PHOSPHORYLATION OF NUCLEAR PROTEINS DURING MUSCLE DIFFERENTIATION IN VITRO

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1. Introduction

Evidence for the involvement of non-histone chromosomal proteins in gene regulation has been reviewed recently [1,2]. In particular, the phosphorylation of these proteins has been implicated in gene activation and specific changes in the pattern of gene transcription [2–4] and the few studies that have so far been carried out with differentiating systems are at least consistent with this notion. These include stimulation of lymphocytes by phytohaemagglutinin [5] and of mammary cells by prolactin [6], both of which are associated with increased phosphorylation activity, and also red blood cells maturation which is accompanied by a decrease in phosphorylation of the nuclear non-histone proteins [7].

Chick muscle cultures present a potentially useful system in which to study the role of nuclear proteins in differentiation. Isolated chick myoblasts differentiate in vitro to form contractile, multinucleate myotubes which show both a profoundly altered pattern of protein biosynthesis [8–10] and an altered pattern of DNA transcription into RNA, as judged by MAK (methylated albumin on kieselguhr) chromatography [11] and DNA–RNA hybridisation [12].

We present here some preliminary observations on the relationship between protein phosphorylation in isolated nuclei and changes in gene expression during muscle differentiation. The rate of phosphorylation of nuclear protein increases steadily throughout the development of cultured muscle cells without any striking temporal relationship to cell fusion or the accumulation of contractile and other muscle proteins. Possible effects of changes in the nuclear ATP pools have been eliminated, and cyclic nucleotides have no apparent effect on the level of phosphorylation.

2. Materials and methods

2.1. *Materials*

Components of tissue culture media were obtained from Flow or Bio Cult Laboratories. ATP-γ-^{32}P (ammonium salt; 16–19 C_i/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. Cyclic AMP (Adenosine 3′:5′-cyclic monophosphoric acid, sodium salt) was obtained from Boehringer and cyclic GMP (guanosine 3′:5′-cyclic monophosphoric acid, sodium salt) from Sigma.

2.2. *Cell culture*

Cells were prepared from thigh muscles of 12-day-old chick embryos by mechanical disaggregation without the use of enzymes as previously described [12]. Cells were plated at an initial density of 15×10^6 per 135 mm collagen-coated Petri dish in 20 ml of Hams F-10, supplemented with 10% selected horse serum, 2% chick embryo extract, penicillin (50 U/ml) and streptomycin (50 μg/ml). They were fed daily, except on the second day, with a complete change of medium.

The degree of cell fusion was estimated by fixing replicate plates as previously described [13].

2.3. *Isolation of nuclei*

Cells were fed with a complete change of medium 2 hr before harvesting. They were then harvested in ice-cold saline G [14] and collected by centrifugation.

Nuclei were prepared by a modification of the method of Rein and Penman [15]. Cell pellets (about 0.1 ml packed cells) were resuspended in 5 ml of RSB (0.01 M NaCl, 0.015 M $MgCl_2$, 0.01 M Tris–HCl pH 7.4) containing 1% Triton X-100 and homogenised by hand with 7 strokes of a tight-fitting Dounce homogeniser. After leaving for 10 min at 0°C, the homogenisation was repeated and the homogenate was centrifuged at 500 *g* for 10 min. The nuclear pellet was then resuspended in RSB without Triton X-100 and the homogenisation and centrifugation steps were repeated as before. Nuclei prepared in this way were examined by phase-contrast microscopy and were judged free of cytoplasmic contamination. All operations were performed at 0–4°C.

2.4. *Phosphorylation assay*

The incorporation of ATP-γ-^{32}P into nuclear proteins was estimated by the method of Rickwood et al. [16]. Nuclei were incubated at 37°C in 0.25 M sucrose, 0.01 M $MgCl_2$, 0.025 M NaCl, 0.1 M Tris–HCl pH 8.0 and 5 or 10 μCi of ATP-γ-^{32}P in a total volume of 1 ml for 5 min, each tube containing the same amount of nuclei (0.3–0.6 A_{260} units). The reaction was stopped by cooling in iced-water and adding 0.5 ml of 0.2 M sodium pyrophosphate pH 7.4 followed by 0.5 ml of 3 M perchloric acid, with 100 μg of bovine serum albumin as protein carrier. The cold acid-insoluble pellets were incubated at 85°C for 20 min in 1 ml of 0.3 M perchloric acid to hydrolyse nucleic acid. After hydrolysis, the concentration of perchloric acid was increased to 0.75 M and protein pellets were collected by centrifugation and then washed 3 times with 5 ml of 0.75 M perchloric acid before suspending in a Triton scintillation fluid (30% Triton X-100, 0.5% 2, 5-diphenyloxazole in toluene). Controls for zero time background incorporation were performed and subtracted from all results.

