Volume 12 Number 13 1984 Nucleic Acids Research

RNA polymerase activity and template activity of chromatin after butyrate induced hyperacetylation of histones in *Physarum*

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Received 9 May 1984; Accepted 4 June 1984

ABSTRACT

We have studied the effect of sodium-n-butyrate on endogenous RNA polymerase in Physarum polycephalum. 1 mM butyrate strongly reduces RNA polymerase activity measured in isolated nuclei or chromatin; both RNA polymerase A as well as the α-amanitin sensitive RNA polymerase B are equally affected. Despite a concomitant hyperacetylation of histone H4 the template activity of chromatin, as analyzed by in vitro transcription of the chromatin with exogenous RNA polymerase from E.coli or RNA polymerase II from wheat germ, remains unaltered as compared to untreated control chromatin, indicating that there is no positive correlation between histone acetylation and template activity of chromatin for transcription in this organism. The results further indicate, that butyrate acts primarily as a quick but reversible inhibitor of protein synthesis in Physarum; the fast decrease of endogenous RNA polymerase activity after butyrate treatment is due to inhibition of enzyme synthesis rather than inactivation of other factors necessary for transcription.

INTRODUCTION

Sodium-n-butyrate, an inhibitor of histone deacetylases, causes hyperacetylation of the core histones and has therefore gained special interest in chromatin research, since acetylation of histones is thought to serve as a possible mechanism for gene expression (for recent reviews see 1,2). In addition butyrate has a variety of effects on cellular function, which cannot be satisfactory explained by its action as a histone deacetylase inhibitor (3-7).

We have previously demonstrated that butyrate has phase-dependent effects on the duration of the cell cycle of Physarum polycephalum (8); in addition butyrate causes a transient depression of RNA synthesis; it also induces a reversible hyperacetylation of histone H4, which follows the same time course as the inhibition of RNA synthesis caused by butyrate (9); the time point of the maximum inhibition of plasmodial RNA synthesis coincides with the maximum acetate content of H4; this finding is somehow unexpected in terms of the model, that histone acetylation is positively correlated with the transcript-

ional activity of chromatin (for review see 1). The fact that we observed inhibition of RNA synthesis in the presence of hyperacetylated H4 urged us to investigate this phenomenon on the enzymatic level.

There are few reports in the literature comparing in vitro transcription of highly acetylated chromatin with control chromatin. An increased elongation of RNA chains was found with chemically acetylated chromatin (10). Moreover an increase in initiation sites was demonstrated for chromatin, which has been extensively acetylated with acetic anhydride, measured by exogenous E.coli RNA polymerase (11) or RNA polymerase B from rat liver (12). The data on transcription of chromatin, which has been hyperacetylated in vivo after application of butyrate, are contradictory. Whereas Lilley and Berendt (13) did not find any difference in RNA synthetic capacity between hyperacetylated and control chromatin in HeLa cells, Dobson and Ingram (14) reported a pronounced increase in RNA chain elongation in the same cell system.

To elucidate the question of a possible correlation between histone acetylation and transcriptional activity of chromatin in Physarum, we studied endogenous RNA polymerase activity and template activity of the chromatin, as measured with exogenous RNA polymerases. In Physarum butyrate has at least two effects on transcriptional activity and chromatin structure; the synthesis of endogenous RNA polymerase A and B is inhibited, leading to a secondary inhibition of RNA synthesis; butyrate causes hyperacetylation, but the hyperacetylation does not alter the template activity of the chromatin for transcription.

MATERIALS AND METHODS

Materials

These were purchased from the following sources: [5,6-^{3}H]uridine 5'-triphosphate, ammonium salt (40 Ci/mmol), [L- 4,5-^{3}H]leucine (57 Ci/mmol), [L- 4-^{3}H]phenylalanine (11 Ci/mmol), [L- 4,5(n)-^{3}H]isoleucine (26 Ci/mmol) from Amersham International plc, U.K.; ATP, GTP, CTP and UTP from Boehringer Mannheim GmbH, W.Germany; RNA polymerase (E.coli) and RNA polymerase II (wheat germ) from P-L Biochemicals, Milwaukee, Wis., USA; α-amanitin, cycloheximide and n-butyric acid from Sigma Chem.Comp.; St.Louis, Mo, USA.

