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Quantification by real-time PCR of developmental and adult myosin mRNA in rat muscles

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Abstract

A real-time RT-PCR assay using newly designed primers was developed to analyze developmental and adult MHC mRNA expression both in skeletal muscles and single fibers. Only 4 ng of total RNA was necessary for the analysis of the relative mRNA expression of MHC genes. Different validation steps were realized concerning both specificity and sensitivity of each primer set, and linearity and efficiency of each real-time PCR amplification. Then, quantification of MHC mRNA in neonatal and adult muscles as well as in single fibers was done by the ΔC_T method, with CycA gene as the reference gene. Due to a higher sensitivity than that of a competitive PCR method, we demonstrated that this assay is suitable to study very low level of MHC mRNA expression as developmental MHC in adult muscle and to quantify mRNA from very small samples.

Keywords: Skeletal muscle; Single fiber; Real-time PCR; Myosin heavy chain; Wistar rats

Skeletal muscle plasticity, which reflects the ability of striated muscles to adapt their phenotypic characteristics according to the functional demand, is mainly investigated from the composition of specific muscle proteins, such as myosin heavy chain (MHC). This protein, which is both an important structural and regulatory protein of the contractile apparatus, can be expressed as different isoforms, which contribute to the physiological differences among muscle fibers [1,2]. In rat skeletal muscles, six distinct MHCs have been identified (for review, see [1]): two developmental (embryonic and neonatal) and four adult MHC isoforms designated as slow type I, fast type IIa, IIx, and IIb. In mammalian muscle fibers, these isoforms are encoded by a highly conserved multigene family [3], whose regulation is under the control of several molecular factors related to contractile activity, neuronal pattern, mechanical loading, and/or hormonal influences [4].

For many years, employing different physiological situations, studies on MHC gene expression have focused on analyses at both protein and mRNA levels to obtain insight into pretranslational/translational processes involved in MHC gene regulation [5–12]. Several methods are commonly used to examine MHC mRNA expression, including slot/dot-blot [13,14,5,7], Northern blot [7,15], RNase protection assay [8,16], in situ hybridization [6,17], and reverse transcriptase reaction coupled to a polymerase chain reaction (RT-PCR) [18–23,12]. The rapid and elegant RT-PCR method developed by different authors ensures measurement of relative amounts of the different MHC mRNAs in whole skeletal muscles [18,20–23,12] or at the myofiber level [19,24–26], in a fast and sensitive way compared to fundamental procedures such as Northern blotting.

A competitive RT-PCR method is commonly used to examine MHCs' mRNA expression, but this method requires simultaneously the amplification of the muscle sample with a synthetic DNA fragment, acting as a "competitor" and representing an internal control to correct

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variability in the amplification reaction. Then, the amount of mRNA is quantified by titration of an unknown quantity of target template against a dilution series of known amounts of internal competitor. Although this method provides a strategy for accurate quantification, intensive and laborious post-PCR manipulations are required. Moreover, possible differences in PCR efficiency between control and target gene products remain difficult to detect and may impair the quantification [27]. Recent developments of real-time PCR, based on fluorescence–kinetic RT-PCR, ensure monitoring PCR product formation continuously during the PCR stage, and lead to improve sensitivity, reduce variability, and eliminate post-PCR manipulations [28]. In the field of muscle plasticity, two recent publications have reported measures of MHC mRNA levels by a TaqMan real-time RT-PCR in skeletal muscles. Indeed, da Costa et al. [29] determined the temporo-spatial expression of MHC mRNA in porcine muscles, and more recently Pattison et al. [30] quantified MHC mRNAs in aging and atrophied soleus muscles of rats. Although these authors demonstrated both sensitivity and accuracy of real-time RT-PCR in comparison with a micro-array analysis in rat muscles, they proposed an incomplete MHC mRNA assay concerning only MHC-IIb and MHC-IIx mRNA expression in slow-twitch muscle. Finally, MHC mRNA expression is weakly studied by real-time PCR, and no completed real-time PCR assay exists to investigate all MHC gene isoforms in skeletal muscles.

Then, the aim of this work was to propose a new procedure to investigate expression in skeletal muscle of both developmental and adult MHC transcripts using real-time RT-PCR method. Because members of the MHC gene family share a high degree of sequence similarity, we designed new primer sets, with special regard to the inter-isoform specificity of MHC. Although this method ensures fast, sensitive, and accurate quantification, different control assays were necessary to control the results' reliability. Furthermore, considering probable disparities among the distinct fiber types in RNA content [31], as well as variations in input RNA or in reverse transcription efficiencies, cyclophilin A (CycA) mRNA was also quantified as an endogenous housekeeping gene [32]. Then, we propose a novel and simple procedure of real-time RT-PCR which can be applied to whole muscle and single fiber analysis for quantification of developmental and adult MHC mRNA isoforms in rat.

Methods

Tissue processing. Skeletal muscles expressing a wide range of MHC mRNA isoform expressions were excised from both neonatal and adult Wistar male rats (IFFA Credo, L'Arbresle, France). Hindlimb muscles, including the slow-twitch soleus muscle and the fast-twitch plantaris muscle, were taken from adult animals, while soleus muscle was also removed from neonatal rats at 15 days of age. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) administered intraperitoneally. Muscles were excised, cleaned of adipose and connective tissues, quick-frozen in liquid nitrogen, and stored at −80 °C until analyses were performed.

