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Reverse transcriptase-polymerase chain reaction detects induction of cardiac-like α myosin heavy chain mRNA in low frequency stimulated rabbit fast-twitch muscle

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Abstract Using reverse transcriptase-polymerase chain reaction we quantified in rabbit skeletal muscles expression levels of the highly homologous cardiac α and β myosin heavy chain (αMHC, βMHC) mRNA isoforms. Masseter muscle displayed highest levels of a cardiac-like αMHC mRNA. This isoform was present at 20-fold lower amounts in slow soleus and at 200-fold lower levels in several fast-twitch muscles. Low-frequency stimulation periods exceeding 20 days drastically induced the αMHC mRNA in fast tibialis anterior. The αMHC mRNA was 140-fold elevated after 60 days when βMHC mRNA had increased 50-fold. Our results demonstrate the wide distribution of a cardiac-like αMHC mRNA in skeletal muscle and its marked induction during fast-to-slow transition as induced by low-frequency stimulation.

Key words: Cardiac-like α myosin heavy chain; Chronic low-frequency stimulation; Rabbit skeletal muscle; Reverse transcriptase-polymerase chain reaction

1. Introduction

Rabbit skeletal muscles are composed of three fast fiber types (IIB, IID, IIA) and the slow type I fibers, characterized by the fast-type myosin heavy chain (MHC) isoforms MHCIIb, MHCIId, MHCIIa and the slow MHCI [1,2]. The latter isoform is thought to be identical with the cardiac βMHC [3], the major isoform in rabbit cardiac ventricle [4]. Contrary to the distribution of βMHC in cardiac ventricle and slow skeletal muscles, the expression of the cardiac αMHC isoform in non-cardiac muscles seems to be limited to special muscles and fibers. To date, the presence of cardiac-like αMHCs has been demonstrated in masticatory and extraocular muscles [5–8], in the diaphragm [9] and in intrafusal fibers [10–12]. A previous immunocytochemical study provided evidence that an αMHC-like isoform is expressed in fast-twitch rabbit muscle under the influence of chronic low frequency stimulation (CLFS) [9], transforming a fast-twitch into a slow-twitch muscle with sequential transitions in MHC isoform expression in the order of MHCIIb → MHCIId → MHCIIa → MHCI [13]. The present study addresses the question as to the assignment of αMHC expression in this sequence. For this purpose, we applied the reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of a sequence specific to αMHC mRNA. Amplifying this sequence, as well as a sequence specific to the βMHC isoform, we studied the distribution of the two MHC isoforms in various muscles. The detection of α-skeletal actin mRNA served as a control of the method. We show the presence of an αMHC-like isoform at low concentrations in several skeletal muscles of the rabbit and demonstrate its pronounced induction during CLFS-induced muscle transformation.

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Abbreviations: ADM, adductor magnus; CLFS, chronic low frequency stimulation; DIA, diaphragm; EDL, extensor digitorum longus; GAS, gastrocnemius; MAS, masseter; MHC, myosin heavy chain; RT-PCR, reverse transcriptase-polymerase chain reaction; SOL, soleus.

2. Materials and methods

2.1. Animals, chronic stimulation, muscles

Adult male White New Zealand rabbits were used. Aductor magnus (ADM), diaphragm (DIA), extensor digitorum longus (EDL), gastrocnemius (GAS), masseter (MAS), psoas (PS), and soleus (SOL) muscles were excised and frozen in liquid nitrogen. Chronic low frequency stimulation (CLFS, 10 Hz, 12 hours daily) of tibialis anterior (TA) muscle was performed as described [14]. Three animals were used for each time point (0d, 6d, 14d, 21d, 30d, 60d).

