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CHROMATIN PROTEIN KINASES AND PHOSPHOPROTEINS DURING MYOBLAST GROWTH AND DIFFERENTIATION

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SUMMARY

Myoblasts, after a period of cell division, undergo differentiation when cultivated *in vitro*. Chromatin protein kinases strongly decreased during this sequence of events. The use of inhibitors of cell division and of cell differentiation suggests that this decrease is essentially related to the arrest of cell division. Chromatin non histone proteins from cells taken at various stages were incubated with (γ-^{32}P)ATP and analyzed by polyacrylamide gel electrophoresis. Radioactive phosphoproteins were present only in the last stages. The use of inhibitors indicates that the appearance of radioactive peaks is related to cell differentiation rather than to cell growth. This observation suggests that phosphorylation of chromatin phosphoproteins could be involved in the process of cell differentiation.

Muscle cells cultivated *in vitro*, after a period of cell division, undergo a specific differentiation characterized by the formation of myotubes (1, 2), by a strong increase in the synthesis of muscle specific proteins (3-6), by the stabilization of the myosin large subunit messenger RNA (7) and by a dramatic change in the pattern of RNA and protein synthesis (8). It has been suggested that chromatin non histone proteins (NHP) could play a key role in the specific regulation of gene expression (8-10). These proteins include protein kinases and phosphoproteins which could be involved in this regulation (11). Modifications of NHP have been reported during differentiation of chondrocytes (12), of intestinal epithelial cells (13) and during maturation of avian erythroid cells (14). It is then very likely that modifications in NHP would also be observed during myoblast differentiation.

In the present study, we have prepared NHP from Yaffé L_6 line of myoblasts (15) at different stages of differentiation and studied variations in protein kinase specific activities and of phosphoprotein phosphorylation pattern. We have used various inhibitors in order to see whether the observed variations could be correlated with the arrest of cell growth or with cell differentiation.

MATERIALS AND METHODS

Culture conditions : Cells from the line L_6 were grown in 100 mm diameter Corning dishes at 37° in a mixture of Eagle's minimum essential medium and of Parker medium 199 (3:1 v/v) supplemented with 10 % fetal calf serum and 0.5 % chick

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embryo extract, at a density of 2.10^5 cells/dish. After 3 days of culture the medium was removed and replaced by the same medium supplemented with 10 % horse serum. The culture medium was replaced every 48 hr. Under these conditions 90-100 % of cell fusion was observed after 8-9 days.

We have defined four stages during *in vitro* myogenesis : Stage I : intensive cell division for 3-4 days ; stage II : confluent lined up myoblasts, appearance of some multinucleated cells, 6th day ; stage III : numerous myotubes, but still some mononucleated cells, 7th day ; stage IV : at least 90 % of cells fused in large multinucleated syncitia, 8-9th day.

Preparation of chromatin NHP : Nuclei were obtained as previously described (16). NHP were prepared by the technique of Kamiyama and Wang (10), except that the final NaCl concentration was 0.14 M NaCl, in order to remove myosin.

Electrophoresis : For the study of the NHP pattern, electrophoreses were performed according to Laemmli (17), with 14 % polyacrylamide-SDS slab gels. The gels were stained with Coomassie Brilliant blue. The phosphoprotein pattern was obtained by electrophoresis performed according to the system 398 of Rodbard and Chrambach (18) modified by Gill and Garren (19). NHP were phosphorylated by incubation with (γ-^{32}P)ATP as described by Kruh *et al.* (16). The gels were sliced into 2 mm-wide sections. The radioactivity of each section was measured by the Cerenkov effect in the Intertechnique SL 32 liquid scintillation counter.

Protein kinase specific activities : The incubation medium contained in a total volume of 0.25 ml : 50 µg of NHP, 100 µg of substrate : phosvitin, casein or histone H_1 at optimal Mg^{++} and pH conditions (16), 2.10^6 cpm of (γ-^{32}P)ATP (Radiochemical Centre, Amersham). The incubations were carried out for 30 min at 37°. Transferred ^{32}P was measured after precipitation by trichloracetic acid and filtration on Millipore filters.

Other techniques : Creatine phosphokinase was assayed by the technique of Rosalki (20). Protein concentrations were determined by the method of Lowry *et al.* (21).

