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Butyrate-Induced Histone Hyperacetylation in Human and Mouse Cells: Estimation of Putative Sites of Histone Acetylation In Vivo

Panagiotis Pantazis and William M. Bonner

Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Human and mouse cells in culture were treated with various concentrations of sodium butyrate. Acid-extracted histones of control and butyrate-treated cells were analyzed by two-dimensional gel electrophoresis. All core histones of the control cells contained modified forms. All core histones of the butyrate-treated cells were hyperacetylated. Depending on the number of acetylation sites per molecule, each histone or histone variant exhibited a characteristic number of acetylated forms. This number was the same for each histone common in human and mouse cells treated with butyrate. Histones 2A.1, 2A.2, and 2A.X have two sites of inner acetylation; 2A.Z has 3; 2B's have 5; and each one of the H3 variants as well as H4 have 4.

Key words: histone hyperacetylation, acetylation sites, mammalian cells

Acetylation-deacetylation is considered to be a putative mechanism for histone modification involved in gene regulation [1–3]. This modification reportedly affects all classes of histones except H1 [3–5]. Acetylation of histones, mediated by histone class-specific or nonspecific acetyltransferase, is either irreversible or reversible [6,7]. N-terminal acetylation is a cytoplasmic event common to several histones and is essentially irreversible, whereas acetylation of the inner lysine residues is a nuclear event and is reversible. Many of the lysine residues that can be acetylated in each histone have been identified [2,8]. In many cases, histone acetylation concomitantly increases with an increase in gene activation triggered by hormonal [9,12] or chemical [13,14] stimulation. In nondividing cells, acetylation of histones appears to be restricted to the template active regions of chromatin [15,16]. However, it is likely that regions of chromatin that are not frequently transcribed also contain acetylated forms, but the acetylated histones in nontranscribed regions may differ from those in transcribed regions in either the rate or extent of acetylation [17]. Whether histone acetylation correlates primarily with active genes or diffuse chromatin which is

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accessible to histone acetyltransferase is still unknown [18,19]. Chromatin, which has been acetylated chemically [20], or chromatin from butyrate-treated cells, is very sensitive to DNAse I [15–17], because of changes in accessibility of DNA sites within the nucleosome core particle [17]. Chromatin from butyrate-treated cells is also transcribed more rapidly by E coli or HeLa RNA polymerase [21]. In addition, butyrate treatment results in reversible decondensation of chromatin clumps in HeLa cells [9].

In this study, mammalian cells were treated with butyrate and histones were analyzed on two-dimensional gels. The number of acetylated forms of both human and mouse histone variants were estimated.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium butyrate from concentrated butyric acid (Fisher, Fair Lawn, NJ) made 1 M and brought to pH 7.2 with concentrated NaOH was filtered and used as a stock solution. Chemical materials used for preparation and electrophoresis of histone extracts have been described [22]. Puromycin was from Sigma (St. Louis, MO) and ^{14}C-acetate (^{14}C-acetic acid; sodium salt, sp. act. 59 mCi/mmole) from Amersham (Arlington Heights, IL).

Gel Electrophoresis

First dimension was on acid-urea-Triton X-100 (AUT) gels, whereas, second dimension analysis was on acid-urea-hexadecyltrimethylammonium bromide (AUC) gels [22]. For high resolution of histones 2A, AUT gels contained 10% acrylamide-8 M urea [23], whereas for high resolution of histones 2B, AUT gels contained 15% acrylamide-6 M urea [24]. AUC gels contained 16.5% acrylamide.

Cells and Cell Cultures

The human leukemic cells, HL60 [25], and K562 [26] were gifts from the Laboratory of Tumor Cell Biology, NCI, National Institutes of Health, Bethesda, MD. Mouse (Friend) erythroleukemia cells, clone C19, were a gift from the Laboratory of Clinical Hematology, NIH, Bethesda. Mouse leukemic cells, L1210, are routinely grown in our laboratories. HL60, K562, and C19 cells were grown in RPMI 1640 medium, and L1210 in RPMI 1630. Both RPMI media were supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, McLean, VA), penicillin, and streptomycin. The cells were grown at 37°C in a humidified incubator containing 5% CO_2. Sodium butyrate was added to exponentially growing cultures to various final concentrations, unless otherwise stated. Butyrate treatment was for various periods of times. To label with ^{14}C-acetate, the cultures were brought to 100 μg/ml in puromycin or 5 mM sodium butyrate for 10 min prior to addition of the label.

