Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Brain Research 156 (2005) 38–45

DEVELOPMENTAL BRAIN RESEARCH

www.elsevier.com/locate/devbrainres

Research report

Housekeeping gene expression during fetal brain development in the rat—validation by semi-quantitative RT-PCR

Maie Dawoud Al-Bader*, Hameed Ali Al-Sarraf

Department of Physiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat, Zip Code 13110, Kuwait

Accepted 26 January 2005
Available online 14 March 2005

Abstract

Mammalian gene expression is usually carried out at the level of mRNA where the amount of mRNA of interest is measured under different conditions such as growth and development. It is therefore important to use a "housekeeping gene", that does not change in relative abundance during the experimental conditions, as a standard or internal control. However, recent data suggest that expression of some housekeeping genes may vary with the extent of cell proliferation, differentiation and under various experimental conditions. In this study, the expression of various housekeeping genes (18S rRNA [18S], glyceraldehydes-3-phosphate dehydrogenase [G3PDH], β-glucuronidase [BGLU], histone H4 [HH4], ribosomal protein L19 [RPL19] and cyclophilin [CY]) was investigated during fetal rat brain development using semi-quantitative RT-PCR at 16, 19 and 21 days gestation. It was found that all genes studied, with exception to G3PDH, did not show any change in their expression levels during development. G3PDH, on the other hand, showed increased expression with development. These results suggest that the choice of a housekeeping gene is critical to the interpretation of experimental results and should be modified according to the nature of the study.

Theme: Cellular & molecular biology
Topic: Gene structure and function, general

Keywords: Housekeeping genes; 18S rRNA; Glyceraldehydes-3-phosphate dehydrogenase; β-Glucuronidase; Histone H4; Ribosomal protein L19; Cyclophilin

1. Introduction

Housekeeping genes are used as internal standards as they are supposed to indicate the rate of transcription of genes which are not affected by experimental conditions. Their levels are presumed to be proportional to the total amount of mRNA being examined. Matched loading based on internal controls is critical for quantitative comparisons of gene expression among different tissue types, varying developmental stages and experimentally treated cells. However, no one single housekeeping gene always manifests stable expression levels under all of these experimental conditions [17].

Accurate quantification of a true reference gene allows the normalization of differences in the amount of amplifiable RNA or cDNA in individual samples generated by: (1) different amounts of starting material, (2) the quality of the starting material and (3) differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the gene of interest.

Housekeeping genes such as G3PDH, albumin, actins, tubulins, cyclophilins, 18S rRNA and 28S RNA have been used extensively but numerous studies have shown that these genes may vary under different experimental conditions [16,23]. The housekeeping genes chosen for the present study are present in various cell organelles or compartments: RPL19 is a ribosomal protein the function of which is unclear [5]; BGLU is a lysosomal enzyme [13]; the protein CY represents 0.1–0.4% of the total cytosolic

* Corresponding author. Fax: +965 5338937.
E-mail address: albader@hsc.edu.kw (M. Dawoud Al-Bader).

0165-3806/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.devbrainres.2005.01.010

Table 1
List of housekeeping genes used in this study: suitability or unsuitability indicated by other investigators

Gene	Suitability	Unsuitability
18S rRNA	Aerts et al. [1], Schmittgen and Zakrajsek [16], Thellin et al. [21]	Unknown
Ribosomal protein L19	Szabo et al. [20]	Aloni et al. [2]
β-glucuronidase	Aerts et al. [1]	Unknown
Cyclophilin	Botte et al. [3], Steele et al. [18]	Jakubowski et al. [10], Vehaskari et al. [22], Zhong and Simons [23]
Histone	Robert et al. [15]	Unknown
Glyceraldehyde-3-phosphate dehydrogenase	Kumar and Joyner [12], Thellin et al. [21]	Aerts et al. [1], Calvo et al. [4], Schmittgen and Zakrajsek [16], Steele et al. [18], Suzuki et al. [19], Szabo et al. [20], Zhong and Simons [23]

protein of most eukaryotic tissues [9], however, its role is unknown; HH4 plays a central role in nucleosome formation [11] and is the most highly conserved of the five histones with respect to amino acid sequence [8]; lastly, G3PDH is a key enzyme in the control of glycolysis [7]. Table 1 shows the housekeeping genes used in this study and their suitability or otherwise as indicated by other investigators under various experimental conditions.

Thus, the objective of this study was to use RT-PCR methodology to measure the mRNA levels of several housekeeping genes during fetal rat brain development in order to identify the genes that are most suitable as endogenous standards for further gene expression studies.