Culture conditions

Physarum polycephalum strain M3b (a Wis1 derivative) was used. Microplasmodia were cultivated in submersed shake culture in semidefined medium (15) supplemented with 0.013 % hemoglobin instead of hematin. To obtain nuclei and chromatin with high endogenous RNA polymerase activity it was necessary to

minimize slime production by the mold. Exponentially growing cultures were therefore diluted 1:7 with fresh nutrient medium approximately 12 hours before they were used for an experiment.

Labelling with radioactive amino acids

Microplasmodia were labelled in separate cultures (4 ml of microplasmodial suspension) with 150 µl (150 µCi) of a mixture of equal amounts of tritiated leucine, isoleucine and phenylalanine to monitor protein synthesis after addition of butyrate (1 or 2 mM) or cycloheximide (0.035 or 0.07 mM). After an incubation period of 0.5 hours microplasmodia were harvested. The plasmodial pellet was washed with distilled water and homogenized by sonication in 4 ml 5% w/v trichloroacetic acid in 50% v/v acetone/water. The pellet was washed with 4 ml 5% w/v trichloroacetic acid and then with 3 ml ethanol. The pellet was solubilized in 1 ml 0.4 N NaOH. Aliquots were analyzed for radioactivity in a liquid scintillation spectrophotometer and protein analysis was done as described (16).

Isolation of nuclei and chromatin

Nuclei were isolated according to the procedure of Mohberg and Rusch (17) with slight modifications: The washed plasmodial pellet was homogenized in a Braun blendor for 15 sec. at half maximum speed (regulated by a thyristor) in homogenizing medium A (0.25 M sucrose, 0.01 M Tris-HCl, pH 7.5 at 25°C, 5 mM $MgCl_2$, 0.04% Triton-X-100). After slow spin centrifugation the homogenate was filtered through 5 layers of Kleenex-tissue. The pelleted nuclei were resuspended in homogenizing medium without Triton and were centrifuged through an underlay of medium B (1.0 M sucrose, 0.01 M Tris-HCl, pH 7.5 at 25°C, 5 mM $MgCl_2$). Nuclei were again washed in homogenizing medium A without Triton. Finally the clean nuclei were suspended in an apropriate volume of buffer 1 (50 mM Tris-HCl, pH 7.5 at 25°C, 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM dithiothreitol, 5 mM KF) for determination of endogenous RNA polymerase activity or for the measurement of exogenous RNA polymerase from E.coli. When nuclei were incubated with RNA polymerase II from wheat germ they were suspended in buffer 2 (50 mM Tris-HCl, pH 7.9 at 25°C, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM $MnCl_2$, 10% v/v glycerol, 5 mM KF, 100 µg/ml bovine serum albumin). Nuclei were counted in a hemocytometer. DNA content of nuclear aliquots was determined according to a published procedure (18) to check the reliability of hemocytometer counting. 1 x 10^7 nuclei are equivalent to 12 µg of DNA.

Chromatin was prepared from isolated nuclei as described (19). An alternative chromatin preparation, which gave a higher yield of endogenous RNA

polymerase activity, was also used (20). The isolated nuclei were washed in a solution containing 0.075 M NaCl and 0.025 M EDTA (pH 7.8 at 25°C). After centrifugation the pellet was resuspended in 0.0375 M NaCl / 0.0125 M EDTA (pH 7.8) and centrifuged again. Finally the chromatin preparation was resuspended in distilled water. For measurement of RNA synthesis the chromatin preparation was dialyzed for 1 hour against the apropriate assay buffer 1 or 2 depending on the experiment.

Assay for endogenous RNA polymerase activity

The incubation mixture contained 0.4 mM of each ATP, CTP and GTP and 0.01 mM UTP (10 µCi [^{3}H] UTP/ml) in a volume of 0.25 ml. Unless otherwise stated the activity was measured in the presence of 0.08 M KCl. The reaction was started by the addition of 0.5 or 1.0 x 10^7 nuclei or the equivalent amount of chromatin. The temperature was set at 25°C. The reaction was terminated by the addition of 1 ml 7.5% w/v trichloroacetic acid containing 1% w/v pyrophosphate at 4°C. After 1 hour the samples were collected onto Whatman GF/C filters under suction. Filters were washed four times each with 5 ml 7.5% w/v trichloroacetic acid - 1% w/v pyrophosphate. Finally the filters were rinsed with 2 ml ethanol and dried, before the radioactivity was counted in a liquid scintillation spectrophotometer. Blanks and 0-min.-values were in the range of 200-1000 cpm. For discrimination of RNA polymerase A and B α-amanitin was added to the reaction mixture (20 µg/ml). The polymerase B activity was taken as the difference between the overall activity and the activity measured in the presence of α-amanitin.

