Analysis of myosin heavy chain mRNA expression by RT-PCR

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Wright, Carola, Fadia Haddad, Anqi X. Qin, and Kenneth M. Baldwin. Analysis of myosin heavy chain mRNA expression by RT-PCR. J. Appl. Physiol. 83(4): 1389–1396, 1997.—An assay was developed for rapid and sensitive analysis of myosin heavy chain (MHC) mRNA expression in rodent skeletal muscle. Only 2 µg of total RNA were necessary for the simultaneous analysis of relative mRNA expression of six different MHC genes. We designed synthetic DNA fragments as internal standards, which contained the relevant primer sequences for the adult MHC mRNAs type I, IIa, IIx, IIb as well as the embryonic and neonatal MHC mRNAs. A known amount of the synthetic fragment was added to each polymerase chain reaction (PCR) and yielded a product of different size than the amplified MHC mRNA fragment. The ratio of amplified MHC fragment to synthetic fragment allowed us to calculate percentages of the gene expression of the different MHC genes in a given muscle sample. Comparison with the traditional Northern blot analysis demonstrated that our reverse transcriptase-PCR-based assay was reliable, fast, and quantitative over a wide range of relative MHC mRNA expression in a spectrum of adult and neonatal rat skeletal muscles. Furthermore, the high sensitivity of the assay made it very useful when only small quantities of tissue were available. Statistical analysis of the signals for each MHC isoform across the analyzed samples showed a highly significant correlation between the PCR and the Northern signals as Pearson correlation coefficients ranged between 0.77 and 0.96 (P < 0.005). This assay has potential use in analyzing small muscle samples such as biopsies and samples from pre- and/or neonatal stages of development.

skeletal muscle, Northern blot, semiquantitative competitive reverse transcriptase-polymerase chain reaction, Sprague-Dawley rats, messenger ribonucleic acid

MAMMALIAN SKELETAL MUSCLE fibers differ widely with respect to their biochemical and functional characteristics and are known to possess a remarkable plasticity in response to various physiological stimuli (25). In fact, muscle fibers can undergo extensive remodeling in their contractile apparatus to meet new functional requirements. This high adaptability appears to reside in the muscle fibers’ ability to transcribe different isoforms of the contractile proteins, each with specific functional characteristics. The polymorphism of the myosin heavy chain (MHC) plays a major role in the diversity and adaptability of muscle fibers. MHC isoforms in mammalian muscle fibers are encoded by a highly conserved multigene family (2). At least six distinct MHCs are known to be expressed in rat skeletal muscles (25). Two developmental isoforms, embryonic and neonatal isoform, as well as four adult MHC isoforms designated as slow type I, fast IIa, IIx, and IIb have been identified. The pattern of expression of these isoforms is developmentally regulated (7, 14, 17) and can be modulated by hormonal, metabolic, and mechanical factors in a tissue-specific manner (3, 8, 11–13, 19, 27).

In most mammals, during the fetal/embryonic stage, all types of skeletal muscles express the embryonic and neonatal MHCs. In the rat, as the animal matures into adulthood, the expression of these two genes becomes downregulated (23, 29, 31), and, by 3–4 wk of age, the protein form for both embryonic and neonatal myosin cannot be detected in the hindlimb muscles of normal rats. However, reexpression of these embryonic/neonatal MHC genes has been observed in regenerating fibers in rat leg muscles (24) and in the masseter muscle of thyroid-deficient rats (14).

Recent studies on MHC gene expression have begun to focus analyses on both protein and mRNA levels to obtain insight on pretranslational/translational processes involved in MHC gene regulation (3, 8, 11–13, 19, 27). MHC protein isoforms can be separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) techniques (15, 28), and their relative distribution can be determined by laser-scanning densitometry of the stained gel. Analysis of mRNA expression has traditionally been accomplished by either slot/dot-blot (3, 11–13, 27), Northern blot (3, 27), or S1 nuclease assays (14, 17, 19). In a steady state, mRNA expression usually parallels the pattern of MHC protein expression; thus it is assumed that MHC expression is chiefly regulated at the pretranslational level (26).

