Histone acetylation in chicken erythrocytes

Rates of acetylation and evidence that histones in both active and potentially active chromatin are rapidly modified

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Of the modifiable histone lysine sites, 2–4 % participate in dynamic acetylation in chicken erythrocytes, suggesting the involvement of no more than 1–2 % of the total genome. The rates and chromatin locality of this dynamic acetylation were studied in both chicken mature and immature red blood cells. In mature erythrocytes, two rates of acetylation of radiolabelled, monoacetylated H4 are observed, with half-lives of ~ 12 and ~ 300 min. In contrast, only one rate with a half-life ($t_{1/2}$) of 12 min is observed in immature cells, and further experiments rule out the possibility of a slow rate of acetylation (with a $t_{1/2}$ of ~ 300 min) for any form of H4 in this cell type. The simplest interpretation of these quantitative results, taken together with the behaviour of H3, H2B and H4 observed on the fluorograms used for rate analysis, is that a portion of the rapidly acetylated histone is converted to a more slowly acetylated form during erythrocyte maturation. The transcriptionally active adult β -globin and H5 nucleohistone, which are presumably converted to potentially active chromatin during the maturation process, remain of the rapidly acetylated form in the mature cell.

INTRODUCTION

Acetate is incorporated into histones in three distinct modes. The *N*-terminal serine residues of H1, H2A and H4 are permanently acetylated during biosynthesis in the cytoplasm (Phillips, 1963). H4 is additionally acetylated in the cytoplasm at two lysine positions, and this modification appears to be required for the deposition of this protein onto the DNA (Louie & Dixon, 1972). Thirdly, all four of the core histones may be acetylated as part of the assembled nucleosome, this modification occurring at approximately 13 different lysine residues within the *N*-terminal portions of these proteins (Hnilica, 1972). It is thought that the deposition-related H4 acetylation is performed by the cytoplasmic acetyltransferase B enzyme (Garcea & Alberts, 1980), whereas a second protein, the nuclear acetyltransferase A, acts on the DNA-bound histones (Belikoff *et al.*, 1980; Garcea & Alberts, 1980). Both deposition-related histone acetylation and acetylation within the assembled nucleosome are reversibly hydrolysed by the histone deacetylase enzyme(s).

The establishment of a function or functions for histone acetylation and deacetylation within the nucleus remains an elusive goal. Although there is evidence that histone acetylation is coupled to a variety of genomic events (for a recent review, see Wu *et al.*, 1986), a key question that remains is its relevance to transcription. If histone acetylation were established as a requirement for transcription, as proposed by Allfrey *et al.* (1964), it is evident that this modification could represent a controlling element in gene expression thereby elevating the nuclear acetyltransferase and deacetylase enzymes to the status of eukaryotic transcription factors.

To establish and strengthen the relationship between

histone acetylation and gene activity, we analysed this histone modification in chicken red blood cells. In anaemic chickens, the peripheral red blood cell population consists predominantly of mid- and late-polychromatic erythrocytes (Williams, 1972). These cells transcribe a restricted subset of the total genetic repertoire, including the adult α - and β -globin genes (Sanders *et al.*, 1973), and the H5 gene (Affolter & Ruiz-Carrillo, 1986). The circulating mature red blood cells of healthy chickens contain condensed (Ruiz-Carrillo *et al.*, 1974), transcriptionally silent (Sanders *et al.*, 1973) chromatin, although previously active chromatin regions remain in the potentially active conformation (Weintraub & Groudine, 1976). DNA synthesis (Williams, 1972) and non-H5 histone translation and deposition (Appels & Wells, 1972; Ruiz-Carrillo *et al.*, 1976) are thought to cease before the mid-polychromatic stage of erythrocyte differentiation. Histone acetylation and deacetylation, however, persist both in the mid- and late-polychromatic erythrocytes, and in the mature red blood cells from healthy animals (Sanders *et al.*, 1973; Ruiz-Carrillo *et al.*, 1976). Ruiz-Carrillo *et al.* (1976) conclude that the active nuclear histone acetylation and deacetylation in the erythrocytes cannot be coupled to chromatin replication or histone deposition. They maintain that histone acetylation is related to gene expression, suggesting that the presence of residual histone acetylation in the transcriptionally silent mature cell indicates a relevance to DNA availability in active chromatin regions, rather than a direct involvement in transcription. In support of this latter hypothesis, the authors demonstrate that transcription inhibitors have no effect on histone acetylation and deacetylation in the mid- and late-polychromatic erythrocytes, as well as in the mature cell.

