Regulation of myosin heavy-chain gene expression during skeletal-muscle hypertrophy

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Changes in the myosin phenotype of differentiated muscle are a prominent feature of the adaptation of the tissue to a variety of physiological stimuli. In the present study the molecular basis of changes in the proportion of myosin isoenzymes in rat skeletal muscle which occur during compensatory hypertrophy caused by the combined removal of synergist muscles and spontaneous running exercise was investigated. The relative amounts of sarcomeric myosin heavy (MHC)- and light (MLC)-chain mRNAs in the plantaris (fast) and soleus (slow) muscles from rats was assessed with cDNA probes specific for different MHC and MLC genes. Changes in the proportion of specific MHC mRNA levels were in the same direction as, and of similar magnitude to, changes in the proportion of myosin isoenzymes encoded for by the mRNAs. No significant changes in the proportion of MLC proteins or mRNA were detected. However, high levels of MLC3 mRNA were measured in both normal and hypertrophied soleus muscles which contained only trace amounts of MLC3 protein. Small amounts of embryonic and neonatal MHC mRNAs were induced in both muscles during hypertrophy. We conclude that the change in the pattern of myosin isoenzymes during skeletal-muscle adaptation to work overload is a consequence of changes in specific MHC mRNA levels.

INTRODUCTION

Vertebrate sarcomeric myosin heavy-chain (MHC) isoenzymes are encoded by a closely conserved multigene family [1–11], of which seven have been identified thus far at both the protein and gene level (reviewed in [1,11]). These include (i) embryonic, (ii) neonatal, (iii) adult fast IIA (fast oxidative), (iv) adult fast IIB (fast glycolytic), (v) adult slow skeletal/β-ventricular, (vi) α-cardiac and (vii) extraocular specific MHC.

Tissue-specific and developmental-stage-specific expression of members of this gene family occurs during muscle development [11–20]. Marked changes in the relative content of myosin isoenzymes also occur in adult skeletal- and cardiac-muscle tissues in response to changes in the hormonal environment [21–28], alterations in innervation and nerve activity [30–36], and imposition of artificial electrical stimulation [29–32,37]. In addition, myosin fast-to-slow transformations occur under more physiological conditions, such as muscle hypertrophy due to work overload, and endurance exercise training, where the nerve is left intact and the pattern of stimulation is not externally imposed [38–44]. The ability of adult muscle cells to modulate the properties of the contractile apparatus in response to environmental and physiological stimuli even after terminal differentiation illustrates their remarkable plasticity. However, little is known about the mechanism by which myosin isoenzyme transitions occur, particularly in differentiated muscle.

We have recently made a detailed investigation of the changes in skeletal-muscle myosin isoenzymes with overload hypertrophy induced by synergist removal and with running exercise [41]. This study showed dramatic alterations in the native myosin isoenzyme profile of the fast plantaris muscle and minor alterations in the slow soleus muscle. In the fast muscle, a transition from fast to slow myosin was observed with work overload hypertrophy with a predominant increase in intermediate (IM) or Type IIA myosin. In addition this transition appears to proceed in a Type IIB → IIA → I sequence, as noted for chronically stimulated fast muscles [37].

In order to investigate the molecular mechanisms by which these changes occur, we have analysed in the present study the expression of different MHC and MLC gene transcripts in plantaris and soleus by S1-nuclease mapping and RNA blot analysis, using the combined hypertrophy and exercise model as in our previous study [41]. Our aim was to assess the degree of correspondence of changes in the relative amounts of myosin isoenzymes and their specific mRNAs as a basis for deciding whether changes in myosin are due to regulation of transcriptional and/or translational events.

MATERIALS AND METHODS

Skeletal-muscle hypertrophy

Hypertrophy of the plantaris and soleus muscles was produced in female Sprague–Dawley rats by surgical removal of the synergist gastrocnemius muscle from both legs, under anaesthesia [41]. The surgical procedure was essentially as described by Baldwin et al. [38], but the soleus was not removed in our experiments. A group of control animals (n = 6) were killed at the beginning of the study to determine whether there were any age-related changes in myosin isoenzymes and MHC mRNA during the course of the study.

