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Dynamically acetylated histones of chicken erythrocytes are selectively methylated

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The relationship between histone acetylation and methylation in chicken immature erythrocytes was investigated. Previous studies have shown that transcriptionally active/competent gene-enriched chromatin fragments are enriched in newly methylated histones H3 and H4. Moreover, newly methylated histone H4 is hyperacetylated. Here, we show that dynamically acetylated histone H4 is selectively engaged in ongoing methylation. While sodium butyrate (an inhibitor of histone deacetylase) does not inhibit ongoing histone methylation, it does affect the acetylation state of newly methylated histone H4 when chicken immature erythrocytes are incubated in its presence or absence. Only one rate of acetylation of labelled newly methylated unacetylated histone H4 with a $t_{\frac{1}{2}}$ of 8 min is observed. Previous studies have shown that the solubility of transcriptionally active/competent gene chromatin fragments in 0.15 M-NaCl is dependent upon the level of acetylated histone species, with induction of hyperacetylation increasing the solubility of this gene chromatin. Here, we show that the low salt solubility of chromatin fragments associated with newly methylated histones H3 and H4 is also dependent upon the level of acetylated histones. These results provide further support for the hypothesis that histones participating in ongoing methylation are associated with transcriptionally active/competent chromatin and suggest that the processes of histone H4 methylation and dynamic acetylation are partially coupled in terminally differentiated erythrocytes.

INTRODUCTION

In most species, including chicken, the ε-amino group of lysine residues 9 and 27 of histone H3 and lysine residue 20 of histone H4 may be methylated (Wu *et al.*, 1986). Histones H3 and H4 may also be acetylated; for example, the ε-amino group of lysine residues 5, 8, 12 and 16 of histone H4 may be acetylated. We presented evidence that histones H3 and H4, which are undergoing methylation, are preferentially associated with transcriptionally active/competent DNA of chicken immature erythrocytes (Hendzel & Davie, 1989). In contrast with total histone H4, where tri- (A_3) and tetra-acetylated (A_4) are minor forms, we observed that a significant proportion of newly methylated H4 was highly acetylated, with A_3 and A_4 forms of H4 being methylated. This observation suggested that there exists a relationship between histone acetylation and methylation.

Highly acetylated histones are complexed to transcriptionally active/competent DNA (Allegra *et al.*, 1987; Zhang & Nelson, 1988*a*; Hebbes *et al.*, 1988; Ip *et al.*, 1988; Ridsdale *et al.*, 1990). Importantly, genetic analysis of histone H4 acetylation has shown that acetylation of H4 has an essential role in chromosome dynamics (Megee *et al.*, 1990). Several reports provide evidence that histone acetylation alters nucleosome structure which in turn may facilitate transcription (Norton *et al.*, 1989; Walker *et al.*, 1990; Oliva *et al.*, 1990).

In chicken immature erythrocytes, approx. 1–2% of the histones participate in rapid acetylation and deacetylation (Zhang & Nelson, 1986). In these cells there is only one rate of acetylation, which has a $t_{\frac{1}{2}}$ of approx. 12 min (Zhang & Nelson, 1988*a*). There are two categories of dynamically acetylated histone H4, one which is acetylated to mono- (A_1) or diacetylated (A_2) forms and the other which becomes hyperacetylated (A_4 form). The A_1 and A_2 forms of H4 are slowly deacetylated, while the A_4 form is rapidly deacetylated (Zhang & Nelson, 1988*a*,*b*).

The acetylation level of the dynamically acetylated histones can be manipulated by incubating cells in the presence or absence of sodium butyrate, an inhibitor of histone deacetylase (Candido *et al.*, 1978). In the presence of butyrate, dynamically acetylated histones become hyperacetylated while in the absence of butyrate, these histones have low levels of the highly acetylated species (Zhang & Nelson, 1988*a*,*b*). Altering the level of chicken erythrocyte hyperacetylated histones has a profound effect on the solubility of active/competent, but not repressed, gene chromatin fragments in buffers containing 3 mM-$MgCl_2$ or 150 mM-NaCl, with increased levels of acetylated histones resulting in enhanced solubility of these gene chromatin fragments (Zhang & Nelson, 1988*a*; Ridsdale *et al.*, 1990). These observations strongly suggest that dynamically acetylated histones are complexed to transcriptionally active/competent DNA.

