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Articles

Exchange of Histones H1, H2A, and H2B in Vivo†

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ABSTRACT: We have asked whether histones synthesized in the absence of DNA synthesis can exchange into nucleosomal structures. DNA synthesis was inhibited by incubating hepatoma tissue culture cells in medium containing 5.0 mM hydroxyurea for 40 min. During the final 20 min, the cells were pulsed with [^{3}H]lysine to radiolabel the histones (all five histones are substantially labeled under these conditions). By two electrophoretic techniques, we demonstrate that histones H1, H2A, and H2B synthesized in the presence of hydroxyurea do not merely associate with the surface of the chromatin but instead exchange with preexisting histones so that for the latter two histones there is incorporation into nucleosome structures. On the other hand, H3 and H4 synthesized during this same time period appear to be only weakly bound, if at all, to chromatin. These two histones have been isolated from postnuclear washes and purified. Some possible implications of in vivo exchange are discussed.

The basic repeating unit of chromatin structure is the nucleosome. As a consequence of the structure of the nucleosome, it would seem to be unavoidable that the nucleosome must be displaced or modified in some manner to permit the passage of enzymes involved in transcription and replication. This modification may be reflected in some identifiable dynamic characteristic of nucleosomes. In this regard, recent experiments have demonstrated that several of the histones (H1, H2A, and H2B) are capable of exchanging in and out of nucleosome structures (Carine & Thomas, 1981; Cremsi & Yaniv, 1980; Louters & Chalkley, 1984). While these studies were done in vitro, there are also several indications that histones also exchange in vivo. First, data concerning the deposition of new histones indicate the likelihood of some exchange of at least histones H1, H2A, and H2B prior to deposition (Jackson, 1978; Jackson & Chalkley, 1981a,b; Jackson et al., 1981). Second, all histones turn over faster than DNA. Different types of histones show different turnover rates: H1 turns over more rapidly than the H2A/H2B pair which turns over faster than H3/H4 (Djondjarov et al., 1983; Commerford & Cronkite, 1982; Gurley et al., 1972). This implies a continual but differential histone replacement process. Finally, histones are synthesized throughout the cell cycle (albeit to a lower degree during non-S-phase periods), and these histones synthesized in the absence of replication have been shown to associate in some way with chromatin (Groppi & Coffino, 1980; Sheinin & Lewis, 1980; Tarnowka et al., 1978; Waithe et al., 1983; Wu & Bonner, 1981; Gurley & Hardin, 1969; Nadeau et al., 1978; Russev & Hancock, 1981; Russev et al., 1980; Wu et al., 1983).

The purpose of this study was to test directly whether histone exchange also occurs in vivo. To do this, histones were radiolabeled in the presence of an inhibitor of DNA synthesis (hydroxyurea). Thus, deposition of new histones can only be by exchange since previous studies have shown that in the presence of hydroxyurea, DNA synthesis is drastically reduced (greater than 95%) while histone synthesis continues at a substantial rate (20–40% of normal) for at least an hour (Nadeau et al., 1978; Russev & Hancock, 1981). We then isolated either nucleosomal fragments or cross-linked, internal core octamers (Louters & Chalkley, 1984) and showed that radiolabeled histones H1, H2A, and H2B are isolated with these nucleosomal structures. We conclude that histones do

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exchange in vivo, but, under the conditions investigated, the exchange is largely restricted to histones H1, H2A, and H2B.

Materials and Methods

Incorporation of [^{3}H]Thymidine in the Presence of 5.0 mM Hydroxyurea. HTC (hepatoma tissue culture) cells in mid-log-phase growth (400000 cells/mL) were exposed to 5.0 mM hydroxyurea. Prior to and at various times after the addition of hydroxyurea, 1.0-mL samples were removed, and 25 μCi of [^{3}H]thymidine was added. After 5 min, the pulse was stopped by adding the cells to 1.0 mL of cold 10% trichloroacetic acid (Cl_3CCOOH).[1] The precipitated material was filtered, and the filters were washed with 5% Cl_3CCOOH and 95% ethanol. The filters were dried, and the acid-precipitable radioactivity was determined by scintillation spectrometry.

