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Gene expression analysis by real-time reverse transcription polymerase chain reaction: influence of tissue handling

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Abstract

Factors such as warm ischemia and time at room temperature before tissue treatment may influence the results of mRNA expression analyses on tissue specimens obtained during surgery. We evaluated the effect of these factors on RNA integrity and mRNA expression levels by incubating freshly obtained mouse liver tissue at 25 or 37 °C for periods of 0–4 h. Changes in the mRNA expression levels of seven genes, *Tbp*, *Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf*, and *Glut2*, were determined by real-time reverse transcription-polymerase chain reaction. Incubation at 25 °C for up to 4 h only slightly altered (by a factor of less than 2) levels of mRNA for *Tbp*, *Eef1a*, *Junb*, *Myc*, *Vegf*, and *Glut2*. This result is consistent with limited RNA degradation at this temperature. Incubation at 37 °C strongly affected the levels of these mRNAs. Four hours of incubation at this temperature resulted in extensive RNA degradation, with mRNA levels falling to 1/10th those before incubation. When relative quantification was performed, i.e., quantification of the target gene transcripts in comparison to an endogenous housekeeping transcript (*Tbp* or *Eef1a*), the changes in mRNA levels were reduced to less than 2.5-fold. *Fos* behaved very differently from the other genes tested on incubation, with *Fos* mRNA levels increasing considerably following incubation at either 25 or 37 °C. Our data suggest that, with the exception of certain genes induced by tissue injury, relative quantification of mRNA, even on degraded RNA samples, can provide a reliable estimate of in vivo mRNA levels.
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Quantitative determination of gene expression at the mRNA level is a powerful approach to the comparative analysis of normal and pathological states. Various methods including Northern blotting [1], RNase protection assays [2], RT[1]-PCR [3–6], branched DNA signal amplification [7], serial analysis of gene expression [8], and DNA arrays [9,10] are available for quantifying mRNA levels. Real-time RT-PCR measures product accumulation during the log-linear phase of the reaction and is currently one of the most sensitive, accurate, and reproducible methods for mRNA expression quantification [3–6]. The advantages of this method make real-time RT-PCR a method of choice for the validation of differentially expressed genes identified using DNA arrays [11].

Banks of frozen normal and pathological tissues obtained from surgical procedures were first established in the 1990s for molecular analyses including expression studies at the mRNA level [12]. The standard surgical excision and processing of tissue specimens are geared toward patient care and samples set aside for molecular studies are frequently suboptimal with respect to RNA integrity. The warm ischemia time in vivo before the surgical removal of the specimen and the time ex vivo at room temperature prior to freezing of the specimen or its treatment with an RNA-preserving solution are variable and this may have significant consequences with regard to the mRNA levels observed.

To evaluate the effects of warm ischemia and time delays at room temperature on RNA integrity and mRNA expression levels, we incubated freshly obtained mouse liver tissue at 25 or 37 °C for periods of 0–240 min before homogenization in guanidine isothiocyanate. We used animal tissue so that we could control incubation temperature and the delay before RNA extraction. As liver is a fairly homogenous tissue, it was possible to compare changes in mRNA expression levels and RNA stability in various fragments of liver tissue exposed to various temperatures for various times. We assessed

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[1] *Abbreviations used:* RT, reverse transcription.

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RNA degradation by following the decrease in 18S and 28S rRNA species and the appearance of degradation products by microcapillary gel electrophoresis [13]. The effects of time and incubation temperature on the mRNA levels of seven genes were measured by real-time RT-PCR. The genes included in this study were three immediate early genes (*Fos*, *Junb*, and *Myc*) [14–16], one hypoxia-induced gene (*Vegf*) [17,18], two housekeeping genes (*Tbp*, *Eef1a*) [19–21], and one tissue-specific gene (*Glut2*) [22].

