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Quantification of Transcript-to-Template Ratios as a Measure of Gene Expression Using RT-PCR

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ABSTRACT

A new protocol was established for the quantitative analysis of gene expression in small muscle biopsies. Reverse transcription-PCR was performed with a preparation of total nucleic acids (DNA+RNA), amplifying the sequences of interest (targets; mitochondrial transcripts: 12S rRNA, cytochrome-c-oxidase [COX I] mRNA) together with an endogenous, non-transcribed reference sequence (template: D-loop region of mtDNA). Synthesis of PCR products at consecutive cycles within the exponential phase was quantified by measuring incorporation of radioactivity. Product accumulation was determined by regression analysis of these data. Gene expression could then be quantified as a ratio of target transcripts to reference DNA. The results revealed a ratio of 12S rRNA:mtDNA and COX I mRNA:mtDNA of 14 and 2, respectively, or a ratio of 12S rRNA:COX I mRNA of 7 in human left ventricle and are in good agreement with previously published values for rat liver and muscle.

In addition to the investigation of mitochondrial gene expression in the steady state and during mitochondrial proliferation, this newly developed method will easily be applicable to expression analysis of any nuclear gene using an intron sequence as endogenous reference.

INTRODUCTION

Mitochondria have retained an autonomously replicating genome, a circular, double-helical DNA. Mitochondrial DNA (mtDNA) is expressed in all eukaryotic cells, though not in a stable fashion, but varying with physical activity (4) or hormone levels (10) in mammals. Additionally, the copy number of mtDNA also varies (11), further complicating the analysis of mitochondrial transcription.

To gain further insight into mitochondrial gene expression, it is essential to be able to easily quantify changes of mitochondrial transcripts relative to the levels of mtDNA and to measure the stoichiometry between mt mRNAs and mt rRNAs. When facing the challenge to perform these measurements in small human muscle biopsies, it was necessary to develop an appropriate reverse transcription polymerase chain reaction (RT-PCR) protocol. To investigate alterations of mRNAs and rRNAs encoded by mtDNA, one representative mt mRNA, encoding subunit I of cytochrome-c-oxidase (COX I), and the 12S mt rRNA were chosen as the sequences of interest, called targets. The rationale of the new approach was to extract total nucleic acids (DNA+RNA) and to transcribe RNAs into cDNAs by RT, thus making them accessible to amplification by PCR together with an endogenous, non-transcribed reference sequence from the non-coding D-Loop region. Quantification of gene expression was then possible by calculating the ratio of target-to-reference molecules.

MATERIALS AND METHODS

Extraction of Total Nucleic Acids

For extraction of total nucleic acids, a published protocol (1) was modified, and the volumes used were adapted to 20 mg of muscle tissue. Samples from human left ventricle were homogenized with 400 μ L of extraction solution (0.5 M LiCl, 1 M urea, 1% sodium dodecyl sulfate [SDS], 0.02 M sodium citrate, 2.5 mM CDTA, final pH 6.8) and digested with 15 μ L of proteinase K (10 mg/mL) for 30 min at 50°C. Nucleic acids were precipitated with sodium acetate/ethanol after phenol-chloroform-extraction and dissolved in 20 μ L of diethyl pyrocarbonate (DEPC)-treated water.

Total nucleic acids as well as DNA and RNA, which were prepared separately by conventional techniques (7) from 20 mg of human left ventricular muscle, were compared in quantity and quality by running aliquots of 2 μ L of all nucleic acid solutions on a 1.5%

agarose gel, stained with ethidium bromide (Figure 1). Results clearly indicated that the preparation of total nucleic acids allows simultaneous extraction of DNA and RNA from muscle tissue comparable in quality and quantity to conventional techniques.

RT-PCR

RT was performed in a total volume of 10 μ L containing 1.5 μ L of total nucleic acid extract from approximately 1.5 mg of tissue, 10 nmol of dNTPs (Pharmacia Biotech, Freiburg, Germany), 0.2 μ g reverse primers and 2 μ L 5 $\times$ reaction buffer (Promega, Madison, WI, USA). Denaturation was at 65°C, for 3 min, before addition of 20 U RNasin® (Promega) and incubation with 20 U AMV-RTase (Promega) for 1 h at 42°C. RTase was denatured before PCR by boiling for 15 min.

PCR was performed with 2.5 μ L of the RT reaction mixture in a total volume of 50 μ L containing in addition 0.05 μ g of forward primers, 2 U of *Taq* DNA Polymerase and reaction buffer (AGS, Heidelberg, Germany) to which 10 μ Ci of [α -³²P]dCTP (NEN Life Science Products, Bad Homburg, Germany) were added. Denaturation temperature was 95°C (30 s), annealing was at 50°C (60 s) and synthesis at 72°C (30 s).

