ELSEVIER

J. Biochem. Biophys. Methods 46 (2000) 69–81

JOURNAL OF
biochemical and
biophysical
methods

www.elsevier.com/locate/jbbm

Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR

Thomas D. Schmittgen*, Brian A. Zakrajsek

Department of Pharmaceutical Sciences, College of Pharmacy, and the Cancer Prevention and Research Center, Washington State University, Pullman, WA 99164-6534, USA

Received 14 June 2000; received in revised form 11 August 2000; accepted 11 August 2000

Abstract

The effects of serum on the expression of four commonly used housekeeping genes were examined in serum-stimulated fibroblasts in order to validate the internal control genes for a quantitative RT-PCR assay. NIH 3T3 fibroblasts transfected with an inducible chimeric gene were serum-starved for 24 h and then induced with 15% serum for 8 h. Serum did not alter the amount of total RNA that was expressed in the cells, however, the amount of mRNA significantly increased over time with serum-stimulation. Both messenger and total RNA from each of the time points were reverse transcribed under two different conditions; one in which the reactions were normalized to contain equal amounts of RNA and another series of reactions that were not normalized to RNA content. The resulting cDNA was amplified by real-time, quantitative PCR using gene-specific primers for β-actin, β-2 microglobulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA. The expression of β-actin and GAPDH increased up to nine- and three-fold, respectively, under all conditions of reverse transcription ($P < 0.01$). The expression of 18S rRNA increased with serum-stimulation when the cDNA synthesized from non-normalized, total RNA was assayed ($P < 0.01$) but not when the reverse transcriptions were normalized to RNA content ($P > 0.05$). The expression of β-2 microglobulin increased up to two-fold when assayed from cDNA synthesized from non-normalized mRNA, but was unaffected by serum when the reverse transcriptions were normalized to mRNA. β-2 Microglobulin

Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, Reverse transcription-polymerase chain reaction; rRNA, Ribosomal RNA

*Corresponding author. Tel.: +1-509-335-3573; fax: +1-509-335-5902.

E-mail address: schmittg@mail.wsu.edu (T.D. Schmittgen).

0165-022X/00/$ – see front matter
PII: S0165-022X(00)00129-9

expression was found to be directly proportional to the amount of mRNA that was present in non-normalized reverse transcription reactions. Thus, β-2 microglobulin and 18S rRNA are suitable internal control genes in quantitative serum-stimulation studies, while β-actin and GAPDH are not. The internal control gene needs to be properly validated when designing quantitative gene expression studies.

Keywords: Real-time PCR; Quantitative RT-PCR; Gene expression; SYBR green; Serum-stimulation

1. Introduction

The response of mammalian cells to serum is a useful model to study complex cellular processes that are influenced by extracellular signaling molecules (reviewed in [1]). Cultured murine fibroblasts (e.g. NIH 3T3, Swiss 3T3, Balb/c 3T3) are particularly useful for these studies. Culture media supplemented with 10–20% serum provides the fibroblasts with the necessary polypeptide growth factors. Serum-starvation, or reducing the amount of serum in the culture media, forces the fibroblasts to enter a quiescent or G_0 phase of the cell cycle. Quiescent cells may be stimulated to reenter the cell cycle by adding 10–20% serum. Serum-stimulation of quiescent fibroblasts induced numerous genes including transcription factors (c-Fos, c-Myc), cytoskeletal and extracellular matrix proteins (β-actin, fibronectin), enzymes (MAP kinase phosphatase-1, nitric oxide synthase) and many others [1]. Qualitative analysis of over 8600 different genes using cDNA microarray technology revealed that genes could be clustered into several different groups based upon their response to serum [2].

Quantitative gene expression assays are typically referenced to an internal control gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin, to account for differences in RNA load. The amount of RNA assayed may fluctuate due to differences in tissue mass, cell number, experimental treatment or RNA extraction efficiency. Ideally, the conditions of the experiment should not influence the expression of the internal control gene. Selecting an internal control gene for quantitative gene expression studies of serum-stimulated fibroblasts is difficult because of the ubiquitous effect that serum has on gene expression [1,2]. We initiated mRNA stability studies using NIH 3T3 fibroblasts that were stably transfected with the serum-inducible fos–glo–myc chimeric gene [3]. Fibroblasts transfected with these chimeric genes are routinely used to study mRNA decay. In order to validate a quantitative RT-PCR assay to study decay of the fos–glo–myc mRNA, we examined the effect of serum on the expression of several candidate internal control genes.

We report a detailed quantitative analysis of the expression of four commonly used housekeeping genes during the early response to serum and the appropriate internal controls to use in quantitative serum-stimulation studies. The methodology reported here may be applied to other quantitative gene-expression studies to evaluate the effects of experimental treatment on the expression of potential internal control genes.