In the present study, we investigated the relationship between histone methylation and histone acetylation in chicken immature erythrocytes. We show that incubation of erythrocytes with butyrate affects the acetylation state of newly methylated histone H4. Only one rate of acetylation of newly methylated H4, with a $t_{\frac{1}{2}}$ of 8 min, is observed. Altering the level of the hyperacetylated histone species affects the low salt solubility of chromatin fragments associated with newly methylated histones and transcriptionally active/competent DNA sequences. These results demonstrate that there exists a relationship between histone H4 acetylation and methylation, with dynamically acetylated H4, which is complexed to transcriptionally active/competent DNA, selectively participating in ongoing methylation.

Abbreviations used: AUT, acetic acid/6.7-M-urea/0.375% (w/v) Triton X-100; A_0, A_1, A_2, A_3 and A_4 denote the un-, mono-, di-, tri- and tetra-acetylated histone forms respectively; DMEM, Dulbecco modified Eagle medium.

MATERIALS AND METHODS

Isolation and labelling of immature chicken erythrocytes

Chicken immature erythrocytes were isolated as described (Hendzel & Davie, 1989). Isolated immature erythrocytes consisting predominantly of mid- and late-polychromatic erythrocytes were incubated in Dulbecco modified Eagle medium (DMEM)-deficient media (pH 7.5) and L-[*methyl*-^{3}H]methionine (70–85 Ci/mmol; Amersham Corp.) as described in Hendzel & Davie (1989) with the following modifications. For incubations of the cells with L-[*methyl*-^{3}H]methionine in the presence or absence of sodium butyrate, cells were pre-incubated for 30 min at 37 °C in DMEM-deficient media with cycloheximide, L-[*methyl*-^{3}H]methionine was added, and the cell suspension was separated into two equal volumes. Sodium butyrate was added to a concentration of 10 mM to the plus-butyrate cells. The cells were incubated for 60 min.

Isolation of nuclei and fractionation of chromatin

Nuclei were isolated as described (Delcuve & Davie, 1989). In some instances, chromatin was fractionated into 0.15 M-NaCl-soluble (fraction S_{150}) and insoluble fractions (P_{150}) as described (Hendzel & Davie, 1989). The relative levels of labelled histone H4 in total chromatin and chromatin fraction S_{150} were determined by electrophoretically separating equivalent amounts of acid-soluble proteins isolated from fraction S_{150} and total nuclei on SDS/15%-(w/v)-polyacrylamide gels, and scanning the Coomassie Blue-stained gels and accompanying fluorograms with a densitometer. The peak-height values for H4 from these densitometric scans were obtained, and the specific activity was a ratio of the peak height (fluorogram) to peak height (stained gel). The enrichment of labelled H4 in fraction S_{150} relative to total labelled H4 was a ratio of these specific activities.

Preparation of protein samples and electrophoresis

Histones were isolated by extraction with 0.2 M-H_2SO_4 as described (Nickel *et al.*, 1987). Acetic acid/urea/0.375% (w/v) Triton X-100 (AUT) and SDS/15%-PAGE was performed as described by Nickel *et al.* (1987). Fluorography was performed as described by Hendzel & Davie (1989).

Measurement of acetylation rates of methylated histones

Cells were incubated with L-[*methyl*-^{3}H]methionine as described (Hendzel & Davie, 1989), and then the labelled cells were resuspended in Swim's S-77 medium (pH 7.5), which contained unlabelled methionine, and chased for 2 h in this medium. This chase period was required to deplete the pools of labelled *S*-adenosylmethionine. This is necessary to ensure that the specific activity of the labelled histones does not change significantly during the butyrate assay period. Cells were resuspended in Swim's S-77 medium (pH 7.5) containing 10 mM-sodium butyrate and samples were collected at various times. The acetylated species of histone H4 were resolved on AUT/15%-polyacrylamide gels. Fluorograms were scanned with a densitometer. The peak heights of the unacetylated and each acetylated species of histone H4 were determined for each time point. The reduction of radiolabel in unacetylated H4 during the incubation in butyrate was used to measure the rate at which newly methylated histone H4 was acetylated (Covault & Chalkley, 1980).