Aliquots of 3 μ L were taken from the reaction mixture before the first

Figure 1. Comparison of extraction protocols. Lane 1: DNA marker λ HindIII; lane 2: DNA prepared by conventional techniques; lanes 3 and 4: total nucleic acids from two independent extractions; lane 5: RNA prepared by conventional techniques. The arrows denote the bands of high molecular weight genomic DNA, 28S rRNA and 18S rRNA, respectively.

cycle (as a measure of background radioactivity) and after consecutive cycles within the exponential phase. The amplification products present in 1 μ L were separated by length in 15% polyacrylamide gels and stained with ethidium bromide. The bands were then cut out, and incorporated radioactivity was determined by liquid scintillation counting.

Primers were: 12S forward [CAGC-CACCGCGGTCACACGA]; 12S reverse [GAACAGGCTCCTCTAGAG-GG], 318-bp product length; COX I forward [TCGCCGACCGTTGACTA-TTC]; COX I reverse [TAAGGGAGG-GTAGACTGTTC], 391-bp product length; Dlp forward [TATTCTCTGTT-CTTTCATGG]; Dlp reverse [AAGT-GTTATGGGCCCGGAGC], 453-bp product length.

RESULTS

Quantification of Product Accumulation in PCR

Product accumulation during PCR

can be described by Equation 1:

$$\log N_n = n \times \log eff + \log N_o \quad [\text{Eq. 1}]$$

N_o is the initial concentration of target DNA at cycle 0, eff is the efficiency of amplification and n is the cycle number. Equation 1, describing product accumulation, can be determined for each individual amplification product by performing regression analysis of product concentrations at several consecutive cycles (11).

For this purpose, the product concentration (mol/ μ L) was calculated from the incorporated radioactivity minus background radioactivity (cpm/ μ L), the specific activity of the precursor dCTP in 1 μ L reaction mixture (cpm/mol) and the number of cytosine moieties in the product (y) according to Equation 2:

$$[\text{mol/mL}] = [\text{cpm/mL}] / [\text{cpm/mol}] \cdot y \quad [\text{Eq. 2}]$$

Regression analysis was then performed for each individual amplification product, thereby determining the equations of product accumulation. The data are shown as product concentra-

Figure 2. Accumulation of products from 12S, COX I and D-loop sequences in RT-PCR vs. cycle number with the respective regression lines and fitting parameters (data from one experiment are shown). In this experiment, ratios for 12S:D-loop and COX I:D-loop were 5.77 and 1.1, respectively.

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tion vs. cycle number in a semilogarithmic plot with the respective linear regression lines (Figure 2).

Quantification of Target Molecules

When quantifying the amount of target molecules as a ratio of target-to-reference molecules, we faced two possible approaches of calculation: (i) Ratios could be calculated for a mid-exponential cycle using the values of target and reference products from the corresponding regression lines for that cycle. (ii) By extrapolation of the regression lines to cycle 0 (intercepts with the y-axis), the concentration of target and template molecules at cycle 0 could be obtained (11), and these data then used to calculate the ratio of target-to-reference molecule.

If the efficiencies of amplification are identical for both target and reference molecules, the values for the two approaches will not differ as the regression lines are running in parallel. However, if there is a systematic difference

between the amplification efficiencies of target and reference, the regression lines will not run in parallel, and the ratio obtained for the mid-exponential cycle and the ratio obtained by extrapolation to cycle 0 will be different. In this case, extrapolation to cycle 0 (11) has to be used to calculate the correct ratio.

In our experiments, no systematic differences between the efficiencies of amplification for the three products were ever observed. Slight differences between efficiencies did not show a uniform direction and are certainly due to unavoidable experimental errors during the experimental procedure (sampling of 1 μ L of PCR mixture, separation by electrophoresis, excision of bands and liquid scintillation counting). Therefore, in our case, we could choose between the two approaches.

The extent to which the slight, non-systematic differences between the amplification efficiencies cause the ratios obtained with the two different approaches to deviate was investigated. For this purpose, from all our measurements (Figure 3), we plotted the values

for $ratio_{mid-exponential}/ratio_{extrapolated}$ vs. the values for the ratio of the efficiencies of target and reference, eff_1/eff_2 . Regression analysis revealed an exponential distribution described by Equation 3:

$$ratio_{mid-exponential}/ratio_{extrapolated} = 10^{5.15 (eff_1/eff_2) - 5.15} \quad [\text{Eq. 3}]$$

This indicates that the ratio obtained by extrapolation deviates from the ratio calculated for a mid-exponential cycle in an exponential fashion, which depends on the ratio of the amplification efficiencies of target and reference. Thus, even with virtually parallel amplification, the slight, nonsystematic differences between the amplification efficiencies of target and reference lead to an error when extrapolation is used as opposed to calculating the ratio of target to reference from the mid-exponential cycle. This effect is particularly pronounced with a mid-exponential cycle >20 (5).

