Histone acetylation in chicken erythrocytes

Estimation of the percentage of sites actively modified

Dong-er ZHANG and Daniel A. NELSON*

Department of Biochemical and Biophysical Sciences, University of Houston-University Park, Houston, TX 77004, U.S.A.

Within the *N*-terminal regions of the DNA-bound histone, 26 lysine residues per nucleosome may be acetylated and deacetylated. In the present paper the percentage of these residues actively acetylated and deacetylated in chicken erythrocytes was measured. This percentage is estimated as 3.7% in chicken immature, and 2.1% in chicken mature, erythrocytes. In metabolically active, dividing, cells one would predict that, after a few generations, each site would at some point in time be modified. We conclude that, in the relatively inactive immature chicken erythrocyte, no more than 1–2% of the genome is composed of dynamically acetylated and deacetylated histone, this percentage decreasing with cell maturity. The active histone acetylation and deacetylation may be confined to transcriptionally active or potentially active erythrocyte domains.

INTRODUCTION

As depicted in Fig. 1, specific core histone lysine residues are reversibly acetylated within the nucleosomal complex *in vivo* (Allfrey *et al.*, 1964). The modification occurs at four positions within the *N*-terminal regions of

histones H4, H3 and H2B, and at one position in histone H2A (Dixon *et al.*, 1975; Isenberg, 1979; Doenecke & Gallwitz, 1982). Acetylation decreases the net positive charge of the *N*-terminal domains of the histone, perhaps weakening histone–DNA interactions for sub-

H2A

H2B

H3

H4

Fig. 1. Sites of acetylation *in vivo* of lysine residues in histones

Histone H2A has one, and H2B, H3 and H4, four, sites of acetylation (from Doenecke & Gallwitz, 1982).

* To whom correspondence should be addressed.

sequent transcription (Allfrey *et al.*, 1964), DNA replication (Cousens & Alberts, 1982; Annunziato & Seale, 1983), DNA repair (Perry & Chalkley, 1982) and displacement by protamines during spermatogenesis (Christensen *et al.*, 1984).

Avian erythropoiesis is a well-defined process characterized by the synthesis of specific macromolecules and by a marked decrease in nuclear function (Tobin *et al.*, 1981). The differentiating definitive erythrocytes retain a mitotically inactive, non-replicating, nucleus (Ringertz & Bolund, 1974) with decreasing transcriptional capacity as the immature erythrocyte (erythroblast) fully develops into the mature form (Gasaryan, 1982). The decrease in transcriptional capacity is accompanied by a decrease in acetate incorporation into histone (Ruiz-Carrillo *et al.*, 1976). Brotherton *et al.* (1981) have reported that only a very small subset of histone is actively acetylated in mature avian erythrocytes, and this result has been verified in the present study. We further estimate that, during erythrocyte maturation, the percentage of lysine sites actively acetylated and deacetylated is decreased from 3.7% in the erythroblasts to approx. 2.1% in the mature cell.

EXPERIMENTAL

Preparation of cells

Adult White Leghorn chickens were purchased from Rich-Glo Lab Animals (El Campo, TX). Chickens were made anaemic by daily injections of 1.0 ml of 1% phenylhydrazine (1% phenylhydrazine hydrochloride in 10 mM-phosphate buffer; the solution was adjusted to pH 7.2 and filter-sterilized). After 7–9 days, maturity of the erythrocytes was assayed by microscopic examination of Brilliant Cresyl Blue-stained cells. Ribosome aggregates were observed in the stained immature erythrocytes; clear cytoplasm was observed in the mature cells (Platt, 1979). Blood from healthy and anaemic chickens was collected by heart puncture using heparinized syringes [approx. 50 units of heparin (Sigma)/ml of whole blood] and centrifuged at 500 *g* for 10 min. Pelleted cells were resuspended and washed twice in Swim's S-77 medium, pH 7.2. After the first centrifugation the white-blood-cell layer (buffy coat) above the pelleted erythrocytes was removed.

HeLa cells grown in Joklik's minimal essential medium were resuspended in Swim's S-77 medium.

