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Dynamic histone acetylation in alfalfa cells. Butyrate interference with acetate labeling

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Dynamic histone acetylation of alfalfa (*Medicago sativa*) was studied in suspension cultures by short-term labeling with radioactive acetate. The relative labeling rates for the acetylated histones were in order of decreasing incorporation; H3.2 > H3.1 > H4 > H2B.1 > H2A.3. Histone H3 showed at least seven sites of acetylation, histone H2B.1 had six sites and histone H4 had five sites. Low numbers of acetylation sites were observed for histone H2B.2 and all histone H2A variants. The mass ratio, steady state acetylation and dynamic acetylation between major variant H3.1 and minor variant H3.2 were approx. 2:1, 1:2 and 2:5, respectively. Treatment of alfalfa cells with 50 mM *n*-butyrate did not lead to histone hyperacetylation, but instead interfered with histone acetylation labeling by acetate. The extent of apparent inhibition increased with time and concentration of butyrate. It is likely that the conversion of butyrate to acetylCoA results in dilution of the specific radioactivity of [^{3}H]acetate in the acetylCoA pool thereby inhibiting the labeling reaction. This interpretation is supported by ^{14}C-labeling of alfalfa acetylated histones by [1-^{14}C]butyrate.

Introduction

Postsynthetic modification of histones by acetylation appears to be an important step in the modulation of chromatin structure for transcription, replication and spermatogenesis (see Refs. 1–4 for reviews). Histone acetylation in transcriptionally active chromatin domains has been characterized by a high steady state level of multiacetylation of core histones [4–6] and by a high rate of acetyl group turnover. In mammalian cells half lives for acetylated lysine residues in core histones as short as 3–30 min have been measured [7–8] with 10-fold slower turnover rates recorded for yeast [9] and *Physarum polycephalum* [10]. In this paper, we present an analysis of dynamic histone acetylation in alfalfa. This study extends the characterization of the dynamics of histone acetylation to higher plants.

In animal cells butyrate is an effective noncompetitive inhibitor of histone deacetylase [11–13] and has been proved to be a useful tool to measure turnover rates of histone acetylation [7,13–17]. Application of butyrate to alfalfa tissue culture cells appeared not to inhibit the histone deacetylase enzyme of this plant, since we were unable to detect induction of histone hyperacetylation in the presence of butyrate. Similar observations have previously been made for tobacco and artichoke plant cells [18,19]. The absence of histone hyperacetylation prevented the use of butyrate to measure turnover rates of histone acetylation in the alfalfa tissue culture cells. However, the addition of butyrate revealed new dynamic characteristics of histone acetylation. In alfalfa plant cells, butyrate appears to be metabolized and to lead directly to histone acetylation. Through this pathway butyrate can interfere with acetate labeling of the histone acetylation reaction.

Materials and Methods

Cultivar strain R4 of *Medicago sativa* was grown as callus and in suspension as described before [20,21]. Suspension cultures, newly initiated by dispersal of 20 g of callus into 60 ml growth medium, were labeled for 60 min by addition of 0.5 ml growth medium with 1 mCi of sodium [^{3}H]acetate (ICN Radiochemicals, 6–27 Ci/mmol) or 125 μCi of *n*-[1-^{14}C]butyric acid (ICN Radiochemicals, 43 mCi/mmol), unless noted other-

Abbreviations: SDS, sodium dodecyl sulfate; AUT, acid urea Triton X-100; CoA and CoA-SH, coenzyme A; TFA, trifluoroacetic acid.

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wise. Labeling was terminated by the start of cell homogenization in pre-cooled nuclear isolation buffer (0.25 M sucrose, 10 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, 12 mM $Na_2S_2O_5$, 1 mM PMSF, 50 mM butyric acid, 0.1% (w/v) Triton X-100, pH 7.2) as described before [20,21]. Histones were fractionated by reversed-phase chromatography on a Dupont Zorbax Protein Plus column (0.4 × 25 cm) by a linear gradient of 20–55% acetonitrile in water with 0.1% TFA, collected and concentrated by lyophilization. Sodium butyrate was added to suspension cultures from a sterile 1 M stock solution (pH 5.8). Histones, prepared from 4 and 12 g callus, were analyzed by electrophoresis in 15 cm long discontinuous SDS and 30 cm long acid-urea-Triton (AUT) polyacrylamide gels [20]. All gels were stained, fluorographed and quantitated as described previously [20,21].