Materials and methods

Tissue samples

Liver tissue was excised from 8-week-old female C57Bl/6 mice (Iffa Credo, L'Arbresle, France) and immediately sectioned into pieces approximately 20 mm^3 in volume. These samples were individually placed in 14-ml tubes (Falcon 352059) and incubated at 25 or 37 °C for various times (0, 5, 10, 20, 30, 40, 60, 80, 120, 180, or 240 min at 25 and 0, 3, 6, 10, 15, 20, 30, 45, 75, 120, 180, or 240 min at 37 °C). Two independent experiments were performed.

Total RNA extraction

After incubation, each liver sample was immediately homogenized in 2 ml Trizol (Invitrogen, Cergy Pontoise, France) with an Ultraturrax T25 homogenizer (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany) and processed for RNA isolation following the manufacturer's recommendations. Total RNA concentration was determined by measuring absorbance at 260 nm.

Assessment of total RNA degradation

RNA degradation was assessed by microcapillary gel electrophoresis with fluorescent detection (Agilent Technologies, Wilmington, DE). We analyzed 250 ng of total RNA from the various liver samples on an Agilent 2100 Bioanalyzer, using an RNA 6000 LabChip kit, following the manufacturer's instructions. Total RNA degradation was evaluated from decreases in 28S and 18S rRNA peak areas and the appearance of peaks corresponding to degradation fragments. The results are displayed as gel-like images and electropherograms.

Reverse transcription

Reverse transcription was performed as described by Kandel et al. [23], with slight modifications [24]. RNA samples (1 μg) were incubated with 200 ng of random hexamers (Roche Diagnostics, Meylan, France) at 65 °C for 5 min in a total volume of 10 μl. The solution was then placed on ice and mixed with 100 U of RNase inhibitor (Amersham–Pharmacia Biotech, Orsay, France), RT buffer (final concentrations: 67 mM Tris, pH 8, 16.6 mM $(NH_2)_2SO_4$, 6.7 mM $MgCl_2$), 1.25 mM each dNTP (Amersham–Pharmacia Biotech), and 200 U of MMLV reverse transcriptase (Invitrogen) in a total final volume of 20 μl. The samples were incubated for 1 h at 42 °C, and the reaction was stopped by heating at 72 °C for 5 min. For each sample, a reaction without reverse transcriptase was performed. A negative control without RNA was included in each series of reverse transcription reactions.

Real-time PCR

The PCRs were performed in an ABI PRISM 7700 real-time thermal cycler using the SYBR Green kit (Applied Biosystems, Foster City, CA). The primer sequences for the seven genes studied are given in Table 1. Primers were obtained from Eurogentec (Seraing, Belgium) or Genset (Paris, France). PCR was performed in duplicate in a final volume of 25 μl containing 1/20 of the cDNA sample, 4 mM $MgCl_2$ (3 mM $MgCl_2$ for *Junb*), 200 nM each oligonucleotide primer, 200 μM dATP,

Table 1
Sequences of primers used in this study

Gene	Primer sequence		Amplicon (bp)	Accession No.
Tbp	Forward	5′-ATGGTGTGCACAGGAGCCAAG-3′	197	NM_013684
	Reverse	5′-TCATAGCTACTGAACTGCTG-3′		
Eef1a	Forward	5′-CTGGAGCCAAGTGCTAATATGCC-3′	219	X13661
	Reverse	5′-GCCAGGCTTGAGAACACCAGTC-3′		
Fos	Forward	5′-GGGACAGCCTTTCCTACTAC-3′	115	NM_010234
	Reverse	5′-GGGATAAAGTTGGCACTAGAG-3′		
Junb	Forward	5′-GCCGCTTGGTACTCAGCCT-3′	81	NM_008416
	Reverse	5′-TCTCCACAGTACTATGCAGAGGG-3′		
Myc	Forward	5′-TAGTGCTGCATGAGGAGACA-3′	104	NM_010849
	Reverse	5′-GGTTTGCCTCTTCTCCACAG-3′		
Vegf	Forward	5′-GTACCTCCACCATGCCAAGT-3′	105	NM_009505
	Reverse	5′-CAGTAGCTTCGCTGGTAGAC-3′		
Glut2	Forward	5′-CAGTTCGGCTATGACATCGGT-3′	109	NM_031197
	Reverse	5′-GTTAATGGCAGCTTTCCGGTC-3′		