Analysis of template activity of nuclei and chromatin by exogenous RNA polymerases

Exogenous RNA polymerase from E.coli or wheat germ was preincubated with nuclei or chromatin for 5 min. at 4°C and for 5 min. at 25°C. The reaction was started by adding 0.4 mM of each ATP, CTP and GTP and 0.01 mM UTP (10 µCi [^{3}H] UTP/ml) in buffer 1, when E.coli RNA polymerase was used. For experiments with RNA polymerase II from wheat germ the concentration of ATP, GTP and CTP was 0.6 mM and 0.6 µM for UTP in buffer 2. The samples were processed as described above.

Analysis of H4 acetylation

Histones were extracted from isolated nuclei as described (21). Histones were electrophoresed in 48 cm long acid-urea-Triton-X-100 slab gels (22) and the gels were evaluated for the relative acetate content (N) of histone H4 as described earlier (9).

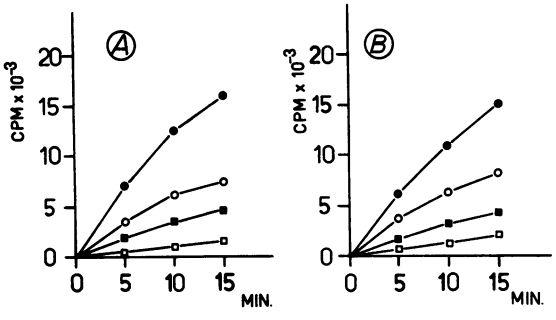

Fig.1. Endogenous RNA polymerase activity in (A) isolated nuclei and (B) chromatin. (A) 10^7 nuclei and (B) the equivalent amount of chromatin (12 µg DNA) prepared as described under Materials and Methods were incubated for the indicated times and incorporated radioactivity was determined. ● control; ○ control in the presence of α-amanitin (20µg/ml); ■ plasmodia treated with 1 mM butyrate for 5 h; □ 1 mM butyrate treated plasmodia in the presence of α-amanitin.

RESULTS

We have measured the endogenous RNA polymerase activity in isolated nuclei and chromatin of Physarum polycephalum. Fig.1A shows the kinetics of the RNA polymerase reaction in controls and butyrate treated plasmodia in the presence and absence of α-amanitin; the kinetics of UMP-incorporation is nearly linear for 15 min. at 25°C. After 15 min. the rate of incorporation declines and reaches a plateau (not shown in Fig.1). α-amanitin decreases the activity to approximately 50%, indicating that 50% of the activity is due to RNA polymerase B. Treatment of plasmodia with 1 mM butyrate for 5 hours reduces the activity to approximately 25%; both enzymes, polymerase A as well as B are equally affected. The same results were obtained when isolated chromatin was used (Fig.1B); it should be noted that the procedure of chromatin isolation did not influence the outcome of the experiment, although the absolute amount of incorporated radioactivity varied with the mode of chromatin isolation; the procedure of (20), which is brievely summarized in Materials and Methods, yielded a slightly higher percentage of polymerase B activity. The relative acetate content (N) of H4 was 1.65 in the plasmodia treated with butyrate for 5 hours as compared to 1.05 in the controls. It is noteworthy, that inclusion of 10 mM butyrate to the RNA polymerase assay increased the activity for approximately 10% when the enzyme was measured in the range of 5-70 mM KCl. The results were unaffected whether nuclei were isolated without or with inclusion of 100 mM butyrate in all buffers.

We investigated whether butyrate treatment of plasmodia changes the de-

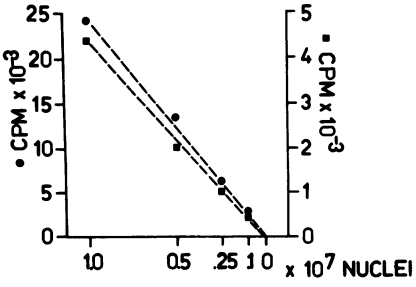

Fig.2. Effect of nuclear dilution on endogenous RNA polymerase activity. Decreasing numbers of nuclei were incubated for 10 min. and incorporated radioactivity was determined. ● control nuclei; ■ nuclei from plasmodia treated with 1 mM butyrate for 5 h.

pendence of the endogenous RNA polymerase activity from ionic strength. We obtained essentially the same salt dependence of the activity as already published (19); this dependence was identical for controls and butyrate treated plasmodia with a maximum of overall activity at 80 mM KCl (result not shown).

