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Non-radioactive reverse transcriptase/polymerase chain reaction for quantification of myosin heavy chain mRNA isoforms in various rabbit muscles

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A method was established for measuring molecule numbers of three different myosin heavy chain (MHC) mRNA isoforms in total RNA preparations. The quantification was based on a combination of primer-directed reverse transcriptase and polymerase chain reactions with 5′-digoxigenin-labeled oligonucleotides, using external standards. The sensitivity of the method allowed the quantitation of mRNA amounts down to the range of 1,000 molecules (detection limit 50 molecules). The numbers determined for eight different rabbit muscles are in the range of 10^3–10^6/μg total RNA. In soleus muscle, the value of 1.11×10^9 MHCI mRNA molecules corresponds to approximately 8% of the total mRNA. With reference to myonuclei, this amount corresponds to $1–2 \times 10^4$ molecules/nucleus. A quantitative comparison of the two fast MHC mRNA isoforms with the distribution of different MHC isoforms at the protein level indicates that one of these two fast sequences is specific to MHCIIb and the other to MHCIId. However, our data point to the existence of additional MHCIId mRNA subtypes.

Muscle fiber type; Myosin heavy chain mRNA isoform; Number of MHC mRNA molecule; Quantitative reverse transcriptase/polymerase chain reaction

1. INTRODUCTION

Relationships have been established between histochemically defined fiber types and their myosin heavy chain (MHC) complement [1]. Although muscle fibers generally express only one MHC isoform, hybrid fibers exist containing more than one HC isoform [2–6]. Moreover, there is evidence that muscle fibers are able to change their phenotype, performing fiber type transitions under the influence of exogenous factors, e.g. altered neuromuscular activity, mechanical activity or hormonal signals. These transitions correspond to alterations in the expression of MHC isoforms. While these changes have been studied down to the level of protein analyses on single fiber fragments [5], little information is available as to the quantitative changes in the amounts of the corresponding MHC mRNA isoforms. In order to monitor alterations in gene expression at this scale, a quantitative approach for assessing absolute amounts or molecule numbers of specific mRNAs would be desirable. The purpose of the present study was to elaborate a standardized, highly sensitive and quantitative assay for mRNAs specific for different MHC isoforms. An appropriate approach for measurements at the microscale appeared to be reverse transcription (RT) of mRNA followed by amplification of the resulting cDNA by the polymerase chain reaction (PCR) [7–10]. This method makes use of specific oligonucleotides derived from isoform-specific cDNA sequences and is superior to conventional hybridization techniques yielding arbitrary numbers or relative amounts.

Different approaches for quantification of mRNA using RT and PCR have been reviewed [11]. In view of the complexity and the kinetics of the amplification reaction, the use of internal or external standards is advisable. In our case, where several mRNA isoforms were of interest, calibration with external standards [12,13] appeared to be most feasible.

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2. MATERIALS AND METHODS

2.1. *Preparation of total RNA*

Total RNA was prepared by the acid guanidinium isothiocyanate method [14] from adductor magnus, diaphragm, extensor digitorum longus, gastrocnemius, psoas, soleus, tibialis anterior, and vastus lateralis muscles of adult male White New Zealand rabbits. RNA concentration was assessed spectrophotometrically. For quantification experiments, RNA stock solutions (0.1 μg/μl) were prepared in 20 mM ribonucleoside vanadyl complexes (RVC; Sigma). Dilution series were prepared from stock solutions as described for cRNA. Aliquots of the dilutions were stored at −70°C.

2.2. *Oligonucleotide primers*

Sequences from three different cDNAs were available: pMHCβ174 [15] was used for the slow MHCI; pMHC20-40 [16] and pMHC24-79 (K. Maeda, A. Wittinghofer, personal communication) were used for two fast MHC isoforms.

Primers (20mer) for isoform-specific segments of the three cDNAs

were synthesized by Microsynth (Windisch, Switzerland). The 5′ ends of primers complementary to mRNA were labeled with digoxigenin [17]. The primers for pMHCβ174 were directed against a 173 nucleotide (nt) sequence of the coding region. For pMHC20-40 and pMHC24-79, the primers were selected to amplify 236 nt and 289 nt segments, respectively, extending from the coding into the non-coding regions at the 3′ end.