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

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Active acetylation is detected on only a small subset of histone in the red blood cells (Brotherton *et al.*, 1981), and we estimate that no more than a few percent of the total histone in the mid- and late-polychromatic erythrocytes is involved in dynamic acetylation (Zhang & Nelson, 1986). This percentage appears to decline as the cells reach maturity. We previously presented evidence that active acetylation is preferentially occurring on the histones associated with the globin genes in the immature erythrocytes (Nelson *et al.*, 1986). We now report the rates of acetylation in the mature and immature red blood cells, and provide evidence that rapid histone acetylation is preferentially occurring on both the transcriptionally active and potentially active chromatin regions in these cell types. The results support the hypothesis that this active histone modification is not coupled to transcription itself, but is involved in the modelling of chromatin structure or in the alterations to DNA accessibility necessary for gene transcription.

MATERIALS AND METHODS

Preparation of cells and nuclei

White Leghorn chickens were purchased from Rich-Glo Lab Animals (El Campo, TX, U.S.A.). For the preparation of immature red blood cells (predominantly mid- and late-polychromatic erythrocytes), chickens were fasted for 24 h followed by daily injections of 1.2 ml of 1% phenylhydrazine (1% phenylhydrazine in 10 mM-phosphate buffer, adjusted to pH 7.2 and filter sterilized). After 7–9 days, greater than 98% of the cells were typically immature erythrocytes as assayed by light microscopy of Brilliant Cresyl Blue-stained cells [1% Brilliant Cresyl Blue in 0.85% saline, filtered and used 1:1 (v/v); Platt, 1979]. Blood was collected from healthy and anaemic birds by heart puncture using heparinized syringes [approx. 50 units of heparin (Sigma) per ml of whole blood]. Cells were pelleted at 500 *g* for 10 min and the plasma removed by vacuum aspiration. Erythrocytes were resuspended in Swim's S-77 medium (pH 7.2) and washed twice by centrifugation and resuspension in the tissue culture medium. During this period the white-blood-cell layer (buffy coat) above the pelleted erythrocytes was removed with a Dispo pipette. Cells resuspended in Swim's S-77 medium were incubated at 37 °C as described in the text and Figure legends. [³H]Acetate (ICN, 5 Ci/mmol) was used at a concentration of 5 mCi/40 ml of Swim's medium.

After incubations, erythrocytes were centrifuged at 500 *g* and resuspended in NIB (Nuclear Isolation Buffer: 0.25 M-sucrose/60 mM-KCl/15 mM-NaCl/3 mM-MgCl₂/1 mM-CaCl₂/15 mM-Mes/10 mM-sodium *n*-butyrate/0.1 mM-phenylmethanesulphonyl fluoride/0.5% Triton X-100, pH 6.6). Samples were vortexed and nuclei pelleted at 2000 *g*. Nuclei were washed twice in the same buffer, resuspended in NIB (without Triton X-100) containing 25% glycerol, and stored at –80 °C.

Histone preparation and analysis

Triton/acid/urea gels. Chicken erythrocyte nuclei were pelleted and resuspended in 0.0375 M-NaCl/0.0125 M-EDTA, pH 8.0, at 1 mg of histone/ml. Histones were then extracted with protamine sulphate as described by Loidl & Grobner (1986) and 20–40 µl of the extract was loaded directly onto pre-electrophoresed 14% poly-

acrylamide gels containing 8 M-urea/5% C₂H₅OH/0.35% Triton X-100 (Urban *et al.*, 1979). The 23 cm gels were electrophoresed at 200 V for 32 h and fluorograms prepared as described previously (Nelson *et al.*, 1986).