RESULTS

Effect of sodium butyrate on histone methylation in chicken erythrocytes

Incubation of HeLa cells for 15 h in the presence of 5 mM-sodium butyrate inhibits histone methylation (Boffa *et al.*, 1981). We determined whether exposure of chicken immature erythrocytes to sodium butyrate for short durations (e.g. 1 h) inhibited histone methylation. Immature erythrocytes pre-incubated with cycloheximide were incubated for 60 min with L-[*methyl*-^{3}H]-methionine in the presence or absence of butyrate. Fig. 1 shows that sodium butyrate does not quantitatively or qualitatively affect the labelling of the histones, with specific activities of histones H3 or H4 of butyrate-treated or untreated cells being

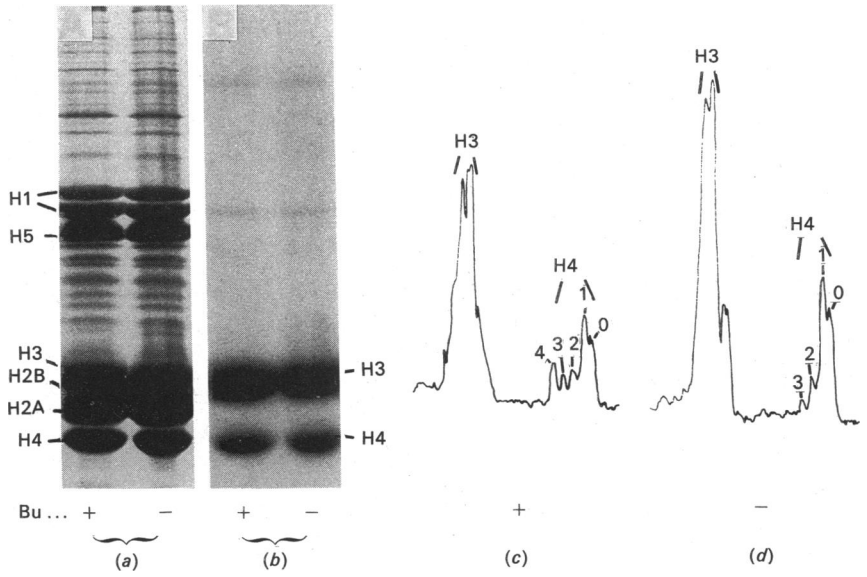

Fig. 1. Effect of sodium butyrate on histone methylation in chicken immature erythrocytes

Acid-soluble proteins (15 μg) of nuclei isolated from chicken immature erythrocytes, which were incubated for 1 h in the presence (+Bu) or absence (−Bu) of 10 mM-sodium butyrate, were electrophoretically resolved on a SDS/15%-polyacrylamide gel. (*a*) and (*b*) show the Coomassie Blue-stained gel pattern and the accompanying fluorogram respectively. The acid-soluble proteins were also electrophoretically resolved on an AUT/15%-polyacrylamide gel, and the labelled histones were revealed by fluorography. (*c*) and (*d*) show the densitometric scans of the fluorograms. The un-, mono-, di-, tri- and tetra-acetylated species of histone H4 are denoted as 0, 1, 2, 3 and 4 respectively.

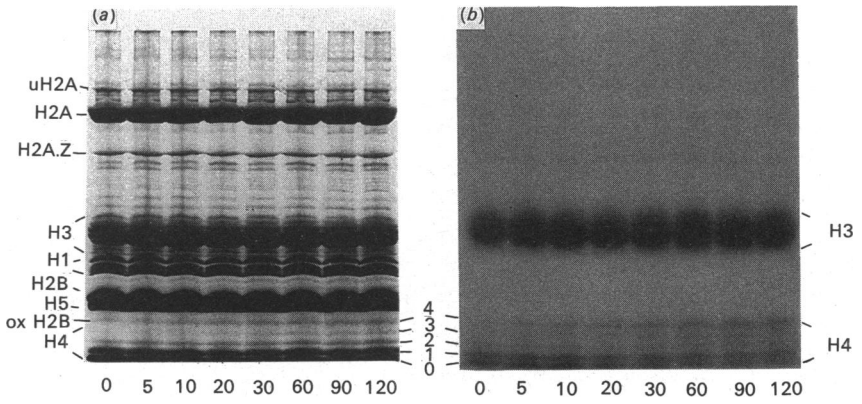

Fig. 2. Dynamically acetylated histone H4 participates in ongoing methylation

Cells were incubated for 1 h with L-[*methyl*-^{3}H]methionine, washed and incubated for 2 h in Swim's medium, and further incubated for 5, 10, 20, 30, 60, 90 and 120 min in Swim's medium containing 10 mM-sodium butyrate. Histones (5000 c.p.m.) were electrophoretically resolved on AUT/15% polyacrylamide gels. (*a*) and (*b*) are the Coomassie Blue-stained gel and fluorogram respectively. 0, 1, 2, 3 and 4 represent the un-, mono-, di-, tri- and tetra-acetylated species of histone H4. ox H2B, which co-migrates with the A_4 form of H4, is oxidized histone H2B. u denotes the ubiquitinated histone species.