2. Materials and methods

2.1. Materials

General laboratory chemicals were purchased from Merck (Dagenham, Essex) and all fine chemicals were obtained from Sigma Chemical (Poole, Dorset). All buffers, enzymes and reagents used in reverse-transcription PCR experiments were purchased from Gibco and AmpliWax PCR gem 50 was purchased from Perkin-Elmer.

2.2. Animal model and tissue collection

Sprague–Dawley rats were obtained from Bantin and Kingman (UK). The rats were housed in the Animal Resources Centre at the Faculty of Medicine, Kuwait University and had free access to food and water. The rats were maintained on a cycle of 12 h light and 12 h darkness at 22 °C. The experiments were carried out in accordance with the rules of laboratory animal care in this institution.

Female rats were mated with males and mating was verified by the presence of sperm in the vaginal smear; this was designated as day 0 of pregnancy. Pregnant dams were stunned and killed by cervical dislocation at 16, 19 or 21 days gestation (dg). Uterine horns containing conceptuses were removed and placed immediately on ice. Fetuses and placentae were separated. After determination of fetal body weight, fetal brains were dissected and weighted. Tissues from each litter were pooled (four pregnancies were obtained at each gestational age [$n = 4$]). Samples were frozen at −70 °C for subsequent analysis.

2.3. RNA isolation and quantification

The method of Chomczynski and Sacchi [6] was used. Briefly, samples were homogenized in denaturing solution (4 M guanidine thiocyanate salt, 25 mM sodium citrate, pH 7.0, 0.5% w/v sarcosyl and 0.1 M 2-mercaptoethanol) using a sterile hand-held homogenizer. To 3.6 ml homogenate, 0.36 ml sodium acetate (2 M, pH 4.0), 3.6 ml citrate buffer-saturated phenol (pH 4.3) and 0.72 ml chloroform/isoamyl alcohol (49:1) were added sequentially, shaking well between each addition. Tubes were vigorously shaken for 15 s after the final addition. Tubes were kept on ice for 15 min, then centrifuged (10,000 × g for 20 min at 4 °C). The aqueous phase was removed, avoiding the DNA interphase, and an equal volume of ice-cold isopropanol was added. After shaking, tubes were kept at −20 °C for >1 h. The precipitate was collected by centrifugation (10,000 × g for 20 min at 4 °C) and dissolved in 0.3 ml denaturing solution. Nucleic acid was re-precipitated by adding an equal volume of ice cold-isopropanol. After >1 h at −20 °C, the samples were centrifuged (10,000 × g for 10 min at 4 °C). The pellet was washed twice with 1 ml 75% (v/v) ethanol (at −20 °C) by suspension/centrifugation. The final pellet was air-dried then dissolved in 0.5% (w/v) SDS (0.25 μl/mg wet weight tissue) at 65 °C for 15 min. Extracted RNA was stored at −70 °C. The quality and quantity of total RNA sample were determined using spectroscopic measurements at 260 and 280 nm. Samples with A_{260}/A_{280} ratios >1.7 were only studied further. The integrity of total RNA was checked by agarose gel electrophoresis and 28S and 18S rRNAs visualized after ethidium bromide staining.

2.4. RT-PCR

SDS was removed from total RNA by precipitation with sodium acetate–isopropanol then resuspended (at ca. 1 μg/μl) in water. The RNA concentration was determined by spectrophotometery and was adjusted to 0.5 μg/μl with water. All samples were DNase-treated before reverse transcription. Briefly, 2 μg of total RNA was mixed on

ice with 40 U of RNasin, 1 U of DNase and 1× DNase buffer in a final volume of 20 μl. The mixture was left at room temperature for 15 min and the reaction was terminated by adding 2 μl of 25 mM EDTA and heating at 70 °C for 10 min. The DNase-treated sample was divided into two 11 μl aliquots, 100 ng random hexamers was added and the mixture was heated at 70 °C for 10 min, immediately chilled on ice for >3 min then briefly centrifuged. With the tube on ice, the following were added: 1× first strand buffer (25 mM Tris–HCl pH 8.3, containing 37.5 mM KCl and 1.5 mM $MgCl_2$), 5 mM DTT and 500 μM dNTP mix. To one tube (RT^+ reaction), 200 U Superscript II RNase H^- reverse transcriptase were added, whereas water was added to the other tube (control RT^- reaction) in a final volume of 20 μl. After gentle mixing, reactions were incubated at room temperature for 10 min then at 42 °C for 50 min. Reactions were terminated by heating at 70 °C for 15 min.

