Biosynthesis and posttranslational acetylation of histones during spherulation of *Physarum polycephalum*

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ABSTRACT

Plasmodia of *Physarum polycephalum* can be induced to differentiate into dormant spherules: DNA-, RNA- and protein-synthesis cease during this process. Analysis of the histone H4 acetylation during spherulation revealed no significant changes of the relative acetate content and percentage of acetylated H4 subspecies. This result does not support a close correlation of histone acetylation and transcriptional activity. Posttranslational incorporation of ^3H -acetate into core histones decreased rapidly after start of spherulation. However, acetate incorporation increased significantly at a late stage of spherulation (30h). To elucidate the role of this elevated acetate incorporation we followed histone synthesis during spherulation. Histone synthesis decreased upon induction of differentiation and stopped after 12 h. After 38 h of spherulation histone synthesis again occurred in the absence of DNA synthesis. The peak of acetate incorporation into core histones clearly preceded this late histone synthesis, indicating acetylation of preexisting histones. We suggest, that this acetate incorporation is part of the mechanism, by which preexisting histones are replaced by newly synthesized histones. Pulse treatment with actinomycin D or cycloheximide during spherulation suggested, that the observed histone synthesis is essential for the germination of spherules. Obviously, new histones have to be synthesized for the coordinate course of the differentiation program.

INTRODUCTION

Histones, the main structural proteins of eucaryotic chromatin, together with DNA, assemble the nucleosome, which represents the basic structural unit of chromosomes (1). The facts that histones exist in different primary structure variants and are subject to posttranslational modifications, indicate a complex functional role of histones in gene regulation rather than a mere structural importance.

A large body of evidence has accumulated for the tight coupling of histone biosynthesis to DNA replication (2). However, several reports demonstrated uncoupling of DNA- and histone-synthesis during the cell cycle of arrested or differentiated cells (3-5). Moreover, it has been shown that, apart from the replication coupled histone synthesis, there is a basal histone

synthesis throughout the cell cycle (6).

Posttranslational modifications of histones include phosphorylation (7), acetylation (8), poly(ADP-ribosylation) (9), glycosylation (10) and ubiquitination (11,12). Modulation of distinct functional activities of histones has been attributed to these reversible modifications.

We have studied histone synthesis and histone acetylation during spherulation of the myxomycete Physarum polycephalum. Since molecular events can be followed in this organism throughout the naturally synchronous cell cycle as well as during various differentiation pathways, it has become a valuable model system to study chromatin structure and function. It has been shown, that histone synthesis is tightly coupled to DNA replication during the synchronous Physarum cell cycle (13,14). Interestingly the histone H4 gene of Physarum is transcribed not only during S-phase but also during late G₂-period without concomitant translation of the message (15). Posttranslational acetylation of histone H4 (16), phosphorylation of H1 (17) and ubiquitination of H2A and H2B (18) undergo distinct cell cycle dependent fluctuations in Physarum.

Spherulation of Physarum represents a vegetative differentiation process from actively growing microplasmodia to dormant spherules. The transition is accompanied by a depression of RNA synthesis (19). For this reason we exploited this differentiation step to study the relation between histone acetylation and transcriptional activity. We have previously shown, that H4 acetylation is not correlated with transcription during the Physarum cell cycle (16). Moreover, the template activity of Physarum chromatin for transcription was not altered upon hyperacetylation of histones (20), supporting our conclusion, that histone acetylation and transcription are not coupled in this organism. This report confirms our earlier conclusion, since we do not find significant changes in H4 acetylation during the spherulation pathway. The analysis of histone synthesis revealed a differentiation specific histone synthesis at a late stage of spherulation, which was not linked to DNA replication. The finding that this histone synthesis is preceded by a considerable incorporation of acetate into preexisting histones suggests a role of histone acetylation in the replacement mechanism of preexisting by newly synthesized histones.