As observed by Rickwood et al [16], little radioactivity is found in nucleic acids before hydrolysis and ^{32}P incorporation into lipids is negligible. These authors also showed that nuclei incorporate very little ^{32}P when ATP-γ-^{32}P is replaced by ATP-α-^{32}P.

3. Results

The time course of incorporation of ^{32}P from ATP-γ-^{32}P into the nuclear protein of isolated nuclei is shown in fig. 1., and is similar to those described by earlier workers using other tissues [16]. For nuclei from both 24 hr and 72 hr cultures the shape of the curve is essentially the same, so that the incorporation after 5 min is representative of both initial rates and final levels. There is no indication of any significant dephosphorylation even after 20 min incubation in nuclei from either 24 or 72 hr cultures. There is a clear increase of over 100% in phosphorylation activity between 24 and 72 hr.

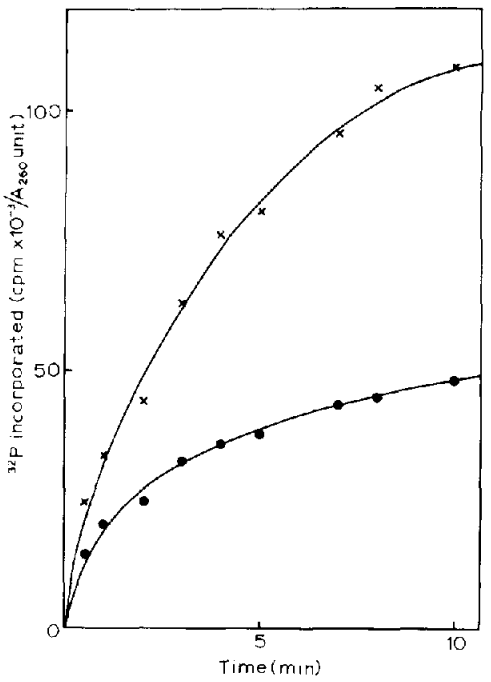

Fig. 1. Time courses of incorporation of ^{32}P from ATP-γ-^{32}P into proteins of isolated nuclei from 24 hr (●——●) and 72 hr (×——×) cultures. Each incubation contains 0.4 A_{260} units of nuclei and a zero time background incorporation of 680 cpm has been subtracted. The maximum range of the duplicate incubations was ± 5%.

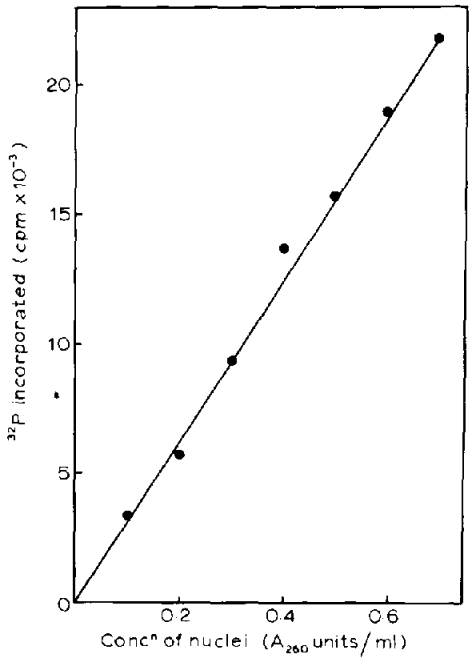

Fig. 2. The relationship between nuclear protein phosphorylation and the concentration of nuclei in the assay. Incubations were performed in duplicate (maximum range ± 10%) and zero time backgrounds were subtracted. Nuclei were obtained from 114 hr muscle cultures.

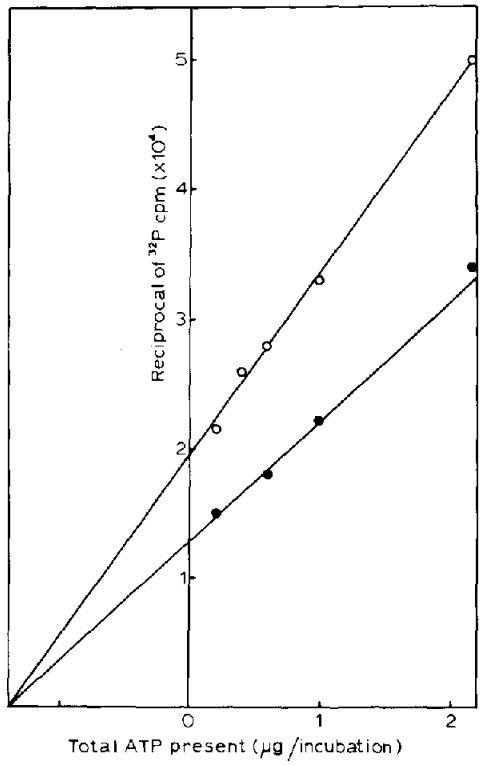

Fig. 3. Estimation of effective ATP pool sizes by isotope dilution in nuclei from 24 hr (○——○) and 120 hr (●——●). The total ATP present includes 0.196 μg (5 μCi) of labelled ATP added to each incubation. The maximum range of the duplicate incubations was ± 10%.