Because of the shorter half-life of the MHC mRNA compared with protein, changes in mRNA expression occur at a faster time scale, and thus it is advantageous to study mRNA expression in short-duration experiments.

Members of the MHC gene family share a high degree of sequence similarity; therefore, classic Northern analysis has been somewhat difficult and most tedious to perform. All MHC mRNAs are about the same size on agarose gels (6.5–7 kb), and cDNA probes for the different MHC isoforms tend to cross hybridize with each other. The S1 nuclease assay has been one solution to this problem (14, 19). An alternative to this
approach was developed by Gustafson et al. (11, 12), who used short oligonucleotide probes from the very diverse 3’ untranslated regions of the different MHC genes in Northern blots and slot blots. Although the assay is specific, it requires a relatively large amount of total RNA, especially if the presence of several MHC isoforms must be determined simultaneously (3, 13), and it lacks sensitivity of detection for low levels of mRNA expression.

Because tissue availability is often limited, particularly in studies in which either small animals or small muscle samples are used, there is a need for a new method that can be applied specifically to analyze small amounts of expressed mRNA, i.e., in cases typically encountered during the neonatal stages of development and in biopsies.

Herein we report a different method to analyze the relative distribution of MHC mRNA isoforms in a muscle sample. We utilized the reverse transcriptase (RT) reaction coupled to a polymerase chain reaction (PCR) to determine relative amounts of the different MHC mRNAs in total RNA samples of rat skeletal muscles. The RT-PCR is a sensitive and powerful method for detection and amplification of low levels of mRNA expression. However, the exponential nature of the PCR reaction makes it difficult to obtain absolute quantitative data of mRNA expression, because the efficiency of the PCR reaction is subject to variations. Thus we developed a new method that enabled us to quickly and reliably quantify relative MHC mRNA expression (abundance) in various rat skeletal muscle samples. Our RT-PCR-based assay has the advantage of allowing simultaneous, rapid analysis and comparison of any rat skeletal muscle sample for the relative levels of expression of six different MHC mRNAs. Furthermore, the high sensitivity of the assay made it very useful when only small quantities of tissue were available.

METHODS

Tissue selection. To examine a broad spectrum of skeletal muscle differentially expressing the various MHCs, RNA was extracted and analyzed from the following Sprague-Dawley rat muscles: 1) the slow-twitch soleus muscle of neonatal rats at 5, 10, and 15 days of age, as well as from an adult rat; 2) the fast-twitch plantaris muscle of neonatal rats at 5 and 10 days of age, including an adult rat; 3) the relatively slow red vastus intermedius muscle and the fast white vastus lateralis muscle of adult rats; and 4) the masseter muscle of both an adult normal and thyroid-deficient (thyroidectomized) rats. The neonatal and adult normal and thyroid-deficient (thyroidectomized) rats were obtained from Taconic Laboratories (Germantown, NY). This spectrum of muscles provided a wide range of MHC mRNA isoform expression resulting in diverse MHC mRNA distribution patterns for analyses. Rats were killed with an overdose of pentobarbital sodium, and specific muscles were dissected out, quick-frozen in liquid nitrogen, and stored at −80°C until subsequent analyses.

Total RNA and protein isolation. Total RNA and total muscle proteins were simultaneously coextracted from frozen muscle samples by using the TRIzol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD) following the company protocol, which is based on the method described by Chomczynski (4). Total proteins were separated in the organic phase and subsequently precipitated with isopropanol, washed with guanidine hydrochloride and ethanol, and suspended in 1% SDS (4); protein concentration was determined by the Bio-Rad protein assay kit using γ-globulin as standard. Samples were adjusted to protein concentration of 1 mg/ml with 1% SDS and were stored at −20°C until later analyzed for MHC distribution pattern by SDS-PAGE. Total RNA was precipitated from the aqueous phase with isopropanol, and after being washed with ethanol it was dried and suspended in a small volume of ribonuclease-free water. The RNA concentration was determined by optical density (OD) at 260 nm (by using an OD260 unit equivalent to 40 µg/ml), and the final concentration was adjusted to 1 µg/ml. This technique provides undegraded RNA, free of DNA and proteins, based on analyses using agarose gel electrophoresis and ethidium bromide stain as well as an OD260/OD280 ratio of ~2.0. Integrity of the RNA was verified by gel electrophoresis of ~1 µg RNA on an 1% agarose 0.5× Tris(hydroxymethyl)aminomethane (Tris)-borate-EDTA buffer (TBE) gel containing ethidium bromide, using 0.5× TBE as the running buffer. For intact samples, we were able to visualize both 28S and 18S (ribosomal RNA) bands with a density ratio of ~2:1 (5). No analysis was performed on any sample with degraded RNA appearance. Samples were stored at −80°C until subsequent analyses.