MATERIALS AND METHODS

Materials

These were purchased from the following sources: ³H-acetic acid sodium salt (4.5 Ci/mmol), 2-¹⁴C-thymidine (54 mCi/mmol), L-4,5-³H-leucine (120 Ci/mmol), L-4,5-³H-lysine monohydrochloride (85 Ci/mmol), 5-³H-uridine (29 Ci/

mmol) from Amersham International plc, U.K.; cycloheximide, L-leucine, pro-tamine sulfate from salmon (grade X) from Sigma Chem Comp., St.Louis, USA; actinomycin D, L-lysine, phenylmethylsulfonylfluoride and uridine from Serva Feinbiochemica, West Germany.

Culture conditions

Physarum polycephalum, strain M₃b FII (a Wis 1 isolate) was used. Micro-plasmodia were cultivated in submersed shake culture in semidefined medium (21) on a reciprocal shaker. The medium was supplemented with 0.013% haemoglobin instead of haematin. The growth temperature was 24°C. Culture techniques were performed under sterile conditions.

Spherulation

To induce spherulation exponentially growing microplasmodia were washed with distilled water. After a second wash in spherulation medium (21) the microplasmodial sediment was transferred to spherulation medium. The medium/sediment ratio was 12. For spherulation the cultures were incubated at 24°C on a reciprocal shaker. The course of spherulation was followed by phase contrast microscopy. Mature spherules were formed within 30 to 36 h.

For germination experiments 5 day old spherules were transferred to nutrient medium (21) diluted 1:1 with distilled water and incubated as described for microplasmodia. 20 h after start of germination the culture was centrifuged and the volume of the plasmodial sediment was determined. The increase of the volume (growth) was taken as a criterion of successful germination.

Labelling and chase

For labelling with radioactive precursors microplasmodia were cultivated for 2 days in medium with decreased amounts of peptone (50%) and yeast extract (25%). The labelling procedure itself was also performed in the reduced medium.

Exponentially growing microplasmodia were labelled with either ³H-leucine (5 µCi/ml medium), or ³H-lysine (30 µCi/ml medium), or ¹⁴C-thymidine (1 µCi/ml medium), or ³H-uridine (40 µCi/ml medium). The labelling period for thymidine was 1 h, for uridine 45 min. The chase was performed in normal nutrient medium, respectively in medium containing 50 µg/ml of nonradioactive uridine for 6 h. In case of ³H-leucine or ³H-lysine the labelling period was 8 h. After careful washing in fresh nutrient medium growth was allowed to continue in the presence of cold leucine (0.053 M) or lysine (0.038 M) for 12 h. In accordance with earlier results (22) control experiments showed, that the presence of amino acids in this concentration does neither affect growth nor encystment. After the chase period microplasmodia were collected by centrifugation and transferred to spherulation medium to induce differentiation. The spherulation medium con-

tained the same amounts of nonradioactive leucine, lysine or uridine as the chase medium. At selected time points during spherulation aliquots were harvested for nuclear isolation or determination of the specific radioactivities of DNA, RNA or protein.

For labelling with ^3H -acetate, microplasmodia were transferred to spherulation medium to induce differentiation. At selected time points during spherulation aliquots of the culture were incubated in spherulation medium containing 0.07 mM cycloheximide for 15 min. Then ^3H -acetate (20 mCi/ml) was added and incubation was continued for 5 min. The cycloheximide pretreatment prevents the cotranslational acetylation of N-acetylserine residues. After incubation with radioactive acetate the aliquots were harvested for nuclear isolation and subsequent histone analysis.

For pulse labelling of cultures with ^3H -lysine during spherulation the spherulating plasmodia were incubated in spherulation medium containing ^3H -lysine (100 $\mu\text{Ci/ml}$) for 30 min. After incubation the culture was harvested for nuclear isolation and histone analysis.

Nuclear isolation, chromatin preparation and histone extraction

Nuclear isolation was performed according to a published procedure (23). 0.2 mM phenylmethylsulfonylfluoride was included in all buffers during nuclear isolation and chromatin preparation. Chromatin was prepared as described (20, 33).

