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Patterns of Histone Acetylation in the Cell Cycle of *Physarum polycephalum*†

Jaap H. Waterborg and Harry R. Matthews*

ABSTRACT: Labeling of histones in the naturally synchronous cell cycle of *Physarum polycephalum* with short pulses of tritiated acetate in vivo clearly showed three distinct patterns of histone acetate turnover. In *G2 phase*, turnover of acetate was observed only in histones H3 and H4, predominantly on the multiple acetylated forms. No acetate turnover was found in histones H2A and H2B. This indicates different functional roles of histones H2A and H2B compared with histones H3 and H4. In *S phase*, intense labeling was seen in all four core histones, in histones H3 and H4 predominantly in the low acetylated forms. In addition, cotranslational acetylation of the amino-terminal serines of histones H4 and H1 was observed during S phase. During *mitosis*, from condensation at prophase to decondensation after telophase, acetate turnover is almost zero. This suggests that within the mitotically condensed chromosomes all potential histone acetylation sites are masked. In G2 phase, when transcription is occurring, only histones H3 and H4 are available for acetate turnover, but in S phase, when both transcription and replication occur, all four histones are available for acetate turnover.

Chromatin is involved in several of the key processes of living cells, particularly transcription, replication, and distribution of the genetic material to daughter cells. These processes are associated with different structural states of the chromatin, and although very substantial progress has been made in understanding the structure of the underlying nucleosome subunit, there remains much speculation about how the nucleosome structure is modified to accommodate different chromatin functions and about higher order packing of nucleosomes in the different chromatin structures [for a review, see Igo-Kemenes et al. (1982)]. There are two key questions being asked about the chromosome structural transitions. First, what signals determine when a particular transition should occur and where it occurs, relative to specific DNA sequences in the chromatin. Second, what are the characteristics of each specific state of the chromatin that stabilize that state and enable it to function in its specific role? We are concerned, here, with the second question and with the role of histone modification in stabilizing specific chromatin structures. Acetylation of core histones has previously been correlated with chromosome replication and transcription and negatively correlated with mitosis [for a review, see Matthews & Bradbury (1982)]. However, many of the systems used to establish these correlations did not have total separation of replicating from transcribing chromatin, and results from chromatin fractionation procedures have shown variable amounts of acetylated histones associated with "active" chromatin. We have reinvestigated the relationship between histone acetylation and chromatin function by using an in vivo, unperturbed system in which three situations are available with a purity of 98–99%. These are the phases of the naturally synchronous cell cycle in the true slime mold *Physarum polycephalum*: mitosis (chromosome condensation and separation; no transcription or replication); S phase (chromosome replication and transcription); and G2 phase (transcription only). The results confirm the correlation of histone acetylation with chromosome structure and function and show, for the first time, that the pattern of acetylation associated with transcription is quite distinct from the pattern associated with replication.

Materials and Methods

Physarum Culture. Physarum polycephalum, strain M3c, was cultured as microplasmodia in shaking flasks or as macroplasmodia on filter papers in petri dishes, essentially as described (Daniel & Baldwin, 1964; Guttes & Guttes, 1964). The semidefined growth medium described by Daniel & Baldwin (1964) was used, with a final hematin concentration of 2.5 mg/L. Exponentially growing microplasmodia were used for fusion to macroplasmodia. Mitosis was observed by phase contrast microscopy in smears taken from the edges of macroplasmodia and fixed in ethanol (Mohberg & Rusch, 1971). The time between the second and third mitoses after fusion was in the range of 8–10 h. All cell cycle times are given relative to metaphase.

For labeling with [^{3}H]acetate at the desired time of the cell cycle ranging from M2 −1 h (1 h before the second metaphase after fusion) to M3 +1 h (1 h after the third metaphase after fusion), we removed the filter paper with the macroplasmodium from the growth medium, allowed it to drain for a few seconds, and then placed the filter paper on 1 mL of semidefined growth medium on a clean petri dish. The medium contained 6.25 mCi of sodium [^{3}H]acetate (New England Nuclear, 2.3 Ci/mmol) per mL. Culture was continued for the desired length of time, as given in the text and figure legends, and the plasmodium was harvested by dropping the

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filter paper and plasmodium into liquid nitrogen. Plasmodia were stored at −76 °C prior to nuclear isolation.

Isolation of Nuclei. Single macroplasmodia were thawed in 50 mL of homogenizing medium [0.25 M sucrose, 0.01 M $CaCl_2$, 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.1% (w/v) Triton X-100, and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.1] (Mohberg & Rusch, 1971) and homogenized in a Potter homogenizer in ice. Nuclei were collected by centrifugation (10 min, 1500*g*), and the pellet was washed twice in homogenizing medium.

Histone Preparation. Histones were prepared essentially as described by Mende et al. (1983). In short, approximately 5×10^7 nuclei from single macroplasmodia were resuspended in 1 mL of 40% GP buffer (40% guanidine hydrochloride, 50 mM KH_2PO_4, and 50 mM K_2HPO_4 adjusted to pH 6.8 with KOH) and sonicated, and the nuclear debris was removed by centrifugation (10 min, 30000*g*). The supernatant was acidified to 0.25 N HCl, allowed to stand on ice for 30 min, and centrifuged (30 min, 40000*g*). Bio-Rex 70 cation resin (0.7–1.0 mL) equilibrated with 5% GP buffer was added, and the suspension was diluted with 100 mM potassium phosphate buffer (pH 6.8) to make its refractive index the same as 5% GP buffer, and the pH was adjusted to 6.8 with KOH. The suspension was gently agitated overnight at room temperature, and the resin was allowed to settle. It was washed with 5% GP buffer until the wash supernatant was clear and packed into a column (0.8 × 2 cm). The column was washed with 15 mL of 5% GP buffer at 1 mL/min and then eluted with 8 mL of 40% GP buffer at 0.2 mL/min. The 40% GP eluate was dialyzed against 100 volumes of 5% (v/v) acetic acid at 4 °C for 2 times 2 h and subsequently overnight. The histones were finally recovered by lyophilization.

