Distribution of the core histones H_{2A}, H_{2B}, H₃ and H₄ during cell replication

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ABSTRACT

The distribution of newly synthesized core histones H_{2A}, H_{2B}, H₃ and H₄ relative to the DNA strand synthesized in the same generation has been examined in replicating Chinese Hamster ovary cells. Cells are grown for one generation in [¹⁴C]-lysine and thymidine, and then for one generation in [³H]-lysine and 5-bromodeoxyuridine (BrUdRib) and a further generation in unlabeled lysine and thymidine. This protocol produces equal amounts of unifilarly substituted and unsubstituted DNA. Monomer nucleosomes isolated from chromatin containing these two types of DNA can be distinguished by crosslinking with formaldehyde and banding to equilibrium in CsCl density gradients. The results indicate that the core histones are equally distributed between the two types of DNA. These findings are discussed in terms of current models for chromatin replication; they do not support any long term association of newly replicated histones with either the leading or lagging side of the replication fork.

INTRODUCTION

The manner in which parental chromosomal proteins are distributed to progeny chromatin during cell replication has been the subject of intensive investigation. Interest in this study stems from the hypothesis that much of the information for the control of gene activity is encoded into the structure of the chromatin via protein-DNA complexes. If this is true, then reproduction of these complexes during DNA replication may be the mechanism by which information for control of gene expression is transmitted from parent to progeny cells.

The core histones H_{2A}, H_{2B}, H₃ and H₄ are the primary proteins involved in the first level of packaging DNA. The histones maintain DNA in a condensed nucleosomal structure consisting of 145 base pairs of DNA coiled around two molecules each of the four histones (the histone octamer) (1). The nucleosome cores are separated by 40-60 bp. of DNA to give a "beads on a string" structure. Under physiological salt conditions this is further condensed into a continuously supercoiled structure. Whether this structure

is maintained during replication and transcription is not known, but there is considerable evidence that both transcriptionally active (2-7) and newly replicated chromatin (8-11) are more accessible than inactive, mature chromatin to attack by a variety of nucleases. The digestion products, however, are similar in size to those obtained from bulk unreplicated, inactive chromatin suggesting that the basic nucleosomal structure is present. Electron microscopic studies have demonstrated that the nucleosomal structure is present on both strands close to the replication fork; no intermediate is apparent (12). How this nucleosomal packaging is maintained and/or rapidly regenerated during replication and whether the histones, once deposited, remain permanently associated with that DNA are unanswered questions.

One currently popular model for assembly of nucleosomes proposes that the preexisting histone octamers cover the leading side (i.e., continuously replicated DNA strand) of the replication fork while newly synthesized histones complex with the lagging (discontinuously synthesized) side (13). A second hypothesis envisions newly synthesized histones segregating with the strand of DNA synthesized in the same generation (14). A third alternative is that histones are randomly distributed to both sides of the fork (15).

Evidence in favor of the conservative distribution to one side of the fork comes from studies of replication in the presence of protein synthesis inhibitors. When SV40 replicates in the presence of cycloheximide, newly synthesized DNA resistant to staphylococcal nuclease hybridizes to the template strand on the leading side of the replication fork (16). This is consistent with the idea that the parental histones preferentially segregate with the leading strand thereby protecting it from digestion. Furthermore, electron microscopic studies of MSB cell chromatin replicating in the presence of cycloheximide reveal replication forks with two beaded (i.e. nucleosomal) sides and one unbeaded side (presumably unpackaged DNA) (13). These results together with a two fold increase in nuclease sensitivity (17) have been interpreted as indicating that one side of the fork is not complexed with protein while the other side exists in the mature nucleosomal configuration. That this may not be the case is suggested by the observation that newly synthesized chromatin has a lighter density in CsCl indicating that it has a higher protein content than unreplicated chromatin (11,18).