dCTP, dGTP, 400 μM dUTP, 1× SYBR Green PCR buffer, and 0.625 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Samples were heated for 10 min at 95 °C and then subjected to 45 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 1 min. A negative control without cDNA template was included in every assay. All PCR products were checked by melting point analysis, using the GeneAmp Detection system (Applied Biosystems). The PCR efficiencies for all genes were determined as previously described [25] and were found to be 100% for *Tbp*, 80% for *Eef1a*, 92% for *Fos*, 100% for *Junb*, 92% for *Myc*, 100% for *Vegf*, and 97% for *Glut2*; the PCR efficiencies were not influenced by the integrity of the RNA (data not shown). For each gene (G), the mRNA level after a given incubation (*n* minutes) was normalized with respect to that of the nonincubated sample, according to the equation

$$(1 + \mathrm{E})^{\mathrm{C_T,G_0} - \mathrm{C_T,G_n}},$$

where $\mathrm{C_T,G_0}$ is the threshold cycle of the nonincubated sample, $\mathrm{C_T,G_n}$ is the threshold cycle of the sample incubated for *n* minutes, and E is the efficiency of the PCR for the gene G. For comparisons of mRNA levels for the target gene (G) and the reference gene (R) (relative expression), the equation of Pfaffl [26] was used,

$$(1 + \mathrm{E_G})^{(\mathrm{C_T,G_0} - \mathrm{C_T,G_n})} / (1 + \mathrm{E_R})^{(\mathrm{C_T,R_0} - \mathrm{C_T,R_n})},$$

where $\mathrm{C_T,G_0}$ and $\mathrm{C_T,R_0}$ are the threshold cycles of the nonincubated sample for genes G and R, respectively, $\mathrm{C_T,G_n}$ and $\mathrm{C_T,R_n}$ are the threshold cycles of the sample incubated for *n* minutes for genes G and R, respectively, and $\mathrm{E_G}$ and $\mathrm{E_R}$ are the efficiencies for genes G and R, respectively.

Results

Degradation of ribosomal RNA as a function of incubation temperature and time

The effect of incubating mouse liver at 25 or 37 °C on the integrity of the RNA was assessed by microcapillary gel electrophoresis. Two representations of RNA analysis were used: gel-like images (Figs. 1A and B) and electropherograms (Figs. 1C and D). For reasons of clarity, only representative electropherograms are shown. Three main RNA bands or peaks corresponding to the 28S, 18S, and 5S rRNA species were seen. Degradation products were observed in both representations after 120 min of tissue incubation at 25 °C and after 75 min at 37 °C. The degradation products were the most prevalent species in the samples incubated for more than 120 min at 37 °C.

We used the electropherogram representation to calculate the amounts of 28S and 18S rRNA and the ratio between these two rRNA species as a function of incubation time (Fig. 2). At 25 °C, the 18S rRNA was stable for up to 4 h, whereas progressive degradation of the 28S rRNA was observed, with about 50% of this species degraded after 4 h of incubation (Fig. 2A). Therefore, at 25 °C, the decrease in 28S/18S ratio paralleled the decrease in 28S rRNA levels (Fig. 2B). At 37 °C, the 28S rRNA was progressively degraded and had almost disappeared after 3 h of incubation. In contrast to what was observed at 25 °C, 18S rRNA was almost completely degraded after 4 h of incubation at 37 °C (Fig. 2A). The 28S/18S ratio decreased much more quickly at 37 °C than at 25 °C, with a 50% decrease observed after 1.5 h of incubation (Fig. 2B).