To extract histones the 10-fold amount of protamine sulfate in relation to the DNA content of the nuclear sample (1×10^7 nuclei is equivalent to 12 μg of DNA) was added to chromatin or isolated nuclei (suspended in a solution of 0.0375 M NaCl, 0.0125 M EDTA, pH 8.0). The suspension was shaken for 5 min. at 4°C . Then the sample was made 8 M in urea and 0.9 M in acetic acid and mixed occasionally for another 5 min. The mixture was centrifuged for 10 min. at $14.000 \times g$ at 4°C . The supernatant was used as the histone sample. The protamine sulfate was used from a stock solution of 100 mg/ml in 8 M urea and 0.9 M acetic acid. For some experiments and also as a control washed nuclei or chromatin were treated with SDS buffer (0.0628 M Tris-HCl, pH 6.8, 3% sodiumdodecylsulfate, 10% glycerol and 5% mercaptoethanol). This SDS-extract or the protamine sulfate extract were further analyzed by SDS-polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

Histones were either analyzed on SDS-polyacrylamide slab gels (16 cm long) as described (24) with slight modifications (25) or on acetic acid-urea-Triton polyacrylamide slab gels (48 cm long) according to a published procedure (26).

Gels were stained with Coomassie Blue, destained by diffusion, photographed and dried. For evaluation of acetic acid-urea-Triton gels for the relative acetate content (N) of H4 as described (16), wet gels were scanned at 572 nm in a Beckman DU-8B spectrophotometer with a gel scanning equipment.

Detection of radioactivity

Liquid samples were analyzed for radioactivity by counting aliquots in a liquid scintillation spectrophotometer. For detection of radioactivity in polyacrylamide gels fluorography was performed as described (27, 28), using preflashed Kodak X-omat RP film. Fluorograms were photographed and scanned at 585 nm in the Beckman DU-8B spectrophotometer. Since exposure times for fluorography were long for certain experiments (up to 12 weeks) we subjected gel slices to liquid scintillation counting, as an alternative. For this purpose, slices from the Coomassie Blue stained gel containing the protein bands, were solubilized in 30% H₂O₂ at 80°C for 3 h and counted after 3 days in the liquid scintillation counter.

Colorimetric procedures

Samples for DNA-, RNA- and protein- analysis were prepared as outlined before (29). Published procedures were followed for estimation of DNA (30), RNA (31) and protein (32).

Actinomycin D and cycloheximide pulse treatment

At selected time points during spherulation actinomycin D (0.3 mM) or cycloheximide (0.07 mM) were applied to spherulating plasmodia. Cultures were allowed to stay on actinomycin D for 1 h or on cycloheximide for 15 min. Then the cultures were centrifuged and the sediment was washed 3 times with fresh spherulation medium to remove last traces of the drugs. Spherulation was continued and after 1 h the medium once more was replaced by fresh spherulation medium. After 76 h spherules were inspected under phase contrast and formation of mature spherules was judged upon morphological criteria. Then the spherules were incubated for another 44 h and germinated as described above after 120 h. After a 20 h period of germination the increase in volume of the plasmodial sediment was measured and used as a parameter for successful germination into growing microplasmodia.

RESULTS

We measured DNA-, RNA- and protein- synthesis during the transition from microplasmodia to spherules with an isotope dilution technique. Microplasmodia were labelled with ¹⁴C-thymidine, ³H-uridine or ³H-leucine, chased and transferred to spherulation medium. Aliquots were harvested during spherulation for

Fig.1. Synthesis of DNA (a), RNA (b) and protein (c) during spherulation of *Physarum*. Exponentially growing microplasmodia were grown on medium containing ^{14}C -thymidine (a), ^3H -uridine (b) or ^3H -leucine (c); after a chase in nonradioactive precursors the plasmodia were transferred to spherulation medium as described in Materials and Methods. At selected time points aliquots were harvested for determination of the specific radioactivity of DNA (a), RNA (b) and protein (c). (●), spherules; (○), microplasmodia as control.