When histones were prepared from total macroplasmodia without nuclear isolation, the plasmodium was thawed and homogenized directly in 2 mL of 40% GP buffer. Subsequent sonication, acidification, centrifugation, and chromatography were as described above.

Gel Electrophoresis and Fluorography. The histones were routinely analyzed by electrophoresis in polyacrylamide slab gels (15 cm × 30 cm, 0.5 or 1.5 mm thick) by using the acid–urea–Triton (AUT) system described by Bonner et al. (1980) with 8 M urea and 8 mM Triton X-100. When the methylene blue dye marker reached the bottom of the gel, the gels were fixed by shaking for 2 h in 5% (v/v) acetic acid and 40% (v/v) ethanol and dehydrated by 10-min washes in 25% acetic acid, 50% acetic acid, and 100% acetic acid. They were impregnated with PPO (2,5-diphenyloxazole) for 2 h in 20% (w/v) PPO in glacial acetic acid, and the PPO was precipitated during a 2-h wash in distilled water. The gel was completely heat dried under vacuum onto Whatman 3MM paper and exposed to preflashed Kodak XR-5 or XAR-5 film at −70 °C (Laskey & Mills, 1975). Exposure times were adjusted so that all peaks to be quantified remained within the linear range of the film sensitivity (below 1.5 absorbance at 550 nm).

After fluorography, gels were reswollen in three to five washes of glacial acetic acid until all PPO had been removed from the gel. Then they were stained with 0.1% Coomassie Brilliant Blue in 5% acetic acid and 40% ethanol overnight and destained in 7% acetic acid and 20% methanol.

Alternatively, gels were stained with Coomassie immediately following electrophoresis, and destained gels were prepared for fluorography. This staining prior to fluorography was never done when low levels of radioactivity were to be quantified from the fluorographs, to avoid quenching.

Fluorographs were scanned at 585 nm (XR-5 film) or 550 nm (XAR-5 film) and Polaroid negatives of stained gels at 500 nm with a Cary 210 spectrophotometer equipped with a gel scanner and a digital interface port. Data were collected directly into a Hewlett-Packard 9845S computer through a 16-bit parallel interface. A digital filter was used to reduce the graininess in the fluorography scans, and areas were determined by numerical integration by using an interactive program written for the purpose, using either trapezoidal or cubic spline methods. The filter and integration programs, in BASIC, are available on request.

Sephadex G75-Superfine Chromatography. Histones were prepared from seven macroplasmodia, labeled for 12 min with [^{3}H]acetate at M2 +20–30 min, and from seven macroplasmodia, labeled for 11 min with [^{3}H]acetate at M2 +5 h. Each lyophilizate was taken into 0.5 mL (v/v) of acetic acid and centrifuged for 2 min at 10000*g*, and the clarified supernatant was loaded on a column (1.2 × 115 cm) of Sephadex G75-Superfine, equilibrated with 5% acetic acid. The column was eluted with 5% acetic acid at a flow rate of 2.3 mL/h. The absorbance at 275 nm and the radioactivity were determined in the 0.9-mL fractions. Aliquots of each fraction were taken, lyophilized, and run on short (15 × 15 cm) AUT gels. The elution position of each histone was determined from the Coomassie pattern of this gel, and from a subsequent fluorograph, and the results obtained were identical.

Label Incorporation from [^{3}H]Acetate into Amino Acids. Macroplasmodia were labeled in S phase (M2 +25 min) and G2 phase (M2 +5 h) for up to 20 min. Histones were prepared, and the specific activity was determined by measurements of radioactivity and protein (Bradford, 1976), with calf thymus histones as standards. The histones were then hydrolyzed for 20 h at 110 °C under vacuum in 6 N HCl and 0.02% 2-mercaptoethanol. This hydrolysis yields free amino acids and free acetate, arising from acid-labile acetyllysine residues. The amount of label in the amino acids was then estimated by removing the volatile acetic acid or by measuring the label in the amino acids directly in their PTH (phenylthiohydantoin) derivatives.

Aliquots containing 0.2 mg of protein were dried in a heated Speed Vac concentrator (Savant) under oil pump vacuum. Blanks were treated in parallel. These contained 40 μg each of glutamic acid, aspartic acid, alanine, glycine, and lysine with 100 000 dpm of either [^{3}H]arginine or [^{3}H]acetic acid. All samples were 3 times resolubilized in 5% acetic acid and redried. The level of radioactivity in the acetic acid blank was then constant, at 10%. The amount of [^{3}H]arginine remained at 100 ± 2% of the initial value. The radioactivity remaining in the samples, supposedly in amino acids, was corrected for 10% nonspecific absorption, as indicated by the [^{3}H]acetic acid blank.

Aliquots of the amino acid hydrolysate were dried and reacted with excess PITC (phenyl isothiocyanate) for 60 min at 45 °C in 50% (v/v) pyridine. The reaction mixture was dried, and thiazolinones were formed in 100% TFA (trifluoroacetic acid) for 30 min at 45 °C. They were dried, extracted with butyl acetate, and converted to PTH (phenylthiohydantoin) derivatives in 20% TFA for 5 min at 80 °C. The PTH-amino acids were extracted with ethyl acetate, and radioactivity was determined (Mende, 1979; Allen, 1981).