Analysis of muscle MHC protein content. Neonatal and adult muscles were subjected to the analysis of MHC isoforms as previously described [33]. Tissue samples were minced with scissors and myosin was extracted in seven volumes of a solution containing 0.3 M NaCl, 0.1 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.01 M $Na_4P_2O_7$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM EDTA, and 1.4 mM of 2β-mercaptoethanol (pH 6.5). After 1 h of gentle shaking at 4 °C, the mixture was centrifuged at 13,500*g* for 15 min and the supernatant containing myosin was diluted with one volume of glycerol. Extracts were stored at −20 °C until the separation process. Electrophoresis was performed using a Mini Protean II system (Bio-Rad, Marne-la-Coquette, France). Separating gel solution contained 30% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris, 0.1 M glycine, and 0.4% sodium dodecyl sulfate (SDS). Stacking gel was composed of 30% glycerol, 4% acrylamide-bis (50:1), 70 mM Tris, 4 mM EDTA, and 0.4% SDS. Gels were run at constant voltage (72 V) for 31 h and then silver stained [34]. The MHC protein isoform bands were scanned and quantified using a densitometer system equipped with an integrator (GS-700, Bio-Rad, Marne-la-Coquette, France).

Oligonucleotide primers. The design of real-time PCR primers was performed in order to avoid primer–dimers or self-priming formation with MacVector software (Accelrys, San Diego, USA). Oligonucleotide primers were synthesized by Eurogentec (Saraing, Belgium). The real-time PCR primer sequences are shown in Table 1. Competitive PCR primers used to test specificity of our real-time PCR primers were previously published [20].

Table 1
Primers designed for real-time PCR amplification and their conditions of use

Gene Unigene ID	Accession Nos.	5′–3′ primer sequence	Primer (μM)	$MgCl_2$ (mM)	Annealing temperature (°C)	Product size (bp)	Product T_m (°C)	Dimer T_m (°C)
CycA Rn.1463	NM_017101	F: AGC ATG TGG TCT TTG GGA AGG TG R: CTT CTT GCT GGT CTT GCC ATT CC	0.4	4	65	92	86.2	—
MHC-I Rn.127778	NM_017240	F: TTG CTC TAC CCA ACC CTA AGG ATG R: TTG TGT TTC TGC CTG AAG GTG C	0.4	4	60	81	83.7	—
MHC-IIa Rn.10092	L13606	F: CTC AGG CTT CAA GAT TTG GTG G R: TTG TGC CTC TCT TCG GTC ATT C	0.4	4	62	265	88.5	—
MHC-IIx Rn.128284	XM_213345	F: GGA GGA ACA ATC CAA CGT CAA CC R: GGT CAC TTT CCT GCT TTG GAT CG	0.4	4	60	178	89.5	79.0
MHC-IIb Rn.34868	XM_340818	F: GAG GTT CAC ACC AAA GTC ATA AGC R: CTT CGC TTA TGA CTT GGA TCC TTG	0.4	4	50	129	78.6	—
Neonatal Rn.34868	K02111	F: TGA AAG TGA GGA GTG AGC ATG TCC R: GGG GTT ACG TGG AAA TTA AGC AGG	0.4	4	62	103	89.7	81.5
Embryonic Rn.98847	NM_012604	F: AGC AGA GGA GGC TGA GGA ACA ATC R: GGC CTC CTC AAG ATG CGT TTA CTC	0.4	4	62	181	83.0	78.5

Preparation of muscle cDNA template. Total RNA was isolated using RNeasy mini kit procedure (QIAGEN S.A, Courtaboeuf, France) following the company protocol, with an optional proteinase K step. Reverse transcription was carried out in a 20 μL final volume from 1 μg total RNA using First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions with 1.6 μg oligo $(dT)_{15}$ primer. The complete mix was incubated for 10 min at 25 °C, then 60 min at 42 °C, followed by 5 min at 99 °C, and chilled on ice.

Preparation of single skeletal muscle fiber cDNA. Recently, our laboratory described an innovative procedure to isolate single muscle fiber, identify fiber types on the basis of the content of MHC isoforms, and perform total RNA extraction and RT reaction at the single fiber level [35,36].

In the present study, fibers were precisely separated from deep plantaris muscles, known to comprise the four main fiber types, with thin tweezers under a stereomicroscope. Single fiber was then cut in two equal parts. MHC protein content was determined on one half-fiber using a method adapted from Talmadge and Roy [33], while the other half-fiber was used for mRNA analyses.

Total RNA isolation was achieved from samples using 50 μL of RNAble reagent (Eurobio, Les Ulis, France), following the manufacturer's instructions. At the end of the procedure, total RNA was suspended in 15 μL of ribonuclease-free water. RT reaction was carried out in a 40 μL final volume containing the whole RNA solution, 300 U M-MLV reverse transcriptase, 30 U recombinant ribonuclease inhibitor (RNasin), and 10 ng oligo$(dT)_{15}$ primer. All RT reagents were purchased from Promega (Charbonnières, France). CycA PCR was carried out using 2 μL cDNA in a 20 μL final volume with the LC Fast Start DNA Master SYBR Green Kit (Roche Applied Science, Mannheim, Germany) as described below.

Real-time PCR. The PCR was carried out with the LC Fast Start DNA Master SYBR Green Kit (Roche Applied Science, Mannheim, Germany) using 0.08 μL cDNA (equivalent to 4 ng total RNA) in a 20 μL final volume. Quantitative PCR was performed using a LightCycler (Roche Applied Science, Mannheim, Germany) for 45 cycles using a 5 s annealing step, and a 10 s elongation step at 72 °C, excluding MHC-IIb (4 s). Detailed conditions are available in Table 1. The ramping temperature was adjusted to 20 °C/s among different stages of the PCR, excepting a 2 °C/s ramping temperature for MHC-IIb between annealing and elongation step.

