Quantitation of TGF- β 1 mRNA in Porcine Mesangial Cells by Comparative Kinetic RT/PCR: Comparison With Ribonuclease Protection Assay and In Situ Hybridization

Monica Ceol,^{1*} Monica Forino,¹ Giovanni Gambaro,¹ Ulrich Sauer,²
Erwin D. Schleicher,³ Angela D'Angelo,¹ and Franca Anglani¹

¹*Division of Nephrology, Department of Medical and Surgical Sciences, University of Padova, Italy*

²*Institute of Pathology, University of Munich*

³*Department of Internal Medicine, Division of Endocrinology, Metabolism and Pathobiochemistry, University of Tübingen, Germany*

Gene expression can be examined with different techniques including ribonuclease protection assay (RPA), in situ hybridisation (ISH), and quantitative reverse transcription–polymerase chain reaction (RT/PCR). These methods differ considerably in their sensitivity and precision in detecting and quantifying low abundance mRNA. Although there is evidence that RT/PCR can be performed in a quantitative manner, the quantitative capacity of this method is generally underestimated. To demonstrate that the comparative kinetic RT/PCR strategy—which uses a housekeeping gene as internal standard—is a quantitative method to detect significant differences in mRNA levels between different samples, the inhibitory effect of heparin on phorbol 12-myristate 13-acetate (PMA)-induced-TGF- β 1 mRNA expression was evaluated by RT/PCR and RPA, the standard method of mRNA quantification, and the results were compared. The reproducibility of RT/PCR amplification was calculated by comparing the quantity of

G3PDH and TGF- β 1 PCR products, generated during the exponential phases, estimated from two different RT/PCR (G3PDH, $r = 0.968$, $P = 0.0000$; TGF- β 1, $r = 0.966$, $P = 0.0000$). The quantitative capacity of comparative kinetic RT/PCR was demonstrated by comparing the results obtained from RPA and RT/PCR using linear regression analysis. Starting from the same RNA extraction, but using only 1% of the RNA for the RT/PCR compared to RPA, significant correlation was observed ($r = 0.984$, $P = 0.0004$). Moreover the morphometric analysis of ISH signal was applied for the semi-quantitative evaluation of the expression and localisation of TGF- β 1 mRNA in the entire cell population. Our results demonstrate the close similarity of the RT/PCR and RPA methods in giving quantitative information on mRNA expression and indicate the possibility to adopt the comparative kinetic RT/PCR as reliable quantitative method of mRNA analysis. *J. Clin. Lab. Anal.* 15:215–222, 2001. © 2001 Wiley-Liss, Inc.

Key words: RT/PCR; RPA; ISH; mRNA quantitation; mesangial cells

Molecular biology techniques are considered one of the most powerful tools to obtain insights into pathogenetic mechanisms of diseases. Analysis of the variation of gene expression seems to have great potential to study in vivo the early phases of pathogenetic processes, and in vitro the effect of different stimuli on cellular models.

Generally, levels of individual mRNAs have been analysed by procedures such as Northern blot, ribonuclease protection assay (RPA), in situ hybridisation (ISH), and, more recently, the reverse transcription polymerase chain reaction (RT/PCR). Northern blot is an essential procedure for transcript analysis, but its sensitivity is relatively low, and large amounts of starting material are required for detection of low-abundance mRNA. RPA assay, on the other hand, is a more sensitive technique but is less straightforward for mRNA quantifica-

tion. The greater efficiency of solution than membrane hybridisation and avoidance of RNA losses during a blotting step are responsible for about a 10-times higher sensitivity than in Northern blot (1).

RT/PCR is an extremely sensitive method of mRNA analysis appreciated for its ability to detect rare mRNA from small number of cells or small amount of tissues. However, because of the multiple amplification steps it may be difficult to ob-

*Correspondence to: M. Ceol, Laboratorio di Biologia e Morfologia Molecolare, Divisione di Nefrologia, Dipartimento di Scienze Mediche e Chirurgiche, Via Giustiniani n°2, 35128 Padova, Italy.
E-mail: giga@ux1.unipd.it

Received 18 January 2001; Accepted 26 February 2001

tain quantitative data with this technique. Different strategies and protocols based on RT/PCR have been described for a quantitative analysis of gene expression. They use an internal standard, to determine the relative level of a particular mRNA (2–6), or a synthetic DNA or RNA competitor to determine the absolute level (7–9).