Both methods to determine labeling in amino acids show essentially the same result (see Table I), relatively low incorporation at short labeling times, increasing by 16% from 2- to 20-min labeling. However, the absolute values for the estimates of label in amino acids differ clearly. We consider

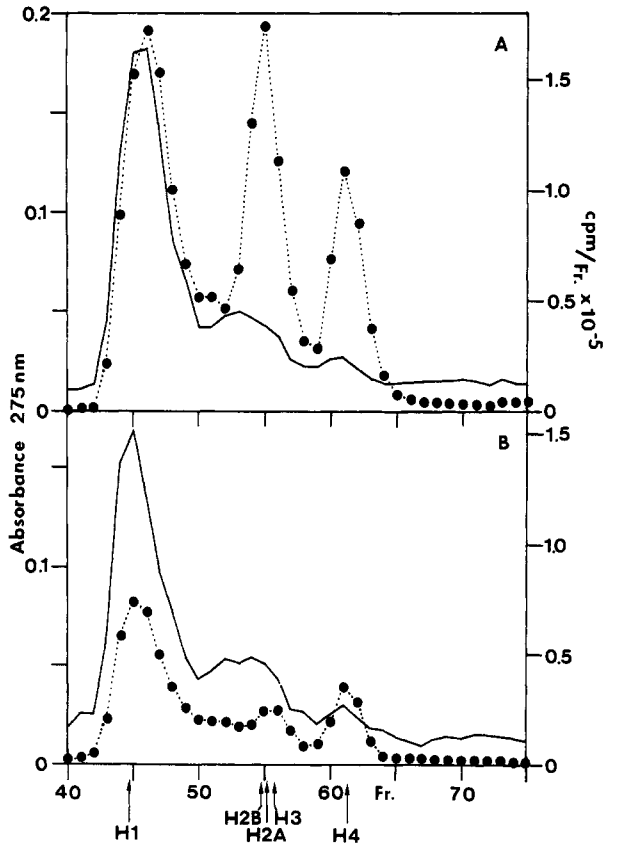

FIGURE 1: Sephadex G75 chromatography of acetate-labeled *Physarum* histones. Macroplasmodia were labeled for approximately 10 min with sodium [^{3}H]acetate during S phase (M2 +25 min) (A) and G2 phase (M2 +5 h) (B), and the histones were chromatographed on Sephadex G75-Superfine in 5% acetic acid as described under Materials and Methods. (—) Absorbance at 275 nm; (●---●) cpm per fraction. The position of histones H1, H2A, H2B, H3, and H4 is indicated as determined after gel electrophoresis. Only a very small percentage of the label in the void volume fractions of S phase labeled plasmodia was present in H1; no label was found in H1 in the G2-phase fractions.

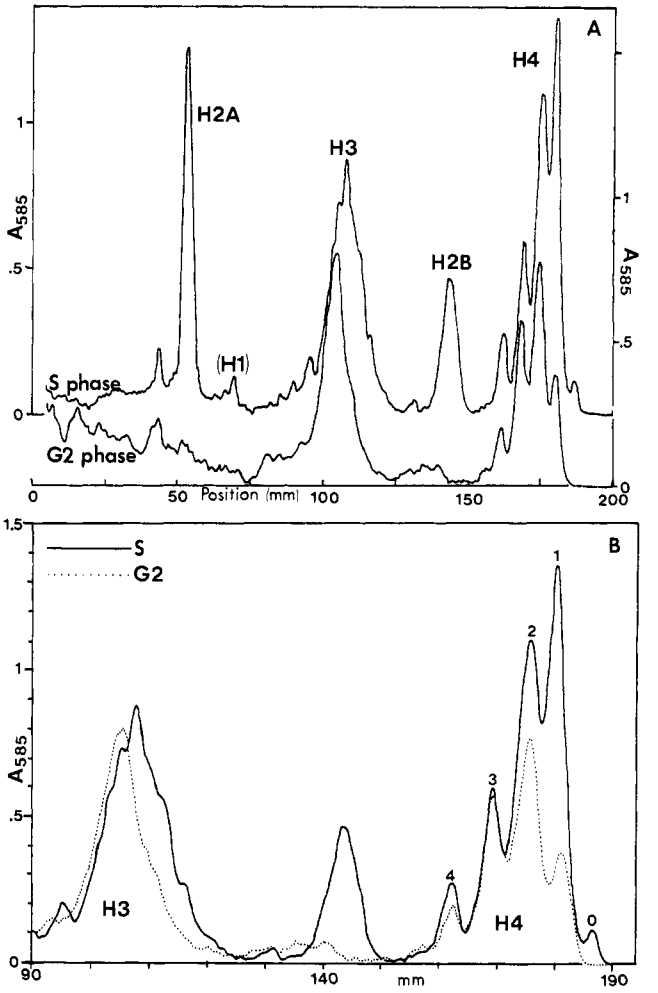

FIGURE 2: Histone acetylation in S phase and G2 phase. Macroplasmodia were labeled for 3 min in S phase (M3 +45 min) and G2 phase (M2 +7.5 h). Histones from 0.4 S phase or 0.8 G2 phase macroplasmodia were electrophoresed in parallel lanes in a 1.5-mm AUT gel. Fluorography for 9 days was followed by Coomassie staining to correct the fluorography scans for slight differences in loading. Migration was from left to right. (A) Scans of equal amounts of histones, acetylated in S and G2 phases. The small peak of cotranslationally acetylated H1 is also indicated. (B) Parts of the scans of S phase (—) and G2 phase (···) were replotted to allow a more direct comparison of quantitative and qualitative differences between the H3 and H4 acetylation patterns. The relative incorporation into nonacetylated through tetraacetylated forms of H4 (0–4) is given in Table II.

the values obtained from the volatility–drying experiment as the upper limit, because [^{3}H]acetic acid remains partially adsorbed, and label into other, nonvolatile forms would by this method also be considered to be in amino acids. We consider the values obtained from the PTH modification experiment as the lower limit since only amino acids could react with PITC and partial amino acid hydrolysis of hydrophobic protein regions into dipeptides may give underestimation, as may partial breakdown of the amino acids themselves during hydrolysis. In addition, the various drying and extraction steps of the PTH conversion may give slight losses.