Initially, the optimal concentrations of magnesium chloride ($MgCl_2$), primers and deoxynucleotide triphosphates (dNTP) were determined. The amount of PCR product over a range of amplification cycles was examined and a cycle number that falls in the linear range was chosen. A pooled sample of 16, 19 and 21 dg fetal rat brain reverse transcribed RNA was used. This pooled cDNA sample was diluted 1 in 6 allowing the same sample to be used for all cycle number optimization experiments. The RT^- sample was used neat. In addition, a water sample was used as a negative control. The amount of PCR product was measured and calibrated and the intensity of the bands optic densities (od) was plotted against the cycle number (detailed below in the Data analysis section). The amount of template dilution was also varied to verify linearity of the reaction with respect to the amount of product. The sample was used neat and at various dilutions: 3, 6, 12 and 24 fold. Again, the RT^- sample was used neat and a water sample was used as a negative control.

The PCR reaction was carried out in a programmable thermal cycler (Perkin-Elmer, model 9700) using a hot start protocol. The lower reaction mixture consisted of: 1× PCR buffer (20 mM Tris/50 mM KCl), 3 mM $MgCl_2$, 0.5 mM dNTPs and 0.3 μM each of upper and lower primers in a final volume of 10 μl. An AmpliWax PCR gem 50 wax bead was added and the tubes were incubated at 80 °C for 5 min then cooled to room temperature. The upper reaction mixture (15 μl volume) consisting of: 1× PCR buffer, 0.5 μl template (RT^+ or RT^-) and 1.25 U recombinant *Taq* DNA polymerase was then added on top of the wax. The PCR reactions were then cycled as follows: 5 min at 94 °C (1 cycle); 30 s at 94 °C (denaturation step), 30 s at the appropriate annealing temperature for the primer set under study (annealing step) and 1 min at 72 °C (extension step) for the required number of cycles (see Table 2 for annealing temperatures and cycle numbers for the different genes studied). Tubes were then incubated for a further 7 min at 72 °C (1 cycle).

2.5. Data analysis

After the PCR reaction, PCR products were electrophoresed alongside a 100 bp DNA marker (100 bp ladder, Gibco) through a 2% (w/v) low electroendosmosis (LE; Boehinger Mannheim) agarose gel stained with ethidium bromide. Images were captured using Gene Genius Bio Imaging System and od values of PCR products were measured using Gene Tools Software. Statistical analysis was performed using SPSS (ANOVA followed by LSD post-hoc analysis when the test for homogeneity of variance was fulfilled and using Games–Howell post-hoc analysis when the homogeneity of variances was not attained). All values are expressed as mean ± SEM and a P value of < 0.05 was taken as the minimum level of significance.

3. Results

When RNA yield/g brain was studied (Fig. 1), it was found that the RNA concentration was significantly lower at

Table 2
Upper and lower primer sequences, annealing temperature, cycle number and expected product size for the different genes studied

Gene	Primer	Sequence (5′–3′)	Nucleotides of coding sequence (bp)	Annealing temperature (°C)	No. of cycles	Expected product size (bp)
18S	Upper	GTCCCCCAACTTCTTAGAG	1436–1454	52	26	419
	Lower	CACCTACGGAAACCTTGTTAC	1834–1855			
RPL19	Upper	ATCGCCAATGCCAACTCT	157–174	52	33	321
	Lower	GAGAATCCGCTTGTTTTTGAA	457–477			
BGLU	Upper	ATCGCCATCAACAACACAC	511–530	52	34	529
	Lower	TGACGCCTTGGAAGTAGAAAG	1019–1040			
CY	Upper	CAACCCCACCGTGTTCTTCG	48–68	59	24	369
	Lower	TTGCCATCCAGCCACTCAGTC	396–417			
HH4	Upper	ACGCCTGTGGTCTTAATCAG	108–129	59	39	250
	Lower	GCGGGTCTCCTCGTAGATGAG	337–358			
G3PDH	Upper	AGGGCTGCCTTCTCTTGTG	135–154	57	26	968
	Lower	GGGTGGTCCAGGGTTTCTTAC	1082–1103			

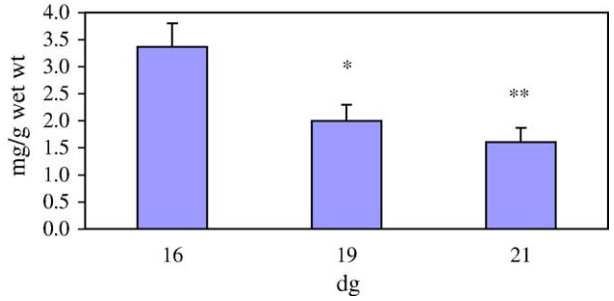

Fig. 1. Figure showing fetal brain RNA concentration. Values are mean ± SEM (n = 4). A significant decrease in RNA concentration was seen between 19 and 16 dg ($P < 0.01$) and between 21 and 16 dg ($P < 0.001$).