2. Materials and methods

2.1. Chemicals

Tissue culture reagents, random hexamers and MMLV reverse transcriptase were from Life Technologies (Gaithersberg, MD, USA). Hygromycin B was purchased from Sigma (St. Louis, MO, USA). The RNeasy Mini RNA isolation kit was from Qiagen (Valencia, CA, USA). The SYBR green I PCR kit was purchased from PE Biosystems (Foster City, CA, USA). SYBR green II was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Tissue culture

NIH 3T3 fibroblasts stably transfected with the fos–glo–myc chimeric gene were generously provided by Dr. Jeffery Ross (University of Wisconsin, Madison, WI, USA). The chimeric fos–glo–myc gene consists of the β-globin gene fused in-frame to a portion of the c-myc 3′ untranslated region. The chimeric gene was placed downstream from the serum-inducible c-fos promoter [3]. The fos–glo–myc cells were cultured in a complete medium consisting of Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml hygromycin B. The cells were cultured at 37°C in a humidified environment of 5% CO_2/air.

2.3. Serum induction experiments

The serum starvation and induction experiments were conducted in a similar manner to those previously reported [3]. Early passage cells (<20) were used in all of the experiments. Cells were plated at low density in six-well plates. Upon reaching 70% confluency, complete medium was removed and the cells were rinsed three times in warm phosphate-buffered saline (PBS). A starvation media consisting of D-MEM/penicillin/streptomycin and 0.5% dialyzed fetal bovine serum was added for 24 h. During the starvation period, the cells were cultured in a humidified environment of 7.5% CO_2/air. Following the starvation period, 15% dialyzed fetal bovine serum was added and the cells were cultured in the humidified incubator, 5% CO_2/air. At various time points following the addition of serum, the media was removed, the cells were lysed in 350 μ1 of buffer RLT (Qiagen). Cell lysates were immediately frozen in a dry ice/ethanol slurry and stored at -80°C. Cells were plated in triplicate for each time point.

2.4. RNA extraction and quantification

Total cellular RNA was isolated from the cells using the RNeasy Mini, RNA isolation kit (Qiagen). Total RNA was eluted from the RNeasy Mini columns with 60 μl of RNase-free water. mRNA was obtained from the total RNA using the PolyATract mRNA isolation system III, according to the manufacturer's instructions (Promega,

Madison, WI, USA). The amount of total or messenger RNA isolated from the cells was quantified using the SYBR green II fluorescent dye. RNA was diluted in a TEF buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 × SYBR green II). Standard curves ranging from 4 to 500 ng/ml were constructed by diluting the total RNA isolated from the NIH 3T3 fibroblasts. To quantify mRNA, standard curves were prepared by diluting rat liver poly $(A)^+$ RNA (Sigma) to produce a standard curve ranging from 4 to 250 ng/ml. Fluorescence was detected in a CytoFluor series 4000 multi-well plate reader (PerSeptive Biosystems, Farmington, MA, USA), with excitation at 460 nm and emission at 530 nm.

2.5. Reverse transcription

A 25-μl volume of the RNA solution (non-normalized conditions) or 800 ng of total RNA (normalized conditions) was added to a reaction containing 50 mM Tris–HCl (pH 8.3), 10 mM dithiothreitol, 0.0225 OD_{260} units of random hexamers, 3.5 μg of bovine serum albumin, 3 mM $MgCl_2$, 0.5 mM of the deoxynucleotide triphosphates, 30 units of RNAguard RNase inhibitor, 500 units of MMLV reverse transcriptase and RNase-free water to 50 μl. Reactions were incubated at 26°C for 10 min, then at 42° for 45 min followed by 75°C for 10 min, to inactivate the reverse transcriptase. cDNA was synthesized from 25 μl of the mRNA solution (non-normalized conditions) or 6 ng of mRNA (normalized conditions) using the identical reaction conditions listed above, except that the reverse transcriptions were primed with 0.31 μg of oligo dT (12–18) (Life Technologies). The cDNA was stored at −80°C.

