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CORRELATION BETWEEN PROTEIN PHENOTYPE AND GENE EXPRESSION IN ADULT RABBIT FAST TWITCH MUSCLES UNDERGOING A FAST TO SLOW FIBER TRANSFORMATION IN RESPONSE TO ELECTRICAL STIMULATION *in vivo*[1]

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SUMMARY: In this report we have defined three distinct stages of the fast to slow transformation of muscle in terms of the myosin isoenzyme pattern in a non-denaturing gel system. In phase I a rearrangement of fast isoenzymes with no increase in slow isoforms took place. Phase II is characterised by a complex pattern of fast and slow isoenzymes and of isoenzymes of intermediate mobility. Phase III shows full conversion to the slow isoform with residual traces of fast and intermediate components. Changes in myosin light chains, as revealed by two dimensional gel analysis, showed good correlation with their corresponding mRNAs as determined by translation of extracted total RNA in a nuclease treated reticulocyte lysate cell-free system. This suggests that in the fast to slow transformation gene expression is regulated at the level of transcription.

Adult skeletal muscle containing fast and slow fiber types is characterised by the presence of distinct myofibrillar protein phenotypes(2). Fast type muscles can be readily transformed to slow type in response to low frequency electrical stimulation, leading to a sequence of changes in sarcoplasmic reticulum (3), an increase in oxidative enzymes on cryostat sections (4,5), a change in the regulatory proteins (6,7) and lastly the appearance of slow type myosin (8,9). Earlier studies with specific anti-myosin antibodies demonstrated the existence of both types of myosin within the same fibers, suggesting a reprogramming of gene expression within a single fiber rather than replacement of the fiber with another (7).During

[1] A preliminary report of this work was presented at the VII International Biophysics Congress and III Pan-American Biochemistry Congress, Mexico City, August 23-28th, 1981. (Ref 1)

ABBREVIATIONS:

TA-Tibialis anterior, EDL-Extensor digitorum longus, HEPES 4-(2-Hydroxyethyl) -piperazine ethanesulfonic acid.

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embryonic development muscle proteins are thought to pass through three distinct isoform stages; embryonic, neonatal and adult (for review see 10). In the newborn animal, the neonatal isoforms are replaced by adult type fast and slow proteins in response to development of the nervous system (11) and aquisition of activity patterns (12). Control of gene expression during these isoform transitions is thought to occur mainly at the level of transcription (11,12). However, a recent report has suggested that in the case of chick embryonic muscle the translation of the mRNA for LC_{3f} may be under additional cytoplasmic control (13).

We report here a correlation between changes in myosin isoenzymes and light chains on the one hand, and the level of translatable mRNA for myosin light chains in total extracted RNA, on the other hand, from muscles undergoing fast to slow transformation. This study suggests that gene expression in adult muscle is regulated mainly at the level of transcription.

MATERIALS AND METHODS

Materials: L-[^{35}S] methionine (Specific activity 800Ci/mmol) was purchased from New England Nuclear. Creatine phosphate, creatine phosphokinase (EC 2.7.3.2) and micrococcal nuclease (EC 3.1.31.1), GTP and ATP were purchased from Boehringer-Mannheim. Ampholines in pH ranges 3.5-10.0, 4-6, and 5-7 were purchased from LKB, ltd.

Methods: Rabbit (3-4 Kg, New Zealand White strain) extensor muscles eg TA and EDL were continuously stimulated via the peroneus nerve at 10 Hz for up to 7 weeks using surgically implanted electrodes as described earlier (14). Myofibrils were prepared from the contralateral control and stimulated muscles and myosin isoenzymes separated by electrophoresis under non-denaturing conditions in pyrophosphate-containing gels as previously reported (15,16,17). Two dimensional gel analysis of myofibrils and translation products was carried out as decribed by O'Farrel (18) with purified muscle proteins as markers. Total RNA was extracted with phenol/$CHCl_3$ (19) and prior to translation DNA and low molecular weight RNA were removed by washing with 3M Na-Acetate pH 5.5 (20). Total RNA was then translated at 30°C using a nuclease treated reticulocyte lysate (21) in a volume of 25µl containing the following; 0.15M K-Acetate, 0.75mM Mg-Acetate, 4mM Creatine phosphate, 4µg creatine phosphokinase, 25mM HEPES pH 7.6, a mixture of 19 unlabelled L-amino acids (-methionine) each 100µM, 5µM L-[^{35}] methionine (specific activity 800Ci/mmol), 40 %(v/v) nuclease treated reticulocyte lysate. Total RNA, up to 40µg per assay, gave linear incorporation for 2h at 30°C and stimulated the translational activity of the reticulocyte lysate 5-8 fold. Translation products were subjected to 2D gel analysis (18) with a pH 4-7 isoelectric focussing range in the first dimension and a 12-20 %linear gradient polyacrylamide gel in the second dimension. After staining and destaining (18) the gels were impregnated with Enhance (New England Nuclear), dried, then placed in contact with Kodak XAR-5 film at -70°C. The film was then developed after an exposure of 5-10 days. Radioactivity associated with specific proteins in the 2D gel was determined by liquid scintillation counting (22).