A threshold cycle (C_T) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated from LightCycler Software v.3.5 (Roche Applied Science, Mannheim, Germany) using the second derivative maximum method. Relative standard curves for each gene were plotted showing C_T (y axis) vs log (initial cDNA) diluted 3.3-fold sequentially (x axis). The slope (a) of the standard curve describes the efficiency of PCR and is defined by the equation:

$$C_T = a(\log Q) + b,$$

where C_T is the threshold cycle, Q is the initial amount, and b is the intercept on the y axis. When PCR amplification is maximally efficient, resulting in a doubling of product in every cycle, the slope will be −3.32 [37].

PCR products were analyzed by agarose gel electrophoresis [4 μL aliquots of a 20 μL PCR loaded on 2% agarose gels in 0.5× TBE buffer (Sigma Aldrich, St Louis, USA) containing 5 μg/ml ethidium bromide] to visualize the PCR products against a 50-bp marker (Promega, Charbonières, France). Pictures of the gels were taken under ultraviolet light using a DC40 camera (Eastman Kodak Company, Rochester, USA) connected to a computer. Intensity of the bands was analyzed by the Kodak Digital Science 1D program (Eastman Kodak Company, Rochester, USA).

Specificity of MHC real-time PCR primers. Specificity of the new primers was checked using as template recombinant DNA of the six MHC isoforms. These recombinant DNAs were PCR products completed by utilization of either forward and reverse competitive PCR primers (MHC-I, MHC-IIa, MHC-IIb, and embryonic) or from forward competitive PCR primer combined to reverse real-time PCR primer (MHC-IIx, neonatal). Each new primer set specificity was verified in three different PCRs with: (1) cDNA muscle templates, (2) a specific MHC recombinant DNA engineered by real-time PCR, and (3) all recombinant MHC DNA, excluding the recombinant MHC DNA corresponding to the primer set tested.

Statistical analysis. All data are presented as means ± SEM. One-way analysis of variance (ANOVA) was used to determine a global effect of either muscle type or muscle fiber type. When appropriate, differences between groups were tested with a Newman–Keuls post hoc test. Statistical significance was accepted at $p < 0.05$.

Results

Primer specificity

Primer sequences are shown in Table 1. Primer specificity was analyzed by a comparison of PCR products obtained from cDNA muscle templates to those obtained using a specific MHC recombinant DNA engineered by real-time PCR. PCR products were documented both with high-resolution agarose gel and melting curve analysis. Examination of agarose gels (Fig. 1A) reveals for each MHC real-time primer set a unique band corresponding to an exclusive PCR product. Furthermore, molecular weight of each MHC gene amplified matched the theoretically designed length. Analysis of melting curves not only permits both identification of amplified products, but also distinction between the target DNA and possible primer–dimers. Melting temperatures of each specific MHC PCR product or primer–dimers are summarized in Table 1. Whatever the MHC isoform considered, melting peaks obtained either from cDNA muscle template or from recombinant DNA were identical (data not shown). Each PCR product engineered by our new primer sets presents a single melting temperature peak (Fig. 1B, blue line) which differs from those of the possible primer–dimers obtained in control real-time PCR without cDNA templates (Fig. 1B, red line). As expected, no PCR product was obtained with a DNA matrix comprising all recombinant MHC DNA, but excluding the recombinant MHC DNA corresponding to the primer set tested.

Sensitivity

High sensitivity of real-time PCR is due to a direct measure of PCR product accumulation by fluorescence emission throughout the four sequential phases of the reaction. During the first cycles of the PCR (linear ground phase), fluorescence does not exceed background. At the beginning of the exponential part of the reaction, fluorescence surpasses background (cycle threshold) and increases during the log-linear phase, and in proportion to PCR efficiency decrease reaches the plateau stage. In contrast with end point PCR, real-time PCR allows detection of amplification product from the early exponential stage formation before the modifications of PCR efficiency [32]. Indeed, comparison of MHC-I quantification from either competitive RT-PCR using cDNA sample corresponding to 200 ng total RNA [20] or real-time PCR using 4 ng total RNA (this study), is, respectively, near the 25th cycle versus 18th cycle (Fig. 2). While the initial amount of total

