Simple, sensitive and accurate method for the quantification of prothrombin mRNA by using competitive PCR

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A method for the quantification of prothrombin (PT) mRNA species in hepatic tissues of rats was developed with the use of competitive PCR. To validate the quantification approach, sequential dilutions of total RNA from one of the samples were reverse transcribed. Their equivalent volumes were amplified together with a known amount of non-homologous competitor cDNA with identical nucleotide primers. The disparate sizes of target and competitor permitted the easy identification and quantification of bands in samples after densitometric analysis of ethidium bromide-stained agarose gels. Ratios of intensities of target and competitor bands were plotted against the initial amounts of total RNA species used, giving a linear relationship. The slope of this line was virtually identical with that obtained when the sample RNA was replaced with recombinant target cDNA, indicating that recombinant cDNA behaved in PCR identically with that made by reverse transcription and permitting the estimation of transcripts in reverse transcription reactions by using the recombinant counterpart of each as a standard. To avoid variation in the final results, the amount of competitor used in the assay was calculated separately from the equivalence

point of the reverse-transcribed total RNA of one of the tissue samples; PCR was performed only for the minimum number of cycles required to detect products. A standard curve was made in each PCR run by amplifying differing amounts of recombinant cDNA species of PT or β -actin together with a constant amount of its competitor. The numbers of transcripts in the tissues were then determined directly by PCR incorporating the same amount of respective competitor (as used in the standard curve) and comparing the ratios of products with the standard curve. Application of this method revealed that the median ratio of PT message to β -actin message in hepatic tissues of 10 normal rats was 0.37, with a mean $\pm$ S.D. of 0.37 ± 0.07 (range 0.27–0.47). Although the method was developed for the quantification of PT transcripts in liver, it can easily be used for non-hepatic tissues as well. The technique is simple, quick and sensitive and requires only a very small amount of substrate.

Key words: calcium oxalate, urinary macromolecules, urinary prothrombin fragment 1, urolithiasis.

INTRODUCTION

Interest in the putative role of prothrombin (PT) derivatives in the pathogenesis of calcium oxalate urolithiasis has intensified since the discovery that PT fragment 1 and its counterpart in urine, urinary prothrombin fragment 1 (UPTF1), are potent inhibitors of calcium oxalate crystallization [1–3]. UPTF1 is present in abundance in the matrix of calcium oxalate crystals generated in human urine *in vitro* [4] and has also been detected in human urinary stones containing calcium [5]. Furthermore, immunohistochemical studies have revealed that the protein is localized in the more lithogenic regions of the human nephron [6]. More recently, expression of the gene encoding PT has been reported in the human kidney [7,8]. Collectively, all available evidence strongly suggests that the inhibitory effects on calcium oxalate crystallization exhibited by UPTF1 *in vitro* might also operate during stone pathogenesis *in vivo*. However, there is as yet no conclusive evidence that UPTF1, PT or indeed any other protein has an indispensable role in the formation or prevention of calcium oxalate stones.

The demonstration of a relationship between the level of UPTF1 or its mRNA and the formation of crystals or calcium stones within the kidney would constitute compelling corroborative evidence for the existence of a cause-and-effect relationship between UPTF1 synthesis and urolithiasis. Although such studies are ethically impossible to perform in humans, they can

be performed with established rat models of stone formation [9–11], particularly since we showed recently that, as occurs in humans, the rat kidney contains mRNA for PT [12,13]. The availability of a quantitative method for determining the level of PT mRNA would permit the measurement of the effects of lithogenic conditions on renal PT synthesis. Thus the main aim of this study was to establish a method for quantifying renal PT mRNA. Because the liver is the primary source of mammalian PT [14–16] and we have shown (P. K. Grover, A. M. F. Stapleton, K. Miyazawa and R. L. Ryall, unpublished work) that it contains significantly higher levels of PT mRNA than does the kidney, liver was used for development of the technique.

EXPERIMENTAL

Materials

Tri-Reagent and 1-bromo-3-chloropropane were purchased from Molecular Research Center (Cincinnati, OH, U.S.A.). Diethyl pyrocarbonate (DEPC), Bromophenol Blue, Xylene Cyanol FF, mineral oil, Trizma base, boric acid, sodium acetate, EDTA and acid-equilibrated (pH 4.7) phenol/chloroform/3-methylbutan-1-ol (125:24:1, by vol.) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Propan-2-ol and glycerol were supplied by Ajax Chemicals (Auburn, NSW, Australia) and AnalaR grade ethanol by British Drug Houses (Merck Pty Ltd, Kilsyth, Victoria, Australia). *Taq* DNA polymerase in storage buffer B,

Abbreviations used: DEPC, diethyl pyrocarbonate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PT, prothrombin; RT-PCR, reverse-transcriptase-mediated PCR; UPTF1, urinary prothrombin fragment 1.

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dNTPs, LE analytical grade agarose and RQ1 RNase-free DNase kits were purchased from Promega Corporation (Madison, WI, U.S.A.). The Superscript™ Preamplification System for First-Strand cDNA Synthesis was supplied by Life Technologies (Grand Island, NY, U.S.A.), aqueous ethidium bromide solution (10 mg/ml) was from Pharmacia Biotech AB (Uppsala, Sweden) and QIA quick PCR purification kit was from Qiagen (Valencia, CA, U.S.A.).