Effect of incubation temperature and time on levels of various mRNAs, as measured by real-time RT-PCR

We assessed changes in mRNA expression levels for seven genes (*Tbp*, *Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf*, and *Glut2*) during the incubation of liver samples for up to 4 h at 25 or 37 °C. The mRNA expression levels of each of these genes were measured by real-time RT-PCR, using a fixed amount of reverse-transcribed RNA (50 ng). For each gene, the mRNA levels measured at each incubation stage were compared with those for the nonincubated samples. At 25 °C (Fig. 3A), mRNA levels for *Tbp*, *Eef1a*, *Junb*, *Myc*, *Vegf*, and *Glut2* were stable for 4 h (variation by no more than a factor of two). In contrast, the levels of mRNA for these genes decreased over time at 37 °C (Fig. 3B), by a factor of 3.3–12.5 after 4 h of incubation. *Fos* behaved very differently from the other mRNAs. At 25 °C, *Fos* mRNA levels increased by a factor of 10 after 3 h of incubation. At 37 °C, they increased by a factor of 2.6 within 30 min and then returned to initial values.

Levels of *Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf*, and *Glut2* mRNAs were then expressed with respect to those for a ubiquitous transcription factor, *Tbp* (Fig. 4). At 25 °C, the results obtained by this method of relative quantification were similar to those obtained without considering reference gene expression (compare Fig. 4A with Fig. 3A). Relative expression, comparing each of the *EeF1a*, *Junb*, *Myc*, *Vegf*, and *Glut2* mRNA species with *Tbp* mRNA, varied by a factor of less than 1.6-fold during the first 4 h of incubation, whereas relative *Fos* mRNA levels increased by a factor of 12 after 3 h of incubation. At 37 °C, the relative levels of *Eef1a*, *Junb*, *Myc*, *Vegf*, and *Glut2* mRNAs showed much smaller variations as a function of incubation time than did the values obtained without considering expression of the reference gene (differing by a factor of less than 2.5, rather than a factor of between 3.3 and 12.5; compare Fig. 4B with Fig. 3B). Thus, the relative levels of *Eef1a* and *Glut2* mRNAs were stable, those of *Junb* and *Vegf* increased by factors of 2.1 and 2.4, respectively, and the relative level of *Myc* mRNA decreased by a factor of 1.8. With *Fos*, an initial increase by a factor of 2.6 was observed with

