Original Articles

The Use of Housekeeping Genes (HKG) as an Internal Control for the Detection of Gene Expression by Quantitative Real-Time RT-PCR

(abl / apoptosis / β2-microglobulin / differentiation / GAPDH / 18S rRNA)

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Abstract. Quantitative real-time RT-PCR is a very useful technique for estimating gene expression at the mRNA level. The expression of a tested gene has to be compared with that of a control gene. Various housekeeping genes have been used as control genes in different systems. In our study we tested several housekeeping genes in the model of gene expression after induction of apoptosis and differentiation. The myeloid cell lines were incubated with phorbol esters, butyric acid and combination of TNFα and IFNγ to induce differentiation. Camptothecin was used for induction of apoptosis. Tested control genes included β2-microglobulin, *GAPDH*, 18S ribosomal RNA and *abl*. *GAPDH* was found to be the best control gene in the apoptotic system. Different control genes were suitable for different systems where differentiation or senescence was induced. Our results show that attention should be paid to the choice of an appropriate control gene of quantitative real-time RT-PCR for different experimental models and various experimental conditions.

In order to estimate the differences in specific mRNA content, quantitative assays are referred to an internal control gene. The amount of assayed mRNA may fluctuate due to differences in tissue mass, cell number, experimental treatment or various efficiency of RNA preparation. Ideally, the conditions of the experiments should not influence the expression of the internal control gene. There are several housekeeping genes, which are commonly used for PCR validation: histone 3.3, β-actin, β2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA, *abl*, *bcr*, aldolase, glucose 6-phosphate dehydrogenase (*G6PDH*), hypoxanthine phosphoribosyltransferase (*HPRT*), porphobilinogen deaminase (*PBGD*) and dihydrofolate reductase (*DHFR*) (Watzinger and Lion, 1998).

The β-actin gene is frequently used for determination of the mRNA content and the efficiency of reverse transcription in analysed samples. However, it has been shown that the human genome contains processed β-actin pseudogenes (Leavitt et al., 1984), which can result in the amplification of fragments from genomic DNA displaying the same size as those generated from the cDNA template. Therefore, β-actin is not a suitable control gene for reverse-transcription polymerase chain reaction (RT-PCR) (Taylor and Heasman, 1994). Additionally, β-actin is a highly expressed gene and yields a strong amplification signal even from very small template amounts. The use of such a control may lead to a wrong interpretation of PCR assays if the investigated gene is expressed at a markedly lower level (Cross et al., 1994). A highly sensitive and specific fluorescence RT-PCR assay for the pseudogene-free part of β-actin was described by Kreuzer (Kreuzer et al., 1999).

A round-table debate on limitations of the use of histone 3.3 (Wells and Bains, 1991), *G6PDH* (Tso et al., 1985), aldolase (Tolan et al., 1987), *HPRT*, *DHFR* (Masters et al., 1983) and β-actin due to their pseudogenes was published in the journal Leukemia in 1997-1998.

Not only β-actin is highly expressed, but also 18S rRNA. Because of the absence of introns in the 18S rRNA molecule, this gene is also susceptible to false results from contaminating DNA.

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Abbreviations: BA – butyric acid, CAM – camptothecin, DHFR – dihydrofolate reductase, G6PDH – glucose 6-phosphate dehydrogenase, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, IFNγ – interferon γ, HKG – housekeeping gene, HPRT – hypoxanthine phosphoribosyltransferase, PBGD – porphobilinogen deaminase, RT-PCR – reverse-transcription polymerase chain reaction, TNFα – tumour-necrosis factor α, TPA – phorbol esters.

Amplification of sequences of the *abl* proto-oncogene has provided a convenient control system for RT-PCR assays in haematopoietic cells displaying the BCR-ABL rearrangement (Melo et al., 1994). *Abl* was also suggested as a suitable control gene for the comparison of the gene expression of normal and leukaemia cells by the Europe Against Cancer group (Gabert, 2002).

All primer pairs used in RT-PCR should be tested whether they give an amplification product from genomic DNA. There are additional factors that may also influence RT-PCR analysis such as different efficiency of mRNA processing (Sanchez and Robins, 1994).

