Levels of Histone H4 Diacetylation Decrease Dramatically during Sea Urchin Embryonic Development and Correlate with Cell Doubling Rate*

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Basic proteins in nuclei and nucleosomes at different stages of development in Arbacia punctulata sea urchins were analyzed directly by in situ protamine release of chromosomal proteins into Triton/acid/urea-polyacrylamide gels. The predominant protein band in the H4 region of 2-cell through 64-cell stage embryos migrates with the mobility expected for diacylated histone H4 (i.e., H4ac), whereas at blastulation (∼300 cells) the predominant H4 species is the unmodified form, H4o. In early embryos this H4o band is highly labeled with [3H]lysine with [3H]acetic acid. The ratio of H4ac:H4o is more than 20-fold greater at the rapidly dividing 2-cell stage than at pluteus stage. This is true for both newly synthesized H4 labeled with [3H]lysine and total H4 (stained). Enhanced acetylation is also found in nucleosomes. The relative amount of this acetylated H4 species correlates roughly with the rate of cell doubling during early embryogenesis, and decreases as the average nucleosomal repeat increases. The results are indicative of a dynamically changing chromatin structure through development, as well as an intimate role of diacylated histone H4 in the maturation of newly replicated chromatin.

Histone proteins are subject to post-transcriptional acetylation that modifies specific lysine residues clustered in the amino-terminal domains (for review, see Refs. 1-4). The H1, H2A, and H4 histones possess a permanent NH2-terminal serine acetylation, and lysyl residues of histones H2A, H2B, H3, and H4 are reversibly modified prior to or subsequent to deposition on the DNA (1, 2, 5). Although histone acetylation has been the subject of intensive research over the past 20 years, its function remains controversial (e.g. see Refs. 6 and 7). Acetylation of the N-terminal domains may modulate higher order chromatin structure by affecting internucleosomal interactions (8-12) or releasing constraints within the nucleosome (13), and is implicated in facilitating both transcription (14-18) and chromatin assembly (19, 20).

Sea urchin development provides unique periods for examining the role of acetylation in differentiation and development. From the 2-cell to 16-cell stages, the sea urchin embryo undergoes extremely rapid replication (21, 22). Slower rates of cell division characterize the embryo from the 16-cell stage to blastula (∼300-cell) stage. Concomitant with this rate reduction, greater embryonic transcriptional activity of gene families like histones (23, 24) and actin (25) occurs. During later stages of development, the overall rates of both replication and transcription decrease.

We have analyzed the basic nuclear proteins of sea urchin embryos at various stages of development with Triton/acid/urea-polyacrylamide gel electrophoresis (26, 27) to determine the levels of histone acetylation during development. We find large amounts of a protein migrating with the mobility of diacylated histone H4 in both nuclei and isolated nucleosomes of early stage embryos (28), with negligible amounts of tri- and tetra-acetylated H4. The amount of this diacylated histone H4 decreases dramatically during development and is proportional to the rate of cell doubling.

These results implicate a role for histone H4 diacetylation in replication and chromatin reorganization, and suggest that, in determining functional roles for acetylation, it may be important to distinguish between the different states of acetylation, with the diacylated form of H4 involved in replicating the chromatin. By deduction, the tri- and tetra-acetylated forms may be necessary for modulating chromatin domains to facilitate transcription (9, 29) in older embryos.