2.6. Real-time quantitative PCR

Primers used for the PCR are listed in Table 1. All of the PCR primers were synthesized by Operon Technologies (Alameda, CA, USA) except the primers for GAPDH, which were purchased from PE Biosystems. Real-time quantitative PCR was performed in the GeneAmp 5700 Sequence Detection System (PE Biosystems) using SYBR green detection as previously described [4]. The endpoint used in the real-time PCR quantification, C_t, is defined as the PCR cycle number that crosses an arbitrarily

Table 1
Primers used in the PCR. The nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR are shown

Gene	Forward primer	Reverse primer
β-Actin	5′ACCAACTGGGACGAT ATGGAGAAGA 3′	5′TACGACCAGAGG CATACAGGGACAA 3′
β-2 Microglobulin	5′GCTATCCAGAAAACC CCTCAA 3′	5′CATGTCTCGATCCCA GTAGACGGT 3′
GAPDH	5′TGCACCACCAACTGC TTAG 3′	5′GATGCAGGGATGAT GTTC 3′
18S rRNA	5′GTAACCCGTTGAACC CCATT 3′	5′CCAT CCAATCGGTAG TAGCG3′

placed signal threshold. Gene expression was presented using a modification of the $2^{-\Delta\Delta Ct}$ method, first described by K. Livak in PE Biosystems Sequence Detector User Bulletin 2 [5]. The expression of each housekeeping gene was presented as $2^{-\Delta Ct}$, where ΔC_t ($C_{t\text{Time X}} - C_{t\text{Time 0}}$) and time 0 represents the $1\times$ expression of each gene.

2.7. Statistics

Least-squares linear regression analysis was performed using Sigma Plot for Windows Version 4.0 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used to study the association between serum-stimulation time and the level of gene expression. Fold increase in mRNA was the dependent variable. A P value ≤ 0.05 was considered to be significant. Analysis was performed using SAS (SAS institute, Carey, NC, USA).

3. Results

3.1. Relationship between serum stimulation and cellular RNA levels

NIH 3T3 fibroblasts were forced to enter a quiescent state by a 24-h period of serum-starvation in 0.5% serum. The cells were stimulated with 15% serum and samples were collected over 8 h. Serum-stimulation produced a steady increase in mRNA synthesis, increasing to a maximum of two-fold over 8 h (Fig. 1). The relationship between the time of serum-stimulation and mRNA content was statistically significant ($P < 0.001$, Table 2). No significant relationship was observed between the serum-stimulation time and the amount of total RNA that was isolated from the cells (Fig. 1 and Table 2). These data demonstrate that the early response of serum-starved fibroblasts to serum is to synthesize mRNA but not total RNA.

3.2. Effect of serum on housekeeping gene expression; analysis of cDNA synthesized from total RNA

The cDNA obtained from the serum-stimulated fibroblasts was amplified by real-time, quantitative PCR using gene-specific primers for β-actin, β-2 microglobulin, GAPDH or 18S rRNA. These genes represent standard housekeeping genes that are commonly used in quantitative gene expression studies. Amplification of cDNA synthesized from non-normalized, total RNA revealed that the expression of GAPDH and β-actin increased by a maximum of two- and four-fold, respectively, over the 8 h of serum-stimulation ($P < 0.01$, Fig. 2). The expression of β-2 microglobulin was unaffected by serum-stimulation ($P = 0.2319$, Fig. 2A). Serum-stimulation increased the expression of 18S rRNA barely above one-fold, however, the relationship was significant ($P < 0.01$, Fig. 2D).

Although the amount of total RNA produced by the cells did not significantly change with serum-stimulation (Fig. 1), we wanted to determine if the effect of serum on gene expression (Fig. 2A–D) was related to the amount of total RNA that was added to the reverse transcriptions. The reverse transcription reactions were normalized to contain

