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Synthesis of Muscle Proteins During the Differentiation of Cultured Chicken Pectoralis Muscle Cells

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培養 鷄胚 筋細胞의 分化에 따른 筋特異 蛋白質의 合成과 젖산탈수소 효소의 活性에 관하여

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요 약

근세포의 분화에 있어서의 근특이 단백질의 합성 순서를 구명하기 위하여 계배 근세포를 2~9일간 배양하면서 단백질합성양상을 SDS-polyacrylamide 겔 전기 영동법, 등전점초점2차원 전기영동법 및 방사자기법으로 분석하였다.

Actin은 분화의 초기부터 활발히 합성되어 그 양이 다량으로 축적되나, myosin은 배양 3일째부터 대량 합성되기 시작하였다. Myosin의 대량합성시기는 배양 근원세포가 융합을 활발히 일으키는 시기와 거의 같았다. Myoglobin은 분화초기부터 서서히 합성축적되기 시작하여 배양 5일에서 최대치에 달하였다. Creatine phosphokinase는 배양 3일만에, 그리고 glyceraldehyde dehydrogenase는 6일만에 전기영동상에 검출되었다. Tropomyosin α와 β, 그리고 troponin C는 분화초기부터 비교적 다량 합성되고 있었다.

젖산탈수소효소의 활성은 배양 2~5일 사이에서 급격히 증가하고 이후 거의 변화가 없었다. 이 효소의 동위효소 조성은 초기 근원세포에서는 H_4와 H_3M형이 많으나 분화가 진행됨에 따라 HM_3와 M_4형이 서서히 출현하였다. 그리고 배양 5일만에 5종의 동위효소가 모두 검출되었다.

INTRODUCTION

The differentiation of myoblasts into contractile muscle fibers involves regulation of gene expression at the transcriptional, translational, and posttranslational levels (Bucking-

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ham, 1977). Although some of these processes are subject to neural control both during embryonic development (Fambrough, 1976; Rubinstein *et al.*, 1977) and in adult animals (Salmons and Sreter, 1976), the fundamental features of the process are faithfully reproduced in cultured muscle cells *in vitro* (Merlie *et al.*, 1977). Thus, in primary cultures of chicken pectoralis muscle the presumptive myoblasts undergo several cycles of cell division, followed on the second and third days of culture by cell-cell contact and fusion into multinucleated myotubes. The synthesis of myofibrillar and sarcoplasmic reticulum proteins initiated during fusion leads to the development of cross striation and by the fifth day spontaneous contraction appears.

Differentiated cells then synthesize characteristic proteins which are expressed at specific times during their differentiation. Some differentiated cell types synthesize large amounts of only one or a few such proteins, whereas other cell types such as muscle cells synthesize a complex array of characteristic proteins. In the latter case it is suggested that there are mechanisms which coordinate gene expression during cellular differentiation.

The most abundant of the contractile proteins in muscle are actin, myosin, troponin and tropomyosin, which are present in a well defined stoichiometry in the myofibrils of skeletal muscle fibers (Potter, 1974). Among these proteins, Emerson (1977) found that the synthesis of the heavy chain subunit of myosin was highly regulated during the differentiation of quail myoblasts in cell culture, being abruptly increased when myoblasts fused. Ha *et al.* (1979) found that muscle specific proteins including myosin, troponin, actin and tropomyosin, showed a large increase in concentration during myoblast differentiation and the increased synthesis of these proteins was inhibited by the inhibition of cell fusion in the environment of low medium calcium concentration, suggesting their coordinate regulation.

The extent to which these proteins are regulated coordinately, however, has not been known. It is, therefore, the purpose of the present experiment to study the temporal sequence of synthesis of contractile proteins during myoblast differentiation. Since the differentiation might well be reflected by the change in the composition of some isozymes, the pattern of lactic dehydrogenase isozyme and its activity during the myoblast differentiation was concomitantly determined in this study.

MATERIALS AND METHODS

1. Muscle cell culture.

The cultures were prepared from superficial breast muscles of 12-day old chicken embryos essentially according to O'Neill and Stockdale (1972), as described elsewhere (Ha *et al.*, 1979). After the required day of culture, the cells were labeled in fresh medium with ^{35}S-methionine as described in the legends of appropriate Figures. Protein in the sample was measured by the method of Lowry (1951).

2. Analysis of protein composition by electrophoresis.

The cultured cells harvested at appropriate time were processed for electrophoresis as described earlier (Ha *et al.*, 1979). Proteins were analysed by one-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis essentially according to Laemmli (1970) using 6~12% gradient slab gels as described elsewhere(Ha *et al.*, 1979). In some cases, proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis according to the method of O'Farrell (1975) using isoelectric focusing with pH gradient as first dimension and SDS polyacrylamide gel electrophoresis as second dimension as described elsewhere (Ha *et al.*, 1979).

After electrophoresis, the gels were stained with 0.2% Coomassie blue in 50% methanol and 10% acetic acid, and destained in 35% methanol, 10% acetic acid and 1% glycerol. The destained gels were either dried immediately under vacuum or processed for fluorography according to Bonner and Laskey (1974). Autoradiograms were made by exposing the dried gels on X-ray films (Kodak X-Omat XR-5 film).

In order to approximate the relative amount of protein and radioactivity incorporated into the protein, wet gels and autoradiograms were scanned in a Transdyne densitometer.

The total protein content of the samples applied for one or two dimensional electrophoresis (usuall 50 μg) was kept constant within each series of experiments. Therefore, the radioactivity of individual bands refers to a fixed amount of total protein.

