Assembly of semihistone A24

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ABSTRACT

Nucleosomal semihistone A24 (uH2A) is composed of histone H2A and ubiquitin peptides. The kinetics of incorporation of nascent H2A and ubiquitin into A24 of transformed chicken lymphocytes (MSB cells) have been examined by peptide mapping, COOH-terminus analysis, density labeling and isopycnic centrifugation of chromosomal proteins. We find that newly synthesized H2A is rapidly conjugated to ubiquitin. SDS-PAGE analysis of fractionated density gradients suggests, however, that newly synthesized ubiquitin becomes bound nonselectively to both new and preexisting H2A molecules.

INTRODUCTION

One level of transcriptional control appears to reside in the protein composition and conformation of nucleosomes (1-4). The evolutionary conservation of histone primary structure and charge distribution (5,6) stands in marked contrast to the wide range of histone covalent modifications known to occur in vivo (7-9) and it has been proposed that the nonrandom occurrence of specifically constituted nucleosomes could serve to define functional domains of chromatin (9-11).

One of the most profound covalent modifications of histone structure is the site-specific conjugation of the nonhistone protein ubiquitin to H2A (12,13). The strong evolutionary conservation of the ubiquitin amino acid sequence (14-16) and the coupling of this peptide to a subset of the nucleosomal H2A molecules (17-19) are consistent with a role for the protein conjugate, semihistone A24 (uH2A), in nuclear function (17). Matsui et al. (20) have recently proposed that the conjugation of H2A with ubiquitin serves to activate chromatin for transcription. Conversely, Ballal and Busch (21) have suggested that A24 assembly plays a role in the transcriptional inactivation of neighboring gene sequences. Consistent with this are the observations of Kleinschmidt and Martinson

(22) who found no increase in nuclease sensitivity or high mobility group nonhistone binding to A24-containing vs. control nucleosomes, features normally associated with transcriptional capacity (4).

The levels of A24 vary with cell cycle position (19) in DON cells, and decrease in the nucleoli of rat liver cells stimulated to hypertrophy by thioacetamide (23) or partial hepatectomy (24). Matsui et al. (19) have shown that A24 may be assembled during puromycin inhibition of protein synthesis in DON cells; suggesting that under these conditions preexisting H2A and ubiquitin become linked. Seale (25) has recently examined the turnover of pulse-labeled A24 in HeLa cells and concluded that newly-made H2A and ubiquitin quickly become incorporated into A24. Moreover, the nascent H2A moiety of A24 turns over less rapidly than the newly synthesized ubiquitin, allowing reconjugation of H2A during a subsequent chase. Unfortunately, in these experiments it was not possible to address the question of whether nascent H2A and nascent ubiquitin were initially incorporated into the same A24 molecule.

Because the covalent attachment of ubiquitin to H2A represents a major postsynthetic remodeling of chromatin structure, we have examined the kinetics of A24 assembly using density labeling and isopycnic centrifugation in conjunction with SDS-PAGE. Our results show that although nascent H2A and ubiquitin are rapidly incorporated into A24, there is no apparent bias favoring the conjugation of newly-made ubiquitin with newly-made H2A. Thus nascent H2A becomes conjugated with either new or preexisting ubiquitin, and nascent ubiquitin may become covalently crosslinked to H2A molecules more than 2-3 hours after histone deposition.

METHODS

MSB-1 cells, a continuous line of chicken lymphocytes transformed by Marek's virus (26), were grown in RPMI-1640 medium supplemented with 5% calf serum and 5% fetal calf serum, under a 10% CO₂ atmosphere at 39°C. Algal ¹⁵N, ¹³C, ²H amino acid hydrolysate (Merck) was filtered through an Amicon UM2 membrane and reconstituted to approximate RPMI-1640. For density pulse experiments, 5 x 10⁶ exponentially growing cells (8-10 hour doubling time) were washed with warmed phosphate buffered saline (PBS) and incubated in 1 ml of warmed density-substituted medium prior to the addition of [³H] lysine (NEN, 73 Ci/mole,

100-200 $\mu\text{Ci/ml}$) or [^{35}S] methionine (NEN, 450 Ci/mmol, 100-200 $\mu\text{Ci/ml}$).