similar. However, butyrate affects the distribution of the methyl label among the acetylated species of histone H4. The majority of the methyl label in H4 of untreated cells is located with the A_0 and A_1 species, with approx. 8% of the label being found in the A_3 and A_4 forms. When cells are incubated with sodium butyrate, approx. 27% of the methyl label in H4 is in the A_3 and A_4 forms. This observation suggests that the small proportion of histone H4 molecules that are participating in active ongoing acetylation are selectively methylated. Also, this demonstrates that altering the level of acetylation does not affect ongoing methylation.

Rate of acetylation of newly methylated histone H4

In the presence of sodium butyrate the acetylation state of dynamically acetylated H4 is rapidly elevated. Histone H4 of chicken immature erythrocytes has only one rate of acetylation, with a $t_{\frac{1}{2}}$ of approx. 12 min (Zhang & Nelson, 1988*a*). This was determined by measuring the reduction in the percentage of label (in this case [^{3}H]acetate) in monoacetylated H4 during the incubation of cells with sodium butyrate (Covault & Chalkley, 1980; Zhang & Nelson, 1988*a*). Since the unacetylated species of H4 is methylated, we were able to measure the rate of acetylation of newly methylated H4 by measuring the reduction in the percentage of methyl label in unacetylated H4.

Histones undergoing methylation were labelled by incubating cycloheximide-treated cells with L-[*methyl*-^{3}H]methionine in the absence of butyrate, and then the labelled cells were incubated in the presence of sodium butyrate. Histones isolated from acid-extracted nuclei were electrophoretically separated on AUT/15%-polyacrylamide gels. Fig. 2(*b*) shows that a significant proportion of methylated histone H4 is rapidly acetylated. The specific activity of unacetylated and monoacetylated histone H4 decreases, while the specific activity of the higher acetylated species increases during sodium butyrate treatment (particularly striking is that of tetra-acetylated histone H4) (Fig. 2*b*). There is also movement of the label into the hyperacetylated species of histone H3 during the time that the cells are incubated with sodium butyrate. However, the change in the labelling of the hyperacetylated forms of H3 during the incubation period is not as pronounced as that seen for H4. A similar behaviour was observed for H3 isolated from cells that were labelled with [^{3}H]acetate and subsequently incubated without label in the presence of sodium butyrate (Zhang & Nelson, 1988*a*).

Fig. 3 shows the change in the content of labelled methylated unacetylated histone H4 as a function of incubation time in the presence of 10 mM-sodium butyrate. The rate of acetylation and the percentage of unacetylated methylated histone H4 that is acetylated during the course of incubation can be determined from this plot. Most acetylation of unacetylated labelled methylated histone H4 takes place within the first 20 min of butyrate incubation. During the first 60 min of butyrate incubation, the

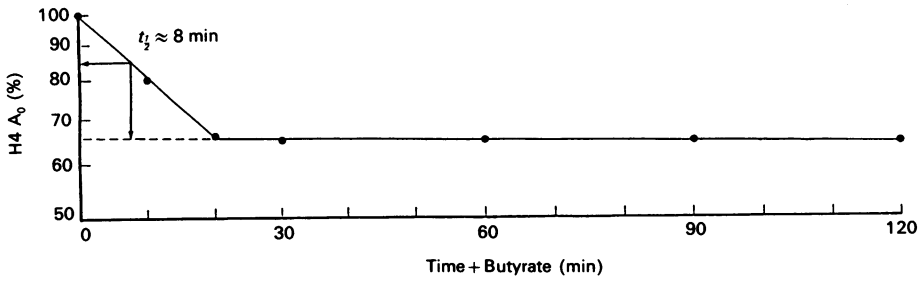

Fig. 3. Rates of labelled methylated H4 acetylation

The histone H4 bands in Fig. 2 were scanned with a densitometer, and the percentage of label in the unacetylated species was determined for H4 in each lane. The percentage of unacetylated H4 at zero time was set to 100% as described in the Materials and methods section. (Note that percentage H4 A_0 is plotted on logarithmic scale.)