Results

Culture of alfalfa cells with radioactive acetate will rapidly incorporate label in postsynthetically acetylated core histone species [21]. The specificity of this labeling reaction is demonstrated by a comparison of the protein profiles determined by staining and the distribution of radioactivity determined by fluorography. Alfalfa cells were incubated with tritiated acetate for 1 h, histones were prepared and fractionated by reversed-phase high-performance liquid chromatography. Fractions were analyzed by gel electrophoresis in AUT (acid-urea-Triton X-100) gels and the protein distribution was quantitated by Coomassie staining (Fig. 1A) and fluorography (Fig. 1B). The majority of the label is localized in the core histones. Less than 20% of the acetate label becomes part of non-histone proteins (Fig. 2B and E). The non-histone protein labeling in part may be due to metabolic conversion of acetate into amino acids with incorporation of label into newly synthesized proteins [22] and in part may be caused by postsynthetic acetylation of non-histone proteins. The high specificity for postsynthetic histone labeling is further demonstrated by the absence of label in the non-acetylated protein bands of histones H4, H2B.1, H3.1, H3.2 and H2A.3 (Fig. 1B).

The fluorographic analysis clearly showed that the histone H3 variants are the major acetylated core histone species (Fig. 2A), and further documented quanti-

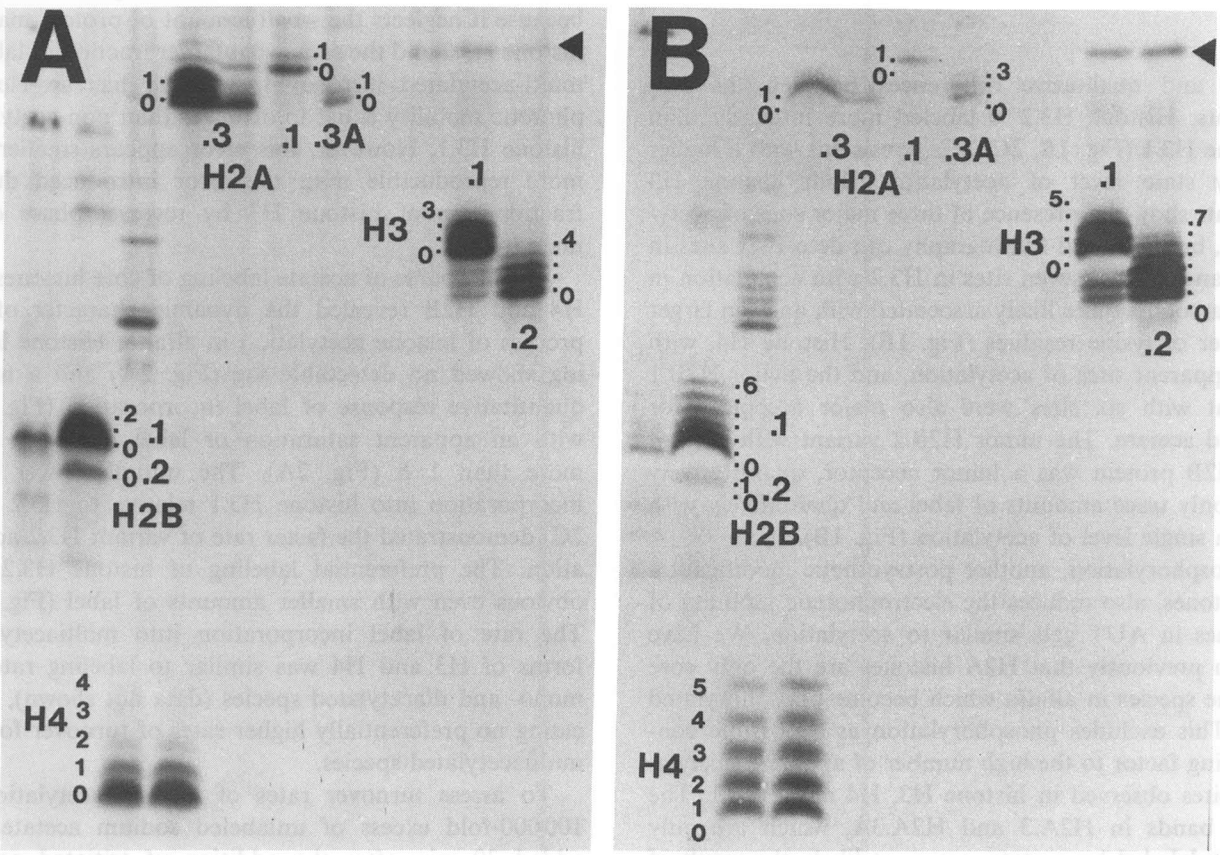

Fig. 1. Acetylation labeling of histone by sodium [^{3}H]acetate. Histones were prepared from alfalfa cells labeled *in vivo* for 60 min by sodium [^{3}H]acetate, fractionated by reversed phase HPLC and analyzed in AUT gels by Coomassie staining (A) and fluorography (B) for 14 days. Histone proteins H4, H2B variants H2B.1 and H2B.2, H2A variants H2A.3, H2A.1 and H2A.3A, and H3 variants