Nuclei were prepared after low-speed centrifugation of cells, a PBS wash and cell lysis in 10 mM NaCl, 5 mM Pipes (piperazine-N,N'-bis [2-ethanesulfonic acid], pH 7.4), 3 mM MgCl_2 , 0.5% NP-40. After two washes in lysis buffer nuclei were resuspended in lysis buffer at an $A_{260} = 10$ and digested for two minutes at 37°C after the addition of Ca^{+2} to 0.1 mM and 50 units of Staphylococcal nuclease (Boehringer) per ml. Nuclei were pelleted at low speed, lysed in ice cold 10 mM Na_2EDTA (disodium ethylenediaminetetraacetate) (pH 7.5) and the supernatant after a 10 minute/10,000 g centrifugation taken as soluble chromatin. Routinely, 25-35% of the initial nuclear A_{260} is recovered.

For crosslinking after reconstitution, chromatin was dissociated in 2.5 M NaCl, 7 M urea, 5 mM Na_2EDTA , 5 mM Pipes (pH 8.0), dialysed to 2.0 M NaCl, 5 mM Na_2EDTA , 5 mM Pipes (pH 8.0) and gradient dialysed to 0.7 M NaCl, 5 mM Na_2EDTA (pH 8.0). Insoluble material was pelleted at 10,000 g for 10 minutes. One-tenth volume of 200 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 9.0) was added to the supernatant to be crosslinked, followed by one-tenth volume DMSO containing dithiobissuccinimidyl propionate (Pierce) at 0.5 mg crosslinker per mg DNA. Acid soluble nuclear proteins were extracted from digested nuclei for 3 hours at 4°C with 0.4 N H_2SO_4 .

Pulse labeling of MSB cells with [^3H] lysine (NEN, 73 Ci/mmol, 400 $\mu\text{Ci/ml}$), [^{14}C] lysine (NEN, 345 mCi/mmol, 50 $\mu\text{Ci/ml}$), or [^{14}C] leucine (Amersham, 6.5 mCi/mmol, 50 $\mu\text{Ci/ml}$) was performed by concentration of a logarithmically growing culture of 2×10^7 cells by low speed centrifugation and resuspension of the culture in 1 ml of fresh lysine-depleted medium for 15-30 minutes. Chromatin was prepared for preparative gel electrophoresis as described above.

Carboxypeptidase Y (Worthington COY, sequencing grade) cleavage (27, 28) was performed on electrophoretically purified A24 or H2A samples eluted from slices excised from 15% polyacrylamide-SDS preparative gels and dialysed extensively against 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 6.0), 150 mM NaCl. Each of these proteins reran as a single band when reelectrophoresed on 12.5%, 15%, 18%, 22% and 5-22% gradient polyacrylamide-SDS gels (29). 0.1-0.2 nmol of A24 or H2A in 150 mM NaCl, 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 6.0) were incubated at 25°C with 0.001 units of carboxypeptidase Y. This enzyme/substrate ratio was chosen as giving continuous digestion of H2A for over two hours. The carboxypeptidase is

not inactivated by the presence of residual SDS under these conditions (27,28). For samples to be analysed by thin layer chromatography, digestion was terminated by mixing each time point aliquot (30 μ l) with 10 μ l of water containing 10 nmol phenylmethylsulfonyl fluoride (PMSF) and 5 μ g each of unlabeled lysine and leucine. The samples were dried and rehydrated to 20 μ l, chromatographed on silica gel TLC plates in propanol:ammonium hydroxide (70:30, v:v). After visualization by ninhydrin, the lysine and leucine standard spots for each sample were eluted from the chromatogram in 0.2 N HCl, 0.1% SDS and counted using Beckman Ready - Solv MP. Alternatively, the enzyme digestion was terminated by pipetting of the reaction mixture into a tube containing 10 nmol of PMSF plus 200 μ g bovine serum albumin (BSA). The samples were then precipitated by adding 0.25 volume of 100% trichloroacetic acid (TCA), cooling at 4°C for 30 minutes and counting the acid - soluble supernatant in Ready - Solv MP. All double label counts were corrected for ^{14}C spill. The band excised as A24 fulfilled the following criteria (not shown): 0.4 N H_2SO_4 extractable from nuclei; sediments with nucleosomes in sucrose gradients containing 0.35 or 0.6 M NaCl; electrophoreses with nucleosome particles on nondenaturing nucleoprotein gels; crosslinks to H2B; labels with [^{35}S] methionine; [^{35}S] methionine label is lost on CNBr cleavage with almost no increase in electrophoretic mobility; yields N-bromosuccinimide and *Staphylococcus aureus* V8 protease peptides in common with H2A and ubiquitin; migrates on SDS and acid-urea gels with mobility of A24 ($M_r \approx 22,500$).

