EFFECTS OF BUTYRATE UPON THE METAPHASE-SPECIFIC DEACETYLATION OF HISTONE H4

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1. Introduction

Several enzymic modifications of the histones are now known to occur during the cell cycle [1–3]. Among these, the most direct link with the physical state of chromatin has been delineated for the acetylation of specific lysine side chains [3]. This modification is dominant in the loose chromatin structures responsible for transcription and replication processes. Conversely, it is lowered as chromatin becomes quiescent and compact during mitosis [4]. Acetylation is restricted to the 4 histones comprising the nucleosomal core particle (H2a, H2b, H3 and H4) and it has been shown convincingly that it serves to release structural constraints within the individual nucleosome [5] and also for internucleosomal contacts [6].

Acetyl contents of the core histones are the result of a dynamic equilibrium between acetylating and deacetylating activities [7]. The recent discovery that deacetylases can be inhibited in the living cell by short fatty acid salts (propionate to valerianate) opened the possibility to study the structural and physiological consequences of histones being in a hyperacetylated state [8]. Usually, these effectors inhibit cell proliferation [9–12] but conditions have also been reported under which cells containing hyperacetylated histones are still capable of traversing the cell cycle [13]. This observation raised doubts about the postulate in [4] that decreased acetyl levels are in fact a prerequisite for chromosome condensation.

Here we have grown a CHO cell line in the presence of butyrate concentrations still permitting proliferation. We will show that mechanisms exist at metaphase which release cells from the butyrate-mediated hyperacetylation found at other stages of the cell cycle.

2. Materials and methods

CHO cells (chinese hamster ovary, ATCC CCL 61) have been purchased from Seromed, München, and cloned to yield a cell line with 23 chromosomes (diploid number: 22). Cells were grown in DMEM medium containing 10% newborn calf serum, non-essential amino acids (Seromed), minimum essential vitamins (Seromed) and 20 mM of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES, pH 7.6). Growth curves were derived from monolayer cultures which had been raised on coverslips positioned in Petri dishes. Each dish was plated with 7×10^4 cells. At fixed intervals, coverslips were removed from the medium, cells were harvested by trypsination and collected by centrifugation ($200 \times g$, 5 min). After their resuspension in phosphate-buffered saline (PBS) containing 0.04% of trypane blue, living cells were counted in a haemocytometer. Each determination was based on triplicate samples from different coverslips and each count covered at least 500 cells.

For a metaphase arrest, CHO cells were grown in 175-cm^2 Roux glass flasks. At a density of 10^7 cells/flask, the medium was adjusted to 1 mM of sodium butyrate. After 16 h, the culture was replenished with a corresponding DMEM medium which in addition contained 0.04 μg/ml of colcemide. At 3-h intervals, the rounded mitotic cells were harvested by gently shaking, pelleted by centrifugation, washed with PBS buffer and collected at 4°C. The procedure was repeated twice; cells remaining attached after 9 h in colcemide were regarded as interphase cells which was confirmed by their mitotic index (see below). After washing with ice-cold PBS buffer, cells were scraped off with a rubber policeman and processed

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like the metaphase population. Cellular pellets were extracted with 100 μl of cold 0.25 M HCl; the extracts were neutralized and analyzed on a 2-gel system (12% acrylamide-SDS slab gel followed by a 12% acrylamide, 6 M urea, 0.9 M acetic acid tube gel) as in [14] and Schröter et al. (submitted). The percentage of cells in metaphase was determined on 0.5 ml of a cellular suspension in 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 20 mM Tris-HCl (pH 7.0). After hypotonic swelling for 5 min, saponine was added to a final concn. of 0.1%. By this procedure, cells quickly turned transparent and could be scored for their metaphase and interphase constituents. Each determination was based on at least 200 cells.

3. Results

3.1. *Butyrate effects on histone hyperacetylation and cell proliferation*

A number of substances is now known which extends the G1 phase of the cell cycle concomitant with alterations in the patterns of transcription [9–12]. In case of the short fatty acids, the hyperacetylation of histones has been suggested as the molecular basis of these effects [15]. However, a statement to the contrary is found in [13] who observed cells to proliferate freely in the presence of 6 mM propionate although the histones were 'hypermodified'.