3. Lactic dehydrogenase isozyme assay.

Cultured cells or breast muscle cells from intact embryos were homogenized in a 4 volume of 0.5 M citrate buffer (pH 6.0) at 4°C and the homogenate was centrifuged at 9,000×G for 30 min. and then at 100,000×G for 1 hour. The supernatant was again homogenized in an aliquot volume of 0.5 M citrate buffer and kept frozen until used.

The enzyme activity was measured photometrically according to Bergmeyer and Bernt (1974). The reaction mixture contained 48 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, 0.1 mM reduced niacinamide adenine dinucleotide (NADH) or reduced niacinamide hypoxanthine dinucleotide (NHXDH), and 10 μg protein in a total volum of 3.15 ml. The enzyme activity that oxidizes 1 μmole of NADH in 1 min. is termed as 1 unit and the unit per mg protein is expressed as specific activity.

The isozyme composition of lactic dehydrogenase was analyzed by the method of isoelectric focusing on polyacrylamide slab gel using 1% ampholine according to O'Farrell (1975). After electrophoresis the gel was stained with Wright's (1971) staining solution in the dark at 37.5 C for 1-2 hours. The staining solution consisted of 2 ml NADH (2.5 mg/ml), 2 ml nitroblue tatrazolium (1.5 mg/ml), 1 ml phenazine methosulfate (0.2 mg/ml), 1.5 ml tris buffer (0.5 M, pH 7.2), 0.5 ml KCN (0.1 M), 0.5 ml lactate (0.5 M, pH adjusted to 7.0 with Na_2CO_3), and 1 ml distilled water.

RESULTS

1. Morphological changes during differentiation

The presumptive myoblasts from 12-day old chicken embryos (pectoralis muscle) were cultured up to 9 days. In this *in vitro* culture, the cells underwent a progressive morphological differentiation including elongation, fusion, formation of multinucleated cells and alignment in a one direction. Fig. 1(A-F) shows these morphological changes with culture time.

After 24 hours of culture the original round-shaped myoblasts elongated into long needle-shaped cells (Fig. 1, A) which continued the elongation up to 48 hours (Fig. 1, B and C). At 48 hours of culture the myoblasts kept proliferate and few cells appeared to commence fusion although most of the cells remained mononucleated. At 60 hours, the elongation of myoblasts was very remarkable and cell fusion was also actively occuring (Fig. 1, D). When the culture reached 72 hours fusion was almost complete and dense long multinucleated fibers were seen aligned in a single direction (Fig. 1, E). After 72 hours, these fibers seemed to undergo little change, at least in morphological aspects, until 120 hours (5 days; Fig. 1, F).

2. Changes in protein patterns during differentiation

The myoblasts cultured for various period of time were analyzed for their protein pattern by SDS-polyacrylamide gel electrophoresis and a typical gel pattern stained with Coomassie blue is given in Fig. 2. In the initial period of the culture (2 days) the myosin content is relatively low compared to other low-molecular weight proteins. Its synthesis seems to burst at 3 days of culture when the fusion is the most notable and to reach saturation until 9 days of culture. On the other hand, actin seems to be present from the very initial stage of differentiation and remain with little change in the relative amount throughout the entire culture period. Other proteins, which are tentatively identified as Ca-ATPase, phosphorylase, creatine phosphokinase (ATP: creatine phosphotransferase), glyceraldehyde dehydrogenase and myoglobin, with appropriate marker proteins, also seem to change little in the amount during the differentiation.

Fig. 3 is a densitometric scanning of the electrophoresis gel given in Fig. 2 to show the change in these protein amounts in the course of differentiation between 2 to 9 days of culture. As obviously seen in the Coomassie blue-stained pattern (Fig. 2), the densitometric scanning of the gel clearly indicates that the myosin peak appears at third day of culture and keeps its amount relatively constant thereafter. The actin peak is the most predominant throughout the culture period. The myoglobin peak gradually increases in its height as the culture proceeds and at third day of culture it exceeds that of actin until 6th day. During this period actin seems to decrease in its relative amount. Creatine phosphokinase appears at third day and exists with little change in the amount through-