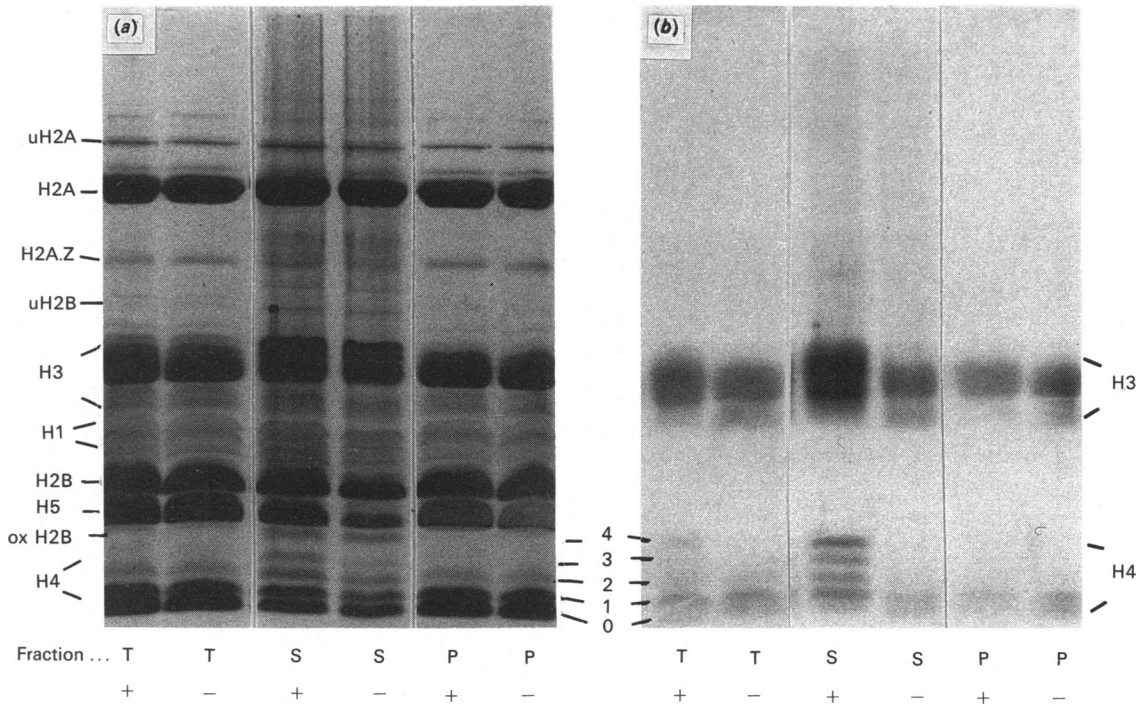

Fig. 4. The solubility of chromatin fragments with labelled methylated histones H3 and H4 is dependent on the level of acetylated histones species

Erythrocytes were incubated in the presence (+Bu) and absence (−Bu) of sodium butyrate for 1 h. Acid-soluble proteins (50 μg) isolated from nuclei (T), 0.15 M-NaCl-soluble (S_{150}; S) and insoluble (P_{150}; P) fractions were electrophoretically resolved on an AUT/15%-polyacrylamide gel. (*a*) and (*b*) show the Coomassie Blue-stained gel and the fluorogram respectively. 0, 1, 2, 3 and 4 represent the un-, mono-, di-, tri- and tetra-acetylated species of histone H4 respectively. ox H2B is oxidized histone H2B (note that this co-migrates with the tetra-acetylated species of histone H4). u denotes the ubiquitinated histone species.

specific activity of the histones remained constant. However, at later times (90 and 120 min) the specific activity of the histones decreased approx. 10%, suggesting a low rate of turnover of methyl groups on the histones.

In agreement with the results of Zhang & Nelson (1988*a*), we observed only one rate of acetylation of methylated H4, with a $t_{\frac{1}{2}}$ of 8 min. This is a representative result of three experiments. Furthermore, the results demonstrate that there exist two biochemically distinct populations within the unacetylated species of histone H4: one which undergoes rapid acetylation and represents approx. 35% of the population of labelled methylated unacetylated histone H4, and one which is essentially refractory to acetylation during the course of incubation in the presence of sodium butyrate. Following 30 or 60 min after initiation of the butyrate chase, approx. 25% of the total labelled H4 remains unacetylated (Fig. 2*b*).

