Only a small fraction of avian erythrocyte histone is involved in ongoing acetylation

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SUMMARY

We have studied histone acetylation in chicken erythrocytes. We find that about 30% of the histone in these cells is acetylated, however the majority of these histones are not in a dynamic steady state typical of other chicken cells and of mammalian cells, but rather are frozen in this state of modification. A very small fraction of erythrocyte histones are being modified normally but cannot be detected as shifting to higher levels of acetylation upon treatment with butyrate because the amount of histone so modified is small. Nonetheless, chicken erythrocytes incorporate ^3H -acetate into histones about 40% as well as seen in the dynamically active HTC cells. This is most likely due to the formation of very high specific activity Acetyl CoA pools in erythrocytes which have very low levels of coenzyme A. We conclude that these genetically inactive cells are involved in only a minor way with histone acetylation.

INTRODUCTION

Histones can be modified extensively by acetylation (1). Three histones exhibit a stable N-terminal modification (2). In addition about 75% of H2B, H3 and H4 can be modified at up to four internal lysine residues with a modification which is both rapidly achieved and with equal vigor removed (3). The study of the acetylation of the non-H1 histones has received considerable impetus recently. This is based on two points. On the one hand histone deacetylases can be effectively inhibited by the addition of sodium butyrate to cell cultures (4,5) and on the other hand histone acetylation has been increasingly invoked as playing a role in the transcriptional competence of chromatin. This last point of view is based on largely correlative evidence linking DNAase I sensitivity of regions of DNA which seem to be associated with hyperacetylated histones (6). However, it is also intriguing that certain invertebrate sperm cells which contain histones show a total absence of acetylation in these transcriptionally inactive systems.

Another cell showing transcriptional inactivity is the avian erythrocyte which has become ever more repressed during its differentiation (7). The

transcriptional activity is also correlated with increasingly condensed chromatin and increased amounts of histone H5 (8,9,10). Accordingly, the acetylation of the avian erythrocyte histones is a matter of considerable interest. The more so as there have been several reports indicating both active modification of such histones by acetylation and its subsequent hydrolysis (8,11). In addition, as much as 40-50% of histone H4 is found in the monoacetyl modification in the steady state as judged by gel electrophoresis.

Since the avian erythrocyte is transcriptionally inactive we wished to probe the nature of the acetylation, particularly asking how extensive a process it is. Utilizing butyrate as a probe we have found that the erythrocyte histones are much less actively modified than was previously thought and that much of the histone is in fact unavailable for either acetylation or hydrolysis of pre-existing histone acetate.

METHODS

Preparation of CRBC. Whole blood was collected from the wing veins of adult chickens into 20% to 30% ACD (acid-citrate-dextrose) (12). Cells were washed two times in equal volumes of Swims S-77 medium plus 10% calf serum. Red cells were separated from other blood constituents by centrifugation in ficoll-hypaque (13) or just ficoll (14). The isolated chicken erythrocytes (CRBS) were washed to remove ficoll and preincubated in S-77 with 10% serum for 45-60 min at 37°C prior to experimental use. All CRBC were treated in this manner, except those used for autoradiography (see figure 6 legend).

Histone Isolation. Histone isolation, electrophoresis and fluorography were exactly as described previously (15). Details of cell handling during specific experiments are provided in figure legends. Nomenclature for histone bands isolated by gel electrophoresis follows that of Zweidler (16).

Determination of CoA and Acetyl CoA pool sizes. The concentration of Acetyl CoA CoASH in neutralized perchloric acid extracts of HTC or chicken erythrocytes was determined by recycling CoASH through a coupled enzyme system catalyzing the following net reaction:

The rate of NADH formation was followed fluorometrically and is proportional to the amount of Acetyl CoA + CoASH added to the coupled enzyme system (17). DNA content of the perchloric acid precipitation was determined utilizing the diphenylamine assay (18).

RESULTS

Extent of Histone Acetylation in Chicken Erythrocyte. A time course of the uptake of radiolabeled acetate into chicken and HTC cells is shown in figure 1a. This represents total acetate and in addition to macromolecular bound acetate includes free acetate as well as small molecules such as acetyl CoA. The pattern of uptake of acetate is quite different in the two systems. HTC cells show an initial rapid uptake followed by a slower but continuous accumulation. The avian erythrocyte also shows rapid initial uptake, however, no further accumulation was noted. At 10 minutes of incubation, uptake into chicken erythrocytes was approximately 25% that of an equal number of HTC cells.