2.3. *Preparation of external standards*

cRNAs were synthesized by in vitro transcription from the following transcription vectors: pB(20-40) and pB(24-79) (gifts of Dr. K. Maeda, Heidelberg) are Bluescribe M13$^+$ vectors containing the 3′ *Pst*I fragments of pMHC20-40 and pMHC24-79, respectively. For cRNA synthesis specific for pMHCβ174, the 173 bp PCR product was cloned into pGEM-4Z (Promega, Madison, WI) according to the supplier's instruction. After linearization of the corresponding plasmids with *Eco*RI, the three cRNA standards (236 nt for pMHCβ174, 416 nt for pMHC20-40, and 579 nt for pMHC24-79) were transcribed in vitro using T7 and T3 RNA polymerase (Boehringer, Mannheim). Digestion of the template was performed with RNase-free DNase (Boehringer, Mannheim). Unincorporated nucleotides were removed by G-50 Sephadex (LKB Pharmacia) gel filtration. The cRNAs were tested for purity by PCR. The length of the transcripts was checked by agarose gel electrophoresis. cRNA concentrations were determined spectrophotometrically taking into consideration the base composition. Copy numbers were calculated using Avogadro's number. For preparing serial dilutions of the cRNAs down to 100 molecules/μl, defined amounts of cRNA (20–35 μg) were mixed with a 50-fold excess of carrier RNA (tRNA from yeast; Boehringer, Mannheim) and then diluted in 20 mM RVC. Aliquots of the dilutions with defined molecule numbers were frozen and stored at −70°C in reaction tubes ready for use in the RT/PCR assay. The reliability of the dilutions and their stability were tested in separate RT/PCR assays.

In comparison to dilutions in water or in dithiothreitol, RVC dilutions yielded the most reliable calibration curves for all three cRNAs, extending to the very low concentrations for studies at the level of single muscle fibers. Moreover, RVC greatly increased the stability of the frozen aliquots which proved to be stable for at least 10 weeks.

2.4. *RT/PCR assay*

A one buffer/one tube system was used for the combined reactions of RT and *Taq* polymerase according to Zafra et al. [18]. Optimum reaction conditions were established for each of the three sequences under study. The assay mixture (25 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ (for pMHC20-40) or 3.5 mM $MgCl_2$ (for pMHCβ174 and pMHC24-79), 0.1 mg/ml gelatin, 0.25 mM dNTPs, 0.12 μM of each primer, 2.5 U human placenta RNase inhibitor, 0.75 U *Taq* polymerase, and 0.5 U AMV reverse transcriptase. All biochemicals were from Boehringer (Mannheim). Mineral oil overlay was unnecessary since the reaction tubes were immersed in glycerol baths of different temperatures using a Robotherm PCR machine (Bühler, Bodelshausen, Germany). The following controls were performed to monitor DNA contaminations: (i) complete assay with *Taq* polymerase, but without template and RT; (ii) complete assay with *Taq* polymerase and template, but without RT.

cDNA synthesis was performed for 30 min at 42°C. Thereafter, PCR was started for 20–45 cycles. The following amplification protocol was used: 1st cycle with 5 min denaturation at 92°C, 2 min annealing at 53.5°C (for pMHC20-40 and pMHC24-79) or 55°C (for pMHCβ174), 30 s synthesis at 74°C. The following cycles consisted of 1 min denaturation, 1 min annealing and 30 s synthesis.

2.5. *Product analysis*

1–2 μl of the reaction mixture were analyzed on a 6% polyacrylamide gel run in 1× TBE (100 mM Tris-HCl, pH 8.3, 83 mM boric acid, 1 mM EDTA). DNA was transferred by electroblotting (LKB Multiphor II) to a nylon membrane (Hybond-Nfp, Amersham). After UV fixation for 2.5 min, the digoxigenin-labeled DNA was detected by an antibody-linked detection assay (Boehringer, Mannheim). The amplified DNA was visualized by chemiluminescence using an anti-digoxigenin antibody coupled to peroxidase (Boehringer, Mannheim) in combination with enhanced chemiluminescence detection reagents (ECL, Amersham). Signals were photographically documented (Hyperfilm-ECL, Amersham, 30 s–3 min exposure). Quantitative evaluation was performed by integrating densitometry, using an image processing system (Syncotec, Asslar, Germany) consisting of a video camera and an image processor. Separate analyses were performed in order to determine linear correlation between product amount and light emission.