RESULTS

Effect of Sodium Butyrate on Cell Growth and Morphology

HL60, K562, C19, and L1210 cells were treated with various concentrations of sodium butyrate for various periods of time. The kinetics of cell growth with respect to butyrate concentration were similar for all cell lines. The data for K562 cells is

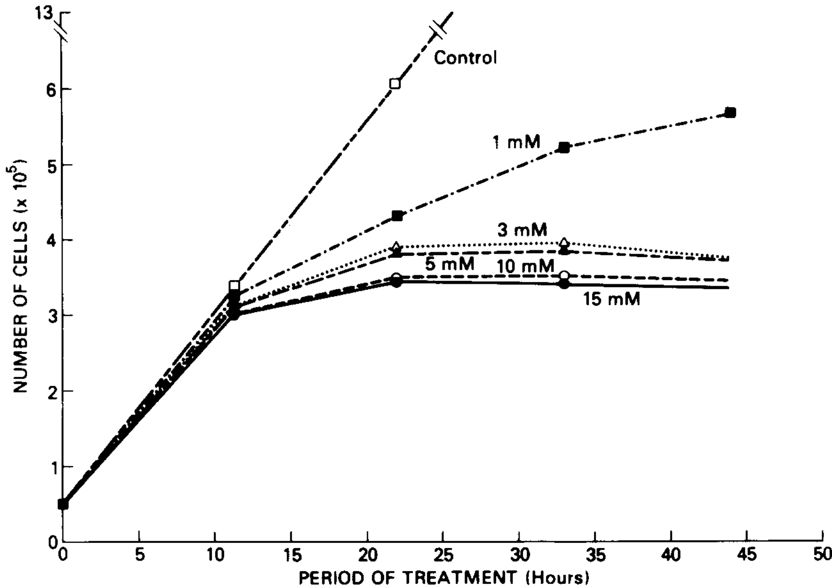

Fig. 1. Effect of butyrate concentration on growth of K562 cells.

shown in Figure 1. The growth rate of K562 cells treated with 1 mM butyrate decreased with respect to that of untreated cells. This correlated with the fact that after four days of treatment about 70% of the K562 cells were found to be hemoglobin-positive [27]. A rapid decrease in the growth rate of K562 cells followed treatment of these cells when increased concentrations of butyrate were present in the culture, ie, concentrations above 3 mM. However, these concentrations of butyrate, when present in the cultures for prolonged periods of time, resulted in changes in the shape of cells, ie, "rugged" instead of "round " normal cell shape cells were observed by light microscopy. It should be noted that appearance of hemoglobin-synthesizing K562 cells after treatment with 1 mM butyrate was not followed by the morphological changes reported by others [28]. Our observations agree with those of Chioe et al [29].

Histone Acetylation Induced by Sodium Butyrate

The presence of sodium butyrate in cell cultures resulted in inhibition of histone deacetylates [4,18] and thus in appearance of acetylated forms of histones. The transition from oligoacetylated histones in control cells to hyperacetylated histones in butyrate-treated cells was studied with respect to time of exposure of the cells to 5 mM butyrate. Gels a to i on Figure 2 show the acetylation patterns of histones of L1210 cells with respect to period of butyrate treatment. The acid-extracted histones were electrophoresed on AUT gels [22]. The position of histone variants and their modified forms have already been identified [22]. Butyrate-treated cells, extracted with acid in the presence or absence of 5 mM butyrate, as a deacetylation inhibitor, did not show significant differences. Perhaps, the presence of butyrate was not required to prevent deacetylation since preparation of nuclei, acid extraction, and preparation of samples for electrophoresis on AUT gels, was shorter than 60–70 min. It is apparent that without salt-treatment of the nuclei, the acid extracts contain a large

