

Histone acetylation in chicken erythrocytes

Rates of deacetylation in immature and mature red blood cells

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We analysed the rates of histone deacetylation in chicken mature and immature red blood cells. A multiplicity of deacetylation rates was observed for the histones and these rates may be subdivided into two major categories based on the extent of histone acetylation. In one set of experiments, cells were labelled with [³H]acetate in the presence of the deacetylase inhibitor *n*-butyrate, thereby accumulating radiolabel in the hyperacetylated forms of the histone. These hyperacetylated forms are deacetylated rapidly. [³H]Acetate-labelled tetra-acetylated H4 (H4Ac4) in mature cells was deacetylated with an initial half-life ($t_{1/2}$) of ~ 5 min (time required for the removal of one-half of the labelled acetyl groups). In immature cells, all [³H]acetate-labelled H4Ac4 was deacetylated with a $t_{1/2}$ of ~ 5 min. Erythrocytes were also labelled with [³H]acetate for extended periods in the absence of the deacetylase inhibitor. During this period, radiolabel accumulated predominantly in the mono- and di-acetylated forms of the histone. Using this protocol, the rate of deacetylation of H4Ac1 was observed to be ~ 145 min for mature cells, and ~ 90 min for immature cells, demonstrating that the less extensively acetylated histone is deacetylated slowly. These results are discussed in the context of the rates of histone acetylation in chicken red blood cells described in the companion paper [Zhang & Nelson (1988) *Biochem. J.* 250, 233–240].

INTRODUCTION

Histone acetylation is often accompanied by histone deacetylation (reviewed by Doenecke & Gallwitz, 1982), making this transient modification a candidate for involvement in dynamic processes such as transcription (Allfrey *et al.*, 1964) and replication (Annunziato & Seale, 1983). We have been particularly interested in the potential involvement of this histone modification in transcription, or transcription-related changes in chromatin structure, and have chosen the chicken red blood cell as a model system for studying this relationship. Chicken red blood cells cease replication before the mid-polychromatic stage (Williams, 1972), hence the residual histone acetylation and deacetylation observed in these cells (Sanders *et al.*, 1973) may be coupled to the maintenance of the active or potentially active chromatin structure as described in the companion paper (Zhang & Nelson, 1988).

Ruiz-Carrillo *et al.* (1976) suggest that histone acetylation is required to increase DNA availability in active chromatin regions, but is not directly involved in transcription. One might envisage the *N*-terminal arms of the core histones in association with the DNA on the outside of the nucleosome core (Van Holde *et al.*, 1974), these arms being accessible to enzymic modification. The affinity of the *N*-terminal arms for the DNA phosphates on the outside of the core particle would then be regulated by the extent of histone modification (Louie & Dixon, 1972). Histone acetylation converts positively charged lysine groups to neutral acetyllysines, with four modifiable groups on H3, H2B and H4, and one on H2A (Dixon *et al.*, 1975). Hyperacetylation of the histones would reduce the positive charge of the histone octamer

by 26, drastically reducing the ionic attraction between histone *N*-terminal arms and the DNA backbone.

Based on the above model, the regulation of the histone deacetylases could contribute directly to the degree of DNA accessibility. By locally reducing deacetylase action, the cell would increase the extent of acetylation within specific chromatin domains, thereby increasing DNA exposure. Reeves & Candido (1980) found that HMGs (high-mobility-group proteins) 14 and 17 inhibited deacetylase action *in vitro*, promoting a mechanism for enhanced DNA accessibility via the binding of these non-histone proteins to active chromatin. In this report, however, we show that all radiolabelled highly acetylated histone (i.e. H4Ac4) in the transcriptionally active immature chicken erythrocyte is very rapidly deacetylated, suggesting that histones are not locally maintained in a hyperacetylated form, but rather are transiently hyperacetylated and deacetylated. Based on the accessibility model, DNA would only be briefly exposed to the nuclear environment during transient acetylation, intimating that histone acetylation plays a dynamic role in active or potentially active chromatin conformation.