3. RESULTS

3.1. *Optimum conditions for the RT/PCR assay*

The combined RT/PCR assay yielded specific signals for the three different MHC mRNAs investigated. The identity of the signals obtained with the selected oligonucleotide primer pairs was verified according to their length, by restriction analysis, and by hybridization with a diagnostic oligonucleotide (A. Uber, unpublished results).

An example for the amplification of mRNA specific for pMHC24-79 is shown in Fig. 1 (upper panel). A

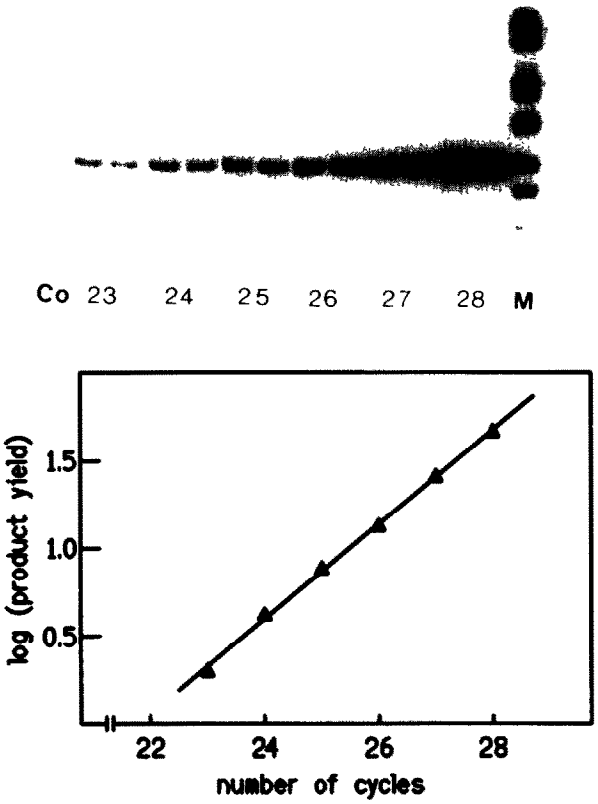

Fig. 1. Accumulation of RT/PCR products in duplicate determinations with increasing cycle numbers during the exponential range of the reaction. The 289 nt fragment specific to pMHC24-79 was amplified from 4 ng of total RNA from rabbit gastrocnemius muscle. Co, control (complete assay with *Taq* polymerase and template, but without RT); M, markers (digoxigenin-labeled DNA molecular weight marker VI; Boehringer, Mannheim). The chemiluminescent signal was documented photographically (upper panel). The lower panel shows the densitometric evaluation of product accumulation during the exponential phase of the RT/PCR. The efficiency of the reaction (E = 86%) was calculated by linear regression analysis.

single band at 289 nt was obtained with cycle numbers falling into the range of the exponential product accumulation. However, additional faint bands were seen at higher cycle numbers, i.e. when the reaction started to plateau and was no longer within the exponential phase. Obviously, the 5′ end-labeling with digoxigenin did not impair the *Taq* polymerase reaction, since amplification efficiencies exceeded 80% during the exponential phase of the reaction (Fig. 1, lower panel). The accumulation of the product is described by the equation: $\log N_n = \log N_0 + n\log(1 + E)$, where N_n is the densitometric value corresponding to the number of molecules at cycle n, N_0 the corresponding number of target molecules at cycle zero, and E the efficiency of the amplification. E is determined by linear regression analysis of the slope, $\log(1 + E)$. Efficiencies above 80% existed for amplification of all three sequences, i.e. 87 ± 5% for pMHCβ174, 81 ± 4% for pMHC20-40, and 86 ± 5% for pMHC24-79 ($n = 5$).

Both the sensitivity and precision of the method was greatly enhanced by chemiluminescent detection of the specific product. As compared to product detection by ethidium bromide, chemiluminescence combined with autoradiography provided an approximately 100-fold higher sensitivity. This enhanced sensitivity was a prerequisite for detecting product accumulation within the exponential range. The reaction product yielded sharp bands with little or no background which greatly facilitated densitometric evaluation (Fig. 1).