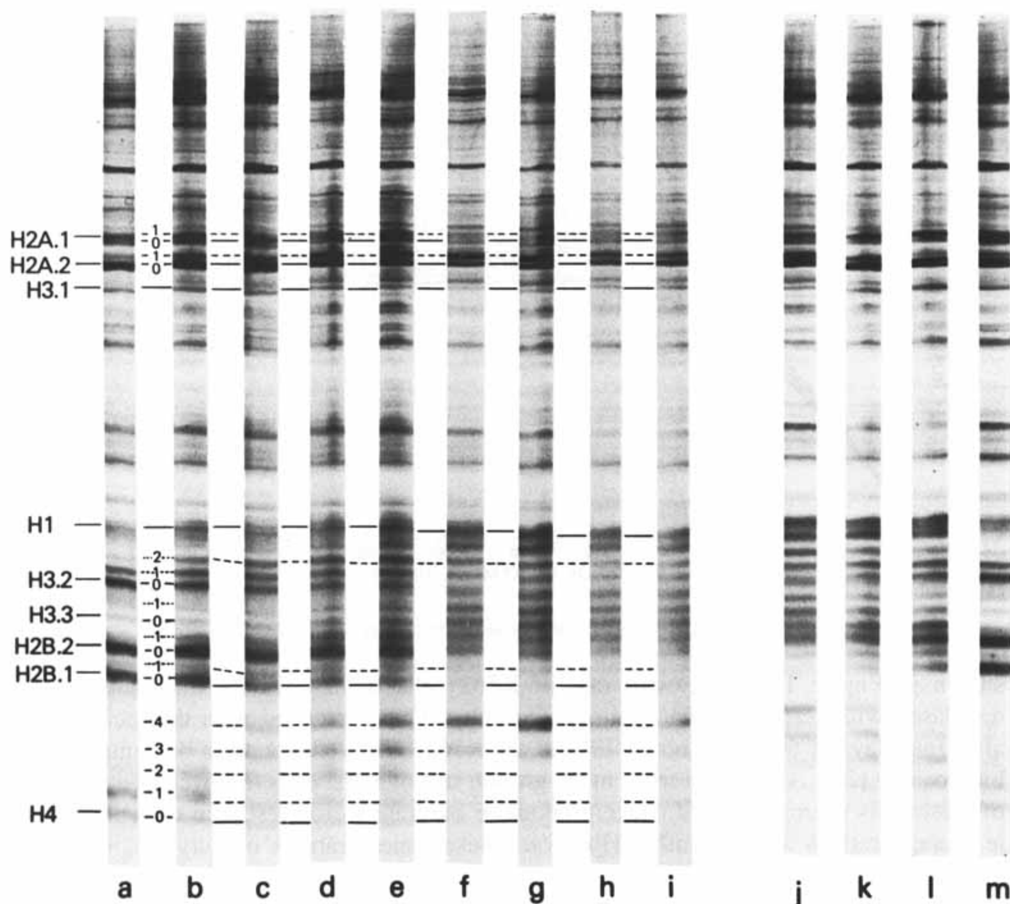

Fig. 2. AUT gels of acid extracts from nuclei of L1210 cells treated with 5 mM sodium butyrate for various periods of time (gels a–i) or released from butyrate treatment (gels j–m). a, 0 min (control); b, 15 min; c, 45 min; d, 90 min; e, 6 hr; f, 1 day; g, 2 days; h, 3 days; and i, 4 days. j, 15-min release; k, 90-min release; l, 6-hr release; and m, 1-day release. AUT gels contained 15% acrylamide-8 M urea.

number of other acid-extractable proteins (Fig. 2). However, one can see that treatment of L1210 cells with butyrate resulted in appearance of more acetylated histone forms within 10 min. After 6 hr of butyrate treatment, the major portion of all histones, except H1, were in a hyperacetylated state. Hyperacetylation of histones was completed after 24 hours of treatment (Fig. 2, gel f). No significant changes in histone hyperacetylation were observed when the cells were treated with butyrate for periods of time longer than 24 hr (Fig. 2, gels g–i). Removal of butyrate from the cultures resulted in deacetylation of the histones to levels of untreated cells (Fig. 2, gels j–m), in agreement with a previous report [30]. When AUT gels were subjected to two-dimensional electrophoresis on AUC gels, the modified histone variants were resolved completely [22]. Figure 3 shows the patterns of L1210 histones before and after butyrate treatment. All core histones are hyperacetylated in the presence of butyrate. Histones 2B, 3, and 4, and their variants, are highly acetylated, and the number of acetylated forms can be distinguished. Figure 4 shows the areas in Figure