Tissue preparation

Ten 7-week-old male hooded Wistar rats weighing 150–200 g were caged in groups of five and were given free access to tap water and standard rat chow. The composition of the diet, as reported by the supplier (Ridley Agriproducts Pty Ltd, Murray Bridge, SA, Australia) was as follows: minimum crude protein, 21%; minimum crude fat, 5%; maximum crude fibre, 10%; maximum added salt, 1%; vitamin A, 22000 i.u./kg; vitamin D₃, 4000 i.u./kg; vitamin E, 135 mg/kg; vitamin B₂, 10 mg/kg; vitamin K, 8.3 mg/kg; niacin, 18 mg/kg. After 8 weeks the animals were fasted overnight and killed in a CO₂ chamber. Their livers were removed and stored at –70 °C for later analyses. The study was approved by the Animal Welfare Committee of the Flinders University of South Australia (code no. 505/99).

Isolation and purification of total RNA

Total RNA was extracted from each of the tissue samples in a single batch experiment with Tri-Reagent in accordance with the manufacturer's instructions, as described previously [12]. To avoid DNA contamination, each RNA sample was treated with RQ1 RNase-free DNase. The samples were purified by extraction with phenol/chloroform (5:1, v/v) and concentrated by precipitation overnight at –20 °C with 0.1 vol. of 3 M sodium acetate, pH 5.2, and 1 vol. of propan-2-ol. They were then centrifuged for 15 min at 14000 *g* and 4 °C; the pellets were resuspended in 100 µl of cold propan-2-ol and centrifuged again. The RNA pellets of all the samples thus obtained were air-dried for 5 min. Because DEPC-treated water has a low pH, which affects the UV absorption spectrum of RNA [17], the samples were solubilized in 50–100 µl of 10 mM Tris/HCl, pH 8.0.

Determination of yield, purity and integrity of total RNA

RNA concentration was determined spectrophotometrically at 260 nm (1 *A*₂₆₀ unit = 40 µg/ml single-stranded RNA) with a GeneQuant RNA/DNA Calculator (Pharmacia Biotech Ltd, Cambridge, U.K.) fitted with a 0.5 mm quartz capillary cell. Purity was also determined by the ratio of *A*₂₆₀ to *A*₂₈₀.

The integrity of the RNA samples was determined by electrophoresis in 1% (w/v) agarose mini-gels as described previously [12].

Treatment with reverse transcriptase

Identical amounts of total RNA species extracted from each of the tissue samples were reverse transcribed in a batch experiment to cDNA species by using the Superscript™ Preamplification System for First-Strand cDNA Synthesis, as described previously [12].

Deoxynucleotides for PT and β-actin

GenBank® was used to design primer sequences; the oligonucleotides were synthesized with a DNA synthesizer

(GeneWorks, Thebarton, SA, Australia). The primer sequences selected were as follows.

For PT, primer 1 (20-mer) was 5'-AGC GGG TCC GCA GAG CCA AC-3' (sense) and primer 2 (22-mer) was 5'-CTG ACC ACA GAC AGG GAT ACT G-3' (antisense), corresponding to bp 116–135 and 546–567 of the PT sequence respectively [18]. The product of this pair of primers matched the nucleotide sequence of the N-terminal fragment of PT contained in the PT fragments 1 + 2 domain (⁴⁰Arg–¹⁸⁹Gln) [19].

For β-actin, primer 3 (22-mer) was 5'-TGT ATG CCT CTG GTC GTA CCA C-3' (sense) and primer 4 (22-mer) was 5'-ACA GAG TAC TTG CGC TCA GGA G-3' (antisense), corresponding to bp 2217–2238 and 2999–3020 of the β-actin sequence respectively [20].

The amplified product lengths were 452 and 592 bp for PT and β-actin, respectively.

Synthesis of recombinant cDNA species of PT and β-actin

The Promega kit was used for all the PCRs. The primer pairs mentioned above were used separately in PCR. A 1.0 µl sample of the tissue cDNA was added to 24 µl of reaction mixture containing 10.64 mM Tris/HCl (pH 9.0 at 25 °C), 53.19 mM KCl, 0.106% (v/v) Triton X-100, 212.76 µM dNTPs, 1.595 mM MgCl₂, 200 ng of each of the primers and 2.5 units of *Taq* polymerase in storage buffer B. Storage buffer B contained 20 mM Tris/HCl (pH 8.0 at 25 °C), 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% (v/v) glycerol, 0.5% (v/v) Tween 20 and 0.5% (v/v) Nonidet P40. Mineral oil (30 µl) was added to the tube and the sample was centrifuged briefly before starting the amplification. PCR amplification was performed with 0.6 ml clear microtubes (AxyGen, Hayward, CA, U.S.A.) in a thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research, Watertown, MA, U.S.A.).