Cell incubations

Erythrocytes from one chicken and HeLa cells from a 1-litre culture were incubated at 37 °C for 30, 60, 120, 240 and 420 min in 200 ml of Swim's S-77 medium, pH 7.2, containing 1 mM-sodium acetate, 10 mCi of [³H]acetate (ICN; 0.5 Ci/mmol) or 100 μCi of [¹⁴C]acetate (NEN; 3 mCi/mmol) and in the presence or absence of 10 mM-sodium propionate or -n-butyrate. Typical cell concentrations were (1–2) × 10⁸ cells/ml. The inclusion or absence of 10% (v/v) chicken serum in the cell incubation medium had no effect on the rate of incorporation of [³H]acetate into histone. Incubation of chicken erythrocytes in 0–50 mM-n-butyrate established that 10 mM-n-butyrate was sufficient to inhibit histone deacetylation fully (D. A. Nelson & C. R. Hauser, unpublished work).

Nuclear isolation

Cells were resuspended in nuclear isolation buffer (0.25 M-sucrose/60 mM-KCl/15 mM-NaCl/3 mM-MgCl₂/1 mM-CaCl₂/15 mM-Mes (pH 6.6)/10 mM-sodium n-butyrate/0.5% Triton X-100/0.1 mM-phenylmethane-sulphonyl fluoride and nuclei prepared by vortex-mixing and centrifuging at 2500 *g*. Nuclei were washed twice in the same buffer and stored at –80 °C in the presence of 30% (v/v) glycerol.

Isolation of nucleic acids and lipids

Total nucleic acid was isolated by phenol extraction as described previously (Ferenz & Nelson, 1985). Lipid was extracted from nuclei by the method of Folch *et al.* (1957).

Protein electrophoresis

Nuclei were resuspended in SDS/polyacrylamide-gel sample buffer and whole nuclear protein was electrophoresed on 18%-(w/v)-polyacrylamide/SDS gels as described previously (Nelson, 1982). The method of Laskey & Mills (1975) was used to prepare gels for fluorography of the ³H-labelled protein. The gels were equilibrated with dimethyl sulphoxide, impregnated with PPO (2,5-diphenyloxazole), soaked in water, dried and exposed to Kodak X-Omat XAR-5 X-ray film at –80 °C.

Scintillation counting

Samples (10–200 μl) were mixed with 10 ml of scintillation cocktail (Hydrofluor; National Diagnostics, Somerville, NJ, U.S.A.) and counted for radioactivity in a Beckman liquid-scintillation counter. All cells and nuclei were disrupted with 1 ml of 1% SDS before mixing with scintillation fluid. Results are presented in c.p.m. with a counting efficiency of 40% for all samples.

RESULTS

The equation below was used to calculate the percentage of the sites modified in each experiment:

$$\text{Percent-} \frac{\left(\frac{\text{Acetate radio-activity (c.p.m.) associated with histone}}{\text{Histone } (\mu\text{g})} \right) \times \left(\frac{\text{Number of molecules of acetate/ml}}{\text{Acetate radio-activity (c.p.m.)/ml}} \right) \times 100\%}{\text{age of sites modified} = \frac{\left(\frac{\text{Total number of modifiable lysine residues}}{\text{Histone } (\mu\text{g})} \right)}$$

The terms in the equation reduce to the number of molecules of acetate that become associated with histone, divided by the total number of modifiable lysine sites. This is the fraction of sites modified during the incubation. Note that the total number of modifiable lysine sites refers only to the sites shown in Fig. 1, and does not include lysine residues in histone H1, histone H5 or the core histones that are never modified.

The first term of the numerator was experimentally determined by measuring the radioactivity (c.p.m.) of [³H]acetate in a portion of nuclei disrupted in SDS. The *A*₂₆₀ for the same sample was then measured and the

Fig. 2. [^3H]Acetate in the protein from isolated nuclei is exclusively associated with core histones

HeLa cells ('HeLa') and immature erythrocytes ('Im. eryth.') were incubated at 37 °C in Swim's S-77 medium containing 1 mM-sodium acetate and 10 mCi of [^3H]acetate for 30, 60, 120 and 240 min. Nuclei were isolated, resuspended in SDS/polyacrylamide-gel sample buffer, electrophoresed on an 18%-(w/v)-polyacrylamide/SDS gel and a fluorogram prepared. Right-hand panel, Coomassie Blue-stained gel. Left-hand panel, fluorogram of the same gel. The positions of histones H1, H5 and the core histones are indicated. Radiolabelled non-histone protein was not detected on the fluorogram.

amount of DNA (μg) calculated. Since histone and DNA are present at a 1:1 (w/w) ratio, the amount of DNA is equal to the amount of histone. The acetate radioactivity (c.p.m.) associated with histone, per μg of histone, is then calculated.