2.2. Preparation of total RNA and oligonucleotide primers

Total RNA was prepared [15,16] and concentrations spectrophotometrically assessed. To amplify sequences specific to α-skeletal actin mRNA [17] and βMHC mRNA, primers were used [18], yielding PCR products of 367 nt and 173 nt length, respectively. For the detection of αMHC mRNA a primer pair was chosen from the 3′-terminal sequence (GenBank accession no. K01867 [19]) to amplify a 227 nt fragment extending from the translated into the untranslated region (Fig. 1). The primer sequences were (a) antisense: TTGCGGGTTAACAAGAGCGG, and (b) sense: TCAAGGCCTACAAGCGCCAG. For controlling the isoform specificity, an additional antisense primer CTGGGCGGATCAAGGCGTCA was chosen for the βMHC mRNA. This primer was derived from an unpublished sequence generously supplied by Dr. R. Zak, Chicago. It corresponds to an independently published sequence [20]. The 5′-ends of the antisense primers of all three sequences were labeled with digoxigenin to allow chemiluminescent detection of PCR products.

2.3. Reverse transcription-polymerase chain reaction

0.2 μg of total RNA were reverse-transcribed according to [21] as previously described [17], using Avian Myoblastosis Virus reverse transcriptase and primer $p(dT)_{15}$ (Boehringer). In order to establish comparable conditions for cDNA synthesis the reaction mixture was: 1 μl aliquots of the RT assay (corresponding to 10 ng of total RNA) were amplified separately for each of the three sequences [17]. The optimized $MgCl_2$ concentrations were 2 mM for αMHC and α-skeletal actin, and 3.5 mM for βMHC. For amplification the following conditions were chosen: denaturation at 94°C, annealing at 60°C and synthesis at 72°C. The number of cycles was adusted to the exponential range of amplification [16]. It was 14 for α-skeletal actin and varied from 16 to 25 for the MHC sequences according to their expression levels. To allow quantitative analyses of gene expression, the amplification reactions were monitored using DNA standards. Therefore, PCR fragments were purified from primers, nucleotides and nonspecific reaction products using the QIAEX DNA gel extraction procedure (QIAGEN) after

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electrophoretic separation of amplification assays on 1.5% agarose gels. The purified DNA was spectrophotometrically quantified, and known amounts of each sequence were amplified in parallel to cDNA aliquots derived from reverse-transcribed RNA. The calculated copy number of standard DNA fragments was 1.1×10^6 for actin and 1.1×10^4, 1.1×10^5 and 1.1×10^6 for αMHC and βMHC.

2.4. Product analysis and quantitative evaluation

Identity and specificity of the PCR products were verified by restriction fragment analysis after 30 cycles. Digestion products were separated electrophoretically and visualized by ethidium bromide staining. For quantitative analysis, 2 μl of each PCR assay were combined and separated electrophoretically. Product anaylsis by digoxigenin-based chemiluminescent detection was performed as described [16]. The photographically documented signal intensities were evaluated desitometrically. Quantitative analyses of gene expression was achieved by comparing the results obtaind from RNA preparations with those obatined from the corresponding DNA standards. For the estimation of specific mRNA molecule numbers the efficiency of first-strand cDNA was calculated from data previously obtained by quantitative RT/PCR using cRNA standards [16].

3. Results and discussion

3.1. Amplification of a sequence specific to cardiac αMHC mRNA

The highest degree of sequence homology among the sarcomeric MHC isoforms most likely exists between αMHC and βMHC [22]. To distinguish the transcripts of these two isoforms by RT-PCR, the sequence specificity of the primers and the amplified DNA fragments had to be demonstrated. For the αMHC mRNA we chose a primer pair amplifying a 227 nt sequence from the 3′-terminal part extending from the translated into the untranslated region, characterized as highly variable between different isoforms [23] (Fig. 1). This sequence is identical with the recently cloned αMHC sequence EOM2, identified in extraocular muscles [7]. In agreement with previous findings [5,6], strong signal intensities were observed for this sequence in cardiac atrium and masseter muscle. A weaker signal was also detected in the ventricle (not shown), known to contain a developmentally regulated fraction of αMHC in combination with βMHC [24]. In addition, a strong signal was obtained with RNA preparations from low-frequency stimulated (60d) fast-twitch tibialis anterior muscle. The possibility that this signal resulted from unspecific cross reaction with the βMHC RNA was highly improbable because soleus muscle, rich in βMHC, yielded a strong signal for the βMHC sequence but only a faint signal for the αMHC (Table 1). Control assays in the absence of reverse transcriptase yielded no amplification products.