The aim of our study is to show how important is the choice of the control gene for the reliable results of quantitative RT-PCR obtained in various experimental conditions. We used two different models where human myeloid cells were induced to apoptosis or differentiation. Our results showed that different control genes are optimal in various experimental models.

Material and Methods

Cells and reagents

Human myeloid leukaemia cell lines ML1, ML2 and ML3 were obtained from Dr. Minowada (Fujisaki Cell Centre, Okayama, Japan). All these cell lines were maintained in RPMI1640^{TM} medium (Sevac, Prague, Czech Republic) supplemented with 10% foetal bovine serum (Invitrogene, Grand Island, N.Y.) at 37˚C in a humidified atmosphere with 5% CO_2.

12-o-Tetradecanoyl-phorbol-13-acetate (TPA), butyric acid (BA), tumour-necrosis factor α (TNFα) and camptothecin (CAM) were obtained from Sigma (St. Louis, MO). Interferon γ (Imukin) was purchased from Boehringer Ingelheim (Wien, Austria). Phenol, chloroform and ethanol were purchased from Merck (Darmstadt, Germany). Syber Green I, II^{TM} and FastStart Taq DNA Polymerase^{TM} were obtained from Boehringer Mannheim (Mannheim, Germany). All the used PCR primers and probes were obtained from TIB MOLBIOL (Berlin, Germany). RNasin, dithiothreitol (DTT), Moloney murine leukaemia virus reverse transcriptase (M-MLV^{TM}), random primers and dNTP were bought from Promega (Madison, WI).

Determination of cell viability, proliferation, differentiation and apoptosis

The cell lines were seeded and allowed to reach exponential growth for 24 h. The cells were plated at an initial density of 5 x 10^5 cells/ml. Phorbol esters (TPA) (120 nM), BA (1 mM), TNFα (10 ng/ml) or IFNγ (1000 U/ml) were added, in indicated final concentrations, to growing cells to induce differentiation. CAM (5 μg/ml) was added to induce apoptosis. Non-induced cells and cells induced by differentiating agents were incubated for 18 and 24 h. Cells induced by CAM to apoptosis were harvested after 4, 8, 16 and 24 h. After that, adherent and non-adherent cells were harvested and counted by the Trypan blue dye exclusion method (at least 200 cells were counted for each assay). Cell viability was calculated as the percentage of cells that excluded the Trypan blue dye. The magnitude of the apoptotic cell pool was estimated from the flow-cytometric profile of the DNA content. A prominent peak in sub-G_1 area appeared in apoptotic cells, which was minimal in the control cells. Measurement of caspase-3 activity was used to confirm the apoptotic results. This measurement was performed according to the manufacturer's protocol (ApoAlert Caspase-3 Fluorescent Assay Kit^{TM}, Clontech, Palo Alto, CA). Appearance of surface CD14 (MY-4) and CD11b (MO-1) was used for determining the myelomonocytic differentiation. Isotypic negative control antibodies determined non-specific fluorescence. The nitroblue tetrazolium (NBT) dye reduction assay was also used to monitor differentiated cells, which were determined as a percentage of stained cells.

Table 1. Primers used for real-time quantitative RT-PCR

Gene	Sequence of primers	Annealing temperature	Final primer concentration	Reference
abl	5′TGGAGATAACACTCTAAGCATAACTAA AGGT 3′ 5′GATGTAGTTGCTTGGGACCCA 3′	60°C	20 μM 20 μM	[a]
18S rRNA	5′CCATCCAATCGGTAGTAGCG 3′ 5′GTAACCCGTTGAACCCCATT 3′	66°C	20 μM 20 μM	Schmittgen 2000
GAPDH	5′TGCACCACCAACTGCTTAG 3′ 5′GATGCAGGGATGATGTTC 3′	61°C	20 μM 20 μM	Schmittgen 2000
β2-micro-globulin	5′ GAGTATGCCTGCCGTGTG 3′ 5′ AATCCAAATGCGGCATCT 3′	60°C	20 μM 20 μM	[a]

[a]primers were designed in our laboratory by the Primer3 Input software.