The above calculation requires that all of the radiolabel incorporated into the nuclei is in the form of histone [^3H]acetate. We verified that the radiolabel associated with the isolated nuclei was exclusively in the form of histone acetate by monitoring the ^3H incorporated into nucleic acid, lipid and non-histone proteins. ^3H was not detected in lipid or nucleic acid extracted from the whole nuclei (results not shown), but was associated exclusively with the core histones for both the chicken erythrocytes and HeLa cells, as shown in Fig. 2.

The second term of the numerator is the inverse of the intracellular specific radioactivity of the [^3H]acetate. The intracellular specific radioactivity of the [^3H]acetate was controlled by the inclusion of 1 mM-sodium acetate in the medium during radiolabelling. The presence of 1 mM-sodium acetate allows maintenance of a constant acetate concentration during the incubation and equalizes the intra- and extra-cellular specific radioactivities, since the cells are permeable to acetate. We demonstrated the permeability to acetate as follows. Medium containing [^3H]acetate (8×10^7 c.p.m./ml) was made 1 or 10 mM with respect to acetate by addition of the unlabelled compound, and the cells incubated for 5, 30, 60, 120, 240 and 420 min at both acetate concentrations. After low-speed centrifugation, the radioactivity (c.p.m./ml) of the packed cells was measured and found to be the same for both the 1 mM- and 10 mM-acetate samples. This value was equivalent to the radioactivity of the

supernatant (8×10^7 c.p.m./ml) and did not change during the 7 h incubation. Regulation of the internal acetate concentration would have resulted in different values (c.p.m./ml) for the two samples. Thus the cells appear to have no mechanism of regulating internal acetate levels when external concentrations are between 1 and 10 mM. By fixing the extracellular specific radioactivity of the [^3H]acetate at a constant value, the intracellular specific radioactivity was controlled and maintained at the same value.

In the denominator we calculated the total number of modifiable lysine residues per μg of histone by using 26 as the number of modifiable residues per nucleosome (13 sites for each set of histones H2A, H2B, H3 and H4; see Fig. 1). There are 1.19×10^{14} potentially modifiable lysine sites per μg of histone.

By using the equation for the percentage of sites modified, we compiled an initial set of erythrocyte data as shown by the open circles in Fig. 3. In agreement with Brotherton *et al.* (1981), only a small percentage of sites are actively modified in the erythrocyte, reaching a steady-state level after 4–7 h of incubation. By comparison, the percentage of sites acetylated in metabolically active HeLa cells (Fig. 3, open squares) increases linearly over a 4 h incubation period, indicating that a steady-state level of radiolabelled histone has not been reached. This latter result is clearly in accordance with the significant genomic activity of the HeLa cells, coupled with the notion that histone acetylation is required for replication (Cousens & Alberts, 1982; Annunziato & Seale, 1983), DNA repair (Perry & Chalkley, 1982) and transcription (Allfrey *et al.*, 1964).

The acetylation of the histone lysine residues is in a

Fig. 3. Percentage of sites acetylated during incubation of HeLa cells (□) and chicken erythrocytes in the absence (○) or presence (●) of 10 mM-*n*-butyrate

(a) HeLa cells and immature erythrocytes. (b) HeLa cells and mature erythrocytes. Cells were incubated at 37 °C in 100 ml of Swim's medium containing 1 mM-sodium acetate and 10 mCi of [3 H]acetate. Nuclei were isolated after 30, 60, 120, 240, and 420 min of incubation and the percentage of lysine residues modified at each time interval was calculated as described in the text.