[^{3}H]Lysine Labeling of Histones Synthesized in 5.0 mM Hydroxyurea. HTC cells (400000 cells/mL) were isolated and resuspended in Swims S-77 medium (lysine free) containing 5.0 mM hydroxyurea for 20 min. The cells then were again isolated, resuspended at 40000000 cells/mL in the same medium plus 0.1 mCi of [^{3}H]lysine/mL, and incubated for an additional 20 min. A portion of the cells was collected by centrifugation and frozen (zero-minute chase). A second portion of the cells was resuspended in radiolabel-free medium containing hydroxyurea, and the label was chased for the times indicated in the figure legends. After the chase, the cells were also collected and frozen.

Purification of H3 and H4 from the Postnuclear Extract. HTC cells were washed 4 times in buffer containing 10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.25 M sucrose, and 0.5% Triton X-100. These postnuclear washes were combined and adjusted to 0.4 N H_2SO_4. The insoluble material was removed by centrifugation at 20000*g* for 10 min and discarded. The soluble portion was dialyzed against 20 mM Tris, pH 8.0, resulting in massive precipitation of proteins. The precipitate was collected and extracted with 0.4 N H_2SO_4. The extract was dialyzed against 0.1 M sodium phosphate, pH 6.5, and adjusted to 5% guanidine hydrochloride. This extract was then loaded onto an IRC-50 ion-exchange column that had been equilibrated with 5% guanidine hydrochloride and 0.1 M sodium phosphate, pH 6.5. The column was washed with 5 volumes of the same buffer, and the histones were then removed from the column with 40% guanidine hydrochloride and 0.1 M sodium phosphate, pH 6.5. The fractions containing histones were combined and adjusted to 0.4 N HCl, and the histones were precipitated with Reinecke salt as previously described (Lindl & Brantmark, 1965). The precipitate was washed with acetone to remove the Reinecke salt, the pellet was dried, and the histones were resuspended in SDS sample buffer.

Exchange of Radiolabeled Histones with Nucleosomal Histones. The methods employed to determine if the radiolabeled histones synthesized in the presence of hydroxyurea had exchanged into nucleosomal structures have been described in detail elsewhere (Louters & Chalkley, 1984). Briefly, nuclei were isolated from [^{3}H]lysine-labeled cells and digested with micrococcal nuclease to about 2% acid solubility. The chromatin fragments were separated in the first dimension on a gel containing 4.5% acrylamide, 0.1% bis(acrylamide), 2.0 mM EDTA (ethylenediaminetetraacetic acid), and 10.0 mM MES, pH 6.5. The individual histones were then separated in the second dimension on a gel containing 18% acrylamide, 0.09% bis(acrylamide), 0.1% SDS, and 0.75 M Tris, pH 8.8. This gel was stained with Coomassie blue, destained, and prepared for fluorography by soaking the gel in 40% methanol for 20 min and then in 40% methanol and 1.0 M sodium salicylate for 1 h. The gel was dried and fluorographed.

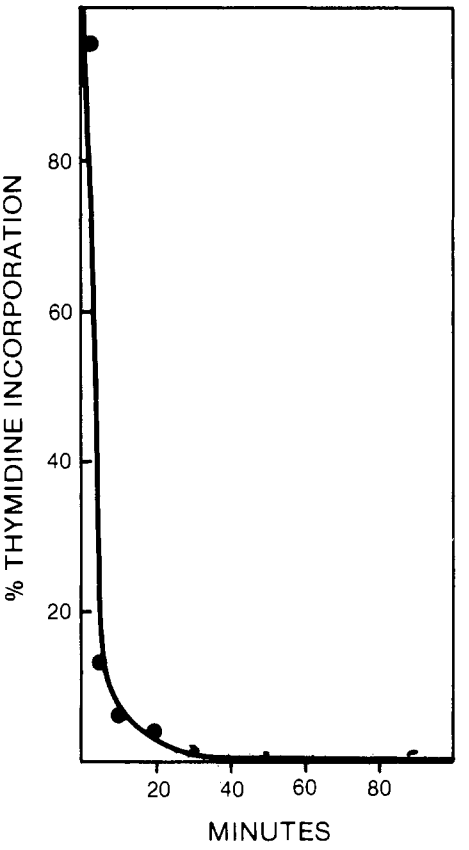

FIGURE 1: Effect of 5.0 mM hydroxyurea on DNA synthesis. HTC cells were adjusted to 5.0 mM hydroxyurea. An aliquot of the cells was removed after the times indicated and pulse-labeled with [^{3}H]-thymidine. The Cl_3CCOOH-precipitable radioactivity is expressed as a percentage of the radioactivity obtained from cells that had been pulse-labeled prior to the addition of hydroxyurea.

