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RAPID COMMUNICATION

Real Time RT-PCR with a Newly Designed Set of Primers Confirmed the Presence of 2b and 2x/d Myosin Heavy Chain mRNAs in the Rat Slow Soleus Muscle

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Summary

In order to re-evaluate the presence and relative quantity of 2b and 2x/d myosin heavy chain (MyHC) transcripts in rat slow soleus muscle by using real time RT-PCR we have compared the available relevant *cDNA* sequences and designed a new set of primers having similar melting temperatures, matching separate MyHC exons in the regions of maximal differences in MyHC coding sequences, and containing G or C at the 3´-end. These also yielded PCR products of corresponding length, which is an important requirement for real time RT-PCR quantification. The experiments were performed on 8-month-old inbred female Lewis strain rats used in our current study of regenerating transplanted muscles. The real time RT-PCR measurement confirmed the expression of all four MyHC *mRNAs* (type 1, 2a, 2x/d and 2b) in both fast extensor digitorum longus and slow soleus muscles, although in the soleus muscle of adult rats, only type 1 and 2a protein isoforms can be usually detected.

Key words

Rat slow soleus muscle • Rat fast EDL muscle • Myosin heavy chain isoforms • Real time RT-PCR

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Introduction

Myosin heavy chain (MyHC) *cDNA* sequences are highly conserved among mammals including human, mouse and rat. Although the full sequence of human and mouse type 1, 2a, 2x/d and 2b MyHC isoforms has been described, the information for rat is still incomplete and often controversial, especially for the 2b isoform. MyHC isoform *mRNA* levels in different muscles and in various species have often been analyzed by reverse transcription followed by polymerase chain reaction (RT-PCR) (Lieber *et al.* 1993, Ennion *et al.* 1995, Peuker and Pette 1995, 1997, Jänkälä *et al.* 1997, Wright *et al.* 1997, Jaschinski *et al.* 1998, Jung *et al.* 1998, Stevens *et al.* 1999, Sakuraba *et al.* 2005, for review see Schiaffino and Reggiani 1996). The RT-PCR proved to be a powerful and sensitive method to determine even a small amount of *mRNA*. Despite many modifications it was, however, difficult to obtain the quantitative data, apparently due to the exponential nature of the PCR. At present, the real time RT-PCR approach is required in order to receive quantitative data of respective transcripts. Regarding MyHC isoform *mRNAs*, the primers used previously in the above studies were inconsistent in their melting temperatures and length of PCR products, which would complicate the experimental design of the real time RT-PCR. They also did not always match separate exons or the sequences with maximal variability, which is required for high specificity RT-PCR measurement.

The soleus is a slow antigravity muscle designated to sustained prolonged activity, consisting in adult rats of a great majority of slow type 1 fibers supplemented by a small number of fast 2A fibers containing type 1 and 2a MyHC isoforms, respectively. The EDL muscle, on the other hand, is a typical fast

Positions of FORWARD primers:

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MyHC 1      ACAGAGGAAGACAGGAAGAACCTACTGCGACTGCAGGACCTGGTGGACAAGCTGCAGTTA
MyHC 2a     ACAGAAGAAGACCGAAAAAATATCCTCAGGCTTCAAGATTTGGTGGATAAACTCCAAGCA
MyHC 2x/d   ACTGAGGAAGACCGCAAGAACGTTCTCAGGCTCCAGGACCTTGTGGACAAACTGCAATCA
MyHC 2b     ACCGAGGAGGACCGCAAGAACGTGCTGAGGCTGCAGGACCTAGTGGATAAATTACAGACT
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Positions of REVERSE primers (in 3' untranslated region):

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MyHC 1      CCCTAAGGATGCCTGTGAAGCCCTGAGACCTGGAGCCTTTGAAACAGCACCTTAGGCAGAAACACAA
MyHC 2a     TAGAATGACCGAAGAGAGGCACAAAATGTGAAGCCTTTGGTCATGTCCCCATGTGATTCTATTTAAT
MyHC 2x/d   AAGTGACCAAAGAGATGAGCAAAATGTGAAGATCTTTGTCACTCCATTTTGTACTTACGACTTTGGG
MyHC 2b     GAAAGGTGACAGAAGAAATCACACAATGTGACGTTCTTTGTCACTGTCCTGTATATCAAGGATCCAA
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Fig. 1. Primers positions. Positions of primers used for MyHC-isoform *mRNAs* RT-PCR quantification are underlined. The reverse prime sequences are reversely complement to respective underlined ones.