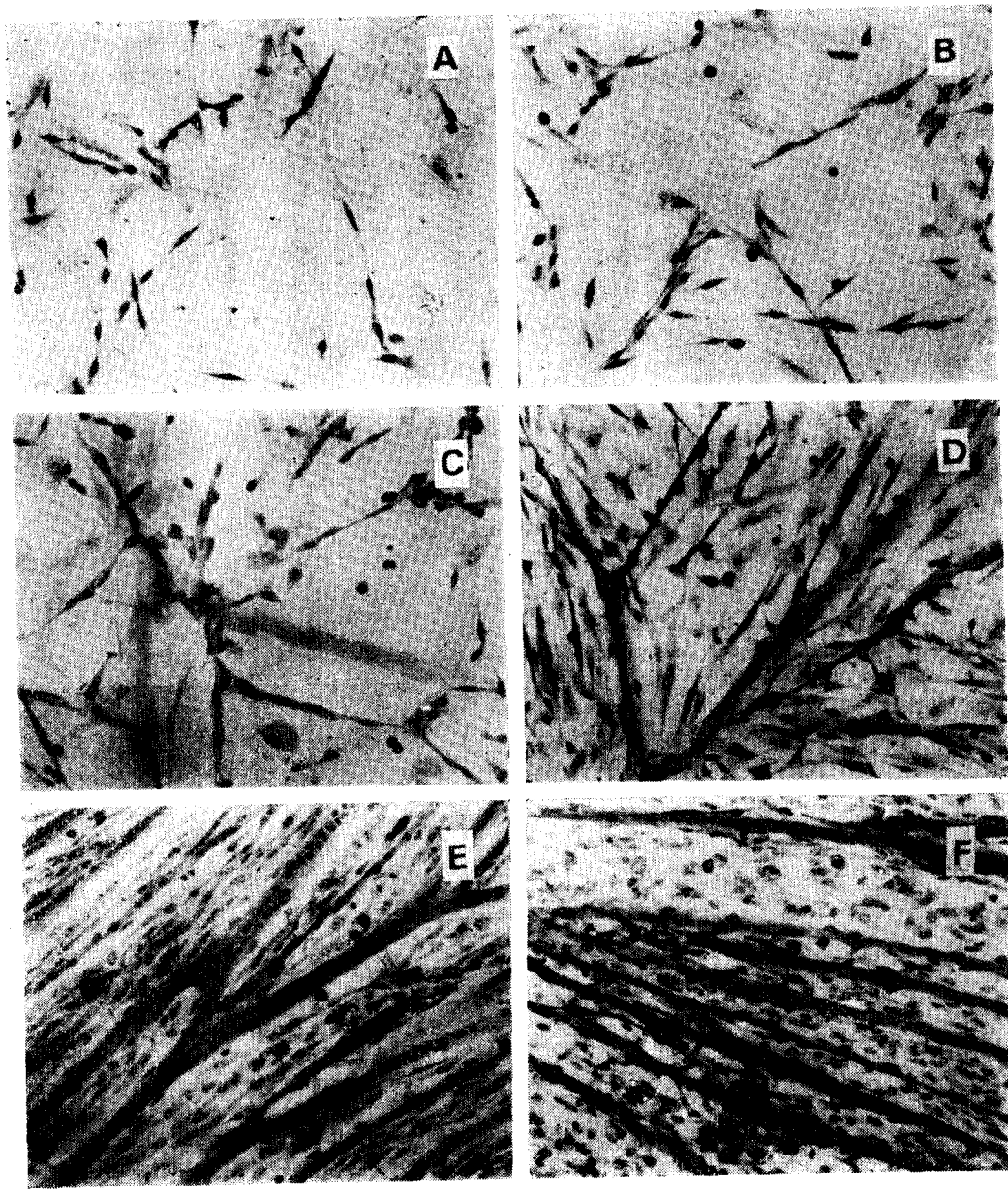

Fig. 1. Morphological changes of chicken pectoralis muscle cells in culture. Cells were harvested after 24 (A), 36 (B), 48 (C), 60 (D), 72 (E), and 120 (F) hours of culture and haematoxylin-stained. ×100.

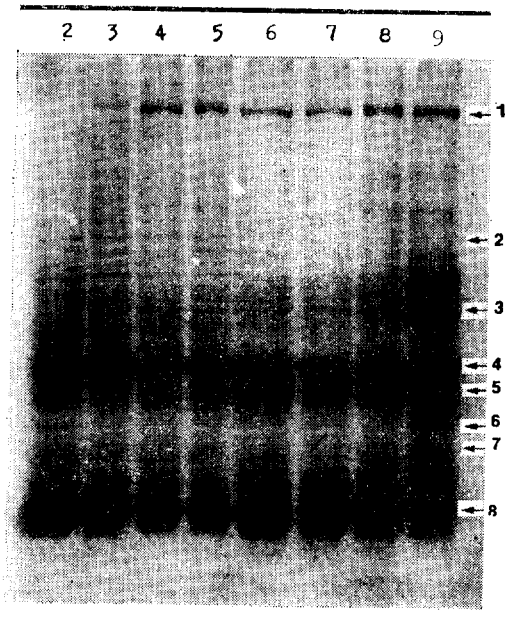

Fig. 2. The protein composition of chicken pectoralis muscle cells in culture. Numerals at the top of each lane indicate the days of culture. Arrows on the right indicate the position of marker proteins; 1, myosin; 2, phosphorylase; 3, serum albumin; 4, actin; 5, creatine phosphokinase; 6, glyceraldehyde dehydrogenase; 7, lactic dehydrogenase; 8, myoglobin.

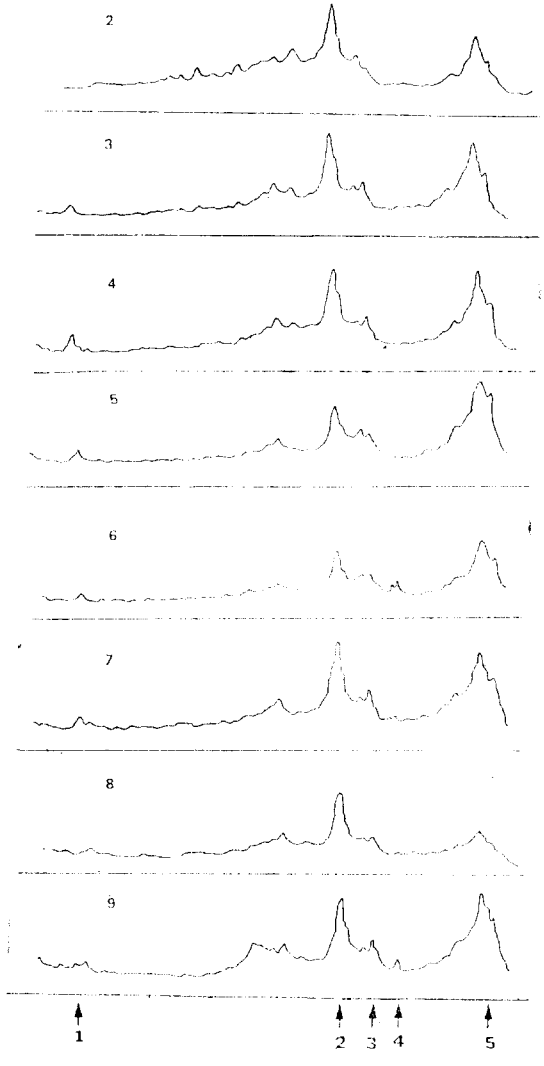

Fig. 3. Densitometric scanning of SDS-polyacrylamide gel electrophoretogram (Fig. 2) of chicken pectoralis muscle cells in culture. Numerals on the left are days of culture. Arrows at the bottom are the position of marker proteins: 1, myosin; 2, actin; 3, creatine phosphokinase; 4, glyceraldehyde dehydrogenase; 5, myoglobin.