N-bromosuccinimide cleavage of A24 and H2A in gel slices was carried out by incubating the stained gel fragment in 1.7 M acetic acid (adjusted to pH 4.0 with 1 M NaOH) containing 10 mg/ml N-bromosuccinimide (Pierce), for 30-60 minutes at 37°C with shaking, in the dark. Slices were rinsed successively with water, 1.5 M Tris-HCl (pH 8.8), 0.5 M Tris-HCl (pH 6.8) and shaken for 10 minutes in SDS-PAGE sample buffer (29) diluted 1:4 with water. The incubation mixture was adjusted to pH 7.0 with 1 M NaOH and after an additional 10 minutes, the slices were loaded directly onto the stacking gel over an 18% polyacrylamide-SDS gel and run according to Laemmli (29).

Cesium formate - guanidine hydrochloride (CsFo-GuCl) density gradients were prepared by mixing 300 μ l of solution A (2.25 M CsFo, 5.82 M GuCl) with 100 μ l of sample and underlayering beneath a mixture of 0.7 ml solution A plus 1.0 ml solution B (6.46 M GuCl). Tubes were

overlaid with paraffin oil and centrifuged at 45,000 rpm for 70 hours at 25°C in the Beckman SW60 Ti rotor. Gradients were fractionated into 60 μ l aliquots from below by pumping paraffin oil onto the surface of the sealed gradient tubes. Fractions were equilibrated against water and then against 0.1% SDS, 62 mM Tris (pH 6.8), 15% glycerol prior to electrophoresis on 15% or 18% polyacrylamide-SDS separating gels (29). After staining, gels were impregnated for fluorography with EN³HANCE (NEN), dried and exposed to preflashed Kodak XR-5 film at -80°C. A parallel gradient containing [³H] lysine labeled, uncrosslinked dense histone and [¹⁴C] labeled, crosslinked light histones was run with each experiment.

RESULTS

Incorporation of nascent H2A and nascent ubiquitin into A24

In order to demonstrate that newly synthesized histone H2A molecules may be incorporated into A24 in MSB cells, A24 was isolated from cells pulse labeled for 15-30 minutes and digested with carboxypeptidase Y. In semihistone A24, lysine 119 of H2A is in isopeptide linkage with a glycine-glycine bridge whose amino terminus forms a peptide bond with the terminal arginine carboxyl of ubiquitin (refs. 19,23 and Figure 1).

Thus, the only carboxyl terminus available for carboxypeptidase Y cleavage is that of histone H2A. When A24 isolated from MSB cells pulsed for 15-30 minutes with [³H] lysine was digested with carboxypeptidase Y, the release of TCA-soluble label was initially rapid, but then reached a plateau with no further release of label for more than two hours

Fig.1 Schematic Diagram of Semihistone A24 (uH2A). The diagram is based on the data of (15,19,20,30). The carboxyl region lysine and leucine residues, and all tyrosine residues are indicated.

(Figure 2A). Based on the substrate specificity of carboxypeptidase Y (27,28) this effect is presumably due to the inability of the carboxypeptidase to proceed beyond the lysine 119 branch point. To demonstrate directly that the enzyme could not hydrolyse beyond lysine 119, nor was the ubiquitin portion of A24 being degraded, MSB cells were pulsed for 15-30 minutes with a mixture of [^{14}C] lysine and [^{14}C] leucine; A24 was isolated, digested with carboxypeptidase Y and the released label analysed by thin layer chromatography. The results of Figure 2A show that the release of [^{14}C] lysine paralleled the release of [^3H] lysine in the previous experiment, while no detectable [^{14}C] leucine was released. Since there are no leucine amino acids carboxyl to lysine 119 in H2A, and no lysine amino acids carboxyl to leucine 67 of ubiquitin (Figure 1), the biphasic release of radiolabeled lysine in the absence of [^{14}C] leucine hydrolysis indicates: (1) that the degradation of ubiquitin is not contributing to the observed released label, (2) that H2A labeled during a brief pulse may be incorporated into A24 and (3) that the digestion kinetics are consistent with the substrate specificity of carboxypeptidase Y and the branched nature of A24. To demonstrate further that it is the lysine 119 branch point which impedes the progress of the carboxypeptidase Y, both A24 and histone H2A were isolated from MSB cells pulse labeled for 15-30 minutes with a mixture of [^3H] lysine plus [^{14}C] leucine. Equal amounts of these proteins were subjected to parallel digestions with carboxypeptidase Y and the release of TCA-soluble radiolabel was monitored (Figure 2B). As expected, A24 showed a biphasic release of [^3H] lysine, with no release of [^{14}C] leucine. In contrast, digestion of histone H2A liberated both lysine and leucine. These results confirm that nascent H2A may be incorporated into A24 within 15-30 minutes of its synthesis.