Association of ^3H -acetate with histones H2A, H2B, H3 and H4 is also presented in figure 1. It can be seen that incorporation into erythrocyte histones is approximately 40% of that into HTC cell histone. The pattern of acetate incorporation is somewhat different than acetate uptake in as much as all fractions continue to incorporate acetate throughout the time course of the experiment in both erythrocytes and HTC cells. Furthermore, the relative extent of acetylation of different histone fractions is essentially the same in the two systems. This observation is in agreement with that reported previously for results gathered in essentially the same way (11).

These results presented an interesting paradox which we felt was necessary to resolve in order to understand more about the function of histone acetylation. It has become increasingly fashionable in this field to consider a possible interrelationship between histone acetylation and gene activity. If this is correct, how could one account for the fact that in the almost totally repressed mature avian erythrocyte, acetylation is so extensive? Two possible explanations were examined. Firstly, there are two kinetic forms of acetate modification, the more rapid of which has been found in active fractions (19). Perhaps the acetate incorporation into the avian erythrocyte histones is only the slow form which may have some function other than in transcription. The second possibility we have considered is that the pools of CoA are very low in the erythrocytes so that ^3H -Acetyl CoA pools develop a very high specific activity and accordingly a very small degree of modification produces the same magnitude of radiolabel incorporation as that seen in HTC cells.

Effect of Butyrate on Erythrocyte Histone Acetylation. One potent means for looking at overall acetylation of both fast and slow forms is to treat the cells with sodium butyrate and to study the extent of ongoing acetylation by

Fig. 1: Comparison of ^3H -Acetate Uptake and Incorporation into Histones in RBC and HTC

1A. Uptake of ^3H -Acetate into Chicken Erythrocytes and Rat HTC Cells.

Freshly resuspended HTC cells (4.5×10^5 cells/ml) and chicken erythrocytes (4.5×10^5 cells/ml) were labeled in medium containing ^3H -Acetate (0.2 mCi/ml) for the indicated times. Samples were then diluted with 5 volumes of ice cold nonradioactive medium, centrifuged and washed repeatedly before freezing. Samples were digested in 30% H_2O_2 prior to counting. HTC cells (— — —), red blood cells (· · · · ·).

1B. Incorporation of ^3H -Acetate into Histones of Chicken Red Blood Cells and HTC Cells

Chicken red blood cells (1.5×10^8 cells) and an approximately equal cell volume of HTC cells (1.1×10^7 cells) were incubated in 5 ml of medium containing ^3H -Acetate (0.2 mCi/ml) for the indicated times. Incubation was stopped by adding 5 volumes of ice-cold medium. After washing, the cell pellet was frozen. Histones were extracted (3,15), separated by SDS-polyacrylamide gel electrophoresis and prepared for counting as described previously (3,15). Counts were normalized for the amount of histone applied by densitometric scanning of the coomassie blue stained gel and are expressed as the relative specific activity.

measuring directly the degree of visible hyperacetylation which is produced by this inhibitor of histone deacetylation (5,20). Accordingly, we have asked what effect sodium butyrate would have on the mass levels of erythrocyte histone acetylation. In contrast to its previously reported effects upon various mammalian cells (4), we see no evidence of hyperacetylation in the erythrocyte cells treated with sodium butyrate for as long as 24 hours, as shown in figure 2. In a parallel experiment, we find that both chicken bursa of fabricius cells and chicken hepatocytes treated with 50 mM butyrate do show the typical mammalian response, so that increased bulk hyperacetylation is observed after 3 to 6 hours of exposure to the short chain fatty acid (results not shown).

Histone Acetate Turnover in Chicken Erythrocytes

One possible explanation for the failure to detect bulk hyperacetylation in chicken erythrocytes might lie either in the presence of a histone deacetylase in erythrocytes which is resistant to butyrate or to a possible inability of the salt of this short chain fatty acid to enter the erythrocyte. Accordingly, we have labeled the histones of the erythrocyte with ^3H -acetate and analyzed the turnover of histone acetate in the presence or absence of sodium butyrate. As shown in figure 3, butyrate clearly inhibits the turnover of acetate groups in these cells. In the absence of butyrate we detect two rates of turnover. One fraction has a very short half life resembling the results we have obtained previously with mammalian cells. In contrast, the other labeled histone acetate turns over with a $t_{1/2} = 2.5$ hr, a rate which is approximately five fold slower than that seen for the slow turnover form in HTC cells (3).