A second method to determine the identity of exchanging histones involved fixation of the micrococcal nuclease digested chromatin with 1% formaldehyde at pH 9.0. Under these conditions, nucleosomal histone octamers can be cross-linked together without covalent linkage to either exogenous histones or DNA. These octamers were extracted with acid and isolated on an SDS–acrylamide gel. The octamer band was cut out, the cross-links were reversed, and the resulting core histones were separated on a second 18% acrylamide–SDS gel. Fluorography was used to determine which of the radiolabeled histones synthesized during exposure to hydroxyurea had exchanged into the octamer.

Results

Effect of 5 mM Hydroxyurea on Replication. The study of the in vivo exchange of histones requires a system in which histones are synthesized but replication is halted. Therefore, we chose to inhibit replication with the DNA synthesis inhibitor, hydroxyurea. This provides us with an easily manipulated system and a sufficient quantity of material with which to perform the experiments.

The inhibitory effect of hydroxyurea on the incorporation of [^{3}H]thymidine into DNA of HTC cells is shown in Figure 1. Logarithmically growing cells were exposed to 5.0 mM hydroxyurea, and at various times, samples were removed and pulsed with [^{3}H]thymidine as outlined under Materials and

[1] Abbreviations: Cl_3CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Methods. The radioactivity of the acid-insoluble material was measured and expressed as a percentage of that obtained from cells pulse-labeled prior to the addition of hydroxyurea. As seen in Figure 1, the inhibitory effect of hydroxyurea is rapid. After 5 min, [^{3}H]thymidine incorporation occurs at only 13% of that seen in untreated cells, and after 20 min, [^{3}H]thymidine incorporation is less than 5%. Exposure of HTC cells to hydroxyurea for the time span of these experiments does not cause cell death (Nadeau et al., 1978).

Histone Synthesis in the Presence of 5.0 mM Hydroxyurea. That a significant measure of histone synthesis occurs in the absence of DNA synthesis was demonstrated by the following approach. HTC cells were treated with 5.0 mM hydroxyurea for 35 min to inhibit DNA synthesis. During the final 15 min of this incubation, the cells were pulsed with [^{3}H]lysine to label the histones. This experiment revealed that H1, H2A, and H2B are indeed synthesized in the presence of hydroxyurea and can be isolated from nuclei. However, in contrast, only small yields of radiolabeled H3 and H4 could be obtained from nuclei of hydroxyurea-treated cells (see Figure 2, panel A). Therefore, if we were to make any conclusions concerning failure of H3 and H4 to exchange, we needed to be sure that they were indeed being synthesized under these conditions.

Accordingly, we isolated histones from the nuclei of hydroxyurea-treated cells which had been cross-linked with formaldehyde before cell disruption to obviate washing losses of H3 and H4 from the nucleus. After nuclear isolation, the cross-links were reversed and histones isolated. The resulting stained gel and also its fluorogram are displayed in Figure 2, panel A. It can be seen that although very little radioactive H3 or H4 is isolated from the nuclei of unfixed cells, nonetheless a substantial amount of radioactive H3 and H4 is isolated from formaldehyde-fixed cells. This is displayed graphically in Figure 2, panel B, in which scans of the fluorogram through the core histone region are shown. It is clear from these data that all the histones are synthesized to about the same extent in the presence of hydroxyurea and are found in nuclei isolated from fixed cells but that very little newly synthesized H3 and H4 can be isolated from the nuclei or chromatin of unfixed cells. While H3 and H4 are clearly vigorously synthesized under these conditions, it is not clear at this time whether such H3 and H4 molecules are loosely associated with chromatin and lost during isolation or if they are not associated with chromatin at all.

Exchange of Histones H1, H2A, and H2B. Since essentially all of the H1, H2A, and H2B synthesized in the absence of DNA synthesis associate with material within the nucleus, we were interested in determining whether these histones had exchanged with bulk histones and were now located within nucleosomal structures. In the course of a previous study in which we demonstrated histone exchange in vitro (Louters & Chalkley, 1984), we developed two methods by which histones located in nucleosomal octamers could be separated from those histones simply bound to the surface of the chromatin. We utilized these same methods in the present study to ask if the histones H1, H2A, and H2B, which are synthesized during exposure to 5.0 mM hydroxyurea, can exchange with nucleosomal histones.