Quantification of gene expression by the real-time RT-PCR technique

Harvested cells (5 x 10^6 cells for one isolation) were washed and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sachi, 1987) modified according to Haškovec et al. (Haškovec et al., 1998) and stored at –80˚C. The quality of the isolated RNA was checked by electrophoresis in 2% agarose gels stained with Syber Green II™. cDNA (aliquot of 2 x 10^6 cells) was synthesized from isolated RNA using random hexanucleotides (25 mM) and M-MLV™ reverse transcriptase (120 U per 10 µl reaction). Quantitative real-time (RQ) RT-PCR of β2-microglobulin and *abl* was performed with 100 mmol each of dNTP, 1 U FastStart Taq DNA polymerase™, 3.5 mM $MgCl_2$, 0.5 µM of each primer and 0.2 µM FAM probe (in a total volume of 20 µl). Primers used in the amplification of control genes are shown in Table 1. There were no non-specific products in optimized amplification after 45 cycles. 6FAM-CCTCCATGATGCTGCTTACATGTCTCp was used for quantification of β2-microglobulin. 6FAM-CCATTTTTGGTTTGGGCTTCACACCATXTp was used for amplification of the *abl* control gene. The thermal cycler parameters for amplification of β2-microglobulin and *abl* were: 1 cycle at 95˚C for 5 min followed by 40 cycles at 94˚C for 20 s, 60˚C for 45 s. Expression of 18S rRNA and GAPDH was measured by Syber Green I™ during the following cycling: 1 cycle at 94˚C for 3 min, 40 cycles at 94˚C for 25 s, 56˚C for 25 s, 72˚C for 25 s and 80˚C for 15 s where fluorescence was measured. Melting curve analysis following PCR contributed to distinguishing the specificity of the PCR products. A RotorGene™ apparatus (Corbett Research, Australia) was used for amplifications, measurements and quantification.

pENTR 1A with inserted cDNA of β2-microglobulin was used as a standard for the construction of the calibration curve. The calibration curve for abl, 18S rRNA and GAPDH was constructed from the amplified serial dilutions of the appropriate PCR product. All results of the real-time PCR were normalized to 5 x 10^6 cells, which was the initial amount for RNA isolation. The amount of total RNA was also checked by quantification by GelBase™ software equipment after agarose electrophoresis to catch the changes in rRNA expression.

Statistical data describing the results (Table 2) were obtained by software analysis (Student t-test). $P \leq 0.5$ was considered to reflect a significant relationship between housekeeping gene (HKG) expression and the induction agent.

Table 2. Relationship between the HKG expression and the induction of apoptosis and differentiation described by the summary of the statistical data. These data were obtained by software analysis (Student t-test). $P < 0.05$ was considered to be a proof of a significant relationship between gene expression and the induction agent.

Induction agent and hours of induction	HKG	P
TPA 18	β-2 microglobulin	0.178
	18S rRNA	0.134
	abl	<0.001
	GAPDH	0.010
TPA 24	β-2 microglobulin	0.087
	18S rRNA	0.201
	abl	$<10^{-5}$
	GAPDH	0.137
BA 18	β-2 microglobulin	0.186
	18S rRNA	0.068
	abl	<0.001
	GAPDH	0.021
BA 24	β-2 microglobulin	0.115
	18S rRNA	0.585
	abl	0.004
	GAPDH	0.352
TNF α + IFN γ 18	β-2 microglobulin	<0.001
	18S rRNA	0.138
	abl	0.002
	GAPDH	0.035
TNF α + IFN γ 24	β-2 microglobulin	0.001
	18S rRNA	0.650
	abl	0.043
	GAPDH	0.924
CAM 4	β-2 microglobulin	<0.001
	18S rRNA	0.080
	abl	0.003
	GAPDH	0.099
CAM 8	β-2 microglobulin	<0.001
	18S rRNA	0.006
	abl	0.002
	GAPDH	0.069
CAM 16	β-2 microglobulin	$<10^{-4}$
	18S rRNA	0.215
	abl	0.011
	GAPDH	0.056
CAM 24	β-2 microglobulin	0.002
	18S rRNA	0.065
	abl	0.008
	GAPDH	0.111

Results

Expression of control genes during apoptosis

Induction of apoptosis by CAM in the studied myeloid cell lines (ML-1, ML-2 and ML-3) was accompanied by cell cycle arrest and 50% apoptotic cells