After surgical recovery the animals were placed in exercise wheels in which they ran voluntarily. The animals
lived in the wheel continuously and had access to food and water without leaving the wheel. A group of eight animals were killed after 4 weeks and a second group (n = 8) after 11 weeks of running. Control animals (4 weeks, n = 8; 11 weeks, n = 8) were maintained in group of four on a 12 h-light/12 h-dark cycle with access to food and water ad libitum. The soleus and plantaris muscles were dissected from each experimental group, frozen in liquid nitrogen, weighed, and stored at −70°C for later analysis.

Pyrophosphate/polyacrylamide-gel electrophoresis

Methods for separation of myosin isoenzymes by pyrophosphate/polyacrylamide-gel electrophoresis were essentially as described in [41,44].

RNA isolation and Northern-blot analysis

Total cellular RNA was isolated from pooled plantaris and soleus muscles by hot phenol extraction, followed by guanidinium chloride precipitation as described in [45]. Northern (RNA) blotting was carried out as described in [12,46] with formaldehyde as denaturing agent.

S1-nuclease mapping analysis

S1-nuclease mapping was performed using a modification of the Berk & Sharp technique [47]. Hybridization probes were 3'-end-labelled with [α-32P]-cysteicin (New England Nuclear, Boston, MA, U.S.A.) using terminal transferase (see the Figure legends). After size separation by polyacrylamide-gel electrophoresis, the desired cDNA fragments were strand-separated and purified [48]. The probes were hybridized in DNA excess to 20 μg of total RNA extracted from the different muscle tissues. Hybridization was carried out in 25 μl of 80% deionized formamide, 0.4 M-NaCl, 10 mm-Pipes, pH 6.4, 1 mm-EDTA and 0.05% SDS. The hybridization mixture was incubated at 65°C for 1 h, the temperature reduced to 42°C, and incubation allowed to continue for 20 h. A portion (150 units) of S1 nuclease (New England Nuclear) in 300 μl of 200 mm-NaCl/30 mm-sodium acetate(pH 4.5)/3 mm-ZnSO4 was added to each sample and incubated at 30°C for 1 h. The reaction was terminated with 10 mm-EDTA and DNA was precipitated with ethanol and 0.3 m-sodium acetate, pH 5.5. Dried pellets were resuspended in 85% (v/v) formamide and electrophoresed on 7%- (w/v)-polyacrylamide sequencing gels in the presence of 8.3 m-urea [48].

Two-dimensional electrophoresis

Two-dimensional gel electrophoresis was performed as described by O'Farrell [49], with Bio-Rad ampholytes at 3% (v/v), pH 5–7, and at 2% (v/v), pH 3–10; Bio-Rad Laboratories, Richmond, CA, U.S.A.) in isoelectric-focusing gels. The second-dimension SDS/polyacrylamide gels contained 12.5% acrylamide, which is optimal for revealing myosin light chains. The gels were stained as previously described [44].

RESULTS

Expression of the fast IIA MHC gene is increased in the fast-twitch plantaris and decreased in the slow-twitch soleus during work-overload hypertrophy

The MHC gene represented by cDNA probe pMHC 40 is expressed in all adult fast skeletal muscles containing fast Type IIA and IIB fibres as well as in slow muscles (e.g. soleus) which contain a small proportion of type IIA fast fibres [9,11,27]. Because of its predominant expression in type IIA or fast oxidative fibres in rat, the probe (pMHC 40) has been designated the ‘fast IIA MHC gene’ [11,27].

To analyse the changes in the pattern of fast IIA MHC gene expression, two different probes were prepared from the cDNA pMHC 40 and hybridized to total RNA from control and hypertrophied muscles of plantaris and soleus. The first probe (Probe A; Fig. 1a) was 360 nt long, single-stranded and contains codons for amino acids 1764–1884 of MHC. S1-nuclease mapping analysis of these samples revealed full protection of the 360-nt probe in all samples, but the relative amounts hybridized were different in the hypertrophied muscle samples (Fig. 1a). In plantaris, the relative amount of fully protected fragment (fast IIA MHC) increased +40% and +60% by 4 weeks and 11 weeks of hypertrophy respectively from their control levels (Table 1). In contrast, the amount of fast IIA MHC mRNA in the soleus decreased significantly, −65% and −85% by 4 weeks and 11 weeks of hypertrophy respectively (Fig. 1a).