Nuclei were prepared from cells labeled briefly with [^{3}H]lysine in the presence of hydroxyurea. The nuclei were treated with micrococcal nuclease to generate nucleosome fragments. These fragments were separated in the first dimension in a 4.5% acrylamide gel, and the nucleosomal histones were separated in the second dimension on an SDS–acrylamide gel. The results of this separation are shown in

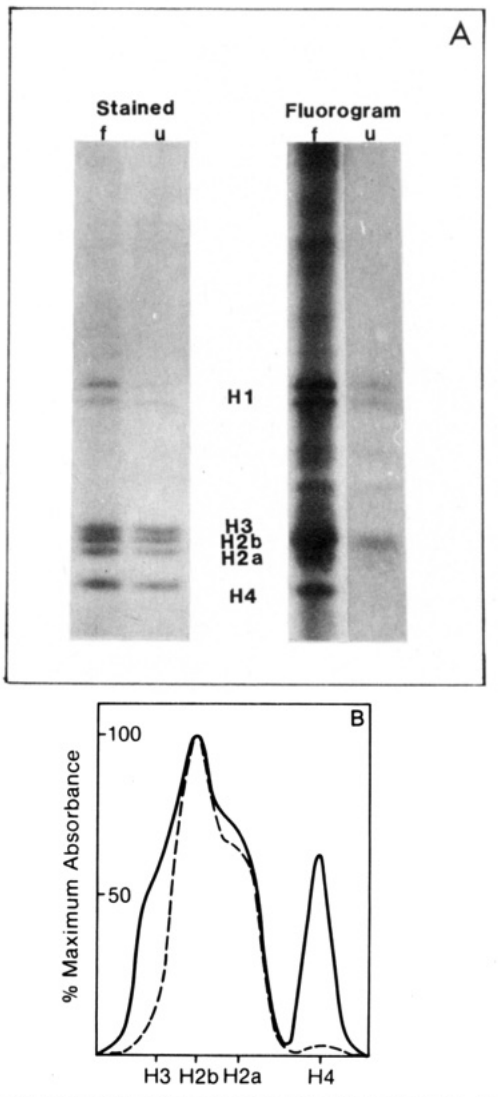

FIGURE 2: Synthesis of histones in the presence of hydroxyurea. HTC cells were exposed to 5.0 mM hydroxyurea for 20 min and pulsed with [^{3}H]lysine for 15 min in the continued presence of hydroxyurea, and a portion of the cells was cross-linked with formaldehyde. Histones isolated from the nuclei of fixed (f) (following reversal of the cross-links) and unfixed (u) cells are shown in panel A. The bulk histones are shown in the stained gel, and the newly synthesized histones are shown in the fluorogram. Panel B is a densitometric scan of both lanes of the fluorogram represented as a percentage of the maximum absorbance in that lane. The solid line represents the core histones from the nuclei of fixed cells, and the broken line represents the histones from the nuclei of unfixed cells.

Figure 3. The fluorogram reveals that radiolabeled H1, H2A, and H2B are isolated with oligonucleosomes. The association of H2A and H2B is especially clear for the core nucleosome region of the gel. Here the trivial explanation that the results are generated by nucleosomes binding extra histones cannot apply since core particles containing additional positive histones would not be expected to have the same electrophoretic mobility as core particles without extra histones. We conclude from these results that histones H1, H2A, and H2B have exchanged with preexisting histones and that H2A and H2B have exchanged into nucleosomes.