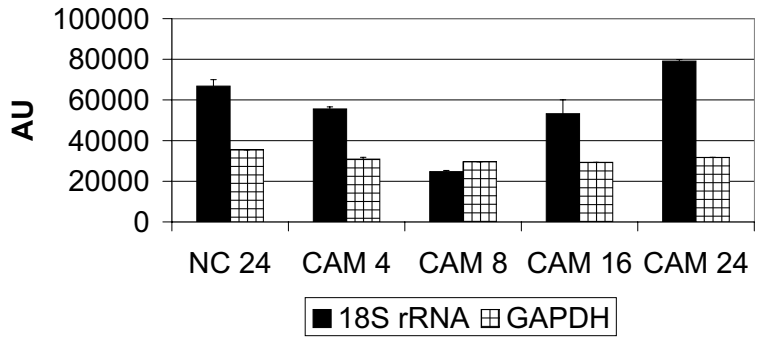

Fig. 1. Expression of GAPDH and 18S rRNA in 5 x 10^6 cells, detected by real-time PCR with Syber Green I. The amount of fluorescence given in arbitrary units was obtained from the calibration curve of the standard. CAM induced the cells to apoptosis and aliquots of the cells were harvested 4, 8, 16 and 24 h after induction (CAM 4, CAM 8, CAM 16, and CAM 24). Results with non-induced control cells (NC 24) are shown only after 24 h.

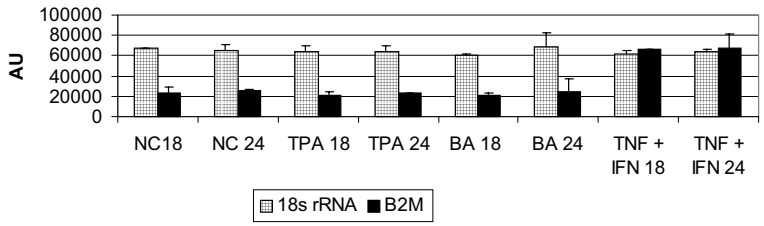

Fig. 2. Expression of β2-microglobulin (B2M) and 18S rRNA in 5 x 10^6 cells, detected by real-time PCR with TaqMan probe and Syber Green I, respectively. The amount of fluorescence given in arbitrary units was obtained from the calibration curve of the standard. The cells were induced to differentiation by TPA, BA and combination of TNFα and IFNγ for 18 and 24 h (TPA 18, TPA 24, BA 18, BA 24, TNFα + IFNγ 18, TNFα + IFNγ 24). NC 18 and NC 24 denote non-induced cells at the indicated time.

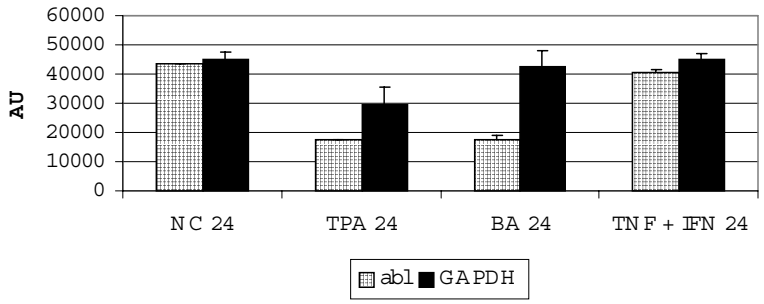

Fig. 3. Expression of abl and GAPDH in 5 x 10^6 cells, detected by real-time PCR with TaqMan probe and Syber Green I, respectively. The amount of fluorescence given in arbitrary units was obtained from the calibration curve of the standard. The cells were induced to differentiation by TPA, BA and combination of TNFα and IFNγ for 24 h (TPA 24, BA 24 and TNFα + IFNγ 24). NC 24 denotes non-induced cells at the indicated time.

appeared after 18 h of the incubation as described earlier (Ullmannová et al., 2003).

GAPDH, whose level was not influenced by CAM in contrast to 18S rRNA (a typical experiment is shown in Fig. 1, Table 2), was found to be the most suitable control gene. A decrease of the 18S rRNA level, which occurred after 8 h, correlated with the maximum of caspase-3 activity and its increase after 16 h might be due to a reversible effect of CAM and restoration of rRNA synthesis.