We therefore chose to calculate the ratio of target-to-reference at a mid-exponential cycle. As the requirement of parallel amplification was met in our experiments, we could thus circumvent the unavoidable experimental errors due to extrapolation.

Consequently, the individual equations describing the accumulation of target and reference products during PCR were determined for each reaction by regression analysis (see Equation 1). From these equations, the concentration values for the middle cycle of the exponential phase sampled were calculated and then used to determine the ratio of target-to-reference. However, for routine work, once the parallel amplification has been established, it is sufficient to sample at one pre-chosen cycle within the exponential phase in several RT-PCRs of the same sample and calculate ratios from these data.

Analysis of Muscle Biopsies

Extraction of total nucleic acids with consecutive RT-PCR and analysis of product accumulation was performed on 5 pieces of the same human left ventricle. The targets, COX I and 12S, were co-amplified with a D-loop sequence as endogenous reference.

Ratios calculated for 12S:D-loop

Figure 3. Relationship between ratios derived from the mid-exponential cycle and from extrapolation to cycle 0. The values for the relationship of the ratios, $ratio_{mid-exponential}/ratio_{extrapolated}$, are shown vs. the ratio of the efficiencies of target and reference, eff_1/eff_2 , in a semilogarithmic plot.

and COX I:D-loop were 8.8 ± 1.0 (standard error of the mean [SEM]) and 1.3 ± 0.1 , respectively ($n = 5$). This clearly demonstrates the high degree of reproducibility of this method.

However, when the accumulation of D-loop product during PCR with and without previous RT was compared in parallel amplifications, a ratio of 1.55 (RT-PCR:PCR, $n = 5$) was observed. Due to the non-coding character of the D-loop region, no synthesis of cDNA had been expected to take place during RT, and amplification should have yielded similar amounts of products resulting in a ratio of approximately 1. With the observed ratio of >1 , products must have been synthesized during RT. Low-abundance transcripts of the D-loop region, which are involved in regulation of mtDNA transcription and replication, have indeed been reported (8), but information on this topic is still scarce and controversial. Other D-loop primers tested also yielded low levels of products in the RT (data not shown).

However, using the data of RT-PCR:PCR for correction, the values for 12S:D-loop and COX I:D-loop could be easily related to cognate D-loop DNA and were 14 and 2 molecules per molecule of mtDNA, respectively. These values are very similar to values obtained by completely independent approaches for rat liver, which are 15 and 4 molecules per mtDNA, respectively (2,3), and rat muscle, which are 9 and 2 molecules per mtDNA, respectively (3). Also, the ratio of 12S rRNA:COX I mRNA in human ventricle of approximately 7 when standardized to mtDNA and of approximately 6 in RT-PCR (see below) is in good agreement with previous findings, which reported ratios of 12S:COX I of 4–5 for rat liver and of 6 for rat muscle (2,3).

In a second approach, we compared the accumulation of products derived from the targets (12S, COX I) during PCR and RT-PCR in two different reactions. Without previous RT, the amplification products would only accumulate from extracted DNA, while PCR after RT would also amplify cDNA originating from transcripts. The ratio of accumulation in RT-PCR to accumulation in PCR would yield information on the amount of DNA+RNA present per DNA for each sequence.

Total nucleic acid extracts were prepared from 4 samples of the same human left ventricle, and for each sample, 12S and COX I were co-amplified by PCR and RT-PCR in individual tubes. The product ratios observed between RT-PCR and PCR were 4.13 ± 1.28 (SEM) for 12S and 1.01 ± 0.34 (SEM) for COX I with variation coefficients of 62% and 67%, respectively.

For the same experiments, we also determined the ratio for 12S:COX I in the four RT-PCRs, which yielded a ratio of 5.6 ± 0.6 (SEM) with a variation coefficient of 20%. The higher variation coefficients in the parallel tubes assay indicate the inaccuracy of comparing and calculating ratios from two separate reactions. Thus, for greater accuracy, the target should be quantified by calculating a ratio to a co-amplified reference sequence.

DISCUSSION

The RT-PCR protocol introduced here quantifies the amount of RNA target molecules in a biological sample as the ratio of target product to an endogenous, co-amplified reference product in the exponential phase of PCR.

The usage of an endogenous, co-amplified reference sequence has distinct advantages over other protocols. Due to the endogenous nature of the chosen reference, target and reference are equally affected by the extraction procedure and RT. Moreover, the co-amplification renders the ratio of target-to-reference independent of tube-to-tube variations, samples remaining comparable even if the amplification efficiencies vary between different reactions.

