Two variants of quantitative reverse transcriptase PCR used to show differential expression of α -, β - and γ -fibrinogen genes in rat liver lobes

Junlong ZHANG, Mina DESAI, Susan E. OZANNE, Cora DOHERTY, C. Nicholas HALES and Christopher D. BYRNE*

University Department of Clinical Biochemistry, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QR, U.K.

Quantitative reverse transcriptase PCR (RT-PCR) is a sensitive method for the measurement of mRNA copy number. However, the methodology has gained a reputation for poor reproducibility, leading to concern over the validity of much of the data generated using this technique. We have developed two variants of quantitative competitive RT-PCR using a synthesized RNA as an internal standard to measure precisely the relative levels of α -, β - and γ -fibrinogen mRNAs in the four lobes of the rat liver. In the first of these variants we altered only the amount of total RNA in the RT-PCR reaction, keeping the amount of internal standard RNA and the number of PCR cycles constant. In the second variant only the number of PCR cycles was altered, and the amounts of total RNA and standard RNA were kept constant. Both variants of RT-PCR allowed calculation of the

number of mRNA copies, which did not differ significantly between the two techniques. Of the two variants, the second gave better reproducibility, and the intra-assay coefficient of variation for this technique was 14% ($n = 20$). Using these two variants we have shown that there are different numbers of fibrinogen mRNAs in the four liver lobes for each of the three genes (α -fibrinogen $F = 14.64$, $P = 0.0003$; β -fibrinogen $F = 3.74$, $P = 0.04$; γ -fibrinogen $F = 3.75$, $P = 0.04$). In conclusion, by using two variants of quantitative competitive RT-PCR we have shown that this technique can be used to give reproducible results, and the low intra-assay coefficient of variation suggests that quantitative RT-PCR should be the technique of choice for accurate measurement of mRNA copy number.

INTRODUCTION

mRNA expression can be studied by a variety of techniques, including Northern blot analysis, ribonuclease protection assays and quantitative reverse transcriptase PCR (RT-PCR). Northern blot analysis is the least sensitive technique, and its lack of sensitivity precludes its use with many genes expressed at a low level. Ribonuclease protection assays are approximately 10 times more sensitive than Northern blot analyses, but do not offer sufficient sensitivity for the study of many genes. The main advantage of quantitative PCR is its extreme sensitivity and specificity [1]. Quantitative RT-PCR is about 1000 times more sensitive than RNA dot blot analysis [2], and is therefore potentially an ideal method for studying genes expressed at a low level. A better understanding of the problems associated with this technique will eradicate many of the problems causing poor reproducibility and should ultimately produce a more robust assay of mRNA copy number.

In the present study of variations in quantitative RT-PCR techniques, we have measured fibrinogen mRNAs. The fibrinogen molecule is composed of three pairs of polypeptide chains, $A\alpha$, $B\beta$ and γ , which form a dimer ($A\alpha$, $B\beta$ and γ)₂. Each of the three chains is encoded by a separate gene [3,4] that is situated in a cluster in the distal one-third of the long arm of chromosome 4 [2]. The three fibrinogen genes belong to a family including the class II hepatic acute-phase proteins, which are regulated at the transcriptional level by members of the interleukin-6 family of cytokines and glucocorticoids [5]. Fibrinogen is synthesized only in the liver, and at present it is uncertain whether: (1) plasma

concentrations are limited by expression of one or more of the α -, β - or γ -fibrinogen genes, (2) levels of expression of the three fibrinogen genes are the same, or (3) each liver lobe has equivalent levels of expression. In the present paper, we report the development of two variants of quantitative competitive RT-PCR that give reproducible results in the study of mRNA copy number for the three fibrinogen genes. We also report that the levels of mRNAs from the three fibrinogen genes differed from each other and also varied between liver lobes.