PCR conditions for the synthesis of recombinant cDNA species of PT and β-actin were identical: after an initial denaturation for 2 min at 94 °C, each cycle consisted of a denaturation step of 30 s at 94 °C, a primer-annealing step of 1 min at 64 °C and a primer extension step of 1 min at 72 °C for a total of 35 cycles. This was followed by a 5 min extension step at 72 °C before the samples were held at 4 °C. The PCR product was diluted 1:100 in DEPC-treated water; 2 µl aliquots were re-amplified under the conditions stated above. The PCR products from several second amplification reactions were pooled separately for the recombinant cDNA species of PT and β-actin and purified with the QIA Quick PCR purification kit in accordance with the manufacturer's instructions. They were then subjected to electrophoresis on 2% (w/v) agarose gel in a mini-sub cell. After being stained in DEPC-treated water containing ethidium bromide, the gel was subjected to trans-illumination with UV, which showed that both preparations contained single bands in the expected size range of 452 and 592 bp for recombinant cDNA species of PT and β-actin respectively. The identities of both purified products were confirmed by sequence analysis with an automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The products were quantified (1 *A*₂₆₀ unit = 50 µg/ml double-stranded DNA) with a GeneQuant RNA/DNA calculator. Spectrophotometric analysis revealed that the ratio of *A*₂₆₀ to *A*₂₈₀ for the products was approx. 1.9, indicating that both were sufficiently pure for downstream applications.

Synthesis of competitor cDNA species of PT and β-actin

The designed sequences of oligomers for competitor PT cDNA were as follows: primer 5 (44-mer), 5'-AGC GGG TCC GCA

GAG CCA ACA TGG GTG TGA ACC ACG AGA AAT AT-3' (sense); primer 6 (44-mer), 5'-CTG ACC ACA GAC AGG GAT ACT GCG GAT ACA TTG GGG GTA GGA AC-3' (antisense).

The designed sequence of oligomers for competitor β -actin cDNA were as follows: primer 7 (46-mer), 5'-TGT ATG CCT CTG GTC GTA CCA CAT GGG TGT GAA CCA CGA GAA ATA T-3' (sense); primer 8 (44-mer), 5'-ACA GAG TAC TTG CGC TCA GGA GCG GAT ACA TTG GGG GTA GGA AC-3' (antisense).

These oligomers were synthesized with a DNA synthesizer (GeneWorks).

The sizes of the amplified competitor cDNA species of PT and β -actin were 370 and 372 bp, respectively.

The oligomer pairs described above were used separately in PCR with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA clone (kindly provided by Dr H. Morris, Hanson Centre for Cancer Research, Adelaide, Australia) as the template. PCR conditions for the synthesis of competitor cDNA species of both PT and β -actin were identical with those detailed above. The PCR products from several second amplification reactions were separately pooled for production of the competitor cDNA species of PT and β -actin. Both pools were purified and subjected to electrophoretic analysis as mentioned above. The preparations contained single bands in the expected size ranges of 370 and 372 bp for competitor cDNA species of PT and β -actin respectively. The identities of the purified products were also confirmed by sequence analysis with an automated DNA sequencer (Applied Biosystems) before quantification of double-stranded DNA as above. Spectrophotometric analysis of both products gave ratios of A_{260} to A_{280} of approx. 1.9, confirming that they were sufficiently pure for subsequent studies.

Analysis for heteroduplex formation

Analysis for heteroduplex formation was performed by the method of McCulloch et al. [21]. Recombinant cDNA of PT or β -actin was mixed in almost equal amounts with its respective competitor. Half of each resulting mixture was retained; the remainder was heated at 95 °C for 5 min and then left to cool to room temperature. Identical volumes of the heated and unheated mixtures were analysed on a 2% (w/v) agarose mini-gel. The gel was stained as mentioned above and photographed on a UV trans-illuminator with a Polaroid GelCam (Polaroid Corporation, Cambridge, MA, U.S.A.) with Polaroid high-speed, coaterless, black-and-white film (type 667) with a no. 15 orange filter. The gel was also scanned on a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and bands were quantified using ImageQuant (Molecular Dynamics). The disparate sizes of the PT and β -actin competitors in comparison with their recombinant counterparts allowed easy identification of the bands.

Validation of the quantification method

Validation of the quantification method was performed by the methods of Zhao et al. [22,23]. Sequential dilutions of one of the RNA samples were reverse transcribed; identical volumes of the resulting mixtures were transferred into PCR tubes. All samples were supplemented with equal volumes of PCR master mixture containing an identical amount of competitor PT cDNA. This was followed by PCR and electrophoretic analysis of identical volumes of all the samples with an agarose mini-gel. The gel was stained, photographed and scanned as described above. The logarithmic ratios of band intensities of native PT product to competitor in all the samples were plotted against the logarithmic

initial amounts of the RNA species used. Similarly, experiments were performed by substituting the reverse-transcribed mixtures with sequential dilutions of known amounts of recombinant PT cDNA template.

Similar experiments were performed with reverse-transcribed mixtures of another sequentially diluted RNA sample of one of the tissues, recombinant cDNA of β -actin and its competitor.

Titration of tissue PT and β -actin cDNA species with their competitors

Titration was performed by a slight modification of the method of Souza et al. [24]. A known amount of competitor PT cDNA was sequentially diluted in PCR tubes. All samples were supplemented with equal volumes of PCR master mixture containing an identical volume of the same reverse-transcribed mixture of one of the sample RNA species. This was followed by PCR and electrophoretic analysis of identical volumes of all the samples. Gels were stained, photographed and scanned as described above. The logarithmic ratios of band intensities of competitor PT product to native counterpart were plotted against the logarithmic initial amounts of competitor PT cDNA species used. From the equation of this line was calculated the amount of competitor PT cDNA that needed to be added to the volume of reverse-transcribed mixture to achieve a band intensity equal to that of the native counterpart.