This conclusion was confirmed by a second method of analysis which involved the isolation of cross-linked octamers. In this method, chromatin was isolated from cells that had been radiolabeled as previously described. The chromatin was reacted with 1% formaldehyde at pH 9.0. Under these pH conditions, histones within nucleosome octamers are cross-linked together free of the encircling DNA. The cross-linked

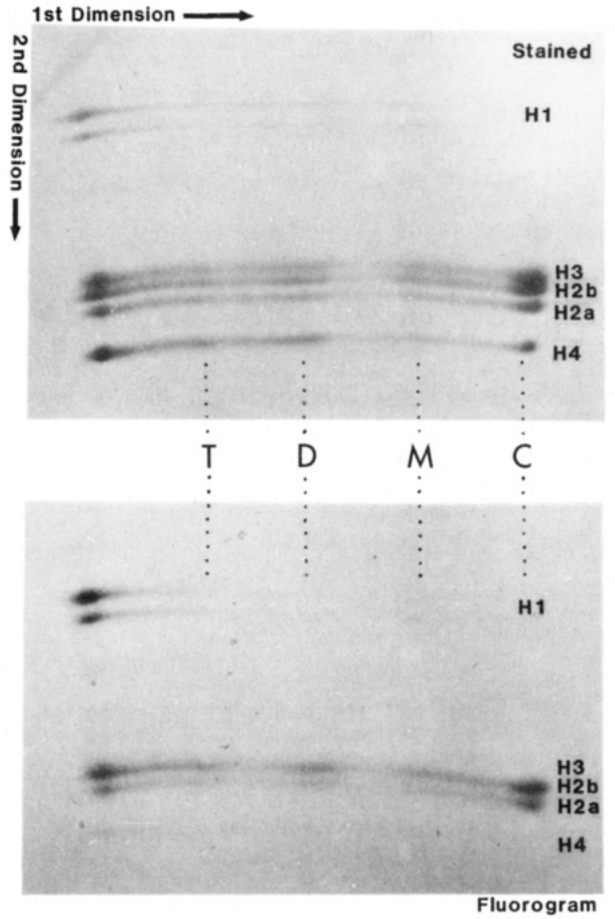

FIGURE 3: Exchange of histones synthesized in the presence of hydroxyurea into nucleosomal structures. Nuclei were isolated from cells labeled with [^{3}H]lysine in the presence of hydroxyurea, and the chromatin was digested with micrococcal nuclease. The nucleosomal fragments were then separated on a 4.5% acrylamide gel and the histones separated in the second dimension on an SDS–acrylamide gel. The stained two-dimensional gel and its fluorogram are shown. The various nucleosomal fragments are indicated as follows: C, core; M, mononucleosome; D, dinucleosome; T, trinucleosome. Their locations were more precisely determined from the more visible bands of the first-dimension gel.

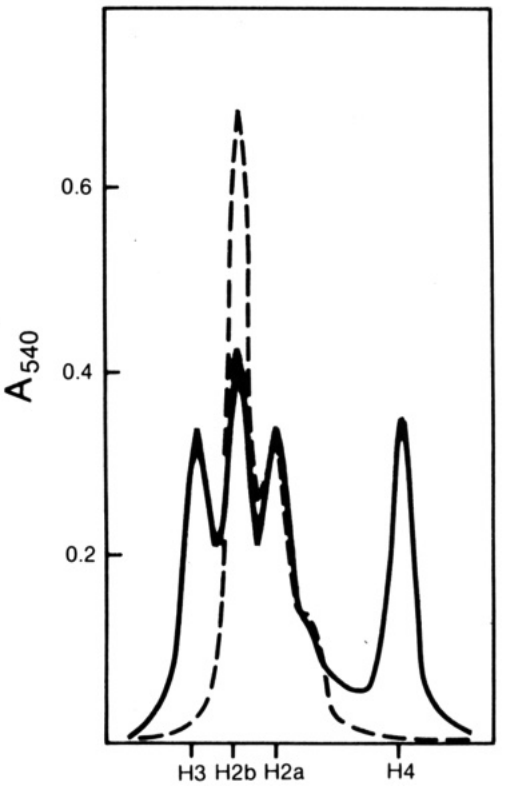

FIGURE 4: Isolation of histones from cross-linked octamers. Chromatin, labeled with [^{3}H]lysine in the presence of hydroxyurea, was fixed with formaldehyde at pH 9.0. The cross-linked octamers were extracted and isolated by electrophoresis. The octamer band was cut out, the cross-links were reversed, and the histones were separated on an SDS–acrylamide gel. The densitometric scan of this stained gel (solid line) and its fluorogram (broken line) are shown in this figure.

octamers were then extracted and isolated by electrophoresis. The cross-links of the octamer were reversed and the core histones separated on a second SDS–acrylamide gel. Fluorography of this gel was used to determine which of the radiolabeled core histones had exchanged with nucleosomal histones. The results of such an analysis are shown in Figure 4. The scan of the fluorogram reveals that only H2A and H2B contain radiolabel. Since we have previously demonstrated that surface-bound histones do not form extractable cross-linked octamers under the conditions employed (Louters & Chalkley, 1984), we conclude that radiolabeled octamers are nucleosomal in origin. This provides further strong support for the idea that H2A and H2B synthesized in the presence of hydroxyurea have exchanged into nucleosomes.