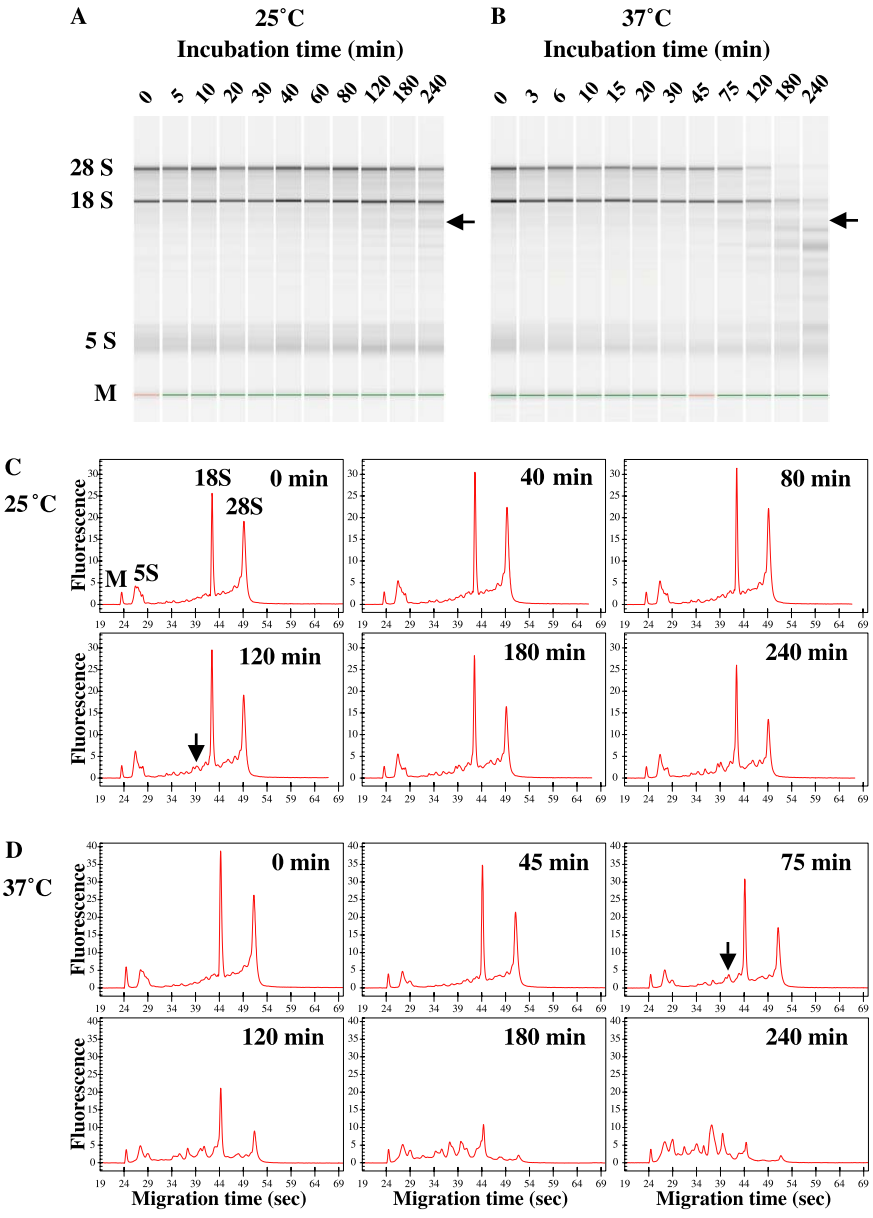

Fig. 1. Analysis of total RNA by capillary gel electrophoresis as a function of incubation temperature and time. Total RNA was isolated from mouse liver samples incubated for various times (0–240 min) at 25 or 37 °C. Aliquots of 250 ng of total RNA were analyzed on the Agilent RNA LabChip. The results are displayed as gel-like images (A and B for incubation at 25 and 37 °C, respectively) and as electropherograms (C and D for incubation at 25 and 37 °C, respectively). 28S, 18S, and 5S ribosomal species and marker (M) are indicated on gel-like images and on electropherograms. The earliest degradation products detected are indicated by arrows.

relative quantification, which was similar to that observed if reference gene expression was not taken into account. At 4 h, relative *Fos* mRNA level had increased by a factor of nine (compare Fig. 4B with Fig. 3B).

Discussion

Transcriptome analysis of normal and pathological tissues will improve our understanding of the molecular events underlying disease processes. It will also identify new diagnostic, prognostic, and therapeutic targets. mRNA analysis has been boosted by rapid progress in the Human Genome Project and by the development of new technologies such as DNA array analysis and laser microdissection [9,10,27,28]. However, as the tissues used in RNA analysis are generally derived from surgical procedures, the results obtained in gene expression analyses may be affected by the various steps of tissue handling: warm ischemia, which occur during surgery, and time delays at room temperature before tissue treatment. In this work, we evaluated the effects of these two factors, in mouse liver tissue, on RNA integrity and mRNA expression levels, as measured by real-time RT-PCR.