RESULTS AND DISCUSSION

NHP ELECTROPHORETIC PATTERN

NHP from cells taken at various stages were analyzed in SDS-polyacrylamide gels (fig. 1). Although several bands were present in all samples, variations in the pattern were observed from one stage to another. The bands around 125,000 and 40,000 tend to disappear between stages I and IV, whereas new bands appear around 50,000 and 25,000. Cells were cultured in the presence of various inhibitors of cell fusion. The inhibitors were added on the 3rd day of culture and the cells were harvested on the 7th day. Creatine phosphokinase activity was estimated as a probe for cell differentiation (Table I). Phospholipase C has little effect on cell growth but prevents cell fusion and molecular differentiation (22). The electrophoretic pattern of NHP from cells grown in its presence shows an intermediate pattern between stages I and II in control cells. Cytochalasin B blocks cell fusion, cell division (23) without affecting the expression of muscle specific genes (6). The electrophoretic pattern of NHP was identical to the pattern of NHP from differentiated control cells. BUdR at a concentration of 10 µg/ml has little effect on cell growth but partly inhibits

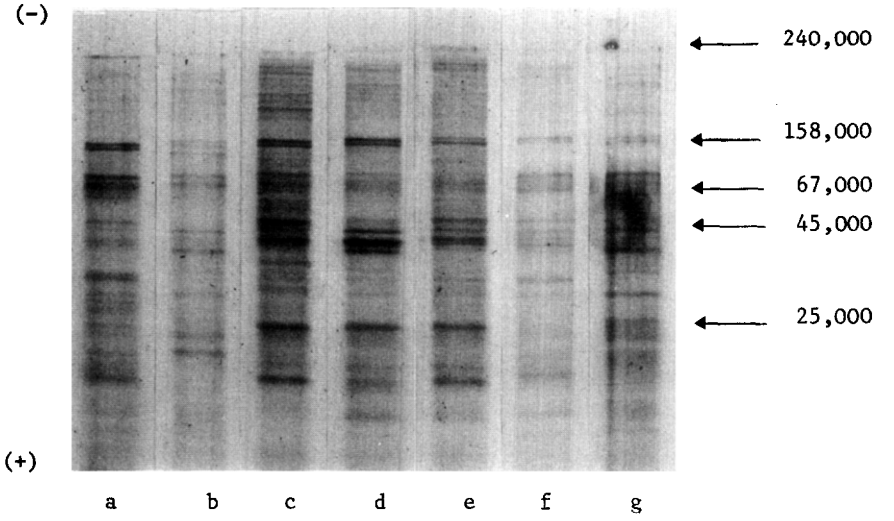

Figure 1 : Electrophoretic pattern of NHP from muscle cells at various stages. Electrophoreses were performed according to Laemmli in 14 % polyacrylamide-SDS gels. (a) Stage I, (b) Stage II, (c) Stage III, (d) Stage IV, (e) cultured in the presence of cytochalasin B, (f) cultured in the presence of BUdR, (g) cultured in the presence of phospholipase C.

The following molecular weight markers have been used : catalase (240,000), aldolase (158,000), serumalbumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000).

Table I : Effect of phospholipase C, BUdR and cytochalasin B on the decrease of chromatin protein kinase specific activity.

Inhibitors were added on the 3rd day of culture, cells were harvested on the 7th day. Protein kinase specific activity was measured before the addition of the inhibitors and after 7 days of culture. The decrease of protein kinase was expressed in p. 100 of the initial activity. Creatine phosphokinase was estimated as a probe for muscle cell differentiation.

ADDITION	CELL GROWTH	CREATINE PHOSPHOKINASE (1 U/mg prot.)	RESIDUAL PROTEIN KINASE ACTIVITY
0		97	37 %
Phospholipase C 10 µg/ml	slightly inhibited	17	59 %
BUdR 10 µg/ml	very slightly inhibited	40	47 %
Cytochalasine B 2.5 µg/ml	total inhibition	187	27 %

cell differentiation (24). It has an intermediate effect between the two other inhibitors. The NHP pattern is intermediate between the patterns of stages III and IV from control cells.