dynamic steady state, i.e., acetylated and subsequently hydrolysed (Sanders *et al.*, 1973). The percentage of sites filled during incubation of the cells in the absence of the deacetylase inhibitor sodium *n*-butyrate (Riggs *et al.*, 1977) represents the percentage of modifiable sites approaching, or at, steady state (Fig. 3, open circles). The inclusion of sodium butyrate at the start of the [3 H]acetate incubation allows the measurement of the percentage of sites acetylated above the steady-state level (Fig. 3, closed circles), since steady-state-level sites are already occupied by unlabelled acetate at the start of the incubation. The total percentage of sites actively acetylated and deacetylated is therefore the sum of the percentage of groups modified at steady state plus the percentage of acetylated groups accumulated in the presence of *n*-butyrate.

The erythrocyte incubations in the presence or absence of *n*-butyrate were repeated four to five times and the average percentage of sites modified detailed in Table 1. The percentage of sites modified in the presence or absence of *n*-butyrate appear to be near, or at, a plateau after 420 min of incubation and thus, by adding these values, an estimate can be made of the total percentage of dynamically acetylated sites in the chicken erythrocytes. The total percentage of actively modified lysine residues is estimated as 3.7% in immature, and 2.1% in mature, erythrocytes.

Two control experiments were performed to substantiate our results further. Both the possibility of *n*-butyrate metabolism and of 3 H exchange with water hydrogen atoms were assessed during the incubation protocols. In the cell, acetate is first coupled to CoA and then transferred to the histone. If *n*-butyrate is metabolized to butyryl-CoA, giving rise to acetyl-CoA, this could decrease the specific radioactivity of the acetyl-CoA present in the cell. To test this possibility, immature erythrocytes were incubated with 10 mM-sodium propionate rather than *n*-butyrate to inhibit histone deacetylation. Propionate is not metabolized to acetyl-CoA and inhibits histone deacetylation approx. 75% as effectively as *n*-butyrate (see Kruh, 1982). The percentage of sites modified in the presence of propionate is slightly less than the percentage in the presence of *n*-butyrate (Table 1). This may be due to the reduced inhibitory capacity of the propionate, and suggests that *n*-butyrate is not a significant contributor to the formation of acetyl-CoA.

Exchange of 3 H with water protons could occur after the formation of acetyl-CoA, resulting in a [3 H]acetyl-CoA specific radioactivity less than that of the [3 H]acetate. The percentage of sites filled during the incubation protocol would then be underestimated. Incubation in the presence of [14 C]acetate yields percentages similar to those obtained with [3 H]acetate and within the experimental-error limits of the protocol (Table 1). Thus exchange of 3 H with water hydrogen atoms does not appear to contribute significantly to a decreased acetyl-CoA specific radioactivity.

DISCUSSION

Brotherton *et al.* (1981) reported that approx. 30% of the chicken mature-erythrocyte histone was in an acetylated form, but was 'frozen' in this state. That is, the bulk of non-acetylated, as well as acetylated, histone was not dynamically acetylated and deacetylated. The shift to higher levels of histone acetate, as detected for tissue-culture cells incubated in *n*-butyrate, was not detectable for the mature erythrocyte samples on stained acid/urea gels. Brotherton *et al.* (1981) also showed that all mature erythrocytes incorporate [3 H]acetate into histone to about the same extent, indicating that the entire population of mature erythrocytes has a decreased capacity for active histone acetylation and deacetylation. Our results demonstrate that in the mature erythrocyte, only 2.1% of the total lysine sites are dynamically modified, confirming the limited histone acetylation and deacetylation in these cells.

The incorporation of radioactive acetate into histone decreases as the erythrocytes mature (Ruiz-Carrillo *et al.*, 1976). Using the percentage of sites modified during the first 30 min of incubation (Table 1), we measured a 3-fold decrease in the rate of incorporation of [3 H]acetate into histone as the cells mature from a population of mid- to late-polychromatophilic erythroblasts (immature erythrocytes) to the fully differentiated erythrocyte. The total number of sites dynamically acetylated and deacetylated decreases from 3.7 to 2.1% during maturation. Since three of the four core histones are acetylated at up to four locations, it is possible that only a few per cent of the immature-erythrocyte genome is actively undergoing histone acetylation and deacetylation, this percentage decreasing with maturity.