3.2. *Calibration of the standard reaction*

The relationship between input of template, number of cycles and amount of product in the RT/PCR was examined on a 1:2 dilution series of the three standards at increasing numbers of cycles (Fig. 2). At increasing dilutions of the template, increasing cycle numbers resulted, due to an efficiency lower than 100%, in slightly lower amounts of product. As illustrated by the semilogarithmic plot in Fig. 2 (lower panel), decreasing the number of target molecules by a factor of 2 could be nearly compensated for by an additional cycle. This constant relation was valid in the case of all three standards for down to approximately 2,000 molecules (Fig. 2, lower panel). Deviations from this constant relationship occurred at lower template numbers where the number of additional cycles had to be progressively increased to maintain the amount of product. Possibly, the decreasing efficiency of the reaction at low template numbers resulted from the appearance of non-specific products (Fig. 2, upper panel). According to these results, we decided to use standards in the range between 3×10^3 and 1.3×10^6 molecules for measurements of MHC mRNAs in preparations of total RNA. This range was two orders of magnitude above the detection limit of the method.

Moreover, it was necessary to establish the range of cycle numbers yielding exponential product accumulation for defined amounts of the standards [19]. Under the chosen conditions, decreasing amounts of cRNA yielded similar amounts of product at increasing number of cycles. As shown in Fig. 3, the lines obtained by linear regression analysis displayed identical slopes, thus indicating comparable efficiencies.

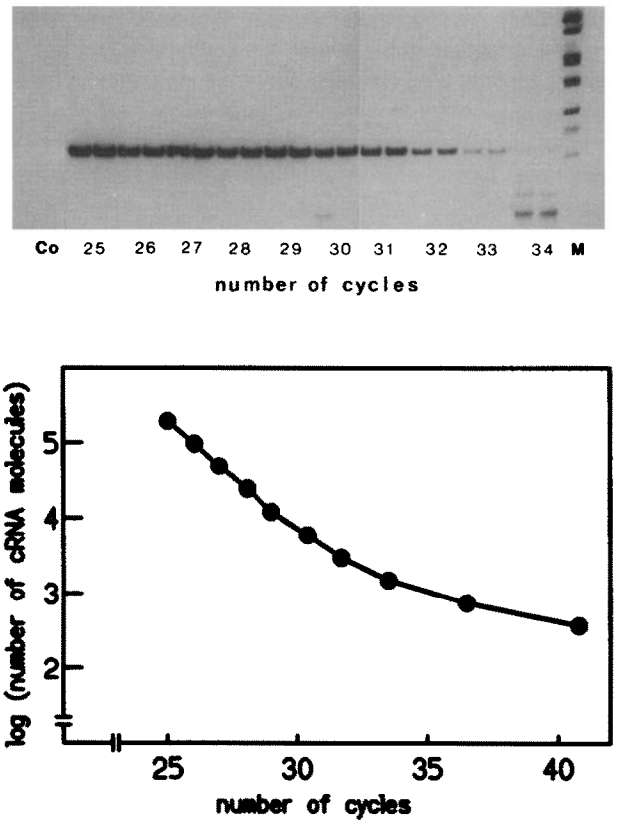

Fig. 2. Relation between number of target molecules, number of cycles and product yield. (Upper panel) A 1:2 dilution series of a stock solution containing 1.4×10^5 molecules of cRNA specific to pMHCβ174 (MHCI) was tested in duplicate in the RT/PCR assay with consecutive cycle number. Co, control; M, markers. The chemiluminescent signal at 173 nt was documented photographically. (Lower panel) Calculation of cycle numbers for defined product yield on the basis of densitometric evaluation of the RT/PCR assay shown in the upper panel. The constant relationship between cRNA input and cycle number extends to approximately 2×10^3 molecules.