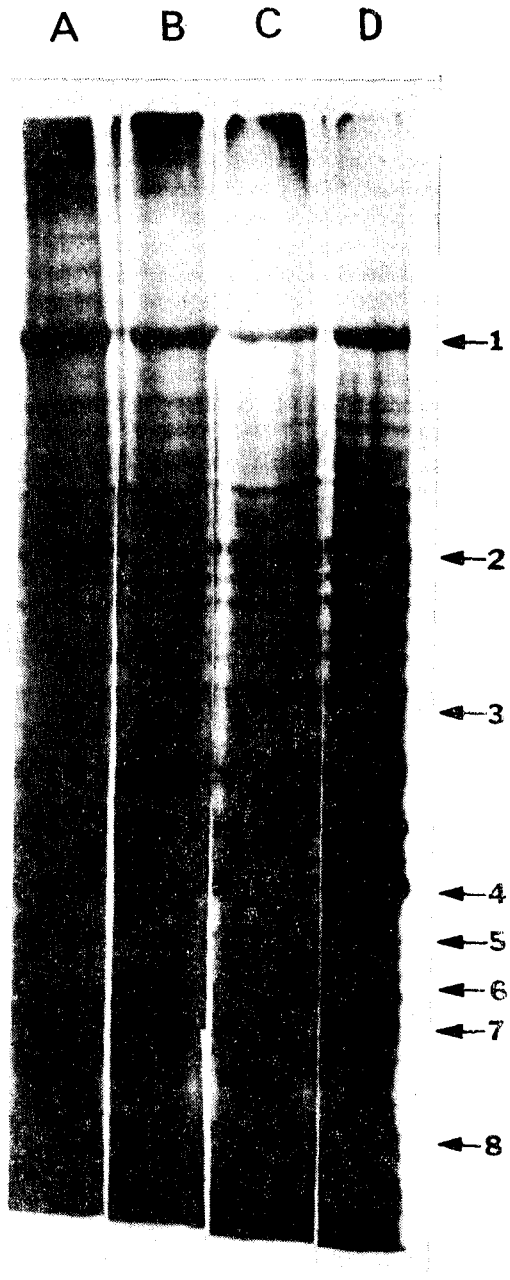

Fig. 4.

Fig. 4. Incorporation of ^{35}S-methionine into proteins of cultured chicken pectoralis muscle cells. The cultures were grown for 3 days (A), 5 days (B), 7 days (C) and 9 days (D). During the last 24 hours of culture, cells were labeled with ^{35}S-methionine, 25μ Ci/ml medium. Autoradiogram was made by fluorography of the dried SDS polyacrylamide slab gel. Arrows on the right indicate the position of marker proteins: 1, myosin; 2, phosphorylase; 3, serum albumin; 4, actin; 5, creatine phosphokinase; 6, glyceraldehyde dehydrogenase; 7, lactic dehydrogenase; 8, myoglobin.

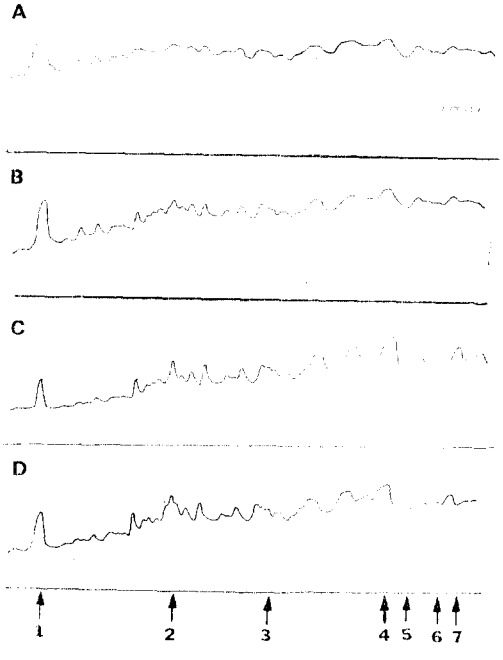

Fig. 5. Densitometric scanning of autoradiogram shown in Fig. 4. Cells were cultured for 3 days (A), 5 days (B), 7 days (C) and 9 days (D). Arrows at the bottom indicate the position of marker proteins as in Fig. 4.

Fig. 6. Incorporation of ^{35}S-methionine into proteins of cultured chicken pectoralis muscle cells. The cultures were grown for 3 days (A), 5 days (B), 7 days (C) and 9 days (D). Before harvesting the cells at appropriate days, the cells were exposed to 25 μCi/ml medium of ^{35}S-methionine for 24 hours. Proteins were separated by isoelectric focusing (IEF) on polyacrylamide tube gel as one dimension (with the pH gradient between 9.2 and 3.2) and then by SDS-polyacrylamide slab gel etectrophoresis (SDS) as two dimension. After two dimensional electrophoresis, the gels were stained, dried and processed for fluorography as described in Methods. Dark column at the left of each plate is one dimensional SDS polyacrylamide gel electropheretogram run concomitantly with the isoelectric focused gel in order to localize two-dimensionally separated spots. Several black spots at far left of C and D are radioactive marker proteins also run one-dimensionally. 1. actin; 2, tropomyosin β; 3, tropomyosin α; 4, myosin light chain 1; 5, myosin light chain 2; 6, troponin C.

