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Histone acetyltransferase activity during the cell cycle

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Histone acetyltransferase activity was measured in isolated nuclei during the synchronous cell cycle of the myxomycete *Physarum polycephalum*. Nuclei were incubated with [^{14}C]acetyl-coenzyme A and an excess of exogenous calf thymus histones. The activity is periodic during the cell cycle; it rises during the S-phase to reach a maximum in the early G_2-period with a decline in mid and late G_2. Comparison of the pattern of enzyme activity with the in vivo acetylation of histones during the cell cycle reveals that the enzyme activity does not wholly determine the acetylation state, indicating that other factors, including possibly the structural state of chromatin, are responsible for the observed cell cycle pattern of in vivo histone acetylation.

Histone; Acetylation; Chromatin; Cell cycle; *(Physarum)*

1. INTRODUCTION

Histones, the main structural proteins of eucaryotic chromatin, undergo various reversible, postsynthetic modifications, among which acetylation of ϵ-amino groups of lysine residues has been extensively studied [1]. Previous work from our laboratory has shown a lack of correlation between histone acetylation and the transcriptional activity of chromatin in *Physarum* [2,3]. Recent results on the functional role of histone acetylation suggest that it serves as a general mechanism in the displacement of histones during structural transitions of chromatin [4]. During the naturally synchronous cell cycle of *Physarum* the steady-state level of acetylation of H4 has a maximum in the S-phase and low levels during the G_2-period [2]. The same is true for cell cycle-dependent [^{3}H]acetate incorporation into core histones (unpublished).

We wanted to address the question of whether the activities of the enzymes maintaining the acetylation state of histones, histone acetyltransferase and histone deacetylase, can account for the periodic pattern of acetylation. Previous results on the cell cycle dependence of histone deacetylase activity of *Physarum* [5] and the results of the present study demonstrate that enzyme activities alone are not at all sufficient to explain the characteristic cell cycle pattern of acetylation during the cell cycle. A distinct cell cycle pattern of acetylation must be attributed to other factors in chromatin or to distinct structural conditions in certain chromatin areas.

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2. MATERIALS AND METHODS

2.1. *Materials*

These were purchased from the following sources: [^{3}H]acetic acid, sodium salt (4 Ci/mmol), from Amersham International; [1-^{14}C]acetyl-CoA (55 mCi/mmol) from New England Nuclear (Boston, USA); and histones (calf thymus, type II-AS) from Sigma (St. Louis, USA).

2.2. *Culture techniques*

Physarum polycephalum strain M_3bFII (a Wis 1 isolate) was used. Maintenance of microplasmo-

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dial stock cultures and preparation of giant macroplasmodia were as described [6].

At selected time points of the cell cycle macroplasmodia or macroplasmodial explants were labelled with [^{3}H]acetate or harvested for determination of histone acetyltransferase activity.

2.3. *Labelling with [^{3}H]acetate during the cell cycle*

Plasmodia were washed three times in nutrient medium lacking peptone and yeast extract and drained on filter paper. Plasmodia were then placed in medium deficient in peptone and yeast extract containing 0.07 mM cycloheximide for 15 min. This cycloheximide preincubation is performed to inhibit cotranslational acetate incorporation into N-terminal serine residues of histones. After cycloheximide preincubation 20 mCi [^{3}H]acetate were added and incubation was continued for 5 min in the presence of cycloheximide. After incubation plasmodia were immediately harvested for nuclear isolation and histone extraction. Equal amounts of the histone fraction (10 μg protein) were counted in a liquid scintillation spectrophotometer.

2.4. *Isolation of nuclei and extraction of histones*

Nuclei were isolated according to [7] with modifications outlined elsewhere [8]. Isolated nuclei were counted in a hemocytometer. Histones were extracted as in [9]. The protein content of the histone sample was determined [10].