19 and 21 dg compared to 16 dg (by 38%, $P < 0.01$ and 51%, $P < 0.001$, respectively).

PCR reactions were carried out for an increasing number of cycle steps (two cycle increments) for all genes studied, however, only the results for 18S are presented here. For 18S rRNA, cycle number was increased from 20–34 cycles and product was first detected at cycle number 24 increasing linearly up to cycle 32 (Fig. 2). From these experiments, a cycle number was chosen that yielded sufficient product for ethidium bromide visualization while falling well within the linear range (for optimal cycle number for the other genes studied see Table 1). In addition, from the optimal template dilution experiments, it was decided that for all genes studied a 6-fold dilution yields enough product and falls within the linear range (Fig. 3 shows 18S results). This dilution was used for all further experiments alongside the standard conditions and optimized cycle numbers.

All genes studied were detected in fetal rat brains from as early as 16 dg (Figs. 4–9). The RT$^-$ reactions did not show any bands for all genes studied (figures not shown), indicating that the amplified products in RT$^+$ reactions are not due to genomic DNA. There was no significant difference in gene expression with age when absolute od values of PCR products were plotted against dg (Figs. 4–9; B). However, because there was no correlation between RNA yield/g brain and gene expression (correlation graphs not shown), it was decided to use 18S ribosomal RNA as an internal standard as at least 70% of the total RNA is 18S and the expression of this gene was constant with age.

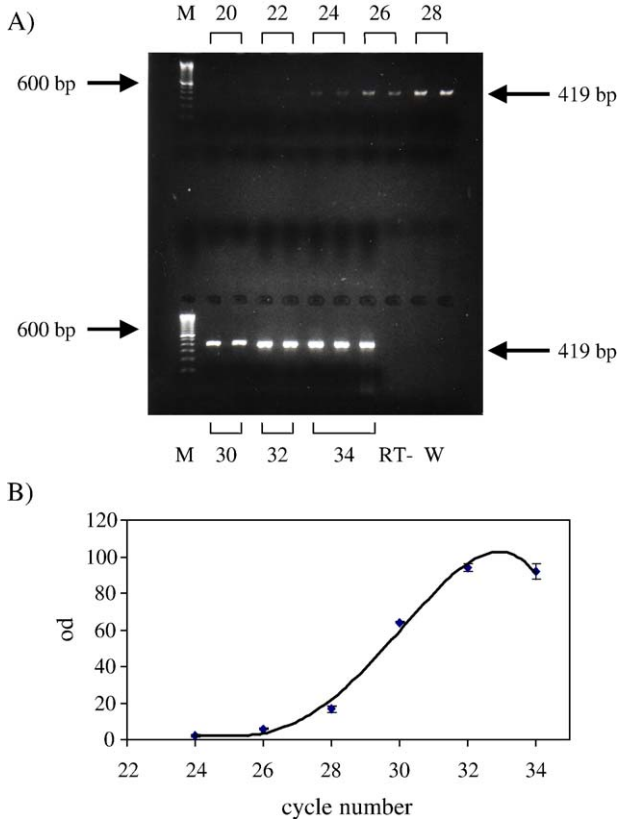

Fig. 2. RT-PCR analysis of 18S rRNA: effect of increasing cycle number. Reactions were performed with a cDNA sample (from 16, 19 and 21 dg fetal brain) for various cycle numbers at 52 °C, using standard reaction conditions. (A) Ethidium bromide stained gel. Cycle numbers are indicated at the top and bottom of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 419 bp). RT$^-$ and water (W) are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate except cycle no. 34 in triplicate. RT$^-$ and water samples were run in singlicate for 34 cycles. (B) Product–input relationship. The amount of product showed a linear relationship with cycle number between cycles 26 and 32.