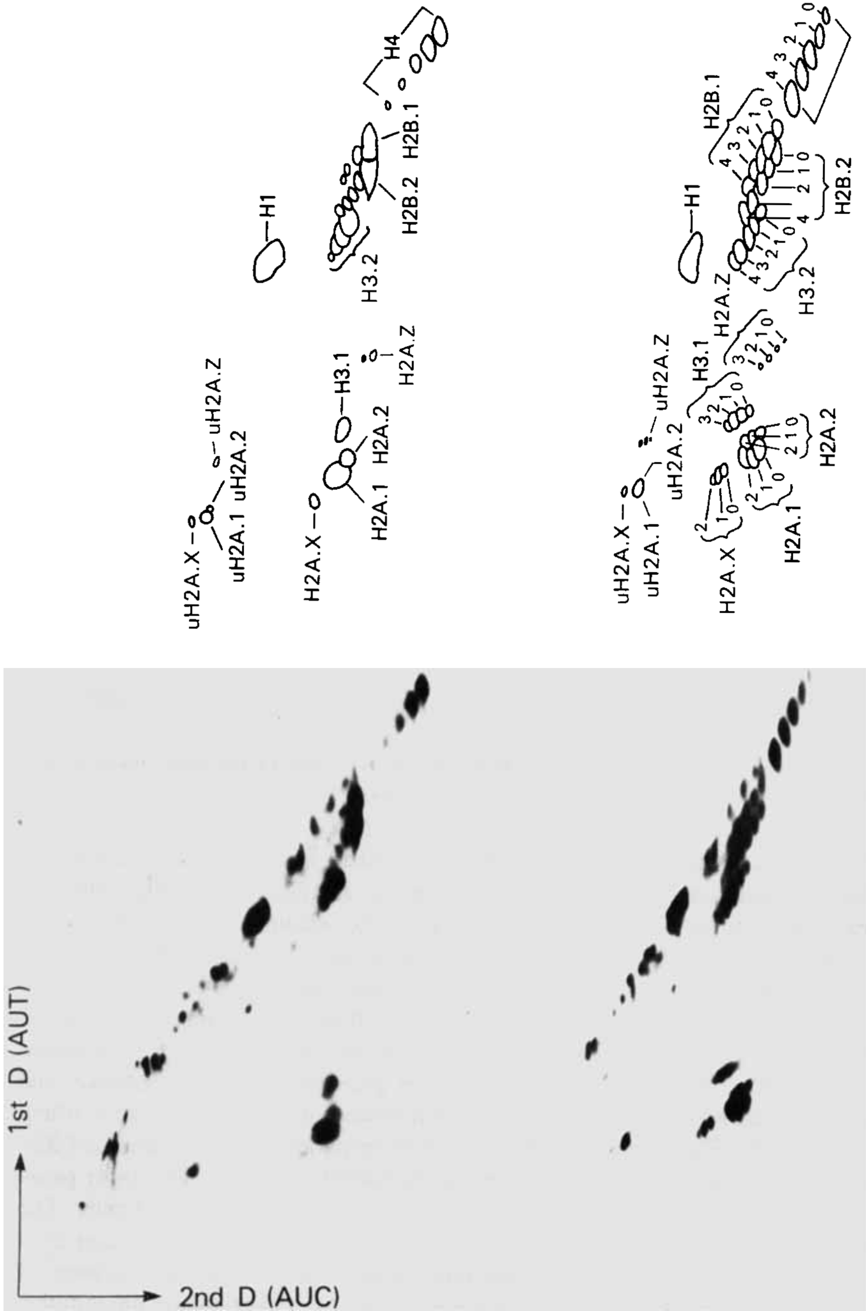

Fig. 3. Analysis of histones on AUT-AUC gels. Upper gel, control L1210 cells; and lower gel, L1210 cells treated with 5 mM sodium butyrate. AUT gels contained 15% acrylamide-8 M urea.