The ideal mRNA quantitation by RT/PCR should rely on the use of an RNA-exogenous standard, the RNA competitor. However, this can constitute a limitation because of the extreme degradability of RNA competitor and consequently the difficulty to maintain stability during the entire duration of the experiment.

The quantitative application of these methods has been limited. Although there is ample evidence that PCR and RT/PCR can be performed in a quantitative manner, considerable debate has revolved around the conditions that must be met to validate the quantitative reliability of such assay (10).

In an ongoing study on the structure-function relationship of heparin and glycosaminoglycans (GAGs), we needed to test if the structure heterogeneity of different GAGs formulations could influence the ability of GAGs to inhibit TGF- β 1 expression in mesangial cells, in which we have previously shown that heparin and GAGs can inhibit TGF- β 1 gene overexpression induced by PMA and high glucose concentration (11). Using primary mesangial cells, 24 experimental conditions had to be simultaneously set and TGF- β 1 mRNA quantified, starting from a low number of cells. Comparative kinetic RT/PCR (12), which uses housekeeping genes as internal standards, was chosen as the strategy of quantitation because of its sensitivity and specificity but in particular because it allows analysis of several samples simultaneously, without utilising an RNA competitor.

The aim of this work is to validate our RT/PCR strategy as a quantitative method to detect significant differences in mRNA levels between different samples with very low levels of mRNA. To this purpose the mesangial cell-TGF- β 1 mRNA expression in control, phorbol 12-myristate 13-acetate (PMA)-stimulated, and PMA+heparin (GAG) culture conditions was evaluated by RT/PCR and RPA, a widely accepted standard methods of mRNA quantification, and the results were compared. Moreover, ISH, a powerful tool for the study of gene expression, was applied for the semi-quantitative evaluation of expression and localisation of TGF- β 1 mRNA in the entire cell population.

MATERIALS AND METHODS

Mesangial Cells Culture

Porcine mesangial cells were isolated and characterised as described previously (13). For RPA and RT/PCR analysis mesangial cells at passage 6 were grown in 154-cm² gelatin-coated dishes (3×10^6 seeded cells) in RPMI 1640 medium containing 10 mM glucose, nonessential amino acids, 1 mM pyruvate, 4 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5% FCS (standard culture media; Seromed,

Berlin, Germany). In a simultaneous, parallel experiment, the same porcine mesangial cells were cultured to subconfluency on collagen-coated 8-well chamber slides and used for the ISH study. To investigate the action of heparin on PMA-induced TGF- β 1 expression, cells were grown for 9 hr in standard culture media (N9: normal condition), in 0.1 μ M PMA (Sigma, St. Louis, MO) (T9: treatment), and in 0.1 μ M PMA + 10 μ g/ml low molecular weight heparin (α -89-289 Alfa Wassermann SpA, Bologna, Italy) (TH9: treatment with heparin).

RNA Extraction

Cells were washed, and total RNA was extracted using the RNazol method (Biotecx, Houston, TX) and quantified. The extracted RNA was used for both RPA and RT/PCR analysis.

Preparation of Riboprobes

cDNA fragments for TGF- β 1 (528 bp) and G3PDH (211 bp) were obtained from porcine mesangial cells RNA by RT/PCR according to the previously described protocol [13]. The cDNA fragments were then cloned into the *Sma*I site of the transcription vector pGEM3Z (Promega, Madison, WI). The TGF- β 1 probe consists of a fragment located near the 5'-terminus of the coding sequence (corresponding to amino acids 32–207). After linearisation of the plasmid, single-stranded RNA probes complementary (anti-sense) or anti-complementary (sense probe, negative control) to cellular mRNA were obtained by run-off transcription using T7 or SP6 polymerase. For ISH the probes were labelled by digoxigenin uridine triphosphate using the DIG RNA labelling kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions; for RPA, [α -³²P]UTP (800 Ci/mM; Hartmann, Braunschweig, Germany) and the MAXIscript kit (Ambion, Heidelberg, Germany) were used.