Results indicative of a random distribution of histones have been obtained by following the fate of ^3H -lysine labeled material and density labeled DNA for several generations (15). On the other hand the observation

that dimethylsulfate preferentially crosslinks newly synthesized histones to the DNA synthesized at the same time suggests a semi-conservative mode of distribution (19). Our previous work (20) has demonstrated that about 10% of the radiolabeled proteins remain with the DNA strand synthesized in the same generation through two subsequent cell divisions. Since 40% of the label is in histones, clearly not all the histones behave this way, but the possibility remains that one or two classes may.

Early experiments designed to examine deposition of proteins at or near the replication fork found that pulse labeled proteins associated predominantly with unreplicated DNA (21,22,23) and were interpreted as favoring a random mode of deposition. More recent studies, however, have indicated that the situation is more complex. Newly synthesized histones H_3 and H_4 are found to appear first in chromatin fractions also containing newly synthesized DNA (11,24,25,26). There is some enrichment of newly synthesized H_{2A} and H_{2B} on newly replicated DNA but the major portion of labeled H_{2A} and H_{2B} is associated with unreplicated DNA (24,25). These experiments indicate that the H_3/H_4 and H_{2A}/H_{2B} pairs are not deposited stoichiometrically (24,25,27) as is required if nucleosomes associated with newly replicated DNA contain only new histones. The results contrast with those of Leffak et al. (28) who found using density labeled histones that a given octamer contains either old or new histones, not a mixture. The evidence in favor of a conservative mode of distribution comes largely from short term experiments which are designed to look at the replicating fork and generally employ protein synthesis inhibitors. Using similar experimental designs other investigators have obtained data suggesting nonstoichiometric deposition of the histone pairs. Experiments following labeled histones through more than one generation have yielded results interpreted as favoring both semi-conservative and random modes of distribution.

In view of the conflicting results described above, we have designed experiments specifically to follow two generations of histones through two subsequent cell divisions in order to determine whether histones remain associated with the DNA synthesized in the same generation. In order to do this, a method for separating monomer nucleosomes containing DNA substituted with 5-bromodeoxyuridine (BrUdRib) from monomers containing unsubstituted DNA has been developed. Using this method, we have followed the fate of labeled histones through several rounds of cell replication. The data provide no evidence for continued segregation of core histones with the DNA strand synthesized in the same generation. These results support the con-

clusion of Jackson and Chalkley (24) that if histones are non-randomly deposited on DNA, they do not remain associated with that DNA through subsequent replication.

EXPERIMENTAL PROCEDURES

Incorporation of Radioactive Tracers and BrUdRib.

Chinese Hamster ovary cells were cultured in 490 cm² roller bottles (Corning) in 50 ml F10 medium (29) containing 5% fetal calf serum (Gibco), 7.5 mM Hepes buffer (Calbiochem), 100 units/ml penicillin and 100 µg/ml streptomycin. The doubling time was 24 h. For labeling the DNA, cells were first cultured in the presence of ¹⁴C-thymidine (0.4 µC/ml) in F10 lacking thymidine for two days, then for two days in the presence of 10⁻⁵ M BrUdRib and 6 µC/ml ³H-BrUdRib and two further days in unlabeled F10. For labeling proteins, cells were cultured in F10 with one-half the usual concentration of lysine (F10-K/2) with 1 µC/ml ¹⁴C-lysine; this was followed by two days in F10-K/2 plus 10⁻⁵ M BrUdRib with 6 µC/ml ³H-lysine and two further days in normal F10. In some cases the order of labeling was reversed with the ³H-lysine being incorporated first followed by the ¹⁴C-lysine. For mixing experiments cells containing ³H labeled unifilarly substituted (HL) chromatin were prepared by growing for two days in the presence of [³H]-lysine (6 µC/ml) and 10⁻⁵ M BrUdRib in F10-K/2 lacking thymidine. Cells containing ¹⁴C labeled unsubstituted (LL) chromatin were prepared by growing for two days in F10-K/2 with 0.4 µC/ml [¹⁴C]-lysine.

Isolation of Nucleosome Monomers.