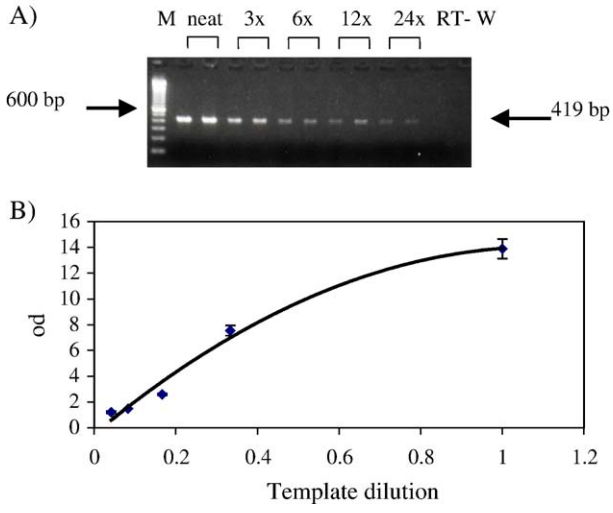

Fig. 3. RT-PCR analysis of 18S rRNA: effect of increasing template dilution. Reactions were performed with a range of dilutions of pooled cDNA sample (from 16, 19 and 21 dg fetal brain) for 26 cycles at 52 °C, using standard reaction conditions. (A) Ethidium bromide stained gel. Template dilution is indicated at the top of the gel; the migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected product size is 419 bp). RT$^-$ and water (W) are reverse transcriptase minus (undiluted) and water controls, respectively. Each dilution of sample was run in duplicate, RT$^-$ and water samples were run in singlicate. (B) Product–input relationship. The amount of product showed a linear relationship with template dilution up to 3-fold dilution. A template dilution of 1 corresponds to undiluted sample.

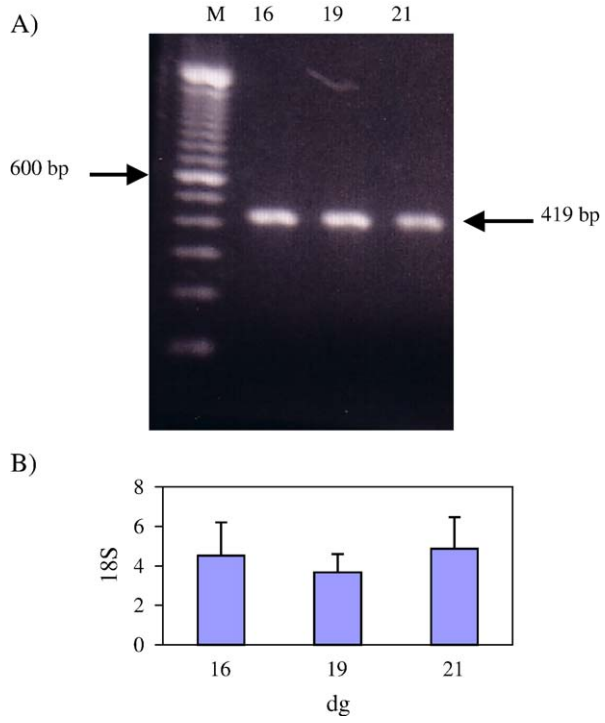

Fig. 4. Expression of 18S rRNA transcripts in fetal rat brain. Reactions were performed for 26 cycles at 52 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 419 bp). (B) Expression level of 18S rRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages.

Thus, the od measurements of PCR products obtained for all housekeeping genes were expressed relative to the od obtained from 18S.

When relative gene expression was plotted (Figs. 5–9; C), it was found that the only gene showing significant variation with age was the G3PDH gene (Fig. 7C). A significant increase in the expression of this gene was detected between 16 and 21 dg ($P < 0.05$).

4. Discussion

In this study, the expression of several housekeeping genes in fetal rat brain was investigated to determine the most suitable gene that can be used as an internal control. The use of the polymerase chain reaction (PCR) has enabled researchers to work with scarce tissue with efficient detection of specific transcripts. However, the amplification plateau phase which appears in later cycles renders the approach more qualitative than quantitative. Thus, a simple approach to prevent over-amplification is to restrict the number of PCR cycles by previously evaluating the optimal cycle number and determining the optimal template concentration/dilution in order to choose both a cycle number and template dilution that yields sufficient product while falling within the linear range of amplification.

The decrease in RNA yield/g brain seen with development indicates that the rRNA, which constitutes the bulk of total RNA, is decreasing. However, when the expression levels of 18S rRNA were studied, there was no sign that the levels decreased. The use of 18S rRNA is therefore recommended as an internal standard for mRNA quantification studies since mRNA variations are comparatively weak and consequently are unable to modify the total RNA level to a great extent. Thus, if mRNA ratios of housekeeping genes relative to 18S rRNA are constant or show no statistical significance then they may be used as standards, but when the ratios vary then it is best to refer to the 18S rRNA as an internal standard [21]. Accordingly, it is necessary to characterize the suitability of various housekeeping genes to serve as internal RNA controls under

