substantially more efficient when the H2A and H2B exist in a free form rather than within nucleosomes (Haas and Jackson, unpublished results).

The studies of this paper do not address the nuclear location for the deposition of these conjugated histones. Previous studies have implicated deposition at transcriptionally active genes (Levinger & Varshavsky, 1982; Nickel et al., 1989). In other studies no such correlation was observed (Huang et al., 1986; Levinger, 1985). It has been suggested that conjugation–deconjugation occurs when histones are in nucleosome structure (Matsui et al., 1982) and functions in preventing mitotic condensation (Matsui et al., 1979) or the formation of higher ordered structure (Levinger & Varshavsky, 1982). Therefore, one of the functions of ubiquitination may be to produce a general relaxation or decondensation of nuclear structure. In this way, a low level of enrichment for these conjugates may be seen on active genes. An additional explanation may be that during replication and transcription or the general replacement of denatured histones a proportional quantity of ubiquitinated histones is incorporated into the dissolved nucleosomes. Those cell systems whose replication is repressed would be predicted to have a greater enrichment of uH2A and uH2B with the active genes. This potential type of incorporation prompts us to suggest a second role for ubiquitination. The conjugation of H2A and H2B when in the free pool argues for a dynamic exchange of these histones into nucleosomes and an involvement at the primary level of nucleosome structure. Specifically, if the incorporation of these conjugates decreases the stability of the nucleosome, a mechanism then exists in which on a random basis regions of the DNA can be opened with a greater frequency than what is produced when nucleosomes dissolve during replication, transcription, or general replacement of denatured histones. The purpose of the increased frequency is to provide a way to generate site-specific interactions with sequence-specific factors, which may potentiate the transcriptional activity of the DNA.

Acknowledgments

We thank Drs. Arthur Haas, John Lough, and Timothy Herman for critical comments and Connie Sawinski for secretarial service in preparation of the manuscript.

Registry No. RNA polymerase, 9014-24-8.

References

Annunziato, A. T., Schindler, R. K., Riggs, M. G., & Seale, R. L. (1982) *J. Biol. Chem. 257*, 8507–8515.

Bandyopadhyay, R., Stein, G., & Stein, J. (1987) *Biochemistry 26*, 2938–2944.

Baumbach, L. L., Marashi, F., Plumb, M., Stein, G., & Stein, J. (1984) *Biochemistry 23*, 1618–1625.

Bonner, W. M., Wu, R. S., Panusz, H. T., & Muneses, C. (1988) *Biochemistry 27*, 6542–6550.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., & Varshavsky, A. (1989) *Science 243*, 1576–1583.

Cox, R. (1976) *Cell 7*, 455–465.

D'Anna, J. A., & Isenberg, I. (1974a) *Biochemistry 13*, 2098–2104.

D'Anna, J. A., & Isenberg, I. (1974b) *Biochemistry 13*, 4992–4997.

Djondjurov, L. P., Yancheva, N. Y., & Ivanova, E. L. (1983) *Biochemistry 22*, 4095–4102.

Eickbush, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964.

Eisenberg, H., & Felsenfeld, G. (1981) *J. Mol. Biol. 150*, 537–555.

Ericsson, C., Goldknopf, I. L., & Daneholt, B. (1986) *Exp. Cell Res. 167*, 127–134.

Goldknopf, I. L., & Busch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 864–868.

Goldknopf, I. L., Taylor, C. W., Baum, R. M., Yeoman, L. C., Olson, M. O., Prestayko, A. W., & Busch, H. (1975) *J. Biol. Chem. 250*, 7182–7187.

Goldknopf, I. L., Sudhakar, S., Rosenbaum, F., & Busch, H. (1980) *Biochem. Biophys. Res. Commun. 95*, 1253–1260.

Groppi, V. E., & Coffino, P. (1980) *Cell 21*, 195–204.

Grove, G. W., & Zweidler, A. (1984) *Biochemistry 23*, 4436–4443.

Gurley, L. R., & Hardin, J. M. (1969) *Arch. Biochem. Biophys. 130*, 1–6.

Haas, A. L. (1988) in *Ubiquitin* (Rechsteiner, M., Ed.) pp 173–206, Plenum Publishing, New York.

Hershko, A., Eytan, E., Ciechanover, A., & Haas, A. L. (1982) *J. Biol. Chem. 257*, 13964–13970.