Fig. 2 shows that the incorporation is directly proportional to the concentration of nuclei in the incubation mixture. Nuclei of high activity (for 114 hr cultures) were used for this experiment. In subsequent experiments, a 5 min incubation time and a concentration of nuclei within the range of fig. 2 were used.

To eliminate the possiblity that the increases in ^{32}P incorporation during myogenesis were due to decreases in the concentration of an intranuclear ATP pool, nuclear ATP was estimated in both early and late cultures by an isotope dilution method.

In fig. 3 the reciprocal of the ^{32}P incorporation is plotted against the total concentration of ATP added (labelled and unlabelled) using a constant amount of labelled ATP. The intercept on the abscissa gives the size of the effective nuclear ATP pool, which is evidently the same for nuclei of both 24 and 120 hr cultures. The decreased slope reflects the increased phosphorylation in the later culture.

The rate of phosphorylation of nuclear proteins increases steadily as in vitro growth and differentiation proceed and fig. 4 shows a typical example of this increase in relation to cell fusion during a 5 day

Table 1
Effects of cyclic AMP and cyclic GMP on incorporation of ^{32}P from ATP-γ-^{32}P into proteins of nuclei isolated from 48 hr muscle cultures. Each incubation contained 0.3 A_{260} units of nuclei. Values are shown ± the range of the duplicate incubations

Additions	^{32}P Incorporation (c.p.m × 10^{-3})	% of control
–	13.0 ± 0.9	100
cyclic AMP 10^{-7} M	13.9 ± 0.1	107
cyclic AMP 10^{-6} M	14.3 ± 0.5	110
cyclic GMP 10^{-7} M	13.5 ± 0.5	103
cyclic GMP 10^{-6} M	12.2 ± 0.3	94

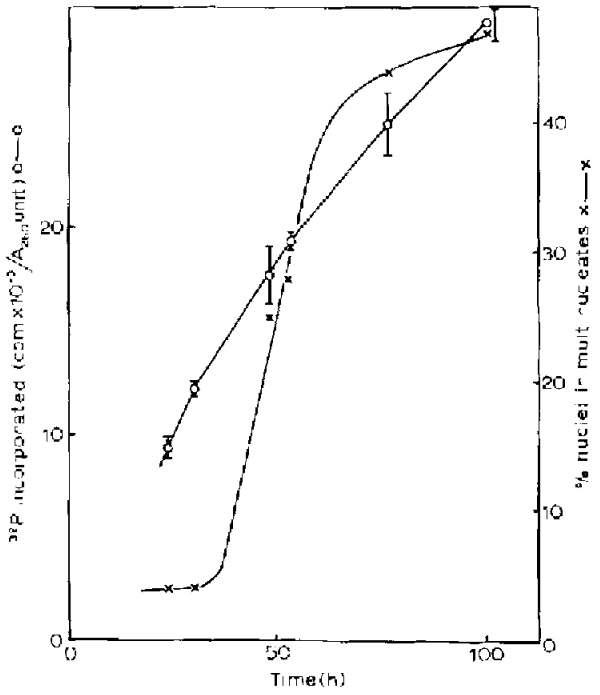

Fig. 4. Quantitative changes in nuclear protein phosphorylation during muscle differentiation in vitro. The incorporation of ^{32}P into protein of nuclei isolated at different times of cell culture (○——○) is shown in relation to cell fusion (×——×). Each incubation contained 0.32 A_{260} units of nuclei and the vertical bars show the standard errors of the means of 3 or 4 incubations.

culture period. Increases of 3–5 fold are usual and an increase is always observed well before the period of rapid cell fusion begins.

Table 1. shows the effects of cyclic nucleotides on nuclear protein phosphorylation. Neither cyclic AMP nor cyclic GMP had any significant effect at concentrations at which cyclic AMP is normally effective on cyclic AMP-dependent kinases in vitro [17].