Isolation of H3 and H4 Newly Synthesized in the Presence of Hydroxyurea. The results presented thus far indicate that histones H3 and H4 are indeed synthesized in the presence of hydroxyurea and are quickly translocated into, or near to, the nucleus. However, these histones are only weakly bound (if at all) to the nuclear material. If this is a correct assessment, it should be possible to isolate newly synthesized H3 and H4 from the postnuclear washings of unfixed cells. There is a technical problem in isolating a relatively small amount of histone from the vast array of other proteins which are found in the cytoplasm; nonetheless, as outlined under Materials and Methods, this goal has been achieved. This is shown in the results presented in Figure 5. Initially, histones were added to the cytoplasmic material to test if they could be observed in the presence of the cytoplasmic protein by oblique illumination using the techniques of Franke and co-workers (Kleinschmidt & Franke, 1982). That this is correct is shown in panel A, lane 2, of Figure 5 in which we see the ability of the oblique illumination approach to specifically detect histones. H4 is evidently unusually sensitive in this detection system (lane 2). After the purification of histones from the cytoplasmic fraction of the treated cells, we obtain bands which migrate in Triton–acid–urea gels in the histone position and which also show up as histone upon oblique illumination as shown in Figure 5B, lanes 1 and 2. Additionally, two-dimensional analysis of these proteins indicates that they migrate identically with histones (data not shown).

Newly synthesized H3/H4 was studied in the postnuclear washes of HTC cells treated with 5.0 mM hydroxyurea for 20 min and labeled with [^{3}H]lysine for 15 min in the continued presence of the drug. The radiolabel was washed out and chased for either 5 min or 5 h in hydroxyurea. At the end of the chase periods, cells were harvested and nuclei prepared. Histones were purified from both the nuclei and the combined postnuclear supernatants (see Materials and Methods). The identity of the purified proteins as histones was confirmed by their location on two-dimensional acrylamide gels. A fluorogram of an SDS–acrylamide gel containing the purified histones is shown in Figure 6, panel A. The postnuclear supernatant after the 5-min chase (lanes 1 and 3) contains essentially all of the newly synthesized H3 and H4. (It should be noted that a portion of the radioactivity found in the H3

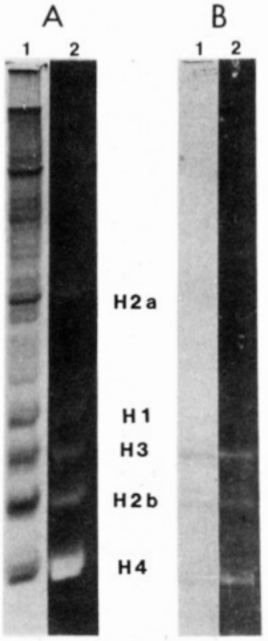

FIGURE 5: Identification of histones purified from postnuclear washes by light scattering. HTC cells were labeled with [^{3}H]lysine in the presence of hydroxyurea. Histones in the postnuclear washes from these cells were partially purified by acid extraction and loaded onto a column containing IRC-50 resin. The column was washed, and histones were eluted as before. Histones in the 40% guanidine hydrochloride eluant were obtained by Reinecke salt precipitation and separated on a Triton–acid–urea gel. Panel A shows a separation of histones that are supplemented with proteins of a postnuclear extract. Panel B shows the histones that are obtained from the 40% guanidine hydrochloride eluant (without supplemented histone). Column 1 is the stained gel illuminated with fluorescent light from below and photographed from the side at an angle of 30°, and column 2 is the stained gel obliquely illuminated.

region of the SDS–acrylamide gel is due to proteins other than H3 as assayed by two-dimensional gels.) This supernatant lacks significant quantities of the new H2A, H2B, and H1 which instead are isolated from the nucleus (lane 5). However, after the 5-h chase, less newly synthesized H3 and H4 is found in the nuclear washes (lane 4), and some is now found in the nucleus (lane 6). This latter point is more clearly seen in panel B where the amounts of radiolabeled histones in the nucleus after 5 min or 5 h are shown relative to histone H2B. The additional radiolabeled H3 and H4 that is found in the nucleus after 5 h most likely represents a measure of histone deposition onto the small amount of DNA which is synthesized during the extended chase period.

