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Control of histone acetylation. Cell-cycle dependence of deacetylase activity in *Physarum* nuclei

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Summary. Nuclei from naturally synchronous plasmodia of *Physarum polycephalum* were assayed for histone deacetylase activity. The substrate for the assay was a peptide comprising the amino terminal region (residues 1–23) of calf thymus histone H4. The deacetylase activity per nucleus remained constant during S phase and early G2 phase and then doubled in a linear fashion during mid and late G2 phase reaching its maximum level at metaphase. The data imply that H4 acetylation is linked to prior chromatin structural changes.

Most, if not all, of the chromatin in a cell nucleus appears to be organized as arrays of nucleosomes [1]. Nevertheless, the function of different regions of chromatin varies from one region to another and from one time in development of the cell cycle to another. These changing functions are probably associated with structural transitions in chromatin which affect the accessibility of DNA in nucleosomes and interactions between nucleosomes. Acetylation of core histones has been positively correlated with transcription and deposition of histones on DNA during chromatin replication [2–5]. Acetylation is negatively correlated with chromosome condensation in mitosis [6, 7]. It is easy to see how acetylation of the lysine-rich *N*-terminal regions of core histones might destabilize nucleosomes or reduce electrostatic internucleosome interactions and the search for such effects is currently underway. In this paper, we are concerned with the possible metabolic controls that determine the number and turnover rate of histone acetyl groups in vivo.

Inhibition of histone deacetylase activity

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by sodium butyrate has a major effect on histone acetyl groups in vivo [8–11]. We asked the question: "Does histone deacetylase activity change normally, in vivo, thus producing changes in histone acetyl content?"

The system chosen was the naturally synchronous cell cycle in *Physarum polycephalum* [12]. *Physarum* has five major histones analogous to those in mammalian cells and histone H4 is indistinguishable from calf thymus H4 on several gel electrophoresis systems and by amino acid analysis [13]. *Physarum* H4 can be post-transcriptionally modified by incorporation of up to four acetyl groups and the changes in proportion of each modified form have been measured through the cell cycle [7]. In the presence of sodium butyrate the proportion of *Physarum* H4 in the highly modified forms increases [7]. The proportion of $(acetyl)_4$-H4 is positively correlated with transcription in the *Physarum* cell cycle [7, 14]. The proportion of highly acetylated forms is low in mitosis [7], as in CHO cells [6]. The turnover rate of H4 acetyl groups, measured by pulse labelling, is about 4 times higher in S phase than in G2 phase [15].

Materials and Methods

Pure samples of peptide 1–23 from calf thymus histone H4, calf thymus deacetylase I and the non-histone protein HMG 17 were generously provided by P. D. Cary, L. S. Cousens [16] and G. W. Goodwin, respectively.

One mg of peptide 1–23 (prepared as described by Lewis et al. [17]) was reacted with 25 mCi [^{3}H]-acetic anhydride (4 μmole) in 1 ml of 0.1 M sodium phosphate buffer pH 7.5 at 0°C for 1 h [18, 19]. Sixty mg non-radioactive acetic anhydride was added and incubation continued for 1 h. The reaction mixture was freeze-dried and 'de-salted' on a Sephadex G-25 column equilibrated with 0.5 M acetic acid [20]. The acetylated peptide was freeze-dried and stored at −20°C. The specific activity was 134 Ci/mole of acetyl groups.

Physarum polycephalum, strain M3C, was grown as microplasmodia or synchronous macroplasmodia (up to 7 cm ∅) as described [12, 21]. Times of metaphase were obtained by microscopic examination of smears of plasmodia as described [12, 22]. Plasmodia were harvested directly into liquid nitrogen and stored at −76°C. Nuclei were prepared essentially as described by Mohberg & Rusch [23] using modified homogenizing medium (30 mM NaCl, 1 mM KCl, 5 mM $MgCl_2$, 0.1% w/v Triton X-100, 10 mM Tris-HCl, pH 7.1) [24]. Each macroplasmodium was thawed in 50 ml homogenizing medium on ice and homogenized in a Potter homogenizer. Nuclei were collected by centrifugation (10 min, 1 500 g) and the pellet washed twice in homogenizing medium. Nuclei were suspended at a concentration of about 5×10^8 nuclei/ml in 10 mM NH_4Cl, 0.25 mM EDTA, 5 mM 2-mercaptoethanol, 20% (w/v) glycerol, 15 mM Tris-HCl, pH 7.9 [25] and their concentration determined by counting a diluted aliquot in a hemocytometer.