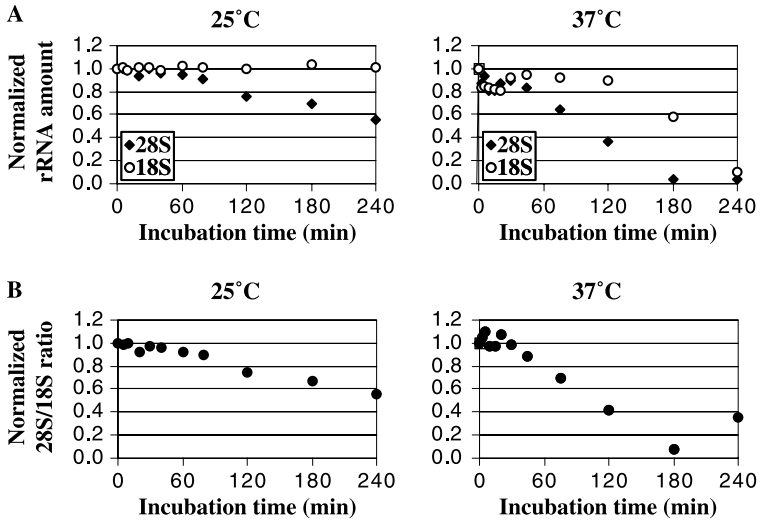

Fig. 2. Changes in 28S and 18S rRNA levels and in the 28S/18S ratio as a function of incubation temperature and time. The signal intensities of the 28S and 18S rRNA species corresponding to the indicated incubation temperature and time were quantified using Agilent Technologies 2100 Bioanalyzer software. Two representations of the results are shown: amounts of 28S and 18S rRNA (A) and the 28S/18S ratio (B) as a function of time. The results are normalized with respect to the values obtained for the nonincubated samples.

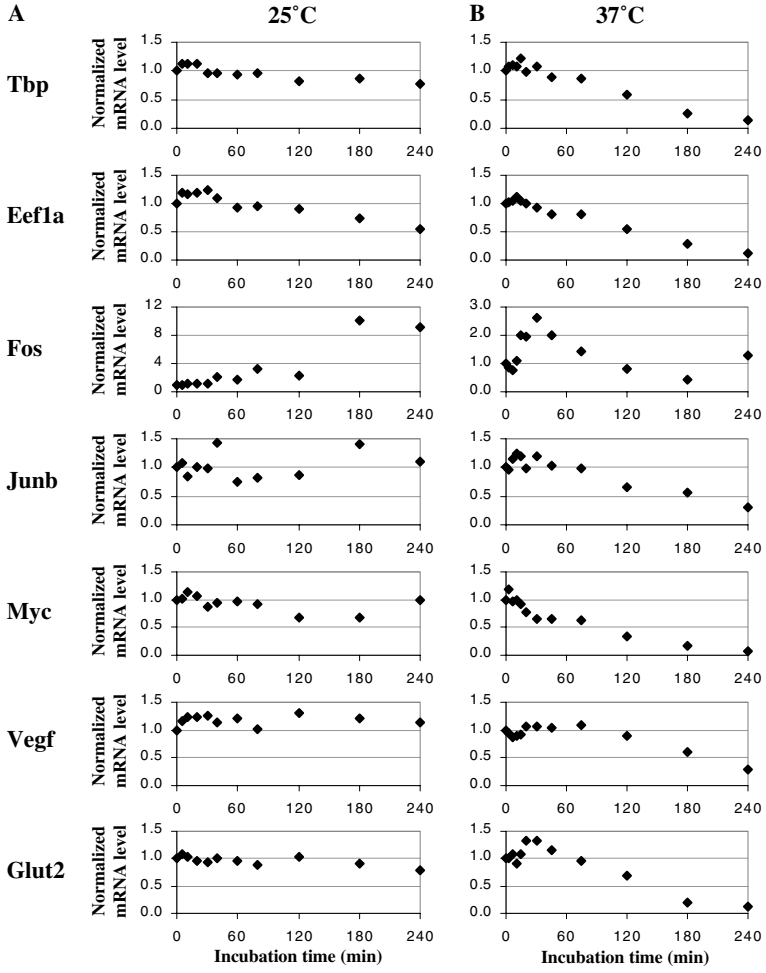

Fig. 3. Quantification of gene expression by real-time RT-PCR as a function of incubation temperature and time. The mRNA levels for *Tbp*, *Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf*, and *Glut2* were quantified by real-time RT-PCR in liver samples incubated for various times (0–240 min) at 25 °C (A) or 37 °C (B). The mRNA levels of the various target genes were measured using a fixed amount of total RNA (50 ng). The results are normalized with respect to the values obtained for nonincubated samples.