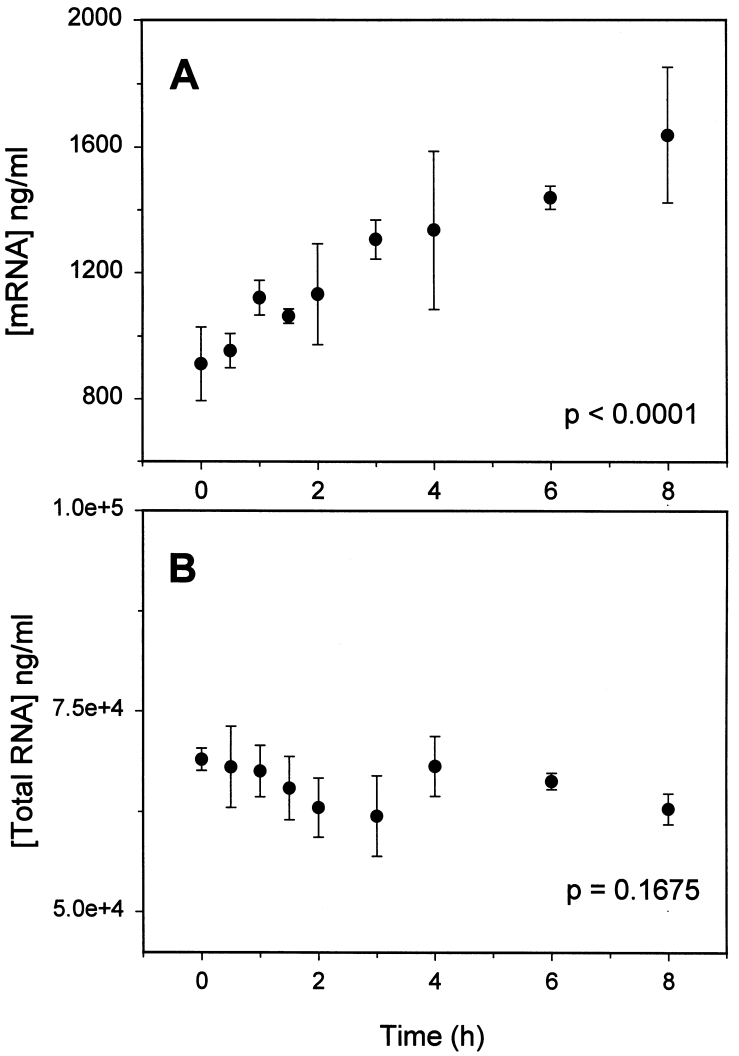

Fig. 1. Relationship between serum-stimulation and cellular RNA levels. 3T3 fibroblasts were cultured in six-well plates, serum-starved for 24 h and induced with 15% serum for 8 h. At each time point, total and messenger RNA were isolated from the fibroblasts and quantified. The data presented on the *y* axis represents the concentration of (A) or (B) RNA that was present in the final solution of isolate. Mean±S.D. ($n = 3$). The *P* values were determined by ANOVA.

equal amounts of total RNA. Amplification of cDNA obtained from normalized, total RNA revealed that the expression of β-actin and GAPDH increased by a maximum of eight- and three-fold, respectively, over the 8 h of serum-stimulation ($P < 0.0001$, Fig. 2). Amplification of the same cDNA with primers for β-2 microglobulin demonstrated that the expression was unaffected by serum ($P = 0.077$, Fig. 2E). Analysis of 18S

Table 2
Relationship between serum-stimulation time and expression of cellular messenger or total RNA[a]

Relationship between serum-stimulation time and fold change in:	Experiment	*P* value
Total RNA	1	0.1675
	2	0.1472
mRNA	1	< 0.0001
	2	< 0.001

[a] Statistical data describing the relationship between time of serum stimulation and the fold-change in messenger or total RNA are presented. The association between serum-stimulation time and the level of RNA was verified by one-way ANOVA. Fold-increase in RNA was the dependent variable. $P \leq 0.05$ was considered to be significant. The data are taken from Fig. 1. Each experiment was performed in duplicate.

rRNA expression from the cDNA synthesized from normalized, total RNA revealed that the expression of 18S rRNA was unaffected by serum ($P > 0.05$, Fig. 2H).

3.3. Effect of serum on housekeeping gene expression; analysis of cDNA synthesized from mRNA

Since β-2 microglobulin, β-actin and GAPDH are expressed as mRNA, we wanted to study the effect of serum stimulation on housekeeping gene expression by amplifying cDNA that was synthesized from mRNA. Amplification of the cDNA obtained from non-normalized mRNA revealed that the expression of β-actin and GAPDH increased by a maximum of six-fold, while β-2 microglobulin increased by about two-fold (Fig. 3). The relationship between serum-stimulation time and expression of all three housekeeping genes was statistically significant (Table 3). Since serum increased the amount of mRNA in the cells (Fig. 1), we wanted to determine if the increase in gene expression observed in Fig. 3A–C was due to the increasing amounts of mRNA that were reverse transcribed. When the cDNA obtained from normalized mRNA was amplified, the relationship between serum-stimulation and expression of β-actin and GAPDH was statistically significant ($P < 0.05$) while the expression of β-2 microglobulin was unaffected by serum ($P = 0.2345$, Fig. 3D–F).

3.4. Relationship between cellular mRNA levels and housekeeping gene expression

The effect of serum on housekeeping gene expression was compared to the amount of mRNA that was isolated from the fibroblasts. The change in mRNA was plotted on the abscissa and the change in gene expression on the ordinate. The concentration of mRNA was determined by fluorescence detection (SYBR green II). The gene expression data were obtained from the amplification of cDNA synthesized from non-normalized mRNA for β-2 microglobulin, β-actin and GAPDH.