Huang, S. Y., Barnard, M. B., Xu, M., Matsui, S., Rose, S. M., & Garrard, W. T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3738–3742.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159–191.
Jackson, V. (1987) *Biochemistry 26*, 2315–2324.
Jackson, V. (1988) *Biochemistry 27*, 2109–2119.
Jackson, V., & Chalkley, R. (1981a) *J. Biol. Chem. 256*, 5095–5103.
Jackson, V., & Chalkley, R. (1981b) *Cell 23*, 121–134.
Jackson, V., & Chalkley, R. (1985a) *Biochemistry 24*, 6921–6930.
Jackson, V., & Chalkley, R. (1985b) *Biochemistry 24*, 6931–6938.
Jackson, V., Marshall, S., & Chalkley, R. (1981) *Nucleic Acids Res. 9*, 4563–4580.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Laskey, R. A., & Mills, A. D. (1979) *Eur. J. Biochem. 56*, 335–342.
Levinger, L. (1985) *J. Biol. Chem. 260*, 11799–11804.
Levinger, L., & Varshavsky, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3244–3248.
Levinger, L., & Varshavsky, A. (1982) *Cell 28*, 375–385.
Louters, L., & Chalkley, R. (1984) *Biochemistry 23*, 547–552.
Louters, L., & Chalkley, R. (1985) *Biochemistry 24*, 3080–3085.
Matsui, S., Seon, B. K., & Sandberg, A. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6386–6390.
Matsui, S., Sandberg, A. A., Negoro, S., Seon, B. K., & Goldstein, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1535–1539.
Meselson, M., & Stahl, F. W. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 671–682.
Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J., & Stein, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 981–985.
Mueller, R. D., Yasuda, H., Hatch, C. L., Bonner, W. M., & Bradbury, E. M. (1985) *J. Biol. Chem. 260*, 5147–5153.
Nickel, B. E., Roth, S. Y., Cook, R. G., Allis, C. D., & Davie, J. R. (1987) *Biochemistry 26*, 4417–4421.
Nickel, B. E., Allis, C. D., & Davie, J. R. (1989) *Biochemistry 28*, 958–963.
Raboy, B., Parag, H. A., & Kulka, R. G. (1986) *EMBO J. 5*, 863–869.
Russev, G., & Hancock, R. (1981) *Nucleic Acids Res. 9*, 4129–4137.
Seale, R. L. (1981a) *Nucleic Acids Res. 9*, 3151–3158.
Seale, R. L. (1981b) *Biochemistry 20*, 6432–6437.
Senshu, T., Fukuda, M., & Ohashi, M. (1978) *J. Biochem. (Tokyo) 84*, 985–988.
Senshu, T., Yomasu, K., & Ohsawa, T. (1985) *Eur. J. Biochem. 150*, 575–580.
Sheinin, R., & Lewis, D. N. (1980) *Somatic Cell Genet. 6*, 225–239.
Stein, A., Whitlock, J. P., Jr., & Bina, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 50000–50004.
Stein, A., Holley, K., Zeliff, J., & Townsend, T. (1985) *Biochemistry 24*, 1783–1790.
Tarnowka, M. A., Baglioni, C., & Basilico, C. (1978) *Cell 15*, 163–171.
Thorne, A. W., Sautiere, P., Briand, G., & Crane-Robinson, C. (1987) *EMBO J. 6*, 1005–1010.
Tsvetkov, S., Ivanova, E., & Djondjurov, L. (1989) *Exp. Cell Res. 180*, 94–105.
Voordoux, G., & Eisenberg, H. (1978) *Nature 273*, 446–448.
Waithe, W. I., Renaud, J., Nadeau, P., & Pallotta, D. (1983) *Biochemistry 22*, 1778–1783.
Wu, R. S., & Bonner, W. M. (1981) *Cell 27*, 321–330.
Wu, R. S., Kohn, K. W., & Bonner, W. M. (1981) *J. Biol. Chem. 256*, 5916–5920.
Wu, R. S., Tsai, S., & Bonner, W. M. (1982) *Cell 31*, 367–374.
Wunsch, A. M., & Lough, J. (1989) *J. Cell. Physiol.* (in press).
Yamasu, K., & Senshu, T. (1987) *J. Biochem. (Tokyo) 101*, 1041–1049.