determination of the specific radioactivity. Fig.1a shows, that the specific radioactivity of DNA decreased rapidly within the initial 12 h of spherulation. This decrease represents DNA replication. Afterwards the specific radioactivity

Fig.2. Acetylation of H4 during spherulation. Histones were extracted from isolated nuclei at selected time points during spherulation (0 until 72 hours) and analyzed by acetic acid-urea-triton polyacrylamide gel electrophoresis. (a), densitometric scans of the Coomassie Blue stained gel; 0-4 indicates the number of acetates per H4 molecule. (b), percentage of acetylated H4 subspecies; (●), H4Ac0; (○), H4Ac1; (▲), H4Ac2; (△), H4Ac3; (□), H4Ac4. (c), the relative acetate content (N) was calculated from the data of (b).

remained almost at the same level until the end of the differentiation process, indicating a slowing of DNA replication with a subsequent stop. In contrast, microplasmodia exhibited a constant decrease of the specific radioactivity due to continuous DNA synthesis (Fig.1a). This is in line with earlier data (19,33).

In Fig.1b the time course of the specific radioactivity of RNA is shown. It decreased during the initial 10 h of spherulation reflecting synthesis of new, unlabelled RNA. The specific radioactivity then remained constant until the end of the differentiation process, indicating the cessation of RNA synthesis. Within the initial 10 h the decrease of the specific radioactivity was faster in growing plasmodia than in spherulating plasmodia (Fig.1b). Thus, spherulating plasmodia, although still active in transcription, respond very quickly to the induction of differentiation by a reduction of RNA synthesis rate.

The pattern of total cellular protein synthesis in spherulating plasmodia is shown in Fig.1c. Protein synthesis continued for at least 20 h of spheru-

lation. After this time a further slight decrease of the specific radioactivity of protein could be observed. The decrease was approximately equal to that in growing plasmodia during the initial 6 h. Then the decrease became more and more attenuated in the spherulating cultures indicating an increasing depression of protein synthesis. Again growing plasmodia exhibited a continuous decrease of the specific radioactivity during the whole course of the experiment (Fig.1c).

Since spherulation was characterized by a cessation of RNA synthesis, we studied the acetylation of histone H4 during this differentiation process to investigate whether there is a correlation of RNA synthesis and histone acetylation during differentiation of Physarum. If such a correlation exists, a dramatic decrease of the acetate content and the percentage of highly acetylated H4 subspecies may be expected.

Fig.2a shows the densitometric scans of the H4 region in an acetic acid-urea-Triton polyacrylamide gel of histone samples isolated at selected time points during spherulation. No major changes occurred in the acetylation of H4 during spherulation. Evaluation of the scans for the percentage of acetylated subspecies revealed only negligible changes (Fig.2b). The relative acetate content (N), calculated as previously described (16), was more or less constant throughout spherulation, decreasing slightly from 1.06 at the start to 1.03 at the end of spherulation (Fig.2c).

A rapid decline of acetate incorporation into the protamine sulfate extract (cpm in 10^7 nuclei) was observed (Fig.3a). After 12 h the incorporation had completely ceased. Interestingly we found an increase of acetate incorporation at 30 h after start of spherulation with a subsequent peak at 35 h and a decline thereafter. At 40 h the incorporation had already returned to very low levels and remained low until the end of spherulation (Fig.3a). We analyzed the histones by SDS polyacrylamide gel electrophoresis with subsequent fluorography. Fig.3b shows the Coomassie Blue stained gel of histones from selected time points of spherulation. Extracted histones of 2×10^7 nuclei were applied to each lane. Fig.3c shows the corresponding fluorogram of Fig.3b. Most of the radioactivity was found in the H2B/H3 band. H2B and H3 of Physarum are not separated from each other due to their very similar molecular weight. There was also incorporation into H2A and H4. The fluorogram clearly shows that the label in core histones almost disappeared within the initial 12 h of spherulation (Fig.3c). At the same time, when the radioactivity began to increase in the protamine sulfate extract (Fig.3a) also increased labelling occurred in the fluorogram. At later stages the label decreased again. This proves that the increase of radioactivity in the protamine sulfate extract between 30 and 40 h