Cells were detached from the roller bottles by incubating for 5 min in 0.01M Tris HCl, 0.001M EDTA pH 7.8, 0.001M phenylmethylsulfonylfluoride (PMSF). The cell suspension was centrifuged for 5 min at 500 x g. All further operations were carried out at 0°C and all solutions were made 0.001M PMSF by a 1:100 dilution of 0.1M PMSF in ethanol. The cell pellet from each bottle was resuspended in 1 ml 0.08M NaCl, 0.02M EDTA, pH 8.0; 0.5 ml of 2% Triton X-100 in the same buffer was added and the suspension was vortexed for 30 sec. The nuclei were recovered by centrifuging through a discontinuous sucrose gradient consisting of 8 ml 1.7M sucrose in 0.01M Tris HCl, pH 7.3, 0.001M CaCl₂ and 1 ml 2.25M sucrose in the same buffer at 35,000 rpm in a Beckman SW41 rotor. The pellet was resuspended in 8 ml of 0.01M Tris HCl pH 7.3, 0.001M CaCl₂, 0.3M sucrose and centrifuged at 2000 rpm for 15 min. The nuclei were resuspended in the same buffer to a concentration of 100-150 µg DNA/ml. The nuclei were digested with 10 units

staphylococcal nuclease/100 μ g DNA for 45 min at 37°C (30). The digestion was stopped and the nuclei were lysed by addition of 1/10 volume of 0.1M EDTA, pH 8.0 and incubation on ice for 30 min. The digest was centrifuged through a 10-35% sucrose gradient in 0.01M triethanolamine HCl, 0.001M EDTA, pH 7.0 in a SW41 rotor at 33,000 rpm for 17 hours. Aliquots of each fraction were spotted on Whatman 3MM filter paper, precipitated with 20% trichloroacetic acid and counted in a toluene based scintillation fluid.

Crosslinking of Monomers.

Monomer nucleosomes were crosslinked with formaldehyde essentially as described by Jackson and Chalkley (31). The fractions of the sucrose gradient under the monomer peak were pooled; 10% formaldehyde neutralized with triethanolamine was added to a final concentration of 1% formaldehyde. The solution was allowed to stand overnight at 0°C and then dialyzed against 0.015M NaCl, 0.0015M sodium citrate, pH 7.0. Control experiments using monomers containing [3 H]-lysine labeled proteins and [14 C]-thymidine labeled DNA showed that after crosslinking under these conditions the two labels cosedimented in CsCl-guanidine HCl gradients (31) at a density of about 1.42 g/cc; when monomers were centrifuged before crosslinking, the DNA went to the bottom of the gradient and free protein remained at the top.

Density Gradient Centrifugation of Monomers.

Enough solid cesium salt was added to the fixed dialyzed monomers to bring the final solution to the appropriate starting density: 1.40 g/cc for CsHCO₃, 1.35 g/cc for Cs₂SO₄ and 1.44 g/cc for CsCl. CsCl and CsHCO₃ gradients were centrifuged in a Ti65 rotor at 42,000 rpm for 60 hr; Cs₂SO₄ gradients were centrifuged at 35,000 rpm for 60 hr. After centrifugation 0.2 ml fractions were collected; the density of each fraction was determined from the refractive index and 0.1 ml aliquots were counted on Whatman 3MM paper after precipitation with trichloroacetic acid as described above.

Polyacrylamide Gel Electrophoresis.

DNA was electrophoresed in 15 cm slab gels of 5% polyacrylamide using the Tris-borate-EDTA buffer system of Peacock and Dingman (32). The DNA was freed of protein by digesting chromatin fractions with 100 μ g/ml pronase in 1% NaDodSO₄ at 37° for 1 hr. Hha I restriction endonuclease fragments of ϕ X174 replication form (a kind gift of Dr. Clyde Hutchinson) were used for size calibration. The gels were stained with ethidium bromide.