To demonstrate by independent means that nascent H2A can be incorporated into A24 we also show that an H2A-specific peptide derived from A24 by N-bromosuccinimide cleavage is labeled during a 30 minute pulse of MSB cells with [^3H] lysine. Cells were incubated with [^3H] lysine and the nucleosomal core histones and A24 isolated by gel electrophoresis (Figure 3A). Each was subjected to partial hydrolysis by N-bromosuccinimide. Due to the aberrant mobility of the histones on SDS-PAGE, the sizes of the A24 peptides were assigned by comparison with the mobilities of the core histone fragments in agreement

Fig.2 Kinetics of Carboxypeptidase Y Digestion of Pulse-Labeled A24. (A) A24 was electrophoretically purified from MSB cells pulse labeled (15-30 minutes) with [³H] lysine. The eluted A24 was digested for increasing times with carboxypeptidase Y and the release of trichloroacetic acid-soluble [³H] lysine measured (o). A24 was electrophoretically purified from MSB cells pulse labeled (15-30 minutes) with a mixture of [¹⁴C] lysine plus [¹⁴C] leucine. The eluted A24 was digested for increasing times with carboxypeptidase Y and the release of [¹⁴C] lysine (x) and [¹⁴C] leucine (Δ) monitored by thin layer chromatography. (B) A24 and histone H2A were electrophoretically purified from MSB cells pulse labeled (15-30 minutes) with a mixture of [³H] lysine plus [¹⁴C] leucine. The eluted A24 and H2A were digested independently for increasing times with carboxypeptidase Y and the release of trichloroacetic acid-soluble [³H] lysine (x, o) and [¹⁴C] leucine (Δ, □) analysed. A24 (x, Δ); H2A (o, □). Notice the difference in scales.

Fig.3 Nascent H2A Is Incorporated Into A24.

(A) Twenty-two percent polyacrylamide-SDS second dimension gel of A24 and core histone bands excised from a preparative 15% polyacrylamide-SDS gel. Fluorogram is shown.

(B) Fluorogram of a second dimension 18% polyacrylamide-SDS gel containing N-bromosuccinimide generated fragments of H2A and A24. 38, 72, 128 and 204 refer to polypeptide length (in amino acids). Bands marked a, b, c are the major [^3H] lysine labeled fragments of A24. Refer to the text for details. The H2A and A24 samples were run alongside one another on the same gel. The H2A track was exposed for 1 week, the A24 track for 4 weeks.

with the respective amino acid sequences (15,19,30,31). The major [^3H] labeled peptides generated by N-bromosuccinimide cleavage of A24 are denoted a, b and c in Figure 3B. Fragment a is a 146 or 148 amino acid fragment arising from cleavage at tyrosine 57 of H2A or tyrosine 59 of ubiquitin (cf. Figure 1). Fragment b is the 58 amino acid N-terminal fragment of ubiquitin (this fragment also appears on cleavage of [^{35}S] methionine labeled A24 [not shown]). Fragment c is the 38 amino acid N-terminal peptide derived from H2A. (Notice also, that due to its attachment to ubiquitin the 72 amino acid fragment generated by cleavage of isolated H2A is absent from the A24 digest.) The appearance of fragment c in labeled form confirms the incorporation of newly synthesized H2A into A24. These results agree with the conclusion by Seale (25), that the H2A moiety of A24 is labeled during a two minute

pulse of HeLa cells with [^3H] lysine.

The demonstration that nascent ubiquitin is incorporated into A24 is simpler than the experiments described above for H2A because ubiquitin contains a single methionine residue located at its amino terminus, while chicken H2A has none (15,30). The experiments described in Figures 5 and 7 show that A24 is labeled during a 30 minute pulse of MSB cells with [^{35}S] methionine.