A direct measurement of label incorporation into histone H4 was made in the following way. Macroplasmodia were labeled for 5 min at M3 +20 min with [^{3}H]acetate, and histone H4 was purified to homogeneity from a total histone preparation on Sephadex G75-Superfine. It was then hydrolyzed at 1 mg/mL in 0.25 N acetic acid for 6 at 110 °C as described by Lewis et al. (1975). The peptides were separated on Sephadex G50-Superfine in 5% acetic acid. The specific activity of the purified peptides was determined by their absorbance at 275 nm and liquid scintillation counting.

Results

Synchronous *Physarum* plasmodia were pulse labeled with [^{3}H]acetate for 10 min either in S phase or in G2 phase of the mitotic cycle. Histones were isolated by extraction of nuclei with 40% guanidine hydrochloride and batch elution from Bio-Rex 70 (Mende et al., 1983) and analyzed by chromatography on Sephadex G75. Figure 1 shows that the optical density profiles of the two preparations were identical which means that there were no differences in the amounts of histones present. The radioactivity profiles showed significant differences, reflecting phase-specific rates of uptake of label by histones. Overall, uptake of label was greater in S phase than in G2 phase. In both phases, uptake of label was low in histone H1 and high in histone H4. Histones H2A, H2B, and H3 eluted together in a single peak, and uptake into this peak was increased in S phase.

These differences were confirmed, and all the histones were separated by running histones on an acid–urea–Triton gel (Mende et al., 1983; Bonner et al., 1980; Borun et al., 1977). The positions of *Physarum* histones on these gels have been established by cutting out bands and determining their amino acid composition and molecular weight (Mende et al., 1983). Figure 2 shows a scan of a fluorograph of histones pulse labeled for 3 min in S phase or G2 phase. The differences between

Table I: Incorporation of [^{3}H]Acetate into *Physarum* Nuclear Basic Proteins[a]

	labeling time (min)	sp act. (^{3}H dpm/μg of protein)	% of label in acid-stable amino acids (A)[b]	(B)[b]
S phase	2.0	126	32.4	7.8
	4.0	644	34.1	12.3
	6.83	2299	42.6	18.6
	10.25	4723	46.6	19.7
	21.0	11372	48.1	23.7
G2 phase	3.0	330	31.2	11.7
	6.0	1002	39.2	18.1
	10.0	2548	44.2	19.9
	20.0	5797	46.2	22.7

[a] Macroplasmodia were labeled in S phase and G2 phase with sodium [^{3}H]acetate, and the label incorporated into total histones was determined. [b] The percentage of label incorporated into amino acids was estimated by the label remaining after removal of volatile acetic acid, derived from hydrolyzed acetyllysine residues (A), and by the label found in PTH-amino acids (B), as described under Materials and Methods.

the two phases are clear, and the same patterns were observed in 11 experiments. The loading of histones on the gel lanes was the same for each phase, and this was checked sometimes by staining a parallel lane of gel, sometimes by staining before fluorography, and sometimes by staining after fluorography. In all cases, there were no differences in the patterns of stain between S phase and G2 phase.

Figure 2 shows that, for this short labeling time (3 min), the major labeled components seen in these preparations are histones. The absence of general labeling of non-histone bands present on the gel suggests that the label was incorporated directly as acetate, and not metabolized into amino acids. The absence of label incorporated pretranslation in stable positions in amino acids was verified directly by carrying out an acid hydrolysis of unfractionated nuclear basic proteins and determining the amount of label released and the amount remaining in amino acids, as described under Materials and Methods. Table I shows that 70–90% of the label incorporated in a 3-min pulse is acid labile although the proportion falls rapidly at longer labeling times. In a separate experiment, using histone H4 labeled for 5 min in S phase, peptides were obtained by aspartic acid cleavage as described under Materials and Methods. More than 80% of the label was found in the peptide analogous to 1–23 of calf thymus where the acetylated lysines are located. For these reasons, the subsequent experiments used mainly the 3-min pulse labeling time.

Histone H1 is not labeled in G2 phase and is only lightly labeled in S phase (Figure 2). In other systems, histone H1 does not undergo reversible acetylation, although there is a cotranslational acetylation of the amino-terminal serine which is not reversible. *Physarum* histone H1 is resistant to Edman degradation (Mende et al., 1983), suggesting that is also has *N*-acetylserine at its amino terminus. Thus, the label in histone H1 in S phase probably arises from irreversible cotranslational incorporation of acetate into *N*-acetylserine at the amino terminus of H1. The absence of this labeling in G2 phase implies either that histone H1 is not synthesized at a rapid rate in G2 phase or that there is little turnover of H1 in G2 phase, or both.

Histones H2A and H2B show substantial uptake of label in S phase but no uptake of label in G2 phase (Figure 2). This S phase specific uptake is not due to labeling of *N*-acetylserine since both histone H2A and H2B from *Physarum* are susceptible to Edman degradation (Mende et al., 1983). The label was probably not incorporated as labeled amino acids during

Table II: Acetylation of Histone H4 in S Phase and G2 Phase[a]

H4 component	relative amount (%) S phase	G2 phase
0	2.1	0.0
1	36.6	16.2
2	36.5	45.1
3	17.2	30.2
4	7.7	8.5

[a] The relative incorporation of acetate into nonacetylated through tetraacetylated forms of H4 (0–4) was determined by integration of the fluorograph densities shown in Figure 2.

synthesis because of (i) the relatively large amount of incorporation compared with histone H1, which we expect to be synthesized at a similar rate, (ii) the short pulse time, and (iii) the fact that histones H2A and H2B take up acetate in plasmodia that have been preincubated with 100 μg/mL cycloheximide for 15 min and then pulse labeled with [^{3}H]acetate in the presence of 50 μg/mL cycloheximide which inhibits protein synthesis in *Physarum*[1] (Muldoon et al., 1971). Hence, we interpret the uptake of [^{3}H]acetate into histones H2A and H2B (Figure 2) as being due to posttranslational modification of the histones. The presence of the acetyl group(s) does not have a large effect on the mobility of histone H2B, and there is no visible effect on the mobility of histone H2A. This is probably due to a combination of a low number of acetates per molecule for H2A and H2B and a relatively small effect of the modification on electrophoretic mobility in acid–urea–Triton gels. However, we cannot rule out the possibility, particularly for histone H2A, that essentially all the histone is in the same modification state. We conclude that histones H2A and H2B are turning over acetate on acetyllysine specifically in S phase and not in G2 phase.