MATERIALS AND METHODS

Preparation of total RNA

'Total RNA' refers to the RNA isolated from rat liver and does not include standard RNA (for explanation of standard RNA, see below). All analytical chemicals for preparation of total RNA were purchased from Sigma. RNA was prepared [6] from each of the four lobes of livers from 90-day-old female rats maintained on a normal chow diet. Rat liver has a median or cystic lobe, a right lobe, a left lobe and a caudate lobe. According to morphology, we designated the left lobe as lobe 1, the median lobe as lobe 2, the right lobe as lobe 3 and the caudate lobe as lobe 4. To study mRNA levels for each of the fibrinogen genes in the different liver lobes, ~100 mg of liver tissue was excised from the same site of each lobe and homogenized on ice. The yield of total RNA was between 1.5 and 3 mg/g of liver tissue. Total RNA was quantified by spectrophotometry, and its quality was checked by gel electrophoresis.

Abbreviations used: RT-PCR, reverse transcriptase PCR; D_t , optical density of target RNA band (pixels) after RT-PCR; D_s , optical density of standard RNA band (pixels) after RT-PCR; R_t , the ratio of D_t to D_s ; X_e , amount of total RNA (ng) when $D_t = D_s$; S_t , size of target RNA band (bp); S_s , size of standard RNA band (bp); K_s , size ratio constant (S_s/S_t); M_t , number of copies of target RNA; M_s , number of copies of standard RNA; M_m , mRNA level (copies/ng).

* To whom correspondence should be addressed.

Figure 1 Strategy used for synthesis of standard RNA

Primer 1 comprises the sequences from the primer 1 binding site (Pri. 1) and the T7 promoter. Primer 3 comprises the sequences from the primer 3 and primer 5 binding sites (pri. 3 and Pri. 5). Primer 5 (not shown) comprises only the sequence from the primer 5 binding site. Standard RNA was synthesized from reverse-transcribed RNA amplified with primers 1 and 3.

Synthesis and preparation of oligonucleotides

Quantitative RT-PCR was performed as described [1]. Three primer binding sites should be identified for the preparation of oligonucleotides. Primer 1 comprised the primer 1 binding site sequence (sense sequence) attached at its 5' end to a T7 sequence. Primer 5 comprised only the primer 5 binding site sequence (antisense sequence). An internal primer site (primer 3 binding site) was identified ~ 100 bp 3' to the primer 5 binding site, and primer 3 comprised the primer 3 binding site sequence attached to the 3' end of the primer 5 binding site sequence (Figure 1). Thus, after RT-PCR of liver RNA with primers 1 and 3, ~ 100 bp was deleted from the amplified DNA product. From this DNA product, the T7 sequence was used to synthesize standard RNA with T7 RNA polymerase. The DNA template was digested with DNase and the standard RNA was purified. Thus, for each of the three fibrinogen genes, three primers were synthesized (primers 1, 3 and 5). Primer pair 1 and 5 was used in the final amplification (after reverse transcription) to amplify both standard RNA and target RNA in the same tube.

To ensure that genomic DNA sequences were not amplified, primer sites were chosen that spanned at least one intron. All primers were synthesized by Genosys Europe, Cambridge, U.K.

The primers for the α -fibrinogen [7] gene were: primer 1 (coding sequence), 5'-TAATACGACTCACTATAGGGGAA-AGGGTTGATTGATGAAG-3'; primer 5 (antisense sequence), 5'-TTAATGCCTTCCACTCTGGG-3'; primer 3 (antisense sequence), 5'-TTAATGCCTTCCACTCTGGGAATGACCTGT-TCAAGTTGCT-3'. Final amplification with primers 1 and 5 resulted in two PCR products: 564 bp from the target RNA and 454 bp from the standard RNA. The 110 bp difference between the two products could be detected easily on an agarose gel.

Primers for the β -fibrinogen gene [8] were: primer 1 (coding sequence), 5'-TAATACGACTCACTATAGGGGACTTTGG-CAGGAAATGGG-3'; primer 3 (antisense sequence), 5'-CACC-CAGTAGTATCTGCCGCTGAAGAACATGCCGTTGT-

3'; primer 5 (antisense sequence), 5'-CACCCCAGTAGTAT-CTGCC-3'. Final amplification with primers 1 and 5 resulted in two PCR products: 497 bp from the target RNA and 386 bp from standard RNA.