We also tested whether there is a linear relationship between the endogenous RNA polymerase activity and the nuclear concentration. Fig.2 shows, that this relationship was absolutely linear for control nuclei as well as for nuclei from butyrate treated plasmodia, leading to a constant value of incorporated radioactivity per nucleus or per equivalent amount of chromatin.

After we had measured this drastic depression of endogenous RNA polymerase activity after butyrate treatment we studied whether the properties of the chromatin as a template for exogenous RNA polymerases were affected by the hyperacetylation of histones after butyrate treatment. Fig.3 shows the results

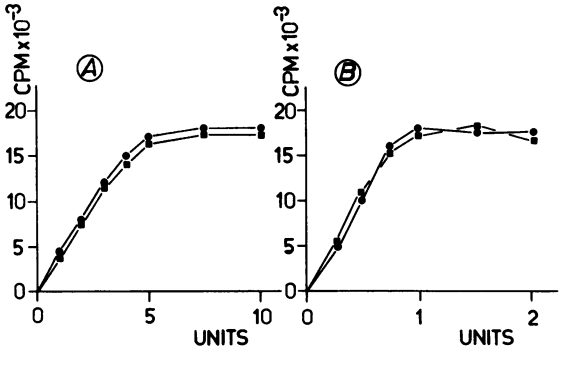

Fig.3. Template activity of chromatin for transcription of controls (●) and butyrate treated plasmodia (■). Chromatin (equivalent to 12 µg DNA) prepared as described under Materials and Methods was incubated with increasing amounts of exogenous RNA polymerase from E.coli (A) or RNA polymerase II from wheat germ (B). After 10 min. the reaction was terminated and the incorporated radioactivity was determined. The plotted exogenous activities were calculated from total activity with exogenous activity minus endogenous activity.

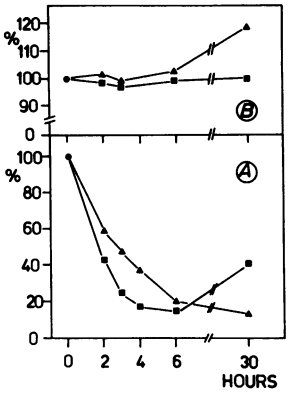

Fig.4. Effect of butyrate and cycloheximide on endogenous RNA polymerase activity (A) and template activity of chromatin for exogenous RNA polymerase from E.coli (B). 1 mM butyrate (■) or 0.035 mM cycloheximide (▲) was added to exponentially growing microplasmodia at 0 h. At the indicated time points plasmodia were harvested and the isolated chromatin (equivalent to 12 µg DNA) was incubated for 10 min. to determine the endogenous RNA polymerase (A) and the template activity of the chromatin (B) measured with 7.5 units of exogenous E.coli RNA polymerase. The control values at 0 h were taken as 100% and measured activities were expressed as % of control.

of an experiment, where a given amount of chromatin (equivalent to 12 µg DNA) was titrated with increasing amounts of exogenous RNA polymerases. When E.coli RNA polymerase was used (Fig.3A) the template activity of the hyperacetylated chromatin (relative acetate content N=1.65) was not different from control chromatin (N=1.05). Both chromatins reached saturation at 5 units of E.coli RNA polymerase; the absolute amount of incorporated UMP was also equal for both chromatins. Essentially the same result was obtained when eucaryotic RNA polymerase II from wheat germ was used (Fig.3B). The saturation point for both chromatins was between 0.8 and 1.0 units of exogenous polymerase II.

Since the template properties of the butyrate treated, hyperacetylated chromatin were not altered, the question remained, why butyrate reduced the endogenous RNA polymerase activity. To test whether the decrease of RNA polymerase activity could be due to inhibition of enzyme synthesis we compared the endogenous RNA polymerase activities in plasmodia after treatment with butyrate and cycloheximide. The results are shown in Fig.4A. Cycloheximide led to a decrease of RNA polymerase activity as a consequence of its action as a protein synthesis inhibitor. Even 30 hours after start of treatment RNA polymerase activity was low. The depression of RNA polymerase activity caused by 1 mM butyrate was faster as compared to cycloheximide, but the minimum activity was in the same range as after cycloheximide treatment. After 30 hours the activity was already slightly increased, but not up to the control level. In parallel we also measured the template activity of the chromatin for in vitro transcription with E.coli RNA polymerase (Fig.4B). Butyrate treatment did not change the template activity at any time point of the experiment. The