2.5. *Standard assay for histone acetyltransferase activity*

Isolated nuclei were resuspended in incubation buffer (15 mM Tris-HCl, pH 7.3, at 25°C, 5 mM EGTA, 3 mM dithiothreitol, 1 mM $CaCl_2$, 15 mM $MgCl_2$). This buffer (the same as that used for isolation of nuclei, but without hexylene glycol and Surfynol) was used, since it provides an optimal environment for isolated nuclei. 5×10^6 nuclei were incubated for 20 min at 25°C in 250 μl reaction mixture, containing 5 μM [^{14}C]acetyl-CoA (55 μCi/μmol, 0.07 μCi/tube), 50 mM sodium *n*-butyrate and 125 μg exogenous histones in incubation buffer. The reaction was started by the addition of [^{14}C]acetyl-CoA and terminated by the addition of 150 μl cold 60% (w/v) trichloroacetic acid. After 30 min on ice the samples were collected onto Whatman GF/C filters under suction. Filters were washed 3 times each with 3 ml of 20% (w/v) trichloroacetic acid, 3 times each with 1 ml ethanol, dried and analyzed for radioactivity in the liquid scintillation spectrophotometer.

Enzyme activities are expressed as amol acetate incorporated into acid-insoluble material/min per nucleus. To convert enzyme activities per nucleus into total enzyme activity per macroplasmodium we determined the total number of nuclei in the plasmodium before mitosis 2 (between 2 and 3×10^8). Since nuclei divide with perfect synchrony in *Physarum* we multiplied the counted number of nuclei by a factor of 2 after the second mitosis and by a factor of 4 after the third mitosis. Accordingly, the enzyme activities per nucleus were multiplied by the total number of nuclei per macroplasmodium.

3. RESULTS

3.1. *Histone acetyltransferase assay*

In our standard assay for histone acetyltransferase activity the kinetics of [^{14}C]acetyl-CoA incorporation were linear for 20 min at 25°C. We used an excess of calf thymus histones as acceptor protein for acetate (10-fold amount in relation to the endogenous histone content of the nuclei in the assay). The Michaelis constant was determined to be 2 μM for acetyl-CoA and the enzyme activity exhibited a broad pH optimum between pH 7 and 8. Table 1 shows the effect of different assay conditions on the enzyme activity. The reaction was temperature-dependent with an optimum at 25°C. Omission from the assay of the deacetylase inhibitor, sodium *n*-butyrate, resulted in a 20% decrease in enzyme activity, due to the action of the histone deacetylase. The activity was proportional to the amount of exogenous histones in the assay; with endogenous histones only (omission of exogenous calf thymus histones) the activity amounts to 15% of that in the standard assay. The measured activity exhibits a linear relationship with the number of nuclei in the assay. A short preincubation of the nuclei at 90°C completely abolishes the enzyme activity (table 1), excluding the possibility of unspecific incorporation of radioactive acetyl-CoA into protein.

Table 1

Reaction conditions for *Physarum* histone-acetyltransferase

Reaction conditions	Relative enzyme activity[a]
Standard assay[b]	1.00
0°C	0.10
15°C	0.60
37°C	0.90
Minus butyrate	0.80
Minus exogenous histones	0.15
62.5 μg exogenous histones	0.75
Minus nuclei	0.00
2.5×10^6 nuclei	0.50
Nuclei denatured (preincubated for 3 min at 90°C)	0.00

[a] For comparison of enzyme activities the activity in the standard assay is taken as 1.00

[b] Standard assay: 5×10^6 nuclei in 250 μl incubation buffer, pH 7.3, 5 μM acetyl-CoA, 50 mM sodium *n*-butyrate and 125 μg exogenous histones incubated for 20 min at 25°C

3.2. *Histone acetyltransferase activity during the cell cycle*

We measured the posttranslational, in vivo incorporation of [^{3}H]acetate as well as histone acetyltransferase activity during the synchronous cell cycle of *Physarum* macroplasmodia starting before mitosis 2 until late S-phase after mitosis 3 (fig.1). It should be mentioned that the cell cycle of *Physarum* macroplasmodia lacks a G_1-period. The S-phase (duration approx. 3 h) follows immediately after mitosis (0.5 h); the G_2-period lasts approx. 6 h. The pattern of [^{3}H]acetate incorporation is phase-dependent with a sharp maximum in early S-phase and rather low levels during late S-phase and the G_2-period (fig.1a). In the late G_2-period, already before the onset of mitosis, the incorporation of [^{3}H]acetate increases again.