MATERIALS AND METHODS

Eggs of the sea urchin Arbacia punctulata were obtained by shedding induced either by injection of 0.5 M KCl or by direct removal of gonadal tissue of scissor-opened females. Eggs were filtered through a nylon net, washed three times in artificial sea water (Instant Ocean) and fertilized in artificial sea water with 1 mM 3-amino-1,2,4-triazole (Sigma). Unless indicated, embryos were grown until the middle of the appropriate cell stage and harveset. Twenty to twenty-five min prior to harvest, [3H]lysine was added (20-30 mCi/liter) to the growing embryos. Nuclei were isolated similar to the procedure described by Cognetti and Shaw (30), with the addition of 6 mM sodium butyrate added to all buffers following removal of the fertilization membrane. Briefly, the fertilization membrane was removed by passage through Nitex (44 μm) and the embryos were washed twice in 0.1 M LiCl, 0.1 M sodium phosphate, pH 6.0, with 24 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 6 mM sodium butyrate. After an additional wash in the same buffer without EDTA, they were homogenized, made 0.1% in Triton X-100, and pelleted at 2000 rpm for 5 min. In some cases, the supernatant (cytosol) was made 0.2 M in H2SO4, and after 30 min on ice centrifuged at 12,000 rpm for 30 min. The acid-soluble proteins of this cytoplasmic fraction were precipitated by the addition of 8 volumes of acetone and stored for several days at -75 °C. Nuclei were washed 3 times in the LiCl/sodium phosphate buffer (without EDTA) and spun through a cushion of 20% glycerol in the same buffer. Chromatin samples were obtained by homogenizing nuclei in 10 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0, followed by centrifugation at 10,000 rpm for 10 min (2 times). [3H]Acetate labeling was performed by growing embryos from mid 1-cell stage to mid 8-cell stage in sea water containing [3H]acetic acid (10 mCi/20 ml of highly concentrated embryos) supplemented with 1 mM glutamic acid and 1 mM aspartic acid. Fluoro-
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Fig. 1. Developmental profile of A. punctulata nuclear proteins. Electrophoresis of proteins from in situ protamine-released nuclei was carried out on Triton/acid/urea-polyacrylamide gels. Embryo stage is indicated above each lane. (The 32- and 64-cell stage are only approximations since certain cell types divide slower than others; MOR, morula; BLAST, blastula (~300 cells); GAST, gastrula; PLUT, pluteus.) H4a and H4 correspond to mobilities expected for diacetylated and monoacetylated H4, respectively. A, fluorograph of newly synthesized nuclear proteins labeled with [3H]lysine for 1/4 h prior to harvest. B, silver-stained proteins indicating bulk protein content of nuclei.

RESULTS

Triton/acid/urea-polyacrylamide gels can resolve the H4 region into five protein bands corresponding to acetylation of 0–4 lysyl residues. Analysis of the basic proteins in nuclei at different stages of development in A. punctulata indicates that the predominant protein band in the histone H4 region changes during development (Fig. 1). At early stages (2-cell through 32-cell) the predominant newly synthesized protein migrates at the position expected for diacetylated H4 (Fig. 1A) with little or no tri- and tetra-acetylated H4 present. At late stages of development, the amount of putative diacetyl-

graphs of [3H]acetate-labeled material required exposure times of two to three months at -70 °C with preflashed films. Either Autofluor (National Diagnostics) or 2,5-diphenyloxazole (31) was used for fluorography of all gels.

Triton/acid/urea-polyacrylamide gel electrophoresis was performed in the presence of 6 mM Triton X-100 and 8 M urea with the in situ protamine release method as described by Richards and Shaw (27). This method avoids the loss of histones commonly associated with acid extraction and allows their direct and quantitative analysis. Silver-staining was performed as described by Mold et al. (32), following staining with Amido Black. Micrococcal nuclease digestions and isolation of nucleosomes on sucrose gradients were performed as described by Shaw et al. (33).
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lated H4 decreases by a factor of more than 20 as compared with early stages, and proteins in the region of tri- and tetra-acetylated species become somewhat more prominent. These trends are true for both newly synthesized histone H4 labeled with [3H]lysine (Fig. 1A) and for bulk (i.e., total) histone H4 stained with either Amido Black (not shown) or silver (Fig. 1B). Thus, our results indicate that the state of modification of the bulk H4 in the nucleus is changing dramatically during development.

Upon analysis on a second-dimensional sodium dodecyl sulfate-polyacrylamide gel, the predominant band in the histone H4 region of early embryos migrates with the mobility expected for doubly modified H4 (Fig. 2). The two other proteins in the H4 region migrate as expected for the unmodified and singly modified H4 species, respectively.

In order to determine if the predominant doubly modified H4 protein was acetylated, cells were labeled with [3H]acetate to the 8-cell stage and harvested, and the nuclear proteins were electrophoresed on Triton/acid/urea gels. As seen in Fig. 3A, one major band is found in the H4 region. This labeled band is the predominant protein on the gel and migrates with mobility corresponding to the doubly modified form described in Fig. 1, indicating that the putative H4a protein in Fig. 1 is an acetylated species. Lesser amounts of the monoacetylated H4a band are also labeled with [3H]acetate, as expected. Fig. 3A also shows that little, if any, of the acetylated protein was isolated from the cytoplasm. We also did not see any change in mobility of the putative diacetylated H4 protein after treatment with bacterial alkaline phosphatase, suggesting that the protein was not phosphorylated, and in agreement with studies showing rapid and complete removal of phosphate groups after the histone enters the nucleus (1).