Our protocol only requires the choice of suitable primers for the sequences under investigation. PCR products should be specific and differ sufficiently in length to allow easy separation by gel electrophoresis. To determine ratios of target-to-reference in the mid-exponential phase of the PCR amplification, efficiencies must be similar. Once the reproducibility of RT-PCR and the parallel amplification have been established, ratios can either be determined from regression lines after sampling at consecutive cycles or by sampling several times at one pre-

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chosen cycle within the exponential phase and calculating the mean ratio of these measurements.

The choice of the reference sequence, being the denominator of the ratio, determines the meaning of the ratio. Gene expression as a transcript-to-template ratio can be determined as shown in this paper with a D-loop sequence for mitochondrial DNA. Unfortunately, the compact nature of this genome and the complexity of regulatory events in this region has not allowed us to find a region that is truly devoid of transcripts. This however should not be a problem when analyzing nuclear gene expression with our protocol, where a second reverse primer located more downstream in an intron region would be used in addition to the primer pair complementary to the transcript. Indeed, an approach using the intron sequence of the cognate gene as an internal standard has been reported (12).

Compared to competitive PCR, the RT-PCR protocol described here has the distinct advantages of neither requiring the construction of external standards nor the material- and time-consuming titration process, for which after all about 5 µg of RNA or DNA are needed (9). Also, competitive PCR determines the starting concentration of a target sequence by comparison to product accumulation in several dilutions of the reference. This practice is only valid if the amplification efficiencies of target and reference are identical and remain constant (6). However, the external standard in competitive PCR has frequently been observed to have an amplification advantage over the target and thus to be amplified more efficiently (9). This suggests the necessity of a critical evaluation of results obtained by competitive PCR.

In conclusion, we have established a new RT-PCR protocol in which the tar-

get is quantified by its stoichiometric relation to an endogenous, co-amplified reference sequence, which, in the best case, is derived from the cognate gene. This RT-PCR protocol should be easily applicable to the quantification of expression of any nuclear gene, making it a powerful tool in the investigation of regulation of transcription. In addition, it is possible to quickly and easily determine changes in the ratio of any two targets under conditions affecting gene expression.

REFERENCES

1. **Birmboim, H.C.** 1993. Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Methods Enzymol.* 216:154-160.
2. **Cantatore, P., M.N. Gadaleta and C. Saccone.** 1984. Determination of some mitochondrial RNAs concentration in adult rat liver. *Biochem. Biophys. Res. Commun.* 118:284-291.
3. **Garstka, H., M. Fäcke, J.R. Escribano and**

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- R.J. Wiesner.** 1994. Stoichiometry of mitochondrial transcripts and regulation of gene expression by mitochondrial transcription factor A. *Biochem. Biophys. Res. Commun.* 200:619-626.
4. **Hood, D.A., R. Zak and D. Pette.** 1989. Chronic stimulation of rat skeletal muscle induces coordinate increases in mitochondrial and nuclear mRNAs of cytochrome c oxidase. *Eur. J. Biochem.* 179:275-280.
5. **Lubin, M.B., J.D. Elashoff, S.-J. Wang, J.I. Rotter and H. Toyoda.** 1991. Precise gene dosage determination by polymerase chain reaction: theory, methodology, and statistical approach. *Mol. Cell. Probes* 5:307-317.
6. **Raeymaekers, L.** 1993. Quantitative PCR: theoretical considerations with practical implications. *Anal. Biochem.* 214:582-585.
7. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
8. **Sbisa, E., M. Nardelli, F. Tanzariello and C. Saccone.** 1990. The complete and symmetrical transcription of the main non coding region of rat mitochondrial genome: in vivo mapping of heavy and light transcripts. *Curr. Genet.* 17:247-253.
9. **Volkenandt, M., A.P. Dicker, D. Banerjee, R. Fanin, B. Schweitzer, T. Horikoshi, K. Danenberg, P. Danenberg and J.R. Bertino.** 1992. Quantitation of gene copy number and mRNA using the polymerase chain reaction. *Proc. Soc. Exp. Biol. Med.* 200:1-6.
10. **Wiesner, R.J., T. Kurowski and R. Zak.** 1992. Regulation by thyroid hormone of nuclear and mitochondrial genes encoding subunits of cytochrome c oxidase in rat liver and skeletal muscle. *Mol. Endocrinol.* 6:1458-1467.
11. **Wiesner, R.J., J.C. Rüegg, and I. Morano.** 1992. Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues. *Biochem. Biophys. Res. Commun.* 183:553-559.
12. **Zenilman, M.E., W. Graham, K. Tanner and A.R. Shuldiner.** 1995. Competitive reverse transcription - polymerase chain reaction without an artificial internal standard. *Anal. Biochem.* 224:339-346.

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