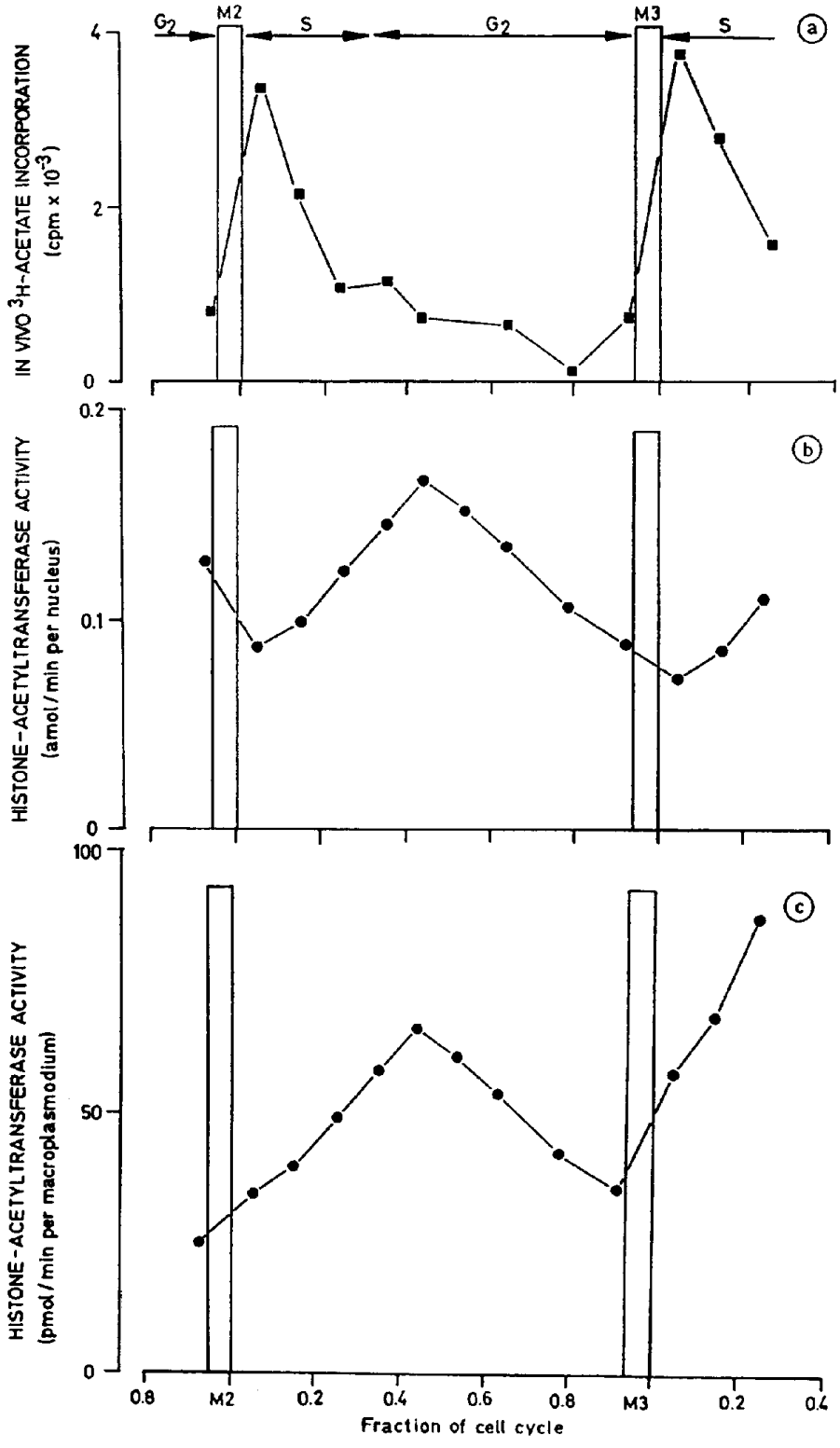
Using the standard assay we measured histone acetyltransferase activity throughout the cell cycle. The enzyme activity is periodic (fig.1b,c). Due to the synchronous division of nuclei the enzyme activity per nucleus (fig.1b) drops after mitosis, since the existing activity is distributed over double the number of nuclei. This misleading decrease in activity can be avoided if the same data are plotted as enzyme activities per whole macroplasmodium, as described in section 2 (fig.1c). The enzyme activity then rises during the S-phase to reach a clear maximum in early G_2-period. During later stages of G_2 the activity decreases until shortly before the onset of the next mitosis. The enzyme activity is already induced before mitosis (fig.1c). It should be mentioned that the cell cycle pattern of the enzyme is the same when the activity is measured without exogenous histones, although the activity values are considerably smaller (not shown).