Acetate-labeled Histone Can be Hyperacetylated. The data of figure 2 indicate that about 30% of H3 and H4 are in the monoacetylated form in control erythrocytes. In mammalian cells this represents a dynamic steady state between acetylation and its hydrolysis, a steady state which can be detected

Fig. 2: Effect of Sodium Butyrate on Histone Modification in Avian Erythrocytes

Electrophoresis of histones from chicken erythrocytes on triton-urea polyacrylamide gels (21). Cells were treated with 50 mM sodium butyrate for 0, 5, 18 and 24 hours. Histones were isolated as described by Jackson et al. (3).

Fig. 3: Acetate Turnover in Chicken Red Blood Cells Exposed to Sodium-Butyrate

Chicken erythrocytes were pulsed with ^3H -Acetate (1 mCi/ml) for 10 min and chased as previously described (3), except that, where indicated, cells were resuspended in medium containing 50 mM butyrate for the chase period. Histones were isolated, and the relative specific activity of ^3H -Acetate associated with histone H4 was determined as described in figure 1B.

by the simple expedient of perturbing it with butyrate. This is clearly not the case for erythrocyte acetylation and suggests two possibilities. First, that acetylation may not proceed beyond the monoacetyl level in these cells, or second, that acetylation may proceed beyond the monoacetyl level but that it is occurring on such a minority of histones that mass shift to higher levels cannot be detected. This last possibility would obviously imply that the 30% monoacetylation observed in erythrocyte histone no longer represents the dynamic steady state seen in other cells, including avian hepatocytes.

Thus, to summarize, although it is clear that the bulk of the histone acetate present in chicken erythrocytes cannot be hypermodified in the presence of sodium butyrate we were interested to know if this meant that most of the histone acetate was metabolically inactive and if that fraction picking up label could be hypermodified in a manner seen previously for mammalian his-

tones. Accordingly, the erythrocyte histones were labeled for 10 minutes, and chased in the presence of 50 mM butyrate for up to 24 hours, with samples collected at appropriate intervals. The histones were isolated, electrophoresed and assayed by fluorography as shown in figure 4. After the initial pulse, radiolabeled acetate is associated with all four acetylation levels of H4, the bulk of the radioactivity being in H4Ac1 and H4Ac2. After a 10 min chase, a significant fraction of the radiolabel has shifted into H4Ac4, indicating that

Fig. 4: Effect of Sodium Butyrate on Acetate Incorporation into Histones

CRBC were pulse-labeled for 10 min and then chased in non-isotopic medium containing 50 mM Butyrate for up to 24 h. Histones were prepared as previously described (3,15) and separated by Triton-urea polyacrylamide gel electrophoresis (21,15). The fluorogram was prepared according to Laskey and Mills (22).

a fraction of radiolabeled H4 (~30%) is hypermodified very rapidly. Hypermodification then proceeds more slowly ($t_{1/2} \cong 60$ min) until 60% of the radiolabeled H4 is elevated to H4Ac₄ after 24 hours as indicated in fig. 5. Clearly, the labeled histone represent a very different set from the pre-existing bulk histone acetate. The latter, although it composes in mass 30-40% of H4 and H3, does not show any hypermodification in butyrate.

Are ³H-acetate-histones in a Special Cell Population? The observation that only a small subset of erythrocyte histones are labeled with ³H-acetate and are capable of hypermodification in butyrate can be explained in one of two ways: first, the erythrocyte population contains a small fraction of cells in which histones are modified along the mammalian lines whereas the bulk of the erythrocytes are metabolically inert for acetate metabolism (i.e., modification and subsequent hydrolysis) or, second, most erythrocytes contain

Fig. 5: Modification of Histone H4 to Tetraacetyl Level.

The fluorogram of figure 4 was scanned and the fraction of radiolabeled histone H4 in the tetraacetyl level was determined.

a small fraction of their chromatin which is metabolically active for acetylation at any given time, the bulk of the chromatin is however inert.