Fig.3. ³H-acetate incorporation into core histones during spherulation. At selected time points during spherulation an aliquot of the culture was grown on spherulation medium containing 20 µg/ml cycloheximide for 15 min. Then ³H-acetate was added for 5 min. After this labelling period the culture was harvested and histones were extracted from isolated nuclei. (a), the protamine sulfate extract of 1×10^7 nuclei was analyzed for radioactivity; (b), the protamine sulfate extract of 2×10^7 nuclei of each sample was analyzed by SDS polyacrylamide gel electrophoresis (Coomassie Blue stained gel); (c), fluorogram of the gel in (b); the numbers 0-45 indicate the time points (h) during spherulation, when aliquots were incubated.

was indeed due to enhanced acetate incorporation into core histones.

To elucidate the role of acetate incorporation into core histones at this late stage of spherulation we analyzed the pattern of histone biosynthesis during the differentiation process. Histones were prelabelled with ³H-lysine and after a chase period spherulation was induced. At selected times aliquots of the culture were harvested for nuclear isolation and the radioactivity in the protamine sulfate extract was measured. Fig.4a shows, that the incorporated

Fig.4. Histone synthesis during spherulation. Microplasmidia were grown on medium containing ^3H -lysine. After a chase in nonradioactive precursor the microplasmidia were transferred to spherulation medium. (a), ^3H -lysine labelled histones were extracted from isolated nuclei at selected time points during spherulation by the protamine sulfate method; the protamine sulfate extract of 5×10^7 nuclei of each sample was analyzed for radioactivity; ($\bullet$), spherules; ($\circ$), microplasmidia as control; ($\blacktriangle$), 1 h actinomycin pulse at 34 h; ($\blacksquare$), 15 min. cycloheximide pulse at 38 h; (b), isolated nuclei were lysed in SDS buffer and the lysate was analyzed by SDS polyacrylamide gel electrophoresis; histone bands were cut off the Coomassie Blue stained gel, dissolved in H_2O_2 and counted in a liquid scintillation counter (the SDS extract of 8×10^6 nuclei of each sample was applied to each lane); (Δ), H1; ($\blacktriangle$), H2A; ($\circ$), H2B/H3; ($\bullet$), H4. (c), Coomassie Blue stained SDS gel (histone extract of 2×10^7 nuclei per lane); (d), fluorogram of (c); the numbers 0-54 indicate the time points (h) during spherulation, when samples were harvested.

radioactivity decreased upon induction of spherulation. There was no further decrease after 12 h indicating a cessation of histone biosynthesis. The radioactivity remained constant until 38 h after start of spherulation. Between 38 and 45 h a significant decrease of radioactivity was observed, indicating another period of histone synthesis. From the results of Fig.1a it is obvious, that this histone synthesis occurs in the absence of DNA replication.

To find out whether all histones were synthesized at that time we analyzed the histone samples by SDS polyacrylamide gel electrophoresis and counted the radioactivity of the excised histone bands. Fig.4b shows the amount of radioactivity present in individual histone bands at various times during spherulation. The radioactivity decreased in all histone bands within the first 12 h; then, as reflected by the constant radioactivity in the individual histone bands, histone synthesis ceased. About 38 h after the start of differentiation the radioactivity in core histone bands again decreased. In contrast, H1 was obviously synthesized at a very low rate throughout spherulation, since there was a small but continuous decrease of radioactivity throughout the differentiation process. The same results were obtained with fluorography of an SDS polyacrylamide gel. The stained gel and the corresponding fluorogram are shown in Fig. 4c and 4d. Again there was a significant decrease of label in histone bands between 38 and 45 h. The results were the same if histones were extracted from isolated chromatin (results not shown). At the time of this late histone synthesis mature spherules had already been formed as judged by microscopic inspection of the culture.