Fig. 7. The protein composition of chicken pectoralis muscle cells in culture. The cultures were grown for 3 days (A), 5 days (B), 7 days (C) and 9 days (D) as in Fig. 6. Proteins were one-dimensionally separated by isoelectric focusing (IEF) on polyacrylamide tube gel and then by SDS-polyacrylamide slab gel electrophoresis (SDS) two-dimensionally. At the left of each plate are one-dimensional SDS polyacrylamide gel electrophoretogram run concomitantly with the isoelectric focused gel in order to localize the protein spots. Several spots at the far left of each plate are marker proteins. 1, actin; 2, tropomyosin β; 3, tropomyosin α; 4, myosin light chain 1; 5, myosin light chain 2; 6, troponin C.

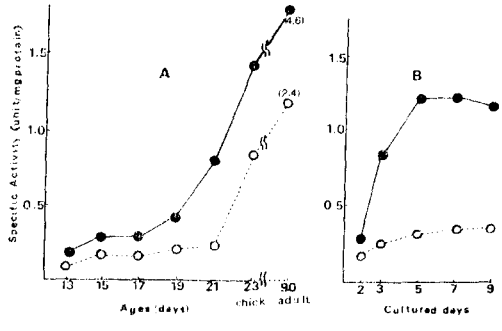

Fig. 8. Specific activity of lactic dehydrogenase of chick pectoralis muscle cells in embryo (A) and in culture (B). The activity was measured for supernatant (——●——) and for pellet(……○……) of 100,000×G contrifugation.

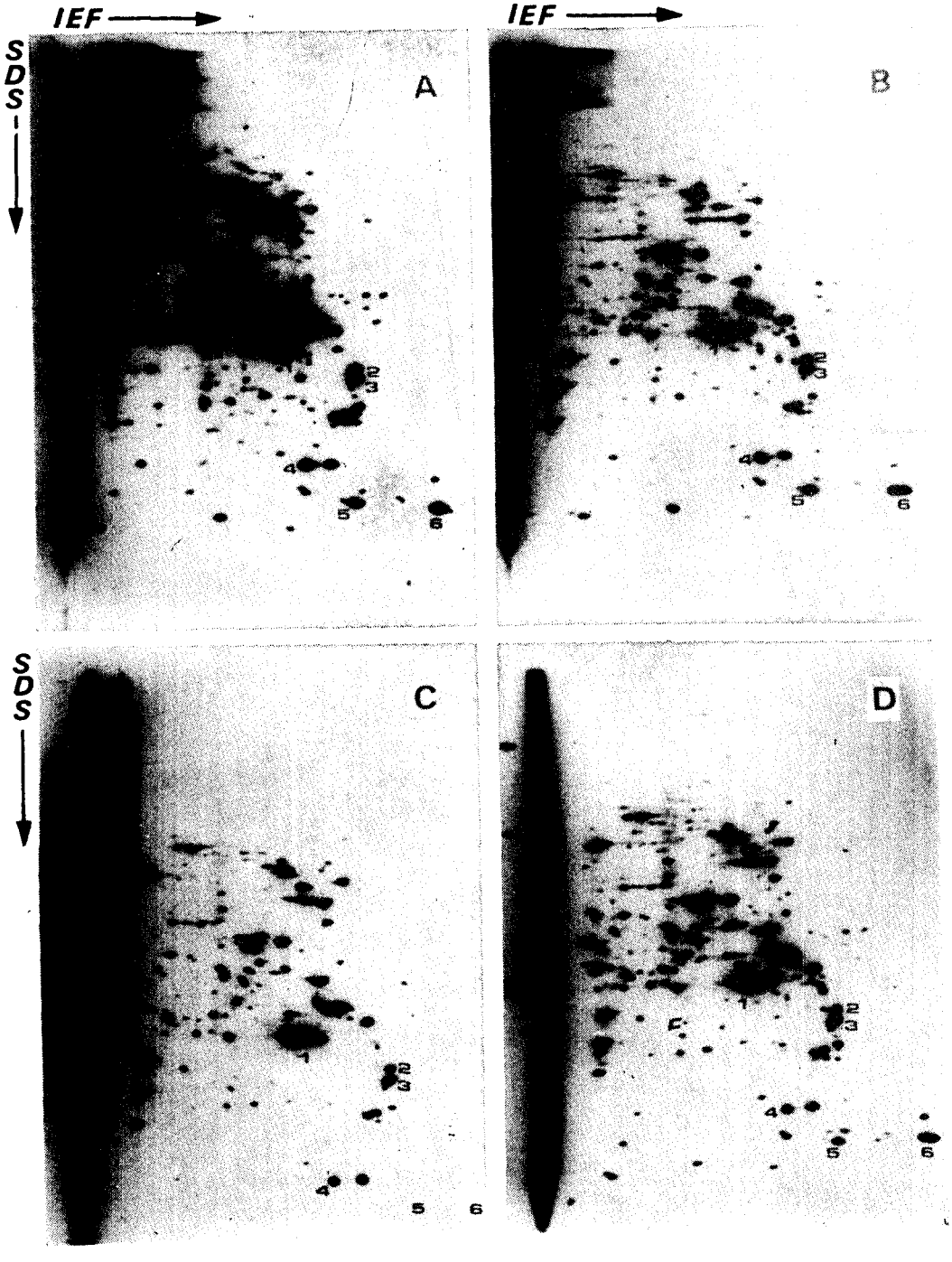

Fig. 6.