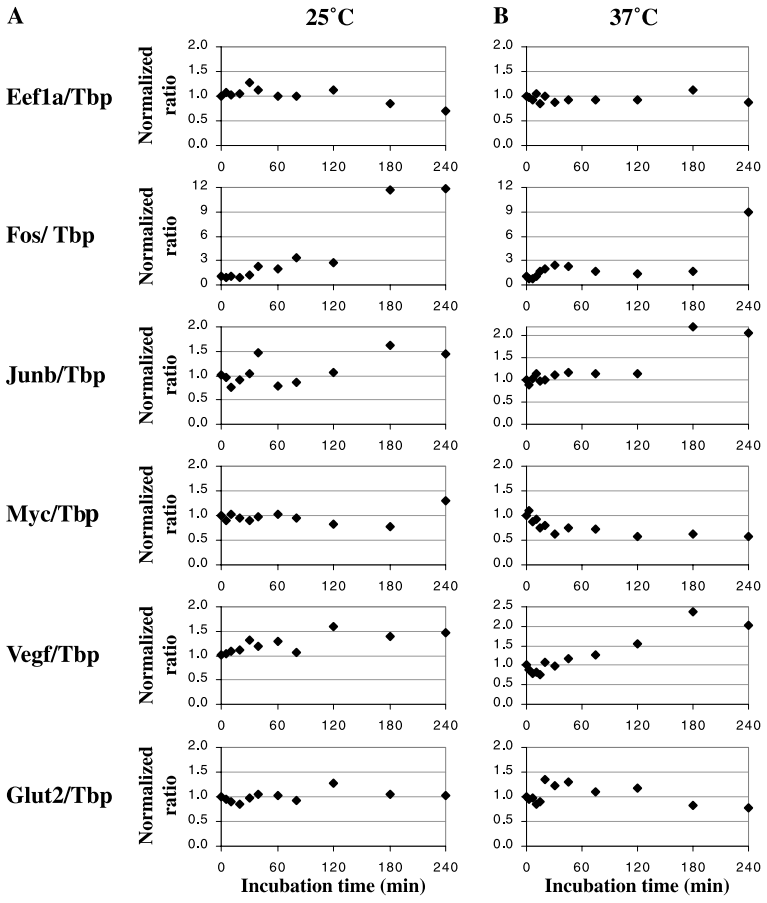

Fig. 4. Relative quantification of gene expression by real-time RT-PCR as a function of incubation temperature and time. The mRNA levels for *Eef1a*, *Fos*, *Junb*, *Myc*, *Vegf*, and *Glut2*, as quantified by real-time RT-PCR, were compared to those for a reference gene *Tbp*. The results for liver samples incubated for various times (0–240 min) at 25 °C (A) or 37 °C (B) are shown. The results are normalized with respect to the values obtained in nonincubated samples.

Working with animal tissues made it possible to control ischemia time precisely, whereas this is not the case for human tissues obtained surgically [29,30].

At 25 °C, RNA degradation was limited, affecting the 28S rRNA species but not the 18S rRNA species. At 37 °C, both the 18S and the 28S rRNA species were affected, being almost completely degraded after 4 h of incubation. The 28S rRNA was degraded more rapidly than the 18S rRNA. The lower stability of the 28S rRNA species has been reported before [31]. We assessed changes in mRNA expression levels for seven genes (*Fos*, *Junb*, *Myc*, *Vegf*, *Eef1a*, *Tbp*, and *Glut2*). These seven genes were selected because their expression levels may be differentially affected by tissue handling under nonphysiological conditions. Incubation at 25 °C for up to 4 h did not significantly alter (factor of less than 2) the mRNA levels for six (*Junb*, *Myc*, *Vegf*, *Tbp*, *Eef1a*, and *Glut2*) of the seven genes tested. This finding is consistent with the limited RNA degradation that occurs at this temperature and with the recent results of Dash et al. [30], who showed, using microarrays, that the expression of less than 0.6% of the more than 9000 genes tested was affected by the incubation of prostate tissue for 1 h at room temperature.

