Fate of newly synthesized histones in G_1 and G_0 cells

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We have shown that quiescent cells as well as those in the G_1 phase of the cell cycle synthesize histones at a reduced but significant rate. Now, we show that the histones synthesized during G_0 and G_1 are stably incorporated into nuclei soon after synthesis. Micrococcal nuclease digestion of nuclei isolated from cells in G_0 and G_1 revealed that the specific histone variants synthesized in these different physiological states are found associated with DNA as nucleosomes. Nucleosomes were separated by polyacrylamide gel electrophoresis in a reducing buffer so that histone spot morphology, particularly that of the H3s was improved.

Histone *Chromatin* *Nucleosome gel* *Cell cycle*

1. INTRODUCTION

A variety of cells synthesize a significant amount of histone during the quiescent (G_0) state and during the G_1/G_2 phases of the cell cycle [1–3]. The synthesis pattern of histone variants during these two physiological states are different from each other and from that found during the S phase of the cell cycle. Since little if any DNA synthesis occurs during G_1/G_2 and G_0, the fate of histones synthesized in these states may or may not be the same as that of histones synthesized in S phase. In G_1 cells, we showed that the histones synthesized during G_1 rapidly accumulated in nuclei and were stable there for at least several cell cycles [1]. In G_0 cells, we showed that the histones synthesized during a pulse in G_0 were stable for at least several days afterwards [2].

Here, we report that histones synthesized during G_1 and G_0 are found as mononucleosomes soon after their synthesis.

2. MATERIALS AND METHODS

2.1. *Cell culture methods*

Chinese hamster ovary (CHO) cells in exponential growth, G_1 and G_0 were obtained as in [2].

2.2. *Nuclei isolation*

CHO cells ($1–2 \times 10^7$) were washed twice with 2 ml 1 mM $MgCl_2$–10 mM Tris (pH 8)–1% mercaptoethanol–1 mM PMSF. They were then resuspended in 2 ml of the same buffer containing 0.3% Triton N-101 and allowed to sit on ice for 15 min. G_1 cells usually lysed under these conditions. G_0 cells required homogenization (10 strokes) in a tight fitting glass dounce. The nuclei were pelleted at 2000 rev./min in a Beckman J6 centrifuge, resuspended in 50% glycerol–1 mM $MgCl_2$–10 mM Tris (pH 8)–1% mercaptoethanol–1 mM PMSF, and stored at −20°C.

2.3. *Nuclease digestion*

Nuclei were washed with 1 mM $MgCl_2$–10 mM Tris (pH 8)–1% mercaptoethanol–1 mM PMSF and resuspended in the same buffer at 10^8 nuclei/ml. Per 100 μl nuclei, 3 μl micrococcal nuclease (0.1 mg/ml) and 1 μl 100 mM $CaCl_2$ was added in that order. The mixture was incubated at 37°C for various times (5–30 min). Enzyme digestion was stopped by adding 6.25 μl 0.2 M EGTA (pH 8) and 3.2 μl 0.8 M EDTA (pH 8) per 25 μl digest. Finally, an equal volume of glycerol was added and the mixture was stored at −20°C.

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2.4. *Nucleoprotein gels*

During early experiments with nucleosome analysis, we found that the H3 was commonly depleted relative to the other histones, presumably due to the oxidation of the cysteines present in H3. This oxidation most likely occurred during electrophoresis in the nucleoprotein gel which lacked reducing agents.

The nucleosome gel recipe [4,5] was modified to include a reducing buffer. Since thiols inhibit polymerization, the gel was polymerized with a Tris–acetate buffer. Then the acetic acid was substituted with thioglycollic acid in the reservoir buffer and the gel pre-electrophoresed for several hours. Thus the nucleoprotein complexes migrated through the gel in the presence of about 3 mM thioglycollate. The gel contained 4.5% acrylamide and 0.15% methylene bis-acrylamide. The buffer in the gel was 10 mM Tris–acetate (pH 8.2), 1 mM EDTA (pH 7) and 0.5 mM EGTA (pH 7). The gel was polymerized with 3 mM TEMED and 4 mM ammonium persulfate.

