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Formation of hybrid nucleosomes containing new and old histones

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ABSTRACT

5 mM hydroxyurea (HU) inhibits DNA synthesis in mouse P815 cells by 95-97% in less than 1 hr. Nevertheless, histone synthesis continues and newly-synthesised histones are incorporated into non-replicating chromatin at a rate of about 20% of that in control exponentially-growing cells. To study the organization of these histones in chromatin, P815 cells were treated with 5 mM HU in medium containing dense (^{15}N, ^{13}C, ^{2}H)-substituted amino acids. After inhibition of DNA synthesis, newly-synthesised histones were labelled with (^{3}H)-arginine. The cells were harvested 90 min later, and mono- and oligonucleosomes were prepared and analysed on metrizamide-triethanolamine (MA-TEA) density gradients. Analysis of the distribution of ^{3}H-labelled histones in these gradients shows that they are incorporated into hybrid mononucleosomes containing both new and old histones. It is also shown that these hybrid nucleosomes are not randomly distributed, but show a certain tendency to be clustered in certain chromatin regions.

INTRODUCTION

There is strong evidence that histone octamers, once organized into nucleosomes, behave as stable structural and metabolic units (1, 2, 3). However, we recently obtained data indicating that this may not always be the case. First, while studying the organization of newly-synthesized histones in chromatin we found that although most of them form entirely new octamers, some are probably incorporated into hybrid nucleosomes containing both old and new histones (3). Second, we found that when DNA synthesis was entirely inhibited in cells treated with HU, histone synthesis, although reduced, still continued and these histones were incorporated into unreplicating chromatin, most probably by displacement of some preexisting nucleosomal histones (4). It is reasonable to suggest that, in this case, individual histones rather than histone octamers were exchanged, because the existence of free histone octamers in the cell nucleus has not been demonstrated, and further because our, as

well as other data (5), show that the four core histones are incorporated into chromatin under conditions of HU-inhibition at similar but not identical rates.

In order to further examine the existence of hybrid nucleosomes, we have labelled the histones synthesized in the presence of HU with dense amino acids. Mononucleosomes were prepared and analysed on the newly-developed MA-TEA density gradients, which are capable of resolving nucleosomes containing dense histones from those containing normal histones (3). Analysis of the gradient patterns shows that a) the dense histones synthesised in the absence of DNA synthesis participate in the formation of hybrid nucleosomes containing both old and new histones, and b) that these hybrid nucleosomes are clustered in chromatin.

MATERIALS AND METHODS

Cells and labelling. P815 mouse cells growing exponentially at 5-6 x 10^5 cells/ml in suspension in Eagle's medium were harvested by centrifugation (1000 rpm, room temp.). They were resuspended at the same cell density in medium containing dense amino acids (2 mg/ml) and dialysed calf serum (5%) and were treated with hydroxyurea (puriss, Fluka), and labelled with (^{3}H)-arginine (Amersham) as described in the appropriate Figure legends.

(^{13}C, ^{15}N, ^{2}H)-substituted amino acids were isolated by acid hydrolysis (6) from about 90% ^{13}C, ^{15}N, ^{2}H-labelled algal cells (Spectometrie Spin et Techniques, Paris).

Isolation of chromatin and digestion with micrococcal nuclease. Chromatin was isolated (7) and suspended in 1 mM $CaCl_2$/10 mM Tris-HCl (pH 8)/0.1 mM phenylmethylsulfonyl fluoride (Serva) at 200-500 µg DNA/ml. It was digested with 1 unit of micrococcal nuclease (Boehringer)/25 µg of DNA at 37°C for 15 min to give about 15% acid-soluble DNA. EDTA (pH 8) was added to 3 mM, and after 1 hr in the cold, samples (0.5 ml) were layered on linear 5-30% sucrose gradients containing 5 mM Tris-HCl (pH 8)/1 mM EDTA with a 1 ml cushion of 2 M sucrose. After centrifugation (SW 40 rotor, 30,000 rpm, 5°C, 16 h), the gradients were unloaded from the bottom and the mono-, and oligonucleosomes separately precipitated with $MgCl_2$ (5 mM final concentration). After 2-3 hr in the cold the precipitates were pelleted by centrifugation (10,000 rpm, 15 min, Sorvall RC-5B) and resuspended in the desired volume of 2 mM EDTA (pH 8). In the case of mononucleosomes this procedure effectively eliminates core nu-

cleosomes and subnucleosomal fragments, and yields a homogeneous population of particles containing all 5 histones in equimolar amounts (8).