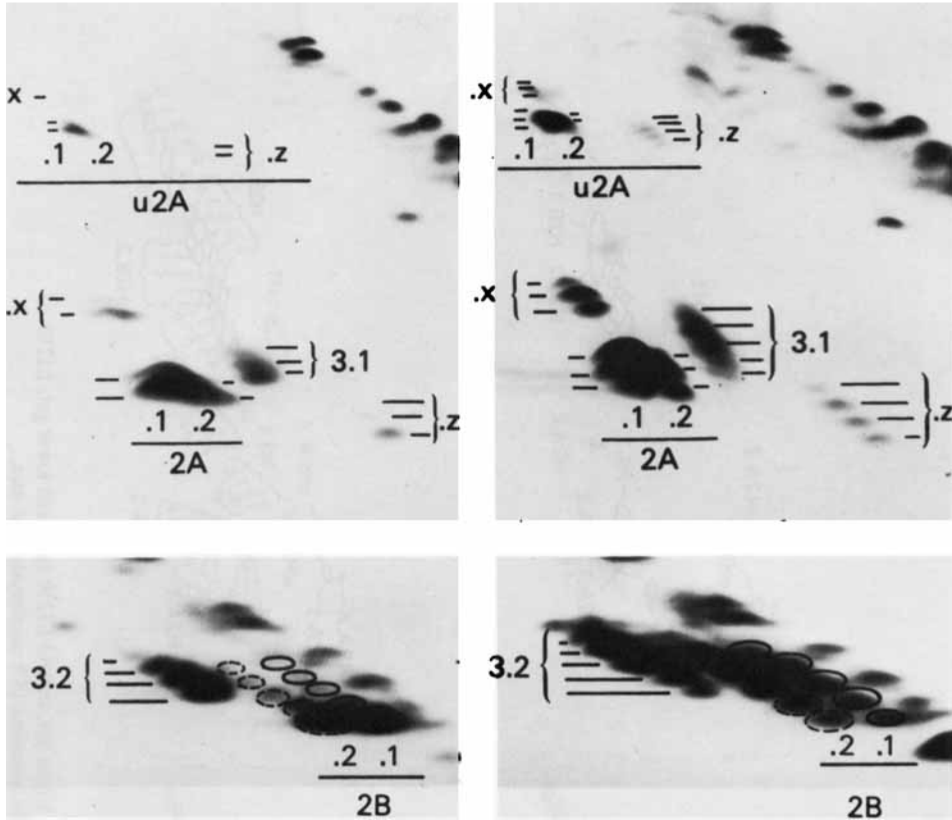

Fig. 4. Higher magnification of selected areas of gels in Figure 3 showing the highly resolved H2A variants. Left panel, control cells, and right panel, butyrate-treated cells.

3 in which histones 2A and 2B have been resolved. Also, it can be seen that histones 2A and their ubiquitinated adducts are modified by acetylation, but the extent of acetylation or the number of acetylated forms can not be adequately resolved on these gels. For this, a modified AUT gel system was used that resolved all the 2As [23]. Histone 2B variants and their modified forms were resolved on AUT gels containing 6 M urea [31], as shown in Figure 5. Unlike 2As, both 2B variants exhibited the same acetylation patterns; in addition, 2Bs seem to be the most extensively acetylated histones. Since H2Bs have not been found to be phosphorylated [5, Pantazis and Bonner, unpublished results], it is likely that their modified forms are induced solely by hyperacetylation. The same AUT-AUC system provided high resolution of histones 3, all of which exhibited the same acetylation patterns (Fig. 5). The upper panel of Figure 5 shows the electrophoretic patterns of histones 2A from L1210 cells. The acetylation pattern is similar for human and mouse cells (not shown). Human cells contain two H2B variants, one major and one minor, whereas the two variants of H2B in mouse cells are about equal in quantity (Fig. 6). Other major quantitative differences on human and mouse variants have been reported by this and other laboratories [24,32]. The similarity of acetylation patterns of the core histones in all gels indicates that human and mouse histones are acetylated at the same positions.