MHC protein isoform distribution. Skeletal MHCs were separated by using a SDS-PAGE technique (28). The gels were run at 275 V for ~22 h under refrigeration. After electrophoresis, the gels were stained for 1 h with brilliant blue G 250 ( Sigma Chemical) and destained with 25% methanol and 10% acetic acid. The separated MHC bands were scanned and quantified by using a Molecular Dynamics densitometer (Sunnyvale, CA). The peaks of interest representing the distinct MHC isoforms were identified on the digitized densitometric data sets. The area of each peak was determined by integration (Image Quant software, Molecular Dynamics). Each specific MHC isoform was expressed as percent of the total MHC expressed in a particular muscle sample, i.e., %type I = 100 × (area of peak I)/sum of area of all the MHC peaks.

RT reaction. Two micrograms of total RNA were reverse transcribed for each muscle sample to be analyzed by using the following protocol: 2 µl GIBCO 5× first strand buffer, 2 µl deoxy nucleic acid triphosphate (dNTP) mix (10 mM each), 1 µl oligo[dT: deoxythymidine] primer (100 ng/µl), 1 µl RNasin (Promega, Madison, WI), 2 µl total RNA (1 µg/µl), and 2 µl Moloney murine leukemia virus ribonuclease H−RT (GIBCO/BRL). The reaction mixture was incubated for 30 min at 37°C, followed by 2 min at 95°C, and then chilled on ice and immediately used in the PCR reaction.

Oligonucleotide primers. The 5’ oligonucleotide for each amplification was designed from a highly conserved region in all known rat MHC genes ~600 base pairs upstream of the stop codon (16). The four adult MHC isoforms are identical in this region, which enabled us to use the same “common primer” with the following sequence: 5’ GAAAGCCCAAGAGGCCCATC3’. To design a perfect match of upstream primers for the embryonic and neonatal sequences, slight modifications of the above common primer were necessary (see Table 1). The optimal annealing temperature of these degenerate common primers was, however, unchanged, and they were used in the same manner as the common primer as 5’-oligonucleotides for PCR reactions. The 3’-oligonucleotides used in the PCR reactions were designed from the 3’-untranslated regions of each of the different MHC genes, where the sequences are highly specific for each MHC gene (7, 11, 12) (Table 1, Fig. 1A).
PCR and yield a fragment of different size than MHC genes.

fragments that are coamplified with each polymerase chain reaction (PCR) product in base pairs (bp); control fragment is the size of amplified exogenous control fragment when used with the specified sets of primers. R, A/G; Y, C/T.

Design of the internal control fragments. Two internal control fragments were constructed by a technique of oligonucleotide overlap extension and amplification by PCR (30).