contracting muscle involved in intermittent bursting efforts and it is composed from type 1, 2A, 2X/D and 2B fibers containing corresponding type 1, 2a, 2x/d and 2b MyHC isoforms. This composition was demonstrated in both muscles by histochemical, immunocytochemical, electrophoretic and immunoblotting methods (e.g. Jaschinski *et al.* 1998, Stevens *et al.* 1999, Smerdu and Soukup 2008, Soukup *et al.* 2002, 2009, for review see Pette 2002).

In previous experiments, using non-real time PCR followed by densitometric evaluation of gel separated reaction products (see Hudecová *et al.* 2004), the presence of all four (1, 2a, 2x/d and 2b) MyHC isoform transcripts was demonstrated in the soleus muscle (Vadászová *et al*. 2006a), similarly as in the EDL muscle (Vadászová *et al*. 2006b). In order to evaluate the presence and relative quantity of *mRNAs* for MyHC isoforms, we have designed a new set of primers and used them for the real time RT-PCR analysis of MyHC isoform *mRNAs* in slow and fast hind limb rat muscles.

Materials and Methods

Animals

Female inbred Lewis strain rats were obtained from the authorized laboratory rat-breeding unit of the Institute of Physiology, Academy of Sciences, Prague, Czech Republic (Accreditation No. 1020/491/A/00). The maintenance and handling of experimental animals was in accordance with the EU Council Directive (86/609EEC) and the investigation was approved by the Expert Committee of the Institute of Physiology, Academy of Sciences, Prague, Czech Republic. Muscles were excised from four 8-month-old rats anesthetized with intraperitoneal injections of Nembutal (sodium pentobarbital 40 mg/kg), placed in the *RNA* stabilizing solution (Qiagen) and stored at -80 °C until used for the real time RT-PCR. The rats were sacrificed by an overdose of the anesthetic.

Primers

The primers have been designed using the Gene Runner program (Hastings Software).

RNA isolation and real time RT-PCR

Total cellular *RNA* was extracted from each muscle sample using the RNeasy Mini kit (Qiagen). The purity and integrity of the *RNA* preparations was checked spectroscopically and by agarose gel electrophoresis. One μg of total *RNA* was converted to *cDNA* using the RevertAid^{TM} H Mius First Strand *cDNA* Synthesis Kit (Fermentas) using oligo(dT) primers according to the manufacturer's instructions. Samples of *cDNA* (1 μl) were amplified in 25 μl of PCR reaction mixtures containing iQ^{TM} SYBR Green Supermix (Bio-Rad) plus 1 μM of each MyHC isoform-specific primers according to the manufacturer's instructions.

PCRs were performed on a Rotor Gene 6000 (Corbett Research) using the following temperature profile: initial denaturation at 95 °C for 2 min, followed by 38 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 54 °C for 30 sec and elongation at 72 °C for 20 sec. Fluorescence was acquired in each cycle after heating the samples to 80 °C eliminating the noise of primer dimers (Pfaffl 2004). At the end of each run melting curve analysis was performed to ascertain the presence of a single amplicon. The data used for calculation are the means of CT values obtained from qPCR performed in triplicates. We have verified that the variation of triplicates did not exceed 0.5 CT. Standard

curves were generated for each pair of primers using 3-fold serial dilution of *cDNA*. The efficiency of the PCR amplification for each primer pair was then calculated from the standard curve to precisely state the relative expression. The level of analyzed transcripts was normalized to the level of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts with regard to the specific PCR efficiency (E) for each gene as:

$$\text{Normalized amount} = (1+E)^{CT\ \text{reference transcript}} / (1+E)^{CT\ \text{target transcript}}$$