We have attempted to distinguish between these two possibilities by labeling erythrocytes with ^3H -acetate for 10 minutes. To ensure that only acetate bound to macromolecules is present, the cells were resuspended in fresh medium containing sodium butyrate for six hours. Obviously, acetate associated with histone during the initial pulse will remain bound but excess acetate will be washed out during the extended chase period. The cells were then fixed and washed, again to ensure that only macromolecule-bound ^3H -acetate remained. Using autoradiographic techniques, we then asked whether a specific population of cells was labeled or if all cells were labeled approximately equally. The results shown in figure 6 indicate that all cell nuclei are labeled to approximately the same degree. Previous studies have indicated that the vast majority of label is associated only with histone (data not shown).

Coenzyme A pools in Avian Erythrocytes. We have come to the conclusion that metabolically active chicken erythrocyte histone acetylation is occurring on only a small population of histone molecules. The most likely explanation for the observation that the level of ^3H -acetate incorporation into erythrocyte histone is only 3 fold less than that seen for HTC histone lies in the possibility that the pool size of CoA is small and so the specific activity is vastly elevated, leading to an elevated degree of radioactivity associated with the histone. To check this idea, we have measured directly the levels of total coenzyme A in erythrocytes and HTC cells. The results shown in table I clearly indicate that the level of coenzyme A is dramatically decreased in the erythrocytes. Thus, even though the rate of uptake into red cells is approximately four fold lower than seen for HTC cells the final specific activity (cpm/acetyl CoA mass) is likely to be substantially higher for the red cells. Assuming the relative extent of activation of acetate to acetyl CoA is comparable in the two systems, the specific activity difference would then be in the range of 30-50 fold, thus accounting for the observations we have reported.

DISCUSSION

The major conclusion from this work is that most of the chromosomal material in the avian erythrocyte population is not involved in extensive modification reactions with acetate, in contrast to observations made in transcriptionally active cells. This conclusion rests upon the inability of sodium butyrate to induce bulk hyperacetylation of chicken erythrocyte his-

Fig. 6: Photomicrograph of ^3H -Acetate Pulsed Chicken Red Blood Cells

Chicken whole blood was washed twice and preincubated in medium for 45 min. The 0.5 ml cell pellet, with buffy coat removed, was suspended in 15 ml fresh medium containing 1 mCi/ml of ^3H -Acetate and incubated for 10 min. The cells were washed 2 times in 15 ml ice cold non-isotopic medium, and then resuspended in fresh warm medium containing 50 mM butyrate. After 2 h, the cells were resuspended in 0.1 M sodium phosphate (pH 7.4) with 10% bovine serum. Smears were made, air-dried and fixed in 3% glutaraldehyde, 0.1 M sodium cacodylate for 1 min. Fixative was rinsed off and the dried smears were coated with NTB-2 emulsion (Kodak) diluted 1:1 with water according to the method of Williams (25). After 8 weeks, the smears were developed with D19 and counterstained with Wright's solution. Control smears consisted of cells similarly treated, except that ^3H -Acetate was absent from the incubation medium. Because of the nature of the counterstain and the need to focus on the nucleus, the cytoplasm does not show up in the photograph. However, even though not shown, the cytoplasm and the plasma membrane were present in all cells autoradiographed.

tones even though it is apparent that butyrate can inhibit the histone deacetylase in these cells. One explanation for the failure to induce bulk hyperacetylation may have been that acetylation was occurring in only a few selected cells. However, the results of the autoradiographic analysis of whole cells, following acetate uptake and an extended chase in sodium butyrate, indicate that all cells are incorporating acetate. Clearly, then, the failure to observe hyperacetylation upon exposure to butyrate must mean that only a small fraction of the total histones are involved in active, ongoing acetylation.

Table I
Amount of CoA Plus Acetyl CoA

	$\mu\text{M}/\text{mg DNA}$	$\mu\text{M}/10^9 \text{ cells}$
Chicken red cells	0.07	0.24
HTC cells	3.56	46

That such a small fraction should give rise to ^3H -acetate incorporation approaching that of active cells such as HTC cells is most probably a result of a reasonably normal uptake of ^3H -acetate combined with very low levels of CoA, thus leading to high specific activity AcetylCoA. Mature avian erythrocytes have on the average only a single mitochondrion (23) and most of their energy metabolism comes from glycolysis. There is most likely no fat biosynthesis and extramitochondrial coenzyme A levels may be even lower than the whole cell values measured. Thus, the maximum degree of histone acetylation may be between 2-5% of that of HTC cell histones, but could be much lower.