Histones H3 and H4 take up acetate both in S phase and in G2 phase (Figure 2), in striking contrast to the other histones. Histone H4 shows five labeled bands in S phase which are interpreted as corresponding to zero to four acetyllysines per molecule, following Chahal et al. (1980). Labeling of "Ac_0H4" is probably due to labeling of *N*-acetylserine since *Physarum* histone H4, like H1, has a blocked amino terminus (Mende et al., 1983). The acetylated components of histone H4 are well resolved, and the area of each of the components was determined by scanning and numerical integration. Table II shows that the proportion of label in each component of histone H4 was different in S phase compared with G2 phase. In particular, label in Ac_1H4 and Ac_2H4 was enhanced in S phase compared with G2 phase. The band of histone H3 is more complex than that of histone H4. The complexity is due to the presence of two subfractions of histone H3 with slightly different mobilities on the acid–urea–Triton gel (Mende et al., 1983). Both subfractions appear to be acetylated, and the various fluorographs we have obtained are all consistent with the presence at least four acetyllysines on each of the two subfractions, giving eight acetylated components of histone H3. We have not attempted to resolve these components quantitatively, but Figure 2 shows qualitatively that in histone H3, as in histone H4, the uptake of label into the Ac_1H3 and Ac_2H3 components is enhanced in S phase compared with G2 phase.

We were concerned to show that these specific acetylation patterns represented the in vivo acetylation patterns rather than changes occurring after cell harvest or changes due to the labeling procedures. The histone isolation procedure is carried

[1] R. D. Mueller, personal communication.

out in 40% guanidine hydrochloride and other denaturing conditions so we did not expect degradation (such as deacetylation) during the preparation. However, butyrate inhibits *Physarum* deacetylase (Waterborg & Matthews, 1982) so butyrate was included in the isolation buffers in some experiments. No differences were observed whether butyrate was included or not. Changes during isolation of the nuclei remained a possibility, but this was ruled out by isolating histones directly from whole plasmodia by the 40% guanidine hydrochloride method. The results obtained with whole plasmodia were the same as those obtained with isolated nuclei, except for a modest excess of Ac_0H4 and Ac_1H4 in the preparation from whole plasmodia which may represent the presence of a pool of histone H4 not bound to DNA.

The labeling protocol involved transferring a plasmodium from its normal growth medium to growth medium plus 3 mM sodium acetate and allowing growth to continue for 3 min, before harvesting into liquid nitrogen. When microplasmodia were exposed to growth medium plus 3 mM acetate continuously, the growth rate dropped. This perturbation of the growth may be due to an effect on the pH gradient between *Physarum* cytoplasm and growth medium (Morisawa & Steinhardt, 1982), and this possibility is being investigated. Incorporation of [^{3}H]acetate was measured in plasmodia prepared from cultures that had adapted fully to growth in growth medium plus 3 mM acetate by successive passage through 1, 2, and 3 mM acetate. The adapted cultures had the same growth rate in growth medium plus 3 mM acetate as normal cultures had in growth medium alone. Similarly, there were no differences in the cell cycle time in macroplasmodia prepared from these microplasmodia and grown in growth medium with or without 3 mM acetate, respectively. Both normal and adapted plasmodia were labeled with 3 mM [^{3}H]acetate in S phase and then harvested and analyzed as above. The resulting fluorographs of histones were scanned. Comparison of scans from normal and adapted plasmodia at short pulse labeling times shows no significant differences, implying that the observed pattern of uptake of acetate in normal plasmodia at short labeling times is not a consequence of any effect on the culture growth rate but represents the pattern of uptake that occurs in normal growth. We used no drugs in the culture medium, either for synchronization or suppression of protein synthesis or for inhibition of bacterial growth. We conclude that the patterns of uptake of [^{3}H]-acetate shown in Figure 2 represent the patterns in unperturbed growth.

Time course data were obtained by incubating plasmodia in medium containing [^{3}H]acetate for periods of 1 min up to 30 min in either S phase or G2 phase, and histones were isolated and analyzed as before. During the longer labeling times, label will be incorporated into proteins via the metabolism of [^{3}H]acetate and the subsequent incorporation of label into stable positions in amino acid precursors of proteins. This incorporation can be inhibited by high concentrations of cycloheximide,[1] but cycloheximide also affects DNA synthesis in *Physarum* (Muldoon et al., 1971) so it is difficult to interpret data obtained from cycloheximide-treated cultures. In both S and G2 phases, long pulse labeling times (>5 min) give substantial general labeling of most bands on the gel. This represents both direct acetylation and incorporation of acetate via metabolism of acetate to amino acids, as already demonstrated in Table I. At short labeling times (<4 min), the label is restricted to acetylated histones, indicating that this label was incorporated directly as acetate. The amount of label was quantitated by integrating the areas of the bands, and examples