RPA (Ribonuclease Protection Assay)

RPA was performed with the RPA II kit (Ambion, Heidelberg, Germany) according to the manufacturer's protocol. For each protection assay, 10 μ g total RNA was hybridised at 45°C overnight with the riboprobes for TGF- β 1 and G3PDH in the hybridisation buffer (80% deionized formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, and 1 mM EDTA) and then digested for 40 min at 37°C with a 1:200 dilution of the Ribonuclease A/T1 mixture (250 U/ml ribonuclease A and 10,000 U/ml ribonuclease T1). Protected RNA fragments were separated on denaturing 5% polyacrylamide gels and detected by autoradiography. Densitometric analysis was performed, and the relative abundance of TGF- β 1 mRNA was expressed as the ratio between OD of TGF- β 1 and G3PDH fragments.

RT (Reverse Transcription)

A 200-ng amount of total RNA was reverse transcribed in a final volume of 20 μ l, using 2.5 U of Moloney murine leu-

kemia virus reverse transcriptase (Perkin-Elmer, Branchburg, NJ) in a buffer containing 5 mM MgCl₂, 2.5 mM random hexamers, 1 U RNase inhibitor, 1 mM dNTP. The reaction was carried out for 30 min at 42°C, 5 min at 99°C in a Thermalcycler (MJ Research). cDNA synthesis was performed for all samples at the same time, and then they were stored at -20°C before PCR amplification.

Although the primers for PCR have been designed to span one or more introns within the genes, control negative reactions without RT were performed during the cDNA synthesis step and were included in the PCR step in order to exclude genomic contamination and simultaneously to control the possible cross-contamination during the PCR.

PCR (Polymerase Chain Reaction)

To minimise the inter- and intra-assay variations between samples, we used a master mix for RT and PCR reactions, containing all components except the template.

To increase the specificity and the efficiency of the PCR reaction, we applied the "hot start" procedure by the use of a Taq specific antibody (Clontech, Palo Alto, CA). 4 μ l of each cDNA were amplified in a total volume of 50 μ l containing 1.5 mM MgCl₂, 0.2 mM dNTP, 2 U Taq polymerase (Perkin-Elmer) from a freshly prepared 28:1 mixture of Taq antibody + Taq polymerase, 0.4 μ M primers specific for TGF- β 1 (162 bp F, GCCCTGGACACCAACTATTGCT; R, AGGCTCCAAATGTAGGGGCAGG) and G3PDH (983 bp F, TGAAGGTCTGGAGTCAACGGATTTGGT; R, CATGTGGGCCA-TGAGGTCCACCCAC) (Clontech). The amplification profile was: an initial denaturation at 94°C for 5 min, followed by 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min.

For each sample, TGF- β 1 and G3PDH cDNA were amplified in different tubes.

Comparative Kinetic RT/PCR Analysis

To quantify mRNAs, we have chosen the comparative RT/PCR strategy that, by employing the housekeeping gene G3PDH as an internal standard, allows determination of relative levels of specific mRNAs by comparing the ratio of PCR products generated from target and standard templates. The kinetic analysis of amplified products, ensuring that signals are derived only from exponential phase of amplifications was determined for each sample proceeding as follows: after the first 23 cycles of amplification, an aliquot of 5 μ l was drawn for electrophoresis and then the tubes were submitted to 1 more cycle of PCR. This procedure was repeated until reaching a total of 30 cycles.

PCR products from each cycle point were separated by polyacrylamide gel electrophoresis (PAGE) in 7% polyacrylamide gel 3% C with 5% glycerol at 150 V in 1 $\times$ TBE buffer for 1.30 hr and then visualised by ethidium bromide staining. Moreover, gels were silver stained according to the following protocol: 10% ethanol for 5 min, 1% HNO₃ for 3 min,

rinse in distilled water, 12 mM AgNO₃ for 20 min, rinse in distilled water, 280 mM Na₂CO₃, and 0.019% formaldehyde until the desired staining is reached and finally 10% CH₃COOH for 2 min.

The quantitation of PCR products was performed by direct densitometric analysis of silver-stained bands using Gel-Pro Analyser software (Media Cybernetics, Silver Springs, MD) and the quantity of TGF- β 1 mRNA was expressed as the ratio between optical density (IOD) generated by PCR products of TGF- β 1 and G3PDH genes.