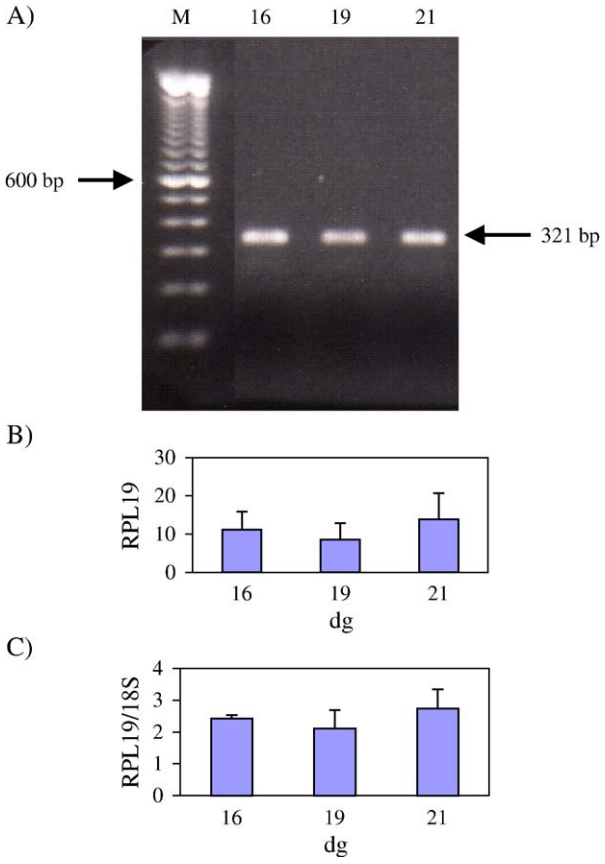

Fig. 5. Expression of RPL19 transcript in fetal rat brain. Reactions were performed for 33 cycles at 52 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 321 bp). (B) Expression level of RPL19 mRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages. (C) Relative RPL19 mRNA level. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between groups.

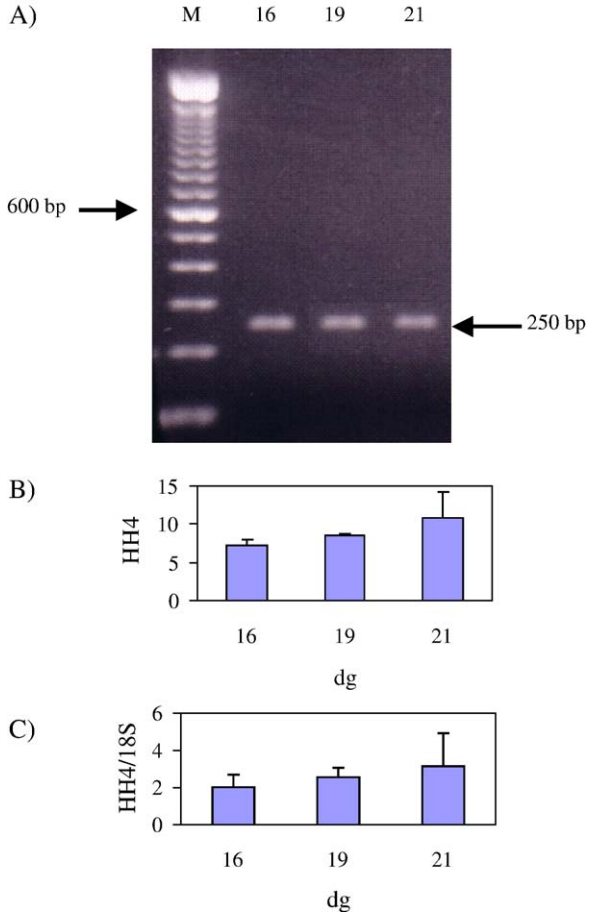

Fig. 6. Expression of HH4 transcript in fetal rat brain. Reactions were performed for 39 cycles at 59 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 250 bp). (B) Expression level of HH4 mRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages. (C) Relative HH4 mRNA level. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between groups.

particular experimental conditions where transcription effects are being tested.

It is indeed very important to study the expression of housekeeping genes during fetal rat brain development as there is lack of research in this area. Many investigators attempt to use housekeeping genes as internal controls however, the suitability of these controls should first be validated under different conditions and especially during ontogeny of various organs. When the levels of housekeeping genes were expressed relative to 18S, it was found that the only gene which showed significant variation with development was that of G3PDH. G3PDH has been used in various studies as a housekeeping gene as it functions as a glycolytic intermediate expected to be present in all cells and exhibiting minimal modulation. However, G3PDH is now known to be involved in other non-glycolytic activities and is therefore prone to show variations in mRNA expression (reviewed in [19]) and has also been shown to be an unsuitable housekeeping gene in many other animal and human models (Table 1). In addition, it has been shown that in fetal organs there is high G3PDH expression due to high rates of aerobic glycolysis during development [14] which could explain the increased expression seen in our study with development. In comparison, studies on postnatal development of rabbit heart have shown that this gene is stable and is indeed a suitable housekeeping gene for that particular study [12].