H3.1 and H3.2 are marked with numbering of all detectable levels of acetylation. Non-acetylated bands are indicated in the fluorograph (B) even if undetectable. The histone H3 dimer is indicated by a solid triangle. Some histone H3.2 is present in the lane with the major amount of histone H3.1.

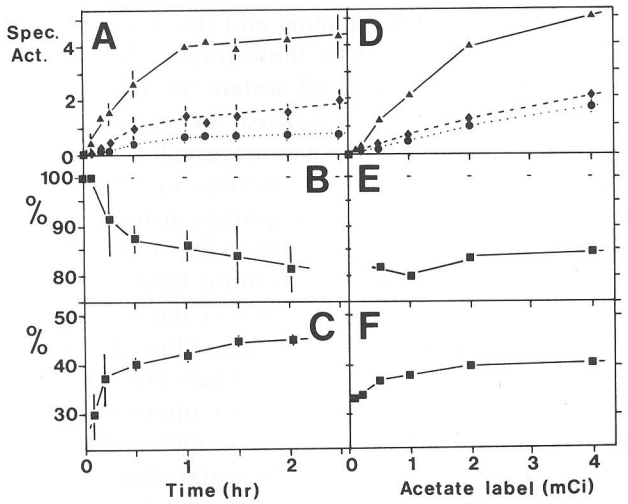

Fig. 2. Dynamic histone acetylation labeling. Acetate labeling was performed as described in Materials and Methods with 1 mCi of label per culture for 4–150 min (A–C) and for 60 min with 0.1 to 4 mCi of label (D–F). Panels A and D show the specific activity of histones H3 (line with triangles), H4 (broken line with diamonds) and H2B (dotted line with circles) in arbitrary units of fluorographic intensity *versus* Coomassie staining intensity. Panels B and E show the percent of the label incorporated into core histones. Panels C and F show the percent of the label incorporated into histone H3.1 relative to the total amount of label in histone H3. Standard deviation errors are shown as vertical bars.

tative and qualitative differences between the two variants. Histone H3.2 is labeled more intensely than histone H3.1 (Fig. 1B, 2C, 2F), consistent with a higher steady state level of acetylation. Both histone H3 variants show the presence of three major sites of acetylation, but extended fluorography can detect six sites in H3.1 and at least seven sites in H3.2 with acetylation in trace amounts quite likely associated with an even larger number of lysine residues (Fig. 1B). Histone H4, with five apparent sites of acetylation, and the major H2B.1 variant with six sites were also major acceptors for labeled acetate. The minor H2B.2 variant with 10% of the H2B protein was a minor acceptor, quantitatively with only trace amounts of label and qualitatively with only a single level of acetylation (Fig. 1B).