Discussion

This study demonstrates directly that some histones synthesized in the absence of DNA synthesis can become a part of the chromosomal nucleosome histone complement. We conclude from these observations that histones can exchange in vivo. This conclusion is consistent with several earlier observations. First, continued histone synthesis throughout the cell cycle combined with the observation that histones turn over faster than DNA implies that there is some sort of exchange process (Djondjurov et al., 1983; Wu & Bonner, 1981). Second, observations of the randomization of some histones prior to their deposition on chromatin of replicating cells supports the concept of histone exchange (Jackson & Chalkley, 1981a,b). Finally, in HTC cells and other cell lines, histones, which are synthesized at a significant rate in the presence of hydroxyurea, have been shown to associate with chromatin (Nadeau et al., 1978; Russev & Hancock, 1981; Wu et al., 1983). The data presented here expand these earlier observations. We show that the histones synthesized in the absence of DNA synthesis not only are associated and coisolated with chromatin structures but also have actually exchanged into the nucleosome structure (see Figures 4 and 5). We also demonstrate that, during the time period of these experiments, this exchange is largely limited to H1, H2A, and H2B.

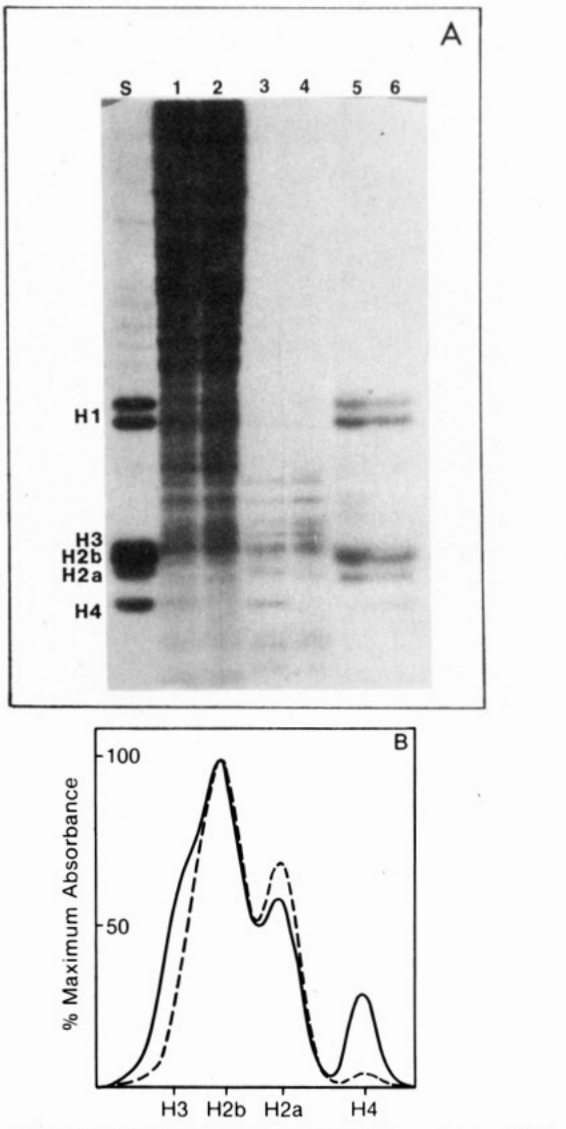

FIGURE 6: Isolation of H3 and H4 from postnuclear extracts. Cells pulse-labeled with [^{3}H]lysine as previously described were chased for either 5 min or 5 h in the continued presence of 5.0 mM hydroxyurea. Histones from both the cytoplasm and nucleus were purified and separated on an SDS–acrylamide gel. The fluorogram of that gel is shown in panel A. Lanes 1, 3, and 5 are proteins from cells that were chased for 5 min, and lanes 2, 4, and 6 are proteins from cells that are chased for 5 h. Lanes 1 and 2 are total postnuclear extracted protein, lanes 3 and 4 are histones purified from the postnuclear extract (see Materials and Methods), and lanes 5 and 6 are nuclear proteins. Panel B is the densiometric scan through the core histone region of lanes 5 (broken line) and 6 (solid line) expressed as a percent of the maximum absorbance found in that lane.