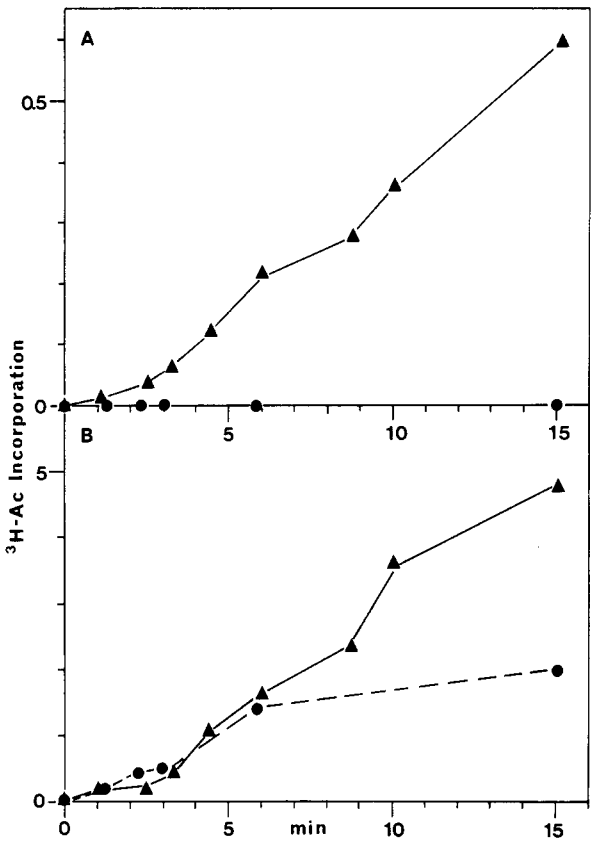

FIGURE 3: Time course of histone acetylation. The time course of label incorporation into individual histone species was determined by scanning of appropriately exposed fluorographs. The gel was reswollen and stained with Coomassie, and determined scanning areas were corrected for differences in loading. (A) Incorporation in nonacetylated H4 in S phase (▲) and G2 phase (●). (B) Incorporation in H3 in S phase (▲) and G2 phase (●). The units of incorporation are arbitrarily chosen but are the same for all data given in this figure. The "kinks" in the incorporation curves at 9–10 min are probably due to experimental error and do not represent a reproducible effect.

of the results are shown in Figure 3. Figure 3A illustrates the absence of label in Ac_0H4 in G2 phase and the presence of label in S phase. The label incorporated in S phase represents cotranslational acetylation of the amino-terminal serine (all labeling times) plus pretranslational incorporation of label into amino acids (longer labeling times only). Figure 3B shows the incorporation of [^{3}H]acetate into total histone H3. Similar amounts of incorporation of acetate were observed at short labeling times in both S and G2 phases, representing overall posttranslational acetylation. The data are not detailed enough (particularly with respect to incorporation of ^{3}H into acid-stable positions, which is difficult to measure) to allow a clear distinction between a rapid and a slow acetate turnover rate in S phase (Jackson et al., 1975). There is no evidence for a substantial slow turnover of acetate in G2 phase: the turnover on H3 and H4 is all rapid.

Having established the existence of two patterns of post-translational histone acetylation, we defined the timing of these events in the cell cycle more closely. Figure 4 shows the results of pulse labeling plasmodia for 3 min with [^{3}H]acetate at 14 defined stages of the mitotic cycle. The S-phase pattern is first seen at 9 min after metaphase, that is, in telophase. This correlates with the onset of DNA synthesis (Funderud & Haugli, 1977; Beach et al., 1980). By 15 min after metaphase, DNA synthesis has reached 80% of its maximum rate, and the S-phase pattern of histone acetylation is at its most intense. DNA synthesis continues at a high rate until 2 h after metaphase (Funderud & Haugli, 1977). The S-phase pattern of