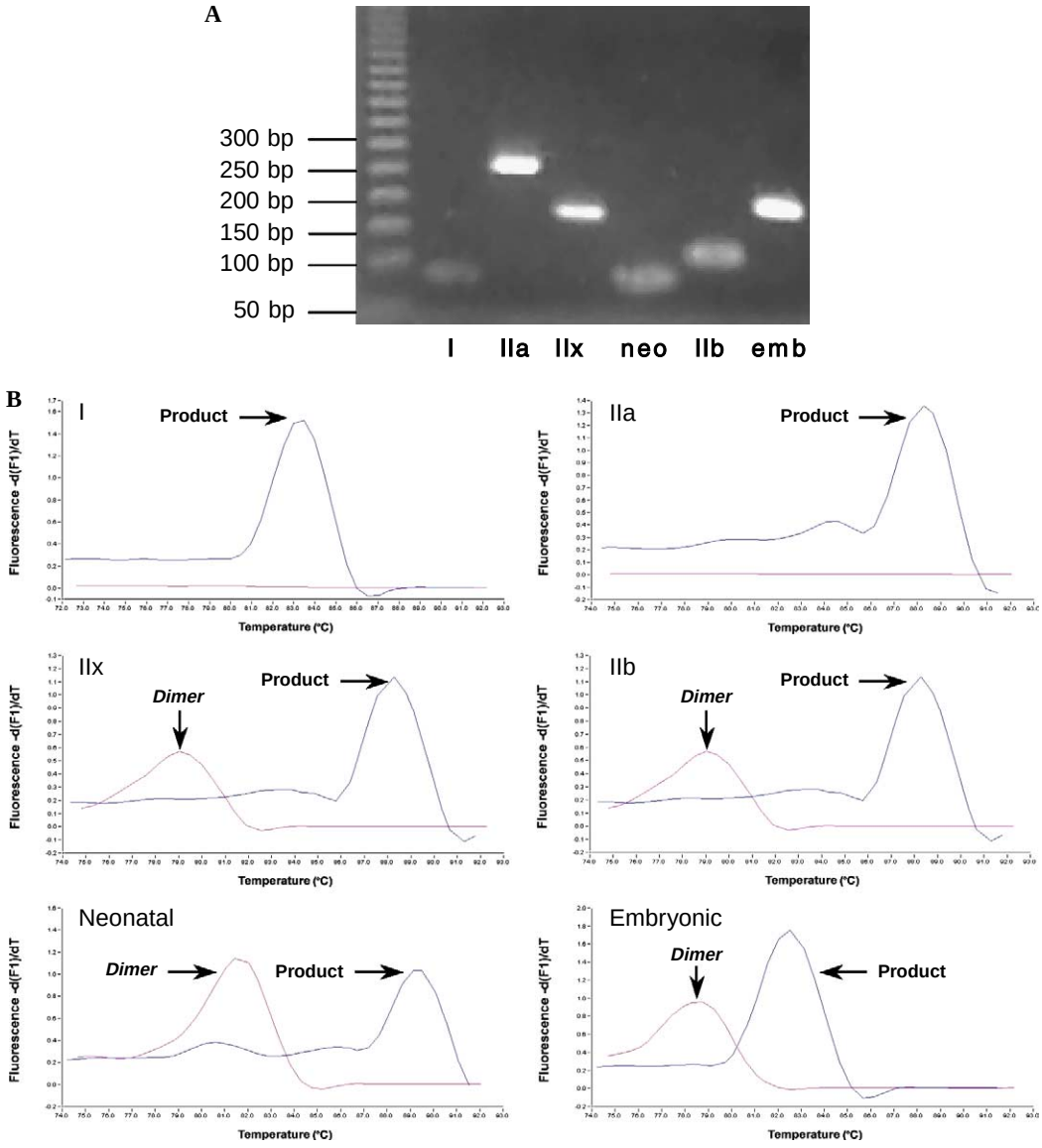

Fig. 1. Identification of real-time PCR products. (A) MHC mRNA signals detected by high-resolution agarose gel. Sizes of PCR products match the theoretically designed length: MHC-I, 81 bp; MHC-IIa, 265 bp; MHC-IIx, 178 bp; MHC-IIb, 129 bp; neonatal (neo), 103 bp; embryonic (emb), 181 bp. (B) Melting curve analysis with LightCycler Software v.3.5 corresponding to specific MHC mRNA (blue line) and possible primer–dimers obtained in control real-time PCR without cDNA templates (red line).

RNA was 50 times less important than in Wright's study, we obtained an earlier detection due to a good efficiency of the PCR, because our real-time PCR conditions were optimized in respect of our primers. Consequently, the specificity of the primer sets developed herein combined to the high sensitivity of real-time PCR would permit detection of very low MHC mRNA levels.

Quantification and normalization

Accuracy of mRNA quantification depends on linearity and efficiency of PCR amplification, which were determined from two recognized methods, i.e., the standard curve analysis and the comparative threshold cycle (C_T) approach (for review, see [38]).

Standard curve analysis

Internal standard curve method ensures an accurate analysis of mRNA quantification, considering the exact efficiency of single PCR amplification for each mRNA measurement. This approach was favored in low mRNA level samples and requires standard DNA utilization. Commonly, recombinant DNA is used to generate standards although PCR efficiencies slightly change with chemical properties of the template. To improve accuracy of this technique, we constituted cDNA standards from a pool of all tested samples whose both RNA extraction and reverse transcription conditions were similar. Then, these standards were diluted over a 100-fold range, in a five-point standard curve, centered on tested sample concentrations. Linearity and efficiency of PCR amplification were

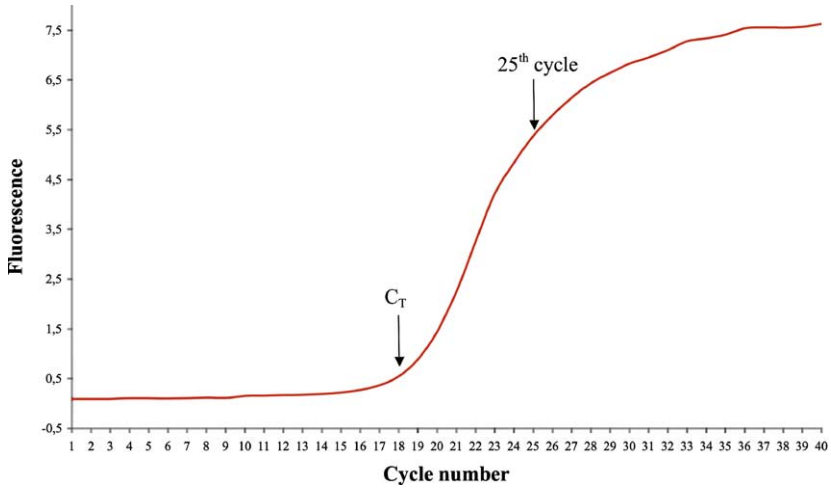

Fig. 2. Sensitivity of real-time PCR for MHC mRNA quantification. Amplification curve of MHC-I gene corresponding to 4 ng cDNA (LightCycler Software v.3.5). C_T determination occurs at the 18th cycle, while in competitive PCR from a 50-fold template (200 ng), MHC-I quantification takes place at the 25th cycle [20].