Nonradioactive ISH (In Situ Hybridisation)

Mesangial cells grown in the different conditions (N9, T9, and TH9) on the 8-well chamber slides were rinsed with PBS containing 1 mM CaCl₂, 1 mM MgCl₂, incubated in 4% paraformaldehyde in ribonuclease-free PBS for 15 min at room temperature and fixed in ethanol/glacial acetic acid (95:5) at -20°C for 5 min. The cells were rehydrated through alcohol series, and the slides were washed with 300 μ g/ml heparin (Thrombophob-2500 heparin-sodium (5000 U/ml), Nordmark, FRG) in RNase-free PBS in order to neutralize the positive charges and to reduce the background during the last steps of ISH signal development. After being rinsed in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA (5 $\times$ TE) the slides were treated with 5 μ g/ml proteinase K in 1 $\times$ TE for 15 min at 37°C. The cells were rinsed with PBS, 0.2% glycine, pH 7.4, for 10 min to inhibit the enzymatic activity of proteinase K and in PBS for 5 min at room temperature. Prehybridisation was performed in the hybridisation buffer for 3 hr at 49°C. The hybridisation buffer contained 50% deionized formamide, 5 $\times$ SSC, 5% dextrane sulphate, 1% Denhardt's solution, 0.5 mg/ml yeast tRNA, 0.2 mg/ml herring sperm DNA, and 11 mM dithiothreitol, pH 7. After dehydration, the prehybridisation solution was replaced by a previously tested amount (1 μ g/ml) of digoxigenin-labeled RNA-antisense TGF- β 1 probe (or digoxigenin-labeled RNA-sense probe for control) in a 100 μ l volume of hybridisation buffer; it was linearised at 95°C for 5 min and then put in ice. Chamber-slides were sealed with a siliconised coverslip and hybridised overnight at 49°C in a humid chamber. The next morning the slides were washed with 5 $\times$ SSC at 49°C for 15 min and once with 2 $\times$ SSC at 42°C for 15 min, and they were then rinsed in 1 $\times$ SSC (15 min), 0.5 $\times$ SSC (15 min) at 37°C, and 0.5 $\times$ SSC (15 min) at room temperature. The cells were then equilibrated in PBST (PBS, 0.1% Tween 20) for 5 min at room temperature and incubated in PBST, 1% BSA containing 2% normal serum (30 min at room temperature) for blocking of nonspecific binding. This was followed by incubation with 1 nm gold antidigoxigenin-Ab complexes in PBST, 0.5% bovine serum albumin for 30 min at room temperature. Three washings in PBST for 5 min each and six washing steps with bidistilled water were followed by incubation with freshly prepared Silver Enhancing Kit (Cameron, Wiesbaden, Germany), developer/enhancer at a ratio of 1:1, for 10 min at

room temperature. The colour reaction was stopped by placing the chamber slides in bidistilled water for 3 min, with six changes, at room temperature, and then the slides counterstained with hematoxylin and mounted; they were observed under light microscopy at 100× and 400× magnification and photographed.

Hybridisation controls were performed as follows: (a) with sense TGF- β 1 probe; (b) with hybridisation buffer without probe.

To obtain quantitative data the morphometric analysis of ISH slides was performed with Image-ProPlus software (Media Cybernetics, Silver Springs, MD). For each condition five fields were randomly selected, the total cells per field were analysed, and the ISH signal was quantified as the integral optical density (IOD). The mean and the standard deviation (SD) were calculated.

Statistical Analysis

RT/PCR and RPA were performed independently at least twice starting from the same RNA. Linear regression analysis was used to estimate the correlation between two different RT/PCR in order to verify the reproducibility of RT/PCR and between RPA and RT/PCR in order to evaluate the reliability of RT/PCR as a quantitative method. For ISH experiments mean and SD were calculated. Comparisons between each experimental condition (N, T, TH) were performed with Student's test. Statistically significant differences were defined as P values <0.05 and are indicated in the legends to figures.