The other genes studied (18S, RPL19, BGLU, CY and HH4) were most consistently expressed during development and can thus be considered as suitable internal controls for this experimental model. Other investigators have shown that CY is a reliable control in human differentiating

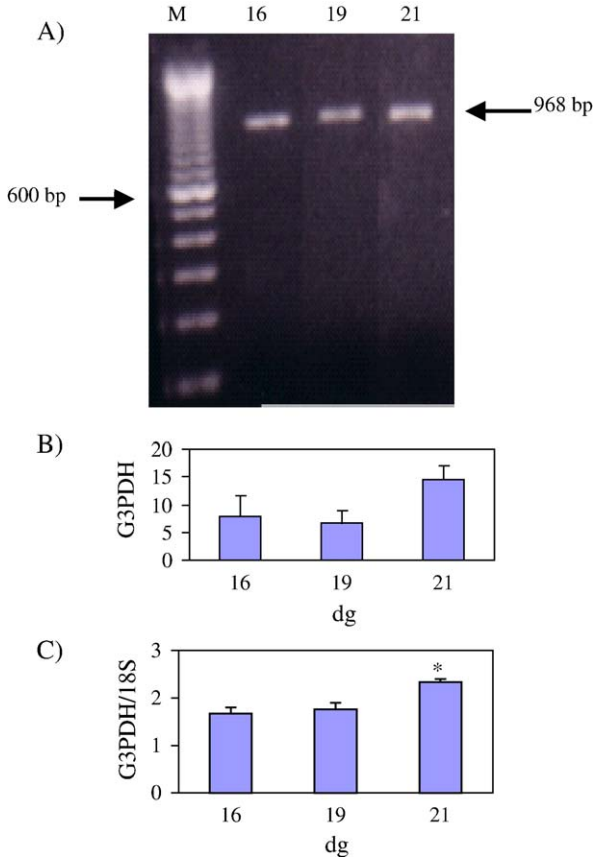

Fig. 7. Expression of G3PDH transcript in fetal rat brain. Reactions were performed for 26 cycles at 57 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 968 bp). (B) Expression level of G3PDH mRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages. (C) Relative G3PDH mRNA level. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± SEM (n = 4). A significant increase in expression of G3PDH was seen between 16 and 21 dg ($P < 0.05$).

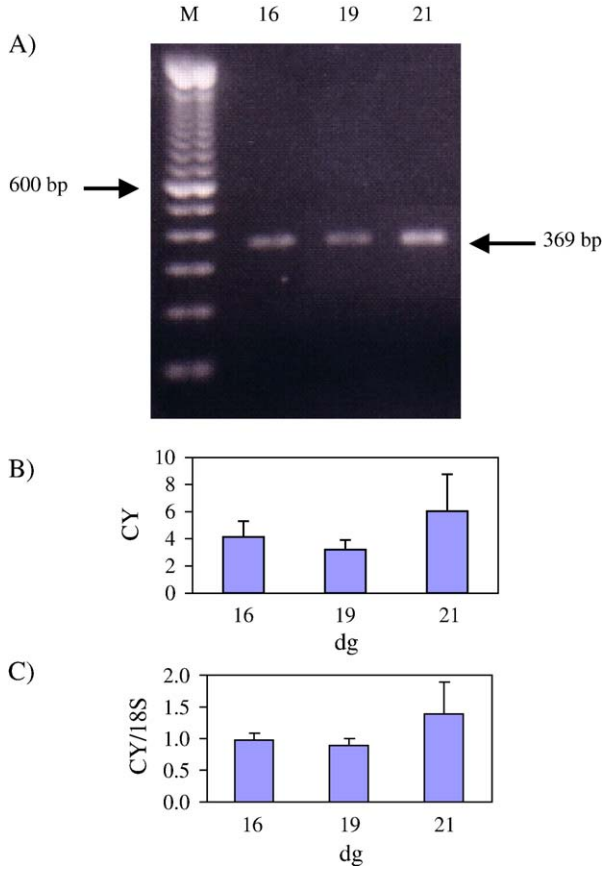

Fig. 8. Expression of CY transcript in fetal rat brain. Reactions were performed for 24 cycles at 59 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 369 bp). (B) Expression level of CY mRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages. (C) Relative CY mRNA level. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between groups.

epithelium [18] and in developing rat testis and ovaries [3], Histone H2a is suitable during the development of bovine pre-implantation embryos [15], RPL19 was stable when studying breast tumor samples [20] and BGLU was suitable in various tumor cell lines [1]. Although these genes have not been studied broadly, rendering it more difficult to draw conclusions on their suitability or otherwise as internal controls, some researchers have shown that they are unsuitable as internal controls in various animal models [2,10,22,23]. On the other hand, G3PDH and 18S have been extensively used as internal controls by many investigators (Table 1).

