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Rapid turnover of the histone-ubiquitin conjugate, protein A24

Ronald L.Seale

Department of Cellular Biology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

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ABSTRACT

The specific activity of protein A24 was found to exceed that of the core histones by 2-3 fold following a brief labeling period. Accordingly, the A24 protein was found to be unstable, with a decay half-life of 90 minutes. When decay of the ubiquitin moiety was measured, it was found to turn over more extensively than the H2A moiety.

INTRODUCTION

The prevailing rationale to the study of chromatin structure is that the knowledge obtained will lead to the understanding of chromatin functions. Since a subset of chromatin undertakes a given function at any time, it would seem fruitful to examine the properties of that subset. A special opportunity toward this goal may obtain with the unique and extreme core histone variant, protein A24 (reviewed in 1).

Among the numerous modifications to histone proteins, the conjugation of ubiquitin to H2A via an isopeptide linkage (2,3) is by far the most site-specific, and the most radical structural modification known. This branched protein is a nucleosomal core histone (4-6); and engages approximately 10% of H2A (4). Such a unique nucleosomal structure would be expected to impart unique function; the data presented by this report indicate that A24 undergoes exceptionally high turnover in exponentially growing HeLa cells.

METHODS

HeLa cells were grown in suspension culture in Eagle's

MEM supplemented with 5% calf serum and 4 g dextrose/l. For uniform labeling, 0.5 μCi/ml of the appropriate labeled amino acid was added to complete medium for 18-24 hours. For pulse-labeling, cells were washed and incubated at a 10-fold increased density for 15 minutes in medium depleted of the appropriate amino acid, followed by a second wash and 15 minute incubation prior to addition of isotope. This treatment did not affect the incorporation of ^{3}H-leucine for over one hour. The labeling period was terminated by addition of unlabeled amino acids to a 10-fold concentration greater than that in the complete medium. In prolonged chase experiments, after 30 minutes the cells were collected by centrifugation and resuspended at a density of 5 x 10^{5}/ml in MEM for the duration.

Cells were ruptured by first swelling in hypotonic buffer (10 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM mercaptoethanol) and then mechanical lysis in a Dounce homogenizer. After two washes in buffer, $CaCl_2$ was added to 10^{-4} M, nuclei were warmed to 37°C for 4 minutes and micrococcal nuclease (Sigma) was added for 105 seconds at 0.1 unit/A_{260} unit of chromatin (measured in 1% SDS). Digestion was stopped by addition of EGTA to 1 mM and cooling to 4°C, and nuclei were collected by centrifugation for 5 minutes at 4°C in an Eppendorf microfuge.

Non-histone proteins, H1, nuclear envelope and nuclear matrix were removed by slow addition of NaCl with vigorous mixing to a final concentration of 0.45 M. Debris was removed by centrifugation for 10 minutes in an Eppendorf centrifuge. The supernatant, containing 95% of the chromatin plus salt-dissociated proteins, was added to 5 ml of stripping buffer (0.45 M NaCl, 2 mM EDTA, 5 mM Tris-HCl, pH 7.6) at 4°C. This was layered over a 2 ml cushion of 50% w/w sucrose in striping buffer and spun at 45,000 rpm for 65 hours in the Beckman Ti50 rotor. The pellet contains stripped polynucleosomes.

Histones were extracted by addition of H_2SO_4 to 0.2 N, centrifugation of precipitated DNA, and removal of the soluble histone supernatant. Histones were precipitated by addition of TCA to 25%, the precipitate was washed with acetone, and then dissolved in electrophoresis buffer containing 5% glycerol

and bromphenol blue. Samples were subjected to electrophoresis in SDS-polyacrylamide gels (20), and stained with coomassie blue. Individual bands were excised, digested with 0.25 ml NCS (Amersham) at 50°C for 3 hours, and then counted.

RESULTS

When total chromatin proteins, or acid extracted proteins (not shown) are resolved in SDS-polyacrylamide gels, protein A24 is closely flanked by proteins of similar electrophoretic mobility (Figure 1). When chromatin is stripped by washing in 0.45 M NaCl, these contaminating proteins are removed, and the specific activity of A24 can be measured directly by excision from the gel.