Sodium acetate (1 mM) was included in all incubations

Table 1. Percentage of sites modified during incubation

Cells were incubated at 37 °C in Swim's medium containing radiolabelled acetate, and the percentage of lysine sites acetylated calculated at each time interval. Standard deviations from the mean values (n = number of measurements) are indicated.

(a) Immature erythrocytes				
Incubation time (min)	Sites modified (%)			[¹⁴ C]Acetate in the absence of short-chain fatty acids
	In the absence of short-chain fatty acids ($n = 4$)	In the presence of <i>n</i> -butyrate ($n = 4$)	In the presence of propionate ($n = 2$)	
30	1.00 ± 0.08	1.25 ± 0.14	1.07 ± 0.18	1.16
60	1.23 ± 0.06	1.60 ± 0.19	1.30 ± 0.14	1.41
120	1.28 ± 0.15	1.82 ± 0.19	1.48 ± 0.01	1.40
240	1.38 ± 0.15	2.03 ± 0.14	1.77 ± 0.07	1.59
420	1.42 ± 0.19	2.29 ± 0.11	1.95 ± 0.04	1.81

(b) Mature erythrocytes		
Incubation time (min)	Sites modified (%)	
	In the absence of short-chain fatty acids ($n = 4$)	In the presence of <i>N</i> -butyrate ($n = 5$)
30	0.24 ± 0.04	0.37 ± 0.06
60	0.38 ± 0.03	0.49 ± 0.09
120	0.49 ± 0.04	0.72 ± 0.12
240	0.69 ± 0.04	1.19 ± 0.13
420	0.87 ± 0.05	1.20 ± 0.05

(c) HeLa cells	
Incubation time (min)	Sites modified in the absence of short-chain fatty acids (%)
30	0.91
60	2.56
120	4.14
240	8.28

to equalize the intra- and extra-cellular acetate specific radioactivity (as described in the Results section) and to maintain a constant acetate concentration during the incubation protocol. Evaluation of the radioactivity (c.p.m.) incorporated into histone (first term of the numerator in the Results-section equation) and the concentration of histone ($\mu\text{g/ml}$) in the incubation medium allows assessment of the percentage of acetate incorporated into histone. For example, after 420 min of incubation of immature erythrocytes in the presence of *n*-butyrate, approx. 10^5 c.p.m. have been incorporated into histone/ml of incubation medium. Comparison of this number with the initial 8×10^7 c.p.m./ml in the medium suggests that about 0.1% of the acetate is incorporated into histone during the 7 h incubation. This result justifies our contention that acetate concentrations remain constant during the protocol. It is unlikely that the presence of 1 mM-acetate has, in itself, an effect on the steady-state levels of histone acetylation, since acetate has little effect on histone deacetylation (Sealy & Chalkley, 1978).

Our results, together with some of our more recent data, suggest that the chicken erythrocytes are specially suited for investigating the relationship between histone acetylation and deacetylation and gene expression. There is a substantial decrease in the percentage of the genome actively acetylated and deacetylated, reflecting the absence of new histone deposition, mitosis, DNA replication and DNA repair. The RNA-synthetic activity of the cells decreases with maturity (Ruiz-Carrillo *et al.*, 1976), as does the number of sites dynamically modified. On the basis of this latter correlation, we hypothesize that dynamic histone acetylation and deacetylation are confined to the transcriptionally active, or potentially active, erythrocyte domains. In support of this hypothesis we find that, when immature chicken erythrocytes are incubated in medium containing *n*-butyrate (to inhibit histone deacetylation), adult globin chromatin is preferentially converted into a more soluble form (Ferenz & Nelson, 1985), this acquired solubility being directly related to hyperacetylation of the globin-associated histones (Nelson *et al.*, 1986). The coupling

between transcription itself and histone acetylation is not tight, however, since inhibition of RNA polymerase activity by α -amanitin or actinomycin D has no effect on the rate of incorporation of [3 H]acetate into histone (Ruiz-Carrillo *et al.*, 1976). This suggests that histone acetylation may be relevant to an 'open' domain structure (Yamamoto & Alberts, 1976), thereby controlling the access of specific transcription factors to the DNA. It may be possible to demonstrate that histone acetylation and deacetylation are required for transcription by using permeabilized erythrocytes (Ruiz-Carrillo *et al.*, 1976) or isolated nuclei. Conditions optimal for histone acetylation and deacetylation in isolated nuclei can be controlled (Garcea & Alberts, 1980) and RNA synthesis monitored (Wiersma & Cox, 1985) during incubations.