RESULTS

TGF- β 1 mRNA Level by Comparative Kinetic RT/PCR

To identify the exponential phase of the PCR reaction we performed a kinetic analysis of amplification for TGF- β 1 and G3PDH cDNA. The exponential phase for G3PDH amplification (Fig. 1a) in all three experimental conditions was located between the 23rd and the 25th cycles; to quantify the PCR products the 24th cycle of amplification was chosen. The exponential phase for TGF- β 1 amplification was located between the 27th and the 29th cycles, and the amplification product was quantified at the 28th cycle (Fig. 1b). Two different RT reactions were performed starting from the same RNA samples and from each RT; both G3PDH and TGF- β 1 cDNAs were amplified, and the amplification products were collected and quantified at 24th and 28th cycles, respectively. The reproducibility of RT/PCR amplification was evaluated by linear regression analysis, comparing the quantity of G3PDH and TGF- β 1 PCR products generated during cycles 23–26 and 27–30, respectively, by the two RT/PCR (RT/PCR 1 and RT/PCR 2) in each experimental condition (Fig. 2a,b). There was a strong correlation between the two different RT/PCR (G3PDH, $r = 0.968$, $P = 0.0000$; TGF- β 1, $r = 0.966$, $P = 0.0000$).

The amount of TGF- β 1 mRNA was expressed as the ratio

Fig. 1. Kinetics of G3PDH (a) and TGF- β 1 (b) amplification. Control (N), PMA-treated (T), and PMA+heparin-treated cells (TH): cDNA underwent sequential cycles of amplification and PCR products were visualised by PAGE and analysed by densitometry.

between IOD generated by PCR product of TGF- β 1 and G3PDH genes. The mean of relative TGF- β 1 levels from the two RT/PCRs were calculated and compared. PMA stimulation induced a 1.66-fold increase of TGF- β 1 mRNA expression compared to basal level; furthermore, heparin treatment normalised the TGF- β 1 expression (Fig. 3a).

TGF- β 1 mRNA Level by RPA

Mesangial cells were grown in control conditions or were treated with PMA and PMA+heparin for 9 hr; this incubation time was chosen because previous time course experiments at 6, 9, and 12 hr indicated that the 9-hr incubation was the best time to show the PMA-dependent TGF- β 1 overexpression. The means of the two experiments were calculated and compared.

Using RPA we have observed a 1.54-fold PMA-dependent TGF- β 1 mRNA over-expression. The addition of 10 μ g/ml heparin prevented the TGF- β 1 upregulation, being the level of TGF- β 1 mRNA in the normal range (Fig. 3b).

Comparison Between RT/PCR and RPA Results

In order to demonstrate that the comparative kinetic RT/PCR is a good strategy to have quantitative information, we compared by linear regression analysis the results obtained

Fig. 2. Correlation of G3PDH (a) and TGF- β 1 (b) results from two different RT/PCR experiments. IOD of G3PDH and TGF- β 1 PCR products, generated during 23–26 cycles and 27–30 cycles, respectively, are compared.

with the two comparative kinetic RT/PCR with those obtained with the two RPA. Significant correlation was observed between the standard methods and RT/PCR ($r = 0.984$, $P = 0.0004$) (Fig. 4).

TGF- β 1 mRNA Level by ISH

The effects of different culture conditions on TGF- β 1 gene expression were also confirmed by ISH. By visual inspection it was clear that in control mesangial cells the signal for TGF- β 1 mRNA, principally located in the perinuclear area, was above the sense control and lower than that observed in PMA-treated cells (Fig. 5a). However PMA-treated cells showed a nonhomogeneous signal intensity: about 60% of cells had a high signal, while lower, variable levels were present in the others, but were still above the control signal (Fig. 6). The TGF- β 1 over-expression was prevented by addition of heparin; indeed, the signal of PMA+heparin-treated cells strictly resembled that one of cells grown in normal conditions (Fig. 5a).

The semi-quantitative estimation of the grains deposition

Fig. 3. Relative level of TGF- β 1 in control (N), in PMA (T), and PMA (0.1 μ M) + heparin (10 μ g/ml) (TH)-treated cells as evaluated by comparative kinetic RT/PCR (a) and RPA (b). Results are expressed as the mean of two experiments.

was performed by morphometric analysis and confirmed these observations. PMA-treated cells expressed a 4.39-fold increase of TGF- β 1 mRNA levels compared to control cells (N vs. T, $P = 0.0023$) (Fig. 5b). The high standard deviation that we obtained averaging the IOD of PMA-treated cells confirmed

Fig. 4. Linear regression analysis of TGF- β 1 expression as determined by comparative kinetic RT/PCR versus RPA. Data, expressed as the ratio between IOD TGF- β 1 and G3PDH, were obtained from two comparative RT/PCR and two RPA.