Primers for the γ -fibrinogen gene [9] were: primer 1 (coding sequence), 5'-TAATACGACTCACTATAGGGTTTCATTCT-ATGCTGGGCGCT-3'; primer 5 (antisense sequence), 5'-TCATGGGTTAGTAACAGTGCTT-3'; primer 3 (antisense sequence), 5'-TCATGGGTTAGTAACAGTGCTTACCTGG-ATGGCTTTAATCAGTT-3'. Final amplification with primers 1 and 5 resulted in two PCR products: 394 bp from the target RNA and 303 bp from standard RNA.

Synthesis of standard RNA

A standard RNA was synthesized for use as an internal standard in the quantification of RT-PCR. Standard RNA was made from a PCR product sharing the same binding sites for primer 1 and primer 5 with the target RNA (Figure 1). To synthesize standard RNA, 1 μ g of total RNA was reverse-transcribed into cDNA and then amplified with primer 1 and primer 3 by PCR. A 10 μ l sample of the RT-PCR product was checked on a 2% (w/v) agarose gel, and 30 μ l of the PCR product was extracted with chloroform/3-methylbutan-1-ol and precipitated with 100% (v/v) ethanol. The purified DNA was dissolved in diethyl pyrocarbonate-treated water, and added to dithiothreitol (10 mM), rNTPs (0.5 mM), 1 $\times$ T7 RNA polymerase buffer (Promega; 40 mM Tris/HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl), RNase (1 unit/ μ l) inhibitor, T7 RNA polymerase (1 unit/ μ l; Promega) and T7 primer (1.25 μ M) to a total volume of 20 μ l. The reaction was incubated at 37 °C for 1 h and then the DNA was digested by incubation with RNase-free DNase (0.1 unit/ μ l) at 37 °C for 15 min. The synthesized standard RNA was extracted with chloroform/3-methylbutan-1-ol, precipitated with 100% (v/v) ethanol and washed with 70% (v/v) ethanol. The standard RNA pellet was vacuum dried,

dissolved in diethyl pyrocarbonate-treated water and quantified by spectrophotometry.

Quantitative RT-PCR using various amounts of total RNA

Two types of RT-PCR were used to check the reproducibility of the quantitative competitive RT-PCR technique. The first of these methods employed a fixed amount of standard RNA and a constant number of PCR cycles with various amounts of total RNA in separate tubes (T-type RT-PCR).

In T-type RT-PCR, total RNA (25, 50, 100, 150, 200 and 250 ng per sample) was added to an equal amount of standard RNA. Both total RNA and standard RNA were denatured at 70 °C for 5 min, and then added to a reverse transcription solution containing 0.5 mM dNTPs, 5 μ M random hexamers, 0.3 unit/ μ l super RT (HT Biotechnology Ltd.), 1 unit/ μ l RNase inhibitor (Promega), 50 mM Tris/HCl, pH 8.3, 50 mM KCl, 4 mM dithiothreitol and 10 mM MgCl₂, in a final volume of 10 μ l. The cDNA synthesis reaction was performed at 42 °C for 60 min. The reaction was stopped by denaturing at 95 °C for 5 min.

For PCR amplification, 40 μ l of the PCR mixture was added to the 10 μ l of reverse transcription solution and covered with about 40 μ l of mineral oil. PCR was performed in 1 $\times$ PCR reaction buffer [Promega; 50 mM KCl, 10 mM Tris/HCl (pH 9.0 at 25 °C), 0.1 % Triton X-100], 0.2 mM dNTPs, 1 pmol/ μ l sense and antisense primers (primer 1 and primer 5) and 0.05 unit/ μ l *Taq* DNA polymerase (Promega). The amplification conditions were: 30 cycles for α -fibrinogen with an annealing temperature of 55 °C, 35 cycles for β -fibrinogen with an annealing temperature of 56 °C, and 25 cycles for γ -fibrinogen with an annealing temperature of 57 °C. For each gene, there was a 1 min denaturing period, a 1 min extension time and a final 10 min elongation time at 72 °C.