Proteins were electrophoresed in 20 cm slab gels of 15% polyacrylamide using the modification of the Laemmli (33) system described by Weintraub et al. (34). Gels were fixed overnight in 50% methanol, 10% acetic acid,

stained with 1% Coomassie blue in the same solution and destained in 5% methanol, 10% acetic acid. For greater sensitivity, the silver stain technique of Oakley et al. (35) was applied after destaining the Coomassie blue. For autoradiography, gels were infiltrated with 2,5-diphenyloxazole as described by Laskey and Mills (36), dried and autoradiographed using Kodak X-Omat R film.

RESULTS

Density Gradient Sedimentation of Crosslinked Monomers Labeled in the DNA

In order to study the distribution of histones, we followed the proteins by labeling with radioactive lysine and the DNA by density labeling with BrUdRib. We chose to examine purified monomer nucleosomes because these should contain only core histones and DNA. We have therefore developed a method for separating density labeled and unlabeled monomers.

Monomer nucleosomes were prepared by staphylococcal nuclease digestion of nuclei from Chinese Hamster ovary cells and isolated by sucrose gradient centrifugation. The monomer nucleosome fraction of the sucrose gradient contained predominantly DNA fragments of about 155 bp associated with core histone (H_{2A} , H_{2B} , H_3 and H_4). SDS gel electrophoresis revealed little histone H_1 or nonhistone protein in this fraction; monomers recovered from CsCl gradients were devoid of all proteins except the core histones.

The sedimentation properties of crosslinked monomer nucleosomes were examined in Cs_2SO_4 , CsCl and $CsHCOO$. Monomers were prepared from cells grown so that they contained DNA bifilarly substituted with BrUdRib (HH DNA) labeled with [3H]-BrUdRib and unifilarly substituted (HL) DNA labeled with [^{14}C]-thymidine; other preparations contained HL chromatin labeled with [3H]-BrUdRib and unsubstituted (LL) DNA labeled with [^{14}C]-thymidine. The positions of the labels were confirmed by CsCl density centrifugation of purified DNA. After crosslinking with formaldehyde, the nucleosomes were centrifuged to equilibrium in the various Cs salts. When conditions of time and rotor speed were optimized for each salt, similar results were obtained with each. As shown in Table 1, the presence of BrUdRib in one strand of the DNA increases the density of monomers by about 0.02 g/cc. Some variation ($\pm$ 0.03/cc) in the absolute densities has been noted with different monomer preparations but the degree of separation of the two species is the same.

Figure 1 shows a typical separation in CsCl of LL monomers labeled with [^{14}C]-thymidine and HL monomers labeled with [3H]-BrUdRib. The 3H -HL monomers band at a density of $1.443 \pm$ g/cc while the ^{14}C -LL monomers band at

Table 1

Densities of Crosslinked Monomer Nucleosomes in Cs Salts

DNA	LL	HL	HH
CsCl	1.424	1.443	1.459
Cs ₂ SO ₄	1.326	1.347	1.363
CsHCOO	1.386	1.407	1.420

Figure 1. CsCl density gradient centrifugation of monomer nucleosomes radioactively labeled in the DNA. CHO cells were grown for one generation in [¹⁴C]-thymidine followed by one generation in [³H]-BrUdRib followed by one generation in unlabeled thymidine. Monomer nucleosomes were crosslinked with 2% HCHO and centrifuged to equilibrium in CsCl as described in Methods. The densities were determined from the refractive indexes. ● ³H cpm; ○ ¹⁴C cpm. ---calculated curve of normalized sum of ³H and ¹⁴C.

1.424 g/cc. This difference in density is reflected in the decrease of the $^3\text{H}/^{14}\text{C}$ ratio with decreasing density. This experiment with material labeled in the DNA moiety demonstrates that the difference in density of HL and LL monomer nucleosomes can be observed.