We have elaborated the correspondence between various degrees of histone acetylation and the cell cycle duration by applying butyrate at concentrations between 1–7 mM (figs.1,2). Fig.1 reflects a gradual extension of population doubling times from 13 h in the absence of butyrate to about 20 h at 1 mM and 40 h at 5 mM of the fatty acid. At 1 mM, the limit acetylation pattern of histone H4 is almost attained (fig.2b) except from some further relative increase of the tetraacetylated component ($H4_4$, see fig.2a). For H3 on the other hand, acetylation is far from complete up to 3 mM of butyrate (fig.2b). The acetylation of histones H2b and H2a finally is not recognizeable below 5 mM of butyrate and requires 10 mM and 15 mM, respectively, for a maximum enhancement. Considering that submaximal acetyl contents reflect the equilibrium between acetylating and deacetylating activities, these results suggest that a deacetylase acting upon H4 is affected somewhat more than an enzyme for H3 and significantly more than activities specific for H2a and H2b. Proliferation is reduced as histones

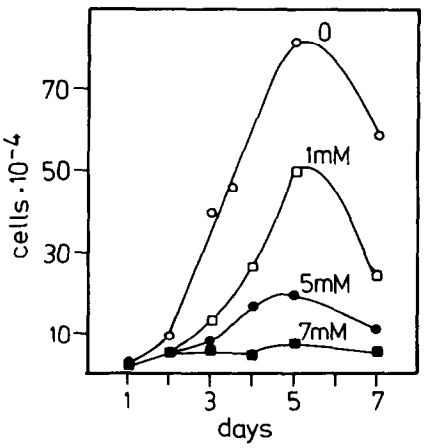

Fig.1. Retardation of cellular growth by butyrate. Numbers next to the curves reflect butyrate concentrations. Growth characteristics have been determined on monolayers of CHO cells as described in section 2.

H3 and H4 become maximally modified but ceases before the remaining histones are involved.

Comparing the acetylation patterns which permit or prohibit proliferation with the related figures in [13] we find striking similarities. The effect of 6 mM propionate is approximated by 1 mM of butyrate in the present study and both additions still allow some proliferation. Therefore we have to conclude that growth is compatible with a certain level of H3 and H4 acetylation but is shut off once this limit is surpassed.

3.2. *Histone H4 deacetylation at metaphase is the presence of butyrate*

To obtain a reasonable yield of metaphase cells and to minimize the cellular damage about by the continued application of colcemide, we chose a compromise concentration of 1 mM butyrate for the following analyses (cf. section 2). At 3-h intervals, the culture flasks were gently shaken to detach the rounded mitotic cells and this procedure was repeated twice to obtain approximately equal populations (5×10^6 cells) enriched in metaphase (supernatant suspension) and interphase (remaining monolayer). Some cross-contamination of the samples was evident from a mitotic index of about 80% in detached cells; it is also the most plausible explanation for some reduction of the amount of acetylated forms in the enriched interphase population of fig.3b if compared with the cells analyzed in fig.2a.