SDS gels. Chicken erythrocyte nuclei were pelleted, resuspended in SDS sample buffer (1% SDS/10 mM-Tris/2 mM-EDTA/1% 2-mercaptoethanol/0.005% Bromphenol Blue/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.0) and 20–40 µl loaded onto SDS/18% polyacrylamide gels as described previously (Nelson, 1982). The 13 cm gels were electrophoresed at 180 V for 10 h and fluorograms prepared.

Fluorograms and Coomassie Blue-stained gels were scanned with a Kontes model 800 scanning densitometer and areas under the curve determined with an attached Hewlett Packard integrator.

Micrococcal nuclease digestion and DNA analysis

Nuclei resuspended in NIB (without Triton X-100) at 70 *A*₂₆₀ units/ml were digested at 37 °C with micrococcal nuclease at 2 units/*A*₂₆₀ unit for 10 min. DNA was purified, incubated with alkali to remove RNA, electrophoresed on 1% agarose gels, transferred to nitrocellulose, hybridized to nick-translated DNA probes and autoradiograms prepared as described previously (Ferenz & Nelson, 1985). The plasmid DNA probes were pHB1001 (containing chicken β^A cDNA sequence; Salser *et al.*, 1979), p2.6H5 (containing 2.6 kb genomic chicken H5 sequence; Krieg *et al.*, 1983) and pls-1 (containing chicken lysozyme cDNA sequence; Sippel *et al.*, 1978).

RESULTS

Rates of histone acetylation in chicken mature erythrocyte

Chicken red blood cell histones can be divided into two major categories with respect to acetylation and deacetylation (Brotherton *et al.*, 1981; Zhang & Nelson, 1986). Approximately 98–99% of the histone in the mature erythrocyte does not participate in active acetylation. These histones are apparently 'frozen' at the levels of modification *in vivo* observed on stained Triton/acid/urea gels. The remaining 1–2% of the histone appears to be dynamically acetylated and deacetylated, and it is the rates of histone acetylation in this small portion of the histone population in the mature erythrocyte that we have measured using the protocol of Covault & Chalkley (1980).

Isolated mature red blood cells were incubated at 37 °C in Swim's medium in the presence of [³H]acetate. The radiolabelled acetate was removed by washing the cells twice in cold Swim's medium plus 10 mM-*n*-butyrate and 0.1 mM unlabelled acetate, and the cells were further incubated at 37 °C for 0–240 min in the presence of *n*-butyrate and unlabelled acetate. During the initial 37 °C incubation, the small percentage of histone population dynamically acetylated and deacetylated is labelled with [³H]acetate. The subsequent incubation at 37 °C requires the presence of *n*-butyrate to inhibit histone deacetylation (Riggs *et al.*, 1977), as well as a small amount of unlabelled acetate to dilute any residual unincorporated radiolabel. The unlabelled acetate may also be required for the formation of acetyl-CoA, one of the substrates of

Fig. 1. Fluorographic analysis of histone acetylation in chicken mature red blood cells

(a) Mature erythrocytes were incubated for 15 min with [^3H]acetate, washed twice with cold Swim's medium containing 10 mM-*n*-butyrate and 0.1 mM unlabelled acetate, and further incubated for 0, 10, 20, 30, 60, 120, 180 and 240 min at 37 °C in the latter medium. Histones were electrophoresed on a Triton/acid/urea gel and a fluorogram prepared. (b) The same protocol was followed as for (a), except that cells were incubated with [^3H]acetate for 180 min.

the reaction. Histone from isolated nuclei was then electrophoresed on Triton/acid/urea gels and fluorograms prepared as shown in Fig. 1. Both short (15 min) and long (180 min) radiolabelling periods are used to observe and quantify rapid and slow rates of histone acetylation.

The fluorograms in Fig. 1 show that radiolabel is incorporated into H3, H2B and H4, and that as the *n*-butyrate incubation progresses, there is the expected increase in the amount of radiolabel in the more highly acetylated forms of the histone. H3, H2B and H4 are acetylated at four sites (Doenecke & Gallwitz, 1982), and thus the rates of acetylation of these histones may be analysed by the method of Covault & Chalkley (1980). H4, however, is most suitable for this analysis because of the consistent high resolution of the H4 bands. H2A is also radiolabelled, but is not included in the Figures since

Fig. 2. Rates of H4 acetylation in chicken mature erythrocytes

The H4 bands in Fig. 1, as well as in samples incubated for 45, 90 and 270 min with [^3H]acetate, were scanned with a densitometer and the percentage of radiolabel in the monoacetylated form was determined for H4 in each lane. The percentage of H4Ac1 at zero time was set equal to 100% as described previously (Covault & Chalkley, 1980).

it is acetylated at only one site and is not suitable for this analysis.