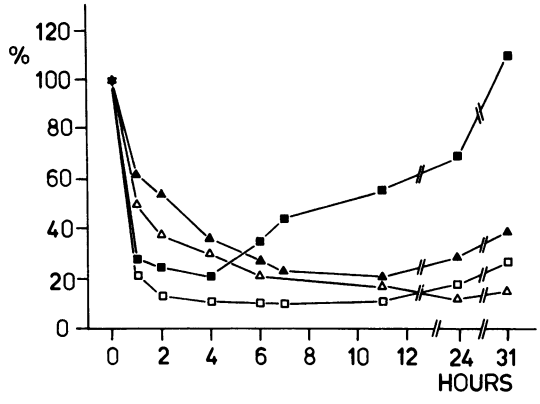

Fig.5. Effect of butyrate and cycloheximide on protein synthesis. Butyrate (■ 1 mM; □ 2 mM) or cycloheximide (▲ 0.035 mM; △ 0.070 mM) were added to exponentially growing microplasmodia at 0 h. At the indicated time points aliquots of the culture were incubated with tritiated amino acids for 0.5 h. Plasmodia were then harvested and processed as described in Materials and Methods. The control value of radioactivity incorporation per mg protein (0 h) was taken as 100% and the values at the different time points were expressed as % of control. The 100% control value measured at 0 h remained constant throughout the experiment as checked at 6,12,24 and 31h.

same was true for cycloheximide treated plasmodia at early timepoints, whereas 30 hours after start of treatment the template activity significantly increased (Fig.4B).

In addition we also measured protein synthesis through incorporation of tritiated amino acids after butyrate and cycloheximide treatment to see whether inhibition of overall protein synthesis caused by butyrate is also faster than the inhibition by cycloheximide. Fig.5 shows that indeed the decrease of incorporation of radioactive amino acids into protein was faster in butyrate treated plasmodia. This decrease was dose-dependent; the inhibition continued for up to 30 hours after start of treatment, when 2 mM butyrate were applied. 30 hours after application of 1 mM butyrate protein synthesis has already recovered, which fits the increase of endogenous RNA polymerase activity at this time point (Fig.4A). In contrast cycloheximide induced inhibition of protein synthesis was not reversible within this time period; this is also reflected in the very low activity of endogenous RNA polymerase 30 hours after start of treatment (Fig.4A). It should be noted that 2 mM butyrate led to the most pronounced inhibition (approximately 10% of control level). Since butyrate obviously leads to an immediate inhibition of protein synthesis in Physarum, it was possible to calculate a half life of RNA polymer-

ase from the data of Fig.4A. This half life was calculated to be close to 2 hours; this half life was the same for RNA polymerase A as well as B as calculated from experiments with and without inclusion of α-amanitin in the assay (data not shown in Fig.4A).

DISCUSSION

Since the first suggestion by Allfrey et al. in 1964 (23), that histone acetylation may play an important role for transcription, numerous studies have been performed to test the validity of this theory. Most of the positive experimental indications for the postulated correlation are indirect in the sense that increased histone acetylation was observed concomitant with enhanced transcriptional activity (24-29). It has also been shown, that susceptibility of chromatin to DNase I, which preferentially digests transcriptionally active chromatin (30), is associated with chromatin regions enriched with highly acetylated histones (31-33). An additional support for acetylation-transcription coupling derived from experiments, where butyrate treatment dramatically increased the digestion rate with DNase (33-35). However , these data represent only circumstantial evidence and there are several recent reports, which do not favor this concept; e.g. in rat liver nuclei transcribing chromatin was not enriched with acetylated histones (36); in Drosophila embryos no correlation between histone acetylation and transcription could be established (37); during spermatogenesis H4 (hyper)acetylation obviously serves other purposes than transcriptional activity as demonstrated in rooster (38) as well as in trout (39). Results with the SV-40 minichromosome (40) and with HTC-cells (41) also cast doubt on the theory of acetylation-transcription coupling.

We tested this hypothesis during the naturally synchronous cell cycle of Physarum polycephalum, where RNA synthesis follows a periodic pattern (for review see 42). We were unable to find a correlation between H4 acetylation and transcription during the cell cycle (9) in contrast to previously published data (43). Moreover we observed a reversible depression of RNA synthesis (8,9) in the presence of butyrate induced hyperacetylation of H4.