PROTEIN KINASE SPECIFIC ACTIVITY

We have previously shown that rat liver (11, 25) and muscle (26) contain several protein kinases which are able to transfer phosphate from ATP into various substrates including phosvitin, casein and histone H_1. We measured the specific activity of the enzymes at the four stages of culture. A decrease in specific activity was observed in all protein kinases when the culture proceeds, the strongest decrease being observed between stages I and II (fig. 2). A decrease in histone kinases has previously been described by Man *et al.* (27).

Since this decrease could be due either to the arrest of cell division or to cell differentiation, we used the inhibitors of cell fusion as described above (table I). In the presence of phospholipase C, an inhibitor of cell differentiation with little effect on cell division, the decrease in protein kinase activity was smaller than with the control cells. BUdR slightly diminished the decrease in protein kinase activity. In the presence of cytochalasin B, an inhibitor of cell division, the decrease in protein kinase was stronger than in control cells.

These observations indicate that the decrease in protein kinase specific activity is related to the arrest of cell division. This result is in agreement with the increase in protein kinase activity observed when cell growth was stimulated (28-30).

PHOSPHORYLATION PATTERN OF CHROMATIN PHOSPHOPROTEINS

We have previously shown that NHP include a protein kinase and phosphoprotein substrates of this enzyme (31). We have studied the electrophoretic radioactive pattern of NHP prepared from cells at the 4 stages and incubated with (γ-^{32}P)ATP (fig. 3). No significant ^{32}P incorporation was observed in phosphoproteins from cells at stage I. A single small very fast moving peak was observed in cells at stage II. Several radioactive peaks appeared at stage III, a large slower moving peak was present in NHP from cells at stage IV.

We performed the same analysis, after addition of each of the inhibitors mentioned above on the 3rd day of culture, the cells being harvested on the 7th day. A small radioactive peak was observed with NHP from cells treated with phospholipase C, a differentiation inhibitor, large peaks were present in NHP from cells treated with BUdR and with cytochalasin B, not significantly different from the peaks observed in differentiated control cells. Several additional slow moving peaks were also observed. It may be concluded from these experiments that the ^{32}P peak in slices 30-40 corresponding to the most