All three graphs produced slopes with a positive value, demonstrating that the increase in mRNA was reflected by the expression of the individual housekeeping gene (Fig. 4). The slopes of the β-2 microglobulin, β-actin and GAPDH plots were 0.73, 2.94 and

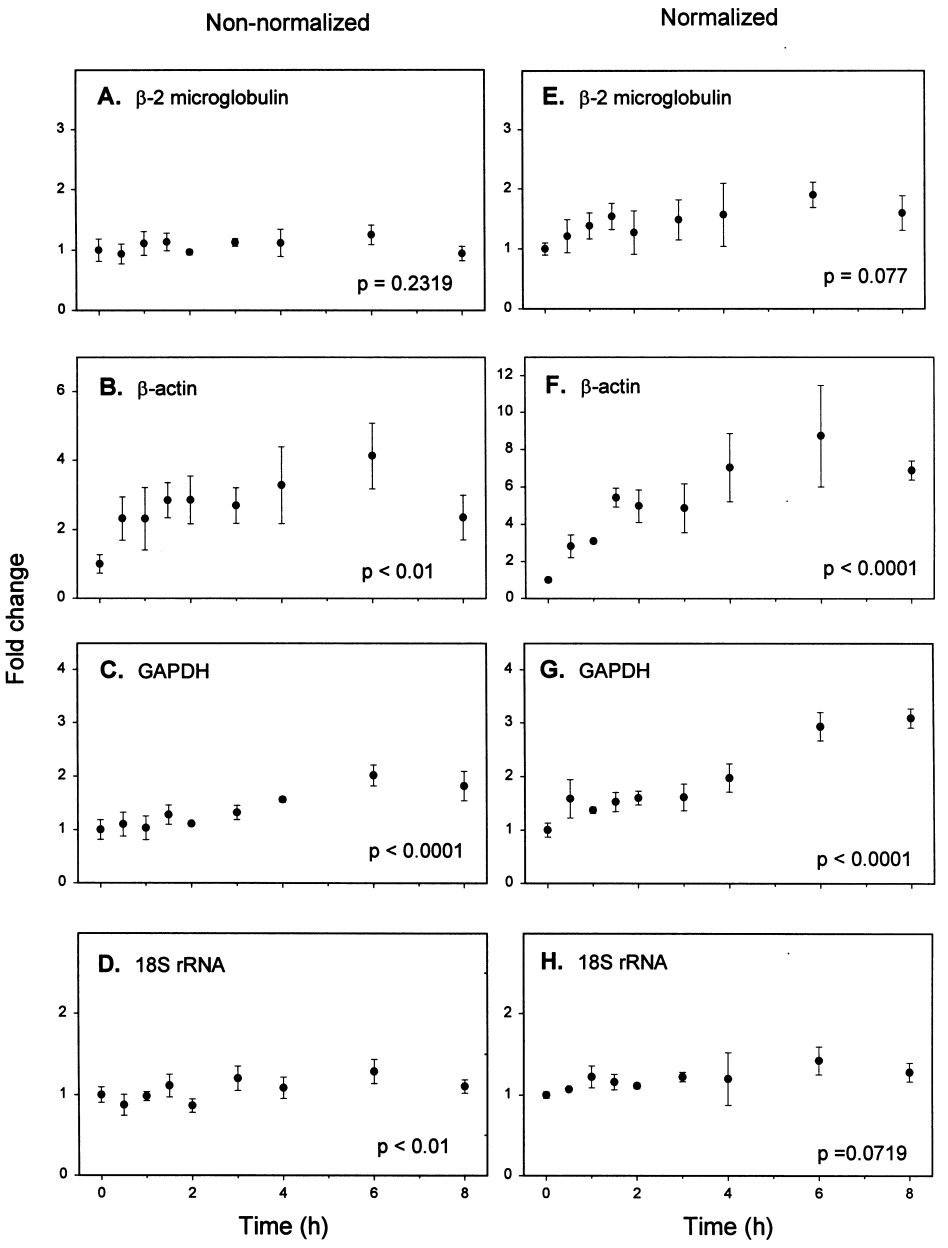

Fig. 2. Effect of serum-stimulation on housekeeping gene expression; reverse transcription of total RNA. NIH 3T3 fibroblasts were cultured in six-well plates, serum-starved for 24 h and induced with 15% serum for 8 h. Total RNA was isolated from the cells and converted to cDNA in reactions that were normalized to contain equivalent amounts of total RNA, or not normalized. Left panel, real-time quantitative PCR of cDNA synthesized from non-normalized total RNA using gene-specific primers for (A) β-2 microglobulin, (B) β-actin, (C) GAPDH and (D) 18S rRNA. Right panel, real-time quantitative PCR of cDNA synthesized from normalized total RNA using gene-specific primers for (E) β-2 microglobulin, (F) β-actin, (G) GAPDH and (H) 18S rRNA. Mean±S.D. ($n = 3$).