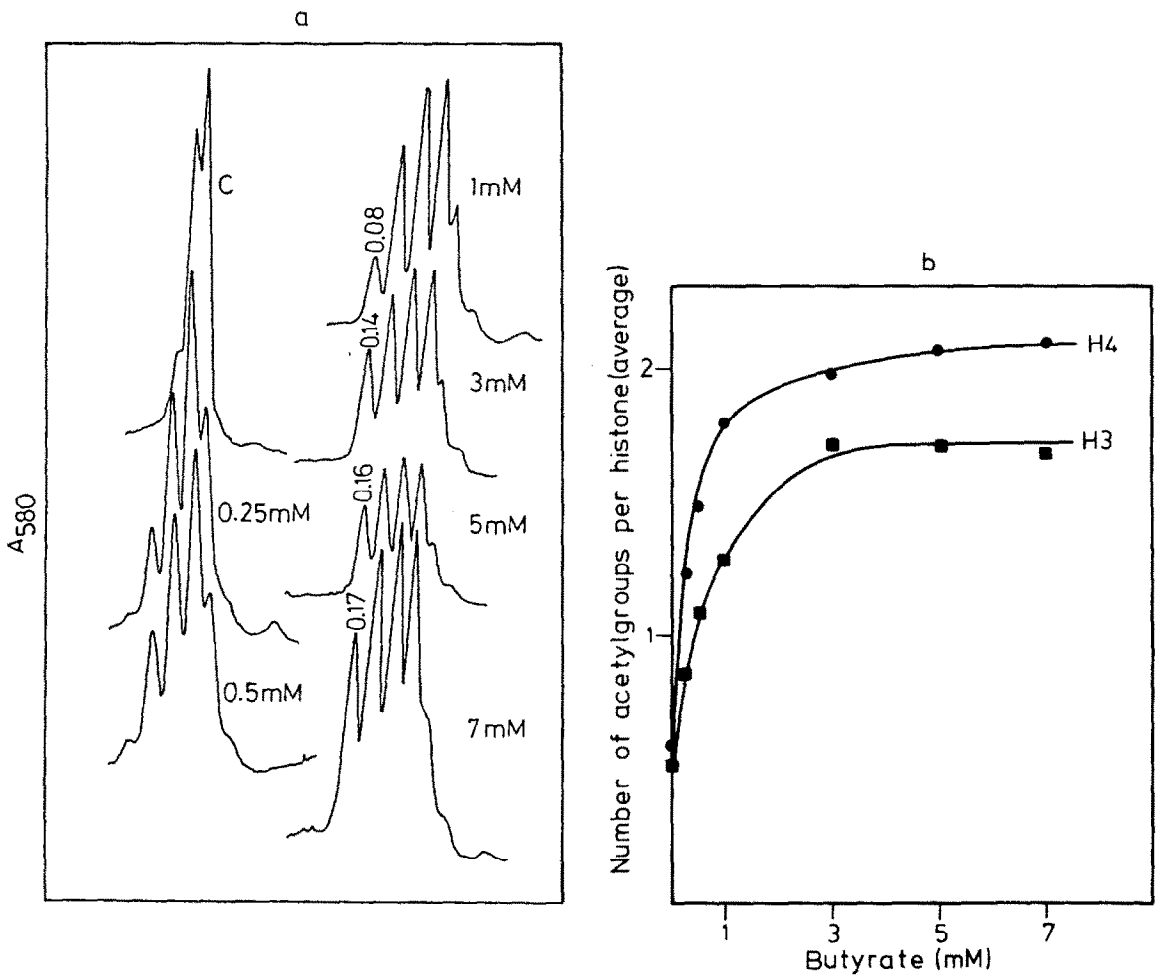

Fig.2. Histone acetylation patterns influenced by different butyrate concentrations. (a) CHO cells have been incubated for 16 h at the indicated concentrations of butyrate; C, control culture, grown in the absence of this addition. Numbers next to the 4-fold acetylated H4 components ($H4_4$) indicate their contribution to the sum of H4 specimens. (b) Average numbers of acetyl groups incorporated into histones H3 and H4 at 0–7 mM of butyrate.

Histone H4 is easily analyzed for its modification because its subfractionation on acid-urea gels is only due to 4 degrees of acetylation ($H4_1$–$H4_4$, cf. [4]). With H3 on the other hand, a ground level of acetylated specimens is superimposed by forms containing a metaphase-specific phosphorylation [1,4]. Although similar conclusions may be drawn for this histone, we will restrict our discussion to the densitograms obtained with H4.

An inspection of fig.3 shows the known phenomenon of hyperacetylation for the cells derived from interphase (fig.3a). However, this does not hold for metaphase cells which even in the presence of butyrate undergo a deacetylation to a state which is very similar to metaphase cells obtained in the absence of the fatty acid (table 1). This becomes particularly evident if the calculations are corrected for an approx. 20% contamination by interphase cells yielding 61% of $H4_0$, 36% of $H4_1$ and 3% of $H4_2$.

Many data are available to support the notion that chromatin in a compact and transcriptionally inactive state contains a minimum number of histone H4 acetyl residues (reviewed in table 1). We have shown in this contribution that cellular growth in the presence of certain deacetylase inhibitors does not invalidate this statement because these inhibitors are relatively inefficient at metaphase.