Similarly, experiments were performed with the same reverse-transcribed mixture as that used above but in which the competitor cDNA of PT was replaced with that of β -actin.

Optimization of PCR cycle number

Titration of samples to estimate the optimum number of PCR cycles was performed as suggested by Tsai and Wiltbank [25]. Equal volumes of the same reverse-transcribed mixture, as used in the previous section, were transferred to PCR tubes and supplemented with equal volumes of PCR master mixture containing an identical amount of competitor PT cDNA template, as determined in the previous section. All samples were subjected to PCR over a range of amplification cycles. This was followed by agarose mini-gel electrophoretic analysis of identical volumes of all samples. Gels were stained and scanned for densitometric analysis, which permitted the determination of the minimum number of amplification cycles required for the adequate detection of bands of native PT and its competitor in the same PCR sample. Similar studies were performed for β -actin.

Quantification of PT and β -actin mRNA species

Identical amounts of total RNA extracted from the tissue samples were reverse transcribed in a batch experiment and the same volumes as those used above were transferred to PCR tubes. The remaining portions of all the reverse-transcribed mixtures were stored at -20 °C for the later quantification of β -actin transcripts. An equivalent volume of a known amount of recombinant PT cDNA was diluted sequentially (as the standard template) in another set of PCR tubes. Samples were supplemented with equal volumes of PCR master mixture containing competitor PT cDNA as determined above, then subjected to PCR for the total number of amplification cycles determined by titration. Identical volumes of all samples were analysed by agarose mini-gel electrophoresis; the logarithm of the ratios of band intensities of recombinant PT to competitor in the standard samples were plotted against the logarithm of the initial amounts of recombinant PT cDNA species used. This plot constituted the standard

curve from which we calculated the amounts of PT transcript in each sample by comparison of the ratios of band intensities of native PT to competitor. The amounts of β -actin transcripts in the same reverse-transcribed mixtures as those used above were determined in a similar manner. The intra-assay and inter-assay coefficients of variation of the method were 6.1 and 7.9% respectively.

To avoid any variations in sample preparation, the amount of PT transcript in each sample was divided by the corresponding amount of β -actin transcript.

Statistical analysis

Each experiment was performed in triplicate and the data are expressed as medians, means $\pm$ S.D. and ranges.

RESULTS

Purity of RNA preparations

The average amount of total RNA isolated from 10 samples of hepatic tissues was 250 μ g/100 mg of tissue. The ratio of A_{260} to A_{280} for all samples ranged from 1.7 to 1.85, confirming that the purity of each sample was satisfactory. Further, the ratios of the intensities of their 28 S and 18 S ribosomal RNA bands were greater than 1, demonstrating that no significant degradation of RNA samples had occurred during isolation.

Analysis for heteroduplex formation

When recombinant PT cDNA was mixed with its competitor, the portion of the resulting mixture that had been heated at 94 °C showed the same band pattern as its unheated counterpart (Figure 1). This was confirmed by densitometric analysis of the band intensities of recombinant to competitor PT in both portions: the ratio of 1.03 remained unchanged in both the untreated and treated samples. Similar observations were noted

Figure 1 Analysis for PT heteroduplex formation

Recombinant PT cDNA (25 μ l; 13.4 μ g/ml) and its competitor (13.2 μ g/ml) were pipetted into a PCR tube. The resulting sample was thoroughly mixed and divided: one half was treated as control and the other was heated at 95 °C for 5 min and then left to cool to room temperature. A 2 μ l sample of each was analysed by electrophoresis on a 2% (w/v) agarose mini-gel and stained with ethidium bromide. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lane 2, untreated sample; lane 3, heated sample; lane 4, positive control for competitor PT (for identification); lane 5, positive control for recombinant PT (for identification); lane 6, DNA markers (pUC19 DNA restricted with *Hpa*II).

Figure 2 Analysis for β -actin heteroduplex formation

Recombinant β -actin cDNA (30 μ l; 13.0 μ g/ml) and its competitor (12.9 μ g/ml) were pipetted into a PCR tube. The resulting sample was thoroughly mixed and divided: one half was treated as control and the other was heated at 95 °C for 5 min and then left to cool to room temperature. Both samples were analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lane 2, untreated sample; lane 3, heated sample; lane 4, positive control for competitor β -actin (for identification); lane 5, positive control for recombinant β -actin (for identification); lane 6, DNA markers (pUC19 DNA restricted with *Hpa*II).

with recombinant β -actin cDNA and its competitor (Figure 2), in which the corresponding ratio of recombinant to competitor band intensity in both portions remained 0.99. The comparable band patterns and ratio of their intensities in untreated and treated samples strongly suggest that when recombinant and competitor cDNA species of PT or β -actin are mixed in the same PCR, there was no formation of heteroduplex, which would have complicated the quantification.

Validation of the quantification method

Figure 3(A) shows an electrophoretogram of the reverse-transcribed mixtures of sequentially diluted tissue RNA of one of the samples amplified together with identical amounts of competitor PT cDNA template. A log-log plot of the ratios of band intensities of native to competitor PT product against the initial amounts of RNA species used yielded a line with the equation $y = -0.22893 + 0.65647x$ (Figure 3B). Similarly, when the logarithm of the ratios of band intensities of recombinant PT product (Figure 3C) to competitor in all samples was plotted against the logarithm of the initial amounts of recombinant PT cDNA species used, a line with the equation $y = -1.8499 + 0.63476x$ was obtained (Figure 3D). The almost identical slopes of 0.65 and 0.63 of the curves in Figures 3(B) and 3(D) respectively indicate that the recombinant PT cDNA behaved identically to its reverse-transcribed counterpart.