Because of the high degree of sequence conservation in the coding region of the probe, a number of partially protected fragments were produced as a result of hybridization with other MHC mRNA (fast IIB, slow MHC). A shorter fragment of about 75 nt corresponds to the β-cardiac/slow skeletal MHC [24] and was the major band in soleus RNA samples (Fig. 1a). Data on other partially protected fragments could not be interpreted, as some of the bands may be artefacts of incomplete S1-nuclease digestion of hybrids formed with partially homologous MHC mRNA. This S1-nuclease pattern is consistent, and the complexity results from highly conserved MHC mRNA isoforms [11].

To validate our observations with Probe A we repeated the S1 mapping analysis with the 3'-end of fast IIA MHC cDNA (Probe B). This probe contains the codons for the last 52 C-terminal amino acids of MHC (156 nt), the entire 3'-non-coding region (124 nt), a short stretch

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>mRNA*</th>
<th>Protein†</th>
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<tr>
<td>4</td>
<td>11</td>
<td></td>
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<tr>
<td>Fast IIA</td>
<td>+40</td>
<td>+60 1M†</td>
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<tr>
<td>Fast IIB</td>
<td>−10</td>
<td>−40 FM§</td>
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<tr>
<td>Slow//MHC</td>
<td>+245</td>
<td>+310 SM</td>
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* Mean percentage changes in MHC mRNA levels (per unit of RNA) were determined by densitometry scanning of multiple autoradiograms of S1-nuclease mapping experiments carried out using both coding and 3'-non-coding probes.
† Abbreviations: 1M, intermediate myosin; FM, fast myosin; SM, slow myosin.
§ Calculated from data in Table 2 of [41].

Table 1. Percentage changes in MHC mRNA and proteins in hypertrophied rat plantaris muscle
of poly(A) and vector sequences. The estimated full protection of the 280–300 bp probe was observed in most RNA samples (Fig. 1b). The smears pattern of the fully protected band results from SI-nuclease ‘nibbling’ of unstable A–T-rich regions of the hybrid formed with the poly(A) segment of the mRNA [9,11]. Densitometry scanning analyses indicated that the changes with hypertrophy in the fast IIA MHC mRNA level of the plantaris (Table 1) and soleus (Fig. 1a and see above) were of the same magnitude as observed with Probe A. A
Fig. 1. Effect of skeletal-muscle hypertrophy on the expression of MHC mRNA

S1-nuclease mapping analysis was performed with total RNA from plantaris and soleus muscles of control and experimental rats. The RNA samples were from: C.O.W, controls ‘0’ week [4-week-old young adults (see the Materials and methods section)]; C.4W, 4-week laboratory controls; H.4W, 4-week hypertrophy; C.11W, 11-week laboratory controls; H.11W, 11-week hypertrophy. S1-nuclease mapping analysis was performed as described in the Materials and methods section using five different MHC cDNA probes. (a) Fast MHC IIA (PMHC 40) Probe A; (b) fast oxidative MHC IIA (PMHC 40) Probe B; (c) fast glycolytic MHC IIB (PMHC 62); (d) slow/βMHC (cMHC 5); (e) embryonic MHC (PMHC 25); (f) neonatal MHC (PFOD 5).

The schematic diagram under the Figures illustrates the origin and length of each probe used. The size markers were generated from 32P-labelled φX HaeIII digests. Abbreviations: N.B, newborn; Ad, Adult; Sk, skeletal.

- Partially protected fragment, ~ 125 nt long, corresponds to the slow/βMHC isoform (Fig. 1b). This MHC mRNA increased several-fold in the hypertrophied plantaris (Table 1) and also increased in the soleus as a function of both age and hypertrophy of the muscle (Fig. 1b). Thus the data obtained with both probes are in close agreement and indicate that the expression of fast IIA MHC mRNA is regulated in opposite directions in the fast-twitch plantaris (increase) and slow-twitch soleus (decrease) with the imposition of work overload.