MATERIALS AND METHODS

Isolation and incubation of chicken erythrocytes

Mature and immature red blood cells were prepared as described in the companion paper (Zhang & Nelson, 1988). Cells were incubated in Swim's S-77 medium in the presence of [³H]acetate (5 mCi/40 ml; ICN, 5 Ci/mmol) and in the presence or absence of 10 mM-sodium *n*-butyrate. For subsequent analysis of deacetylation

Abbreviations used: H4Ac1, H4Ac2, H4Ac3 and H4Ac4, mono-, di-, tri- and tetra-acetylated histone H4.

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rates, cells were washed twice in cold (4 °C) Swim's medium (centrifugation at 500 *g* followed by gentle resuspension of the pellet), resuspended in the same medium containing 0.1 mM-sodium acetate at 37 °C, and portions removed during a time course of incubation at 37 °C.

Isolation and storage of nuclei: Triton/acid/urea- and SDS-gel electrophoresis

These methods are as described in the companion paper (Zhang & Nelson, 1988).

Fluorography and densitometry

As required, stained gels were scanned with a scanning densitometer (Kontes model 800) with an attached Hewlett Packard integrator. Gels were then prepared for fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975) and fluorograms also scanned with the Kontes densitometer.

RESULTS

Typically, cellular rates of histone deacetylation are measured by monitoring the rate of removal of [³H]acetate from the histone during a time course of incubation. The first step in these experiments is therefore to incubate the cells in medium in the presence of [³H]acetate to incorporate radiolabel into the histone. The cellular enzymic systems convert the [³H]acetate to [³H]acetyl-CoA, and subsequently transfer the acetyl moiety to a histone lysine residue. Cells are then further incubated in the absence of [³H]acetate and the specific activity of the histone monitored during this time course of incubation. One of the potential complications of measuring the rates of histone deacetylation is the re-utilization of [³H]acetate during the deacetylation incubation period. That is, after removal of [³H]acetate from the histone by the endogenous histone deacetylase, this radiolabel may again be converted to [³H]acetyl-CoA and re-incorporated into the histone, reducing the measured rates of deacetylation. We found this effect to be particularly pronounced in chicken red blood cells, in which CoA and acetyl-CoA concentrations are very low (Brotherton *et al.*, 1981). To prevent [³H]acetate re-utilization, 0.1 mM unlabelled acetate was included in the incubation medium during rate measurements, reducing the specific activity of the [³H]acetate within the cell to a negligible level before re-incorporation. The presence of the acetate in the Swim's medium does not appear to affect the action of the histone deacetylase. In a separate set of experiments, we found that the presence of 0.1 mM-acetate *in vitro* neither enhanced nor inhibited the activity of the partially purified chicken erythrocyte deacetylase (D.-E. Zhang & D. A. Nelson, unpublished work).

As described above, the rate of deacetylation of the radiolabelled erythrocyte histones was measured by monitoring the relative specific activity of these proteins during a time course of incubation of the cells in Swim's medium. At each incubation time point, cells were chilled on ice, nuclei isolated, and the samples electrophoresed directly on SDS/18% polyacrylamide gels. Coomassie Blue-stained gels were scanned with a densitometer, fluorograms prepared and also scanned, and the relative areas under the curves for the histones used to calculate the relative specific activity. These data were then used to generate graphs as shown for H4 in Fig. 1. The non-