Similarly, recombinant β -actin cDNA behaved identically to its reverse-transcribed counterpart. Electrophoretograms for native and recombinant cDNA species for β -actin and their curves are presented in Figure 4.

Titration of tissue PT and β -actin cDNA species with their competitors

Figure 5(A) shows a representative electrophoretogram of sequentially diluted competitor PT cDNA amplified together

Figure 3 Validation of the method for quantification of PT transcripts

(A) Total RNA (2.5 μ g) from one of the samples was diluted sequentially (1:1) six times; all seven samples were reverse transcribed. Samples (2 μ l) of the mixtures were supplemented with 23 μ l of PCR master mixture containing 900 fg of competitor PT cDNA. All samples were subjected to PCR after an initial denaturation for 2 min at 94 $^{\circ}$ C. Each cycle consisted of a denaturation step of 30 s at 94 $^{\circ}$ C, a primer-annealing step of 1 min at 64 $^{\circ}$ C and a primer extension step of 1 min at 72 $^{\circ}$ C for a total of 25 cycles. This was followed by a 5 min extension step at 72 $^{\circ}$ C before the samples were held at 4 $^{\circ}$ C. The resulting samples were analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–8, 0.04, 0.08, 0.16, 0.31, 0.63, 1.12 and 2.50 μ g of the tissue total RNA processed as described; lane 9, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) A plot of the logarithms of the ratios of band intensities of native PT to competitor PT in the samples in (A) against the logarithms of the amounts of total RNA used. The equation of the straight line is $y = -0.22893 + 0.65647x$; $R^2 = 0.991$. (C) Recombinant PT cDNA (2003.49 fg) was diluted sequentially (1:1) six times. All seven samples were amplified together with competitor PT cDNA as in (A) and the resulting samples were analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–8, 31.30, 62.61, 125.22, 250.44, 500.87, 1001.75 and 2003.49 fg of recombinant PT cDNA processed as described; lane 9, DNA markers (pUC19 DNA restricted with *Hpa*II). (D) A plot of the logarithms of the ratios of band intensities of recombinant PT to competitor PT in the samples in (C) against the logarithms of the amounts of recombinant PT cDNA species used. The equation of the straight line is $y = -1.8499 + 0.63476x$; $R^2 = 0.995$.

with identical volumes of reverse-transcribed tissue RNA, from one of the samples. A log–log plot of the amounts of competitor PT cDNA species against the ratios of the band intensities of competitor to native PT was linear (Figure 5B), the equation of the line being $y = -0.94223 + 0.46664x$. From this equation it was calculated that 104.52 fg of competitor PT cDNA needed to be added to 1 μ l of the reverse-transcribed mixture to achieve a band intensity equal to that of native cDNA. This experiment was repeated three times and an average value of 100.16 fg was obtained.

Similar experiments were also performed for β -actin with the same reverse-transcribed mixture as that used above and the results are illustrated in Figure 6. The equation of the line was $y = -1.4142 + 0.68535x$, from which it was calculated that 115.74 fg of competitor β -actin cDNA needed to be added to 1 μ l of the reverse-transcribed mixture to achieve a band intensity equal to that of native cDNA. An average value of 118.43 fg was obtained from three replicate determinations.

Optimization of PCR cycle number

We found (results not shown) that adequate detection of native and competitor bands of PT or β -actin, in the same PCR sample, required 24 and 23 amplification cycles respectively.

Quantification of PT and β -actin mRNA species

Figures 7(A) and 7(B) show electrophoretograms of reverse-transcribed mixtures of tissue RNA samples and recombinant PT cDNA (as the standard templates) respectively, amplified together with 100.16 fg of competitor PT cDNA. From the log–log plot of the amounts of recombinant PT cDNA species against the ratios of band intensities of recombinant to competitor PT cDNA (Figure 7C) we calculated the amounts of PT transcript in the experimental samples. Similarly, β -actin transcripts were quantified in the same reverse-transcribed mixtures as those used above (Figure 8).

Figure 4 Validation of the method for quantification of β -actin transcripts

(A) Total RNA (2.5 μ g) from one of the samples was diluted sequentially (1:1) four times. All five samples were reverse transcribed; 2 μ l of each was amplified together with 893 fg of competitor β -actin cDNA with PCR conditions as described in the legend to Figure 3(A) before analysis as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–6, 0.16, 0.31, 0.63, 1.25 and 2.50 μ g of the tissue total RNA processed as described; lane 7, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) A log–log plot of the ratios of band intensities of native β -actin to competitor β -actin in the samples in (A) against the amounts of total RNA used. The equation of the straight line is $y = -0.25861 + 0.66883x$; $R^2 = 0.955$. (C) Recombinant β -actin cDNA (450.0 fg) was diluted sequentially (1:1) four times and the resulting samples were amplified together with competitor β -actin cDNA as described in the legend to Figure 3(A) and analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–6, 28.13, 56.25, 112.50, 225.0 and 450.0 fg of recombinant β -actin cDNA processed as described; lane 7, DNA markers (pUC19 DNA restricted with *Hpa*II). (D) The logarithm of the ratios of band intensities of recombinant β -actin to competitor β -actin in the samples in (C) plotted against the logarithm of the amounts of recombinant β -actin cDNA species used. The equation of the straight line is $y = -1.8288 + 0.67097x$; $R^2 = 0.978$.