PCR. Ten picograms of each of the control fragments (adult/developmental) were added to the reverse-transcribed RNA reaction tube, and total volume was brought up to 100 μl with nuclease-free pure water. Ten microliters of this mixture (containing ~200 ng of reverse-transcribed total RNA and 1 pg of the control fragments) were used for each 50-μl PCR reaction. The PCR reaction mixture contained 5 μl 10× PCR buffer (GIBCO), 2 μl dNTP mixture (10 mM each), 3 μl 50 mM MgCl2, 500 ng of each of the two appropriate primers (Table 1), 1 μl (1 unit/μl) Taq polymerase (GIBCO), and water to a final volume of 50 μl. Amplification was carried out in a Stratagene Robocycler with an initial denaturation step of 1 min at 94°C, followed by 25 cycles, with each cycle consisting of 45 s at 94°C, 60 s at 50°C, 90 s at 72°C, and a final step of 3 min at 72°C. The number of cycles was optimized so that the amplified signal was still on the linear portion of a semilog plot of the yield expressed as a function of the number of cycles. PCR products were analyzed by agarose gel electrophoresis (20-μl aliquots of a 50-μl PCR reaction loaded on a 1.5% agarose gels (in 1× Tris-Acetate-EDTA buffer) containing 0.2 μg/ml ethidium bromide) to visualize the PCR products. Pictures of the gels were taken under ultraviolet (UV) light using Polaroid instant film number 55 to generate both a negative and a positive printed image of the gel (see Fig. 2).

Analysis of gels. Bands were analyzed by laser-scanning densitometry of the obtained negatives (Molecular Dynamics personal densitometer). The volume of the OD of a DNA band as determined by the Image Quant software (Molecular Dynamics) and corrected to local background was directly proportional to the amount of DNA over a wide range. Intensity (volume of the OD) of the MHC band was divided by the intensity of the control fragment, thereby correcting for any differences in the efficiency of the PCR reactions. The percent content of each MHC gene was calculated based on the corrected values and based on the fraction of specific MHC mRNA-corrected value relative to the total sum of expressed MHC mRNA isoforms in a given sample. For example, %type I MHC mRNA = 100 × (type I MHC band intensity/ corresponding control band intensity)/sum (MHC band/ control band) for all six MHC mRNA isoforms).

Northern blots. Approximately 5 μg of total RNA were electrophoresed in 0.8% agarose gel in a buffer containing 8% formaldehyde, 20 mM 3(N-morpholino)-propanesulfonic acid, 5 mM sodium acetate, and 0.5 mM EDTA, pH 7.0. Because six different probes (for each specific MHC isoform) were required to be tested, each sample was loaded six times, i.e., on six separate gels. A series of the different samples to be tested were loaded on the same gel to be processed simultaneously for the same MHC probe. The RNA was transferred to a quartz (Quiagen) nylon membrane by the capillary method using 10× saline-sodium citrate and subsequently covalently cross-linked to the nylon membrane by UV light (UV crosslinker, Fisher Scientific). After drying at 80°C for 1 h to evaporate the formaldehyde, blots were stored at 4°C until used for hybridization.

Hybridization. For hybridization we used the same 3′antisense oligonucleotides as those used for the PCR reactions (see Table 1). Probe labeling and hybridization conditions were performed as described previously (3, 8, 13, 27). Hybridization with each of the specific probes, washing, and exposing to the autoradiographic film were done simultaneously for all the samples to be included in the analyses. This simultaneous treatment is important if one is to compare the intensity of the signal of one isoform across the different samples. After signal detection with the use of autoradiography, the probes were washed off the blots by...
being boiled for 10–15 min in 1% SDS. The blots were then rehybridized with an excess of a 32P end-labeled 18S oligoprobe (18), which hybridizes to 18S ribosomal RNA. The signal of 18S rRNA in each sample is directly proportional to the amount of total RNA on the membrane and thus can be used to correct the MHC signal for differences in the total amount of RNA in each of the samples. Autoradiograms were analyzed by laser-scanning densitometry (Molecular Dynamics). For each specific MHC band, the OD was normalized to its corresponding 18S signal.

RESULTS

We have used a new PCR-based method to analyze MHC mRNA distribution in a wide spectrum of rodent skeletal muscles. Results were compared with those generated by the more commonly used method of Northern blot hybridization. Also, in adult control tissue, in which mRNA expression is at a steady state, %MHC mRNA as generated by the new method were compared with %MHC protein as an additional way to validate this approach.