Fig. 5. (a) In situ localisation of TGF- β 1 mRNA expression. (N) Hybridisation of normal control porcine mesangial cells with antisense TGF- β 1 riboprobe shows few, but significant cytoplasmic signals; (T) PMA-treated cells exhibited enhanced TGF- β 1 mRNA expression; (TH) PMA+heparin-

treated cells show TGF- β 1 mRNA expression similar to control; (S) control cells hybridised with the sense riboprobe (original magnification $\times 400$). (b) Evaluation of the in situ hybridisation data (*N vs. T, $P = 0.0023$; **T vs. TH, $P = 0.00012$).

the high variability of the cellular signal, which ranged from 3.28- to 5.57-fold over the control level. Heparin treatment prevented TGF- β 1 mRNA induction, normalising mRNA level to control (N vs. TH, $P = 0.524$) (Fig. 5b).

DISCUSSION

While RT/PCR is an extremely sensitive method of mRNA analysis, obtaining quantitative information with this technique can be difficult. The exponential nature of the PCR and the practical aspects of performing PCR pose the most serious obstacles to obtaining quantitative information. With some adaptations, however, RT/PCR can yield quantitative results.

Comparative kinetic RT/PCR can be used to estimate the relative amount of target mRNA in two or more samples. This can only be achieved when the amplification efficiencies are the same between samples and the data are collected before the reaction begins to reach the plateau phase. Moreover, in the attempt to correct tube-to-tube variation in amplification efficiency, an endogenous sequence that is normally present in samples should be used as an internal standard. G3PDH, which we decided to utilise in the study, allowed us not only to normalise target mRNA levels be-

tween samples to be compared but also to verify indirectly the RNA integrity (12).

To prove the accuracy of our RT/PCR strategy, the level of TGF- β 1 mRNA in mesangial cells grown under control conditions, PMA, or PMA+heparin were evaluated and compared to the results obtained by classical standard methods of mRNA quantitation, such as RPA.

Unlike the RPA method, obtaining quantitative data by RT/PCR requires the assessment that amplification efficiency is equal for each gene in each sample. Plotting PCR product IOD versus cycle numbers allowed us to determine that the amplification efficiency of both G3PDH and TGF- β 1 in each experimental condition was the same (Fig. 1a,b). Moreover, kinetic studies allowed us to recognise the exponential phase of reaction, within which it is necessary to work to obtain quantitative data.

The reproducibility of RT cannot be measured directly, although an estimate of the combined variability of the RT/PCR procedure can be obtained by comparing the RT/PCR results derived from two separate cDNA syntheses from a single RNA sample. The strong direct correlation we found between RT/PCR 1 and RT/PCR 2 in all experimental condi-

Fig. 6. Different degree of ISH signal intensity in PMA-treated porcine mesangial cells (original magnification $\times 100$).

tions demonstrated the good reproducibility of the method (Fig. 2a,b).

The quantitative capability of the RT/PCR strategy we adopted was verified by comparing the data of TGF- β 1 mRNA expression obtained with the RPA to the data obtained analysing the same RNA by comparative kinetic RT/PCR (Fig. 4). There was a strong correlation ($r = 0.984$, $P = 0.0004$), indicating that RT/PCR can give reliable quantitative data. Although they originated from quite different quantity of total RNA (200 ng for RT/PCR vs. 10 μ g for RPA) and although the two methods were completely different ways of processing RNA (amplification versus hybridisation), the levels of TGF- β 1 mRNA measured by the two methods in the three experimental conditions were very similar.

ISH that is normally performed to obtain qualitative information on gene expression was also used for semi-quantitative evaluation. This approach confirms the results obtained by RT/PCR and RPA, i.e., that PMA stimulation upregulated the TGF- β 1 expression and that heparin lowered its over-expression, but it has also given us two further pieces of information: (a) the PMA effect on mesangial cells is not homogeneous and is probably related to the cells' proliferative state (asynchronous cultures) (Fig. 6); (b) in every cell the heparin effect decreases the TGF- β 1 mRNA level, but it never goes below the basal level of expression.