The ratios of PT to β -actin transcripts in each sample are presented in Table 1.

DISCUSSION

A variety of methods, including Northern blotting, *in situ* hybridization, dot-blot hybridization, nuclear run-on assay and RNase protection assay, are currently available to quantify tissue mRNA. However, where only very small quantities of tissue samples are available, reverse-transcriptase-mediated PCR (RT–PCR) offers significant advantages because it can be performed relatively rapidly and easily and can quantify mRNA where other procedures are usually inadequate [26,27].

Although several variations of RT–PCR are available for the quantification of mRNA, the most precise method is competitive RT–PCR (reviewed in [28–30]). Considering the hundreds of papers published on the use of the technique, it is not surprising that many different experimental protocols exist. In its simplest form, the method involves the amplification of serial dilutions of known amounts of internal standard mRNA (also known as competitor) together with a constant amount of target with the use of a common set of primers. The technique is based on

the premise that if the efficiencies of amplification of the target and its competitor are identical, the ratio of their products after PCR will directly reflect the initial amounts present. This principle requires that the efficiency of reverse transcription and the subsequent amplification of target and competitor should be similar, with the latter being more crucial because 30–40 PCR cycles can cause even small differences in amplification efficiencies to be magnified into large variations in product [21]. Both of these requirements can be satisfied by the use of a competitor that is a mutant with a similar size to that of its target but containing or lacking a restriction site. However, this runs the risk of heteroduplex formation as a result of sequence similarity between target and competitor and the ensuing increased likelihood of competitor and target strand pairing during the denaturation and annealing steps of PCR [31]. In addition, the use of a mutant competitor often requires the digestion of PCR products with a restriction enzyme before its identification by electrophoretic analysis [32]; although heteroduplexes are resistant to digestion [21], the efficiency of restriction enzymes can be variable. Furthermore, the amount of target is measured as an equivalent amount of its competitor [33–35]. These limitations, taken together, are sufficient to yield unreliable data from this

Figure 5 Titration of tissue cDNA with competitor PT cDNA template

(A) Competitor PT cDNA (500.80 fg) was diluted sequentially (1:4) four times and the resulting samples were supplemented with 24 μ l of PCR master mixture containing 1 μ l of the reverse-transcribed mixture obtained from 4 μ g of total RNA from one of the tissue samples. Samples were then subjected to PCR as described in the legend to Figure 3(A) and analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–6, 0.80, 4.00, 20.03, 100.16 and 500.80 fg of competitor PT cDNA processed as described; lane 7, positive control for competitor PT; lane 8, positive control for native PT; lane 9, PCR negative control; lane 10, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) The logarithm of the ratios of band intensities of competitor PT to native PT in the samples in (A) plotted against the logarithm of the amounts of competitor PT cDNA species used. From the equation of the line it was calculated that 104.52 fg of competitor PT cDNA needed to be added to 1 μ l of the reverse-transcribed mixture as used in (A) to achieve a band intensity equal to that of its native counterpart. This experiment was repeated three times; an average value of 100.16 fg was obtained. The equation of the straight line is $y = -0.94223 + 0.46664x$; $R^2 = 0.901$.

type of competitive RT–PCR unless performed by a highly experienced experimenter.

In addition to the shortcomings discussed, the type of competitive RT–PCR assay described requires several reactions for each sample [36], which in turn requires additional tissue, reagents and time. As a whole, the assay is therefore less than ideal for limited amounts of samples and is certainly not suitable for the routine processing of large numbers of specimens. To decrease the number of reactions per sample, a method was devised [37] that required only a single reaction per sample and resolution of the products by HPLC. Although simple, the procedure relied on a comparison of results with a quantified competitor that, as mentioned above, again raised the possibility that any amplification differences between target and competitor would produce non-competitive results [21]. To overcome this problem, Tsai and Wiltbank [25] developed what is commonly known as the standard curve method, which requires one reaction per sample but not an equivalence of the amplification efficiencies of target

Figure 6 Titration of tissue cDNA with competitor β -actin cDNA template

(A) Competitor β -actin cDNA (986.94 fg) was diluted sequentially (1:3) three times and the resulting samples were supplemented with 24 μ l of PCR master mixture containing 1 μ l of reverse-transcribed mixture from the same reaction as that described in the legend to Figure 5(A). Samples were then subjected to PCR as described in the legend to Figure 3(A) and analysed as described in the legend to Figure 1. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–5, 15.42, 61.68, 246.73 and 986.94 fg of competitor β -actin cDNA processed as described; lane 6, positive control for competitor β -actin; lane 7, positive control for native β -actin; lane 8, PCR negative control; lane 9, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) The logarithm of the ratios of band intensities of competitor β -actin to native β -actin in the samples in (A) plotted against the logarithm of the amounts of competitor β -actin cDNA species used. From the equation of the line it was calculated that 115.74 fg of competitor β -actin cDNA needed to be added to 1 μ l of the reverse-transcribed mixture as used in (A) to achieve a band intensity equal to that of its native counterpart. This experiment was repeated three times; an average value of 118.43 fg was obtained. The equation of the straight line is $y = -1.4142 + 0.68535x$; $R^2 = 0.992$.

and competitor, thus permitting the use of a competitor different in size and sequence (non-similar) from its target but possessing a shared primer sequence. Unlike a mutant competitor, which often requires treatment with a restriction enzyme for identification [32], non-similar competitors of different sizes can easily be distinguished by gel electrophoresis; the lower sequence similarity decreases the probability of heteroduplex formation, which when present can seriously affect results [21,31]. Although there are several established methods available for synthesizing non-homologous competitors [33,38,39], they traditionally involve cloning, and can thus be time-consuming and complicated, especially for quantifying multiple target mRNA species.