As described by Covault & Chalkley (1980), the reduction in the percentage of radiolabel in monoacetylated H4 (H4Ac1) during the incubation in *n*-butyrate, is one measure of the rate of acetylation. Acetate groups attached to the histone during the *n*-butyrate incubation are unlabelled. Thus, as radiolabelled H4Ac1 is further modified, there is no contribution to the H4Ac1 band intensity on the fluorogram from the non-acetylated protein. The H4 regions of the fluorograms shown in Fig. 1 were scanned with a densitometer and the fraction of radiolabel in H4Ac1 determined for H4 in each lane. The percentage of radiolabel in H4Ac1 was then plotted against *n*-butyrate incubation time using a series of initial radiolabelling periods as illustrated in Fig. 2.

The two slopes on the log plot indicate two rates of acetylation of radiolabelled H4Ac1. As would be predicted for a system with fast and slow components, the rapid and slow proportions of labelled H4Ac1 depend on the length of labelling time. The rapidly labelled form of H4Ac1 is no longer visible in Fig. 2 when [^3H]acetate incubation periods are 90 min or longer. The slow rate of H4Ac1 acetylation can be estimated from the 270 min labelling period, and yields a $t_{1/2}$ of ~ 300 min (time at which one-half of the radiolabelled, slowly acetylated, H4Ac1 is removed from the H4Ac1 band).

To reliably quantify the fast rate of acetylation, a short, 10 min [^3H]acetate labelling period was used as

Fig. 3. Rates of H4 acetylation in mature erythrocyte after 10 min of [^3H]acetate labelling

Mature erythrocytes were incubated for 10 min with [^3H]acetate and then further incubated for 0–240 min in the presence of *n*-butyrate and unlabelled acetate as described in Fig. 1. A fluorogram was prepared, the H4 region of the fluorogram scanned with a densitometer, and the percentage of H4Ac1 determined as in Fig. 2.

illustrated in Fig. 3. The fast rate is apparent during the first 30 min of *n*-butyrate incubation, and the $t_{1/2}$ is calculated to be ~ 12 min. This rate is similar in magnitude to the fast rate of acetylation reported for hepatoma tissue culture cells ($t_{1/2}$ 7 min; Covault & Chalkley, 1980).

In summary, there are two rates of acetylation of radiolabelled H4Ac1 in mature erythrocytes with half-lives of ~ 12 and ~ 300 min. Although rates of acetylation were not calculated for other H4 forms, or for other histone species, the results nevertheless provide a satisfactory quantitative measure of the rates of acetylation. Visual inspection of the other labelled histones in the fluorograms (Fig. 1) suggests that their rates are similar. As described previously (Zhang & Nelson, 1986), the dynamically acetylated and deacetylated histones comprise no more than 1–2% of the total mature cell histone, thus representing a special class of protein within this cell type. Of this special class of dynamically acetylated histone, it is clear that only a portion is of the rapidly acetylated variety.

Rates of histone acetylation in chicken immature erythrocyte

The red blood cells isolated from anaemic birds consist of approximately 40% mid- and 55% late-polychromatic erythrocytes (Williams, 1972). Most of the remainder of the cells in this population are mature erythrocytes, with only 0.4% of the immature cells actively synthesizing DNA and histone at earlier stages of erythroid differentiation.