Phosphorylation, another postsynthetic modification of histones, also reduces the electrophoretic mobility of histones in AUT gels similar to acetylation. We have shown previously that H2A histones are the only core histone species in alfalfa which become phosphorylated [21]. This excludes phosphorylation as a possible contributing factor to the high number of apparent acetylation sites observed in histone H3, H4 and H2B.1. The three bands in H2A.3 and H2A.3A, which are only weakly labeled by acetate, are most likely the result of phosphorylation combined with at least one site of acetylation (Fig. 1B).

Quantitative densitometry of Coomassie-stained total histone patterns in AUT gels was used to determine the steady state level of acetylated lysines in histone H2B.1 at 0.32 ± 0.05 ($n = 9$) and of histone H4 at 0.73 ± 0.13 ($n = 25$). For a similar determination of histone H3, it is necessary to separate the two histone H3 variants due to the overlap of multi-acetylated H3.2 bands with bands of H3.1 (Fig. 1). In five independent analyses of separated histone H3 variant proteins by Coomassie staining of AUT gels, histone H3.1 contained between 0.44 and 0.56 acetylated lysines per protein and histone H3.2 showed a range from 0.97 to 1.01 acetylations per molecule. The observed variations appeared to be due to a partial fractionation of histone H3 protein on the basis of differences in steady state acetylation levels during reversed-phase chromatography. To exclude this experimental variability from the determination of the specific activities of the two histone H3 variants, unfractionated histone preparations were analyzed on AUT gels as before [21]. Under these conditions, histone H3.1 contained $63.4 \pm 1.9\%$ ($n = 35$) of the protein, quantitated by Coomassie staining, and only $40.8 \pm 3.0\%$ ($n = 23$) of the label, measured by fluorography. This indicates that acetate label incorporation *in vivo* for 1 h results in a specific activity of histone H3.2 which is more than 2.5-fold higher than that of histone H3.1. This measurement contains a small, systematic error because it neglects the small amount of protein mass of histone H3.2 and the somewhat larger fraction of labeled multi-acetylated histone H3.2 which has an electrophoretic mobility equal to or lower than non-acetylated histone H3.1. However, this error appears smaller and more reproducible than the error introduced during fractionation of histone H3 by reversed-phase chromatography.

A time course of acetate labeling of core histones H3, H4 and H2B revealed the dynamic character of the process of histone acetylation in alfalfa. Histone labeling showed no detectable lag (Fig. 2A) and a nearly quantitative response of label incorporation (Fig. 2D) with an apparent saturation or label depletion after more than 1 h (Fig. 2A). The initially lower label incorporation into histone H3.1 relative to H3.2 (Fig. 2C) demonstrated the faster rate of variant H3.2 acetylation. The preferential labeling of histone H3.2 was obvious even with smaller amounts of label (Fig. 2F). The rate of label incorporation into multiacetylated forms of H3 and H4 was similar to labeling rates of mono- and diacetylated species (data not shown), indicating no preferentially higher rates of turnover for the multiacetylated species.

To assess turnover rates of histone acetylation, a 100 000-fold excess of unlabeled sodium acetate was added 30 min after the addition of tritiated acetate when half of the apparent steady state level of acetylation was reached (Fig. 2A), in order to follow the decay of the specific activity of the incorporated label. Contrary to expectation the added acetate did not affect the