RESULTS AND DISCUSSION

Myosin isoenzyme patterns of myofibrils isolated from a series of animals stimulated for 3-7 weeks are presented in Figure 1. Three distinct phases characterise this transformation: After 3-4 weeks of stimulation there is a redistribution of fast isoenzymes (Fig 1, gel 2), with the slowest migrating isoform predominating (phase I). The difference in the mobility within fast isoenzymes reflects their alkali light chain composition (23). At this stage a small amount of slow isoenzyme is present which may represent the intrinsic slow, type I fibers present in these muscles prior to stimulation (Fig 1, gel 1). After 4-5 weeks there was a considerable increase in the content of slow isoenzymes (Fig 1, gel 3) together with the appearance of 2-3 isoform bands having mobility between those of fast and slow isoenzymes (phase II). The slower mobility of slow isoenzymes is a reflection of differences in the primary sequence between fast and slow myosin heavy chains (24). These

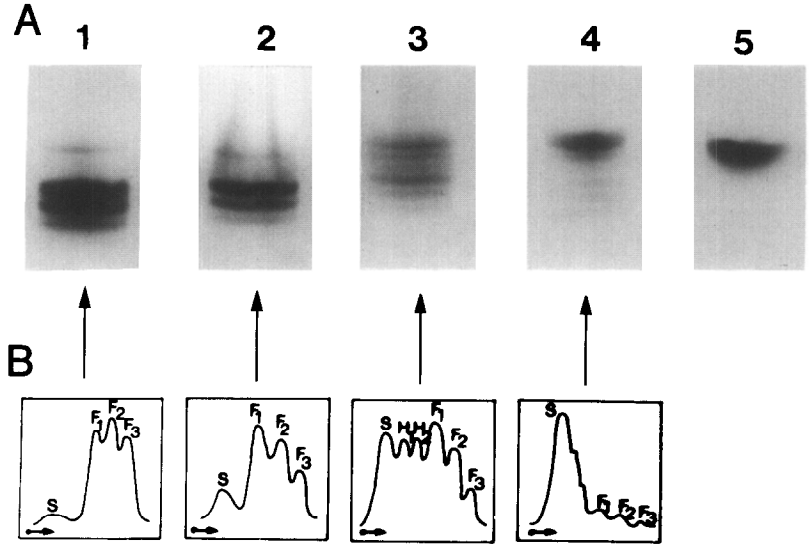

Figure 1
Pyrophosphate gel analysis of myofibrils from contralateral and stimulated muscles. Panel A: Myosin isoenzyme pattern of control and stimulated TA muscles; pyrophosphate gel electrophoresis. Myofibrils (10-20μg) were loaded onto a 0.5x10 cm gel containing 3.6 %acrylamide prepared as described earlier (16,17). Electrophoresis was carried out with buffer recirculation at 4°C for 24h at a constant voltage of 40V. The gels were then stained in 0.04% Coomassie brilliant blue (G-250) in 3.5 %perchloric acid (16). Gels; 1) control TA, 2) 3-4 weeks (phase I), 3) 4-5 weeks (phase II) and 4) 5-7 weeks (phase III) stimulated TA muscles. Gel 5 shows control soleus for comparison.
Panel B: Laser densitometric scan (LKB Ltd.) of the stained gels in the above series.

molecules are most probably combinations of fast and slow components which are present in the muscle fiber at this stage of transformation. After 5-7 weeks there was full conversion to the slow myosin isoenzyme (Fig 1, gel 4. phase III) which had the same mobility as control soleus (Fig 1, gel 5). At this stage there were still some residual traces of intermediate and fast isoenzymes which became undetectable after longer periods of stimulation (Results not shown).