In recent years the use of a fluorogenic probe in quantitative competitive PCR has become increasingly popular. The principle of this approach is that a probe is designed so that it hybridizes within the target sequence and its signal accumulates during PCR cycling in a manner proportional to the concentration of the amplification product [40,41]. The fluorescent signal can be

Figure 7 Quantification of PT transcripts

Identical amounts (4 μ g) of total RNA extracted from the samples were reverse transcribed in a batch experiment; 1 μ l was transferred into PCR tubes. Similarly, 1 μ l of recombinant PT cDNA containing 100.18 fg was diluted sequentially (1:1) five times (as the standard templates) and the samples (unknown and standards) were supplemented with 24 μ l of PCR master mixture containing 100.16 fg of competitor PT cDNA template, as calculated from Figure 5(B). Samples were then subjected to PCR in a batch experiment with conditions as described in the legend to Figure 3(A) except that the number of cycles was 24. A 3 μ l portion of each sample was analysed as described in the legend to Figure 1. (A) Photomicrograph of gel from the liver samples. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–11, liver samples; lane 12, positive control for competitor PT; lane 13, positive control for native PT; lane 14, PCR negative control; lane 15, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) Photomicrograph of gel from the standard templates. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–7, 3.13, 6.26, 12.52, 25.04, 50.09 and 100.18 fg of standard samples processed as described; lane 8, positive control for competitor PT; lane 9, positive control for recombinant PT; lane 10, PCR negative control; lane 11, DNA markers (pUC19 DNA restricted with *Hpa*II). (C) The logarithm of the ratios of band intensities of recombinant PT to competitor PT in the samples in (B) plotted against the logarithm of the amounts of recombinant PT cDNA species used. The equation of the straight line is $y = -1.2013 + 0.68094x$; $R^2 = 0.989$.

measured either at the end of the PCR or during the amplification phase (real-time PCR). Although the sensitivity and accuracy of these methods are comparable with those of other conventional

Figure 8 Quantification of β -actin transcripts

Aliquots (1 μ l) of the same reverse-transcribed mixtures as those used in Figure 7(A) were transferred into PCR tubes. In addition, 1 μ l of recombinant β -actin cDNA containing 210 fg was diluted sequentially (1:1) five times in PCR tubes (as the standard templates). Samples (unknown and standards) were supplemented with 24 μ l of PCR master mixture containing 118.43 fg of competitor β -actin cDNA template, as calculated from Figure 6(B). All samples were subjected to PCR in a batch experiment with conditions as described in the legend to Figure 3(A) except that the number of cycles was 23. A 3 μ l portion of each sample was analysed as described in the legend to Figure 1. (A) Photomicrograph of the gel from the liver samples. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–11, liver samples (in the same sequence as described in the legend to Figure 7A); lane 12, positive control for competitor β -actin; lane 13, positive control for native β -actin; lane 14, PCR negative control; lane 15, DNA markers (pUC19 DNA restricted with *Hpa*II). (B) Photomicrograph of the gel from the standard templates. Lane 1, DNA markers (pUC19 DNA restricted with *Hpa*II); lanes 2–7, 6.56, 13.13, 26.25, 52.50, 105.0 and 210 fg of standard samples processed as described; lane 8, positive control for competitor β -actin; lane 9, positive control for recombinant β -actin; lane 10, PCR negative control; lane 11, DNA markers (pUC19 DNA restricted with *Hpa*II). (C) The logarithm of the ratios of band intensities of recombinant β -actin to competitor β -actin in the samples in (B) plotted against the logarithm of the amounts of recombinant β -actin cDNA species used. The equation of the straight line is $y = -1.6629 + 0.83066x$; $R^2 = 0.999$.

Table 1 Ratios of PT to β -actin transcripts in 1 μ l of the same reverse-transcribed mixtures obtained from 4 μ g of total RNA of tissue sample

The median ratio of PT to β -actin message in hepatic tissues of 10 normal rats was 0.37, with a mean $\pm$ S.D. of 0.37 ± 0.07 (range 0.27–0.47). Ratios shown are averages for three experiments.

Sample no.	Ratio, PT to β -actin
1	0.38
2	0.46
3	0.41
4	0.29
5	0.45
6	0.27
7	0.34
8	0.31
9	0.35
10	0.47

techniques, their main advantage is that they minimize carry-over contamination between samples because the fluorescent signal is measured in sample tubes with closed optical caps. However, their main disadvantage is that fluorogenic probes are expensive and measuring a fluorescent signal requires a dedicated instrument.