Metrizamide-triethanolamine density gradient analysis. The gradients, made in nitrocellulose tubes for the Beckman Ti-50 rotor, consisted of three layers of (from bottom to top) : 37% (w/v) metrizamide (MA) (centrifugation grade, Nyegaard, Oslo), in 18% (v/v) triethanolamine (TEA) (Merck) (1.5 ml); 32% MA in 16% TEA (1.6 ml); 28% MA in 14% TEA (1.4 ml). The middle layer also contained the sample. The tubes were filled with paraffin and centrifuged (Ti 50 rotor, 32,000 rpm, 4^{o} for 65 h). The gradients were fractionated from the bottom, 0.1 ml aliquots were diluted with 0.1 ml of water, and mixed with 2 ml of Lumagel (Fakola A.G., Basel) for radioactivity determinations. The density of the fractions was calculated from the refractive index after correction for the presence of TEA (9).

Isolation and fractionation of histones. Histones were isolated by extraction of chromatin with 0.3 N H_2SO_4 for 2 hr in the cold followed by ethanol precipitation. They were dissolved in electrophoresis sample buffer at about 1 mg/ml and fractionated by SDS-polyacrylamide gel electrophoresis in 22 cm long slab gels (10). The gels were stained with Coomassie Brilliant Blue, scanned at 530 nm with a Zeiss scanning spectrophotometer and the individual histone fractions excised. These were dissolved in 0.5 ml of 30% H_2O_2-25% NH_4OH (4:1) and counted after mixing with 5 ml of Lumagel scintillation cocktail. The specific radioactivity of each fraction was determined in arbitrary units as the ratio between the counts and the area under the scanned peak.

RESULTS

To check if hybrid nucleosomes containing new and old histones are formed in chromatin, the following experiments were carried out.

Assembly of newly-synthesised histones into nucleosomes. Exponentially-growing P815 cells were prelabelled with (^{14}C)-dT and then transferred to medium containing heavy amino acids. After 1 hr the cells were given (^{3}H)-arginine for 90 min and were harvested for isolation of chromatin. This was digested with micrococcal nuclease and the isolated mononucleosomes were centrifuged in MA-TEA density gradients(Fig. 1). The old ^{14}C-labelled nucleosomes form a peak at $\rho = 1.218$, which is the characteristic density of normal nucleosomes in these gradients (3). This peak is asymetric due to the fact that

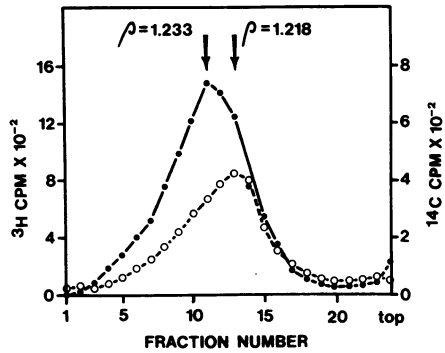

Figure 1

Analysis of nucleosomes containing new, dense histones in MA-TEA density gradients.
P815 cells were prelabelled with 10 nC/ml of (methyl-^{14}C)-dT (Amersham) for 90 min and were resuspended in fresh medium containing (^{13}C, ^{15}N, ^{2}H) amino acids. After 1 hr at 37°C they were labelled with 10 µC/ml of (2,3-^{3}H)-arginine for 90 min. Chromatin was isolated (7), digested with micrococcal nuclease, and the isolated mononucleosomes (Materials & Methods) were centrifuged in MA-TEA density gradients. ●--●, ^{3}H-labelled new histones; o--o, ^{14}C-labelled old DNA. The densities of the peaks are shown by arrows.

^{14}C-prelabelled DNA associates with newly-synthesised dense histones. The ^{3}H label forms a peak at ρ = 1.233, the characteristic density of nucleosomes containing only dense histones (3). However, this peak has a clear shoulder in the density region of normal nucleosomes. This result shows that in replicating chromatin 70-80% of the new histones are assembled into entirely new nucleosomes, while the remaining 20-30% form hybrid nucleosomes containing both new and old histones.