deduced from the given slopes by LightCycler Software v.3.5 (Roche Applied Science, Mannheim, Germany). Fig. 3 represents the relation between C_T and logarithm of cDNA concentration. For all primer sets standard curve analysis led to a high linearity ($r = 0.998$). PCR efficiencies (E_x) were calculated from the equation. $E_x = 10^{-1/\text{slope}} - 1$. A slope value of −3.32 implies a PCR efficiency of 100%, while lower values mean a PCR efficiency lower than 100%, even slope values down to −4.1 are tolerated [39,40]. CycA and MHC amplification slopes were −3.40 (CycA), −3.51 (MHC-I), −3.70 (MHC-IIa), −3.47 (MHC-IIx), −3.63 (MHC-IIb), −3.77 (embryonic), and −3.62 (neonatal), corresponding to a range of efficiencies between 84% (MHC-emb) and 97% (CycA). These comparable slopes obtained both for each MHC gene and CycA gene indicate very similar amplification efficiencies for target and reference genes. Then, we considered that the high linearity and the reproducible amplification efficiencies of the six MHC PCR assays (Fig. 3A) ensure an accurate quantification of target gene expression.

A reliable quantitative RT-PCR method requires corrections for experimental variability among different samples, i.e., cDNA amount and minor differences in PCR efficiencies. In contrast with end point PCR, real-time PCR quantification is based on C_T values, which are determined rapidly in the exponential phase of the reaction. Thus, inter-sample variability in amplification efficiencies is not a major problem [32] for real-time PCR quantification. However, variations in input RNA or in reverse transcription efficiencies must be taken into account. Normalization to a housekeeping gene is currently the most acceptable method to avoid these discrepancies [32]. The choice of an appropriate gene is crucial for reliability of results and because its transcription level remained unaffected by many physiological conditions [41,42,35,43], we assessed the cyclophilin A (CycA) mRNA expression in different single muscle fibers. As shown in Fig. 4, there is no statistical difference in CycA mRNA expression between fiber types. Then, we considered that for studying MHC mRNA expression both in myofibers and in whole control muscles, CycA gene was revealed as a good housekeeping gene.

The comparative C_T method (ΔC_T method)

As an alternative to the standard curve method, the ΔC_T method is a fast and simple way for mRNA quantification, which is based on very close amplification efficiencies between target and reference genes [44]. This must be checked by determination of the ΔC_T variability with template dilutions. The validity of the ΔC_T variability calculation implies a slope value close to zero. Fig. 3B represents the ΔC_T variability calculation for the whole MHC genes versus CycA gene in a cDNA dilution over a 100-fold range in three independent experiments in soleus muscle. Slope values for each MHC gene vary between −0.082 and +0.027, but were inferior to 0.1, leading to the validation of this method to quantify MHC mRNA levels in rat skeletal muscles [45,46,38].

MHC mRNA detection in skeletal muscles and single fiber

After these steps of validation, a real-time PCR with each new MHC primer set was applied to both neonatal and adult whole skeletal muscles and single fibers for detection of developmental and adult MHC mRNA isoforms. Quantification of MHC mRNA was performed using the ΔC_T method due to its simplicity and rapidity for setup and analysis, using CycA gene as the housekeeping gene. Then, MHC mRNA was also expressed as a percentage of the total distribution, whereby each MHC mRNA quantification was divided by the sum of all of the six MHC mRNA isoform quantifications in a given sample (Table 2).

Unexpectedly, all MHC transcripts were detected in both young and adult skeletal muscles. Nevertheless, the MHC mRNA distribution varied among muscles with