The reservoir buffer contained 10 mM Tris base, 3.6 mM thioglycollic acid, 1 mM EDTA (pH 7) and 0.5 mM EGTA (pH 7). The gel was pre-electrophoresed at 300 V for about 2 h; bromophenol blue migrated about 10 cm during this time. The reservoir buffer was changed and the nucleoprotein samples added. The gel was electrophoresed overnight at 50 V.

The gels were stained with ethidium bromide diluted into reservoir buffer. Bands were cut from the gel on the UV light box and soaked in 50 mM sodium thioglycollate for 1 h.

The bands were then loaded onto a second dimension acetic acid–urea–Triton X-100 (AUT) gel and embedded with 1% agarose (Sigma type I) containing 1 M acetic acid and 50 mM NH_3. The pieces of nucleoprotein gels were overlaid with the above 1% agarose solution containing 0.2% protamine [6]. The AUT gel was run as in [7]. The appropriate samples were excised from the AUT gel and electrophoresed on acetic acid–urea–cTAB (AUC) as in [7].

3. RESULTS

3.1. *G_1 period*

Cells in G_1 synthesize a reduced but significant amount of histone [1]. This histone synthesis has a

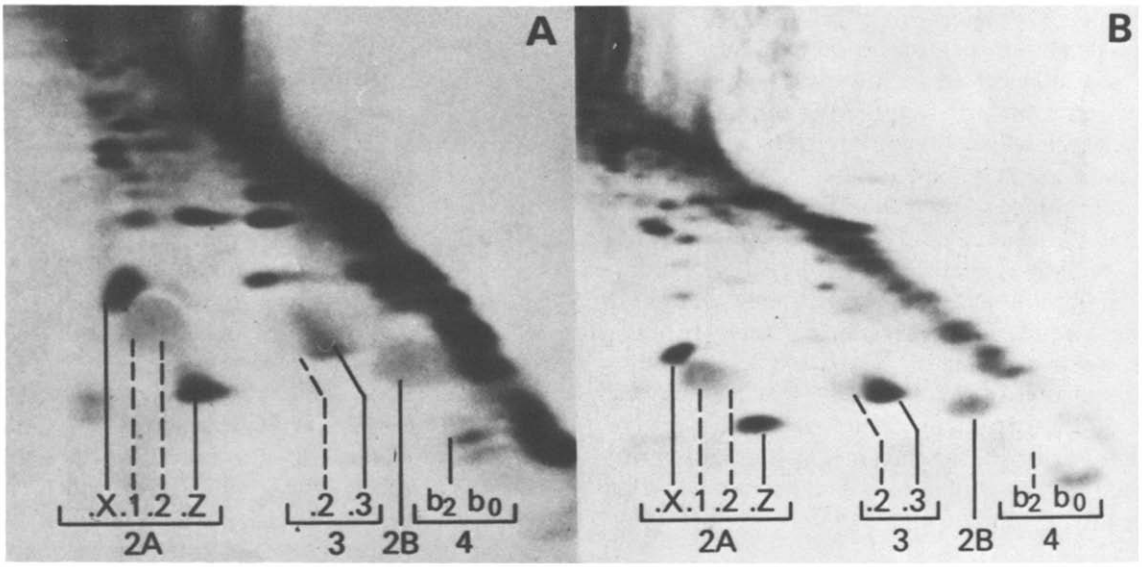

Fig.1. Accumulation of newly synthesized histones in nuclei of G_1 phase CHO cells. CHO cells were labeled for 1 h in arginine-free RPMI 1640 containing 10% fetal calf serum and 10 μCi/ml of [^{14}C]arginine. At the end of the labeling period, one flask of cells was processed and histones extracted from the nuclear pellet as in section 2. The other flask of cells was chased for 1 h in RPMI 1640 containing 10% fetal calf serum and 10 times the normal concentration of arginine. Histones were extracted from the nuclear pellet. The nuclear acid extracts were analyzed on two-dimensional gels as in section 2: (A) pulse; (B) chase.

variant pattern which distinguishes it from that found in S phase. Fig.1 shows the two-dimensional gel analysis of newly synthesized histones found in nuclei of CHO cells in G_1 phase of the cell cycle. The histones synthesized during a 1 h labeling period (fig.1A) are found in the nuclei of these cells; little if any radioactive histones are found in the cytoplasmic fraction of G_1 cells (not shown). The pattern of histone variant synthesis is similar to that in [1]. The small amounts of labeled H2A.1/.2 and H3.2 found in these samples can be attributed to contamination by S phase cells [1].