- Fast IIB MHC gene expression is decreased in hypertrophied plantaris and soleus muscles

The cDNA pMHC 62 has been shown to encode the fast IIB MHC mRNA. This fast IIB MHC gene begins to be expressed late in development and is retained only in adult muscles containing fast IIB (fast-glycolytic) fibres [11].

Expression of the fast IIB MHC gene was studied by using a 213-nt probe from the coding region (amino acids 1795–1865) of the MHC gene. S1-nuclease mapping analysis (Fig. 1c) revealed a fully protected 213-nt fragment, indicating that this gene is expressed at high levels in the plantaris and low levels in the soleus. In hypertrophied plantaris the relative amount of fast IIB MHC mRNA decreased −10% and −40% by 4 weeks and 11 weeks of hypertrophy respectively (Table 1). Changes in fast IIB MHC expression in the soleus muscle were difficult to estimate, owing to its low-level expression.

A short, partially protected, fragment approx. 75 nt long corresponds, on the basis of nucleotide conservation, to fast IIA MHC [11]. This gene transcript increased in hypertrophied plantaris, as shown with both fast IIA MHC probes (Figs. 1a and 1b).

Thus the data obtained clearly demonstrate that the fast IIB and fast IIA genes are regulated in the opposing directions to the hypertrophy stimuli. The changes observed in the IIA and IIB mRNA levels were qualitatively similar to changes in the corresponding myosin protein isoforms detected on pyrophosphate gels (Table 1).

- Slow/βMHC gene expression is induced in hypertrophy in both plantaris and soleus

The predominant MHC isoform expressed in the soleus muscle is the slow/βMHC, whereas in the fast plantaris muscle the slow/βMHC comprises only about 5% of the total myosin population.

To detect changes in the slow MHC mRNA level that
occur during work-overload hypertrophy, we used a cDNA clone, CMHC-5, identified as β-cardiac slow skeletal MHC [4,11,24]. The probe used was 347 nt long, (Fig. 1d) maps at the 3'-end of the cDNA and contains the codons for amino acids from 1865 to the C-terminal end and the 3'-untranslated region, including the poly(A) tail and the poly(dG) cloning linker.

A fully protected fragment ~310 nt long was observed in all muscle samples (Fig. 1d). Densitometric scanning analysis revealed that expression of the slow/β-MHC gene increased +245% and +310% in 4 weeks and 11 weeks hypertrophied plantaris muscles respectively (Table 1). The expression of slow/β-MHC was also increased, to a lesser extent in the hypertrophied soleus muscle, 35% and 27% after 4 and 11 weeks respectively. Similar changes were found when probes from the coding region were used. These results were in close agreement with the increases observed in the partially protected fragments corresponding to slow/β-MHC identified with the fast IIA MHC probes (Fig. 1a and 1b). Thus the expression of the slow/β-MHC gene transcript is increased in both fast and slow skeletal muscles during hypertrophy.

Induction of embryonic and neonatal MHC isoforms during skeletal-muscle hypertrophy

We were interested in investigating whether the myosin transitions in adult skeletal muscle subjected to work overload involved the expression of embryonic or neonatal MHC genes which normally are expressed only transiently during the muscle differentiation and maturation.

The MHC gene mRNA transcript represented by cDNA probe pMHC 25 is expressed throughout embryonic life and continues to be present in neonatal life [13]. However, by 2 weeks of postnatal life, this gene is deinduced and is not detectable in adult plantaris and soleus muscles of the rat. The probe for this gene used in the present study was 220 nt long [including the 16-nt poly(G) linker] and contains the codons for amino acids 1805–1870 of the MHC protein. S1-nuclease mapping analysis using this probe yielded full-length protection of the probe (194 nt) in RNA isolated from differentiated L6E9 cells (rat muscle cell line) and from 20-day-old fetal skeletal muscle in which the embryonic MHC gene is expressed (Fig. 1e). To our surprise, the embryonic MHC gene transcript (194 nt) was induced in the samples of hypertrophied soleus and plantaris muscles, albeit at a relatively low level. A partially protected fragments(s) of ~72 bp observed is due to strong homology of the probe with IIA and IIB MHC mRNA, as determined from their nucleotide sequences.