Quantitative RT-PCR using various numbers of PCR cycles

Quantitative RT-PCR with various PCR cycle numbers was also performed (C-type RT-PCR). Both the total RNA and standard RNA amounts were fixed and increasing numbers of PCR cycles were carried out. In C-type RT-PCR, 500 ng of total RNA was added to 12.5 fmol of standard RNA; the mixture was incubated at 70 °C for 5 min and then added to the cDNA master mixture (the composition was the same as for T-type RT-PCR) in a total volume of 20 μ l. After completion of cDNA synthesis, 18 μ l of cDNA was added to the PCR master mixture (composition as above) in a total volume of 90 μ l. The mixture was divided into six tubes, with 15 μ l of reaction solution in each tube. The PCR reaction conditions were the same as above, except that the annealing temperature was 57 °C. For each sample, the PCR was undertaken for 21, 22, 23, 24, 25 and 26 cycles, and the PCR products from each of the six different cycles were analysed (C-type RT-PCR was only undertaken with γ -fibrinogen).

Quantification of RT-PCR products

The RT-PCR products were analysed in a 2 % (w/v) agarose gel containing EtBr. DNA bands were photographed using an Eagle Eye II Still Video System (Stratagene). The image was exported in a TIFF file and DNA bands were quantified using NIH Image 1.55 software for Macintosh. The quantification value of the bands in the gel was designated the 'optical density' (pixels). Linear amplification was observed up to 120 pixels (see below).

In each case the target RNA/standard RNA optical density ratio was obtained and, since the number of copies of standard RNA added to the reaction was known [1 mole = 6×10^{23}

molecules (Avogadro constant)], the number of copies of target RNA could be calculated (see below). The amount of mRNA was expressed as number of mRNA copies per ng of total RNA.

Statistical analyses

All statistical calculations were performed using Stata statistical software (Statacorp, TX, U.S.A.). The significance of differences in fibrinogen mRNA copy number between lobes was examined using ANOVA (*F* values and *P* values). Specific comparisons between mean mRNA levels from two different lobes were undertaken by unpaired Student's *t* tests. Results in all cases are presented as means $\pm$ S.E.M.

RESULTS

Preparation of total RNA and standard RNA

Total RNA was prepared from the four different liver lobes. All the RNA samples were quantified using a spectrophotometer, and had a A_{260}/A_{280} ratio of ~ 1.8 . Quantification was carried out in duplicate. From 100 mg of liver tissue, $\sim 150 \mu$ g of total RNA was obtained.

The standard RNA prepared was quantified as above. Approx. 58 pmol of α -fibrinogen, 100 pmol of β -fibrinogen and 140 pmol of γ -fibrinogen RNA was obtained. The amount of standard RNA used in the RT-PCR was 0.11 fmol (6.6×10^7 copies) for α -fibrinogen, 0.01 fmol (5.6×10^6 copies) for β -fibrinogen and 12.5 fmol (7.5×10^9 copies) for γ -fibrinogen.

Study of factors affecting RT-PCR results

It was important to define the RT-PCR conditions to ensure reproducible results, because various factors could affect the results. Variation in PCR yield and quantification of this yield was affected by: (1) amount of total RNA, (2) PCR efficiency, and (3) amount of PCR products loaded on to the agarose gel.

Effect of total RNA amount on RT-PCR yield

The appropriate amount of total RNA added to the RT-PCR was determined in preliminary experiments. If too little total RNA was used, the optical density of target RNA band was below the detection limit after amplification. Conversely, if too much total RNA was added, amplification was not exponential and saturation occurred. When the amount of total RNA was increased from 25 ng to 500 ng, the increase in yield was exponential. If the amount of total RNA was greater than 500 ng, amplification was not exponential. Therefore the amount of total RNA was fixed below 500 ng (but was greater than 25 ng).