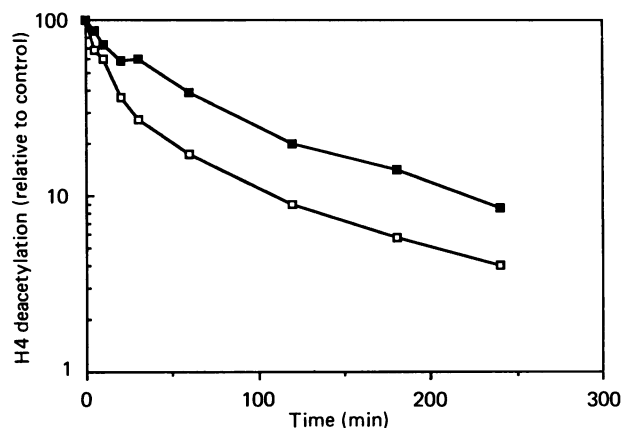


Fig. 1. Rates of H4 deacetylation in chicken erythrocytes

Mature (■) and immature (□) chicken erythrocytes were incubated with [³H]acetate and 10 mM-*n*-butyrate in Swim's S-77 medium for 180 min. Cells were then washed twice with cold Swim's medium and further incubated for 0, 5, 10, 20, 30, 60, 120, 180 and 240 min at 37 °C in the latter medium containing 0.1 mM unlabelled acetate. Nuclei were isolated, resuspended in SDS sample buffer, histones electrophoresed on SDS/18% polyacrylamide gels (stained with Coomassie Blue), and scanned with a densitometer. Fluorograms were prepared, densitometer-scanned and the relative specific activity of H4 calculated by dividing the area under the curve from the fluorogram, by the area under the curve obtained from scanning the stained gel. Values were normalized to an assigned relative specific activity of 100 at the start of the incubation. Each data point is the mean value obtained from scanning three separate polyacrylamide gels and fluorograms. Values are approximately ± 10%.

linearity of the log profile in Fig. 1 indicates that more than one rate of deacetylation is present for H4 in both the immature and mature red blood cells. We have specifically concentrated on the rate of deacetylation of H4, since it is well resolved from all other proteins in the densitometer scans. It appears that H4 from immature cells is slightly more rapidly deacetylated compared with H4 from the mature population.

To explore the nature of the multiple rates of deacetylation in the red blood cell, we made use of the following observations. 1) The more highly acetylated histone is more rapidly deacetylated in hepatoma tissue culture cells (Covault & Chalkley, 1980), and this result should apply to the chicken erythrocytes. 2) The extent of acetylation of radiolabelled histone can be controlled by the presence or absence of *n*-butyrate in the incubation medium during the [³H]acetate-labelling period. The latter observation is illustrated in Fig. 2, where cells have been radiolabelled in the presence or absence of *n*-butyrate, the histones electrophoresed on a Triton/acid/urea gel (which separates according to charge as well as size and shape), and the levels of radiolabelled H4 acetylation observed directly on a fluorogram. When cells are labelled in the presence of *n*-butyrate a considerable portion of the radiolabel is in the tetra-acetylated band (H4Ac₄). It should be pointed out that since cells were labelled in the presence of *n*-butyrate for H4 in Fig. 1, the multiplicity of deacetylation rates observed applies to H4 initially in a highly acetylated

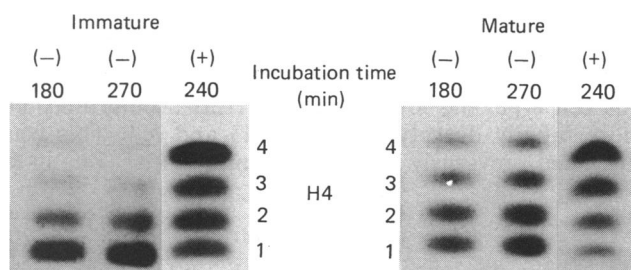


Fig. 2. Relative levels of acetylation of radiolabelled H4 in chicken erythrocytes incubated in the absence (-) and presence (+) of 10 mM-*n*-butyrate

Immature and mature chicken erythrocytes were incubated at 37 °C for the times indicated in the Figure (180, 270 and 240 min) in Swim's medium in the presence of [³H]acetate. Histones were electrophoresed on Triton/acid/urea gels and fluorograms prepared as shown in the Figure. The numbers 1, 2, 3 and 4 mark the positions of mono-, di-, tri- and tetra-acetylated H4, respectively.

state. On the other hand, when cells are [³H]acetate-labelled in the absence of *n*-butyrate, the radiolabel is predominantly in the mono- and di-acetylated H4 bands (Fig. 2). Thus this labelling protocol should allow the measurement of the rates of deacetylation of these lower levels of histone-acetate.