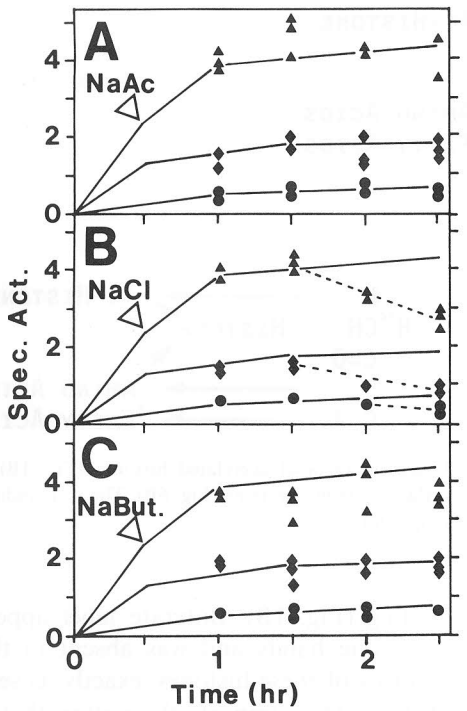

Fig. 3. Salt effects on histone acetylation labeling. Unperturbed acetate labeling for 15–150 min (see Fig. 2A) is indicated by lines and compared to the specific activity of histone H3 (triangles), H4 (diamonds) and H2B (circles) obtained when sodium acetate (A), chloride (B) or butyrate (C) was added to a final concentration of 50 mM at 30 min after the addition of tritiated label (0.5 μM acetate) (open triangles). Significant deviations from the unperturbed pattern are shown by a broken line.

course of label incorporation (Fig. 3A). The control experiment is shown in Fig. 3B where NaCl was added to mimic the increase in ionic strength without increases in acetate concentration. The increased NaCl concentration also did not inhibit acetate incorporation up to 1 h after addition (Fig. 3B), but then showed a decay in label specific activity.

Addition of sodium butyrate in the same amount as sodium acetate and chloride to alfalfa cells labeled for 30 min with tritiated acetate remained without effect on the specific activity of the acetylated histones for at least 2 h (Fig. 3C). At these concentrations, butyrate will rapidly induce histone hyperacetylation in mammalian cells due to a complete inhibition of histone deacetylase activity [11–13]. To extend the observation of butyrate effects alfalfa cells were exposed to 50 mM butyrate for 24 h. Histone acetylation levels remained unaffected, as shown for histone H4 (Fig. 4A). Pulse labeling with tritiated acetate for 1 h prior to preparation of histones showed significant to complete inhibition of label incorporation (Fig. 4B). This inhibition was clearly dependent on butyrate as shown in Fig. 4C. Co-addition of butyrate and tritiated acetate reduced labeled acetate incorporation to less than 25% of normal levels of incorporation. Only preincubation with labeled acetate for at least 30 min prior to the addition of butyrate led to apparently normal levels of label incorporation (Fig. 4B), consistent with the effect of butyrate seen earlier in Fig. 3C.

These observations suggested that butyrate interference with labeled acetate incorporation might involve the presumed intermediate for histone acetylation, acetylCoA (Fig. 5A). To evaluate the possibility that plant cells may catabolize butyrate through an acetylCoA intermediate (Fig. 5B), *n*-[1-^{14}C]butyrate was added to alfalfa cells for 1 h, histones were prepared,