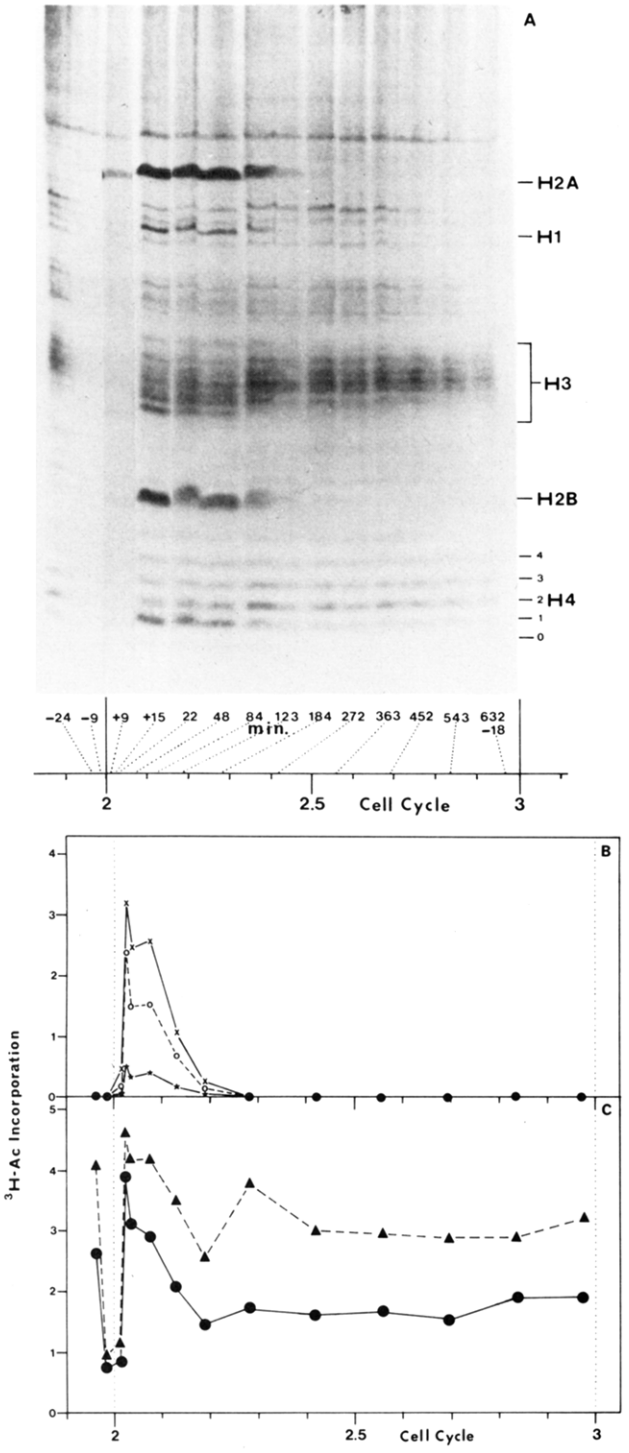

FIGURE 4: Histone acetylation in the cell cycle. Macroplasmodia were labeled for 3 min at the indicated positions of the 10.8-h cell cycle between M2 and M3. (A) Histones from 0.2 macroplasmodium were electrophoresed in parallel lanes in a 0.5-mm-thick AUT gel. Migration was from top to bottom. Fluorography was for 31 days, followed by Coomassie staining to correct for slight differences in loading. The position of H2A, H1, H3, H2B, and H4, nonacetylated through tetraacetylated (0–4), is indicated. At M2 −24 min, prophase condensation is beginning; at −9 min, prophase condensation is clear; at +9 min, telophase; at +15 min, nucleolar reconstruction and decondensed nonnucleolar chromatin. (B) Incorporation into H2A (×), H2B (○), and H1 (solid stars). The units of incorporation are arbitrarily chosen but are the same for all data given in this figure. (C) Incorporation into H3 (▲) and H4 (●).

histone acetylation remains fairly intense up to 1.5 h after metaphase but has practically disappeared by 2 h after metaphase. The G2-phase pattern, with intense labeling of highly acetylated histones H3 and H4, reduced labeling of the rest of H3 and H4, and no labeling of H2A and H2B was observed throughout G2 phase, up to prophase. In prophase, a very faint G2-phase pattern was observed. The G2-phase pattern may be present in S phase, superimposed on the S-phase pattern, or it may be restricted to G2 phase. In the stages of mitosis where chromatin was highly condensed, a third pattern was observed in which very low incorporation of [^{3}H]acetate into histone occurred. These data were quantitated by integration of areas under scans of the fluorograph. Figure 4B,C shows examples of the incorporation of acetate into specific histone bands through the mitotic cycle.

Discussion

The nuclear division cycle in *Physarum* plasmodia appears to be a steady-state process, under the conditions used in these experiments, and there is no evidence of any ongoing differentiation or development process. Moreover, this is a stage of growth that very closely approximates the growth of the plasmodium in the wild and can thus be maintained without the use of drugs or other means of perturbing the culture. A major characteristic of this steady-state growth, both in the wild and in the laboratory, is the naturally synchronous nuclear division cycle. Metaphase occurs within a period of a few minutes in essentially all the nuclei throughout the plasmodium, and all the nuclei enter S phase as early as telophase (Beach et al., 1980). During S phase, the nuclei are engaged in both chromosome replication and RNA synthesis (transcription). S phase lasts approximately 2 h and is followed by G2 phase. DNA synthesis is largely confined to S phase but continues at a very low level during G2 phase due to replication of the ribosomal genes, which is not restricted to S phase, ligation of DNA fragments produced during S phase, and DNA repair (Evans, 1982). RNA synthesis is high in S phase and mid G2 phase, drops slightly in early G2 phase, and is absent in mitosis. It appears that both peaks of RNA synthesis involve both polymerases I and II although polymerase II is more important during S phase while polymerase I is more important during G2 phase (Braun & Seebeck, 1982).

We have identified three distinct patterns of histone acetylation correlated with the three well-defined stages of the nuclear division cycle in *Physarum*. The main characteristics of these patterns are as follows: (1) S phase, rapid acetylation of each of the four core histones, acetylation of H3 and H4 concentrated in the less-modified forms; (2) G2 phase, rapid acetylation of histones H3 and H4 (concentrated in the more highly modified forms), no acetate turnover on histones H2A and H2B; (3) mitosis (late prophase, metaphase, and anaphase), no acetate turnover on histones. Some differences between acetylation or deacetylation of H3 and H4 compared with H2A and H2B were observed in previous studies (Shepherd et al., 1971; Vidali et al., 1978; Moore et al., 1979; Covault & Chalkley, 1980; Schroter et al., 1981; Marian & Wintersberger, 1982).

G2-Phase Pattern. The main implication of these results arises from the characteristics of the G2-phase pattern. The major chromosomal activity during G2 phase is RNA synthesis, and this clearly does not require acetate turnover on histones H2A and H2B. This has only been shown for *Physarum* but may well hold true for other cells since previous studies have not achieved sufficient separation of the processes of S phase and G2 phase in normally growing cells to make the observations reported here. This result provides clear in vivo evidence of differing functional roles for the histones H2A and H2B compared with histones H3 and H4.

How is specific acetylation of histones H3 and H4 achieved? The histone acetyltransferases and deacetylases that have been solubilized from mammalian nuclei have not been shown to have specificity for specific histone substrates, in vitro. We previously concluded, on the basis of cell cycle measurements of histone deacetylase activity in *Physarum*, that histone acetylation is probably controlled by substrate availability and not by gross changes in the acetyltransferases and deacetylases (Waterborg & Matthews, 1982). This conclusion is consistent with other data from mammalian cells. It remains possible that the enzymes responsible for histone acetate turnover in G2 phase have not been solubilized and that in vivo they do show the required specificity for histones H3 and H4 as substrate. The alternative hypothesis, consistent with the properties of the solubilized enzymes, is that the N-terminal regions of histones H2A and H2B are masked in transcriptionally active chromatin while the N-terminal regions of histones H3 and H4 are available for acetylation. In fact, even in bulk nucleosomes, several authors have found a distinct preference for acetylation of histones H3 and H4 vis-à-vis acetylation of H2A and H2B, using isolated acetyltransferases to acetylate isolated nucleosomes or oligonucleosomes (Horiuchi et al., 1978; Cano & Pestana, 1979; Estepa & Pestana, 1981; Bohm, J., et al., 1980; Otto et al., 1980; Dod et al., 1982). The data suggest that, in nucleosomes, the N-terminal domains of histones H2A and H2B are inaccessible to acetyltransferase while histones H3 and H4 are accessible. Shewmaker et al. (1978) (Cohen et al., 1980) made similar observations with the chemical acetylating agent acetyl adenylate, which specifically acetylated histones H3 and H4 but not histones H2A and H2B in nucleosomes, although free histones were acetylated nonspecifically (Ramponi et al., 1975). This is in contrast to NMR data that have been interpreted as showing the N-terminal regions of H2A and H2B being more mobile than H3 and H4 (Cary et al., 1978) and to trypsin digestion studies showing that H2A and H2B N-terminal domains are available for digestion (Whitlock & Simpson, 1977; Bohm, L., et al., 1980, 1982).