To detect expression of the neonatal MHC gene, an internal coding endonuclease-PstI fragment (amino acids 1794–1865) 213 nt long from cDNA clone pFOD5 [12] was used as a probe. Expression of this neonatal MHC is restricted to late fetal life up to 4 weeks postnatal life, and is not detectable in most adult muscles [11,12,27]. The expression of this MHC mRNA, detected by a fully protected fragment ~210 nt long, was observed with RNA from fetal skeletal muscle and to a similar level in hypertrophied soleus muscles, but not in control soleus (Fig. 1f). Expression also was detectable to a lesser extent in hypertrophied plantaris samples. To confirm the expression of the neonatal MHC mRNA in the hypertrophied plantaris, the S1-nuclease mapping was repeated with several different fragments of the cDNA clone. The results (not shown) were identical and showed a low, but detectable, level of neonatal MHC mRNA. These results suggest that the skeletal muscles subjected to work overload induced low amounts of the development-stage-specific isoforms.

Pyrophosphate/polyacylamide-gel electrophoresis of MHC proteins

We carried out an analysis of the myosin proteins of control and hypertrophied muscles in order to evaluate the significance of the observed changes in MCH mRNA levels. Myosin isoenzymes from portions of the same muscles used in the RNA analysis were examined in their native state by using non-denaturing pyrophosphate/polyacrylamide-gel electrophoresis. This method separates isoenzymes composed of different heavy chains as well as those formed by combinations of heavy chains with different light chains [41,44].

The results of this analysis were the same as that reported previously [41] and for comparison are expressed as a percentage change with hypertrophy in Table 1. The IM band (fast IIA) increased by ~70% and 50% after 4 and 11 weeks of hypertrophy respectively relative to controls, values similar to the increases in fast IIA mRNA (+40% and +60%). A good correlation between FM myosin bands and Fast IIB mRNA level was also observed. Slow myosin increased markedly, as did slow mRNA, but there was less correspondence in the changes as was found with the protein and mRNA of the other myosins (Table 1). In the soleus the IM band was decreased (result not shown), as was IIA mRNA (Fig. 1a). The proportion of slow myosin was correspondingly increased, as was its mRNA.

![Fig. 2. Expression of MLC mRNAs during skeletal-muscle hypertrophy](image-url)

A portion (10 μg) of total RNA from plantaris and soleus muscles were size-fractionated on 1% (w/w) agarose/3% (v/v) formaldehyde denaturing gels, transferred to nitrocellulose filters and hybridized to nick-translated [46] cDNA probes of MLC1, MLC2, and MLC3, [51,52]. The RNA samples were the same as in Fig. 1. Abbreviations: Hypert., hypertrophy; W, weeks.
**DISCUSSION**

Our aim was to determine if changes in the proportion of myosin isoenzymes that occur during muscle hypertrophy are reflected in changes in the levels of the specific mRNAs which code for the component heavy chains. Since myosin heavy isoforms are encoded by a highly conserved multigene family, we chose to use a very discriminative S1-nuclease mapping technique to assess relative MHC mRNA levels. We intended originally to conduct both RNA and protein analyses for individual muscles from each animal. However, there was insufficient tissue, and, accordingly, muscles from each experimental group were pooled and multiple analyses were performed on each group. The disadvantage of this experimental design is that it is not possible to express a measure of the variability of the data. We have previously reported a detailed analysis of the variability of changes in myosin isoenzyme levels in response to hypertrophy using the above model [41].