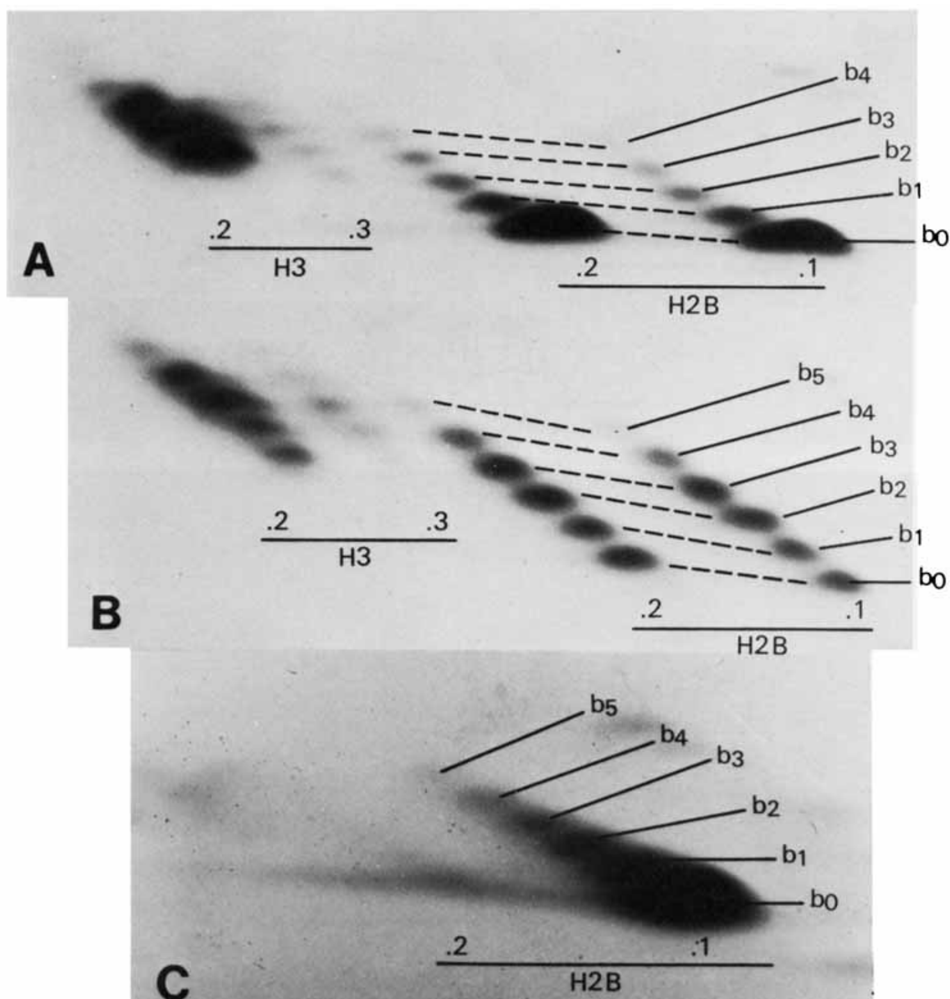

Fig. 5. Two-dimensional electrophoresis of acid extracts from nuclei of mouse (C19) and human (K562) cells treated with 0.4 NaCl prior to extraction. First dimension, 15% acrylamide-6 m urea AUT; and, second dimension, 16.5% acrylamide AUC. Panels A and B: C19; Panel C: K562.

Hyperacetylation of 2A Histones

Figure 6 shows the electrophoretic analysis of histones 2A from L1210 cells treated with butyrate and labeled with ^{14}C-acetate. Mass changes in H2A variants and their ubiquitinated forms caused by butyrate-induced hyperacetylation can be seen in the upper panel of Figure 6. Also, the number of acetylated forms for each 2A histone can be distinguished. Since the fastest migrating form of each variant is not acetylated in the presence of butyrate [23], variants 2A.1, 2A.2, and 2A.X contain molecules that are monoacetylated or diacetylated, whereas variant 2A.Z contains monoacetylated, diacetylated and triacetylated molecules. The ubiquitin adducts of H2As, ie, u2As, are also hyperacetylated. The number of acetylated forms of the ubiquitinated