The modification pattern of the newly synthesized H4 found during the pulse is different from that seen in the mass pattern; b_2H4 dominates the former while b_0H4 dominates the latter [1]. During the chase period most of the dimodified (b_2) form of H4 is processed and converted to the unmodified (b_0) form (fig.1B) by removing the phosphate and acetate groups. As shown in [8], newly synthesized H4 is found in the nucleus as the dimodified form and converted to the unmodified (b_0) form (fig.1B) in dividing cells. In dividing cells, however, b_2H4 dominates the pattern only in very short pulses of the order of a few minutes. These results suggest that the pathway of H4 processing is the same in G_1 and S phase cells, but that the kinetics are different.

In order to ascertain whether the histones synthesized during G_1 are associated with chromatin, nucleosomes were prepared from nuclei. Fig.2A shows the nuclease digestion pattern displayed on a polyacrylamide gel. This gel system is modified from that in [4,5] and better preserves the integrity of the histones, especially the H3s, for subsequent protein analyses. Fig.2B shows the typical mass pattern of core histones that are associated with the mononucleosomes fractionated on the gel shown in fig.2A. Very little, if any, non-histone protein appears on the diagonal of this gel. The histone variant synthesis pattern observed in the nuclei of the pulse sample (fig.1A) is duplicated in the histone variant synthesis pattern found in the mononucleosome fraction of the pulse sample (fig.2C). H3.3, H2A.X and H2A.Z are the respective H3 and H2A basal variants [1] found associated with G_1 nucleosomes. In addition, the dimodified (b_2) form dominates the pattern of newly synthesized H4 in nucleosomes. Likewise the histone variant synthesis pattern found in mononucleosomes after a chase (not shown) is the same as that found in chased nuclei (fig.1B). The dominant form of H4 in the nucleosomes of the chased sample is the unmodified (b_0) species. This result indicates that the H4 is processed after being incorporated into nucleosomes. Such processing

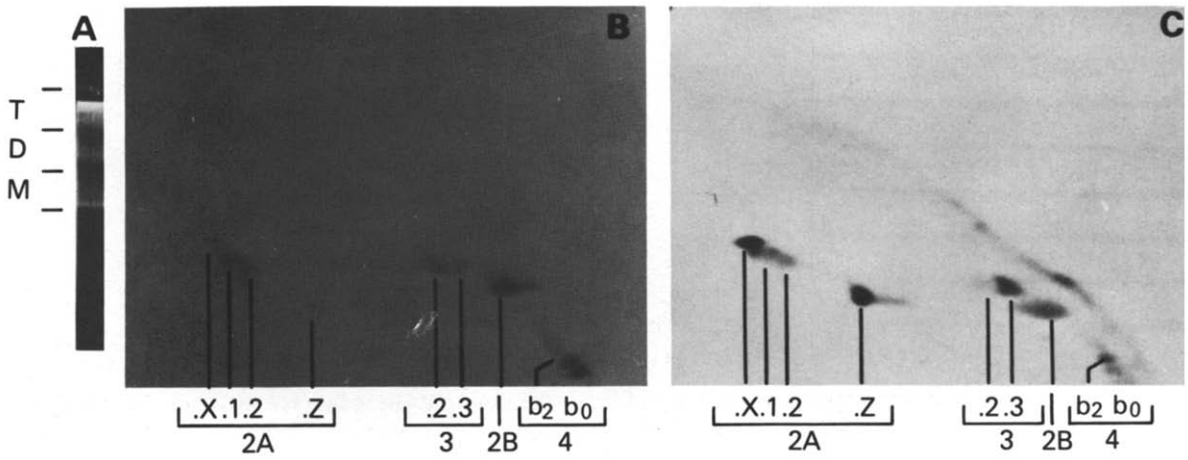

Fig.2. Micrococcal nuclease digestion pattern of G_1 nuclei and analysis of proteins associated with the mononucleosomes. Nuclei derived from labeled CHO cells in fig.1 were digested with micrococcal nuclease and the proteins associated with the mononucleosome band were analyzed as in section 2: (A) ethidium staining pattern of micrococcal nuclease digest; (B) Coomassie blue staining pattern of proteins associated with the mononucleosome band; (C) radioactive proteins associated with the mononucleosome band. Only the analysis for the pulse sample is presented here.

may be involved in a nucleosome maturation process [9,10].