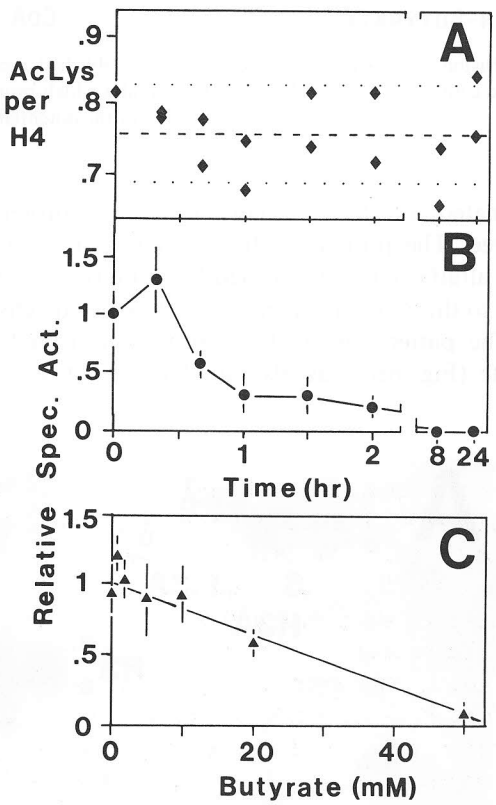

Fig. 4. Butyrate effects on histone acetylation labeling. (A) Alfalfa suspension cells were cultured in the presence of 50 mM butyrate for 0–24 h, and tritiated acetate was added 1 h before the start of histone preparation. Histones were prepared and electrophoresed in AUT gels. The steady state level of histone H4 acetylation was determined by quantitative analysis of the Coomassie staining pattern of non-through multi-acetylated histone H4 bands and expressed as molecules of acetylated lysine per protein molecule (diamonds). The average values and standard deviations range calculated from all determinations are indicated by broken and dotted lines, respectively. (B) The specific activity of histones H3, H4 and H2B was determined by quantitation of protein and label profiles, expressed relative to the specific activity obtained for acetate labeling without butyrate, and averaged (circles) with calculation of standard deviation error (bars). (C) Tritiated acetate was added together with butyrate to a final concentration of 0–50 mM to suspension cultures of alfalfa. The cells were cultured for 1 h, collected, histones were prepared and analyzed by gel electrophoresis. The specific activity of histones H3, H4 and H2B was determined by quantitation of protein and label profiles, expressed relative to the specific activity obtained for acetate labeling without butyrate, and averaged (triangles) with calculation of standard deviation error (bars).

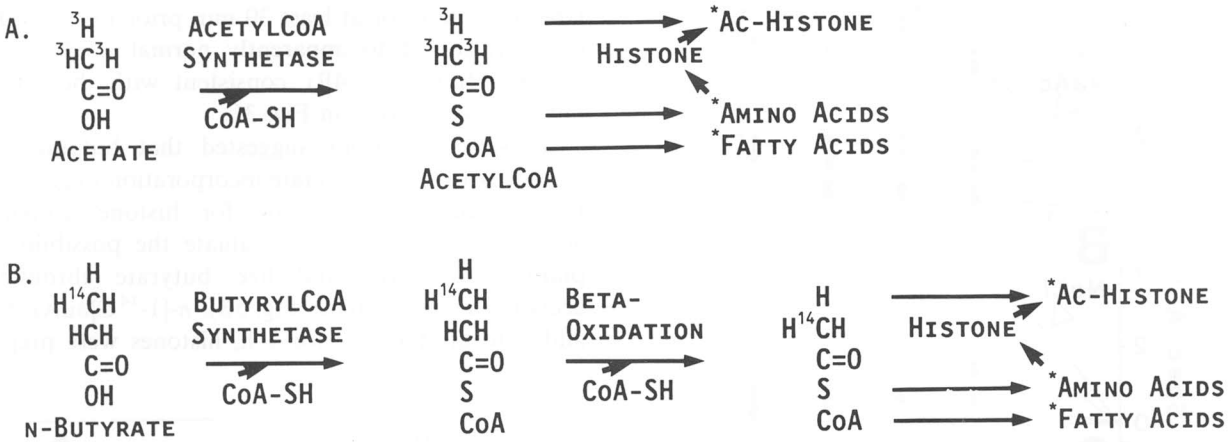

Fig. 5. Histone acetylation labeling. (A) The established metabolic conversion of [^{3}H]acetate to label acetylated histones (Fig. 1B). (B) The presumed conversion of *n*-[1-^{14}C]butyrate in alfalfa which resulted in the labeling of acetylated histone proteins (Fig. 6B). The stars indicate some of the potentially radioactively labeled compounds.

fractionated and the ^{14}C incorporation into protein was measured. The protein profiles prepared after incubation of alfalfa cells with labeled butyrate (Fig. 6A) were similar to those obtained from acetate labeled cells (Fig. 1A). The pattern of label incorporation from labeled butyrate (Fig. 6B) was also similar to that seen after acetate labeling (Fig. 1B). Butyrate label appeared in acetylated histone bands and was absent in the non-acetylated forms of these histones, exactly as seen after acetate labeling. This supports the notion that acetate and butyrate have a metabolic intermediate in common which participates in the acetylation of histones (Fig. 5).