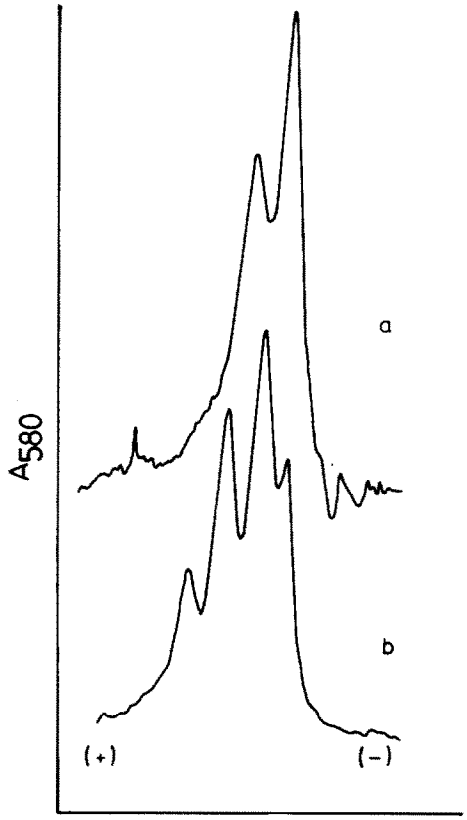

Fig.3. Histone H4 subfractionation of CHO cells grown at 1 mM of butyrate. a, metaphase cells (mitotic index 80%); b, interphase cells (contaminated to 20% by metaphase cells). Both cell populations were derived from a single culture flask as described in section 2.

4. Discussion

The acetylation of the core histones leads to an unravelling of the nucleosome [5] and to a general decondensation of chromatin [6]. As the presence of acetyl residues appears to be an obstacle to the condensation processes which occur during spermiogenesis [16], erythropoesis and nuclear inactivation [16,17] and at metaphase [4,18], a deacetylation has to occur prior to these events. There are several reports suggesting that this is accomplished by enhancing deacetylase activities because these activities appear elevated comparing erythrocytes with reticulocytes [20], rat liver with either regenerating liver [21] or hepatoma cells [22] and early with late stages of meiosis [23].

We have inhibited deacetylases by butyrate which in an in vitro assay proved to be a highly efficient, non-competitive inhibitor with an apparent K_i value of 60 μM [19]. Cells were capable of proliferating at reduced rates in the presence of 1 and 5 mM of the fatty acid (fig.1) which according to these figures should inhibit 94 or 99% of the activity, respectively. Nevertheless, at metaphase mechanisms operate which strongly reduce acetylation to almost uninhibited levels while the interphase population shows the known phenomenon of hyperacetylation. A mere increase in the amount of a butyrate-sensitive deacetylase cannot account for this result under conditions of nearly complete inhibition. On the other hand, there is growing evidence for a differential action of butyrate upon distinct deacetylase activities (see the graded effect

Table 1
Acetylation pattern of histone H4 in condensed chromatin in comparison to physiologically active chromatin (in parenthesis)

	$H4_0$	$H4_1$	$H4_2$	$H4_3$	$H4_4$	Ref.
Sea urchin						
Sperm (blastula)	100 (46)	(34)	(13)	(7)		16
Tetrahymenea						
Micronucleus						
(macronucleus)	77 (37)	14 (33)	5 (21)	4 (9)		17
Avian red cells						
Mature (immature)	65 (58)	26 (29)	6 (8)	3 (5)		16
Physarum						
Metaphase (late G2)	20 (15)	66 (55)	11 (23)	2 (3)	(3)	18
CHO cells						
Metaphase (Interphase)						
–Butyrate	69 (51,50)	25 (34,41)	5 (11,9)	(4)		4,5
+ 1 mM butyrate	55 (15,23)	37 (29,35)	8 (28,27)	(20,12)	(8,3)	This work (figs. 2,3)

on the core histones as discussed above) which is either due to diverse enzyme entities or to specific responses of the various physiological effectors which are now known to exist [24–27]. In conclusion, histone deacetylation is strictly correlated with the condensation of chromosomes at metaphase and the maximum acetyl content which is compatible with this compact state appears rather well defined.

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