Formation of hybrid A24 molecules

To examine directly the incorporation of nascent H2A and nascent ubiquitin into A24 a density labeling approach was employed. When MSB cells are cultured in medium similar to RPMI-1640 but containing ^{15}N , ^{13}C , ^2H substituted amino acids, the polypeptides synthesized during this incubation exhibit an average 4-5% increase in density relative to unsubstituted preexisting proteins, as analysed by isopycnic centrifugation in denaturing cesium formate-guanidine hydrochloride (CsFo-GuCl) density gradients (32).

If a covalent crosslink is introduced (in vivo or in vitro) between a density substituted (dense) protein and an unsubstituted (light) protein, the dimer will exhibit a hybrid density upon isopycnic centrifugation. Alternatively, crosslinking of two dense proteins will yield a uniformly dense dimer. We have taken advantage of the in vivo crosslinking of H2A and ubiquitin to examine whether the nascent H2A and nascent ubiquitin synthesized during a density labeling pulse become assembled into the same A24 molecule.

MSB cells were incubated for 60 minutes in density substituted medium containing tracer amounts of [^3H] lysine to radiolabel specifically the newly made proteins. After such a pulse approximately 5-10% of the total histone of these cells is density labeled (33). Chromatin was prepared from these cells (Methods) and, in order to include internal markers of hybrid density on the eventual CsFo-GuCl gradients, an aliquot of this chromatin was added to a 10-fold excess of unlabeled chromatin and the mixture dissociated in 2.5 M NaCl, 7 M urea. This procedure does not disturb the in vivo crosslink preexisting between ubiquitin and H2A but does dissociate essentially all of the histones away from the DNA and one another (31). Subsequent decreasing salt gradient dialysis reconstitution of the density labeled histone in the presence of a 100-fold excess of unlabeled histone ensures that in the reconstituted nucleoprotein, any dense histone will have become

associated only with light histones. Thus, after partial *in vitro* crosslinking (with dithiobissuccinimidyl propionate) of the reconstitute to optimize formation of histone-histone dimers, this protocol provides internal markers for the positions of hybrid density (heavy-light dimers) and fully dense (uncrosslinked monomer) proteins. It is to these markers that the density of the monomeric A24 is compared.

The crosslinked reconstitute and uncrosslinked chromatin were mixed in the presence of cesium formate and guanidine hydrochloride and centrifuged to equilibrium. On these denaturing gradients the histones (either crosslinked or uncrosslinked) are dissociated from the DNA (which pellets) and the histones not crosslinked to one another band independently (32). The gradient was fractionated, and the fractions desalted and electrophoresed (in the absence of β -mercaptoethanol) on an SDS-polyacrylamide gel. The stained gel of Figure 4 shows the distribution of unsubstituted A24; as expected banding occurs in the light region of the gradient, near the core histones. It was previously shown that crosslinking *per se* does not affect the equilibrium position of histones on these gradients (32). We observe, however, that histone H1 reproducibly bands lighter than the nucleosome core histones (and exogenous BSA bands denser; not shown) on these gradients. The characteristic banding of H1 is independent of density substitution and is presumably due to a differential affinity for Cs^+ ion (34). It is important, therefore, to note that the distribution of A24 is not aberrant, and that unsubstituted A24 bands with the unsubstituted core histones (Figure 4A). The fluorogram of this gel (Figure 4B) shows that the [^3H] labeled A24 band peaks midway between the [^3H] labeled dense core histones and the position of light histones or light A24. Its density is comparable to that of the H2A-H2B dimer [which, due to the mixing of heavy and light histones during reconstitution must be heavy-light; compare Figure 4A]. We conclude that this A24 is of hybrid density on the basis of its banding relative to these markers and to the totally dense A24 which accumulates during a 3 hour density pulse (see below). Since nascent H2A and nascent ubiquitin may both be incorporated into A24 the present results suggests that most nascent ubiquitin has become bound to preexisting H2A and most nascent H2A has become bound to preexisting ubiquitin.