In order to verify isoform specificity, we compared the selected αMHC sequence with the corresponding part of the βMHC sequence (Fig. 1). The comparison indicated that the antisense primer is specific to the αMHC, whereas the sense primer is identical for both isoforms. To test whether the primer specificity was reliable under the applied conditions of amplification, we selected an additional βMHC-specific antisense primer to amplify a corresponding 210 nt fragment of the βMHC that should include an additional *Alu*I restriction site (Fig. 1a). Based on this sequence data, the PCR fragment derived from the βMHC isoform could be distinguished from the corresponding αMHC sequence by four instead of three restriction fragments of predictable length. Fig 1. shows that this was the case for the βMHC-specific fragments obtained from SOL and stimulated TA muscles (Fig. 1b, lanes 1, 2 undigested and lanes 3, 4 digested) and also for the αMHC-specific fragments, amplified from MAS and stimulated TA muscles (Fig. 1b, lanes 5, 6 digested and lanes 7, 8 undigested). Moreover, the restriction fragment pattern of the βMHC and αMHC sequences were identical in normal and stimulated muscles. The completenes and purity of the digestion assay demonstrated the isoform specificity of the amplified PCR products. The specificity of the previously characterized 173 nt fragment [18] used for the detection of the βMHC transcripts was re-insured by the same approach. The corresponding αMHC sequence was distinct by a unique *Pst*I restriction site, while the 173 nt βMHC fragment was not digested (not shown). The unequivocally established isoform specificity of the amplified αMHC and βMHC sequences was a prerequisite for their separate detection. Nevertheless, it seemed justified to assign this mRNA not as αMHC but as cardiac-like αMHC because its identification was based only on a restricted region of the molecule.

In our view, the RT-PCR technique is superior to other methods such as S1 nuclease mapping or in situ hybridization for the distinction of highly homologous mRNA isoforms. Obviously, the sequence specifity is more reliable with the use of short primers instead of long hybridization probes. Due to the high sequence homology between αMHC and βMHC it should be considered that previous data on the expression of βMHC