Effect of amount of standard RNA on RT-PCR results

Standard RNA added to the RT-PCR reaction not only acted as an internal control, but also allowed calculation of the number of copies of target RNA. As discussed below, the number of target RNA copies was calculated by obtaining the ratio (R_t) of the optical densities of target RNA and standard RNA. By changing the amount of standard RNA added to the reaction, R_t was changed accordingly, so that the calculated results represented real expression levels. When 0.0125 fmol and 0.025 fmol of standard RNA was added to 150 ng of total RNA, after RT-PCR the mean R_t was 1.125 ± 0.34 and 0.625 ± 0.26 respectively, confirming the 2-fold difference between standard RNAs after amplification.

Figure 2 T-type RT-PCR

Upper panel: results of T-type RT-PCR showing amplification of both target RNA and standard RNA with increasing quantities of total RNA. In all lanes the amount of standard RNA in the reaction was kept constant (e.g. 0.01 fmol for β -fibrinogen). The amount of total RNA was increased from 25 ng (lane 1) to 250 ng (lane 6). Lower panel: regression line for amplification of β -fibrinogen mRNA by T-type RT-PCR. Where $y = 1$ (i.e. $D_t/D_s = 1$), $X = 137$ ng. Thus the amount of total RNA can be defined for which $D_t = D_s$, and therefore the number of copies of target RNA can be expressed per ng of total RNA.

Data from RT-PCR experiments with other genes in which the difference in size between the target RNA and standard RNA was greater than for each of the three fibrinogen genes showed that the PCR amplification efficiency of reverse-transcribed target RNA and standard RNA in the same reaction depended upon the ratio of copies of mRNA rather than the ratio of sizes of target RNA/standard RNA (results not shown). When the appropriate amount of standard RNA was fixed, the PCR amplification efficiency of reverse-transcribed target RNA and standard RNA was similar provided that the amount of total RNA in the reaction was within a certain range. This range was determined for each gene studied, because each gene expressed unique amounts of mRNA and thus different amounts of total RNA were required in the RT-PCR reaction. Outside of this range, reverse-transcribed target RNA and standard RNA were not amplified with similar efficiency. There was little difference in amplification efficiency of both reverse-transcribed target and standard RNAs for each of the three fibrinogen genes studied (e.g. the difference in amplification efficiency between reverse-transcribed target and standard RNAs was $\sim 3\%$ for γ -fibrinogen).

Detection limit of agarose gel

A 2% agarose gel was used (7.5 cm $\times$ 10 cm, made with 50 ml of 1 $\times$ TBE solution) to check the linear range. Different amounts of the same PCR product were loaded on to the gel and the optical density (pixels) of the bands was plotted against volume loaded

(results not shown). The detection of DNA bands was linear between 6 and 120 pixels, and the saturation point was reached above 120 pixels. Consequently values of > 120 pixels were not considered.

Interpretation of data

Interpretation of data was somewhat dependent upon the method used to undertake quantitative RT-PCR. With T-type RT-PCR, different amounts of total RNA (from 25 to 250 ng) were added to a fixed amount of standard RNA (Figure 2, upper panel). The ratio (R_t) of the optical density of the target RNA band (D_t) to that of the standard RNA band (D_s) was plotted against the amount of total RNA in each reaction (Figure 2, lower panel). A straight line was obtained, and the equation for the regression line was $y = KX + b$ [y is the value of R_t ; R_t values change with x value; X is the amount of total RNA (Figure 2, lower panel)].

From this equation, the amount of total RNA was calculated where $D_t = D_s$ (i.e. $y = 1$). The value (X) of total RNA at this point was designated X_e (Figure 2, lower panel).

The number of target RNA molecules was calculated from the number of standard RNA molecules (N.B. 1 mole of standard RNA = 6×10^{23} molecules) and was corrected for the difference in size between target RNA and standard RNA.

We knew that D_t and D_s were proportional to the number of copies multiplied by the size, i.e.:

$$D_t \propto M_t \times S_t$$

and:

$$D_s \propto M_s \times S_s$$

Therefore:

$$D_t/D_s = M_t S_t / M_s S_s \quad (1)$$

When $D_t/D_s = 1$, then:

$$M_t S_t = M_s S_s \quad (2)$$

where M_t is the number of molecules of target RNA, M_s is the number of molecules of standard RNA, S_t is the size of target RNA (bp) and S_s is the size of standard RNA (bp).