3.3. *Conditions for mRNA quantification and calibration by external standards*

Quantitative measurements on RNA preparations required preliminary experiments in order to estimate concentration ranges of the three mRNAs of interest. The amount of total RNA used for the quantitative RT/PCR assays was individually chosen (62.5 pg–0.4 μg) according to the results of the preliminary measurements and the calibration curves for the standards described above. Combining dilution series and sequential cycles, the following procedure was routinely used: RNA preparations were tested in duplicate at 2–3 concentrations in parallel to 2–3 amounts of standards in

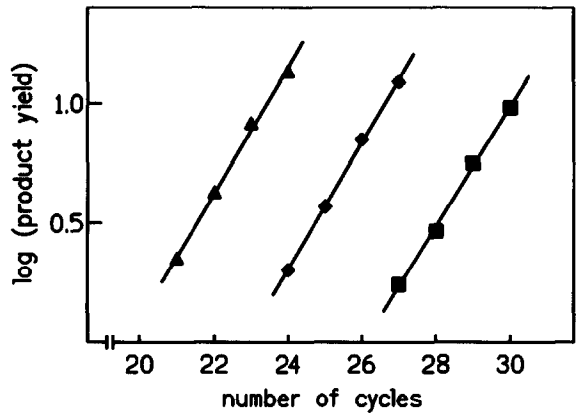

Fig. 3. Linear regressions of densitometrically evaluated product accumulation of an RT/PCR assay performed with three defined amounts of cRNA at increasing cycle numbers. The following amounts were used: 6.1×10^3 (▲), 6.1×10^4 (◆), 6.1×10^5 (■).

4–5 sequential cycles using a range of cycle numbers yielding the exponential relationship. In order to establish identical conditions, all measurements were performed by pipetting freshly prepared assay mixture (buffer, primers and enzymes) into the tubes containing standards or samples (thawed aliquots). The results of a typical quantification experiment for pMHC24-79 are presented in Fig. 4. The data indicate identical efficiencies and show that product yields were in the exponential phase for both standard and sample. This was a prerequisite for the evaluation of the sample data.

3.4. *Quantification of three specific MHC mRNA isoforms in various rabbit muscles*

Measurements of the three MHC mRNA species under study were performed on eight muscles (Table I). Pronounced differences existed between the amounts of the three isoforms. Soleus muscle exhibited the highest amount of mRNA specific to pMHCβ174 (slow MHCI), i.e. $1.11 \times 10^9/\mu g$ total RNA, whereas the mRNAs specific to the two fast isoforms, pMHC20-40 and pMHC24-79, were lower by a factor of 10^6 and 10^5, respectively. However, low amounts of MHC mRNA specific to pMHCβ174 (MHCI) were found in muscles with a predominance of fast fiber types. The lowest amounts of this isoform (approximately 10^6 copies per μg of total RNA) were detected in psoas and vastus lateralis muscles. Conversely, muscles with a high percentage of fast-twitch fibers contained high amounts of mRNA specific to the two fast clones. Adductor magnus and vastus lateralis exhibited the highest amounts of mRNA specific to the fast pMHC20-40. Fast-twitch extensor digitorum longus, tibialis anterior, psoas (white portion), and gastrocnemius muscles were characterized by higher amounts of mRNA specific to the fast pMHC24-79 as compared to the fast pMHC20-40.

The absolute detection limit of the method was determined for the three sequences to be in the range of 30–50 mRNA molecules. Measurements on soleus muscle for mRNA specific to pMHCβ174 (MHCI) showed that reproducible signals could be obtained down to a value of 30 fg total RNA. By extrapolation and using the data of Table I, this corresponded to 33 molecules of specific MHCI mRNA.