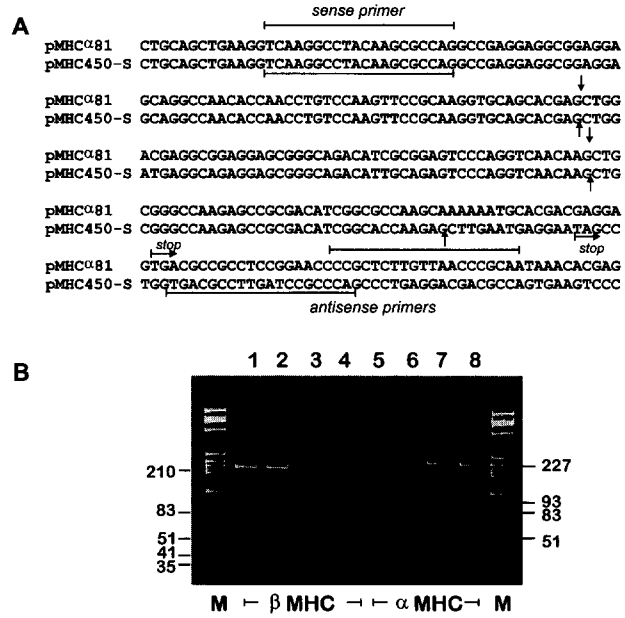

Fig. 1. Comparison and isoform specificity of the sequences specific to rabbit αMHC and βMHC mRNAs. (A) presents a sequence comparison of two cDNA clones, pMHCα81 (bases 1–250) specific to αMHC, and pMHC450-S (bases 109–358) specific to βMHC. The sequences extend from the translated into the variable, untranslated region. 20-mer sense and antisense primers are marked. Arrows indicate *Alu*I restriction sites. The *Alu*I-restriction fragment analysis of the two amplified sequences, 227 nt specific to αMHC and 210 nt specific to βMHC, is documented by the ethidium bromide-stained gel shown in (B). Obtained fragment lengths were as expected. Lanes 1 and 2, 210 nt product from soleus and 60d stimulated tibialis anterior; lanes 3 and 4, corresponding digests. Lanes 7 and 8, 227 nt product from masseter and 60d stimulated tibialis anterior; lanes 5 and 6, corresponding digests. M, DNA molecular weight marker V (Boehringer Mannheim).

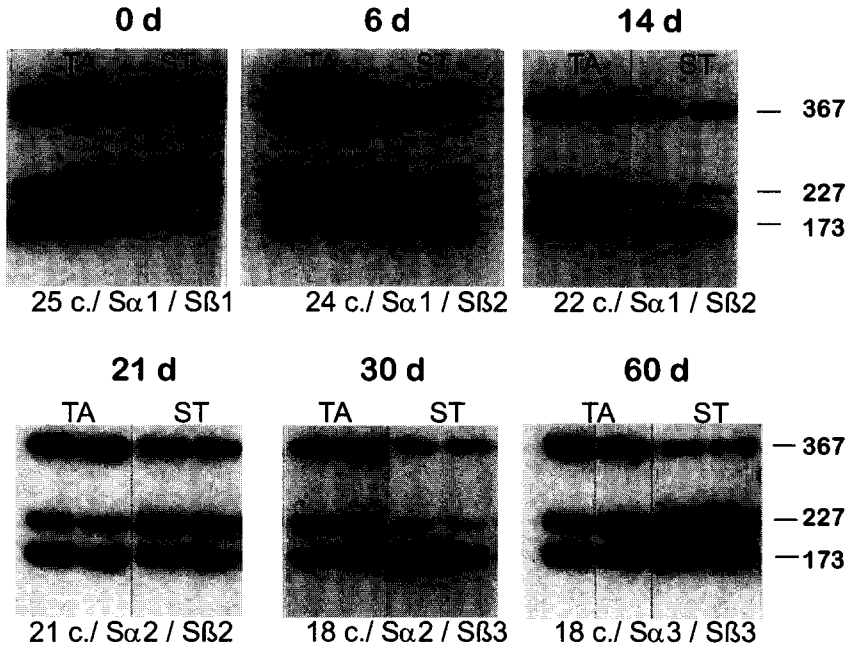

Fig. 2. Signal detection and quantitative evaluation of αMHC and βMHC PCR products detected by chemiluminescence. Signals specific to α-skeletal actin (367 nt), αMHC (227 nt), and βMHC (173 nt) were obtained from RNA preparations of tibialis muscle (TA) stimulated for different time periods at low frequency (10 Hz, 12 h daily). RNA samples from TA muscles were amplified together with DNA standard dilutions (ST) matching the corresponding signal intensities. For actin, number of cycles (14), as well as standard amounts (1.1×10^6 molecules) were the same for all measurements. Indications in the bottom lines refer to αMHC and βMHC only and give number of cycles (c.) and specifiy different standards: Sα1, Sβ1, 1.1×10^4 molecules; Sα2, Sβ2, 1.1×10^5 molecules; Sα3, Sβ3, 1.1×10^6 molecules. Signal intensities were evaluated by densitometry, and template numbers were calculated according to the intensities of the corresponding standards.

(MHCI) obtained by these methods [20,25–27] may have related to both isoforms.