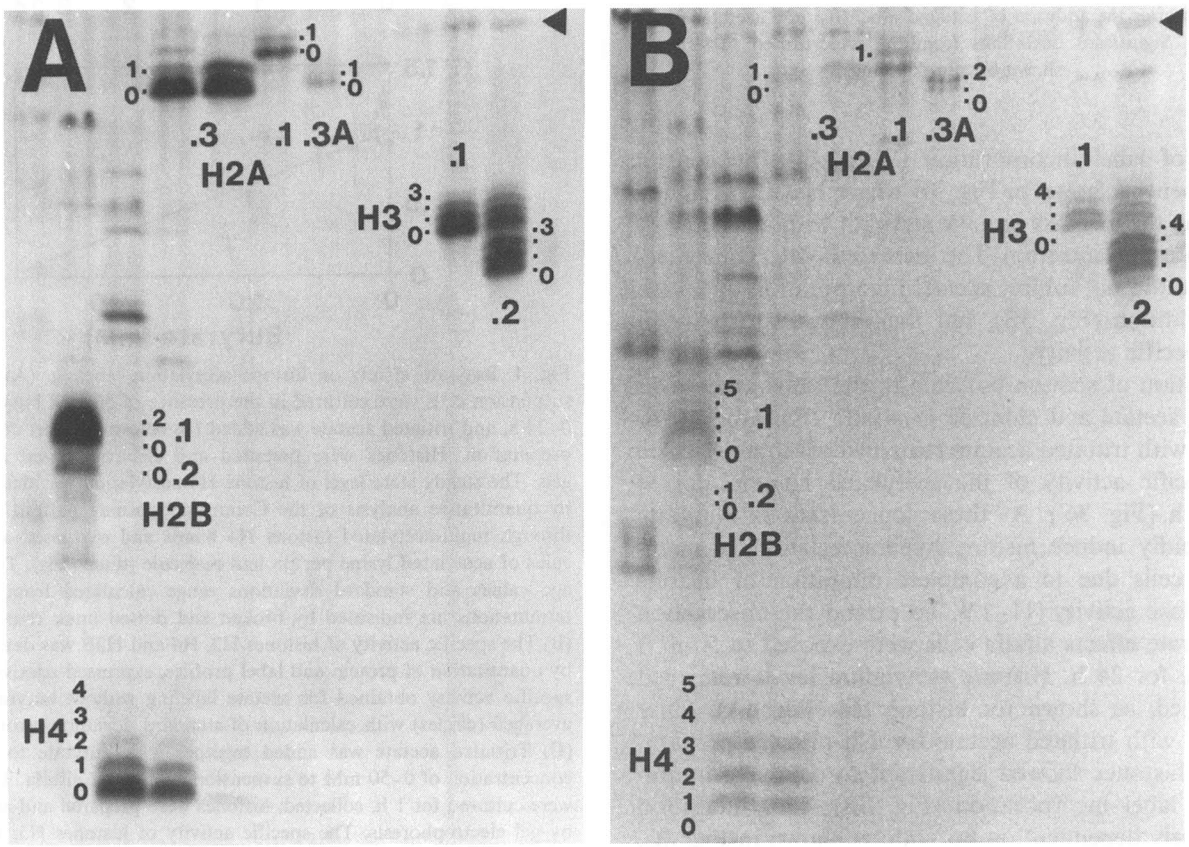

Fig. 6. Acetylation labeling of histone by sodium *n*-[1-^{14}C]butyrate. Histones were prepared from alfalfa cells labeled in vivo for 60 min by sodium *n*-[1-^{14}C]butyrate, fractionated by reversed phase HPLC and analyzed in AUT gels by Coomassie staining (A) and fluorography for 67 days (B). Histone species and modified bands are marked as in Fig. 1. Histone H3.1 is present in the lane with the major amount of histone H3.2.