Here we report a quick and simple general PCR-based approach for synthesizing non-similar competitor cDNA species, which were then used in a simple technique modified from the original version of Tsai and Wiltbank [25]. The accuracy, reproducibility and applicability of the method were demonstrated by using it to quantify PT mRNA in the livers of male Wistar hooded rats. To control for variations resulting from sample preparation, a second competitive PCR was also developed to measure transcripts of the housekeeping gene β -actin from the same reverse transcription reactions as internal control. The amount of PT transcript was then normalized to the amount of β -actin transcript.

The synthesis of non-similar competitor cDNA with shared pairs of primers involved the preparation of composite primers 5'-appended to those of non-similar PCR products. The latter in this particular case was G3PDH with the chosen primer sequences 5'-ATG GTG AAC CAC GAG AAA TAT-3' (sense) and 5'-CGG ATA CAT TGG GGG TAG GAA C-3' (anti-sense). These correspond respectively to bp 421–444 and 727–748 of the nucleotide sequence of G3PDH [42]. The oligomers thus designed were synthesized and used in PCR with rat G3PDH cDNA clone as template. After several amplification reactions, the samples were pooled and the product was purified and analysed by agarose-gel electrophoresis. The product showed single bands in the expected size range of 370 and 372 bp for competitor cDNA species of PT and β -actin respectively, the identities of which were also confirmed by DNA sequencing. A spectrophotometric analysis to determine purity and concentration demonstrated their suitability for further applications.

As expected, heteroduplex formation did not occur, probably because the competitors of PT and β -actin that we designed contained non-similar bases except for the shared primers. Validation of our approach was vital for accurate results, because we intended to substitute recombinant cDNA for the tissue cDNA of PT and β -actin in the standard curve: any differences would have rendered the assay non-quantitative [32]. Initial studies demonstrated that recombinant PT and β -actin cDNA species behaved identically with their corresponding tissue reverse-transcribed counterparts and confirmed that the initial PT and β -actin mRNA species in the reverse transcription

reaction can be reliably estimated with recombinant products as standards.

It has previously been shown [24,43,44] that the lowest intra-assay and inter-assay coefficients of variation are found near the equivalence point, namely where the concentrations of target and competitor are equal. To maximize the reproducibility of the assay, it was therefore necessary to ensure that the amount of PT or β -actin competitor added to the samples was as close as possible to the quantity of its respective target. This in turn required the estimation of the amounts of PT and β -actin messages in all samples by using reverse transcription. In the present study, although a single, randomly selected sample was used, we regarded this as being representative of the entire batch of tissues because they had all been harvested from rats of the same age that had been fed with an identical diet for an equal duration. The amounts of PT and β -actin transcripts in this randomly selected sample were determined by titrating identical volumes of the reverse-transcribed mixture from the same reaction with PT and β -actin competitors and determining their respective equivalence points.

Although PCR offers high sensitivity for the quantification of transcripts from very small specimens, it is now well accepted that large numbers of PCR cycles increase experimental variability in final results [25]. To decrease this problem we determined the amount of competitor to be added and amplified it together with an identical volume of the same reverse-transcribed mixture (from the randomly selected sample) over a range of amplification cycles. This permitted the determination of the minimum number of PCR cycles required for adequate detection of native and competitor bands, in the same PCR sample, of PT or β -actin. The use of fluorimaging was helpful because it permitted the accurate assessment of low intensity bands.

In the method we have described, the amount of a target is measured as the equivalent amount of its recombinant counterpart. Although this requires that the amplification efficiencies of both species be identical, this is easily achieved by selecting the same size and sequence for both target and recombinant. The method is also independent of the amplification efficiencies of a target and its competitor because the amount of competitor is not used in any of the calculations. This feature of the method is particularly useful because it still permits the use of a competitor that differs in both sequence and length from its target: the former decreases the likelihood of heteroduplex formation and the latter permits the easy identification of products after PCR without digestion by restriction enzymes. The linear standard curve guarantees that there is a constant relationship between the amplification efficiencies of target and competitor. However, because the slope of the curve depends on the relative amplification efficiencies of both amplicons, it is important to add identical amounts of competitor to all standards and experimental samples in any batch analysis. This was ensured in the present study by adding competitor to the PCR master mixture. Because unavoidable variations always exist between different PCR preparations and because the sensitivity of the technique is very high, it is necessary to include a standard curve in every PCR run.

In the present study the practicability of the technique was demonstrated by applying it to the quantification of PT mRNA in hepatic tissues of normal male Wistar hooded rats. We have also recently used the technique for the quantification of PT mRNA in renal tissues of normal male Wistar hooded rats and compared it with their counterparts fed with a lithogenic diet: the results revealed a significant decrease in the latter group (P. K. Grover, A. M. F. Stapleton, K. Miyazawa, R. L. Ryall, unpublished work). A possible drawback of the method is that it

requires prior reverse transcription of the tissue RNA and quantification of the resulting cDNA with competitive templates. This does not take into account the variability of reverse transcription steps such as annealing with oligo(dT) and the efficiency of reverse transcriptase and might thus not be suitable for absolute quantification of transcripts in RNA samples. However, the technique is very simple and has much to offer for a comparison of transcripts between different samples.

In summary, we have described a quick and simple general PCR-based method for the synthesis of a non-similar competitor cDNA. We have also developed a quick, sensitive and accurate method based on competitive PCR, which we have used to measure the expression of PT in rat liver but which can also be used to quantify PT message in other rat tissues. Most importantly, the principles on which the technique is based can be applied to the quantitative measurement of mRNA in any tissue from any source.

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