Twenty μl of nuclear suspension were made 1 mM in PMSF and added to 20 μl 0.1 M Tris-HCl, pH 7.5, containing 26 000 cpm of ^{3}H-peptide 1–23 and incubated at 30°C for 2 h. The reaction was stopped by cooling and adding 0.02 ml 0.5 N HCl, 0.08 M acetic acid containing 0.1 mg total calf thymus histone as carrier [16, 26]. Ethylacetate (0.3 ml) was added, mixed and allowed to equilibrate for 30 min at room temperature. 0.2 ml of the upper phase was removed and its radioactivity determined by liquid scintillation counting.

Results

Peptide 1–23 from calf thymus H4 was labelled with [^{3}H]acetic anhydride and analysed by gel electrophoresis. Approximately equal amounts of label were present in each acetylated form. *Physarum* nuclei, extracts of *Physarum* nuclei or purified calf thymus deacetylase [16] released radioactive acetic acid from this substrate. The linearity of the assay was tested by incubating the substrate with varying concentrations of nuclei isolated from asynchronous microplasmodia. The amount of acetate released after either 2 or 4 h incubation was proportional to the number of nuclei in the range $(3–36)\times10^6$ nuclei per 0.04 ml. Ninety percent of the activity could be released from nuclei by high-salt washes and sonication, but the enzyme has not been purified further. The activity in nuclei was heat-labile above 37°C.

Nuclei were isolated from *Physarum* macroplasmodia at defined stages of the mitotic cycle and deacetylase activity meas-

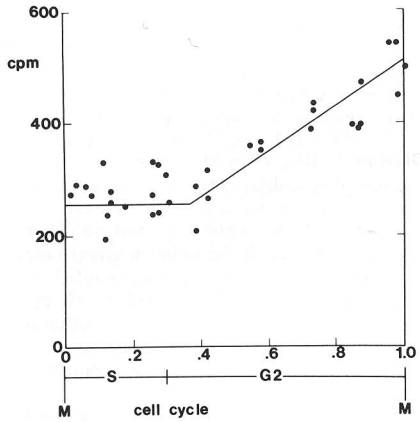

Fig. 1. Cell cycle dependence of nuclear histone deacetylase activity. Deacetylase activity is shown as cpm [^{3}H]acetate released from acetylated peptide 1–23 of calf thymus histone H4. 200 cpm corresponds to approx. 3 pmole acetate released by 10^7 nuclei during a 2 h incubation at 30°C, or approx. 2×10^{-19} mole acetate per nucleus per hour. The cell cycle times were 8–10 h and plasmodia were harvested between M3+2 h and M4+4 h. The cell cycle is shown here as going from metaphase (M) to metaphase and the time in the cell cycle is given as a fraction of the time between consecutive metaphases in each culture. The solid line is the function $A=F(T)$, where $A=256$ ($0<T<0.37$); $A=256+406\,(T-0.37)$ ($0.37<T<1.0$).

ured on aliquots of approx. 10×10^6 nuclei. The concentration of nuclei was determined by counting in a hemocytometer. The activity per nucleus is shown as a function of time in the mitotic cycle in fig. 1. Visual inspection of the data indicated that there is no major activation or repression of total deacetylase activity at any time in the cycle. The simplest interpretation is that the activity per nucleus doubles in G2 phase (possibly as deacetylase enzyme is synthesized) and otherwise remains constant, except when the nuclei divide. The data was fitted by minimizing the sum of squares of residuals using the function $A=F(T)$ where

$A=A_0$, $0<T\leq T_1$
(constant low activity)
$A=A_0+A_0\cdot(T-T_1)/(T_2-T_1)$ $T_1<T\leq T_2$
(enzyme 'synthesis')
$A=2A_0$, $T_2<T\leq1.0$
(constant high activity)

where A is the deacetylase activity at time T in the cycle (T being expressed as a fraction of one cell cycle). The values of the parameters A_0, T_1 and T_2 which gave the minimum sum of squares of residuals were determined numerically and found to be: $A_0=256$ cpm/10^7 nuclei; $T_1=0.37$; $T_2=1.00$. The function with these parameters is shown as the solid line in fig. 1.

