Acetylation sites in histone H3 from *Physarum polycephalum*

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Histone H3 from *Physarum polycephalum* was labelled with [^{3}H]acetate in G2 phase of the cell cycle. Only histones H3 and H4 were labelled and the H4 was removed by chromatography. Sequential Edman degradation of labelled H3 showed that acetate was incorporated into residues 9, 14, 18 and 23 which correspond to the sites of acetyl-lysine determined in histones H3 from other organisms. The results confirm the sequence conservation of H3 and support the notion that data on H3 acetylation, obtained with *Physarum*, can be extrapolated to higher eukaryotes.

Histone H3 *Acetylation site* *Acetyl-lysine* *H3 sequence conservation* *Higher eukaryote* (Physarum polycephalum)

1. INTRODUCTION

Histone acetylation has long been regarded as a likely part of the mechanisms for making genes accessible for transcription [1] and for depositing newly synthesized histones onto DNA during chromosome replication [2,3]. The use of butyrate to obtain highly acetylated histones and the purification of acetyl transferases and deacetylases [5,6], (review [3]) is allowing this field to develop rapidly. In *Physarum*, acetylation of histones H2A and H2B is restricted to S phase, the time of chromosome replication, while acetylation of H3 and H4 occurs in both S and G2 phases, but not at metaphase [7]. Experiments using inhibitors and other approaches have shown that a specific pattern of acetylation is associated with transcription while a different pattern is associated with newly synthesized histones during chromosome replication [8]. To assess the significance of these results for eukaryotes in general, it is necessary to characterize the *Physarum* histones to determine their similarity to mammalian histones. An overall similarity, at the level of amino acid composition and M_r has been established although both H2A and H1 show substantially larger M_r-values in *Physarum* [9]. A sequence study of histone H4 from *Physarum* established the identity of the sequences of *Physarum* and pea H4 in the N- and C-terminal regions with a few variations in the central region [10]. The acetylation sites in *Physarum* H4 were identical with those of higher eukaryotes.

Sequence studies of histone H3 in *Physarum* have been hampered by the difficulty of purifying it in sufficient quantity. Preparative gel electrophoresis has been used and chromatography on Bio-Gel P columns [9,11] but preparative gel electrophoresis yields only moderate amounts and the Bio-Gel P columns give H3 contaminated with H2B. Thus, we have not undertaken a sequence study of *Physarum* H3. To determine the sites of acetylation in *Physarum* H3 we chose a specific labelling protocol that labelled only acetate groups on histones H3 and H4 and then separated H3 and H4 by chromatography on Sephadex G-75. The positions of the acetate label were then determined by Edman degradation. The results were confirmed using electrophoretically purified H3.

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2. MATERIALS AND METHODS

2.1. *Histone isolation*

Physarum polycephalum was maintained in submerged culture and grown as synchronous cultures on filter paper suspended in about 20 ml semi-defined growth medium by a stainless steel grid [9,12,13]. Cultures were harvested by plunging in liquid nitrogen and stored frozen for a short time or used immediately. The cultures were scraped off the filter papers into homogenizing medium (0.25 M sucrose, 0.01 M $CaCl_2$, 0.01 M Tris–HCl, 0.1% Triton X-100 (w/v), 1 mM phenylmethane sulphonylfluoride) and nuclei prepared as in [9,14,15]. Histones were isolated by extracting nuclei with 40% guanidinium hydrochloride, as in [9].

Column chromatography on Sephadex G-75 superfine was carried out in 5% acetic acid on a 130 cm long × 1 cm diam. column eluted at 0.03 ml/min. The eluant was monitored for absorbance at 230 nm and for radioactivity. Fractions of interests were pooled and lyophilized. Gel electrophoresis was carried out in polyacrylamide gels containing 15% acrylamide, 8 mM Triton X-100, 8 M urea, 1 M acetic acid, 50 mM NH_4OH [9].

Histone bands in gels dried on Whatman 3MM paper were localized by fluorography assisted by residual Coomassie staining and cut out. The dried gel pieces were reswollen 2 times for 15 min in 10 vol. glacial acetic acid with 0.001% Coomassie blue. Most of the PPO (2,5-diphenyloxazole used for fluorography) was removed from the gels but residual amounts were seen as white precipitates on the reswollen gel pieces. PPO does not interfere with the subsequent electrophoretic elution of the histones [9] following [16]. The gel pieces were equilibrated 2 times for 30 min in 7 M acetic acid, 50 mM NaOH, electrophoretically separated overnight from the Coomassie stain and diffused from the agarose into 0.02 M HCl–0.1% (w/v) cysteamine. The diffusate was lyophilized, desalted on Sephadex G25 and re-lyophilized as in [9].

2.2. *Sequencing*

For micro-manual sequencing [17,18], H3 containing 17000 cpm ^3H was coupled to 80 mg isothiocyanato–glass (Pierce Chemical Co.). Sequential Edman degradations were carried out and the radioactivity in the dried thiazolinone of each step was determined. A small proportion (about 1%) of the glass beads were lost at each step and this was monitored by weighing the glass beads every third step. The data shown are corrected for the loss of glass.