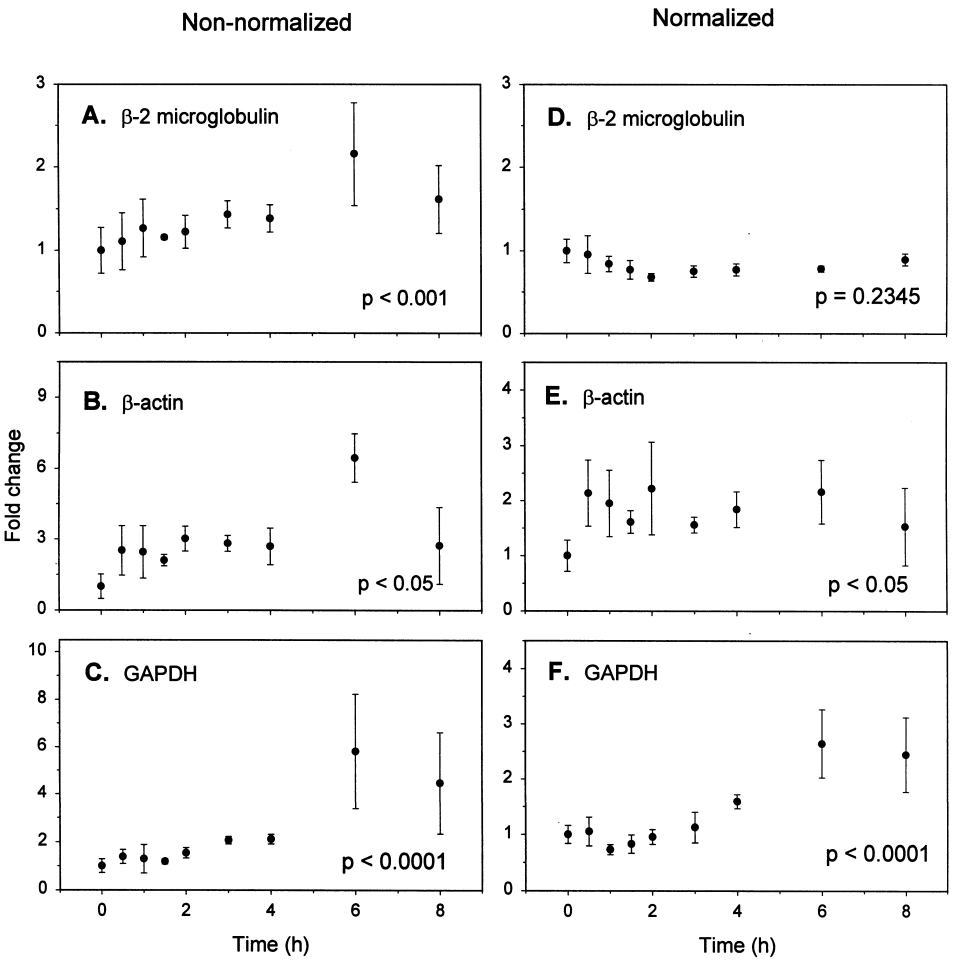

Fig. 3. Effect of serum stimulation on housekeeping gene expression; reverse transcription of mRNA. NIH 3T3 fibroblasts were cultured in six-well plates, serum-starved for 24 h and induced with 15% serum for 8 h. Messenger RNA was isolated from the cells and converted to cDNA in reactions that were normalized to contain equivalent amounts of mRNA, or not normalized. Left panel, PCR of cDNA synthesized from non-normalized mRNA using gene-specific primers for (A) β-2 microglobulin, (B) β-actin and (C) GAPDH. Right panel, PCR of cDNA synthesized from normalized mRNA using gene-specific primers for (D) β-2 microglobulin, (E) β-actin and (F) GAPDH. Results are expressed as the mean±S.D. ($n = 3$).

3.18, respectively. A slope with a value of one confirms that serum affected the expression of the housekeeping gene to the same extent as it did cellular mRNA. A slope with a value greater than one demonstrates that serum stimulated the expression of the housekeeping gene greater than it affected cellular mRNA. Another interpretation is that a slope of one demonstrates that the quantity of the expressed gene at any given time point is equal to the amount of mRNA that was added to the reverse transcription reaction, while a slope greater than one implies that the quantity of the expressed gene is

Table 3
Relationship between serum-stimulation time and housekeeping gene expression[a]

Relationship between serum-stimulation time and housekeeping gene expression. Quantitative PCR analysis of cDNA synthesized from:	Gene	*P* value
Non-normalized, total RNA	β-2 microglobulin	0.2319
	β-actin	< 0.01
	GAPDH	< 0.0001
	18SrRNA	< 0.01
Normalized, total RNA	β-2 microglobulin	0.077
	β-actin	$< 0.000\ 1$
	GAPDH	< 0.0001
	18SrRNA	0.0719
Non-normalized mRNA	β-2 microglobulin	< 0.05
	β-actin	< 0.0001
	GAPDH	< 0.001
Normalized mRNA	β-2 microglobulin	0.2345
	β-actin	< 0.05
	GAPDH	< 0.0001