4. DISCUSSION

We have shown that the histone acetyltransferase activity is highly periodic during the cell cycle with a peak in early G_2-period. The enzyme activity is induced already before mitosis; this premitotic induction is a common feature with other enzymes involved in DNA replication, such as thymidine kinase and thymidylate synthase [11], other nuclear proteins, like histones [8], or ADP-ribosyltransferase [6]. Enzyme activities [6,11] begin to increase before mitosis and exhibit a peak of activity in the S-phase; histone acetyltransferase activity also increases before mitosis, but the increase in activity lasts longer in comparison to the other enzymes, resulting in a maximum in early G_2-period. Obviously, there is a common triggering event for premitotic induction of a variety of enzyme activities during the *Physarum* cell cycle.

The aim of this investigation was to elucidate whether the in vivo acetylation pattern during the cell cycle, as reflected by acetate incorporation into histones or by the steady-state level of acetylation [2], can be explained in terms of the activities of

Fig.1. Histone acetyltransferase activity and in vivo acetate incorporation into histones during the cell cycle. (a) At various time points during the cell cycle plasmodia were labelled with [^{3}H]acetate and the incorporated radioactivity was determined in equal aliquots (10 μg protein) of the histone fraction. In parallel, histone acetyltransferase activity was determined using the standard assay and the activities were plotted as activity per nucleus (b) or activity per whole macroplasmodium (c) as described in section 2. Arrows in (a) indicate the duration of the S-phase and G_2-period. The columns (M2, M3) indicate the duration of the second and third mitosis.


G2
M2
S
G2
M3
S
a
IN VIVO 3H-ACETATE INCORPORATION (cpm x 10-3)
4
2
0
b
HISTONE-ACETYLTRANSFERASE ACTIVITY (amol/min per nucleus)
0.2
0.1
0
c
HISTONE-ACETYLTRANSFERASE ACTIVITY (pmol/min per macroplasmodium)
100
50
0
0.8
M2
0.2
0.4
0.6
0.8
M3
0.2
0.4
Fraction of cell cycle

the enzymes involved, histone acetyltransferase and histone deacetylase. The latter has been studied during the *Physarum* cell cycle [5]; the enzyme activity was shown to be constant during the S-phase with a subsequent, steady increase during the G_2-period. It therefore follows the synthesis pattern of the majority of the *Physarum* proteins [12]. In contrast, histone acetyltransferase activity exhibits a distinct, highly periodic cell cycle pattern. The time point of maximum histone acetyltransferase activity is characterized by rather low in vivo acetate incorporation into histones; at the same time histone deacetylase activity begins to increase [5]. On the other hand, the time point of maximum in vivo acetate incorporation is not coincident with the peak of histone acetyltransferase activity, which is located much later in the cell cycle. For this reason the in vivo acetylation of histones is not regulated via the enzyme activities of histone acetyltransferase and histone deacetylase. Therefore, it is problematic to study histone acetylation by in vitro incorporation of acetyl-CoA into isolated nuclei.

It has already been shown that the in vitro pattern of histone acetylation differs considerably from the corresponding pattern obtained by in vivo acetate labelling with respect to the preferred substrate histone species [13]. The authors suggested specific factors or structures that determine the acetylation of certain histone species. We extend this suggestion to the phase-specific cell cycle pattern of histone acetylation and conclude that other factors, possibly the structural state of chromatin, determine the in vivo acetylation pattern rather than the activities of the involved enzymes.

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