Fig.5 shows results of an experiment, where we followed incorporation of ^3H -lysine and ^3H -acetate into histones of isolated chromatin. At different time points during spherulation we pulse-labelled aliquots of the same spherulating culture with ^3H -lysine or ^3H -acetate, isolated chromatin and analyzed histones by gel electrophoresis and subsequent fluorography. The fluorogram shows, that ^3H -lysine incorporation decreased to very low levels during spherulation (Fig.5a). Between 38 and 45 h an increase of lysine incorporation into chromatin occurred. After 45 h incorporation again decreased to almost undetectable levels. The peak of ^3H -acetate incorporation was located around 35 h and clearly preceded the ^3H -lysine peak (Fig.5b). During maximum lysine incorporation (40-45 h) only very little ^3H -acetate was incorporated. Note, that ^3H -lysine was incorporated mainly into histones at 43 h, whereas it was incorporated into numerous other proteins at 0 h (Fig.5a).

When actinomycin D was applied to the culture 34 h after start of spherulation, late histone synthesis was repressed; the same repression was observed

Fig.5. Incorporation of ^3H -lysine and ^3H -acetate into histones of chromatin. At selected time points during spherulation aliquots of the same culture were pulse-labelled with ^3H -lysine or ^3H -acetate. Aliquots were harvested immediately after labelling and chromatin was isolated. Histones were extracted from chromatin and analyzed by SDS-polyacrylamide gel electrophoresis with subsequent fluorography. (a), fluorogram of ^3H -lysine labelled histones (histone extract of chromatin equivalent to 1×10^7 nuclei per lane); (b), fluorogram of ^3H -acetate labelled histones (histone extract of chromatin equivalent to 2×10^7 nuclei per lane). The numbers 0 - 61 indicate the time points (h) during spherulation, when samples were harvested.

when cycloheximide was applied 38 h after start of spherulation (Fig.4a). Interestingly, spherules, treated with actinomycin D or cycloheximide in this way, were unable to germinate into microplasmodia when exposed to nutrient medium after 5 days. This suggested to us, that the late synthesis of histones or probably of other proteins was necessary for the viability of the spherules rather than for spherulation itself. To test this assumption we studied spherulation and germination after actinomycin D or cycloheximide pulses applied at various times during spherulation. Table I shows, that actinomycin D treatment inhibited the spherulation process up to at least 6 h after start of spherulation. Cycloheximide had the same inhibitory effect up to 12 h after start of spherulation. At later stages actinomycin D or cycloheximide no longer affected the formation of mature spherules (Table I). When the spherules, which had been treated with pulses of actinomycin D or cycloheximide, were tried to be germinated into growing microplasmodia, it was recognized that treatment with actinomycin D between 6 and 32 h or with cycloheximide between 12 and 34 h respectively, did not affect the viability of the spherules. However, when actinomycin D was applied between 32 and 40 h or cycloheximide between 34 and 45 h respectively, spherules were no more able to germinate into microplasmodia.

TABLE I. Effect of actinomycin D and cycloheximide pulses during various times of spherulation on the formation and germination of spherules.

time of pulse (h after start of spherulation)	actinomycin D		cycloheximide	
	spherulation	germination	spherulation	germination
0	-	-	-	-
2	-	-	-	-
4	-	-	-	-
6	-	-	-	-
12	+	+	-	-
17	+	+	+	+
24	+	+	+	+
26	+	+	+	+
32	+	+/-	+	+
34	+	-	+	+/-
38	+	-	+	-
40	+	+/-	+	-
45	+	+	+	+/-
48	+	+	+	+
54	+	+	+	+
64	+	+	+	+
72	+	+	+	+

At various times of spherulation actinomycin D (1 h) or cycloheximide (15 min.) was applied. Formation of mature spherules was judged by morphology under phase contrast 76 h after start of spherulation. To decide whether germination of spherules had occurred, the volume of the spherule sediment was determined at the begin of germination and 20 h later. Successful germination resulted in a doubling of the sediment volume within 20 h.