To measure the rates of deacetylation of the lower levels of H4-acetate, mature and immature red blood cells were labelled for 180 min with [³H]acetate in the absence of *n*-butyrate, the cells resuspended in fresh Swim's medium in the absence of radiolabel, and the relative specific activity of H4 monitored during a time course of incubation. The rate of H4 deacetylation using this protocol is shown graphically in Fig. 3 and can be directly compared to that illustrated in Fig. 1, where the

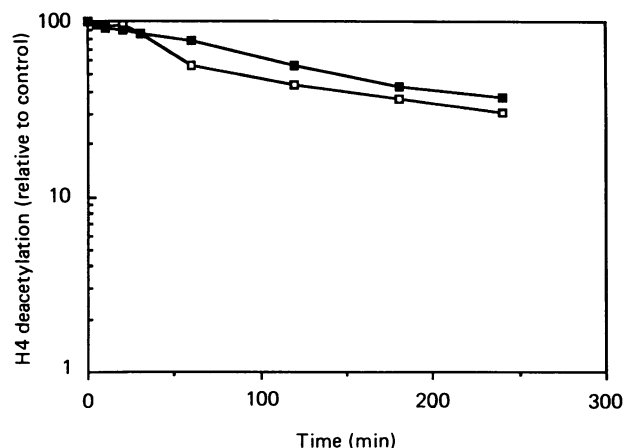


Fig. 3. Slow rates of deacetylation of H4 in chicken erythrocytes

Mature (■) and immature (□) chicken erythrocytes were incubated in Swim's medium for 180 min at 37 °C in the presence of [³H]acetate. Cells were washed twice with cold Swim's medium and further incubated for 0, 5, 10, 20, 30, 60, 120, 180 and 240 min at 37 °C in the latter medium containing 0.1 mM unlabelled acetate. Histones were electrophoresed on SDS/polyacrylamide gels, fluorograms prepared and the relative specific activity of H4 determined during the time course of incubation. Values are normalized to a relative specific activity of 100 at the start of the incubation.

radiolabelling incubation medium included *n*-butyrate. We observe a very slow rate of deacetylation of the low levels of H4-acetate, as observed previously for H4 in hepatoma tissue culture cells (Covault & Chalkley, 1980). The *t*_{1/2} (the time required to release one-half of the labelled acetate) is ~ 90 min for the immature and ~ 145 min for the mature erythrocyte. From these results,

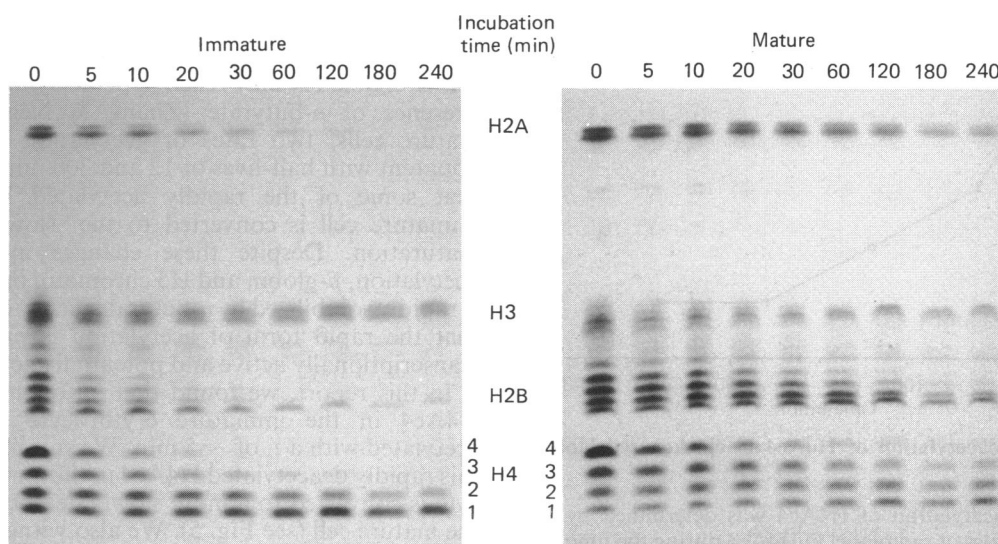


Fig. 4. Fluorographic analysis of histone deacetylation in chicken red blood cells