The same protocols were followed to measure the rates of histone acetylation for the immature erythrocytes, and Fig. 4 depicts the resultant fluorograms for the 15 and 180 min [^3H]acetate labelling periods. The immature cells in Fig. 4(a) were labelled with [^3H]acetate for 15 min. It is evident that after this short labelling period, there is an increase in the amount of radiolabel in tetra-acetylated H4 (H4Ac4) after only 10–20 min incubation in *n*-butyrate. Thus a fast rate of histone acetylation is present

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

(a) Immature erythrocytes were incubated for 15 min with [^3H]acetate, washed twice with cold Swim's medium containing 10 mM *n*-butyrate and 0.1 mM unlabelled acetate, and further incubated for 0, 10, 20, 30, 60, 120, 180 and 240 min at 37 °C as in Fig. 1. Histone was electrophoresed on Triton/acid/urea gel and a fluorogram prepared. (b) The same protocol was followed as for (a), except that cells were incubated with [^3H]acetate for 180 min.

in the immature red blood cells. However, continued incubation in the presence of *n*-butyrate (for 30–240 min) does not substantially alter the pattern of radiolabelled H4; an indication that a slow rate of acetylation may be absent in this cell type. In contrast to the mature erythrocyte results (see Fig. 1a), approximately 35% of the radiolabelled H4 remains in the monoacetylated form for the duration of the *n*-butyrate exposure.

The absence of a slow rate of acetylation in the immature red blood cells becomes more apparent in Fig. 4(b). For this experiment, cells were labelled for 180 min with [^3H]acetate, and it is clear that most of the radiolabelled H4 remains in the mono- or di-acetylated form, even after exposure to *n*-butyrate for 240 min. There is no movement of radiolabelled H4 to the more highly acetylated forms; a strong indication that a slow rate is absent. A number of other features are also notable in the fluorogram depicted in Fig. 4(b). First,

Fig. 5. Rates of H4 acetylation in chicken immature erythrocytes

The H4 bands in Fig. 4, as well as in samples incubated for 45, 90 and 270 min with [^3H]acetate, were scanned with a densitometer and the percentage of radiolabel in the monoacetylated form was determined for H4 in each lane. The percentage of H4Ac1 at zero time was set equal to 100%.

there is a substantial reduction in the relative specific activity (band density on the fluorogram/ μg of histone) of H2B, compared to H3 and H4. This reduced band intensity appears to be related to the length of the [^3H]acetate labelling period (180 min). Secondly, it is peculiar that much of the radiolabelled H4Ac1 is not rapidly acetylated to higher forms during the *n*-butyrate incubation. Both of these points will be addressed in the Discussion section. Thirdly, the slow rate of acetylation ($t_{1/2} \sim 300$ min) not only appears to be absent for H4Ac1, but also for all H3, H2B and H4 subtypes. Beyond 30 min incubation in *n*-butyrate, there is no observable increase in the amount of radiolabel in hyperacetylated forms of these proteins.

The resolution of the H3 and H2B bands in Fig. 4 is not as satisfactory as in Fig. 1, but rather, is typical for this gel system. The H4 bands, however, are fully resolved and amenable to an acetylation rate analysis. The H4 portions of the fluorograms in Fig. 4 were scanned and the percentage of radiolabel in H4Ac1 presented as a function of *n*-butyrate incubation time, as shown in Fig. 5. Three other [^3H]acetate labelling periods, 45, 90 and 270 min, are included in the quantitative analysis. These data for immature red blood cells are directly comparable to those presented for mature erythrocytes in Fig. 2. For all [^3H]acetate labelling periods, the percentage of radiolabel in H4Ac1 remains constant

Fig. 6. Absence of a slow rate of acetylation of H4 in immature erythrocytes

Immature erythrocytes were incubated for 0–240 min at 37 °C in Swim's medium containing [^3H]acetate and 10 mM-*n*-butyrate. Isolated nuclei were resuspended in SDS gel sample buffer, electrophoresed on an 18% polyacrylamide SDS gel and a fluorogram prepared. The graph shows the relative specific activity of H4 during the incubation. The relative specific activity was calculated by dividing the area under the curve from scanning the H4 band in the fluorogram, by that of the area under the curve from scanning the H4 band in the Coomassie Blue-stained SDS gel.

after approximately 30 min, graphically illustrating the absence of a slow rate of acetylation of radiolabelled H4Ac1 in the immature cells. The rapid rate of acetylation of H4Ac1 is identical to that observed in the mature cells, with a $t_{1/2}$ of ~ 12 min.