3.2. *Quiescence or G_0*

Quiescent CHO cells also had a reduced but significant level of histone synthesis exhibiting a variant pattern different from that found in either the G_1 or S phases of cycling cells [2]. In quiescent cells as in G_1 cells the H3.1 and H3.2 variants were not synthesized. However, the H2A.1 and .2 variants were still synthesized in quiescent cells, and the H2A.1 and .2/H2A.X and .Z ratio was similar to that found in exponentially growing cells. Fig.3 shows that the pattern of histone accumulation in the G_0 nuclei (fig.3A) is similar to that for G_0 cells [2], while fig.3B shows that these histones are associated with mononucleosomes. No other proteins including H1 and uH2A are apparent on the mass pattern of G_0 nucleosomes (not shown). However, the fluorograph does show some uH2a and two other proteins (on the diagonal of the upper part of fig.3B), suggesting that some heavy mononucleosomes were included in this fraction.

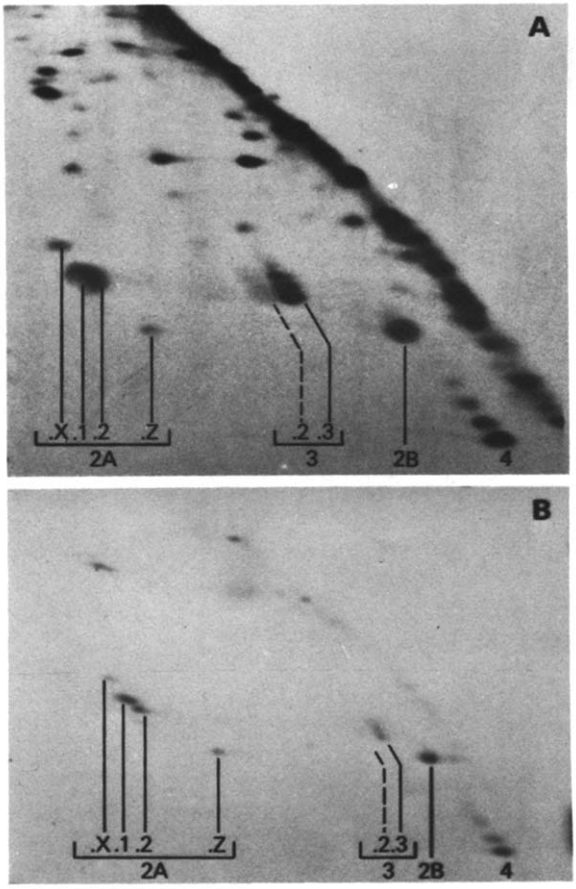

Fig.3. Accumulation of newly synthesized histones in nuclei and mononucleosomes of G_0 cells. G_0 CHO cells were grown as in section 2 and labeled for 2 h with 10 μCi/ml of [^{14}C]arginine in arginine-free RPMI 1640 supplemented with 2% fetal calf serum. Nuclei and mononucleosomes were prepared and their respective proteins analyzed as in section 2: (A) radioactive proteins found in the nuclear HCl extract; (B) radioactive proteins found associated with mononucleosomes.

The modification pattern of newly synthesized H4 presented in fig.3A,B shows b_0 as the major form, while in G_1 (fig.1A) b_2 was the major form. Since the labeling in G_1 was shorter (1 h) compared to G_0 (2 h), shorter pulses were also used for G_0. These results (not shown) indicated that in shorter pulses in G_0, b_2 was also the major form of H4. The conclusion from these experiments is that newly synthesized H4 in G_1, G_0, or S cells always seems to be present initially as the b_2 form. However, the kinetics of conversion of b_2H4 to b_0H4 may differ among various cell states.