4. Discussion

We have shown that both the rate and final level of protein phosphorylation in isolated chick muscle nuclei increase steadily during myogenesis in vitro and that this increase is not due to a decrease in nuclear ATP pools. Further experiments [18] show that quantitative changes in phosphorylation of chromosomal proteins occur, and in the non-histone fraction rather than the histones, as observed in nuclei from other tissues [16].

The fact that increases in nuclear protein phosphorylation are occurring in cell cultures supposedly undergoing processes of gene activation does not, of course, necessarily mean that the two processes are directly or causally related. Experiments in progress may reveal the precise significance of these results, but, for the moment, fig. 4 clearly suggests that the increase in phosphorylation is not closely related to cell fusion and the accumulation of characteristic muscle proteins, nor is it dependent on cell density [18]. We cannot, however, as yet rule out a relationship with either withdrawal of nuclei from the cell division cycle, which may occur before cell fusion [19], or a possible pre-fusion developmental stage postulated by Yaffe [20].

Interpretation of quantitative data on total non-histone proteins is complicated by the heterogeneity and limited tissue specificity of this protein fraction and of its phosphorylated components [16,21]. It will be interesting, therefore, to look for the appearance of new phosphorylated components during myogenesis or, at least, for increases in phosphorylation of individual nuclear proteins. It seems likely that non-histone chromosomal proteins can show different degrees of phosphorylation at multiple amino acid sites [16], and our preliminary studies suggest that such changes in the phosphorylation pattern do occur during myogenesis [18].

The observation that phosphorylation is not stimulated by cyclic AMP is consistent with similar results in rat liver [22]. Cyclic GMP was also ineffective in our experiments. This does not necessarily eliminate a role for cyclic nucleotides in a model of gene regulation by phosphorylation of non-histone chromosomal proteins, since multiple protein kinases have been shown to exist in liver nuclei [23], some of which may be greatly stimulated by cyclic AMP while others are correspondingly inhibited [24], and each kinase may show a different specificity [24].

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References

[1] Stein, G. S., Spelsberg, T. C. and Kleinsmith, L. J. (1974) Science 183, 817.
[2] Spelsberg, T. C., Wilhelm, J. A. and Hnilica, L. S. (1972) Sub-cell Biochem. 1, 107.
[3] Shea, M. and Kleinsmith, L. J. (1973) Biochem. Biophy. Res. Comm. 50, 735.
[4] Platz, R. D., Stein, G. S. and Kleinsmith, L. J. (1973) Biochem. Biophys. Res. Comm. 51, 735.
[5] Kleinsmith, L. J., Allfrey, V. G. and Mirsky, A. E. (1966) Science 154, 780.
[6] Turkington, R. W. and Riddle, M. (1969) J. Biol. Chem. 244, 6040.
[7] Gershey, E. L. and Kleinsmith, L. J. (1969) Biochim. Biophys. Acta 194, 519.
[8] Paterson, B. and Strohmann, R. C. (1972) Dev. Biol. 29, 113.
[9] Morris, G. E., Cooke, A. and Cole, R. J. (1972) Exptl. Cell. Res. 74, 582.
[10] Heywood, S. M., Havarinis, A. S. and Herrmann, H. (1973) J. Cell. Physiol. 82, 319.
[11] Mán, Nguyen Thi and Cole, R. J. (1972) Exptl. Cell. Res. 73, 429.
[12] Mân, Nguyen Thi and Cole, R. J. (1974) Exptl. Cell. Res. 83, 328.
[13] Morris, G. E. and Cole, R. J. (1972) Exptl. Cell. Res. 75, 191.
[14] Puck, T. T., Cieciura, S. J. and Robinson, A. (1958) J. Exptl. Med. 108, 945.
[15] Rein, A. and Penman, S. (1969) Biochim. Biophys. Acta 190, 1.
[16] Rickwood, D., Riches, P. G. and McGillivray, A. J. (1973) Biochim. Biophys. Acta 229, 162.
[17] Langan, T. A. (1968) Science 162, 579.
[18] Mán, Nguyen Thi, Morris, G. E. and Cole, R. J., unpublished observations.
[19] Bischoff, R., and Holtzer, H. (1969) J. Cell. Biol. 41, 188.
[20] Yaffe, D. (1971) Exptl. Cell. Res. 66, 33.
[21] McGillivray, A. J. and Rickwood, D. (1974) Eur. J. Biochem. 41, 181.
[22] Takeda, M., Yamamura, H. and Ohga, Y. (1971) Biochem. Biophys. Res. Comm. 42, 103.
[23] Ruddon, R. W. and Anderson, S. L. (1972) Biochem. Biophys. Res. Comm., 46, 1499.
[24] Kish, V. M. and Kleinsmith, L. J. (1972) J. Cell. Biol. 55, 138a.