From eqn. (2):

$$M_t = M_s S_s / S_t$$

S_s/S_t is the ratio of the size of the standard RNA fragment to that of the target RNA fragment. Once primers have been designed, this ratio will not change. Thus the ratio is a constant, and was defined as the size ratio constant (K_s), i.e. $K_s = S_s/S_t$. Hence:

$$M_t = M_s K_s \quad (3)$$

The relationship established in eqn. (3) is correct only when $D_t/D_s = 1$. We also defined the mRNA copies in 1 ng of total RNA to represent the expression level. Hence this mRNA level (M_m) was calculated as:

$$M_m = M_t / X_e = M_s K_s / X_e \quad (4)$$

In eqn. (4), the M_s value and the K_s value were known, and the X_e value could be calculated individually (when $y = 1$, $X = X_e$; see Figure 2, lower panel). For example, in Figure 2 (lower panel), K_s is 386 bp/497 bp (β -fibrinogen) = 0.77666; M_s is 5.6×10^6 copies; and, from $y = 0.008X - 0.098$, when $y = 1$ then $X_e = 137$ ng. Therefore:

$$M_m = 5.625 \times 10^6 \times 0.7766 / 137 = 3.2 \times 10^4 \text{ (copies/ng)}$$

Figure 3 C-type RT-PCR

Top panel: results of C type RT-PCR. The amount of both total RNA and standard RNA was kept constant in each reaction (e.g. for γ -fibrinogen, total RNA = 500 ng and standard RNA = 12.5 fmol). The number of PCR cycles was increased from 21 (lane 1) to 26 (lane 6). Middle panel: exponential amplification of target RNA and standard RNA with increasing number of PCR cycles. Exponential amplification occurred for both target RNA and standard RNA products from cycle 21 to cycle 24. Bottom panel: regression lines for amplification of target RNA and standard RNA RT-PCR products. Exponential phases were used to calculate the efficiency of PCR amplification.

Table 1 Comparison of the two variants of quantitative RT-PCR

A comparative study of two types of quantitative RT-PCR was undertaken by calculation of γ -fibrinogen mRNA copy number (mean $\pm$ S.E.M.) in two different liver lobes using the two methods. *P* values compare means of the two variants for each lobe.

	$10^{-3} \times$ Copy number		<i>P</i> value
	T-type RT-PCR	C-type RT-PCR	
Lobe 1	10369 $\pm$ 627	9710 $\pm$ 302	0.50
Lobe 3	11657 $\pm$ 1198	10584 $\pm$ 342	0.36

Therefore the β -fibrinogen mRNA copy number in 1 ng of total RNA was 3.2×10^4 .

C-type RT-PCR was undertaken by fixing the amounts of both total and standard RNA and performing six separate reactions with an increasing number of amplification cycles [e.g. 21–26 cycles (Figure 3, top panel)]. The mRNA copy number was calculated in the following way.

First, the exponential amplification phase was defined. This was achieved by plotting the log values of both D_t and D_s against cycle number (Figure 3, middle panel). Two straight regression lines were obtained, and these represented exponential amplification (Figure 3, bottom panel). It should be noted that if the actual D_t and D_s values, rather than their log values, were plotted against number of PCR cycles, exponential amplification was depicted by a curve.

Secondly, the R_t values were calculated, i.e.:

$$R_t = D_t/D_s$$

Note that R_t values obtained outside the exponential phase were not considered. Then we calculated the mean values ($\bar{R}_t$) of those R_t values (at least three consecutive PCR cycles were used) within the exponential phase:

$$\bar{R}_t = (R_{t1} + R_{t2} + \dots + R_{tn})/n$$

where $n \geq 3$ and ≤ 6 .