We have also studied changes in myosin light chains by two dimensional gel analysis in Figure 2, and correlated them with the 2D gel distribution of

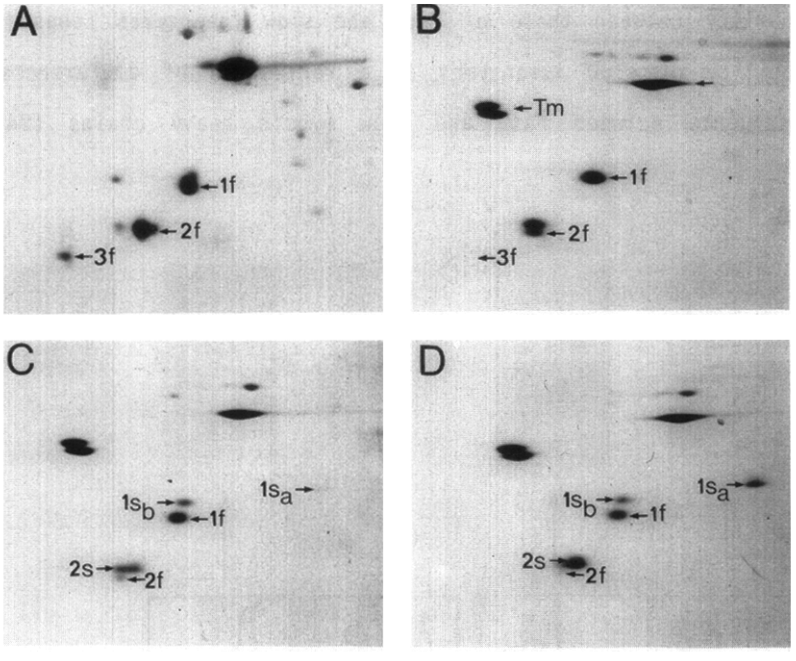

Figure 2
Two dimensional gel analysis of the light chain content of myofibrils prepared from normal and stimulated TA muscles. Myofibrils were dissolved in 9.5M urea, 2%(v/v)Nonidet P-40 containing 2%ampholines (0.4%pH 3.5-10, 0.8%pH 4-6, 0.8%pH 5-7) and loaded (100µg) onto a 0.3x10 cm gel prepared and prerun as described by O'Farrel (18). After 16h electrophoresis at 400v the voltage was raised to 800v for 1h. We used a total Vhr integral of 6832 using a Pharmacia power supply and Vhr integrator to acheive reproducible focussing conditions. A blank gel was then removed and cut into 2mm slices which were then placed in degassed 0.1M NaCL for pH measurement. A linear pH gradient was reproducibly obtained over a pH 4-7 range. The gel cylinders were then processed as described by O'Farrel (17) with a 12-20%linear gradient polyacrylamide gel in the second dimension. After electrophoresis the gels were stained with Coomassie brilliant blue and destained according to published procedures.Gels; A) control TA, B) 3-4 weeks (phase I), C) 4-5 weeks (phase II) and D) 5-7 weeks (phase III) stimulated TA muscles. Only the light chain area is included.