[a] A summary of the statistical data describing the relationship between time of serum stimulation and the fold-change in housekeeping gene expression is presented. The association between serum-stimulation time and the level of gene expression was verified by one-way ANOVA. Fold-increase in gene expression was the dependent variable. $P \leq 0.05$ was considered to be significant. The data were taken from Figs. 2 and 3.

greater than the amount of mRNA that was reverse transcribed. β-2 Microglobulin is the only gene assayed whose slope approaches a value of one (Fig. 4).

4. Discussion

4.1. Appropriate internal control genes for quantitative serum-stimulation studies

Ideally, the internal control gene for quantitative gene expression studies should not be influenced by the conditions of the experiment. We demonstrate that serum-stimulation influenced the expression of several commonly used housekeeping genes. Serum stimulation for 8 h significantly increased the expression of β-actin and GAPDH (Figs. 2–3 and Table 3). Furthermore, serum stimulated the expression of β-actin and GAPDH to a greater extent than it influenced cellular mRNA levels (Fig. 4). These data demonstrate that the expression of β-actin and GAPDH does not reflect the amount of total or messenger RNA that was added to each reverse transcription reaction. We conclude, therefore, that β-actin and GAPDH should not be used as internal controls in quantitative gene expression studies using serum-stimulated fibroblasts.

Amplification of the cDNA that was synthesized from normalized, total RNA demonstrated that no relationship existed between the time of serum stimulation and expression of 18S rRNA ($P > 0.05$, Fig. 2H and Table 3). A significant relationship

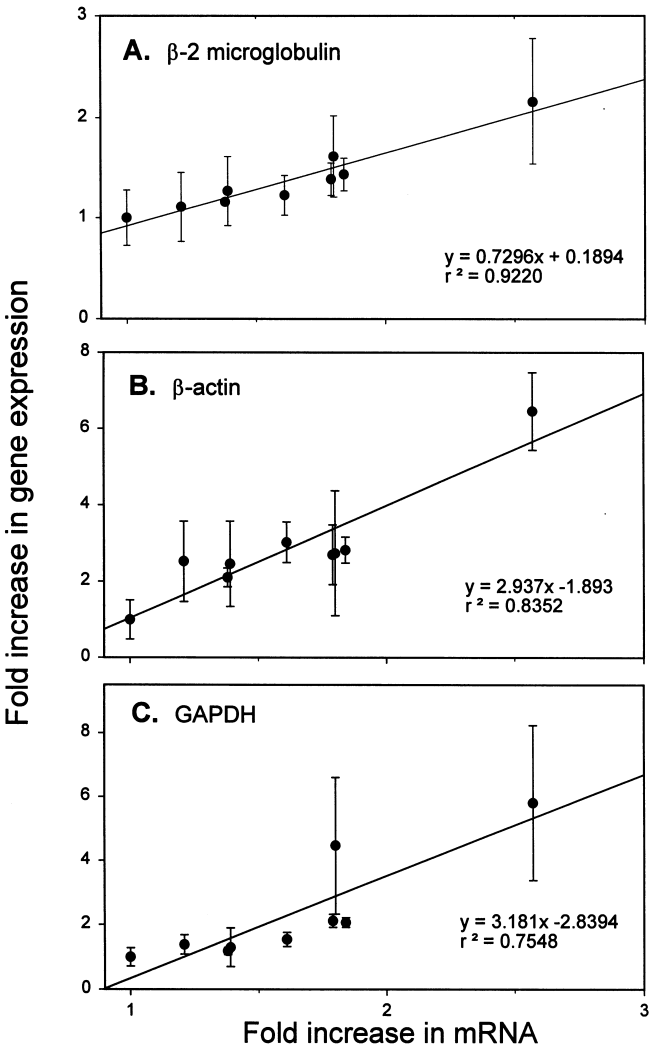

Fig. 4. Relationship between the cellular mRNA levels and housekeeping gene expression. NIH 3T3 fibroblasts were serum-starved for 24 h and induced with 15% serum over 8 h. The mRNA was isolated from the fibroblasts, quantified and converted to cDNA; the mRNA content was not normalized in these reactions. The cDNA was amplified by real-time PCR using gene-specific primers for β-2 microglobulin, β-actin and GAPDH. The change in mRNA over 8 h of serum-stimulation versus the change in expression of (A) β-2 microglobulin, (B) β-actin and (C) GAPDH is presented. The lines represent the least-squares linear regression. Results are expressed as the mean±S.D. ($n = 3$).

existed between serum-stimulation time and 18S rRNA expression when cDNA obtained by reverse transcribing unequal amounts of total RNA was analyzed ($P < 0.01$, Fig. 2D). We conclude that 18S rRNA may be used as an internal control in quantitative gene

expression studies of serum-stimulated fibroblasts only when the reverse transcriptions contain equal amounts of RNA.