In a second experiment, the relative specific activity of H4 during a simultaneous incubation with [^3H]acetate and *n*-butyrate was measured to verify the absence of a slow rate of acetylation ($t_{1/2} \sim 300$ min) for total H4. If a slow rate of acetylation was present for H4 in the immature cell, then radiolabelled acetate would continue to be incorporated into this protein after long periods of incubation in *n*-butyrate and [^3H]acetate. The results of such an experiment are presented in Fig. 6, and demonstrate that the incorporation of [^3H]acetate into H4, plateaus after approximately 2 h. Thus although different rates of acetylation might exist for the different forms of H4 (i.e. non-acetylated form to H4Ac1, H4Ac1 to H4Ac2, H4Ac2 to H4Ac3, etc.), a slow rate with a $t_{1/2}$ of ~ 300 min is not possible, since the relative specific activity of H4 plateaus after 120 min.

In summary, only one rate of acetylation of radiolabelled H4Ac1 was detected in immature red blood cells, with a $t_{1/2}$ of ~ 12 min.

Regions of dynamic acetylation within the red blood cell genome

In appropriate buffers, enhanced solubility of polynucleosomes is coupled to histone hyperacetylation (Nelson *et al.*, 1980; Perry & Chalkley, 1981, 1982). Thus when cells are incubated with *n*-butyrate for a short period of time, nuclei isolated, mildly digested with micrococcal nuclease, and the nucleohistone centrifuged in the presence of MgCl_2 , only those polynucleosomes containing highly acetylated histones are soluble. We

Fig. 7. Regions of active histone acetylation within the red blood cell genome

Immature (I) and mature (M) chicken red blood cells were incubated at 37 °C for 1 h in Swim's S-77 medium in the absence (–) or presence (+) of 10 mM-*n*-butyrate. Nuclei were isolated, digested with micrococcal nuclease, and fractionated into soluble (S) and insoluble (P = pellet) components as described previously (Ferenz & Nelson, 1985). DNA was isolated, incubated with alkali to remove RNA, and electrophoresed, 40 µg per lane, on 1 % agarose gels. DNA in the agarose gels was transferred to nitrocellulose, hybridized to ³²P-labelled chicken adult β-globin, H5, and lysozyme DNA probes (see the Materials and methods section) and autoradiograms prepared as shown in the Figure.

have previously demonstrated that this soluble chromatin from chicken immature red blood cells contains hyperacetylated H4 and is highly enriched for DNA sequences from the β-globin domain (Nelson *et al.*, 1986). This information suggests some form of coupling between dynamic histone acetylation and active chromatin conformation.

In the immature red blood cell, both non-transcribed and transcribed regions of the β-globin domain are solubilized by the *n*-butyrate incubation (Nelson *et al.*, 1986). We have interpreted the solubilization of non-transcribed regions of this active domain as an indication that dynamic histone acetylation is involved in the formation or maintenance of an active chromatin structure, rather than transcription by itself. Since regions such as the β-globin chromatin are maintained in a potentially active conformation in the mature erythrocyte (Weintraub & Groudine, 1976), we were interested in testing this hypothesis further by analysing the solubility behaviour of this potentially active chromatin.

The experimental protocol used has been described previously (Ferenz & Nelson, 1985; Nelson *et al.*, 1986). Red blood cells were incubated for 1 h with 10 mM-*n*-butyrate to constrain the rapidly acetylated histone to the hyperacetylated form. After the isolation of soluble and insoluble chromatin fractions, DNA was prepared free of contaminating RNA, electrophoresed on an agarose gel, transferred to nitrocellulose, and autoradiograms prepared after hybridization to nick-translated DNA probes. The results are presented in Fig. 7, where chicken adult β-globin, lysozyme and H5 DNA were used as the probe sequences. In the portions of the autoradiograms labelled I, immature erythrocyte DNA was transferred to the filter. Incubation of the immature red blood cells with *n*-butyrate for 1 h (+) solubilizes the transcriptionally active adult β-globin chromatin [left hand panel, lane (+)S], but not the inactive lysozyme chromatin [right hand panel, lane (+)S]. In the absence

of *n*-butyrate during the incubation (–), neither chromatin region becomes hyperacetylated and solubilized. These results were presented previously (Nelson *et al.*, 1986), and serve to illustrate the reproducibility of the method. The behaviour of the transcriptionally active chromatin in the immature red blood cell was confirmed with an H5 DNA probe, as shown in the middle panel of Fig. 7. H5 chromatin, which is transcriptionally active in the immature erythrocyte, is preferentially solubilized by the *n*-butyrate incubation.