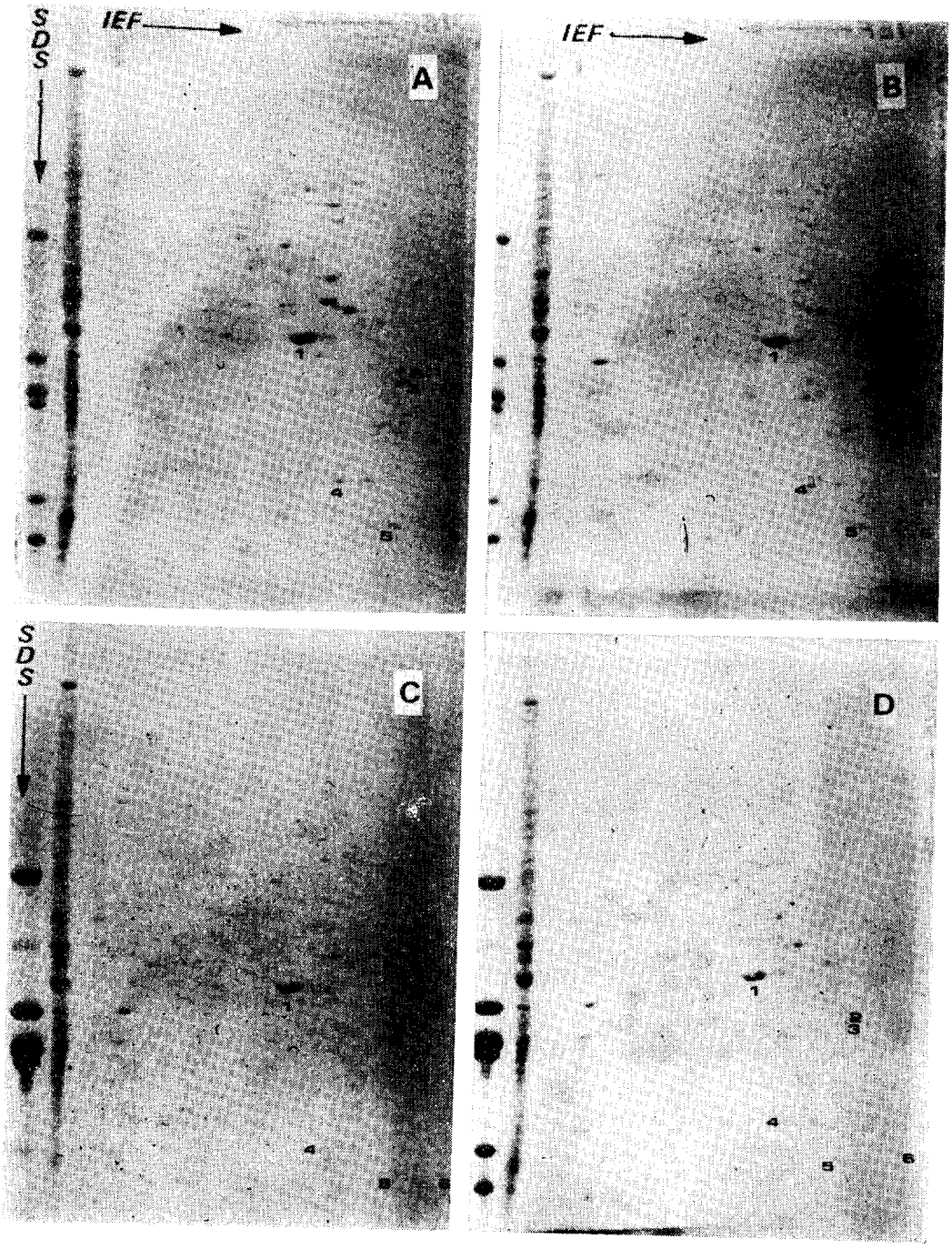

Fig. 7.

out all the culture period. Glyceraldehyde dehydrogenase appears at 6th day.

3. The incorporation of ^{35}S-methionine into muscle proteins during differentiation

The change in the relative amount of few muscle-specific proteins during cell differentiation as revealed in Fig. 2 represents that of the myoblasts in the steady state during culture. The gradual increase in the myosin content as the myoblasts proceed morphological differentiation (Fig. 2) is revealed in the autoradiogram (Fig. 4), where the incorporation of ^{35}S-methionine into myosin and other proteins are clearly seen. The radioactivity of the myosin band of different age of myoblasts is the highest at third day culture indicating that the synthesis of myosin in the myoblast is the most active at age of 3 days, when the cell fusion is most remarkable.

The incorporation of radioactivity into myosin after third day of culture decreases slightly and this rate of incorporation is kept until 9 days. On the other hand, the rate of ^{35}S-methionine into actin seems to be fairly constant from 3 to 9 days possibly indicating that this protein is synthesized from early period of culture.

Fig. 5 shows the densitometric scanning of the autoradiogram shown in Fig. 4 to reveal the degree of radioactivity incorporation into various species of proteins in the muscle cell, although the degree may only be an approximation of incorporation because the scanning pattern does not necessarily represent the incorporation quantitatively. At 3 and 5 days of culture (Fig. 5, A and B), the incorporation of ^{35}S-methionine into myosin is very remarkable reaching almost the same rate of other low-molecular weight proteins including actin. This high incorporation rate, however, declines after 7 days of culture (Fig. 5, C and D).

The protein bands resolved by one-dimensional electrophoresis are frequently heterogeneous, leading to ambiguities in the interpretation of the rate of synthesis of individual proteins. For this reason the influence of age upon the rate of synthesis of muscle proteins was analyzed by two-dimensional electrophoresis of proteins labeled in cell cultures with ^{35}S-methionine. A typical two-dimensional pattern of this autoradiogram is given in Fig. 6 (A-D). A 24 hour-exposure of cells to ^{35}S-methionine leads to intense labeling of several hundred muscle proteins. Only a few of these are detectable by Coomassie-blue staining (Fig. 7, A-D). Many muscle-specific proteins were intensively labelled. Tropomyosin (α and β) and troponin C which were not identified in one dimensional gel were clearly seen. They were synthesized from early period of differentiation in relatively constant rate. Myosin light chains 1 and 2 were also labeled intensively throughout all the period of culture.