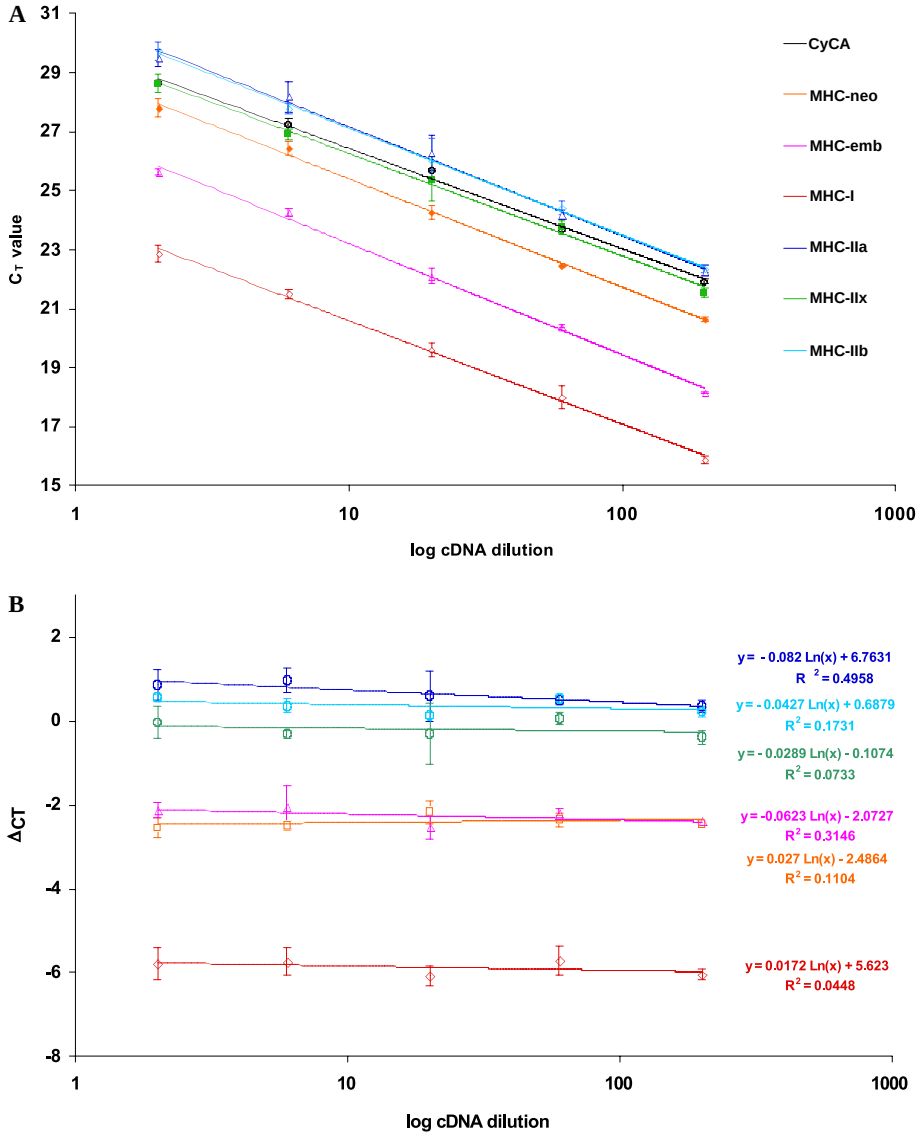

Fig. 3. (A) Representative standard curves of each MHC isoform. Threshold cycle values are plotted against input cDNA concentration. Each value is the mean of three independent MHC quantifications. Correlation coefficient (r) is equal to or greater than 0.998. Amplification slopes are −3.508 (MHC-I), −3.697 (MHC-IIa), −3.469 (MHC-IIx), −3.627 (MHC-IIb), −3.766 (embryonic), and −3.675 (neonatal). Data are means ± SEM. (B) Validation of the $2^{-\Delta C_T}$ method for adult MHC mRNA isoform quantification. The ΔC_T method for relative quantification requires that efficiencies of target (MHC) and reference (CycA) amplified in different tubes are approximately equal. Serial dilutions of cDNA from soleus muscles were amplified by real-time PCR using gene-specific primers for MHC isoforms and CycA. The difference (ΔC_T) between cycle threshold (C_T) from target and reference was calculated for each dilution and the value of the slope of ΔC_T in relation to logarithm of cDNA concentration should be less than 0.1 [45]. Data were fitted using least-squares linear regression analysis. Data are means ± SEM.

respect to both phenotype maturation and muscle contractile properties (Table 2). Fifteen days after birth, soleus muscle already expressed mainly the MHC-I transcript (52%), while developmental and fast MHC mRNA corresponded to 30% and 18%, respectively, of total MHC mRNA expressed. Whereas MHC-emb and MHC-neo mRNA expression was similar, among fast MHC transcripts type IIa was predominant (Table 2). Adult soleus muscle comprised a high proportion of MHC-I mRNA (93%), a small proportion of fast MHC mRNA (5%), and surprisingly 2% of developmental MHC transcripts. On the other hand, adult plantaris muscle, known as a fast-twitch skeletal muscle, comprised 96% adult fast isoform MHC transcripts, types II-a (13%), II-x (52%), and II-b (31%), a small proportion of MHC-I mRNA (4%), and very low levels of developmental MHC mRNA (0.1%).

Because MHC-emb and MHC-neo mRNA were poorly expressed in adult plantaris muscle, these primer sets were not assessed in single fibers isolated from this muscle (Fig. 5). Then, we tested our new primer sets and their real-time PCR conditions on mRNA expression for only adult MHC isoforms in pure and hybrid fibers from plantaris muscle, classified according to their MHC protein profile. Although MHC isoforms co-existed in hybrid fibers in combinatorial patterns according to the "next neighbor rule," previously established from sequential transitions in

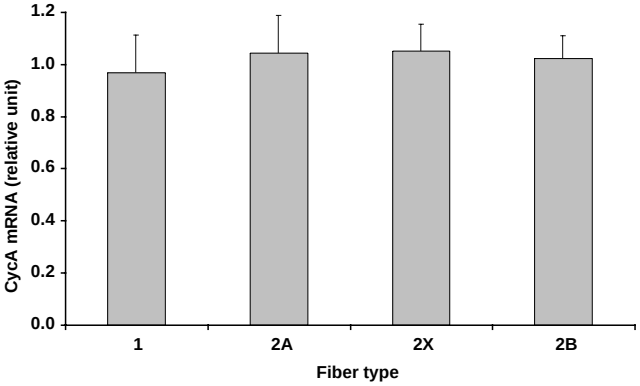

Fig. 4. Total CycA mRNA among single muscle fibers. Individual fibers were categorized regarding MHC isoform content into types I ($n = 13$), IIa ($n = 11$), IIx ($n = 21$), and IIb ($n = 27$). Data are means ± SEM. CycA mRNA expression is similar whatever the fiber type.