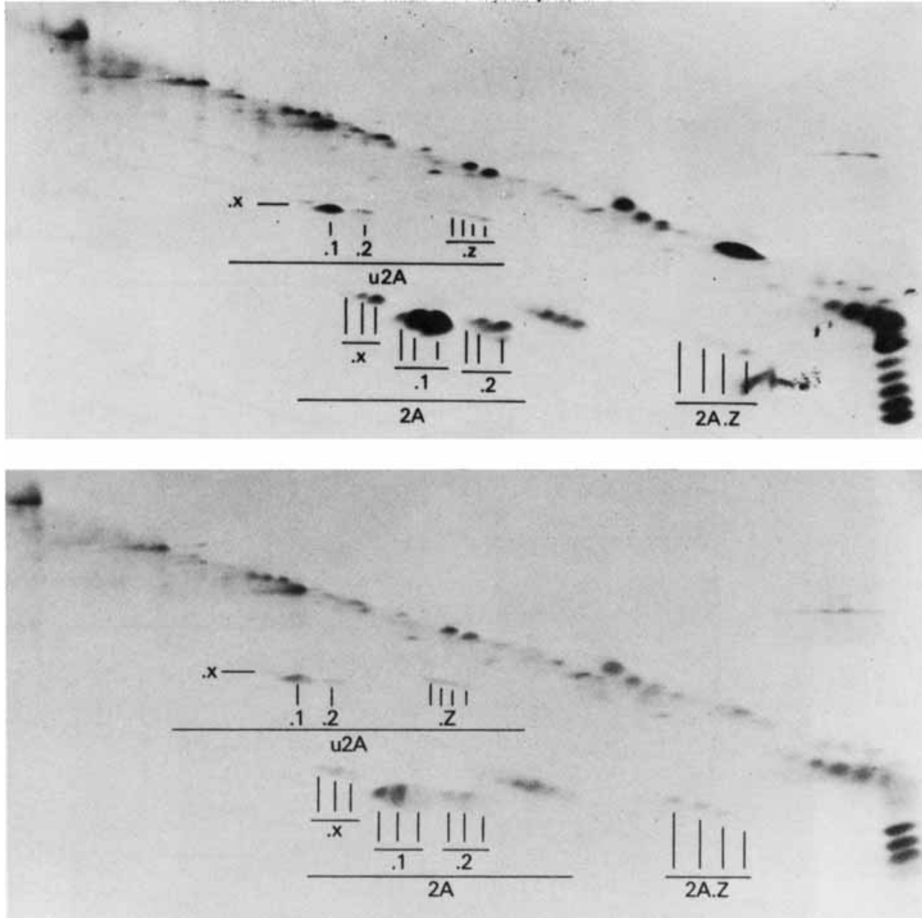

Fig. 6. Electrophoretic analysis of histones from L1210 cells treated with sodium butyrate and labeled with ^{14}C-acetate on AUT-AUC gels. First dimension, AUT gel containing 10% acrylamide-8 M urea; and second dimension, AUC gel containing 16.5% acrylamide. Upper gel, histones stained with Coomassie blue; and lower gel, fluorogram of the same gel.

H2As cannot be distinguished easily on the gels, since uH2As represent only a small portion of the parental histones [24]. Incorporation of ^{14}C-acetate into the acetylated forms of H2A variants is shown in the fluorogram of Figure 6. The pattern of labeled H2As is similar to that of the stained gel. However, in the stained gel, the front running form of each variant that is the newly synthesized histone is not labeled [23].

All 2A histones but H2A.Z can be both acetylated and phosphorylated [5,23, 33]; however, these two modifications appear to act antagonistically on H2As [33, Pantazis and Bonner, unpublished results]. The forms of H2A modified by acetylation, phosphorylation, or acetylation and phosphorylation cannot be distinguished by their rates of migration on the electrophoresis gels used in this study. However, knowing that acetylation antagonizes the phosphorylation of H2As [33], it is likely that the additional forms of H2A observed after butyrate treatment of cells are caused by hyperacetylation of these histones.

Histone Acetylation in Absence of Protein Synthesis

Acetylation of the unmodified form, b_o, of all core histones is a cellular event that is due to acetylation of the N-terminal serine residues of histones. Addition of acetyl group to this terminal residue takes place while the histone molecule is still synthesized. Consequently, this type of acetylation is coupled to histone synthesis. Recently, we showed that the unmodified form of H2As, ie, b_0H2A, contains histone molecules acetylated only at the N-terminal serine residue. Treatment of cells in culture with 5 mM butyrate prior to addition of ^{14}C-acetate resulted in a small but detectable incorporation of ^{14}C-acetate in the b_o form of histones (Fig. 6). Apparently, butyrate did not completely shut off the synthesis of histones. We then treated the cells in culture with puromycin, which was used previously to demonstrate that acetylation of the N-terminal serine residues of histones are tightly coupled to histone synthesis [23,34]. When L1210 cells were treated with puromycin prior to labeling with ^{14}C-acetate, the resulting electrophoretic pattern of histones on gels subjected to fluorography indicated absence of ^{14}C-incorporation in the fastest migrating or b_o form of all histones modified by acetylation [23]. However, it is apparent that treatment of the cells with puromycin did not inhibit incorporation of ^{14}C into the other forms of histones, ie, b_1 to b_5 (Fig. 3), indicating that all histone forms modified by inner acetylation result from a postsynthetic acetylation mechanism, as suggested previously [23]. It should be noted that, although both minor variants of histone 2A (.X and .Z) can be acetylated, we have shown that their patterns of acetylation were not similar [23].