Immature and mature chicken erythrocytes were incubated for 180 min at 37 °C in Swim's medium containing [³H]acetate and 10 mM-*n*-butyrate. Cells were washed twice in cold Swim's medium and further incubated 0, 5, 10, 20, 30, 60, 120, 180 and 240 min at 37 °C in the latter medium containing 0.1 mM unlabelled acetate. Histones were electrophoresed on Triton/acid/urea gels and fluorograms prepared as shown in the Figure.

it is evident that the highly acetylated histone must be of the rapidly deacetylated form to yield the rapid rates of deacetylation observed in Fig. 1.

To confirm that the hyperacetylated histones are rapidly deacetylated, we directly measured the rate of deacetylation of H4Ac4 by monitoring the loss of radiolabel from this band during a time course of incubation. Cells were labelled with [³H]acetate in the presence of *n*-butyrate for 180 min (as for Fig. 1), resuspended in fresh Swim's medium, and incubated at 37 °C from 0–240 min. During the time course of incubation, portions were placed on ice and nuclei prepared. Histones were then electrophoresed on Triton/acid/urea gels and fluorograms prepared as shown in Fig. 4. The loss of radiolabel from the H4Ac4 band during the time course of incubation is shown graphically in Fig. 5, and this rate can be compared to those illustrated in Figs. 1 and 3. It is clear that the highly acetylated histone is rapidly deacetylated, with H4Ac4 from the immature cells deacetylated with a $t_{1/2}$ of ~ 5 min. The initial rate of deacetylation of H4Ac4 in the mature cell also has a $t_{1/2}$ of ~ 5 min; however, it is evident that not all of H4Ac4 is deacetylated at this rate. As the incubation continues, slower rates are observed for these hyperacetylated species. The reasons for this rate reduction in the mature erythrocyte are not known, but may be related to the reduced genomic activity as the cell becomes fully differentiated.

Visual inspection of the fluorograms in Fig. 4 suggests that all four core histones are deacetylated in a manner similar to that observed for H4. We have not, however, quantified these rates for the other three histones pictured in the fluorogram. One distinction already discerned between H4 in the mature and immature cells, which is the overall reduction in the deacetylation rate for mature

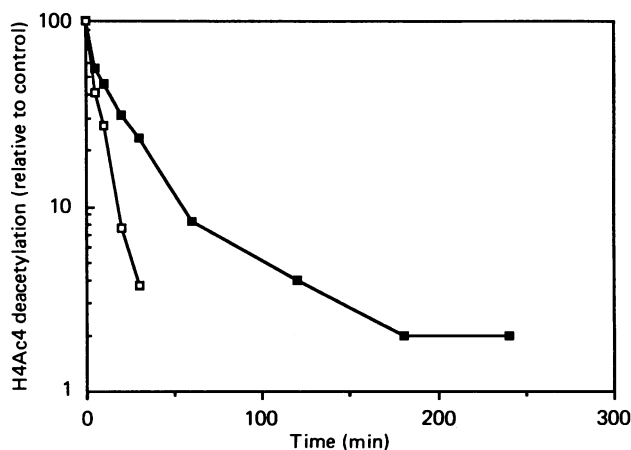


Fig. 5. Rates of deacetylation of H4Ac4 in chicken red blood cells

The rate of deacetylation of H4Ac4 was determined by monitoring the loss of radiolabel in H4Ac4 during the time course of incubation of mature (■) and immature (□) erythrocytes. The area under the curve for H4Ac4 in the fluorogram (Fig. 4) was divided by the total area under the curve for H4 on the Coomassie Blue-stained gel. These values were then normalized to a value of 100 at the start of the incubation.

H4, is also apparent for the other core histones. Secondly, most of the radiolabel in the H2B bands in the immature erythrocyte disappeared after 240 min, indicating that in this cell type, the lower levels of H2B-acetate may be more rapidly deacetylated than H3 and H4. This was suggested previously in the companion paper (Zhang & Nelson, 1988).