From studies on avian erythroid and hepatoma culture cells, it is known that newly synthesized histone H4 in the cytoplasm is in a doubly modified form that is subject to further processing by nuclear enzymes upon entering the nucleus (1, 5). Thus, it was important to establish that the diacetylated H4 in cleavage stage embryos was not simply free H4 arising from histone pools (34). The retention of such large amounts of diacetylated H4 in nuclei that had been washed in buffers of physiological ionic strength (i.e., 200 mM monovalent cat-

ions, cf. Fig. 1), as well as in chromatin washed in low ionic strength (data not shown), argues against this. Furthermore, as shown in Fig. 4, nucleosomes generated by a micrococcal nuclease digestion of 16-cell stage nuclei, and isolated from sucrose gradients, showed high levels of diacetylation, whether labeled from fertilization or just prior to isolation. Therefore, the changes in H4 are not simply due to differences in the pool size, but reflect changes in the bulk acetylation levels of nucleosomes during development.

Since the early cell stage proteins in Fig. 1 were isolated midway between cell divisions, it was possible that this H4 acetylation reflected a brief temporal process occurring during

**Fig. 2.** Fluorograph of second dimensional analysis of 8-cell stage A. punctulata nuclear proteins labeled with [3H]lysine from fertilization until harvest. First dimension on Triton/acid/urea (TAU); second dimension of histone region on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. H4a and H4 correspond to diacetylated and monoacetylated H4, respectively.

**Fig. 3.** A, fluorograph of [3H]acetate-labeled proteins from A. punctulata 8-cell stage cytoplasm, nuclei, and chromatin. The cytoplasmic fraction was acid extracted (27), as were the SP2 proteins that originated from S. purpuratus blastula stage embryos labeled with [3H]leucine and isolated in the absence of sodium butyrate. In this polyacrylamide gel system, H4 from A. punctulata and S. purpuratus have identical mobility. B, fluorograph of [3H]lysine-labeled proteins of 16-cell stage nuclei from A. punctulata (AP) and S. purpuratus (SP) embryos.
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16-Cell
Nucleosomes
1 2 3

16-Cell
Whole Nuclei
4 5 6

H2A
H3
H2B
H4α
H4α
H4α

FIG. 4. Fluorograph of proteins of nucleosomes after sucrose gradient centrifugation of a micrococcal nuclease digest of 16-cell stage A. punctulata embryos. Lanes 1-3 are nucleosomes; lanes 4-6 are whole nuclei. Nuclear proteins of lanes 1 and 4 were labeled with [3H]lysine from fertilization to harvest at the 16-cell stage and digested for 2 min; nuclear proteins of lanes 2 and 5 were labeled from 8-cell to harvest at the 16-cell stage and digested for 2 min; nuclear proteins of lanes 3 and 6 were labeled from 8-cell to harvest at the 16-cell stage and digested for 5 min. All cultures were chased with 10 mM lysine for 5 min prior to harvest.

rapid replication. However, the relative amount of H4 protein in the diacetylated state is constant throughout the cell cycle for a highly synchronous population of 8-cell stage embryos labeled and harvested at five different times during 1-cell doubling (data not shown).

The ratio of diacetylated H4 to unmodified H4 (Fig. 1) is more than 20-fold greater at the 2-cell stage than at pluteus stage for A. punctulata, as determined from densitometric scans of both gels shown in Fig. 1. Fig. 5 shows that the relative amount of diacytelylated H4 (as compared with total H4) correlates roughly with the average rate of cell division during early embryogenesis, suggesting that diacytelylation levels are closely related to the rate of cell doubling or proportion of chromatin undergoing replication at any one time in the embryo.

Whereas high levels of diacytelylated H4 are found in the rapidly replicating A. punctulata embryo, considerably smaller amounts of diacytelylated H4 are found in the early cell stages of the more slowly replicating species, Strongylocentrotus purpuratus, having 60–70 min/division during cleavage stages, i.e. twice as long as Arbacia (see Fig. 3B), even when the nuclear isolation is carried out in the presence of sodium butyrate. The preponderance of the diacytelylated form of histone H4 in the early cleavage stages of A. punctulata might be due to an inability of deacytelases to keep pace with the rapid replication or an adaptation by the organism to permit rapid replication of its chromatin.