4. The changes in lactic dehydrogenase isozymes during differentiation.

The enzyme activity of lactic dehydrogenase was measured in the myoblast during *in vivo* embryonic development and in the myoblast during in vitro culture. In the in vivo embryonic myoblast, the fraction of $100,000 \times G$ supernatant was much higher in the

enzyme activity than in the pellet. The enzyme activity in the supernatant of the embryo was increased gradually in the earlier period of the development (13-15 days of incubation) and then increased sharply at about 19 days of incubation. The sharp increase continued after hatching and the enzyme activity was the highest in the adult (Fig. 8, A).

The enzyme activity in the cultured myoblast was also higher in the supernatant than in the pellet. The activity in the supernatant increased sharply form earlier period of culture (2-5 days) and then kept constant (Fig. 8, B).

There was a remarkable difference in the enzyme activity between *in vivo* embryo and *in vitro* cultured myoblasts. The activity of 5-day cultured myoblasts was almost the same with that of hatched chicken muscle cells and was more than five times higher than that of 17 day embryo. The myoblast cultured for 5 days is equivalent to 17 day-old embryo with respect to the age.

The isozyme composition of embryonic and cultured myoblasts was analyzed and the electrophoretic pattern is presented in Fig. 9. Isozymes of H_4 and H_3M were present in embryos of 13, 15, 17, and 19 days and in adult. On the other hand, H_2M_2 type first appeared at 15 day embryo and HM_3 and M_4 at 19 day embryo. In other words, the embryo of 19 day incubation was equipped with all five isozymes.

The isozyme pattern of lactic dehydrogenase of cultured myoblasts is also shown in Fig. 9. Isozymes of H_4 and H_3M types were present from the earliest period of culture (2

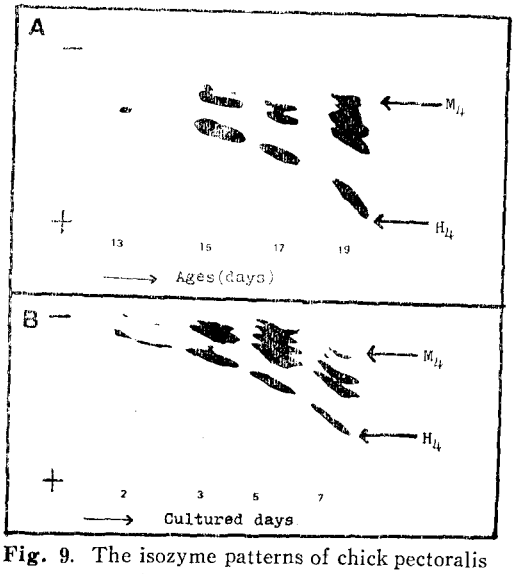

Fig. 9. The isozyme patterns of chick pectoralis muscle cells in embryo (A) and in culture (B). Isozymes were separated by isoelectric focusing on polyacrylamide slab gel and stained as described in Methods.

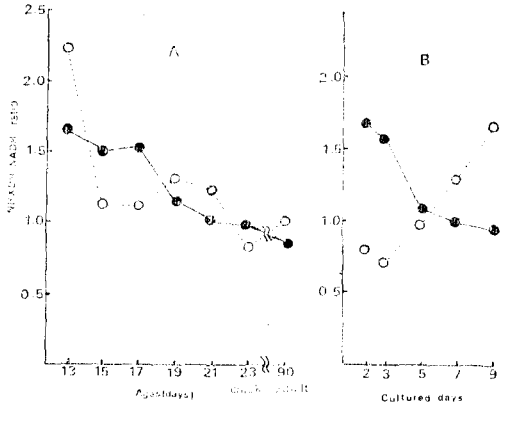

Fig. 10. The NHXDH/NADH ratio of lactic dehydrogenase of chick pectoralis muscle cells in embryo (A) and in culture (B). The ratio was measured for supernatant (——●——) and for pellet (……○……) of 100,000×G centrifugation.

days) while H_2M_2 first appeared at 3-day culture and HM_3 and M_4 at 5-day culture. Thus, all five types appeared only at 5-day cultured myoblasts.

If niacinamide hypoxanthine dinucleotide (NHXDH), an analog of niacinamide adenine dinucleotide (NAD), is used as coenzyme instead of NADH, H form gives higher activity than M form. Therefore, the ratio NHXDH/NADH reflects the subunit composition, the higher the ratio the more H subunit than M subunit in a single molecule of the enzyme. This ratio in the embryonic and in the cultured myoblasts is shown in Fig. 10. In the supernatant fraction of the embryo, the ratio tended to decrease from 13 days until hatching and adult. The ratio in the pellet also decreased in general as the embryo proceeded developing, but in this case the decrease was very sharp during the initial period and then rather slowly with wide fluctuations.

In the cultured myoblasts, the ratio in the supernatant fraction also decreased as the culture was prolonged; the decrease being abruptly during 2 to 5 days of culture and then slowly. The ratio in the pellet, on the other hand, showed an opposite change to that of supernatant; it increased steeply until 9 days of culture.

DISCUSSION

The morphological changes during the myoblast differentiation in culture observed in the present study (Fig. 1) are very similar to those which have been well established and widely observed. Many reports describing biochemical changes in association with the myoblast differentiation were based on this typical morphology and results described in the present paper therefore may be directly compared with previous reports.