Northern analyses of MHC mRNA expression. The MHC mRNA signals generated by the traditional Northern hybridization, and as corrected to the 18S rRNA signal, are reported in Table 2. These data reflect the abundance of specific MHC mRNA isoforms in each individual muscle, and the results generally agree well with the distribution of MHC protein. For example, the adult soleus muscle expresses almost exclusively the type I (85–95%) and type IIA (5–15%) MHCs. The Northern data show that in the normal adult soleus, type I MHC mRNA is the most abundant MHC isoform expressed, whereas IIA MHC is the only other detectable mRNA signal. Also, from the Northern data involving the adult skeletal muscle, where protein expression is in a relative steady state, the signal intensity for a specific MHC type across the different samples corresponds well to %MHC protein expression. Linear regression and correlation analyses between %MHC protein expression and MHC mRNA signal relative to 18S demonstrate a high correlation coefficient, with statistical significance across all the adult samples (Table 3). However, this correlation becomes weaker in performing analyses on muscles in developmental stages or when muscles are undergoing transformation in the early stages of an imposed perturbation, such as after 3 wk of thyroid deficiency.

RT-PCR analyses of MHC mRNA expression. A total of six separate PCR reactions were run simultaneously on each reverse-transcribed total RNA sample by using the specified primers (Table 1). Each reaction resulted
in two products that could be separated by agarose gel electrophoresis, stained by ethidium bromide, and quantified by scanning densitometry of the clear negative image on the film. The higher molecular weight product (Fig. 2) corresponds to the amplified MHC mRNA existing in the total RNA sample, and its intensity depends on the abundance of the specific MHC mRNA in the total RNA sample. In contrast, the lower molecular weight product of each PCR reaction corresponds to the amplified, externally added, synthetic DNA fragment when the same primers were used as those used for amplifying the MHC mRNA. Because the amount of synthetic fragment relative to MHC cDNA is constant in each PCR reaction for a given sample, variability in the intensity of this band depends on the PCR priming efficiency, especially that of the specific primer (antisense primer), and thus can be used as an internal control to correct for variability in the amplification reaction due to slightly different optimal annealing temperatures of the specific primers and possibly variable conditions from one run to another. Therefore, the ratio of the amplified MHC signal to that of the amplified fragment is used to represent the abundance of the specific MHC mRNA. With the use of this approach, the mRNA was also expressed as a percentage of the total distribution, whereby each corrected MHC mRNA signal was divided by the sum of all of the six MHC mRNA isoform-corrected signals in a given sample.

As an initial attempt to validate the PCR method as a suitable method of quantification of MHC mRNA distribution in a sample, %MHC protein was correlated with %MHC mRNA as generated by PCR for the adult samples, in which RNA/protein ratios are in a relative steady state. Correlation between the two was highly significant for all the MHC isoforms tested (Table 4), demonstrating that this method is as reliable as the Northern blots for analyzing MHC mRNA expression in a muscle sample.

As an additional approach to validate the PCR method, the RNA signals as generated by Northern blot hybridization were correlated with %MHC mRNA as calculated by the PCR method. These correlations were highly significant for all the six different MHC mRNA signals when studied over a broad range of expression (see Fig. 3). Correlations for the neonatal and type IIa isoforms, however, were lower compared with the other MHCs tested. For type IIa analysis, the lower $r^2$ value may be due to the overall low expression of this MHC isoform in muscles. Northern signals are generally low for this isoform and, therefore, more subject to errors. The RT-PCR assay (exponential amplification of the target sequence) increases the sensitivity for detection of this message and may help to generate more consistent signals when the appropriate controls are also included. In the case of the neonatal MHC, the reason for low correlation is not known; however, correlation improved significantly when samples with the highest Northern signal were excluded ($r^2 = 0.81; P < 0.002$).

We speculate that the slight discrepancy between PCR and Northern in this case is due either to our choice of primers or to some suboptimal Northern hybridization conditions. Despite these small deviations between the two procedures (Northern vs. PCR), and based on $P < 0.005$ for the regression analyses including those on IIa and embMHC mRNAs, the use of the RT-PCR method is highly reproducible with 10% variability from run to run, when the same sample was retested.