**DISCUSSION**

Our results show that in A. punctulata embryos the diacytelylated state is by far the most predominant form of histone H4 in chromatin during the stages of development when the embryo is rapidly dividing. This diacytelylated state is not a transient phenomenon but is a comparatively long-lived form existing throughout most (if not all) of the cell cycle during the early cleavage stages. By pluteus stage, the ratio of H4α to H4 has decreased at least 20-fold and the acetylation pattern approaches that seen in chromatins of differentiated tissues. The marked difference in levels of diacytelylation of H4 between early and late embryos provides one of the most striking examples of the association between histone acetylation and replicational activity. The diminished proportions of the diacytelylated form in a sea urchin species experiencing much slower growth rates (S. purpuratus)1 (Fig. 3) suggest that this state might be either an adaptation to permit very rapid replication rates or a consequence of the inability of deacytelases to keep pace with very rapid replication. The hypothesis is supported by the observation that

1 S. A. M. Chambers and B. R. Shaw, unpublished data.
long exposure (2 h) of 16-cell stage Arbacia embryos to temperature extremes (0 °C) that inhibit cell division results in a substantial reduction of the diacylated state for histone H4.2

The presence of the diacylated form in nucleosomes (Fig. 4) indicates that the acetyl groups of nascent H4 molecules are not removed until after formation of the nucleosome, and that diacylated H4 may assist in forcing a configuration for correct formation of the nucleosome during early development. The high levels of diacylation in cleavage-stage nucleosomes indicate that the temporal changes in the steady state modifications of H4 are not due solely to internal pools accompanied by artifactual sticking of free H4 molecules to chromatin. Whereas it has been demonstrated that isolated nucleosomes can bind additional histones, such complexes are insoluble (40) under the low ionic strength conditions employed in our sucrose gradients (i.e. 10 mM Tris, 1 mM EDTA).

To solubilize complexes containing excess histones, it is necessary to raise the ionic strength much higher than that this form is important not only in the deposition of histones onto DNA but that nascent H4 remains diacylated in the nucleosome of certain cells after assembly. Relevant to our findings, recent studies indicate that histone deacylation is required for the maturation of newly replicated chromatin.

Previous work indicated that nascent H4 is rapidly modified and transported into the nucleus of replicating cells (1, 5, 41). The prominence of diacylated histone H4 in the bulk nucleosome population of 8-cell stage embryos (Fig. 4) implies that this form is important not only in the deposition of histones onto DNA but that nascent H4 remains diacylated in the nucleosome of certain cells after assembly. Relevant to our findings, recent studies indicate that histone deacylation is required for the maturation of newly replicated chromatin.

It is important to distinguish between three different states of H4 acetylation and the general roles that each state might play. 1) In mature chromatin, H4 exists mainly in an unacylated (i.e. hypoacylated) form. Terminally differentiated or quiescent nuclei have hypoacylated chromatin (38, 39). 2) When lysines in the NH2-terminal tails are acetylated, such that tri- and tetra-acetylated forms of H4 can be readily visualized on gels, the chromatin is hyperacetylated and thought to be primed for transcription (4, 29). 3) Intermediate between these extremes is the diacylated form of H4. The predominance of the diacylated form of histone H4 in early embryos in the studies presented here suggests that it is important to consider the role of histone H4 diacylation in chromatin structure as distinct from that of tri- and tetra-acetylation, since very low levels of tri- and tetra-acetylated H4 are detected in our studies. There may be several related roles for diacylation. Elegant early studies from the laboratories of Allfrey and Chalkley clearly indicated that histone H4 initially enters the nucleus in a diacylated or monoacetyl-monophosphorylated form which is rapidly degraded to the parental form (1, 5). Retention of this latter transport form may explain the doublet observed in the singly modified region of H4, in Fig. 1A. Following that early work, it has been assumed that the dimodified state is associated with the process whereby proteins enter the nucleus and are integrated into the chromatin (41). Our studies further imply that the diacylated form of histone H4 is intimately involved in replication and cell development.

The strong correlation of diacylation of H4 with higher replicational activity in early stage embryos (Fig. 5) suggests that the diacylated state of H4 may be involved in chromatin remodeling, possibly involving nucleosome transfer (43), or sliding. Formation of two acetyllysines in H4 results in a 14% decrease in the (+14) net average charge on each NH2 terminus. This reduction in charge upon diacylation may release constraints on the nucleosomes and allow a more open chromatin structure and/or permit shuttling of nucleosome octamers about the nucleus at a lower energy cost, thus facilitating rapid replication and chromatin reorganization in early embryos. Studies of chromatin structure in four other species of sea urchins (34-37 and Footnote 4) all indicate that the average nucleosomal repeat length increases during early development, which necessitates a wholesale reorganization of the chromatin structure. We note that in A. punctulata the time period of large decreases in levels of H4 diacylation corresponds with that of the large increases in nucleosomal repeat and suggest that diacylation may allow for transposition of the core along the DNA to produce the different repeat lengths observed during development. Changes in the repeat length and level of diacylation could be important prerequisites of growth and differentiation.

REFERENCES


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