Since M_s (number of copies of standard RNA) and K_s (size ratio constant) were known, and the mean value of R_t ($\bar{R}_t$) was obtained, the M_m value was calculated from eqn. (5) (in which M_t could be deduced from eqn. 1), i.e. M_t value divided by the amount of total RNA in the reaction (T , in ng):

$$M_m = M_t/T = \bar{R}_t K_s M_s/T \quad (5)$$

For example, for γ -fibrinogen, $M_s = 7.5 \times 10^9$ copies, $K_s = 303 \text{ bp}/394 \text{ bp} = 0.769$ and $\bar{R}_t = 1.58 \pm 0.23$; therefore:

$$M_m = 1.58 \times 0.769 \times 7.5 \times 10^9 / 500 = 1.8 \times 10^7 \text{ copies/ng}$$

It should be noted that, in C-type RT-PCR, the mean value of R_t ($\bar{R}_t$) was different from that in T-type RT-PCR, where $R_t = 1$.

Calculation of PCR efficiency

When PCR amplification was exponential, PCR products accumulated according to the equation [2,10]:

$$P = M_0(1 + E)^n$$

Here P represents the amount of PCR product, M_0 is the number of molecules in the reaction prior to amplification, E is the PCR efficiency and n is the number of PCR cycles. Taking log values of the above equation, we get:

$$\log P = n \times \log(1 + E) + \log M_0$$

When the $\log P$ value was plotted against PCR cycle number (as discussed for C-type RT-PCR above), PCR amplification efficiency could be calculated from the equations of the two regression lines (see Figure 3, bottom panel). For example, for Figure 3 (bottom panel) the calculated PCR amplification efficiency was 98% for target RNA and 100% for standard RNA, i.e. the difference in efficiency between target RNA and standard RNA was 2%. Under our experimental conditions, the efficiencies were generally above 80%, and the difference in efficiency between target RNA and standard RNA was $\sim 3\%$.

Table 2 Comparison of α -, β - and γ -fibrinogen mRNA copy numbers in each liver lobe

mRNA copy numbers (means $\pm$ S.E.M.) were compared between lobes for each of the three genes by ANOVA (F values and level of significance as shown, $n = 3$ animals).

	$10^{-3} \times$ Copy number				F value	P value
	Lobe 1	Lobe 2	Lobe 3	Lobe 4		
α -Fibrinogen	550 $\pm$ 110	930 $\pm$ 42	436 $\pm$ 60	1325 $\pm$ 139	14.6	0.0003
β -Fibrinogen	85 $\pm$ 9	36 $\pm$ 3	47 $\pm$ 8	58 $\pm$ 18	3.74	0.04
γ -Fibrinogen	10040 $\pm$ 136	21323 $\pm$ 6414	11538 $\pm$ 865	19709 $\pm$ 3117	3.75	0.04
Ratio α/β	6:1	26:1	9:1	23:1		
Ratio α/γ	18:1	23:1	26:1	15:1		
Ratio β/γ	118:1	592:1	245:1	340:1		

Comparison of the two types of RT-PCR

A comparative study was undertaken with both variants of quantitative RT-PCR using total RNA from liver lobes 1 and 3 to determine if both techniques gave reproducible results. There were no significant differences in calculated mRNA copy numbers from the two techniques (Table 1). C-type RT-PCR gave slightly more reproducible results, and the intra-assay coefficient of variance of this variant was 14% ($n = 20$).

Differential expression of fibrinogen genes in rat liver lobes

The two variants of RT-PCR were used to investigate mRNA copy numbers of the α -, β - and γ -fibrinogen genes in the four different liver lobes (Table 2). It was found that the mRNA levels of the three fibrinogen genes were different from each other, and each also varied between liver lobes.

DISCUSSION

We have developed two variants of quantitative RT-PCR to examine the reproducibility of this PCR-based methodology. Our results show that there were no significant differences in calculated mRNA copy numbers between the two variants, even though these variants were dissimilar and the methods of calculating mRNA copy numbers were different. Since quantitative RT-PCR is the most sensitive technique that has been developed so far for RNA analysis, our data suggest solutions that will be useful for a number of applications. In our experience, the most important determinant affecting the reproducibility of results was the quality of the original RNA preparation and not the technique itself. Given that extraction of good-quality RNA from liver tissue can be difficult, we believe that this is the factor that will most be likely to affect results.