Quantitation of Acetylation Sites

Two-dimensional electrophoretic analysis of acid extracts of human and mouse nuclei was utilized to estimate the number of potential sites of acetylation in the core histone variants. The number of acetylation sites was indicated by the number of acetylated forms on stained gels, which were resolved according to their charge. Acetylation sites in human and mouse histones were estimated from AUT-AUC gels in which AUT gels consisted of 10% acrylamide-8 M urea or 15% acrylamide-6 M urea. Acetylated forms of human and mouse H2B variants were best resolved when AUT gels were made of 15% acrylamide-6 M urea (Fig. 6). Only sites of postsynthetic (inner) acetylation were considered in this quantitation. Estimation of the acetylation sites in each core histone or histone variant is presented in Table I. In this table, it is noted that the electrophoretic systems used in the present study reveal a

TABLE I. Estimation of Internal Acetylation Sites of Core Histones

	Number of acetylation sites	
Histone/variant	HL60, K562 (human cells)	L1210, C19 (mouse cells)
H2A.1	2	2
H2A.2	2	2
H2A.X	2	2
H2A.Z	3	3
H2B.1	5	5
H2B.2	5	5
H3.1	4	4
H3.2	4	4
H3.3	4	4
H4	4	4

higher number of acetylation sites in histones than those previously reported [reviewed in 35].

DISCUSSION

The presence of sodium butyrate in cultures of human and mouse cells affected the growth of these cultures. Sodium butyrate at concentrations of 5 mM or more resulted in cessation of cell growth of all cell lines used in this study. Light microscopy of the treated cells revealed that observed changes in cell shape and size were concomitant with decreases in growth. Cell shrinkage was observed with increasing periods of exposure to butyrate. Upon removal of sodium butyrate, the cells progressively recovered their normal round shape and acquired the growth rate of control cells. Recovery of the initial cell shape and growth rate were faster in cultures treated with butyrate for shorter periods of time. Other investigators have also reported that the concentration of butyrate administered to the cells inhibits cell growth [5,36]; however, this growth inhibition appears to be independent of butyrate-induced histone hyperacetylation.

In this study, we showed that all core histone variants can be acetylated internally. Under conditions of complete histone hyperacetylation, each variant exhibited a characteristic number of acetylated forms that differ in charge and thus could be resolved on acrylamide gels. Although the extent of acetylation per variant is not known for any specific cellular or nuclear event, the present study showed that, at least in continuously dividing cells, all the core histone variants are capable of being acetylated to some extent. Perhaps this acetylation is associated with histone variants localized in transcriptionally active regions of chromatin similar to acetylation patterns in nondividing cells [15,16]. However, the acetylation of histones localized in the transcriptionally inactive regions of chromatin has not been excluded [17]. In addition, it seems likely that there is a correlation between increased chromatin transcription by procaryotic or eucaryotic RNA polymerase [21], and the decondensation of heterochromatic nuclear regions [9] in butyrate-treated cells. In general, histone acetylation has been postulated to be involved in histone deposition or chromatin assembly [38,39], transcriptional activity [38,39], induction of specific proteins in leukemic [40] or chemically transformed [41] cells in culture, and the inhibition of cell differentiation [42] or specific gene activation induced by hormones [43]. Also, acetylation can modulate modification of histones by phosphorylation [33, 44, Pantazis and Bonner (unpublished results)]. The extent of acetylation per histone variant, that is, the number of acetylated forms per histone variant, is not known for each one of the events reported above. Recently, we observed that when the human leukemic cells, HL60, are converted to more differentiated cells by dimethylsulfoxide or tetradecanoylphorbol-13-acetate the number of acetylated forms of histone 4 is decreased [Pantazis and Bonner, unpublished data]. In this case, it is likely that the decrease in acetylated forms correlates with a decrease in the DNA synthesis. It has been reported that histone acetylation decreases in aging cells in culture [45–47], but this modification has not been shown to correlate with biochemical mechanisms involved in the process of aging. Other studies showed that the chromosomal proteins are more tightly bound to DNA in older rather than in young animal cells [48–50], and these findings indicate that there is a general age-related repression in transcription [49,51,52]. Thus, it is likely that histone acetylation could control the association of chromosomal proteins with DNA and consequently result in altered transcriptional activity of the genome. Studies on the acetylation of histone variants in aging cells in culture are currently in progress.

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