Abl and β2-microglobulin were not suitable genes due to their fluctuation in apoptotic and normal cells during the course of incubation (data not shown). In our experimental model of CAM-induced apoptosis, *GAPDH* therefore seems to be the most suitable control gene for a quantitative estimation of gene expression by real-time RT-PCR.

Expression of control genes during differentiation

As described earlier (Tso et al., 1985), TPA, TNFα and the combination of TNFα and IFNγ induced differentiation. The effect of BA was classified as cell senescence (Terao et al., 2001; Ullmannová et al., 2003).

Induction of both differentiation and senescence was accompanied by cell cycle arrest (Ullmannová et al., 2003).

The expression of β2-microglobulin was not influenced in cases of TPA and BA induction, but it was changed in the case of induction by the combination of TNFα and IFNγ (Fig. 2). On the other hand, *GAPDH* and *abl* were suitable controls in this system because their mRNA level did not vary. *GAPDH* was also suitable for PCR validation after induction by BA. This agent similarly as TPA influenced the level of abl mRNA and this gene therefore could not be used as a control in these systems (Fig. 3).

Although slight fluctuations in the expression of 18S rRNA were detected, this gene was in general suitable in all tested differentiating systems (Fig. 2).

β2-Microglobulin (but not *abl*) was a suitable control gene for comparing gene expression in non-induced cells with that induced by TPA or BA. On the other hand, *abl*, but not β2-microglobulin, was a suitable control after induction by the combination of TNFα and IFNγ (Figs. 2, 3). P-values, which also characterize the

relationship between inductional agents and HKG expression, are noticed in Table 2.

The results clearly showed that different control genes are optimal in different differentiation models.

Discussion

A parallel quantification of a housekeeping gene is a commonly used approach for the validation of the expression of tested genes. Real-time PCR offers a more exact and easier way to estimate the amount of the template. The first comparisons of the levels of gene expression were based on the assumption of constant expression of housekeeping genes in all the tissues of a multicellular organism. Recently, it became clear that various incubating conditions (e.g. serum stimulation) could also strongly influence the expression of a housekeeping gene (Schmittgen and Zakrajsek, 2000). All of the microarray technologies, which are currently one of the hot topics, include several housekeeping genes for the general normalization of the array to avoid mistakes due to different levels of control gene expression (e.g. Clontech Atlas Human Apoptosis cDNA Array^{TM}).

The amount of cDNA could be confirmed by an independent technique such as Nothern blot. However, one of the disadvantages of this techniques is the necessity of huge amounts of initial material and handling with labile RNA.

In spite of the fact that *abl* was suggested as a suitable gene for testing the expression of leukaemia cells (Gabert, 2002), this gene was not suitable in most of our models. The only model where its level was not influenced was after induction of differentiation by TNFα + IFNγ. It contrasted with the experiments with other differentiating agents such as BA or TPA (Fig. 3), where an increased number of apoptic cells was detected (data not shown). It was shown that abl regulates p73 in the apoptotic response to DNA damage (Yuan et al., 1999) and they collaborate in the induction of apoptosis (Agami et al., 1999). These results may provide an explanation of the fluctuations of abl in our apoptotic system.

18S rRNA is a widely used control gene. It may give false results in the systems where in contrast to rRNA, mRNA is degraded, or they are metabolized by different ways. There are also the limitations as described above. Except for the case of the apoptotic system, 18S rRNA was suitable in all the tested differentiating models.

GAPDH appeared to be a useful control gene in most experimental variants (non-induced cells versus apoptotic and differentiated cells) except for the cells induced by TPA. Also β2-microglobulin seemed to be a suitable control gene for the detection of expression in all the systems except for those induced with TNFα + IFNγ. Both TNFα and IFNγ have their responsive elements in the promotor of β2-microglobulin as described earlier (Lee et al., 1999).

Statistical significance of the induction effects on the expression of the tested HKGs is mentioned in Table 2. There are also differences between P-values in a time-dependent manner (e.g. in the expression of GAPDH). It could reflect a time-dependent expression of this gene after stimulation of differentiation.

Our results confirm the need to optimize not only the PCR conditions of the tested gene, but also to find the optimal internal control gene. Expression of different housekeeping genes varies according to the experimental conditions (e.g. for the induction of apoptosis or differentiation in our systems), and choosing a suitable control gene is therefore very important for reliable quantification.

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