The chromatin solubilization patterns seen on the autograms for the mature red blood cell samples (Fig. 7, M = mature) are virtually identical to those for the immature erythrocytes. Both H5 and β-globin chromatin are solubilized by the *n*-butyrate incubation, whereas lysozyme chromatin remains insoluble. The short, 60 min *n*-butyrate incubation preferentially solubilizes the active chromatin in immature erythrocytes, and the potentially active chromatin in mature erythrocytes. We conclude that dynamic, rapid histone acetylation preferentially occurs in both transcriptionally active and potentially active chromatin regions.

DISCUSSION

Brotherton *et al.* (1981) originally reported that only a small portion of the chicken erythrocyte genome is actively modified, and we verified this result by demonstrating that only an estimated 3.7 % of the modifiable lysine sites in immature, and 2.1 % of the modifiable lysine sites in mature erythrocytes are available for acetylation (Zhang & Nelson, 1986). In view of the slow rate of acetylation ($t_{1/2} \sim 300$ min) in mature cells, the value of 2.1 % may be a slight underestimate, since our original experiments were only conducted over a period of 7 h. During the first 30 min of exposure of the erythrocytes to [³H]acetate, the amount of radiolabel

incorporated into the histones is 3–4-fold greater in the immature compared to the mature erythrocytes (Zhang & Nelson, 1986). A reasonable explanation for this reduction in the amount of acetate incorporated is that a substantial portion of the rapidly acetylated histone in the immature erythrocyte is converted to the slowly acetylated form in mature cells.

Radiolabelled H4Ac1 in mature erythrocytes has two rates of acetylation, and examination of the fluorograms in Fig. 1 suggest that a slow rate of acetylation is found in all three histones (H4, H2B and H3). This result differs from that obtained for the immature cells, in which the slow rate of acetylation is absent for these proteins. Also, as noted in the Results section, and clearly visible in Fig. 4(a), the specific activity of H2B relative to H3 and H4 in the immature cells is reduced after extended [^3H]acetate labelling periods. Secondly, it is also evident in Fig. 4(b) that not all of the radiolabelled H4 (or H3) is rapidly acetylated, but rather remains at low levels of histone-acetate during the *n*-butyrate incubation. We feel that the simplest explanation for these results is the following. Although the results reveal only one rate of acetylation of H4Ac1 in immature erythrocytes, there may be two categories of rapid acetylation; one in which histone is acetylated at only one or two positions to generate radiolabelled H4Ac1 and H4Ac2, and one in which the histones become hyperacetylated and subsequently deacetylated. In the former case, since H4 (and H3) containing only 1–2 acetyl groups is slowly deacetylated (see companion paper Zhang & Nelson, 1988), it is clear that there will be a build-up of the lower forms of H4Ac1 and H4Ac2 during the [^3H]acetate labelling period. This is not true for H2B. The only visible form of H2B on the stained Triton/acid/urea gels is the non-acetylated form (results not shown), suggesting that all acetylated species of H2B are rapidly deacetylated. Hence, during the extended incubations of the immature cells in the presence of [^3H]acetate, but before *n*-butyrate addition, radiolabel will accumulate in the lower levels of H3 and H4, while the relative specific activity of H2B concomitantly declines. After the addition of *n*-butyrate, the majority of the mono- and di-acetylated H4 (and H3) in the immature cells cannot be further hyperacetylated, as is clearly shown in Fig. 4(b).