Non-template and non-RT reactions were performed as controls. The quantitative data are the means of four experiments and these are presented as ratio among values for individual *mRNAs.*

Results and Discussion

Design and characterization of new primers

We have designed a set of primers with similar melting temperatures, matching separate MyHC exons with maximal differences in MyHC coding sequences and containing G or C at the 3´end (Fig. 1). These have also yielded PCR products of corresponding length, which is an important requirement for real time RT-PCR quantification. The primers used in our study were as follows:

MyHC1 _ F, AGAGGAAGACAGGAAGAACCTAC;
MyHC1 _ R, GGCTTCACAGGCATCCTTAG;
MyHC 2a_F, TCCTCAGGCTTCAAGATTTG;
MyHC 2a_R, TTAAATAGAATCACATGGGGAC;

MyHC 2x/d_F, AAGACCGCAAGAACGTTCTC;
MyHC 2x/d_R, TCGTAAGTACAAAATGGAGTGAC;

MyHC 2b_F, GAGGACCGCAAGAACGTG;
MyHC 2b_R, TGTGTGATTTCTTCTGTCACC;

GAPDH_F, GCTGAGTATGTCGTGGAGTC;
GAPDH_R, GTCAGATCCACAACGGATAC.

The information about the exact composition of rat MyHC isoforms is still incomplete in databases and often controversial, especially for the 2b isoform. In our previous study (Žurmanová *et al*. 2007), we separated the MyHC 2b isoform by SDS-PAGE from the rat EDL muscle, identified its composition using mass spectrometry (MALDI-TOF) and sequenced it using the liquid chromatography-tandem mass spectrometry (ESI LC/MS/MS). The dataset that we obtained has allowed us to select the respective 2b *mRNA* transcript from its alternatives present in databases and thus to design the most appropriate primers.

We used the pairs of primers and reverse transcribed *RNA* samples isolated from both soleus and EDL muscles also in non-real time PCR and separated their respective reaction products using gel electrophoresis to ensure that only a single product is amplified in each reaction. After extraction from the gel, the *DNA* products were sequenced (Center of DNA Sequencing at the Institute of Microbiology, Prague) in order to check their sequence identity. Sequencing confirmed the expected lengths of PCR products; the lengths of 1, 2a, 2x/d, and 2b MyHC isoform PCR products were 285, 309, 318, and 285 bps, respectively.

Real time RT-PCR analysis

Using the new set of primers and real time PCR technique, we have identified all four transcripts encoding MyHC 1, 2a, 2x/d and 2b isoforms both in the slow soleus and fast EDL muscles. The difference of CT values obtained from *cDNAs* and background (corresponding *mRNAs* or water) confirmed that both 2x/d and 2b isoform *mRNAs* were present in the slow soleus muscle of normal adult rats (Fig. 2). In general, the relative proportion of fast 2a : 2x/d : 2b MyHC isoform transcripts in the soleus muscle were by two to three orders of magnitude lower than that of slow MyHC 1 isoform. This result is in agreement with the dominant content of MyHC 1 protein isoform supplemented by a small amount of MyHC 2a isoform in the adult rat soleus muscle. Our SDS-PAGE and immunohistochemical (by specific monoclonal antibodies) analyses of soleus muscle (Soukup *et al.* 2002, 2009) have confirmed the prevalence of the type 1 MyHC isoform over a minority of 2a isoform (about 95 and 5 %, respectively), as well as of slow fiber type 1 over fiber type 2A (97.5 and 2.5 %, respectively).

In the EDL muscle the relative ratio of 1 : 2a : 2x/d : 2b MyHC isoform transcripts differed much less compared to the soleus muscle. The 2b isoform *mRNA* was roughly two orders higher compared to isoform 1 and one order of magnitude higher compared to 2a and 2x/d ones. The lowest relative amount of the MyHC type 1 isoform transcript corresponds to a minor representation of about 0-5 % of slow type 1 MyHC isoform (determined by SDS-PAGE) and of slow type 1 fibers