The observations that the rather sizable amount of histone acetate is metabolically inert in these cells is different from the state of histones in the transcriptionally inactive invertebrate sperm cells in which all the histone exists only in the unacetylated parental forms. However, a better analogy may lie in the histone of the SV40 virion which is also not involved in transcription in its final packaged form. Here, the histones are frozen in a form containing both histone acetate and unmodified parental histone (24). It seems likely that the events leading to cessation of transcriptional activity in the developing reticulocytes and erythrocytes also lead to a cessation of histone modification activity which is then frozen in the steady state in which it was maintained prior to the changes induced by the erythroid differentiation.

These studies certainly do not prove that acetylation plays a role in transcriptional capacity, however, the observation that the decrease in transcription is accompanied by a drastic decrease in histone acetate metabolic activity is intriguing and deserves further attention.

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REFERENCES

1. Pogo, B.G.T., Allfrey, V.G., and Mirsky, A.E., (1966) *Proc. Natl. Acad. Sci., USA* **55**, 805-812.
2. Hnilica, L.S. (1972) in *The Structure and Biological Functions of Histones*, pp. 79-92. Chemical Rubber Co. Press, Cleveland, Ohio.
3. Jackson, V., Shires, A., Tanphaichitr, N., and Chalkley, R., (1975), *J. Biol. Chem.* **250**, 4856-4863.
4. Riggs, M.G., Whittaker, R.G., Neumann, J.R., and Ingram, V.M. (1977) *Nature* **268**, 462-464.
5. Sealy, L., and Chalkley, R., (1978) *Cell* **14**, 115-121.
6. Sealy, L., and Chalkley, R., (1978) *Nucleic Acids Res.*, **5**, 1863-1876.
7. Bruns, G.A.P. and Ingram, V.M. (1973) *Phil. T. Roy. Soc. London B* **266**, 225-325.
8. Ruiz-Carrillo, A., Wamgh, L.J., Littau, V.C. and Allfrey, V.G. (1974), *J. Biol. Chem.* **249**, 7358-7368.
9. Appels, R., Wells, J.R.E. and Williams, A.F. (1972) *J. Cell, Sci.* **10**, 47-59.
10. Billet, M.A. and Hindley, J. (1972) *Eur. J. Biochem.* **28**, 451-462.
11. Sanders, L.A., Schechter, N.M. and McCarty, K.S., (1973), *Biochemistry* **12**, 783-791.
12. Aster, R.H., Jand, J.H. (1964) *J. Clin. Invest.* **43**, 843-855.
13. Cutts, J.H. (1970) *Cell Separation Methods in Hematology*, Academic Press, New York, N.Y.
14. Noble, P.B. and Cutts, J.H. (1967) *Can. Vet. J.* **8**, 110.
15. Covault, J., and Chalkley, R., (1980) *J. Biol. Chem.*, **255**, 9110-9116.
16. Zweidler, A., (1978) *Methods Cell Biol.*, **17**, 223-233.
17. Allred, J.B. and Guy, D.G. (1969) *Analytical Biochem.* **29**, 293-299.
18. Burton, K. (1956) *Biochem. J.* **62**, 315-323.
19. Nelson, D., Covault, J. and Chalkley, R., (1980) *Nucleic Acids Res.*, **8**, 1745-1764.
20. Cousins, L.S., Gallwitz, D., and Alberts, B.M. (1979) *J. Biol. Chem.*, **254**, 1716-1723.
21. Alfageme, C.R., Zweidler, A., Mahowald, A. and Cohen, L.H. (1974) *J. Biol. Chem.* **249**, 3729-3736.
22. Laskey, R.A. and Mills, A.D. (1975) *Eur. J. Biochem.* **56**, 335-341.
23. Small, J.V. and Davies, H.G., (1972) *Tissue And Cell* **4**, 341-378.
24. Schaffhausen, B.S., and Benjamin, T.L. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1092-1096.
25. Williams, M.A. (1977) *Practical Methods in Electron Microscopy*, Vol. 6, pp. 1-100, North Holland Publishing Co., New York.