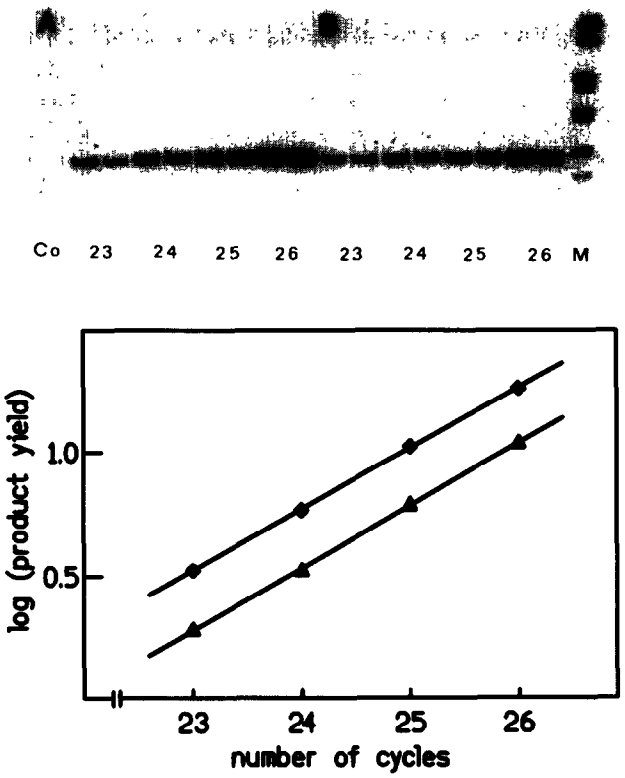

Fig. 4. Documentation of mRNA quantification in the RT/PCR assay. Product accumulation for pMHC24-79 was followed in duplicate determinations at 4 consecutive cycles from (A) 4 ng of a total RNA preparation from rabbit gastrocnemius in parallel, to (B) 6.1×10^4 molecules of the corresponding cRNA standard. The chemiluminescent signals at 289 nt were documented photographically. (Lower panel) Linear regression analysis of the densitometric evaluation of product accumulation shown in the upper panel. Amplification from total RNA (◆) and from the corresponding cRNA (▲).

4. DISCUSSION

The present RT/PCR assay serves for non-radioactive quantification of specific MHC mRNA isoforms in total RNA preparations. This method permits the comparison of molecule numbers and allows the assessment of variations in mRNA amounts over a range of six orders of magnitude. The quantification of specific mRNA is reliable down to numbers of approximately 1,000 molecules. This sensitivity exceeds the value published by Hoof et al. [12] for P-glycoprotein mRNA by two orders of magnitude. It is high enough for future application to analyses at the level of single muscle fibers. Such measurements will be performed on muscle

fiber fragments with 50–100 ng dry weight. According to the data for soleus muscle in Table I, this would correspond to approximately 2×10^5 molecules mRNA in a 100 ng fragment of a type I fiber.

The combination of RT and PCR within the same test tube provides conditions for an efficient activity of the RT. In agreement with Wang et al. [20], a sufficiently high concentration of $MgCl_2$ seems to be important. The extremely low detection limit suggests that previously reported inhibitory effects of RVC on the RT reaction [21] did not play a role in our assay. An additional experimental series (data not shown) did not provide evidence for an appreciable interference between RT and *Taq* polymerase [22]. Similar results were obtained when RT and *Taq* polymerase reactions were performed separately or run within the same test tube. The use of a one tube/one buffer method is advantageous because pipetting errors and contamination risks are reduced. Finally, sample-to-sample variations are largely excluded by using batches of the assay mixture. As compared to direct incorporation of Dig-11-dUTP in the *Taq* polymerase reaction (results not shown), a higher efficiency of the amplification is obtained by 5′ labeling of the primers. The high specificity of the reaction allows direct labeling of the PCR product, thus providing the possibility of direct product detection and avoiding an additional hybridization step. Chemiluminescence for detection of the final product greatly increases the sensitivity.

The use of external vs. internal standards in mRNA quantification is controversially discussed in the literature. In our view, sequence-specific variations of both the RT and polymerase reactions make it advisable to establish optimum conditions for each sequence. In addition, co-amplification experiments (data not shown) yielded non-reproducible results, most likely due to interferences between different primer pairs, e.g. dimerization (as assessed by computer analysis), and competition between the amplified sequences. Therefore, the use of external standards was preferred in the present study.

According to the results in Table I, MHC mRNA represents only a small fraction of total RNA. For soleus muscle, in which the slow MHCI predominates (95% relative concentration [6]), the following calculations can be made. Assuming a length of 6,000 nt for a MHC mRNA molecule [23], its mass is approximately 3.34×10^{-18} g. The number of mRNA molecules specific to pMHCβ174 (MHCI) amounts to 1.11×10^9 per μg of total RNA, which corresponds to 3.7 ng mRNA/μg total RNA or to 0.37% of total RNA. Assuming that total poly(A)$^+$ RNA amounts to approximately 5% of total RNA [24], the fraction of MHCI mRNA accounts for approximately 8% of total mRNA. This value is in close agreement with that of 3% published by Wiesner [25] for the fraction of mRNAs encoding α- and β-cardiac MHC isoforms in rat heart.