The approximate error in an individual value was estimated as $\pm12\%$ from the statistics of counting nuclei and the differences between duplicate enzyme assays. Application of the chi-squared test showed that the data are consistent with the above function ($p>0.5$). The ranges of the parameters are: $230<A_0<280$ cpm/10^7 nuclei ($p>0.05$); $0.55<T_1<0.15$ ($p>0.05$); $0.9<T_2$ ($p>0.05$).

The experiment was repeated using a diphenylamine assay for DNA [27] instead of counting nuclei. The scatter observed with this method was about twice that observed with counting of nuclei, possibly due to variable ploidy levels which do occur in *Physarum* [28]. The complete set of 89 data points collected was consistent ($p>0.3$) with the results obtained above, i.e. $A=\Phi(T)$ where $A=2A_0-A_0\times T/T_0$ ($0<T\leq0.3$, DNA synthesis); $A=A_0$ ($0.3<T\leq0.37$, constant activity); $A=A_0+A_0\,(T-0.37)/0.63$ ($0.37<T\leq1.0$, enzyme 'synthesis'). The DNA content per nucleus was taken as 10–12 g in G2 phase giving $A_0=256$ cpm/0.01 mg DNA. The rate of DNA synthesis was assumed constant for $0<T<0.3$ and zero for $T>0.3$.

The effect of possible inhibitors of deacetylase activity was tested. Sodium butyrate or sodium acetate at 50 mM gave 70 or 40% inhibition, respectively. Sodium

or potassium chloride were less inhibitory. Calf thymus HMG-17 at concentrations up to 1 mg/ml had no significant effect on the activity.

Discussion

Histone deacetylase activity in *Physarum* nuclei is constant during S phase and rises linearly during G2 phase to reach a maximum value in mitosis. The change in activity is thus inversely correlated with DNA synthesis. The fact that the activity just doubles during one cell cycle is consistent with the hypothesis that histone deacetylase is a stable enzyme whose synthesis is restricted to G2 phase but direct measurement of enzyme synthesis is needed to prove this. The deacetylase activity is not correlated with changes in the overall acetyl content of histone H4 nor with changes in amount of high or low acetyl forms of H4 [7, 13]. It is inversely correlated with the turnover rate of acetyl groups on H4, which is 4-fold higher in S phase than in G2 phase, but the correlation is not precise [15]. We conclude that changes in histone acetyl content or acetyl turnover rate are not caused mainly by changes in overall histone deacetylase activity.

The changes in $(acetyl)_4$-H4 that are correlated with transcription may be associated with localized inhibition of deacetylase activity as suggested by Reeves & Candido [29]. We found no inhibition of *Physarum* deacetylase by calf thymus HMG-17 but it remains possible that a *Physarum* HMG-like protein could be a specific inhibitor.

The lack of major changes in histone deacetylase activity in the cell cycle makes an interesting contrast to the major activation of histone kinase that precedes phosphorylation of histone H1 in G2 phase [30, 31]. In the case of H1 it seems that phosphorylation is a response to an extra-chromosomal signal and that phosphorylation then initiates a chromosome structural transition (chromosome condensation) [32]. In the case of H4 it seems more likely that changes in acetyl content or turnover rate are localized to regions of the chromatin that have already undergone a structural change and hence the function of acetylation is to complete the structural transition and/or to stabilize or maintain the altered conformation.