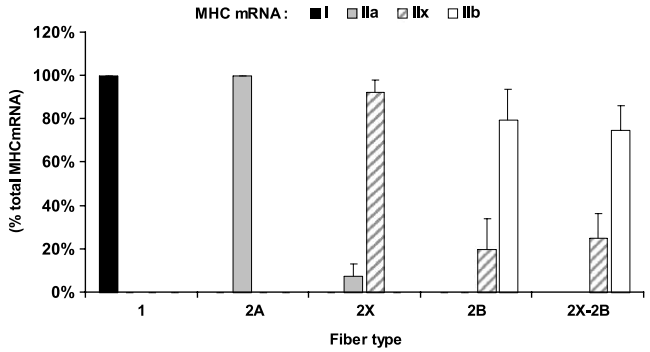

Fig. 5. Distribution of MHC mRNA isoforms in single fibers ($n = 20$).

the order of MHC-I → MHC-IIa → MHC-IIx → MHC-IIb, we only identified in our single fiber population, hybrid fibers containing MHC-IIx and MHC-IIb protein. This is probably due to the small number of fibers studied ($n = 20$). while other hybrid fiber subpopulations exist in plantaris muscle. Among our selected fibers, type 1 and 2A fibers expressed exclusively MHC-I and MHC-IIa mRNA, respectively (Fig. 5), while pure type 2X fibers displayed the co-existence of MHC-IIx mRNA (92%) and MHC-IIa mRNA (8%). Pure type 2B fibers expressed mainly MHC-IIb mRNA (80%) and a lesser percentage of MHC-IIx mRNA (20%). Hybrid 2X-2B fibers comprised MHC-IIx mRNA (25%) and a high proportion of MHC-IIb mRNA (75%).

Discussion

Until now, competitive RT-PCR has proved to be the most accurate and sensitive method to study MHC mRNA expression among different physiological situations either in skeletal muscle or in single fiber [18–20,24,12]. This method presents some drawbacks such as time consuming, post-PCR manipulations, or an impaired quantification due to either a lack of sensitivity in gel quantification or variations in PCR efficiencies between internal control and target cDNA [27]. In contrast, real-time PCR allows to bypass these disadvantages by increasing sensitivity and lowering variations [28]. Real-time PCR assays offer a lot of benefits including, a wide range of quantification (7–8 logarithmic decades), a high detection sensitivity (5–10 copies), the best precision (<2% standard deviation), a high throughput, and the lack of post-PCR manipulations [47,48]. This method allows direct detection of PCR products during the exponential phase of the reaction, by combining amplification and detection in a single step [38]. Sophisticated chemistries have been developed to directly measure PCR product accumulation by fluorescence emission, such as hybridization probes or minor groove binder probes. Recently, a TaqMan quantitative real-time RT-PCR was used to measure MHC mRNA levels in porcine muscles [29], and Pattison et al. [30], using a micro-array analysis, quantified both type IIx and IIb MHC mRNA in rat skeletal muscle. Nevertheless, these authors recognized a lack of precision of micro-arrays compared to a real-time PCR with a TaqMan probe. Then, the main difficulty of this novel procedure to study MHC mRNA expression was in how to design new primer sets, with a special care toward inter-isoform specificity of MHC, because members of the MHC gene family share a high degree of sequence similarity. Primer sets previously described for MHC PCR assays [18,20] were not suitable for real-time PCR in respect to the considerable length of the amplicons (600 bp). Then, real-time PCR primer sets were designed in the 3′ untranslated region (3′UTR), which is the most specific part of MHC isoform mRNA, in respect to their: (1) ability to differentiate MHC mRNA, (2) a melting temperature (T_m) greater than 55 °C, and (3) a GC-content close to 50 percent. Also, 5′ primers were

Table 2
MHC protein and mRNA distribution in neonatal and adult skeletal muscles

	Embryonic	Neonatal	Type I	Type IIa	Type IIx	Type IIb
MHC protein						
15-Day soleus	21.2 ± 1.2	17.0 ± 1.2	46.3±2.30	15.2 ± 1.3	0.0 ± 0.0	0.3 ± 0.3
Adult soleus	ND	ND	92.8 ± 1.2	7.2 ± 1.2	ND	ND
Adult plantaris	ND	ND	9.0 ± 0.9	20.2 ± 0.8	31.3 ± 0.7	39.5 ± 2.1
MHC mRNA						
15-Day soleus	16.8 ± 1.1	13.0 ± 1.4	52.4 ± 3.2	17.4 ± 2.3	0.4 ± 0.0	0.1 ± 0.0
Adult soleus	1.9 ± 0.9	0.04 ± 0.02	93.1 ± 2.2	2.3 ± 1.3	1.4 ± 0.8	1.2 ± 0.6
Adult plantaris	0.1 ± 0.0	0.01 ± 0.0	3.9 ± 0.4	12.7 ± 1.9	51.8 ± 5.5	31.5 ± 4.7

Data are means ± SEM in percentage of total MHC expression. ND, not detectable.

designed in respect to their: (1) compatibility with 3′ primers, (2) a 90–250 bp amplicon length, and (3) the ability to differentiate MHC mRNA. This approach ensured us to obtain a primer set highly specific to each MHC isoform as shown by melting temperatures in a range of 78.6 °C (MHC-IIb) to 89.7 °C (MHC-neo, see Table 1), and a GC-content between 46% (MHC-IIb) and 54% (MHC-emb). Furthermore, the small amplicon sizes (81–265 bp, Fig. 1A) ensure a high PCR efficiency, which coupled to the high specificity of each primers couple (Fig. 1B) lead to accurate quantification of MHC isoforms.