3.2. *Electrostimulation-induced expression of αMHC mRNA detected by quantitative evaluation of RT-PCR*

Our method used known amounts of purifired PCR fragments as external DNA standards for monitoring the amplifications of different cDNAs by PCR and the subsequent chemiluminescent detection (Fig. 2). This approach permitted the quantitative analysis of gene expression of the different sequences with regard to possible variations in amplification efficiencies and allowed reliable evaluation of signal intensities. Using DNA standards, it was possible to determine initial first-strand cDNA template numbers introduced into PCR. Thus, reverse transcription was not included into the standardization procedure, and slight differences in the efficiency of first-strand cDNA synthesis for the different sequences could not be excluded. However, this may have affected only the comparison of data related to different sequences, but not the results for the same sequence. Based on previously determined molecule numbers of specific MHC mRNA isoforms with cRNA standards [16], the efficiencies of reverse transcription in the present study were determined to be in the range of 25 to 30%. Therefore, the applied method is suitable for determining relative expression levels, but it may also allow estimations of mRNA molecule numbers.

α-Skeletal actin has already been proven to be a suitable internal standard for determining relative mRNA expression levels of different MHC isoforms in muscle fiber fragments [17]. In a similar manner actin served as a control for RT-PCR performed on total RNA to monitor the validity of RNA preparations and endogenous degradation events. In control TA muscle, α-skeletal actin first-strand cDNA amounted to $3.9 \pm 0.7 \times 10^8$ molecules/μg of total RNA. The present study showed only minor variations in its expression level (1.6-fold elevation), most likely due to increases in total RNA [28]. Therefore, actin mRNA content in normal TA muscle was used as reference for normalizing the αMHC and βMHC levels.

Fig. 2 demonstrates signal intensities of PCR products visualized by chemiluminescence. Results from muscles exposed to CLFS for increasing time periods (up to 60d) are shown for the three sequences, i.e. α-skeletal actin (367 nt), αMHC (227 nt) and βMHC (173 nt) in combination with corresponding amounts of standards. The increase in the expression of αMHC and βMHC is evident from the increase in the number of standard molecules (10^4 to 10^6) and the decrease in the number of

Table 1
Initial amounts of first-strand cDNA ($\times 10^6$/μg total RNA, means ± S.D., $n = 4$) present in PCR assays for αMHC and βMHC isoforms in various rabbit muscles

Muscle	αMHC	βMHC
Adductor magnus	1.8 ± 0.5	2.9 ± 1.1
Diaphragm	40.0 ± 10.4	129 ± 23
Extensor digitorum longus	6.2 ± 1.9	12.3 ± 1.9
Gastrocnemius	9.1 ± 2.7	31.7 ± 2.8
Masseter	265 ± 15	13.4 ± 6.4
Psoas (white portion)	0.4 ± 0.1	0.5 ± 0.2
Psoas (red portion)	1.1 ± 0.3	2.7 ± 1.1
Soleus	19.5 ± 3.9	279 ± 34
Tibialis anterior	1.4 ± 0.6	7.1 ± 2.2

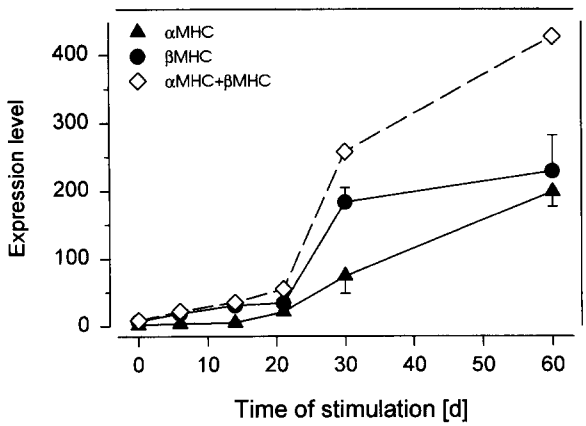

Fig. 3. Time course of low frequency stimulation-induced increases in cardiac-like αMHC (filled triangle) and βMHC (filled circles) mRNA isoforms of rabbit tibialis anterior muscle. Data (means ± S.D.) were calculated as described in legend to Fig. 2. The sum of the two isoforms is represented by the dotted line and open diamonds. Three animals were investigated for each time point. Four independent analyse were performed on each animal.

cycles (25 to 18) necessary to obtain similar signal intensities. Conversely, for actin the amount of standard and the number of cycles were the same up to 60d.