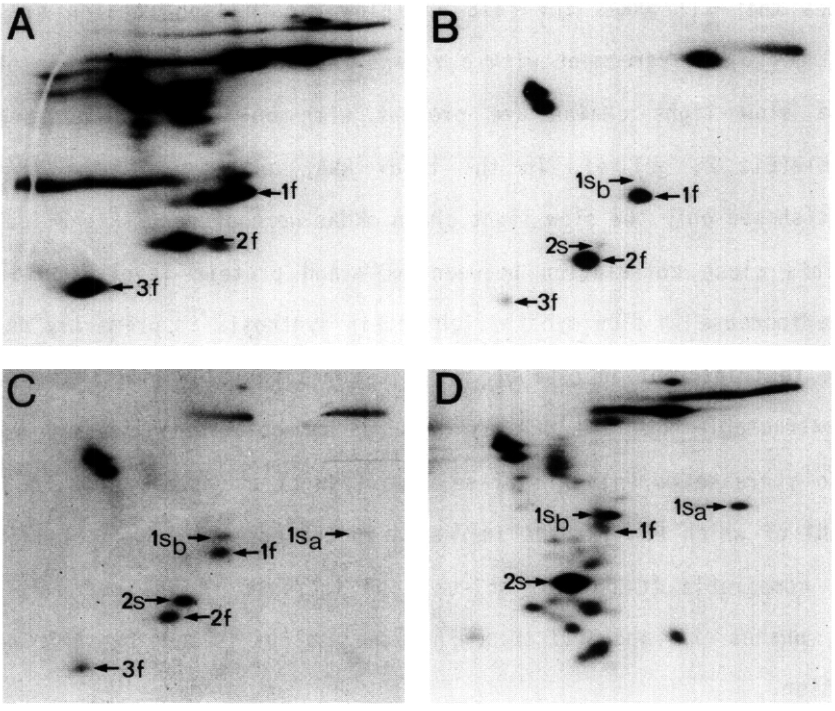

Figure 3
Two dimensional gel analysis of cell-free translation products of total RNA extracted from contralateral and stimulated TA muscles. Total RNA (10-20μg) was incubated in a 25μl assay system as described in Methods. [^{35}S] methionine labelled translation products (1-2x 10^{-5} cpm) were loaded onto isoelectric focussing gels as described in Fig 2 and separated by two dimensional gel analysis as described by O'Farrel (18). The destained gels after impregnation with autoradiography enhancer (NEN Ltd.) were processed for fluorography as described in the Methods. Gels; A) control TA, B) 3-4 weeks (phase I), C) 4-5 weeks (phase II) and D) 5-7 weeks (phase III) of stimulation.

proteins synthesized from total RNA translated in a cell-free system (Figure 3). In this comparison we have extracted total RNA which upon translation in a cell-free system yields labelled products which give a qualitative indication of the content of mRNAs in the total RNA sample. During phase I chiefly fast light chains are present with only trace amounts of slow light chains (Fig 2, gel 2). The distibution of the total RNA translation products closely parallels the protein picture at this stage (Fig 3, gel 2). As the transformation proceeds into phase II the level of fast light chains LC_{3f} and LC_{2f} is greatly reduced (Fig 2, gel 3) in relation to LC_{1f}. The slow light chains LC_{2s} and LC_{1sb} are now present with only trace amounts of LC_{1sa} detectable. At this stage analysis of the translation products

indicates that all mRNAs for fast and slow myosin light chains are present (Fig 3, gel 3) in agreement with a recent report (25). Finally, in phase III all the slow light chains are present with only trace amounts of LC_{1f} detectable(Fig 2, gel 4). In the total RNA, analysis of the translation products showed only the slow light chain mRNAs were present (Fig 3, gel 4).

This close correlation between mRNA and protein levels would suggest that the increase in slow myosin light chain synthesis is primarily due to the increase in transcription rate of specific genes for slow fiber type proteins. Total extractable RNA and DNA as measured by colorimetric assay were also found to increase during this phase, the results are summarised in Table 1. Total RNA of which 93 % is rRNA increased at the onset of phase II and reached a level comparable to control soleus. The increase in DNA was more gradual through phases II and III reaching the soleus value by 6-7 weeks of stimulation.

In conclusion, we have used the fast to slow fiber type transformation resulting from electrical stimulation in vivo to investigate regulation of gene expression in adult muscle. These results presented would suggest that after 4 weeks of stimulation there is an overall increase in the transcription rate and a selective activation of slow fiber type specific gene activity.