Effect of altering histone acetylation levels on distribution of newly methylated histones among salt-soluble and salt-insoluble chromatin fragments

The solubility of active/competent gene chromatin fragments in 150 mM-NaCl is dependent on the level of acetylated histone species. Elevating the level of hyperacetylated histone species increases the salt solubility of the active/competent chromatin fragments, while reducing the level of acetylated histone forms decreases the solubility of these fragments. Based partially on this observation, we postulated that histone acetylation alters the capacity of the H1 histones to form compact higher-order structures such that active/competent gene chromatin is maintained in a less folded state than the bulk of chromatin (Ridsdale *et al.*, 1990).

We determined whether altering the level of acetylated histone species affected the salt solubility of chromatin fragments associated with labelled methylated histones H3 and H4. Fig. 4(*a*) shows that incubation of immature red blood cells with sodium butyrate for 1 h affects the level of acetylated histones located in chromatin fraction S_{150}, with fraction S_{150} of butyrate-treated cells having a greater level of acetylated histones species than fraction S_{150} of untreated cells. Fig. 4(*b*) shows that altering the level of hyperacetylated histones by incubating cells in the presence or absence of sodium butyrate also affects the level of labelled methylated histones H3 and H4 in chromatin fraction S_{150}. The levels of labelled methylated histones H3 and H4 are greater in fraction S_{150} (+butyrate) than in fraction S_{150} (−butyrate). Furthermore, the hyperacetylated species of H4 (A_3 and A_4) are labelled in S_{150} (+butyrate), but not in S_{150} (−butyrate), where the lower A_0 and A_1 species of H4 are labelled. Comparing lanes P (+ and −butyrate) in Fig. 4(*b*) shows that fraction P_{150} (+butyrate) has a lower amount of labelled methylated histones H3 and H4 than fraction P_{150} (−butyrate). These results indicate that manipulating the level of acetylated histones affects the solubility of chromatin fragments with newly methylated histones.

Table 1 shows the percentage of newly methylated labelled histone H4 that is in fraction S_{150}. The specific activity of histone H4, which migrates well away from the other histones on SDS/polyacrylamide gels, was determined as described in the Materials and methods section. Relative to histone H4 of total chromatin, the specific activity of H4 of fraction S_{150} was increased by 5.1 ± 0.4-fold (+butyrate; $n = 7$) or 1.5 ± 0.6-fold (−butyrate; $n = 7$). The distribution of the labelled methylated histone H4 among the salt-soluble and salt-insoluble chromatin fragments parallels that of transcriptionally competent DNA sequences (e.g. vimentin and ϵ-globin). Fraction S_{150} (+butyrate)

Table 1. The effect of incubating immature red blood cells with sodium butyrate on the 0.15 M-NaCl solubility of chromatin fragments with newly methylated histone H4

The content of newly methylated histone H4 (Me H4) in low salt-soluble chromatin fragments isolated from immature chicken erythrocytes incubated in the presence (+Bu) or absence (−Bu) of sodium butyrate was determined. The percentage of total DNA in the 0.15 M-NaCl was determined by absorbance at 260 nm, and the percentage of total labelled methylated histone H4 associated with the low salt-soluble chromatin fragments was determined as described in the Materials and methods. Each value represents the mean ± S.E.M. from seven measurements.

Fraction	DNA (%)	Me H4 (%)
S_{150} (+Bu)	9.8±0.4	48.3±3.6
S_{150} (−Bu)	7.0±1.6	8.8±3.3
Ratio (+/−)	1.4	5.5

chromatin fragments contained approx. 42% of the total competent DNA sequences, while fraction S_{150} (−butyrate) had approx. 7% (Ridsdale *et al.*, 1990). These results demonstrate that elevating the level of acetylated histones by incubating cells in the presence of sodium butyrate increases the level of salt-soluble chromatin fragments containing labelled methylated histones and transcriptionally competent DNA sequences.