The information given by ISH on the behaviour of single cells under a particular stimulus are unique and relevant also for its application in *in vivo* studies (14).

The highest level of PMA-induced TGF- β 1 expression estimated by the semi-quantitative analysis of IHS signal could be attributed to (a) the different cellular response to PMA-

stimulation in IHS experiment which was run in parallel but independently from RT/PCR and RPA experiments; (b) the different substances used for coating chamber slides and dishes (collagen and gelatin respectively) that could have conditioned the cellular growth behaviours; (c) the signal development system of ISH, since during silver enhancer reaction, the spontaneous self-nucleation of silver precipitates might increase in positive samples.

In conclusion, the results we obtained demonstrate the close similarity of RT/PCR and RPA methods in giving quantitative information on mRNA expression and the possibility to adopt our comparative kinetic RT/PCR as a reliable quantitative method of mRNA analysis. The method does not require preparation of competitive templates and is accurate and reproducible.

REFERENCES

1. Wang T, Brown M J. mRNA quantification by real time Taq Man polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem* 1999;269:198–201.
2. Iwano M, Akai Y, Fujii Y, Dohi Y, Matsamura N, Dohi K. Intraglomerular expression of transforming growth factor-beta 1 (TGF- β 1) mRNA in patients with glomerulonephritis. Quantitative analysis by competitive polymerase chain reaction. *Clin Exp Immunol* 1994;97:309–314.
3. Guiffre A, Atkinson K, Kearney P. A quantitative polymerase chain reaction assay for Interleukin 5 messenger RNA. *Anal Biochem* 1993;212:50–57.
4. Nicoletti A, Sassy-Prigent C. An alternative quantitative polymerase chain reaction method. *Anal Biochem* 1996;236:229–241.
5. Chen Z-P, Malapetsa A, Mohr G, Brien S, Panasci LC. Quantitation of ERCC-2 gene expression in human tumor cell lines by reverse transcription-polymerase chain reaction in comparison to Northern blot analysis. *Anal Biochem* 1997;244:50–54.

6. Halford WP, Falco VC, Gebhardt BM, Carr DJJ. The inherent quantitative capacity of the reverse transcription-polymerase chain reaction. *Anal Biochem* 1999;266:181-191.
7. Gilliland G, Perrin S, Blankard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantification by competitive polymerase chain reaction. *Proc Natl Acad Sci U S A* 1990;87:2725-2729.
8. Ross R, Kleinz R, Reske-Kunz AB. A method for rapid generation of competitive standard molecules for RT/PCR avoiding the problem of competitor/probe cross-reaction. *PCR Methods Appl* 1995;4:371-375.
9. Didier A, Hubert V. The use of the reverse transcription-competitive polymerase chain reaction to investigate the in vivo regulation of gene expression in small tissue samples. *Anal Biochem* 1997;245:141-148.
10. Raeymakers L. Quantitative PCR: theoretical consideration with practical implication. *Anal Biochem* 1993;214:582-585.
11. Ceol M, Gambaro G, Sauer U, Baggio B, Anglani F, Forino M, Facchin S, Bordin L, Weigert C, Nerlich A, Schleicher ED. Glycosaminoglycan therapy prevents TGF- β 1 over-expression and pathological changes in renal tissue of long-term diabetic rats. *J Am Soc Nephrol* 2000;11:2326-2336.
12. Del Prete D, Forino M, Gambaro G, D'Angelo A, Baggio B, Anglani F. A comparative kinetic RT/PCR strategy for the quantitation of mRNAs in microdissected human renal biopsy specimens. *Exp Nephrol* 1998;6:563-567.
13. Kolm V, Sauer U, Nerlich A, Lehmann R, Schleicher ED. High glucose-induced transforming growth factor β 1 production is mediated by the hexosamine pathway in porcine glomerular mesangial cells. *J Clin Invest* 1998;101:160-169.
14. Ceol M, Nerlich A, Baggio B, Anglani F, Sauer U, Schleicher E, Gambaro G. Increased glomerular α 1(IV) collagen expression and deposition in long-term diabetic rats is prevented by chronic glycosaminoglycan treatment. *Lab Invest* 1996;74:484-495.