TABLE I Total extracted RNA and DNA of control and stimulated TA and EDL muscles

	RNA[1] (µg/g Muscle)	DNA[2] (µg/g Muscle)
Control[3]	190.8±30.7(8)[4]	136.0±13.1(8)
Stimulated		
Phase I[5]	276.3±28.6(4)	194.8±59.3(4)
Phase II	526.5±32.2(4)	251.0±18.2(4)
Phase III	548.3±12.6(4)	330.0±7.2(4)
Control Soleus	559.5±27.6(4)	375.0±10.4(4)

1) Total RNA was extracted from the above muscles as described in Methods. We assume E_{260} (1µg/ml, 1cm)= 0.025. 2) DNA was estimated by the colorimetric method described by Giles and Myers (26) using calf thymus DNA as a reference. 3) Values of TA and EDL muscles were pooled to give the above table. 4) Mean±standard deviation with number of animals shown in parenthesis. 5) Stage of transformation as judged by pyrophosphate gel analysis of myosin isoenzymes (see Fig1)

Isoforms of myosin of intermediate mobility between fast and slow presumably contain combinations of fast and slow components. These molecules are present during a phase in which the genes for all fiber type components are active and myosin molecules containing combinations of both component are assembled in vivo. The elucidation of the nature of the pathway linking electrical stimulation to the above increase in transcription will require further study.

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REFERENCES

1)Pluskal, M.G. and Sreter, F.A. (1981) Abstract VII International Biophysics Congress and III Pan-American Biochemistry Congress, Mexico City, August 23-28th, pg 178.
2) Jolesz, F and Sreter, F.A. (1981) Ann. Rev. Physiology 43: 531-552.
3) Ramirez, B.U. and Pette, D. (1974) FEBS lett 49: 188-190
4) Pette, D., Smith, M.E., Staudte, H.W. and Vrbova, G. (1973) Plugers Archiv. 338: 257-272
5) Romanul, F.C.A., Sreter, F.A., Salmons, S. and Gergely, J. (1974) in Exploratory Concepts in Muscular Dystrophy, ed A.T. Milhorat, pg 334.
6)Sreter, F.A., Romanul, F.C.A., Salmons, S. and Gergely, J. (1974) in Exploratory Concepts in Muscular Dystrophy, Ed A.T. Milhorat, pg 338.
7) Rubinstein, N.A., Mabuchi, K., Pepe, F., Salmons, S., Gergely, J and Sreter, F.A. (1978) J. Cell Biol. 179: 252-261
8) Pette, D., Staudte, H.W. and Vrbova, G. (1972) Naturwissenschaften 59: 469-470.
9) Sreter, F.A., Gergely, J., Salmons, S and Romanul, F. (1973) Nature 241: 17-19.
10) Bandman, E., Matsuda, R. and Strohman, R.C. (1982) Devel. Biol. 93: 508-518.
11)Strohman, R.C., Moss, P.S., MicouEastwood, J., Spector, O. and Przybyla, A. (1977) Cell 10: 265-273
12)John, H.A., PatinouGeorgoulas, M. and Jones, K.W. (1977) Cell 12: 501-508
13)Roy, R.K. and Sarkar, S. (1982) FEBS lett 149: 22-28.
14)Sreter, F.A., Pinter, K., Jolesz, F. and Mabuchi, K. (1982) Exptl. Neurol. 75: 95-102.
15)Sreter, F.A., Holtzer, S., Gergely, J and Holtzer, H.J. (1972) J. Cell. Biol. 55: 586-594.
16)Pinter, K., Mabuchi, K. and Sreter, F.A. (1981) FEBS lett 128: 336-338.
17)Mabuchi, K., Szvetko, D., Pinter, K. and Sreter, F.A. (1982) Am. J. Physiol. 242: 373-381.
18)O'Farrel, P.H. (1975) J. Biol. Chem. 250: 4007-4021.
19)Monson, J.M. and Goodman, H.M. (1978) Biochemistry 17: 5122-5128.
20)Palmiter, R.D. (1973) J. Biol. Chem. 248: 2095-2106
21)Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67: 247-256
22)Stockdale, F.E., Raman, N. and Baden, H. (1981) Proc. Natl. Acad. Sci. USA 78: 931-935.
23)D'Albis, A., Pantaloni, G. and Bechet, J.J. (1979) Eur. J. Biochem. 99: 261-272.
24)Lowey, S., Benfield, P.A., Silberstein, L. and Lang, L.M. (1979) Nature 282: 522-524.
25)Heilig, A and Pette, D. (1983) FEBS lett 151: 211-214.
26)Giles, K.W. and Myers, A. (1965) Nature 206: 93.