Our data can also be used to calculate the number of mRNA molecules per myonucleus. Published values for myonuclear DNA content vary between 6 and 10 pg DNA/nucleus [26,27]. Using 900 μg/g muscle as the average DNA content of soleus, the number of myonuclei is in the range of 5×10^7–10^8 per g wet weight. The value of 1.11×10^9 molecules mRNA for HCI per μg of total RNA in Table I corresponsd to 1.06×10^{12} molecules of mRNA per g wet weight, yielding a value of $1–2 \times 10^4$ transcripts per myonucleus in soleus muscle. Values of

Table I

Molecule numbers of specific MHC mRNA isoforms in various skeletal muscles of rabbit

mRNA isoform	Number of mRNA molecules/μg of total RNA		
	Slow pMHCβ174	Fast pMHC20-40	Fast pMHC24-79
Adductor magnus	$1.07 \pm 0.20 \times 10^7$ ($n = 14$)	$4.43 \pm 0.85 \times 10^8$ ($n = 18$)	$2.00 \pm 0.40 \times 10^7$ ($n = 16$)
Diaphragm	$4.78 \pm 0.76 \times 10^8$ ($n = 16$)	$5.71 \pm 0.55 \times 10^5$ ($n = 13$)	$1.37 \pm 0.15 \times 10^7$ ($n = 14$)
Extensor digit. long.	$3.32 \pm 0.43 \times 10^7$ ($n = 12$)	$5.59 \pm 0.66 \times 10^4$ ($n = 10$)	$3.11 \pm 0.47 \times 10^7$ ($n = 14$)
Gastrocnemius (red)	$1.05 \pm 0.18 \times 10^8$ ($n = 14$)	$1.91 \pm 0.25 \times 10^7$ ($n = 14$)	$2.73 \pm 0.44 \times 10^7$ ($n = 12$)
Psoas (white)	$5.83 \pm 0.85 \times 10^5$ ($n = 12$)	$1.54 \pm 0.10 \times 10^6$ ($n = 10$)	$7.32 \pm 1.06 \times 10^6$ ($n = 12$)
Psoas (red)	$8.02 \pm 1.34 \times 10^6$ ($n = 12$)	$7.83 \pm 0.60 \times 10^6$ ($n = 8$)	$6.68 \pm 0.78 \times 10^6$ ($n = 10$)
Soleus	$1.11 \pm 0.17 \times 10^9$ ($n = 14$)	$1.34 \pm 0.67 \times 10^3$ ($n = 25$)	$2.65 \pm 0.43 \times 10^4$ ($n = 11$)
Tibialis anterior	$2.32 \pm 0.30 \times 10^7$ ($n = 14$)	$2.98 \pm 0.23 \times 10^6$ ($n = 22$)	$2.82 \pm 0.39 \times 10^7$ ($n = 22$)
Vastus lateralis	$9.60 \pm 1.07 \times 10^5$ ($n = 18$)	$8.82 \pm 1.05 \times 10^7$ ($n = 16$)	$2.68 \pm 0.24 \times 10^7$ ($n = 22$)

Values are means ± S.D.

2.6×10^4 and 6×10^3 molecules per rat heart myonucleus were recently determined by Wiesner [25] for cardiac α- and β-MHC mRNA.

The total amounts of the three mRNAs differ in various muscles between 10^7 (psoas) and 10^9 (soleus) molecules per μg total RNA (Table I). However, it must be taken into account that only three sequences were investigated and that information concerning other isoforms, e.g. the fast HCIIa, is missing. Substantial evidence exists that pMHC20-40 is specific to the fast HCIIb and that pMHC24-79 represents a sequence specific to an as yet unidentified subtype of HCIId (A. Uber, D. Pette, unpublished results). According to protein analysis, adductor magnus and vastus lateralis contain high amounts of HCIIb, which agrees with the results obtained at the mRNA level. Conversely, extensor digitorum longus and tibialis anterior are characterized by a predominance of HCIId with only minute amounts of HCIIb [6,28], which is in accordance with their relatively high content of mRNA specific to pMHC24-79.

A discrepancy between protein and mRNA data exists for psoas muscle. Its very high content of HCIId (96% relative concentration) [6,28] is in contrast to the extremely low amount of mRNA specific to pMHC24-79. This discrepancy might indicate the presence of additional HCIId mRNA isoforms which are of different specificity than that assessed by the oligonucleotide primers used.