3. RESULTS

Physarum plasmodia were grown as naturally synchronous cultures and, during G2 phase, cycloheximide (10 μg/ml) was added for 15 min followed by the addition of [^{3}H]acetate. Acetate was kept below 2 mM to avoid perturbing the growth of the plasmodium [7]. After 10 min incubation in cycloheximide and [^{3}H]acetate the plasmodia were harvested and histones isolated. In [7] we determined that the presence of butyrate in the isolation buffers did not affect the measured acetylation of histones so butyrate was not used in the experiments described here. The histones were analyzed by Triton–acid–urea gel electrophoresis. The pattern of Coomassie stain was similar to that for *Physarum* histones in [7,9] but fluorography of the gel showed that only histones H3 and H4 were labelled with [^{3}H]acetate (fig.1). Histones H2A and H2B are not acetylated during G2 phase in *Physarum* [7] and the cycloheximide present during the labelling had eliminated the faint background of label incorporated into newly syn-

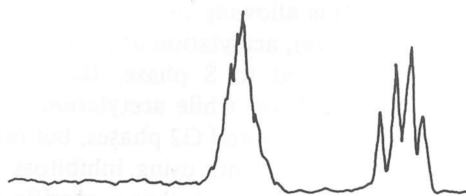

Fig.1. Triton–acid–urea gel electrophoresis [7,9] of *Physarum* histones labelled with [^{3}H]acetate in G2 phase in the presence of cycloheximide. The figure shows a scan of a fluorograph [20] of the gel. Electrophoresis was from left to right. The group of peaks near the center of the gel is histone H3 partially separated into its modified forms; the group of 4 peaks on the right is histone H4 separated into components with 4, 3, 2 or 1 acetyl lysines per molecule. Histone H4 is resistant to Edman degradation [9,10], but, to avoid any possible problems, it was removed by Sephadex G-75 chromatography before the H3 was subjected to Edman degradation (fig.2).

thesized non-histone proteins [7]. The histones were fractionated by chromatography on Sephadex G-75 superfine which separated histones H3 and H4 as in [7]. Fractionation on a Bio-Gel P column was not used as we have observed some trailing of highly acetylated H4 into the H3 region on these columns and 5% acetic acid is a more convenient eluant than HCl and NaCl. The peaks of radioactivity corresponding to acetylated histones H3 and H4 were pooled separately. The H3 peak was coupled to isothiocyanato glass beads to improve the yield of successive cycles of Edman degradation and then subjected to manual liquid phase Edman degradation with phenylisothiocyanate using the protocol described for DABITC (4-*N,N*-dimethylaminoazobenzene-4′-isothiocyanate) micro-manual sequencing [18] omitting the reaction with DABITC. For each degradation cycle, the extracted thiazolinone derivative in *n*-butanol was dried and the radioactivity determined by liquid scintillation counting. Fig.2 shows the radioactivity released at each step, together with the sequence of calf histone H3. Thirty cycles of Edman degradation were carried out and after the final step no radioactivity remained on the beads. This showed that all the H3 molecules were unblocked at their N-terminus and that all the acetylation sites had been determined.

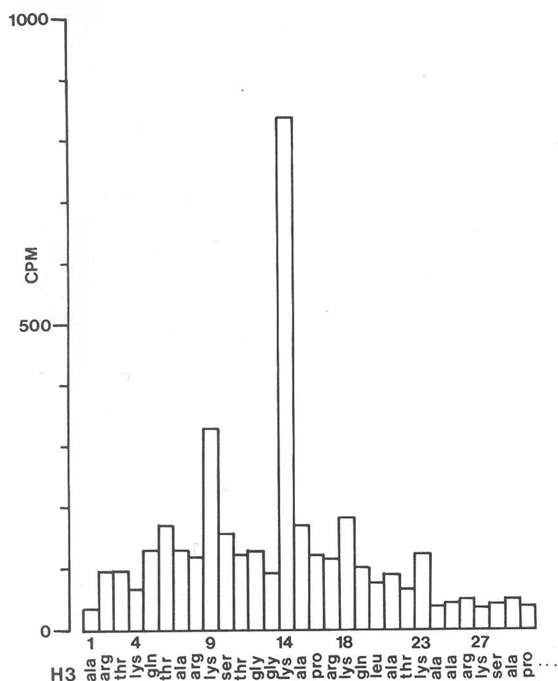

Fig.2. [^{3}H]Acetate released by Edman degradation of [^{3}H]acetate-labelled *Physarum* histone H3. The radioactivity (cpm) in the released derivatized amino acid at each of the first 30 cycles of manual Edman degradation is shown, together with the amino acid sequence of the first 30 residues of calf thymus H3. Note the absence of label in residues 4 and 27 (both lysine in calf H3) and the presence of label in residues 9, 14, 18 and 23 (the other lysines in calf H3).

To confirm the acetylation sites on H3 in cultures not perturbed by cycloheximide, H3 was labelled in G2 phase with [^{3}H]acetate, isolated and purified by gel electrophoresis. The isolated H3 was subjected to automated spinning cup sequencing and the radioactivity liberated in each step was determined. Only a small proportion of the radioactivity in the proteins was released as PTH-amino acids. The remainder of the protein appeared to be protected from Edman degradation, presumably due to modification during gel electrophoresis or electrophoretic elution. Such modification has been described in [19] but the Quadrol procedure for removing the block was not effective in this case. The radioactivity that was released was determined and the same four residues (-9, -14, -18 and -23) as shown in fig.2 were selectively labelled.

4. DISCUSSION

The acetylation sites determined for *Physarum* histones H3 are the same as those determined for higher eukaryote H3. This means that residue 4 (lysine in calf H3) is protected from acetylation in *Physarum* as in higher eukaryotes and acetylation does not occur on residue 27 (lysine in calf H3) again in common with higher eukaryotes [1–3]. The 4 lysines between residues 5 and 26 are each subject to acetylation with residue 14 showing by far the largest acetate content. This identity of acetylation sites between *Physarum* H3 and higher eukaryote H3 confirms the sequence conservation of H3 and gives support to the contention that results obtained with *Physarum* can be extrapolated to higher eukaryotes.

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