Validation of our real-time MHC PCR assay is founded on a fast and simple quantification by the comparative C_T method (ΔC_T method), showing high sensitivity and great accuracy. Indeed, whatever the MHC mRNA tested, both close PCR efficiencies and linearity of standard curves (Fig. 3) are distinctive of a rapid, high throughput, and reliable mRNA analysis for MHCs. Accuracy of real-time PCR is related to normalization through an internal control. Because skeletal muscle phenotype is regulated by many factors (contractile activity, neuronal pattern, mechanical loading, and/or hormonal influences), normalization of results appears to be a critical step in the field of muscle plasticity (for review, see [49]). In our experiment, utilization of a consensus MHC primer as an internal control was ruled out due to possible differences in PCR efficiency between MHC isoforms [27]. We normalized our results through CycA gene expression, which was previously demonstrated to be stable in rat muscles during aging [41,42], hypoxia [43], or exercise [35]. Although, Pattison et al. [30] suggested that CycA gene was not an excellent internal control according to changes in micro-array mRNA expression in aging and/or atrophied muscles, these authors recognized in their study that CycA mRNA variations were not statistically different and that real-time PCR is most sensitive than micro-arrays. Furthermore, skeletal muscles used in this study were excised from control animals, then transcript expression was not influenced by nuclear modifications. The constancy of CycA mRNA levels both in various MHC fiber types (Fig. 4) and in control muscles validates this gene as a good internal standard in our experiment.

The final step to validate these new real-time PCR primer sets was to measure MHC mRNA distribution in different skeletal muscles expressing various amounts of MHC both at protein and mRNA levels, i.e., neonatal versus adult muscles for developmental MHC, and slow versus fast contracting muscle for adult MHC. Then, mRNA expression of MHC in single fiber fragments was examined to confirm that these primers allow detection and quantification of MHC mRNA from very small samples.

An interesting finding is the detection of the six skeletal muscle MHC transcripts in young and adult hindlimb muscles of rats employing real-time PCR (Table 2). As expected, MHC mRNA expression is influenced by the developmental stage and contractile properties. Indeed, neonatal soleus presented a high proportion of developmental MHC transcripts (30%) compared to adult muscles (<2%), and MHC-I mRNA is the most abundant MHC isoform expressed both in immature and adult slow soleus muscles (52% and 93%, respectively) while fast MHC mRNA is predominant in fast plantaris muscle in comparison with slow neonatal and adult muscles (96%, 18%, and 5%, respectively). Although MHC-emb have already been found by others using competitive PCR in adult soleus [21,12] or adductor longus muscle [12], we detected for the first time, MHC-neo mRNA in adult muscles. Expression of MHC-neo transcript represents less than 0.1% of total MHC mRNA in adult skeletal muscles, but this result demonstrates the high sensitivity of our method as regards the quantification of very low amounts of mRNA. Findings concerning plantaris muscle are in accordance with those of previous studies, using different technologies such as RNase protection assay [16], Northern blotting [20,15], or competitive PCR [18,23]. For soleus muscle, the unique discrepancy concerns MHC-IIb transcript distribution, which is detected in our real-time PCR testing of both neonatal and adult muscles, but not in Northern blotting experiments of Huey and Bodine [8] or Wright et al. [20], or in competitive PCR works of Jung et al. [21] or Huey et al. [12]. Two hypotheses and their combination would explain this difference: (1) the best sensitivity of real-time PCR compared to those of Northern blot or competitive PCR techniques and (2) the strain of rats used in these studies (adult Sprague–Dawley animals). Indeed using competitive PCR, Jankala et al. [18] and Stevens et al. [23] have measured MHC-IIb mRNA expression in soleus of Wistar male rats.

Application of real-time PCR method to single fibers reveals numerous populations of fibers containing one or two different MHC mRNA isoforms in variable ratios (Fig. 5). Due to the low number of fibers tested in the present study, we are conscious that this subpopulation does not reflect the entire population of fibers existing in a plantaris muscle. Nevertheless, our results are in line with previous competitive PCR studies on normal fast-twitch rabbit muscle [19], or soleus muscle of rats [25] in which some fibers, classified according to their MHC protein content as pure, proved to be hybrid at the level of MHC mRNA isoforms expressing until four different MHC transcripts. Finally, the use of these new real-time PCR MHC primers confirms the mismatch between MHC mRNA and protein isoform distribution both in whole muscle (Table 2) and in single fiber (Fig. 5) described by other authors in control and transforming fibers or muscles [19,25,10,12] and strengthens the fact that MHC gene regulation required different post-transcriptional levels.

In summary, although competitive RT-PCR was until now the most sensitive and accurate way to quantify MHC mRNA, this approach is time consuming in post-PCR manipulations and less sensitive and accurate than new real-time PCR. Herein we report a real-time RT-PCR assay using newly designed primers developed to analyze developmental and adult MHC mRNA expression

both in skeletal muscles and single fibers. Due to the high sensitivity and precision, we demonstrated that this assay is suitable to study very low level of MHC mRNA expression as developmental MHC in adult muscle and quantify mRNA from very small samples. The use of real-time PCR in control and transforming muscles or single fibers will lead to new considerations concerning post-transcriptional processes involved in MHC gene regulation.

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