### DISCUSSION

A common problem in quantitative PCR analysis is inaccuracy due to variability in the starting amounts of

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<th>Table 3. Linear regression and correlation analyses of protein vs. Northern signal for the adult samples</th>
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Linear regression was determined by using statistical software package (Graphpad Prism 2.0); $n = 5$ independent observations for each isoform.

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RNA. Because of the exponential nature of the PCR reaction [PCR final product amount = initial amount \times (1 + efficiency)^{(no. of cycles)}], small differences in the starting amounts of target DNA can lead to large changes in the amounts of the final products. Furthermore, the PCR amplification depends on the reaction efficiency, which can be quite variable, and small changes in efficiency can lead to major differences in the final product. To add to this complication, RT-PCR products are even more difficult to quantify because of the sequential enzymatic reactions involved: RT followed by PCR.

One way to overcome these problems is the use of differential/competitive PCR. In this approach, a constitutively expressed gene is amplified simultaneously in the same reaction as the target mRNA sequence, and correction is done relative to the constitutive gene. The two PCR products can be differentiated by gel electrophoresis based on size difference (10). On the other hand, to correct for variability in PCR reaction efficiency, known amounts of a control DNA fragment (internal control or competitive template) are added to the reaction and amplified simultaneously with the mRNA of interest, by using the same set of primers as the target mRNA, but yielding a product of different size (1, 30). When the above two methods of correction are combined, one can accurately estimate the exact absolute amount of a specific mRNA species in a given sample (6).

The method reported herein does not provide absolute quantities of the mRNA expressed in a sample;
therefore, correction to the amount of starting material is not necessary. It provides, however, accurate comparative data on the relative level of mRNA expression of each of the MHC isoforms in a given muscle. The construction of the internal fragment and simultaneous amplification made it possible to correct for possible variation in efficiency (30). These comparative data are sufficient for most biological purposes and compare well with the way MHC protein expression is studied in muscle samples (3, 8, 13, 27, 28).

Other studies have attempted to determine MHC mRNA expression in skeletal muscle by using the RT-PCR method (9, 20–22). Ennion et al. (9) used RT-PCR to detect specific MHC mRNA expression in human single fibers. That particular study was mainly qualitative; it determined the presence or absence of the MHC mRNA species in a given sample. The amplified α-skeletal actin mRNA was used as a mere positive control for the PCR reaction. α-Actin as a marker, however, cannot be used for normalizing the data for two reasons: 1) the relative expression of the α-actin mRNA might be variable among muscle samples because of the experimental conditions imposed; and 2) since different primer pairs are used to amplify the MHC than those to amplify the α-actin, one cannot correct for efficiency of the PCR amplification.

Peuker and Pette also used the RT-PCR approach to analyze specific MHC mRNA expression in either rabbit single fibers (20) or in whole muscles (21, 22). These studies attempted to quantify the absolute amounts of specific MHC mRNA expressed in a muscle, by using the α-actin as internal positive control and by comparison to known amounts of a cDNA standard (20, 22). In these studies, correction for the efficiency of the PCR reaction was not considered, despite its likely contribution to significant error in the quantitative aspect.

These previous papers (9, 20–22) clearly demonstrate the sensitivity of the RT-PCR method in detecting low-expression genes and its applicability to small sample size. However, these approaches lack simplicity and/or inclusion of appropriate controls; therefore, they cannot be applied to study quantitative changes in MHC mRNA expression among different muscle samples, as commonly studied by using S1 nuclease or Northern blot assays.

In conclusion, we describe a new approach to the RT-PCR method, which can be used for the analysis of six MHC mRNAs in skeletal muscle samples. This approach is simple and quantitative and thus offers a new alternative to the standard Northern analysis. Statistical analysis of the signals generated by both methods shows that the signals are highly correlated to one another. Whereas the Northern blot remains the more straightforward technique for mRNA analysis (no manipulation of the RNA and a direct correlation between signal intensity and the number of mRNA copies in the sample), our RT-PCR assay makes analysis of MHC mRNA possible where the Northern technique is difficult or impossible, i.e., when sample size is small and the amount of available RNA is insufficient.

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