Absence of Detectable Exchange of Proteins

In order to ensure that exchange of proteins does not occur during our procedures, a mixing experiment was performed. Cells containing [^3H]-lysine labeled proteins and HL DNA were mixed with cells containing [^{14}C]-lysine labeled proteins and LL DNA. Monomers were prepared, crosslinked and centrifuged to equilibrium in CsCl. As shown in Figure 2a, there is a clear difference in the densities of the two species. The ^3H (HL) monomers sediment in a band centered at a density of around 1.45 g/cc while ^{14}C (LL) monomers sediment around 1.439/cc. The pattern obtained when the monomers are first isolated and crosslinked before the different types are mixed (Fig. 2b) is identical to that seen when cells are mixed initially. This demonstrates that no significant exchange of proteins from one type of chromatin to the other is occurring during the experimental manipulations. Thus exchange of proteins cannot account for the results presented below.

Distribution of Core Histones

In order to examine the distribution of core histones, an experiment was performed in which the radiolabels were introduced into the protein components of the monomers and the DNA was density labeled as in Figure 1. In this experiment if newly synthesized core histones remain with the DNA made concomitantly for more than one generation, the distribution of ^3H and ^{14}C should be identical to that obtained with DNA labeled monomers (the filled and open circles in Figure 1). If core histones are randomly deposited on the DNA or do not remain nonrandomly associated with the DNA, the ^3H and ^{14}C curves should be superimposable. The shape of each curve should be that described by addition of the normalized ^3H and ^{14}C curves obtained with DNA labeled material; the curve predicted for this outcome is given by the dotted line in Figure 1.

To execute this experiment, Chinese hamster ovary cells in culture were first radiolabeled with ^{14}C -lysine for one generation followed by ^3H -lysine for a second generation. During this second generation, BrUdRib was added to density label the DNA strand synthesized concomitantly with the ^3H proteins. The cells were then grown for a third generation in unlabeled amino acids and thymidine. This protocol produces cells in which half of the DNA has a density of 1.487 g/cc (that of fully substituted HL DNA) and the rest

Figure 2. CsCl density gradient centrifugation of reconstituted mixtures of lysine-labeled monomer nucleosomes. A. Cells containing [^3H]-lysine labeled proteins and HL DNA were mixed with cells containing [^{14}C]-lysine labeled proteins and LL DNA. Monomers were isolated crosslinked and centrifuged to equilibrium in CsCl. B. [^3H]-lysine labeled HL monomers were crosslinked and mixed with crosslinked [^{14}C]-lysine LL monomers. The mixture was centrifuged to equilibrium in CsCl. —●— ^3H cpm; —○— ^{14}C cpm.

has a density of 1.705 g/cc (that of unsubstituted LL DNA). Thus the DNA has replicated one time in the presence of BrUdRib and one subsequent time in thymidine. Control experiments reported previously (20) have established that radioactive label added as lysine is not incorporated into DNA.

Monomers isolated from these protein labeled cells were crosslinked and sedimented in CsCl gradients. The distribution of labeled material in this gradient is shown in Figure 3. The ^3H and ^{14}C peaks are superimposable

Figure 3. CsCl density gradient centrifugation of monomer nucleosomes labeled in the histones. CHO cells were grown for one generation in [^{14}C]-lysine followed by one generation in BrUdRib and [^3H]-lysine followed by one generation in unlabeled amino acids and thymidine. Monomer nucleosomes were crosslinked with 2% HCHO and centrifuged to equilibrium in CsCl as described in Methods. --●-- ^3H cpm; --○-- ^{14}C cpm.

and the $^3\text{H}/^{14}\text{C}$ ratio does not change along the gradient. This contrasts with the results obtained with material labeled in the DNA (Figure 1) which shows that HL and LL monomers are being separated. The coincidence of the ^3H and ^{14}C labeled proteins, but not the HL and LL DNAs indicates that both old and new proteins are associated with both types of DNA. Since the only proteins which remain in this preparation of monomers are core histones, this experiment demonstrates that core histones do not remain associated with the DNA strand synthesized in the same cell generation. Rather, each daughter helix has both parental and newly synthesized histone complexed with it.