Serum stimulation did not greatly influence the expression of β-2 microglobulin (Figs. 2 and 3). β-2 Microglobulin expression significantly increased when cDNA synthesized from non-normalized mRNA was amplified (Fig. 3A). However, when these data were plotted against the change in cellular mRNA, the expression of β-2 microglobulin directly paralleled the change in mRNA (Fig. 4A). Therefore, β-2 microglobulin may be used as an internal control in quantitative serum-stimulation studies. This includes situations when total or messenger RNA is reverse transcribed and whether the reactions are normalized or not.

4.2. Appropriate methods of cDNA synthesis for quantitative serum-stimulation studies

Various methods to synthesize cDNA for quantitative RT-PCR analysis have been reported in the literature. These include methods to amplify cDNA obtained from total [6–8] or messenger RNA [2,5]; some reports normalized the RNA content in the reverse transcription reaction [2,6,7], others did not [5,8]. As demonstrated here, internal control genes may be used to quantify cDNA synthesized from non-normalized messenger or total RNA, as long as the conditions are properly validated. Since RNA is an unstable molecule, it is advantageous to perform the quickest and least complicated method when preparing RNA for RT-PCR. The isolation of mRNA from total RNA requires the binding of the poly A RNA to oligo dT and is somewhat more complicated and expensive to perform than the isolation of total RNA. Normalization of RNA content adds two additional steps to the procedure, i.e., (i) quantification of the RNA (typically by optical density or a fluorescence assay) and (ii) pipeting different amounts of RNA and water into individual reaction tubes. Our long-term objective is to develop high-throughput quantitative RT-PCR assays in a 96-well format using serum-stimulated fibroblasts. Assays designed using cDNA synthesized from non-normalized RNA will increase throughput and reduce error.

4.3. Conclusions

We report a detailed investigation of the effects of serum on the expression of standard housekeeping genes that are typically used as internal controls in quantitative RT-PCR assays. This analysis was facilitated by the sensitivity and efficiency of real-time quantitative PCR. The methodology reported here may be applied to other quantitative RT-PCR studies where the experimental treatment affects the expression of the internal control genes. We demonstrate that certain housekeeping genes (18S rRNA and β-2 microglobulin) may be used as internal controls in quantitative gene expression studies in serum-stimulated fibroblasts, while others (β-actin and GAPDH) may not. Proper validation of internal control genes is necessary when designing quantitative gene-expression studies.

Acknowledgements

We thank Dr. Jeff Ross for providing us with the 3T3 fos–glo–myc cells and Dr. David A. Sclar for his assistance with the statistical analysis.

References

[1] Winkles JA. In: Moldave K, editor, Prog Nucleic Acid Res Mol Biol, Vol. 58, San Diego: Academic Press, 1998, pp. 41–78.
[2] Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JCF, Trent JM, Staudt LM, Hudson Jr. J, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO. The transcriptional program in the response of human fibroblasts to serum. Science 1999;283:83–7.
[3] Herrick DJ, Ross J. The half-life of c-myc mRNA in growing and serum-stimulated cells: influence of the coding and 3′ untranslated regions and role of ribosome translocation. Mol Cell Biol 1994;14:2119–28.
[4] Tirmenstein MA, Nicholls-Grzemski FA, Schmittgen TD, Zakrajsek BA, Fariss MW. Nitric oxide production by isolated rat hepatocyte suspensions. Toxicol Sci 2000;53:56–62.
[5] Winer J, Jung CK, Shackel I, Williams PM. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 1999;270:41–9.
[6] Chen CY, Del Gatto-Konczak F, Wu Z, Karin M. Stabilization of interleukin-2 mRNA by the c-Jun NH_2-terminal kinase pathway. Science 1998;280:1945–9.
[7] Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, Vidaud M. Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. Cancer Res 1999;59:2759–65.
[8] Lenz HJ, Hayashi K, Salonga D, Danenberg KD, Danenberg PV, Metzger R, Banerjee D, Bertino JR, Groshen S, Leichman LP, Leichman CG. p53 point mutations and thymidylate synthase messenger RNA levels in disseminated colorectal cancer: an analysis of response and survival. Clin Cancer Res 1998;4:1243–50.