The first indication that A24 may have a unique metabolism was found during measurement of the kinetics of association of pulse-labeled histones with chromatin (Figure 2). Cells labeled 18 hours with ^{14}C-lysine were incubated with ^{3}H-lysine for 2 minutes. To initiate chase conditions, unlabeled lysine was added at the zero time. This chase is effective for at least 8 hours (7), as determined by whole cell radioactivity, and the specific activity of the core histones. Specific

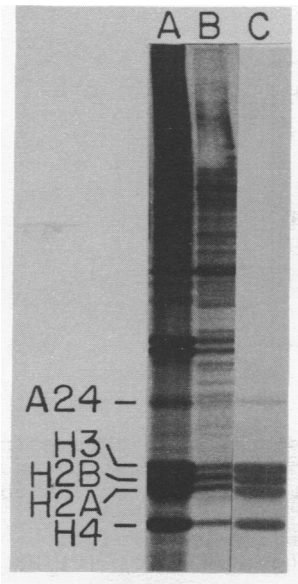

Figure 1. Electrophoretic separation of nuclear proteins in SDS-polyacrylamide gels (20). After labeling with ^{3}H-lysine, chromatin was fractionated into micrococcal nuclease-soluble (Panel A) and insoluble chromatin fractions (Panel B). Panel C shows the proteins remaining after a 0.45 M NaCl wash of nucleosomes. After electrophoresis, the gel was prepared for fluorography and exposed to film (21).

activity is expressed as $^{3}H/^{14}C$ for individual proteins. At the onset of the chase, about 40% of histones were already chromatin-associated. H2A and H2B enter more rapidly than H3 and H4; the duration of accumulation is about 10 minutes. This temporal difference (24) is possibly a reflection of the tenuous association of these newly synthesized histones in chromatin (24,25). Although the period of A24 accumulation was similar, the specific activity attained was 2.5-3 times that of the core histones. Since the specific activity of the core histones represents metabolic stability (8-11), it was suspected that A24 undergoes turnover.

In order to measure turnover, the pulse-chase protocol was extended 7 hours. Cells prelabeled with ^{14}C-lysine were pulse-labeled with ^{3}H-lysine for 2 minutes as in Figure 2. The specific activity rose immediately as the newly synthesized protein accumulated in the nucleus in the initial 10 minutes, and after that time the A24 specific activity decayed rapidly (Figure 3). Decay was biphasic; the half-life in the initial phase was 90 minutes, and the second decay half-life was 7.5 hours and was apparently still underway at the termination of the experiment. The stability of pulse-labeled radioactivity in histone H3 is also shown in Figure 3. After initial

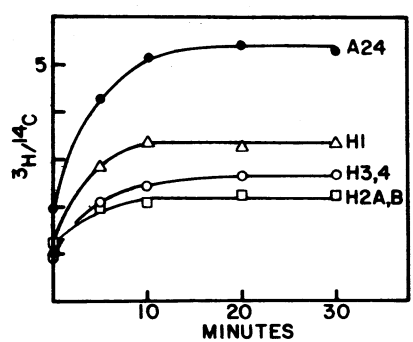

Figure 2. Kinetics of association of core histones with chromatin. HeLa cells were prelabeled with ^{14}C-lysine for one generation and then with ^{3}H-lysine for 2 minutes. At this time excess unlabeled lysine was added and sampling begun. Nuclei were isolated, digested, and the proteins separated as in Figure 1, Panel C. Individual bands were excised and counted. (□) H2A, H2B; (O) H3, H4; (Δ) H1; (●) A24.

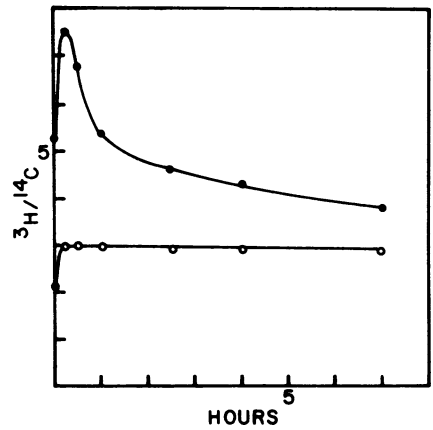

Figure 3. Decay of protein A24. Cells were labeled as in Figure 2, and samples were taken over 7 hours. (●) A24; (○) H3.

accumulation of H3 in chromatin, the radioactivity was stable. The stability of radioactivity in histone H3 also demonstrates the efficacy of chase conditions toward suppression of further ^{3}H-lysine incorporation.