DISCUSSION

Erythropoiesis in chickens produces nucleated mature red blood cells that are inactive both in transcription and DNA synthesis. At the developmental stages just before cell maturity, DNA synthetic capacity is absent, but transcription is occurring on specific genes such as the α - and β -globins (Sanders *et al.*, 1973). These immature mid- and late-polychromatic erythrocytes may be produced in large quantities by inducing anaemia in otherwise healthy chickens. The immature and mature red blood cells represent a model system for investigating the relationship between histone acetylation and transcription, or transcription-related events, in the absence of other genomic activities such as mitosis and DNA replication.

The amount of [³H]acetate incorporated into histone during a 30 min incubation period is reduced in the mature compared with the immature erythrocyte (Sanders *et al.*, 1973), and we have confirmed a 3–4-fold reduction in the amount of acetate incorporated during this period (Zhang & Nelson, 1986). Only 3–4% of the total acetyltable lysine sites in the immature erythrocyte histone can be modified, this number of modifiable sites decreasing to 2–3% in the mature cell (Zhang & Nelson, 1986). It is likely that no more than 1–2% of the total histone is undergoing acetylation and deacetylation in the immature and mature erythrocytes, suggesting that only 1–2% of the erythrocyte genome is participating in dynamic histone acetylation.

There are concomitant changes in the rates of acetylation as the erythrocyte matures. In the immature cells, all of the H4 available for modification is of the rapidly acetylated form ($t_{1/2}$ ~ 12 min), although not all of this available H4 may reach hyperacetylated states in the presence of *n*-butyrate (Zhang & Nelson, 1988). In mature cells, two rates of acetylation of H4Ac1 are apparent with half-lives of 12 and 300 min. We postulate that some of the rapidly acetylated histone in the immature cell is converted to the 'slow' form during maturation. Despite these changes in the rates of acetylation, β -globin and H5 chromatin in both cell types is rapidly solubilized by *n*-butyrate incubation, suggesting that the rapid form of acetylation is present on both transcriptionally active and potentially active chromatin.

In this report, we found that all of the radiolabelled H4Ac4 in the immature erythrocyte is rapidly deacetylated with a $t_{1/2}$ of ~ 5 min. We surmise that some of this rapidly deacetylated H4Ac4 in the immature erythrocyte is converted to a more slowly deacetylating form in the mature cell (see Fig. 5). We also witnessed an overall reduction in the rates of deacetylation of total mature cell H4, as illustrated in Fig. 1, and in the slow rate of deacetylation, as shown in Fig. 3. We conclude that although the rate of histone deacetylation in the mature erythrocyte is reduced, the histones are not maintained in a hyperacetylated form. In both cell types, acetylation

and deacetylation are dynamic events, preferentially occurring on the active and potentially active chromatin.

We subscribe to the notion that histone acetylation regulates DNA accessibility. If the *N*-terminal arms of the histones bind to the outside of the nucleosome core as suggested by Van Holde *et al.* (1974), then histone acetylation would weaken histone-DNA interactions, exposing more of the DNA to the nuclear environment. Experiments with hyperacetylated chromatin and core particles demonstrate 1) increased DNAase I accessibility (Simpson, 1978), 2) enhanced chromatin solubility (Nelson *et al.*, 1980), presumably due to an increase in the overall net negative charge on the nucleohistone, 3) only a subtle weakening in core particle structure (Yau *et al.*, 1982; Imai *et al.*, 1986) and 4) only subtle changes in chromatin higher-order structure (McGhee *et al.*, 1983). These results are consistent with the idea that histone hyperacetylation does not drastically alter chromatin structure, but rather plays a role in enhancing the exposure of the DNA to the nuclear environment. Since dynamic histone acetylation and deacetylation are preferentially occurring on the active and potentially active regions in the red blood cell, we suggest that acetyltransferase and deacetylase enzymes, by modulating DNA accessibility, should be considered as potential transcription factors in this cell type.

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