This research was supported by a grant (no. GM29442) from the National Institute of General Medical Sciences and by a Biomedical Research Support Grant from the University of Houston-University Park.

REFERENCES

- Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **51**, 786–794
- Annunziato, A. T. & Seale, R. L. (1983) *J. Biol. Chem.* **258**, 12675–12684
- Brotherton, T. W., Covault, J., Shires, A. & Chalkley, R. (1981) *Nucleic Acids Res.* **9**, 5061–5073
- Christensen, M. E., Rattner, J. B. & Dixon, G. H. (1984) *Nucleic Acids Res.* **12**, 4575–4592
- Cousens, L. S. & Alberts, B. M. (1982) *J. Biol. Chem.* **257**, 3945–3949
- Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., Macleod, A. R. & Sung, M. T. (1975) *Ciba Found. Symp.* **28**, 229–258
- Doenecke, D. & Gallwitz, D. (1982) *Mol. Cell. Biochem.* **44**, 113–128
- Ferenz, C. R. & Nelson, D. A. (1985) *Nucleic Acids Res.* **13**, 1977–1995
- Folch, J., Lees, M. & Stanley, G. H. S. (1957) *J. Biol. Chem.* **226**, 497–509
- Garcea, R. L. & Alberts, B. M. (1980) *J. Biol. Chem.* **255**, 11454–11463
- Gasaryan, K. G. (1982) *Int. Rev. Cytol.* **74**, 95–126
- Isenberg, I. (1979) *Annu. Rev. Biochem.* **48**, 159–191
- Kruh, J. (1982) *Mol. Cell. Biochem.* **42**, 65–82
- Laskey, R. A. & Mills, A. O. (1975) *Eur. J. Biochem.* **56**, 335–341
- Nelson, D. A. (1982) *J. Biol. Chem.* **257**, 1565–1568
- Nelson, D. A., Ferris, R. C., Zhang, D.-E. & Ferenz, C. R. (1986) *Nucleic Acids Res.* **14**, 1667–1682
- Perry, M. & Chalkley, R. (1982) *J. Biol. Chem.* **257**, 7336–7347
- Platt, W. R. (1979) *Color Atlas and Textbook of Hematology*, 2nd edn., J. B. Lippincott Co., pp. 259–260, Philadelphia and Toronto
- Riggs, M. G., Whitaker, R. G., Neumann, J. R. & Ingram, V. M. (1977) *Nature (London)* **268**, 462–464
- Ringertz, N. R. & Bolund, L. (1974) in *The Cell Nucleus*, vol. 3 (Busch, H., ed.), pp. 417–446, Academic Press, New York
- Ruiz-Carrillo, A., Wangh, L. J. & Allfrey, V. G. (1976) *Arch. Biochem. Biophys.* **174**, 273–290
- Sanders, L. A., Schechter, N. M. & McCarty, K. S. (1973) *Biochemistry* **12**, 783–791
- Sealy, L. & Chalkley, R. (1978) *Cell* **14**, 115–121
- Tobin, A. J., Hansen, D. H., Chapman, B. S., McCabe, J. B. & Seftor, E. A. (1981) in *Hemoglobins in Development and Differentiation* (Stamatoyannopoulos, G. & Nienhuis, A. W., eds.), pp. 203–213, Alan R. Liss, New York
- Wiersma, P. A. & Cox, G. S. (1985) *Arch. Biochem. Biophys.* **242**, 90–103
- Yamamoto, K. R. & Alberts, B. M. (1976) *Annu. Rev. Biochem.* **45**, 722–746

Received 26 June 1986/27 August 1986; accepted 4 September 1986