DISCUSSION

These experiments were designed to determine whether core histone segregate. Figure 4 shows the expected distribution of histones under the conservative and semiconservative models. When cells are labeled according to our protocol, both models predict that ^{14}C histones will be associated with LL DNA while ^3H histones are associated with HL DNA. The experimental data presented here are not in agreement with the predictions of either of these

Figure 4. Schematic representation of predictions of two models for protein distribution. Solid lines represent DNA containing thymidine; dotted lines represent BrUdRib substituted DNA; C and H refer to ^{14}C -lysine and ^3H -lysine labeled proteins.

models. When examined after two rounds of replication, each daughter DNA molecule is found to be equally complexed with histones synthesized in both generations, as expected under the random distribution model.

The data appear to be in direct conflict with those presented by Russev and Tsanev (19) which showed that newly synthesized histones preferentially crosslink to the newly synthesized strand of DNA. In their experiments the Ehrlich ascites cells were grown in mice in the presence of DNA density label for only one round of DNA replication; thus all the chromatin should have contained one newly synthesized (density labeled) DNA strand and one old (unlabeled) strand. The current concept of nucleosome structure makes it difficult to envision how newly synthesized histones could be preferentially crosslinked to the heavy strand of the DNA double helix while "old" histones are crosslinked to the light strand. An alternative explanation for their data is that some of the chromatin containing "old" histones did not replicate in the presence of density label. If this were the case, their preparation would contain two types of chromatin, one with density labeled DNA and "new" histones and a second with light DNA and "old" histones. Crosslinking of these two types of chromatin would produce the observed results.

Our experiments do not examine deposition of histones at the replicating fork, but rather ask whether asymmetrical distribution, if present, is maintained through several rounds of replication. Accordingly, the data do not rule out models of nonrandom deposition of histones at the replication fork, but they do indicate that if such an asymmetrical distribution occurs, the association of the histones with the DNA is not maintained through subsequent replication events. In this regard our results confirm those recently reported by Jackson and Chalkley (24).

Our results and those of Jackson and Chalkley (24) provide no support for the conservative distribution model; however, they can not definitively rule it out, since this would require that the origins of replication used during the second cell cycle be precisely those used during the first. There is good evidence that small eukaryotic DNAs have unique origins of replication (37-41) and a recent indication that the same may be true for active genes (16). That unique origins may not be required for all eukaryotic DNA replication is suggested by the observation that a variety of sequences will replicate after microinjection into unfertilized *Xenopus* eggs (42); replication in this system is independent of the presence of a viral origin. In addition, synthesis of RNA for Okazaki fragments appears to

lack sequence specificity (43). Under our labeling protocol, initiation of replication at different origins during the two cell cycles would randomize nucleosomes which had been deposited nonrandomly. This, in fact, is what Jackson and Chalkley (24) have observed. They noted that histones H_3 and H_4 deposit specifically on newly synthesized DNA while H_{2A} and H_{2B} are slightly enriched on new DNA. These histones remain associated with the same DNA for the remainder of the cell cycle but are redistributed during the second round of replication producing a random pattern. Both our experiments and those of Jackson and Chalkley (24) used density labels incorporated into the DNA. The effect of these labels on normal patterns of replication is not clear; the possibility remains that the observed randomization of label reflects a change in origins perhaps caused by the density label. Comparison of the results of several methods for examining deposition at the replicating fork, however, indicates that this process is not affected by the use of density labels. The picture which emerges from all these experiments is that under normal circumstances newly synthesized H_3 and H_4 are deposited predominantly on newly synthesized DNA, while H_{2A} and H_{2B} are deposited on both newly synthesized and unreplicated DNA. During subsequent rounds of replication, the histones redistribute so that the distribution becomes random. In the presence of cycloheximide, this redistribution may not occur, thus producing the results observed by Seidman et al. (16).

Our earlier work using crude chromatin containing large amounts of non-histone proteins (a non-histone protein:DNA ratio of 1.6:1) indicated that a small percentage of chromosomal proteins do remain with the DNA synthesized at the same time through subsequent generations. Since the core histones clearly do not behave this way, we conclude that the segregating proteins are most probably non-histone proteins. The identification of these proteins and their relationship to active chromatin is presently being pursued.

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