4. DISCUSSION

We conclude that the histones synthesized during G_0 and G_1 become stably associated with chromatin as nucleosomes. All 4 newly synthesized core histones are found in the mononucleosomes and approximately equimolar quantities of each seem to be synthesized [1,2].

Nucleosomes have a net negative charge at neutral pH and therefore migrate toward the anode. Histones have a positive charge at neutral pH and if free would not be found anywhere in a nucleosome gel. Thus histones found in a mononucleosome band must be tightly bound to a negatively charged molecule such as DNA. Also the fact that these labeled histones are found in the fastest migrating mononucleosome band indicates that they are not bound in some non-nucleosomal fashion to an already complete mononucleosome, since the migration of such complexes would be retarded.

Only part of the digested chromatin is found as mononucleosomes (fig.2A). Thus we have not attempted to quantitate what fractions of the newly

synthesized histones found in nuclei are also in nucleosomes. However, because of the amounts of material used, at least a major portion of the labeled histone found in isolated nuclei was probably present as nucleosomes. Since extracts of nuclei or of whole cells generally yielded similar amounts of radioactive histones, these results suggest that histones are rapidly incorporated into nucleosomes whether the cells are in S, G_1 or G_0.

By definition, the basic difference between S phase on the one hand and G_1 and G_0 on the other is the lack of DNA replication in the latter two states. Since the regions of 'free' newly synthesized DNA available for histone association are presumably not present in G_1 or G_0, different mechanisms of nucleosome assembly may be involved. However, we have found no evidence of this so far. For example, the change of newly synthesized H4 from the b_2 form to b_0 form in S phase cells [8] also occurs in G_1 phase and G_0 cells, albeit at somewhat slower rates.

If there is no net DNA synthesis, then newly synthesized histones could incorporate into chromatin by exchanging with existing histones either singly, as dimers, tetramers or whole octamers. While our results do not differentiate among these possibilities, the maintenance of nucleosomal ratios of newly synthesized histone in G_1 and G_0 as well as S suggests that whole histone octamers may be involved. If exchange of single histone species occurred, there would be no obvious need to maintain nucleosomal ratios of newly synthesized histone.

Another possibility is that nascent nucleosomes could bind to chromatin leading to a shortening of the nucleosome repeat. Such a situation has been noted in several rather specific instances involving differentiated cells [11] or inhibition of DNA synthesis [12,13].

However, this latter mechanism by itself could not accommodate continuous, long term quiescent (G_0) histone synthesis. However, there is some evidence for histone exchange. The half-life of liver histones in mice was 117 days as compared to 318 days for DNA, thus indicating some turnover of histones [14]. If nucleosomes do slowly turn over, the histone composition of the core particles should ultimately reflect any changes in the synthesis pattern of histone variants. Many adult mouse tissues, such as liver, kidney and brain, which show little or no DNA synthesis, have H3.3 as the major H3 (mass) variant [15]. Also, the mass fraction of H3 in H3.3 increases from 15% in livers of newborn mice to 55% in livers of adult mice [16]. Similar findings were made in chickens [17]. In human T-lymphocytes, cells which are in G_0, the H3.3 variant is the major H3 (mass) variant [3]. Upon activation, human T-lymphocytes change their H3 variant (mass) pattern; H3.1 and H3.2 become the dominant mass variants, as found in most tissue culture cell lines [2]. Since lymphocytes are capable of surviving in vivo for an average of 4–6 months [18], the higher mass fraction of H3 as H3.3 in resting lymphocytes may be accounted for by nucleosome turnover.

Since G_1 cells are poised to enter S phase and will thus be soon undergoing DNA replication, it is not excluded that the nascent histones could be accommodated by the existing chromatin without nucleosome turnover. Newly synthesized histones may associate with non-replicating regions of chromatin [10,19].

Some histone synthesis and histone turnover for repair may occur independently of DNA synthesis in non-proliferating cells [20]. However, quiescent histone synthesis is not increased when repair DNA synthesis is induced [3,21], but is present continuously at the same level. This finding suggests that histone synthesis and turnover in quiescent cells may also be involved in other more continuous processes such as transcription.

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