However, differences between acetate and butyrate are apparent. The level of label incorporation into non-histone proteins is much higher with labeled butyrate (Fig. 6B) than with labeled acetate (Fig. 1B). We cannot exclude the possibility of direct butyrate modification of labeled proteins in the experiments shown in Fig. 6B.

Discussion

Our study has shown that radioactive acetate was rapidly taken up by alfalfa cells and a high fraction of the label becomes localized specifically in acetylated core histone proteins (Figs. 1 and 2). The increase in histone specific activity reaches a plateau after approx. 1 h (Fig. 2A). Addition of a second aliquot of tritiated acetate at this time resulted in a doubling of specific activity after one additional hour of incubation (results not shown), consistent with the near-linear response between 1 and 2 mCi of tritiated acetate seen after 1 h of labeling (Fig. 2D). This indicates that exhaustion of the labeled acetate pool, which probably exists as acetylCoA (Fig. 5A), rather than a real steady state labeling is reached after 1 h. Complete steady state labeling conditions were not obtained in this study.

It was not possible to interfere with the course of the acetate labeling reaction by the addition of a large excess of unlabeled acetate (Fig. 3A) or butyrate (Fig. 3C). It remains unclear why addition of an excess of acetate or butyrate, both compounds which effectively and rapidly label histones by acetylation (Figs. 1B, 6B), fail to have any apparent influence on the radioactive acetate pool. One possible explanation could be a distinction in cellular response to micromolar concentrations of acetate (0.5–2.2 μM in this study) or butyrate (40 μM) *versus* a concentration of 50 mM of these salts. It appears that the effect of 50 mM butyrate becomes noticeable in histone acetylation labeling only after 30 min (Fig. 4B). Addition of 50 mM sodium chloride also affects histone acetylation after 1 h (Fig. 3B). This may indicate a slow cellular equilibration of salt by alfalfa cells. Thus, addition of a large excess of unlabeled acetate (Fig. 3A) or butyrate (Fig. 3C) requires a significant delay to equilibration with the existing radioactive acetate pool (Fig. 3).

Interference by butyrate in establishing a high specific activity tritiated acetate (or acetylCoA) pool, is apparent when increasing concentrations of butyrate are supplied together with tritiated acetate (Fig. 4C) or when butyrate is added prior to label addition (Fig. 4B). Based on the labeling of acetylated histones by *n*-[1-^{14}C]butyrate (Fig. 6B), we interpret the interference as the result of a catabolic conversion of butyrate to an expanded acetylCoA pool (Fig. 5B). This expansion dilutes the specific activity of the acetylCoA pool either when butyrate is added in large amounts together with tritiated acetate (Fig. 4C) or when butyrate is added before the addition of labeled acetate (Fig. 4B).

Application of butyrate to mammalian cells in culture is very effective in inducing a persistent pattern of histone hyperacetylation due to continued inhibition of histone deacetylase [13,23]. Our observations in alfalfa indicate two significant differences between plant and animal cells. Alfalfa cells appear to convert added butyrate rapidly to precursors for histone acetylation (Fig. 6B) and fail to induce detectable histone hyperacetylation in the presence of butyrate (Fig. 4A). These results, combined with preliminary observations in tobacco [18] and artichoke [19], suggest that plant histone deacetylase is not inhibited by butyrate. The low butyrate sensitivity of histone hyperacetylation in the lower eukaryotes *Tetrahymena* [24] and *Physarum* [25] appears to be intermediate between highly butyrate-sensitive mammalian cells and butyrate-insensitive plant cells. Extension of this general pattern of butyrate responses to other phyla like fungi and insects could have major implications for the application of butyrate in gene transformation methodologies [26].

Acknowledgements

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