Association of hybrid nucleosomes with nonreplicating chromatin. In a previous paper we showed that during normal chromatin replication 70-80% of the new histones associate with newly-replicated DNA, while the remaining 20-30% associate with old DNA (3). In order to establish whether the hybrid nucleosomes we observed here formed on new or old DNA, we inhibited DNA synthesis with HU and followed the distribution of the new histones synthesised in the absence of DNA replication. In full agreement with our previous results obtained with Ehrlich ascite tumour cells (4), and with published data for other cell lines (5, 11), 5 mM HU inhibits DNA synthesis in P815 cells by 95-97% in 1 hr (Fig. 2). Since at least part of the residual incorporation is

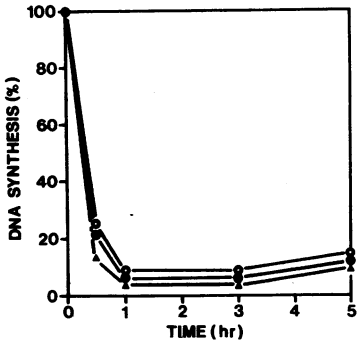

Figure 2

Inhibition of DNA synthesis by HU.
P815 cells were incubated with 2.5 mM (o), 5 mM (●), or 10 mM (▲) HU. At the designated times aliquots were taken, incubated with 20 nC of (^{14}C)-dT per ml for 15 min, precipitated with 10% trichloroacetic acid in the cold, and counted.

due to mitochondrial DNA synthesis or/and repair, which are less sensitive to HU, the actual level of DNA replication should be even lower. At the same time histone synthesis and incorporation into chromatin continues at 10-20% of the control rate (Table 1). When mononucleosomes were prepared from these cells, in which DNA was prelabelled with ^{14}C-dT and the histones synthesised in the presence of HU were labelled with ^{3}H-arginine, and analysed on MA-TEA density gradients, the two labels coincide precisely and form a symmetrical peak at $\rho = 1.218$ (Fig. 3A). This shows that the histones synthesised under conditions of HU block are assembled into nucleosomes having a normal histone to DNA ratio (4), and are not deposited onto old nucleosomes as extra histones.

To study this assembly process we prelabelled DNA with (^{14}C)-dT and transfered the cells into medium containing dense amino acids and 5 mM HU. After 1 hr, when DNA synthesis was maximally inhibited (Fig. 2), the cells were labelled with (^{3}H)-arginine for 1 h. Mono- and oligonucleosomes were prepared and analysed on MA-TEA density gradients. The bulk of the ^{14}C-labelled mononucleosomes formed a peak at $\rho = 1.218$ (Fig. 3B), as in the previous case. The mononucleosomes containing dense, ^{3}H-labelled histones formed a somewhat broader peak situated midway between the positions of fully-dense and normal nucleosomes (Fig. 3B). This shows that the dense histones synthe-

TABLE I

Incorporation of individual histones into chromatin of HU-treated P815 cells. Cells were treated with 5 mM HU and after 1 hr were labelled with 10 µC/ml of (^{3}H)-arginine for 90 min. Control (untreated) cells were labelled in exactly the same way. Isolated histones were fractionated by SDS-polyacrylamide gel electrophoresis; the specific radioactivity of each individual histone from HU-treated cells is expressed as the percentage of the specific radioactivity in the control for 4 different experiments.

Expt	H3	H2B	H2A	H4
1	11	10	9	6
2	37	34	28	12
3	22	19	19	12
4	19	21	21	15
Mean	22	21	19	11

sised in the absence of DNA replication join old chromatin and form hybrid nucleosomes with an approximately 1:1 mean ratio of new to old histones.