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References

1. McGhee, J D & Felsenfeld, G, Ann rev biochem 49 (1980) 1115.
2. Allfrey, V G, Faulkener, R M & Mirsky, A E, Proc natl acad sci US 51 (1964) 786.
3. Allfrey, V G, Chromatin and chromosome structure (ed Li & Eckhardt) p. 167. Academic Press, New York (1977).
4. Johnson, E M & Allfrey, V G, Biochemical actions of hormones (ed G Litwac) vol. 5. Academic Press, New York (1978).
5. Louie, A J, Candido, E P M & Dixon, G H, Cold Spring Harbor symp quant biol 38 (1973) 803.
6. D'Anna, J A, Tobey, R A, Barham, S S & Gurley, L R, Biochem biophys res commun 77 (1977) 187.
7. Chahal, S S, Matthews, H R & Bradbury, E M, Nature 287 (1980) 76.
8. Riggs, M G, Whittaker, R G, Neumann, J R & Ingram, V M, Nature 268 (1977) 462.
9. Sealy, L & Chalkley, R, Cell 14 (1978) 115.
10. Candido, E P M, Reeves, R & Davie, R, Cell 14 (1978) 105.
11. Vidali, G, Boffa, L C, Bradbury, E M & Allfrey, V G, Proc natl acad sci US 75 (1978) 2239.
12. Aldrich, H H & Daniel, J W (ed), Cell biology of *Physarum* and *Didymium*. Academic Press, New York (1981).
13. Matthews, H R & Bradbury, E M, The cell biology of *Physarum* and *Didymium* (ed H Aldrich & J W Daniel). Academic Press, New York (1981).
14. Matthews, H R, The cell cycle (ed P John) pp. 223–246. Cambridge University Press, UK (1981).
15. Chahal, S S, Waterborg, J W, Mueller, R D, Mende, L & Matthews, H R. Submitted for publication (1981).
16. Cousens, D G, Gallwitz, D & Alberts, B M, J biol chem 254 (1979) 1716.

17. Lewis, P N, Bradbury, E M & Crane-Robinson, C, Biochemistry 14 (1975) 3391.
18. Riordan, J F & Vallee, B L, Methods in enzymology (ed S P Colowick & N O Kaplan) vol. 25 (ed C H W Hirs & S N Timasheff) pp. 494–499. Academic Press, New York (1972).
19. Ostrowski, K, Barnard, E A, Sawicki, W, Chorzelski, T, Langner, A & Mikulski, A, J histochem cytochem 18 (1970) 490.
20. Heinegard, D K & Hascall, V C, J biol chem 254 (1979) 921.
21. Daniel, J W & Baldwin, H H, Methods in cell physiology (ed D M Prescott) vol. 1, pp. 9–41. Academic Press, New York (1964).
22. Guttes, E & Guttes, S, Methods in cell physiology (ed D M Prescott) vol. 1, pp. 43–54. Academic Press, New York (1964).
23. Mohberg, J & Rusch, H P, Exp cell res 66 (1971) 305.
24. Polman, B J J, Ph.D. thesis, University of Nijmegen, The Netherlands (1974).
25. Sures, I & Gallwitz, D, Biochemistry 19 (1980) 943.
26. Kervabon, A, Mery, J & Parello, J, FEBS lett 106 (1979) 93.
27. Burton, K, Biochem j 62 (1956) 315.
28. Mohberg, J, Babcock, K L, Haugli, F B & Rusch, H P, Dev biol 34 (1973) 228.
29. Reeves, R & Candido, E P M, Nucleic acids res 8 (1980) 1947.
30. Bradbury, E M, Inglis, R J, Matthews, H R & Sarner, N, Eur j biochem 33 (1973) 131.
31. Bradbury, E M, Inglis, R J & Matthews, H R, Nature 247 (1974) 257.
32. Matthews, H R, Recently discovered systems of enzyme regulation by reversible phosphorylation (ed P Cohen) pp. 235–254. Elsevier/North-Holland, Amsterdam (1980).

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Sequential staining with Hoechst 33258 and quinacrine mustard for the identification of human chromosomes in somatic cell hybrids

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Summary. We have analysed metaphase chromosomes of man–mouse somatic cell hybrids using a sequential staining procedure involving the fluorescent DNA-binding stains, Hoechst 33258 and quinacrine mus-