The result that H3 and H4 synthesized under these conditions do not exchange or exchange only to a very small degree is reflected in the observation that we can purify these histones from the postnuclear extracts even when cells are lysed very gently, indicating that these proteins are not bound very tightly, if at all, to chromatin (see Figure 3). However, we cannot be absolutely sure that such newly synthesized H3 and H4 were even located within the nucleus. The observation that they are isolated from a nuclear preparation from fixed cells is not entirely conclusive, since one could argue that H3 and

H4 are not transported into the nucleus but are in a nearby, albeit peripheral, pool and that they become cross-linked to nuclear material during the fixation process. Nonetheless, there is good reason to suspect that H3 and H4 are indeed located in the nucleus and are washed out during the isolation procedure. We base this on the following reasoning. First, it is well documented that there can be extensive loss of nuclear proteins during nuclear isolation (Paine et al., 1983). Second, it is clear from Figure 3 that hydroxyurea does not entirely prohibit the nuclear transport of H3 and H4 (lane 6). We realize that these data do not exclude the possibility of a hydroxyurea-induced decrease in the rate of H3/H4 transport. However, since there is currently no evidence to support this notion, a more plausible interpretation of the data is that H3 and H4 are transported to the nucleus where they can neither deposit due to the lack of new DNA nor exchange, and therefore are easily lost during nuclear isolation. These conclusions are consistent with observations made upon H3 and H4 immediately after synthesis in replicating cells in the absence of hydroxyurea (Jackson et al., 1981; Seale, 1981).

We can also infer from these data that H3 and H4 are bound to some other factor. Both from our own observations and from the observations of others, we know that naked exogenous histones bind chromatin very tightly (Eisenberg & Hardin, 1981; Stein, 1979; Voordouw & Eisenberg, 1978). Since the H3 and H4 synthesized in the presence of hydroxyurea are easily dissociated, it seems unavoidable that these histones must be bound to something which mutes the strong binding normally seen between histones and chromatin. Such an agent may serve to inhibit unwanted interactions with bulk chromatin, thus ensuring that H3 and H4 only become associated with newly synthesized DNA at the point of replication. The report of an acidic protein which binds H3 and H4 may be pertinent to this point (Kleinschmidt & Franke, 1982).

The results in this paper are consistent with other studies which have also reported a differential behavior between H3/H4 and H2A/H2B (Jackson & Chalkley, 1981a,b; Gurley et al., 1972). We do not know if the exchange of these histones is limited to certain structures or regions of the chromatin or if it is a general property of all chromatin. However, in view of recent data, it is likely that a certain portion of this exchange occurs on contiguous nucleosomes (Russev & Hancock, 1981). One attractive possibility is the idea that histones exchange into regions of transcriptionally active chromatin. However, it is clearly not a function of the transcriptional event itself inasmuch as exchange proceeds in the presence of actinomycin D (data not shown). This does not exclude the possibility that the whole transcription unit may have an altered nucleosome structure (Bloom & Anderson, 1982) and as such may permit exchange more efficiently. In this regard, it is of interest to note the recent report indicating that a part of the transcriptional chromatin lacks H2A and H2B (Baer & Rhodes, 1983). Perhaps this reflects a decreased binding and an increased ability to exchange. If exchange were indeed limited to specific zones in this manner, we could explain the apparent disagreement between ourselves and Leffak, who reported that the core histones synthesized in the absence of replication remain together as an octamer, indicating that there is no exchange (Leffak, 1984). He employs 60-min pulse periods, so that if exchange is restricted to a limited portion of the chromatin, that zone could approach a steady state of label incorporation over such a time period. As a consequence, the labeled H2A and H2B synthesized near the end of the pulse period would exchange with labeled H2A and H2B that had exchanged into that zone at the start of the pulse. Therefore, much of the H2A and H2B that is later deposited with labeled H3 and H4 would also be labeled even though these histones sets were not made at the same time.

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