DISCUSSION

Our observation that labelled newly methylated histones of chicken immature erythrocytes are enriched in the acetylated species of histones H3 and H4 (Hendzel & Davie, 1989) led us to investigate the relationship between histone acetylation and methylation. Our results demonstrate that the dynamically acetylated histones selectively participate in ongoing histone methylation. This conclusion is supported by the following observations. First, the acetylation state of the newly methylated histone H4 is altered by incubating cells in the presence or absence of sodium butyrate. The majority of the H4 of immature erythrocytes, which is unacetylated (A_0) or monoacetylated (A_1), is not affected by incubating cells in the presence or absence of sodium butyrate. In contrast, incubation of cells with sodium butyrate results in a significant shift in the distribution of methyl label among the acetylated species of H4, with 85% of the methyl label being located in the A_0 and A_1 H4 species at the onset of butyrate treatment, and this is reduced to 52% after 1 h of butyrate incubation. If we assume that the A_2, A_3 and A_4 forms of H4 participate in active ongoing acetylation, then at least 48% of the newly methylated H4 is engaged in dynamic acetylation. In comparison, only 1–2% of the total H4 population participates in dynamic acetylation (Zhang & Nelson, 1986). Secondly, the newly methylated histone H4 undergoes only one rate of acetylation, with a $t_{\frac{1}{2}}$ of 8 min. This rate of acetylation is similar to that obtained by Zhang & Nelson (1988*a*), who reported one rate of acetylation with a $t_{\frac{1}{2}}$ of 12 min.

Although our results demonstrate that newly methylated H4 participates in dynamic acetylation, we show that H4 that is not acetylated is also methylated. Following 30 or 60 min after initiation of the butyrate chase, approx. 25% of the labelled H4 remains unacetylated. Moreover, butyrate incubation of cells neither reduced nor enhanced the level of ongoing histone methylation. These results suggest that acetylation of H4 is not a requirement for methylation.

In contrast with the results of Boffa *et al.* (1981), we observed that sodium butyrate does not inhibit histone methylation. It should be noted that Boffa *et al.* (1981) used much longer exposure times (15 h versus 1 h) of the cells to butyrate. Furthermore, the HeLa cells incubated with butyrate have ceased replicating (Boffa *et al.*, 1981). Thus, in comparing ongoing histone methylation in HeLa cells incubated in the presence or absence of sodium butyrate, two physiologically different cell populations are being compared.

Recent evidence suggests that histones undergoing methylation are associated with transcriptionally active/competent DNA (Hendzel & Davie, 1989). The results presented here provide further support for this hypothesis. We show that the low salt solubility of chromatin fragments with newly methylated histones is dependent upon the acetylation state of the histones. Transcriptionally active/competent, but not repressed, gene chromatin fragments exhibit the same dependency (Ridsdale *et al.*, 1990). Thus altering the histone acetylation levels has the same affect on the low-salt-solubility properties of chromatin fragments associated with active/competent DNA or with newly methylated histones.

The physiological significance of histone methylation remains to be elucidated. The site of methylation in histone H4 (Lys-20) occurs within a basic domain which is bound to the sharply bent region of nucleosomal DNA (Ebralidse *et al.*, 1988). This basic histone H4 domain is a 'hotspot' for post-translational modifications, including acetylation (Lys-16), phosphorylation (His-18) and methylation (Lys-20). Although methylation of lysine does not change its charge, methylation significantly reduces its affinity of binding to DNA. The binding affinity of lysine for DNA is inversely proportional to the number of methyl groups per residue (Grandos & Bello, 1980). Tetra-acetylated methylated H4 would thus have reduced electrostatic interactions between DNA and the basic domain when both Lys-16 and Lys-20 are modified. These modifications could alter nucleosome structure and function (Ebralidse *et al.*, 1988; Norton *et al.*, 1989; Megee *et al.*, 1990; Oliva *et al.*, 1990; Ridsdale *et al.*, 1990; Walker *et al.*, 1990)

Histone methyltransferase and histone acetyltransferase activities are enriched in transcriptionally active/competent gene-enriched chromatin fractions of immature erythrocytes (Hendzel & Davie, 1989; Chan *et al.*, 1988). Histone methyltransferase activity is associated with the nucleosome, while histone acetyltransferase activity is found in the linker DNA regions. Although the location of the histone deacetylase activity in transcriptionally active/competent gene chromatin domains has not been shown, it is reasonable to assume that this enzyme activity must also be located here to account for the dynamic acetylation of these histones. Interestingly, histone deacetylase activity is associated with the nucleosome (Mold & McCarty, 1987). The centralization of the histone methyltransferase, histone acetyltransferase and histone deacetylase in active/competent chromatin domains would ensure the maintenance and dynamics of histone modifications which are important in the structure and function of transcriptionally active/competent gene chromatin.

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