+ ... spherulation/germination was observed in 5 independent experiments

- ... spherulation/germination was not observed in 5 experiments

+/-... in some experiments germination occurred, in some it did not occur.

The begin of this time period (32 h for actinomycin D, 34 h for cycloheximide) obviously is a crucial point, since we got different results from experiment to experiment. The same is true for the end of this period. The effect at the time points in between was the same within 5 experiments. Actinomycin D or cycloheximide applied at later stages of spherulation did not affect the germination of the spherules (Table I).

DISCUSSION

It has been a commonly accepted theory, that histone synthesis is tightly coupled to DNA replication (2). This idea was mainly based on the following observations: 1) histone synthesis was shown to be restricted to the S-phase of the cell cycle (34, 35); 2) histone mRNA is present during S-phase, but greatly reduced or even absent in G₁-period (36-39); 3) inhibitors of DNA synthesis inhibit histone synthesis (40,41). Although this interrelationship between the two synthetic processes exists, some reports in the literature demonstrate, that this linkage is not obligatory. Thus, histones may be synthesized at equal rates in G₁- and S-phase (42); moreover, histone synthesis can be separated into S-phase synthesis and basal synthesis throughout the cell cycle (6). During development and differentiation, histone synthesis can occur in the absence of DNA replication as demonstrated during frog oogenesis (43), in neuron cells of newborn rabbits (44,45), in mouse brain (46) and in rat spermatogenic cells (5).

One of the important implications originating from the theory of coupling of histone synthesis to DNA replication was the unique metabolic stability of histone proteins. However, it has been demonstrated, that even in terminally differentiated cells histones undergo continuous turnover (4).

Our results show, that histone synthesis can occur also independently from DNA replication during differentiation of Physarum. During growth of Physarum macroplasmodia, histone synthesis was shown to be tightly coupled to DNA replication (13,14); this coupling seems to persist during early stages of spherulation, since the initial decline of histone synthesis follows the decline of DNA replication. Later during differentiation the two synthetic processes become uncoupled; this occurs at a time when most of the differentiation specific events have already taken place (40 h after start of spherulation). At that time histone synthesis takes place, though the synthesis of DNA, RNA and total cellular proteins is repressed. The results of experiments with actinomycin D or cycloheximide rather suggest that this histone synthesis is essential for the germination of spherules. However, we can not exclude the possibility that other proteins, essential for the germination process, are synthesized at the same time. It is worth mentioning, that the germination of spherules occurs when the organism is introduced into an appropriate environment, i.e. when the spherules are supplied with nutrients under laboratory conditions. Germination can occur years or even decades after spherulation. It is a striking feature that the organism prepares for the possible germination event already during spherulation and not, as could be expected, after in-

duction of germination. The fact, that the newly synthesized histones are assembled into chromatin (Fig.5) rules out the possibility that histones are stored until germination occurs. We cannot decide, whether the observed late histone synthesis is due to enhanced turnover of the histones at this particular stage of spherulation or to the synthesis of specific histone variants. So far histone primary structure variants have not been reported in Physarum. We could not detect new histone variants during spherulation, at least in 48 cm long acetic acid-urea-Triton gels (results not shown). The fact that all histones are synthesized during the same, relatively short time period does not necessarily presuppose a simple turnover since individual histone species are characterized by quite different turnover rates in other systems (3,4). It should be noticed that no histone turnover was observed between 12 and 38 h of spherulation.