The experiment described in Figure 3 measured decay in both moieties of A24. It is possible to measure the decay of the ubiquitin moiety, since it has an N-terminal methionine, whereas the H2A does not. For this experiment, cells were prelabeled with ^{3}H-lysine, and pulse-labeled for 5 minutes with ^{35}S-methionine (Figure 4). Specific activity is expressed as $^{35}S/^3H$. The decay of the ubiquitin moiety in A24 was also biphasic, and the two rates had similar half-lives to those in Figure 3, but decay in the first period was more extensive, resulting in a loss of nearly 70% of the initial radioactivity in the same period (Figure 3). The $^{35}S/^3H$ ratio for histone H3 in Figure 4 provides an internal reference for pulse-chase effectiveness.

DISCUSSION

From these data we are able to reach several important conclusions regarding the metabolism of protein A24 in exponentially growing HeLa cells. Newly synthesized ubiquitin

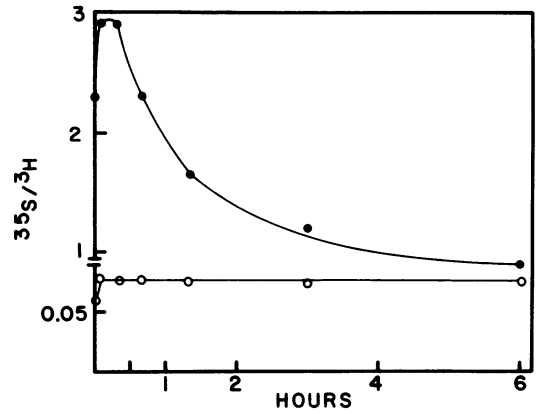

Figure 4. Decay of the ubiquitin moiety of A24. Cells were pre-labeled with ^{3}H-lysine and pulse-labeled with ^{35}S-methionine for 2 minutes. (●)A24; (O) H3.

and newly synthesized H2A [and H2B (22)] are preferentially conjugated, resulting in the high initial specific activity. The ubiquitin moiety turns over at a higher rate than the H2A moiety; thus, some H2A must be re-conjugated following ubiquitin cleavage. The radioactivity in the free H2A histone is depressed initially, reflecting preferential incorporation of new H2A into the A24 protein. However, persistence of radioactivity in A24 indicates that pre-existing A24 is also conjugated. Long-term labeled histones have an H2A radioactivity which reflects the stoichiometry of the core histones. This implies, but does not prove, that H2A freed from A24 by cleavage of the isopeptide linkage becomes a stable nucleosomal histone. There is another interpretation of this result which merits further consideration. The conjugation of ubiquitin to cytoplasmic proteins is a marker for turnover of those proteins (12,13); it remains possible that ubiquitin is performing the same function in chromatin. Thus, following nucleosome assembly, chromatin may be remodeled in a post-replicative event to produce the proper genetic disposition of the cell. In this alternative pathway, H2A synthesis would be correspondingly greater than that of the other core histones by 5-10%, to compensate for the destruction of that histone. An increase in the specific activity of H2A during

A24 decay would not discriminate between replacement due to cleavage by A24 lyase (23) and compensatory synthesis. Gurley and Hardin (9) demonstrated the metabolic stability of core histones; however, the labeling period would not detect short-term decay and stable replacement. These considerations also apply to H2B, since this histone has recently been demonstrated to be conjugated to ubiquitin also (22).

If the core histones conjugated to ubiquitin are not destroyed in A24 turnover, then this extreme structural variant could provide a signal for metabolic function within a chromatin domain. It would be of interest to know whether A24 marks such a domain, or whether A24 is common to the nucleosomes within that domain.

A24 is lost from chromatin prior to metaphase (15). Metaphase in HeLa cells occurs in one hour, out of a 24-hour cycle; thus, the turnover measured in this report is not explained by 4-5% metaphase cells within the population.

The presence of A24 has been correlated with a state of transcriptional quiescence. A24 content is lowered in rat liver nucleoli in cells stimulated by partial hepatectomy (16) or by thioacetamide administration (17). Correlations between decreased A24 content and transcriptional activity in chromatin fractions have been noted (18,19). In one report of conflicting evidence, erythropoiesis in phenylhydrazine treated chickens is accompanied by the appearance of A24 in chromatin (14).

This report indicates that A24 is continuously turned over in dividing cells and accentuates the unique metabolism of this structural nucleosomal protein, and indicates that chromatin remodeling occurs in a post-assembly event.

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