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REFERENCES

[1] Pette, D. and Staron, R.S. (1990) Rev. Physiol. Biochem. Pharmacol. 116, 1–76.
[2] Danieli-Betto, D., Zerbato, E. and Betto, R. (1986) Biochem. Biophys. Res. Commun. 138, 981–987.
[3] Biral, D., Betto, R., Danieli-Betto, D. and Salviati, G. (1988) Biochem. J. 250, 307–308.
[4] Staron, R.S., Gohlsch, B. and Pette, D. (1987) Pflügers Arch. 408, 444–450.
[5] Termin, A., Staron, R.S. and Pette, D. (1989) Eur. J. Biochem 186, 749–754.
[6] Aigner, S., Gohlsch, B., Hämäläinen, N., Staron, R.S., Uber, A., Wehrle, U. and Pette, D. (1993) Eur. J. Biochem. 211, 367–372.
[7] Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Ehrlich, H.A. and Arnheim, N. (1985) Science 230, 1350–1354.
[8] Harbarth, P. and Vosberg, H.-P. (1988) DNA 7, 297–306.
[9] Chelly, J., Kaplan, J.-C., Maire, P., Gautron, S. and Kahn, A. (1988) Nature 333, 858–860.
[10] Kawasaki, E.S., Clark, S.S., Coyne, M.Y., Smith, S.D., Champlin, R., Witte, O.N. and McCormick, F.P. (1988) Proc. Natl. Acad. Sci. USA 85, 5698–5702.
[11] Volkenandt, M., Dicker, A.P., Banerjee, D., Fanin, R., Schweitzer, B., Horikoshi, T., Danenberg, K., Danenberg, P. and Bertino, J.R. (1992) Proc. Soc. Exp. Biol. Med. 200, 1–6.
[12] Hoof, T., Riordan, J.R. and Tümmler, B. (1991) Anal. Biochem. 196, 161–169.
[13] Ballagi-Pordány, A., Ballagi-Pordány, A. and Funa, K (1991) Anal. Biochem. 196, 89–94.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[15] Sinha, A.M., Umeda, P.K., Kavinsky, C.J., Rajamanickam, C., Hsu, H.-J., Jakovcic, S. and Rabinowitz, M. (1982) Proc. Natl. Acad. Sci. USA 79, 5847–5851.
[16] Maeda, K., Sczakiel, G. and Wittinghofer, A. (1987) Eur. J. Biochem. 167, 97–102.
[17] Höltke, H.-J. and Kessler, C. (1990) Nucleic Acids Res. 18, 5843–5851.
[18] Zafra, E., Hengerer, B., Leibrock, J., Thoenen, H. and Lindholm, D. (1990) EMBO J. 9, 3545–3550.
[19] Murphy, L.D., Herzog, C.E., Rudick, J.B., Fojo, A.T. and Bates, S.E. (1990) Biochemistry 29, 10351–10356.
[20] Wang, R.-F., Cao, W.-W. and Johnson, M.G. (1992) Biotechniques 12, 702–703.
[21] Puskas, R.S., Manley, N.R., Wallace, D.M. and Berger, S.L. (1982) Biochemistry 21, 4602–4608.
[22] Sellner, L.N., Coelen, R.J. and Mackenzie, J.S. (1992) Nucleic Acids Res. 20, 1487–1490.
[23] Sinha, A.M., Friedman, D.J., Nigro, J.M., Jakovcic, S., Rabinowitz, M. and Umeda, P.K. (1984) J. Biol. Chem. 259, 6674–6680.
[24] Gagnon, J., Kurowski, T.T., Wiesner, R.J. and Zak, R. (1991) Mol. Cell. Biochem. 107, 21–29.
[25] Wiesner, R.J. (1992) Nucleic Acids Res. 20, 5863–5864.
[26] Gibson, M.C. and Schultz, E. (1983) Muscle Nerve 6, 574–580.
[27] Jackowski, G. and Liew, C.-C. (1980) Biochem. J. 188, 363–373.
[28] Hämäläinen, N. and Pette, D. (1993) J. Histochem. Cytochem (in press).