Our earlier studies of histone acetylation (16) suggested that newly synthesized H4 in S-phase has a higher degree of acetylation in comparison to H4 during G₂-period. In contrast, no elevation of H4 acetate content or ³H-acetate incorporation into core histones could be observed at the time of histone synthesis during late spherulation; instead we found a considerable incorporation of ³H-acetate into core histones 5-10 h prior to histone synthesis. This time shift was always the same in repeated experiments. In an experiment, where we followed histone synthesis and histone acetylation by pulse-labelling with ³H-lysine and ³H-acetate, we could conclusively show that lysine incorporation starts at a time when the incorporation of acetate has already ceased again (Fig.5). In both cases the radioactivity was found in histones of isolated chromatin. This experiment proves that the radioactive acetate is incorporated into 'old' histones, since the assembly of 'new' histones into chromatin is restricted to a later time period. For this reason we assume that the acetylation of preexisting histones may be part of the replacement mechanism by newly synthesized histones. This mechanism could operate similarly as during spermatogenesis, where H4 hyperacetylation assists in the replacement of histones by protamines (47-49). It is possible, that acetate incorporation does not occur uniformly over the whole chromatin, but is clustered in certain domains, giving rise to even hyperacetylated regions of chromatin. The existence of histone acetylation which is not related to gene expression, but to histone deposition and chromatin assembly was recently demonstrated for *Tetrahymena* (50).

The pattern of H4 acetylation during the whole spherulation process of Physarum supports our earlier results (16,20), that histone acetylation is not correlated with transcriptional activity because we observed an almost invari-

able acetate content of H4 during the differentiation process. It is remarkable, that the organism is able to stabilize the degree of H4 acetylation even during the transition from active growth to quiescence.

It should be mentioned that we performed our experiments without inclusion of the deacetylase inhibitor sodium-n-butyrate during nuclear isolation and histone extraction. In contrast to other systems the presence of butyrate during the isolation procedures does not significantly influence the acetylation pattern of Physarum plasmodia (16,20,51). The same is true for acetylation patterns during spherulation as we checked in control experiments. We used 100 mM sodium-n-butyrate, since unlike other eucaryotic systems, Physarum deacetylase is less sensitive to butyrate (52).

It has been pointed out recently, that cycloheximide pretreatment (15 min. preincubation prior to a 5 min. labelling with ^3H -acetate) completely changes the pattern of ^3H -acetate incorporation into histones of Physarum plasmodia (53). The cycloheximide preincubation led to the disappearance of label in H1, which is cotranslationally acetylated at the N-terminal serine residue. For this reason it is possible during prolonged ^3H -acetate pulses, that the cotranslationally labelled H1 is transported into the nucleus and then appears as label in the fluorogram. This can be suppressed by cycloheximide preincubation. However, under our labelling conditions radioactivity is not incorporated into H1 within a 5 min. pulse either in the presence or absence of cycloheximide. Nevertheless we included cycloheximide pretreatment in our experimental protocol as an additional precaution. The same authors (53) also reported the disappearance of ^3H -acetate label in H2A and H2B after cycloheximide pretreatment. They explain this by the complete lack of posttranslational acetylation of pre-existing H2A and H2B. They claimed, that only newly synthesized H2A and H2B are posttranslationally acetylated. As can be clearly seen from our fluorograms ^3H -acetate is incorporated to a large extent into preexisting H2A and H2B (Fig. 3 and 5), since this incorporation occurred in the absence of histone synthesis. We have performed some of our experiments in parallel with and without cycloheximide pretreatment, but did not observe significant differences, provided that the labelling time did not exceed 5 min. We therefore conclude, that contrary to published data (53), posttranslational acetylation of H2A and H2B is not restricted to newly synthesized molecules.

We were able to demonstrate for the first time that histone synthesis can occur in the absence of DNA replication in Physarum polycephalum. This differentiation-specific histone synthesis may be required for the development or the maintenance of a certain differentiated state; in the case of Physarum spheru-

lation it is most likely, that this histone synthesis serves as a prerequisite for the viability of the spherules. The synthesis might be related to special requirements for nucleosome stability in dormant cells. Histone acetylation obviously assists the replacement mechanism of histones during this differentiation pathway.

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