The change in the protein composition during the myoblast differentiation (Figs. 2 and 3) suggests that most of muscle proteins are synthesized at relatively constant rate during development of myoblast into contractile muscle fibers. There were more than hundred proteins resolved in SDS polyacrylamide gel electrophoresis (Figs. 2, 4 and 6) and the concentrations of most of these proteins seemed to be essentially constant during the myoblast development. However, some "muscle specific" proteins showed changes in the concentration and in the rate of synthesis (Figs. 3 and 5) as myoblasts developed. The content of myosin during the first 2 or 3 days of culture was very low but increased after 3 days, while that of actin remained relatively constant in much higher concentration from the very early period of culture. This suggests that the synthesis of actin and hence the expression of actin genes in the myoblast is actively occuring far earlier than that of myosin. It may be speculated therefore that accumulation of actin to a certain degree in the myoblast induces the expression of genes for myosin. Devlin and Emerson (1978) reported that the contractile proteins were regulated coordinately during myoblast differentiation. A futher study is required to shed light on this speculation. Paterson and Strohman (1972) early suggested that the potential for myosin synthesis during myogenesis

and cell fusion potential should be developed sequentially. On the other hand, Moss and Strohman (1976) and Vertel and Fischman (1976) suggested that cell fusion was not a prerequisite for myosin synthesis and accumulation during myogenesis *in vitro*. Ha *et al.* (1979), however, presented results that myosin synthesis was greatly inhibited by inhibition of cell fusion with low medium calcium concentration. In the present experiment, the synthesis of myosin was very slow before cell fusion possibly suggesting that the fusion might trigger the myosin synthesis.

The content of myoglobin also increased gradually as the culture time elapsed and reached its maximum at about 4th day of culture, when it exceeded that of actin (Fig. 3). Because of the far abundant content of myoglobin during 4 to 6 days of culture, the relative concentration of actin during this period decreased (Fig. 3).

A muscle specific enzyme, creatine phosphokinase (ATP: creatine phosphotransferase) was first detected in SDS polyacrylamide gel electrophoresis at 3rd day of culture after which it remained rather constant in the concentration (Fig. 3). This is in well agreement with the finding of that the enzyme activity is sharply increased at about 2 days after cell plating (Turner *et al.*, 1976). Glyceraldehyde dehydrogenase appeared much later (at 6th day of culture) than other proteins mentioned above, indicating late expression of its gene(s).

The temporal consequence of the synthesis of other muscle specific proteins, tropomyosin and troponin, could not be analyzed in the present experiment because of their early synthesis in the myoblast. The incorporation of ^{35}S-methionine into tropomyosin and troponin was so intense from early period of culture as revealed in Fig. 6. Much shorter period of radioactive labeling of the culture seemed to be necessary for the analysis of their synthesis. Allen *et al.* (1978) reported that small amounts of tropomyosin were detectable during fusion and that as fusion approached a maximum tropomyosin accumulation began to increase. They also reported that the increased synthesis of tropomyosin after the initiation of muscle cell fusion was consistent with the increased synthesis of other proteins characteristic of muscle, including myosin. In our experiment, tropomyosin, both α and β, was radioactively labeled from the 3rd day of culture and remained rather constant thereafter, partially supporting their conclusion.

With regards to lactic dehydrogenase isozymes, Philip and Vesell (1962) reported that during embryonic development of the chick the activity of anodal migrating lactic dehydrogenase isozymes decreased as the activity of the cathodal isozymes increased and that in chick embryo cells grown in culture an isozyme pattern identical in all chick tissues developed within a few days and the intensity of the anodal bands decreased with relatively increasing intensity of the cathodal bands.

Data of the present study indicated that both in chick muscle cells during embryonic development and in culture the sequential alterations consisting of a redistribution of total enzyme activity among the isozymes occurred. As myoblasts in intact embryo and in

culture underwent differentiation the isozyme composition was gradually shifted in such a way that the M subunit was increased (Fig. 9). This tendency was also seen in the result of th measurement of NHXDH/NADH ratio during the development. Since the ratio reflects the subunit composition, the lower ratio indicating the more M subunit, the measurement of the ratio will provide the change in the isozyme activity. In the present study, this ratio both in embryonic and cultured myoblasts kept decreasing as the myoblasts aged, indicating more appearance of M subunit during the differentiation of myoblasts into contractile myofibrils. The pattern of this decrement was very similar in both systems.

SUMMARY

In order to study the temporal sequence of synthesis of several muscle-specific proteins during the differentiation of myoblasts, the composition of proteins was analyzed by one- and two-dimensional polyacrylamide gel electrophoresis in chick embryonic pectoralis muscle cells in culture. The lactic dehydrogenase activity and the isozyme composition were also analyzed during the myoblast differentiation.

Actin was present from the very initial stage of differentiation and the rate of its synthesis as determined by the incorporation of ^{35}S-methionine was relatively constant throughout the entire culture period (2-9 days). Myosin, on the other hand, was increased in its content abruptly from 3rd day of culture when the cell fusion was most remarkable and kept rather constant until later period of culture. Myoglobin appeared more and more as the myoblast develops and reached maximum at about 5th day of culture. Creatine phosphokinase appeared at 3rd day and glyceraldehyde dehydrogenase at 6th day of culture. Tropomyosin α and β and troponin C seemed to be synthesized from early period of differentiation in relatively constant rate.

The lactic dehydrogenase activity increased sharply from earlier period of culture (2-5 days) and then kept constant until 9th day of culture. The isozyme composition of this enzyme changed as the myoblasts differentiated into contractile muscle cells. Isozymes of H_4 and H_3M types were predominant in early myoblasts and HM_3 and M_4 types appeared much later. All five types of isozymes appeared only at 5th day cultured myoblasts.

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