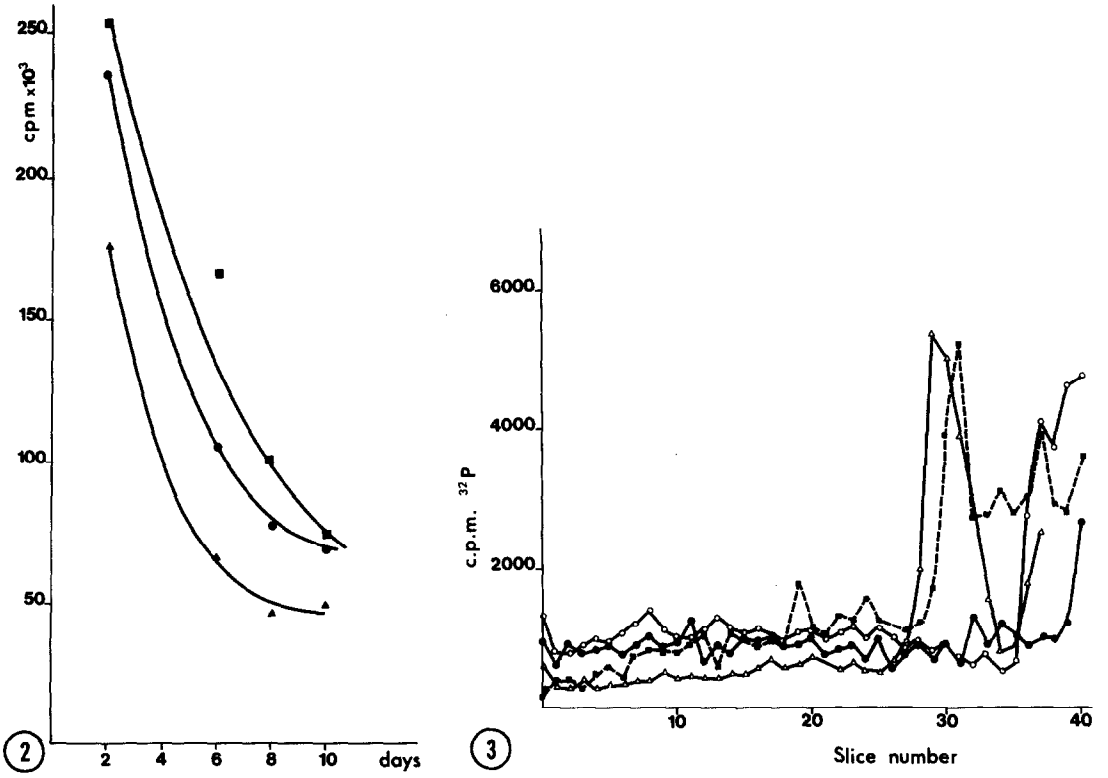

Figure 2 : Protein kinase specific activities in NHP from muscle cells. NHP were prepared from cells at each of the four stages and assayed for protein kinase specific activity with : phosvitin (■——■), histone H_1 (●——●) and casein (▲——▲) as substrates. Incubation medium included 50 µg of NHP and 100 µg of substrate, 2 x 10^6 cpm of (γ-^{32}P)ATP.

Figure 3 : Electrophoretic pattern of ^{32}P incorporated *in vitro* into chromatin NHP from muscle cells. NHP were prepared from cells at Stage I (●——●), II (o——o), III (■----■) and IV (Δ——Δ), incubated with (γ-^{32}P)ATP and submitted to electrophoresis on 7.5 % polyacrylamide gel according to Rodbard and Chrambach. The gels were sliced and the radioactivity in each 2 mm-wide section measured.

acidic proteins is characteristic of cell differentiation, it is not present when differentiation is completely abolished.

These changes in the ability of NHP to be phosphorylated *in vitro* could be interpreted in several ways : (a) synthesis of new molecular species of phosphoproteins, (b) changes in phosphoprotein conformation which could increase the number of phosphorylating sites, (c) increase in the turnover of protein bound phosphate which would increase the number of phosphorylation sites available *in vitro*, (d) increase of a protein kinase specific for endogenous substrates. Such an increase was observed by Man *et al.* (28). A general increase

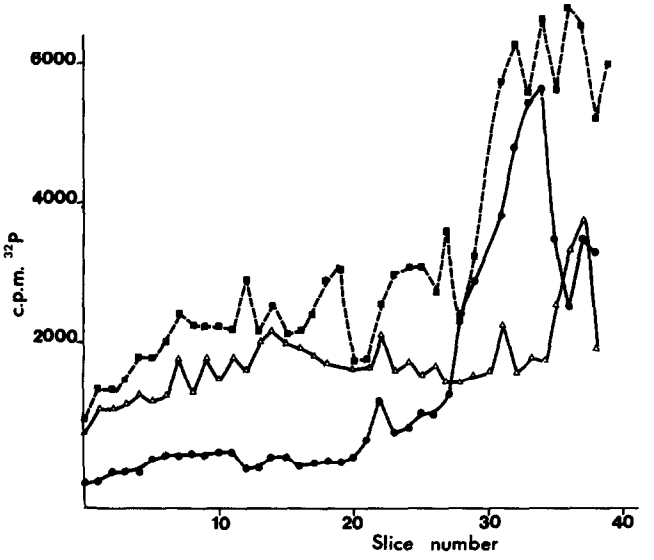

Figure 4 : Electrophoretic pattern of ^{32}P incorporated *in vitro* into chromatin NHP in muscle cells cultured in the presence of various cell fusion inhibitors. Phospholipase C, 10 µg per ml (Δ——Δ), BUdR, 10 µg per ml (■----■) and cytochalasin B, 2.5 µg per ml (●——●) were added on the 3rd day of culture. Cells were harvested on the 7th day. NHP were prepared, incubated with radioactive ATP and analyzed as described under fig. 3.

in NHP phosphorylation in intact nuclei was observed by Man during muscle cell differentiation.

These results confirm and extend previous observations concerning the possible role of chromatin phosphoprotein phosphorylation in the control of cell growth and differentiation (9, 11, 16, 32). The question remains open whether the modification in chromatin phosphorylation is a prerequisite of the changes in the pattern of gene expression or if it is a consequence of it.

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