There are three main factors that will affect the reproducibility of results. The first of these is the efficiency of cDNA synthesis, the second is PCR efficiency and the third is quantification and interpretation of data. Quantitative RT-PCR techniques require an internal standard, and this can be in the form of a synthetic RNA (standard RNA) added to the tissue RNA prior to cDNA synthesis. Alternatively, a mimic DNA can be added to the PCR reaction, or a 'housekeeping' gene can be amplified in the same reaction using different primers. Although it is assumed that efficiency of cDNA synthesis is the same between reactions, in reality cDNA synthesis efficiency may vary between 5 and 90% [11]. Consequently, quantitative competitive RT-PCR undertaken with a standard RNA acting as an internal standard provides greater accuracy than techniques based upon either

addition of DNA mimics or amplification of housekeeping genes.

C-type RT-PCR provided an easy way of monitoring PCR amplification of both target RNA and standard RNA. By plotting the log of the optical density of either the target RNA or the standard RNA against the number of cycles, both the exponential amplification phase and the saturation point could easily be determined for both RNAs. We consider that identification of the exponential amplification phase is important, as it allowed correct quantification of both RT-PCR products, although it has been previously considered unimportant [12]. In our study, if either the target RNA or the standard RNA reached saturation point, the D_t/D_s ratio changed, affecting the accuracy of the results.

It has been suggested that different-sized RT-PCR products result in unequal amplification efficiency [13]; however, our data suggested that this is not always the case. Although the standard RNA was designed to be of a different size (usually shorter) than the target RNA, it was the ratio of the number of target RNA copies to standard RNA copies, not the ratio of the amount of total RNA to standard RNA, nor the ratio of the size of target RNA to standard RNA, that predominantly affected the amplification efficiencies of both the target RNA and the standard RNA. We have introduced a size ratio constant (K_s) into the calculation of numbers of mRNA copies, because the number of mRNA copies was calculated from the relative D_t/D_s ratio after PCR amplification, as it was known that the difference in the size of PCR products affects their optical density. By using K_s , the potential error in calculating the number of mRNA copies has been corrected.

Both T-type and C-type PCR techniques gave reproducible results; however, there were some differences between them. In T-type RT-PCR, reduced efficiency of amplification of both target RNA and standard RNA did not affect the result, because the D_t/D_s ratio was calculated. The disadvantage was that a series of dilutions had to be undertaken with multiple pipetting steps, possibly resulting in errors. In C-type RT-PCR, both total RNA and standard RNA were added in larger amounts (compared with T-type RT-PCR) to a master mixture, and so errors attributed to the greater number of pipetting steps could be reduced. Secondly, since all tubes originated from the same master mixture, there was no reagent variation between tubes. Thirdly, it is easy to monitor the PCR amplification process for both target RNA and standard RNA by plotting the log values for target RNA and standard RNA against cycle number (Figure 3, middle panel). Therefore we favour C-type quantitative competitive PCR, as this technique was simpler and more robust.

Gene expression may vary greatly between tissues for certain genes [14]. We have observed a 2–3-fold variation in mRNA

copy number for each of the fibrinogen genes in different liver lobes. The data suggest that other genes may be differentially expressed within liver lobes, and accurate quantification of the mRNA transcripts needs to be considered in terms of the specific liver lobe. In preliminary experiments, in which biopsies were taken 'blind' from rat liver and so the site of biopsy was unknown, we observed a large variation in α -fibrinogen mRNA levels between rats. In subsequent experiments where α -fibrinogen mRNA levels were compared in matched lobe samples, most of the variation between rats disappeared. Thus the data suggest that the site of liver biopsy may contribute to some of the variation in mRNA levels between individuals.

In conclusion, we have demonstrated by using two variants of quantitative competitive RT-PCR that this technique can be usefully employed to determine precisely mRNA copy numbers in liver tissue. We have shown that there are different numbers of α -, β - and γ -fibrinogen mRNA copies in each of the liver lobes.

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