Distribution of hybrid nucleosomes in chromatin. In order to understand whether the formation of hybrid nucleosomes occurs in specific chromatin domains or at random, the oligonucleosome fraction from the previous experiment, which consisted of fragments containing 5 to 9 mononucleosomes (3), was also analysed by equilibrium centrifugation in MA-TEA density gradients. If the hybrid nucleosomes are distributed at random, there would generally not be more than 1 such nucleosome per fragment, which would not be enough to noticably increase the buoyant density of the fragment. In this case the ^{3}H and ^{14}C labels would coincide to form a single peak at the density of normal nucleosomes. On the other hand, if the hybrid nucleosomes are clustered in specific regions of chromatin, all oligonucleosomes originating from this region would be composed of hybrid nucleosomes only, and would band at a heavier density than normal nucleosomes. We observed that the ^{3}H-labelled oligonucleosomes band at a density higher than the normal, ^{14}C-labelled oligonucleosomes (Fig. 4), but less than that expected for fully dense oligonucleosomes. This shows that there is a tendency for nucleosomes containing dense

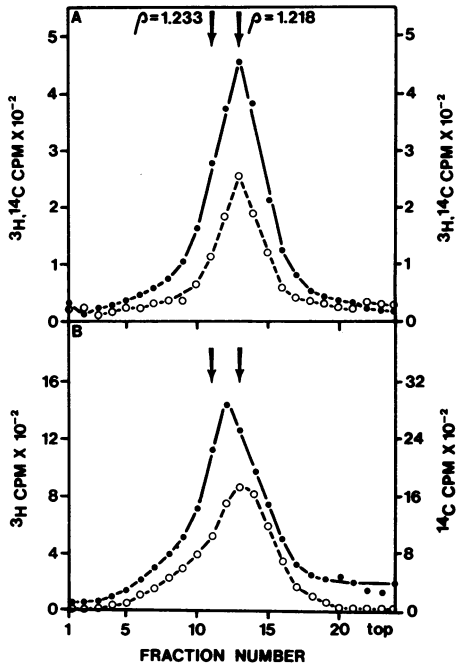

Figure 3

Analysis of nucleosomes from cells treated with HU in the presence of normal (A) and dense (B) amino acids.
Cells were prelabelled with 10 nC/ml of (^{14}C)-dT for 90 min and resuspended in medium containing 5 mM HU and normal (A) or dense (B) amino acids. After 1 hr the cells were labelled with 10 µC/ml of (^3H)-arginine for 60 min. Nucleosomes were prepared from the two samples and analysed on MA-TEA density gradients. Designations as in Fig. 1.

histones to be clustered adjacent to each other.

DISCUSSION

From the results described here, the conclusion can be drawn that in exponentially-growing cells newly-synthesized histones are assembled into chromatin in 2 different manners. 70-80% of them associate with newly-replicated DNA, where they become assembled into nucleosomes containing new histones only. The remaining 20-30% join unreplicated chromatin by displacing some old histones, leading to the formation of hybrid nucleosomes. This process continues when assembly by the first pathway is supressed after inhibi-

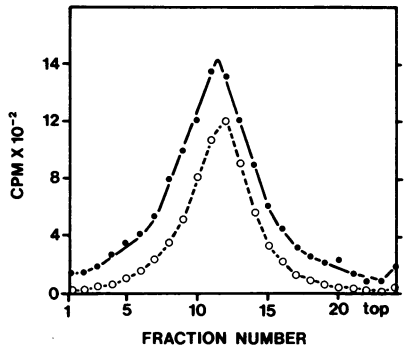

Figure 4

Analysis of oligonucleosomes from cells treated with HU in the presence of dense amino acids.
The oligo (5-9)-nucleosome fraction from the cells in Figure 3B was analysed on a MA-TEA density gradient. Designations as in Fig. 1.

tion of DNA synthesis, as in the experiments described here. Although the mean ratio of new to old histones in these hybrid nucleosomes is about 1:1, both the different rates of incorporation of individual histones into chromatin of HU-blocked cells (Table I), and the buoyant density profile of mononucleosomes in MA-TEA gradients (Fig. 3B) suggest that the hybrid nucleosomes are heterogenous both in respect to the amount and the type of new histones which they contain.

The biological significance of the first assembly pathway is obvious; newly-replicated DNA is furnished with the necessary histone complement to assemble nucleosomes. The biological significance of the second pathway of histone incorporation is for the time being obscure. However, the observation that histone exchange occurs to a certain extent in adjacent nucleosomes makes it tempting to suggest that it is maybe connected with changes in nucleosomal organization in certain regions of chromatin, for example regions undergoing transcription. This idea is in agreement with the finding that extensive histone replacement takes place in chromatin during development and differentiation (12, 13).

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