The values resulting from densitometry (Fig. 3) demonstrated pronounced increases in αMHC and βMHC mRNAs during prolonged CLFS. Up to 14d, both isoforms increased only slightly (αMHC 3.5-fold, βMHC 4.2-fold). At 21d, the level of the αMHC mRNA was 50-fold elevated, whereas that of the βMHC mRNA was only 6.5-fold augmented. At this time, the rise in αMHC seemed to precede that of βMHC mRNA. Thereafter, the increase in βMHC mRNA was steeper than that of the αMHC mRNA, reaching at 30d a level 30-fold elevated over control. This steep increase was in agreement with previous results obtained by S1 nuclease mapping [25]. During the same time period the level of αMHC mRNA was 53-fold augmented over control. Longer stimulation periods up to 60d led to further increases in αMHC mRNA at a similar rate, whereas the increase in βMHC mRNA became smaller. At 60d, the total increases in αMHC and βMHC mRNAs amounted to 140-fold and 50-fold, respectively. The attained levels (αMHC = $2.0 \pm 0.2 \times 10^8$, βMHC = $2.3 \pm 0.5 \times 10^8$ first-strand cDNA molecules/μg total RNA) were in the range of SOL and MAS, respectively (Table 1). The sum of both transcript levels has been included in Fig. 3 (dotted line) to indicate the time course that would have been measured using a probe reacting with both isoforms. Due to the stronger induction of αMHC, the αMHC/βMHC ratio was shifted to higher values. It was 0.20 in the control muscle and amounted to 0.85 in 60d stimulated muscle. Therefore, the αMHC reached an expression level similar to that of βMHC. Obviously, αMHC mRNA expression in skeletal muscle, especially during fast-to-slow MHC isoform transition, has escaped detection up to now. Our results suggest, that the αMHC represents an important isoform during the last step of low-frequency-induced muscle transformation. Therefore, the αMHC can be assigned as an additional element in the sequential MHCIIb → MHCIId → MHCIIa → αMHC/βMHC isoform transition. Whether its high level is transient or persists with ongoing stimulation can not be decided on the basis of the present results. However, it may be noted that SOL, a typical slow muscle, displayed a significantly lower level of αMHC mRNA (Table 1) than 60d stimulated TA muscle.

3.3. Expression of αMHC mRNA in additional muscles

Recently the expression of αMHC mRNA and protein was shown for masticatory and extraocular muscles [5–8]. Moreover, αMHC is also present in muscle spindles [10–12]. However, this isoform was never shown to represent a sarcomeric compound in skeletal muscles other than masticatory and extraocular muscles. Its low value in normal TA may reflect its expression in intrafusal fibers. This assumption is in agreement with similar low expression levels detected in other typical fast-twich muscles, such as ADM and PS (Table 1). Previous methods used for the assessment of αMHC mRNA in skeletal muscles may not have been sensitive enough to detect these very low expression levels [6]. EDL and GAS muscles display slightly higher levels of αMHC mRNA than TA muscle (Table 1). The higher content of βMHC mRNA in these two muscles possibly indicates that αMHC is associated with βMHC in extrafusal fibers. This assumption is corroborated by the twofold higher levels of αMHC mRNA in DIA than in SOL muscle (Table 1), and matched previous immunocytochemical findings on the presence of αMHC-positive fibers in adult rabbit DIA and low frequency stimulated muscle [9]. It will be interesting to investigate single fibers of transforming muscles in order to examine in more detail the sequence of αMHC and βMHC mRNA expression and their distribution with regard to pure and hybrid fibers.

In summary, we have identified a mRNA specific to a cardiac-like αMHC isoform present at low concentrations in normal skeletal muscles, as well as its pronounced induction during fast-to-slow transition of low frequency stimulated skeletal muscle. Posttranscriptional modifications of the αMHC gene transcript were recently demonstrated in rat heart to result from alternative splicing and three different polyadenylation sites [29]. As our data can not rule out possible diversities between the cardiac αMHC mRNA and the isoform under study, its identity remains to be established.

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