Cousens et al. (1979) have concluded that chromatin contains regions of differing accessibility to acetyltransferases. If this is the case, we suggest that in G2 phase of the cell cycle there are two classes of chromatin that differ in their accessibility to acetyltransferase. The first class, the bulk of the chromatin, is folded into a higher order structure (Chahal et al., 1980; Thoma et al., 1979) in which all the core histones are inaccessible to acetyltransferase. The second class, transcriptionally active chromatin, is formed either from newly replicated chromatin in S phase (Fouquet et al., 1975; Weintraub, 1979) or directly from inactive chromatin. A signal, whose molecular nature remains unknown, recognizes the correct region of chromatin and modifies its structure to make the nucleosomes in this region accessible to acetyltransferase. Histones H3 and H4 become acetylated, and the structure is stabilized, or further modified, and becomes more sensitive to DNase I and available for transcription. This change can be rationalized by postulating that the unacetylated N-terminal domains of histones H3 and H4 stabilize the higher order structure of inactive chromatin (Allan et al., 1982). The inaccessible N-terminal domains of histones H2A and H2B could be involved in interactions that stabilize the structure of active chromatin when the interactions of H3 and H4 are released by acetylation.

In support of this hypothesis, we note that active nucleosomes from *Physarum* do have a different structure from bulk, inactive, nucleosomes and that micrococcal nuclease produces "peak A particles" from active chromatin in *Physarum*. Peak A particles have a normal nucleosome complement of histones H2A and H2B but are partially depleted in histones H3 and H4 which may have been selectively released during the isolation (Johnson et al., 1978). Berkowitz & Doty (1975) also observed a partial loss of histones H3 and H4 from active chromatin prepared from chicken reticulocytes.

Turnover of acetate on Ac_1H4 and Ac_2H4 occurs in G2 phase on many H4 molecules (Figure 4) and is not specifically associated with transcription (Chahal et al., 1980). This may represent turnover due to an acetyltransferase different from that which acetylates H4 on active chromatin or may represent some "leakiness" in the inaccessibility of H4 in inactive chromatin.

S-Phase Pattern. During S phase, all four core histones show rapid acetate turnover. In the case of histones H3 and H4, the S-phase patterns can be distinguished from the G2-phase patterns by increased acetylation of the less modified species in S phase. The data are consistent with the hypothesis that the G2-phase pattern persists in S phase and has an S phase specific acetylation superimposed upon it. This would imply that the transcription-associated (G2-phase pattern) acetylation remains basically unchanged throughout interphase. The remaining, S phase specific, acetylation of all four core histones is then correlated specifically with chromosome replication.

The precise role of histone acetylation in chromosome replication is not clear. Dixon suggested acetylation may be required for correct assembly of histones and DNA (Candido & Dixon, 1972). More recently, Seale (1981) has shown that nucleosomes containing newly synthesized histones are more salt labile than mature nucleosomes, consistent with the binding scheme proposed by Dixon and the presence of acetylation in all core histones. Our data are fully consistent with this proposal and with the data of Ruiz-Carrillo et al. (1975) on acetylation of newly synthesized histone H4 in erythrocytes. Such a role for histone acetylation is also consistent with the recent finding by Perry and Chalkley that acetylation occurs on histones associated with all DNA sequences, in non-synchronous proliferating cells (Perry & Chalkley, 1982). However, none of these experiments rules out an additional function for acetylation in preparing the chromatin for replication.

Several investigators in Chalkley's laboratory have reported a fast and a slow turnover of acetate on histones (Jackson et al., 1975; Moore et al., 1979; Covault & Chalkley, 1980). We were prevented from analyzing slow turnover rigorously in the present experiments by incorporation of label into non-acetylated amino acids at longer labeling times in S phase. Nevertheless, in G2 phase where histone synthesis did not interfere, we found only a rapid turnover (shown in Figure 3 for histone H3) in which labeling reached a steady state after 6 min.

Mitotic Pattern. For about 15 min in late prophase, metaphase and anaphase *Physarum* nuclei stop turning over acetate in histones. Previous studies have correlated low levels of highly acetylated H4 with mitosis in *Physarum*, HTC, and CHO cells (Chahal et al., 1980; Moore et al., 1979; D'Anna et al., 1977). The lack of acetylation could be due to gross inactivation of acetyltransferase and deacetylase, although the activity, in vitro, of the solubilized deacetylase from *Physarum* remains high in mitosis (Waterborg & Matthews, 1982). Alternatively, the histone substrates could be masked in condensed chromatin. Condensed chromatin is not totally inaccessible to enzymes because histone phosphatase acts on

metaphase chromosomes so it is likely that the N-terminal regions of the core histones are involved in specific interactions in metaphase chromosomes that make the histones inaccessible to acetyltransferase and deacetylase. The degree of accessibility in metaphase chromatin is less than that in inactive G2-phase chromatin, for histones H3 and H4, since even the turnover of acetate on Ac_1H4 stops.

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