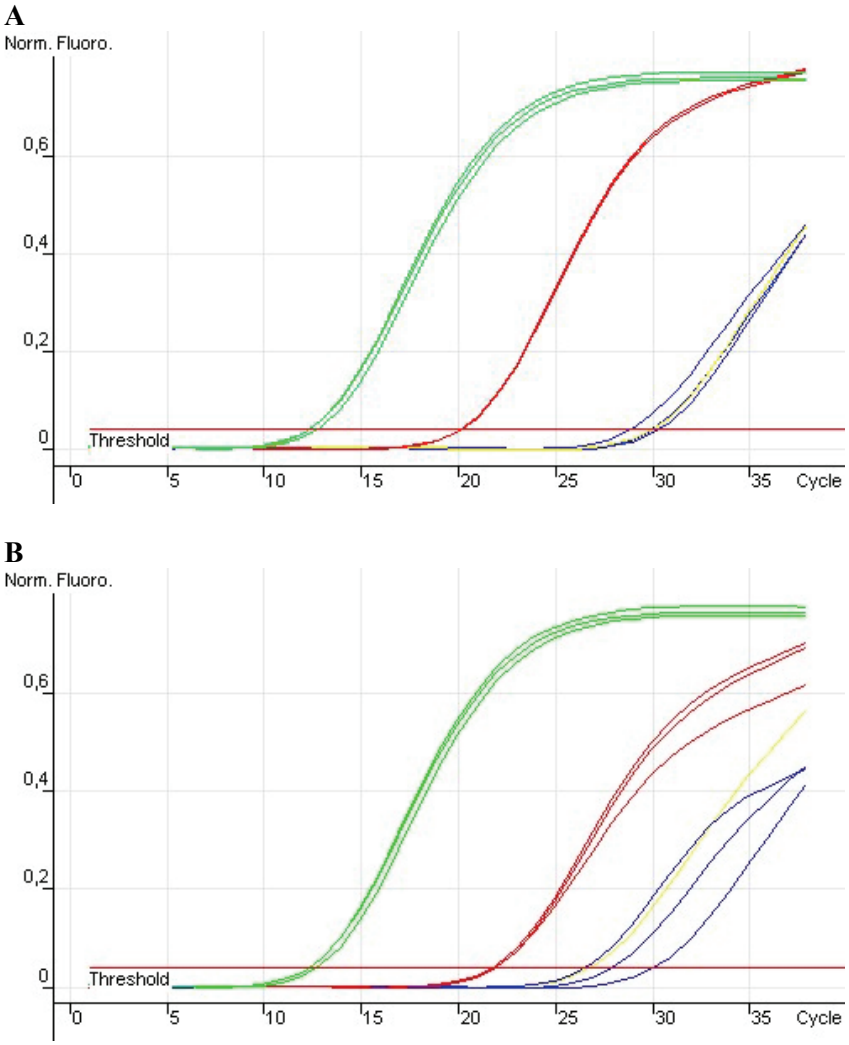

Fig. 2. The soleus muscle contains both 2b and 2x/d isoform mRNAs. The curves represent the fluorescent signal of GAPDH (in green) and MyHC obtained from soleus muscle *cDNA* (in red) or corresponding mRNA (in blue), compared to water (in yellow) using PCR amplification and SYBR-Green detection. **A**, MyHC 2x/d; **B**, MyHC 2b.

(determined by immunohistochemistry) in Lewis strain rats (Soukup *et al.* 2002, 2009). The amount of *mRNAs* of fast MyHC isoforms increased in the same order as the content of MyHC protein isoforms and fiber type percentage of the adult rat EDL muscle, i.e. 2a < 2x/d < 2b (Soukup *et al.* 2002, 2009).

This study, together with semi-quantitative Elisa MyHC method (Říčný and Soukup, 2006) and quantitative determination of the fiber type and MyHC isoform content (Zachařová *et al.* 2005, Vadászová-Soukup *et al*. 2006c, Vadászová-Soukup and Soukup 2007, Soukup *et al.* 2009), should help us analyze the regulation of MyHC isoform expression in regenerating transplanted muscle in more details (for review see Soukup and Jirmanová 2000, Vadászová *et al*. 2004).

Conflict of Interest

There is no conflict of interest.

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