Our study demonstrates that changes in different MHC mRNA levels during skeletal-muscle hypertrophy parallel those of the corresponding isoenzymes, suggesting that mRNA abundance is of major importance in determining the relative contents of individual MHCs. A similar correlation between changes in α- and β-MHC isoenzyme content and mRNA levels has been observed in the heart muscle [24,53–55]. Two fast MHC isoforms, IIB MHC (fast glycolytic; most abundant), IIA MHC (fast oxidative) and a small amount of slow myosin (5% of the total) are expressed in the plantaris of the normal rat. When subjected to work overload, this muscle undergoes a substantial increase in size (hypertrophy), and a fast-to-slow myosin transformation occurs [41]. Whether this transformation occurs at the single-fibre level has not been demonstrated. However, the results presented here show a redistribution of the entire isomyosin profile and MHC mRNA population, and indicate that most of the muscle fibres must be involved. Furthermore, the increase in slow myosin is accompanied by an increase in fast IIA MHC and accompanying decrease in fast IIB MHC, suggesting that the expression of fast IIA myosin is an important intermediate step in the conversion from fast to slow myosin, as noted in chronically stimulated fast muscles during fast-to-slow transformation [37]. An obligatory intermediate step in fast-to-slow conversion suggests a defined genetic programme for myosin gene switching during fibre transformation. The results of the present study also provide evidence, albeit circumstantial, that intermediate myosin is the product of the fast IIA gene.
since the changes in fast IIA mRNA levels are of the same magnitude and direction as changes in the intermediate-myosin protein level (Table 1 and [41]).

It is known that fetal isoforms of actin, tropomyosin and myosin (βMHC) are expressed in cardiac-muscle hypertrophy [23,24,58]. Thus it was also important to know whether the transformations observed with hypertrophy in the present study involved the re-expression of embryonic and neonatal MHC isoforms and, in particular, whether existing fast fibres de-differentiate and subsequently follow a normal differentiation programme before finally expressing slow myosin. The SI-nuclease mapping results indicate that a small amount of embryonic and neonatal MHC RNAs were induced during work overload hypertrophy, especially in the soleus muscle. These results are puzzling, particularly in the absence of evidence that the corresponding proteins are synthesized. However, low amounts of embryonic and neonatal isoforms may not have been detected by the gel techniques we used [41]. Because our analyses were of whole muscle rather than of single fibres, we do not know whether those fibres which undergo fast-to-slow transformation express these developmental MHC isoforms. Given the extreme change in contractile activity during work overload, it is conceivable that some existing fibres were lost and replaced by new ones. Thus an alternative explanation is that (i) these genes are expressed in new fibres formed from satellite cells [56], either to replace those that are lost due to muscle damage or to support the increased functional demands, and (ii) that these fibres express the developmental MHCs during their differentiation process. In light of the small amount of embryonic and neonatal mRNA observed, we believe the latter explanation is the most likely. In any event, the expression of developmental MHC isoforms during overload hypertrophy requires further investigation in order to determine their physiological significance.

A number of hormonal and other factors are known to affect MHC gene expression differentially in skeletal muscle [21–28,31,32]. Of particular relevance to our studies are the results of Izumo et al. [27], who showed that the same myosin heavy-chain gene can be regulated by thyroid hormone in opposite directions, depending on the specific muscle in which the gene is expressed. Thus, during skeletal-muscle hypertrophy, we found the expression of the fast IIA MHC gene is regulated in opposite directions in the fast plantaris and slow soleus muscles. The slow/βMHC gene, however, is up-regulated in both muscle types. This indicates that differences in local environment are important, or more likely, that individual muscles respond differently to common signals.

Our studies indicate that there are no marked alterations in the level of fast MLCs in hypertrophy muscles, even though slow MLCs increased. Thus the disappearance of type IIB myosin on native gels during hypertrophy of plantaris cannot be due solely to changes in light-chain ratios. It may be that MLC transitions in overload hypertrophy are slower to occur than the MHC transitions, as has been found in skeletal muscle in response to chronic electrical stimulation [59]. In the present study we have observed significant expression of MLC3, mRNA in both normal and hypertrophied soleus muscle, although two-dimensional gel analysis does not reveal a comparable level of the MLC3 protein. It would appear that the expression of this protein may be regulated at the level of mRNA translation [57].

In summary we interpret our results to indicate that: (1) changes in isomyosin composition during muscle adaptation to work overload are caused by changes in the relative mRNA contents coding for each of the isoenzymes; (2) the pattern of expression of an individual MHC gene in response to skeletal-muscle hypertrophy and exercise is controlled by the phenotype of the muscle and/or its immediate environment; (3) skeletal-muscle hypertrophy induces the expression of the genes for embryonic and neonatal MHC isoforms; and (4) the disproportionate levels of MLC3, mRNA and protein in the soleus muscle suggest that translational control mechanisms may be involved in regulating the expression of this gene.

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