The same amounts of adult β -globin and H5 chromatin in mature cells are solubilized by the 1 h *n*-butyrate incubation, as in immature erythrocytes. Although no direct evidence is presented, it is possible that those histones in the immature cells estimated to be acetylated to the mono- or di- levels, but unable to undergo further modification, are converted to undergo the slow rate of acetylation in the mature erythrocyte. If this were the case, this would allow the rapidly acetylated histones capable of hyperacetylation and in association with active chromatin in the immature cell, to remain in the rapidly acetylated form in the potentially active chromatin in the mature erythrocyte. That is, based on the rapid solubilization of adult β -globin and H5 chromatin (within the first hour of *n*-butyrate incubation; see Fig. 7) in both immature and mature cells, we propose that those histones that become rapidly hyperacetylated during the *n*-butyrate incubation are localized in both transcriptionally active, and potentially active regions of chromatin. Thus, rapid histone acetylation is in some fashion coupled to gene expression.

How reliable is the evidence that histone hyper-

acetylation is directly coupled to enhanced chromatin solubility after *n*-butyrate incubation of the erythrocytes, as we have implied in our interpretation of the results in Fig. 7? First, we have previously demonstrated that approximately 40–45 % of the adult β -globin chromatin is solubilized by a 1 h *n*-butyrate incubation (Nelson *et al.*, 1986). This solubilization depends entirely on the presence of *n*-butyrate; removal of the short-chain fatty acid from the cell incubation medium results in the instantaneous loss of the adult β -globin chromatin from the soluble fraction. Secondly, the soluble polynucleosomes enriched in adult β -globin DNA contain hyperacetylated H4. We do not detect non-acetylated or low levels of H4-acetate in this chromatin. Thirdly, we have completed experiments (Alonso & Nelson, unpublished results) which provide further direct evidence for the coupling between enhanced adult β -globin polynucleosome solubility and histone hyperacetylation. The soluble β -globin polynucleosomes isolated from *n*-butyrate incubated cells were exposed to a purified histone deacetylase, during which time some of the nucleohistone aggregates can be pelleted by centrifugation. The histones in the pelleted chromatin were shown to be deacetylated by the action of the enzyme. The DNA in the pellet was found to be predominantly polynucleosome size and it contained the adult β -globin sequences. In other words, the removal of acetyl groups from the hyperacetylated histone caused a loss in solubility of the adult β -globin chromatin. The evidence is very compelling that histone hyperacetylation is occurring on this active chromatin during the *n*-butyrate incubation, and also that this is the reason for the enhanced adult β -globin chromatin solubility.

If rapid histone acetylation is preferentially occurring on active and potentially active chromatin, is it restricted to these regions because of the fortuitous accessibility of the histones in this 'open' chromatin, or is it an intimate part of the process that generates and maintains a more open or accessible chromatin conformation? Although this question remains unanswered, it seems plausible that histone acetylation is playing some fundamental role in active chromatin structure. Histone hyperacetylation is known to enhance chromatin solubility (Nelson *et al.*, 1980; Perry & Chalkley, 1981, 1982) and DNAase I sensitivity (Simpson, 1978; Nelson *et al.*, 1978; Vidali *et al.*, 1978), the latter being an indication of increased DNA accessibility. The acetyltransferase (Belikoff *et al.*, 1980) and deacetylase (Kaneta & Fujimoto, 1974) enzymes are chromatin-bound during nuclear isolation, and approximately 30 % of the erythrocyte deacetylase is very tightly bound to the chromatin (D.-E. Zhang & D. A. Nelson, unpublished work), as reported for HeLa cells (Hay & Candido, 1983). We feel that these bound histone-modifying enzymes, whose activities are specifically confined to active chromatin regions, have a positive role in promoting an accessible chromatin structure.

Our evidence suggests that the rapid histone acetylation observed in active regions of the immature erythrocyte continues to occur on potentially active chromatin in the mature red blood cell. This lends support to the notion that this histone modification is not coupled to transcription itself, but is relevant to the modulation of DNA accessibility or chromatin structure in active regions. If the function of the *N*-terminal histone arms is to bind to the DNA, then it is reasonable to suggest that this interaction is modulated by histone acetylation and

deacetylation, thereby controlling accessibility of the DNA to other proteins. The acetyltransferase and deacetylase enzymes should therefore be considered as potential transcription factors, implying a need for the direct characterization of the function of these enzymes.

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