At 37 °C, the levels of *Junb*, *Myc*, *Vegf*, *Tbp*, *Eef1a*, and *Glut2* mRNAs were substantially affected by incubation. After 4 h of incubation, RNA was extensively degraded and a decrease in mRNA levels by a factor of up to 10 was observed. If target gene transcripts were quantified with respect to transcripts of an endogenous housekeeping gene (*Tbp*), the relative mRNA levels of incubated samples and nonincubated samples were found to differ by a factor of less than 2.5. Very similar results were obtained when another housekeeping gene, *Eef1a*, was used instead of *Tbp* as the reference gene (data not shown). The stability of relative mRNA levels as a function of incubation time suggested that the various mRNAs were degraded at similar rates at 37 °C, although there were differences between genes: the relative mRNA levels for *Eef1a* and *Glut2* varied less (by a factor of less than 1.3) than did the relative mRNA levels for *Junb* and *Vegf* (doubling) and *Myc* (decrease by a

factor of two). These differences may be accounted for by *Junb* being an immediate early gene, *Vegf* being a hypoxia-inducible gene, and Myc mRNA being particularly unstable [15,17,18,32]. Our results are consistent with those of Castensson et al. [33], who showed that relative mRNA levels in human brain samples were similar for tissues obtained by biopsy and autopsy. These authors did not assess RNA degradation in their study. Performing RNA analysis using DNA arrays and degraded RNAs would probably result in much larger errors than are observed with RT-PCR as, in most cases, the DNA array method involves a labeling reaction beginning at the 3′ poly(A) tail and subsequent hybridization to a probe that may bind at some distance from this 3′ end. In the real-time PCR technique used here, reverse transcription was performed with random hexamers and the PCR amplicons were short (less than 200 bp), minimizing the effect of RNA degradation. It should be noted that, in addition to the possibility of quantifying mRNA levels for samples containing degraded RNA, relative quantification based on a reference transcript has several other advantages: it can compensate for RT efficiency and cDNA sample loading variations (this is particularly important for microdissected samples, in which the amount of RNA is often too small to be measured). 18S and 28S rRNA are sometimes used as internal standards [34]. We found that the difference between mRNA levels in incubated samples and nonincubated samples was always greater if 18S or 28S was used as an internal control than if *Tbp* or *Eef1a* was used (data not shown). This is probably due to differences in the sensitivities of rRNA and mRNA to degradation.

Fos mRNA behaved differently from the other mRNAs; its levels increasing following incubation at both 25 and 37 °C. This increase was already observed when RNA degradation was very limited (increases by a factor of 12 after 3 h at 25 °C and by a factor of 3 after 30 min at 37 °C). Perou et al. (unpublished data cited in [35]) using an array of 8102 genes, found that a cluster of genes including *FOS* and *JUNB* were induced by prolonged handling of breast cancer samples after surgical resection. In another study, Soriano et al. [36], using an array of 750 genes, showed that ischemia in the rat brain affects the expression of only 24 genes, with very few of these genes underexpressed. Most of the upregulated genes were immediate early genes, including *Fos*, which was the gene most strongly upregulated, and *Junb*, which, as shown here, presented only a limited increase in expression. A list of genes particularly sensitive to stressful conditions should be established, as has been done for yeast [37]. This list of genes may be tissue dependent and could be determined using DNA arrays in the absence of RNA degradation.

The time for which samples are stored at room temperature can be minimized, but the time under ischemic conditions at 37 °C during surgery cannot. It is therefore reassuring that for all but one of the mRNA species tested, including the mRNA for *Junb*, an immediate early gene, *Myc*, which has a very short half-life, and *Vegf*, which is induced by hypoxia, real-time RT-PCR quantification of target gene transcripts relative to a reference gene transcript, even if performed on degraded RNA, can be used to provide a reliable estimate of in vivo mRNA levels. Similarities in the rates of degradation of target and reference mRNAs are probably responsible for this. However, some target mRNAs may be particularly stable or labile, and the results of PCR quantification on degraded RNA should therefore always be interpreted with caution.

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