In order to test whether there is a cause and effect relationship between histone acetylation and transcriptional activity of chromatin in Physarum we measured the endogenous RNA polymerase activity and the template activity of chromatin for exogenous RNA polymerases in the presence and absence of hyperacetylated H4. The results undoubtedly show that the chromatin template is not altered for its capacity to support RNA synthesis; this is

demonstrated for procaryotic and eucaryotic RNA polymerase. The constant properties of the template are somehow surprising, since the endogenous RNA polymerase activity decreases after butyrate treatment. A comparison of the behavior between endogenous polymerase in butyrate treated plasmodia and cycloheximide treated plasmodia revealed that the synthesis of the enzyme is inhibited. The idea that butyrate disturbs enzyme synthesis is supported by the fact, that both polymerase A as well as the α-amanitin sensitive polymerase B are equally affected. Moreover the inhibition of overall protein synthesis follows the same pattern as does RNA polymerase activity; in both cases butyrate acts faster than cycloheximide. It has been previously reported that butyrate inhibits the synthesis of other proteins in Physarum, as shown for thymidine kinase and thymidylate synthetase (44). From these experiments we conclude, that apart from its action as a deacetylase inhibitor, butyrate acts as a quick, but reversible inhibitor of protein synthesis in Physarum; this is a new aspect of this compound.

Interestingly the template activity of chromatin for transcription with exogenous enzyme considerably increased in plasmodia, which have been treated with cycloheximide for 30 hours. This increase above the control level could be explained by the continuous inhibition of the synthesis of chromosomal proteins, which will lead to histone depletion of the chromatin and other disturbances of chromatin integrity. One can therefore expect free areas of DNA, which would facilitate transcription by exogenous RNA polymerases.

In an earlier report (13) it was shown that nuclear dilution decreased the amount of incorporated radioactivity per mg of DNA, a finding which was explained by the existence of diffusable activation factors in HeLa cell nuclei, the authors observed this effect in controls as well as in hyperacetylated nuclei. We tested whether such factors are also present in Physarum nuclei, since it appeared interesting to study this phenomenon with respect to the butyrate-action as a protein synthesis inhibitor. However, we were unable to verify this phenomenon, since we could neither get evidence for such factors in controls nor in nuclei from butyrate treated plasmodia.

The question remains why previous investigations of in vitro RNA synthesis with highly acetylated chromatin revealed positive results supporting a tight connection of acetylation and transcriptional capacity (10-12, 14). The majority of these studies was undertaken with chemically acetylated chromatin (10-12). Acetic anhydride will introduce acetyl-groups into lysine and tyrosine residues of all histones and certainly other nonhistone chromosomal proteins. Since histone H1, which is never acetylated postsynthetically

in vivo, is also extensively acetylated by acetic anhydride, whereas H4 is only slightly modified, the results on transcription of such chromatin are questionable. It seems possible that acetylation of H1 leads to a partial loss of H1, a fact that would cause an increase in template activity for transcription (45). Only a small H1 depletion of chromatin, which would be hardly detectable, could result in a considerable increase in RNA synthesis. Recent results on histone depletion of chromatin regions during active transcription are in line with this explaination (46); it was shown for transcription of hsp 70 genes, that moderate transcription is paralleled by removal of most of the H1, while highly transcribed genes are completely depleted of all histones.

Our results are in accordance with an earlier report (13), where no correlation between histone acetylation and transcriptional activity of chromatin could be found in HeLa cells; however, there is an unfolding discrepancy to the results of Dobson and Ingram (14), who verified such a correlation in the same cell system. The contradictory results are probably due to other "factors" necessary for transcription rather than hyperacetylation. These factors could depend on the functional state of the cell, which is affected in quite different ways by butyrate. The unique feature of our system is that we can study template activity of chromatin for exogenous enzymes under conditions of strongly suppressed endogenous RNA polymerase activity, whereas Dobson and Ingram (14) found enhanced endogenous activity together with enhanced template activity in their experiments.

From our data on histone acetylation and transcription in Physarum we conclude, that there is no correlation between the two events. Support for this conclusion comes from structural data of hyperacetylated chromatin, which show that the physical properties do not change significantly as a response to hyperacetylation (47,48). It has been conclusively demonstrated, that structural transitions of chromatin and folding into higher order structures are not affected by histone hyperacetylation (48).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the expert technical assistance of Mrs.Herta Denifl. This project was supported by the Dr.Legerlotz-Foundation.

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