To examine specifically the fate of nascent ubiquitin, cells were labeled with [^{35}S] methionine (H2A has no methionine in these

Fig.4 Incorporation of Density-Labeled H2A and Ubiquitin Into A24. Cells were pulsed with [^3H] lysine during a one hour incubation in density-substituted medium. Chromatin was extracted, dissociated, reconstituted, crosslinked and centrifuged to equilibrium on a denaturing isopycnic cesium formate-guanidine hydrochloride density gradient (Methods). (A) Coomassie stained 15% polyacrylamide-SDS gel showing the distribution of light proteins. (B) Fluorogram of (A) showing the distribution of tritiated proteins labeled during the density pulse. DENSE ($\rho = 1.29 \text{ gm/ml}$) and LIGHT ($\rho = 1.24 \text{ gm/ml}$) represent peak positions of [^3H] and [^{14}C] markers in parallel gradients. Assignment of crosslinked and uncrosslinked bands was on the basis of second-dimension gels run after reversal of crosslinks with β -mercaptoethanol (not shown).

cells) for the final 30 minutes of a 1 hour incubation in the presence of density-substituted amino acids, chromatin was prepared and submitted to CsFo-GuCl isopycnic centrifugation. Figures 5 and 6 show that essentially

Fig.5 One-Hour Incorporation of Density-Labeled Ubiquitin Into A24. Cells were pulsed with $[^{35}\text{S}]$ methionine during the final 30 minutes of a one hour incubation in dense medium. Chromatin was extracted and centrifuged to equilibrium on CsFo-GuCl gradients (Methods). (A) Coomassie stained gel. (B) Fluorogram of (A) showing the distribution of $[^{35}\text{S}]$ methionine incorporated into protein.

all of the $[^{35}\text{S}]$ labeled A24 bands in the hybrid density region of the gradient. If only nascent H2A and nascent ubiquitin were condensed together, all of the $[^{35}\text{S}]$ labeled A24 should appear in the dense region of the gradient. If nascent ubiquitin had been distributed equally between new and old H2A [e.g. near the replication fork (33,35-40)], 50% of the $[^{35}\text{S}]$ labeled A24 would appear in the dense region of the gradient and 50% in the hybrid region. Clearly, the data satisfy neither of these models; nearly all the nascent ubiquitin is bound to preexisting H2A.

When the $[^{35}\text{S}]$ methionine was added for the final 30 minutes of a three hour density pulse (Figure 7), densitometry of the A24 band across the fluorogram (Figure 6) indicated 25-30% of the $[^{35}\text{S}]$ label in the dense region. This agrees with an estimated 27% of H2A density-labeled in a 3 hour incubation, suggesting that nascent H2A is incorporated into A24 in proportion to its fraction of total H2A, and that nascent ubiquitin may become crosslinked to H2A more than 3 hours after nucleosome assembly.

Fig.6 Quantitation of A24 Distributions After Isopycnic Centrifugation. The relative distribution of A24 across (A) the Coomassie blue stained gel of Figure 5A; (B) the fluorogram of Figure 5B; (C) the fluorogram of Figure 6. Densitometry was performed using a Gelman Model ACD-18 densitometer.

Fig.7 Three-Hour Incorporation of Density-Labeled Ubiquitin Into A24. Fluorogram of a 15% polyacrylamide-SDS gel showing the distribution of [³⁵S] methionine incorporated during the final 30 minutes of a three hour density pulse.

DISCUSSION

We have utilized peptide mapping, COOH-terminal analysis and equilibrium density gradient ultracentrifugation of density-labeled proteins to study the assembly of semihistone A24. By adding SDS-polyacrylamide gel electrophoresis to the latter technique, the resolution with respect to specific crosslinked products is substantially improved, as demonstrated by the ability to distinguish the half-dense H2A-H2B dimer from dense H2A (H2B) or hybrid A24 (35:65, dense ubiquitin:light H2A) from completely light H2A (or A24). These results confirm the previous report of the sensitivity of these gradients (32). Electrophoretic analysis furthermore permits inclusion of internal standards on each gradient.

Our data suggest that newly synthesized histone H2A molecules are not preferential targets for assembly into semihistone A24. Conversely, H2A molecules are assembled into A24 more than 2-3 hours after their deposition on DNA. Our present results are therefore consistent with the conclusions of Seale (25) and Matsui et al. (19) that H2A molecules may acquire ubiquitin independently of DNA replication. The conjugation of ubiquitin with nucleosomal H2A molecules therefore represents a major modification of nucleosomal structure which may occur on both newly assembled and preexisting chromatin.

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