In summary, it is necessary to characterize the appropriateness of various housekeeping genes to serve as internal RNA controls in RT-PCR experiments for the comparison of mRNA levels. Our results confirm the need to optimize not only the PCR conditions of the tested gene, but also the cycle number and cDNA template dilution. It can be concluded from this study that G3PDH is not a suitable housekeeping gene to be used during fetal brain development while 18S, RPL19, BGLU, CY and HH4 are reasonably stable housekeeping genes suitable for normalization when quantifying mRNA levels of genes expressed in developing fetal brain.

Acknowledgments

Financial support for this study was provided by the Kuwait University Grant No MY02/99 and the Department of Physiology, Faculty of Medicine.

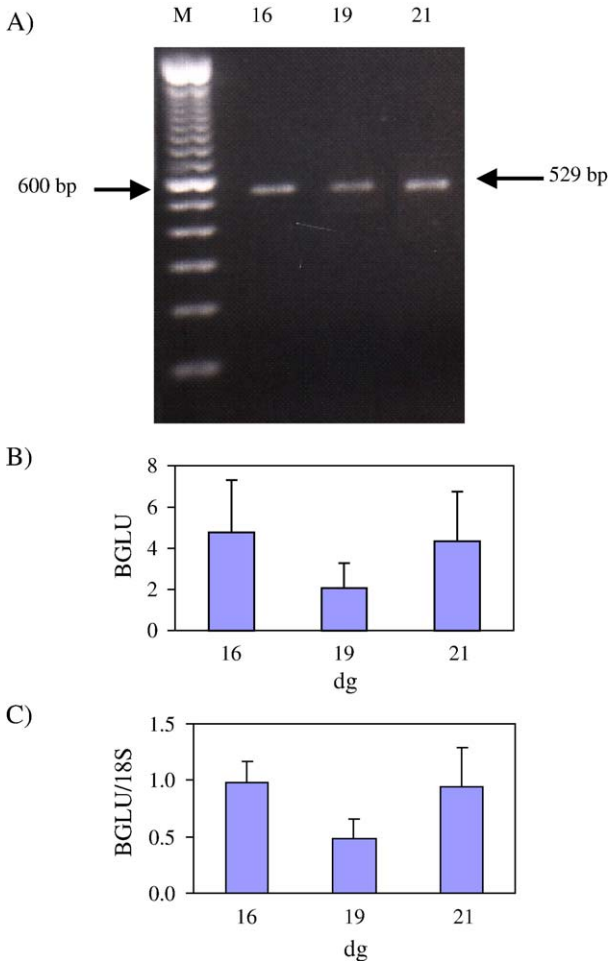

Fig. 9. Expression of BGLU transcript in fetal rat brain. Reactions were performed for 34 cycles at 52 °C, using standard reaction conditions. (A) Representative ethidium bromide stained gel. The migration of the 100 bp marker (M) is shown on the left-hand side and the calculated size of the signal indicated on the right-hand side of the gel (expected size is 529 bp). (B) Expression level of BGLU mRNA. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between ages. (C) Relative BGLU mRNA level. The amount of product was expressed relative to 18S. Samples were run in duplicates; results shown are mean ± SEM (n = 4). No significant difference was detected between groups.

References

[1] J.L. Aerts, M.I. Gonzales, S.L. Topalian, Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR. Biotechniques. 36 (2004) 84–86, 88, 90–91.

[2] R. Aloni, D. Peleg, O. Meyuhas, Selective translational control and nonspecific posttranscriptional regulation of ribosomal protein gene expression during development and regeneration of rat liver, Mol. Cell. Biol. 12 (1992) 2203–2212.

[3] M.C. Botte, A.M. Chamagne, M.C. Carre, R. Counis, M.L. Kottler, Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary, J. Endocrinol. 159 (1998) 179–189.

[4] E.L. Calvo, C. Boucher, Z. Coulombe, J. Morisset, Pancreatic GAPDH expression during ontogeny and acute pancreatitis induced by caerulein, Biochem. Biophys. Res. Commun. 235 (1997) 636–640.

[5] Y.L. Chan, A. Lin, J. McNally, D. Peleg, O. Meyuhas, I.G. Wool, The primary structure of rat ribosomal protein L19. A determination from the sequence of nucleotides in a cDNA and from the sequence of amino acids in the protein, J. Biol. Chem. 262 (1987) 1111–1115.

[6] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[7] P. Fort, L. Marty, M. Piechaczyk, S. el Sabrouty, C. Dani, P. Jeanteur, J.M. Blanchard, Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family, Nucleic Acids Res. 13 (1985) 1431–1442.

[8] S. Grimes, P. Weisz-Carrington, H. Daum III, J. Smith, L. Green, K. Wright, G. Stein, J. Stein, A rat histone H4 gene closely associated with the testis-specific H1t gene, Exp. Cell Res. 173 (1987) 534–545.

[9] M.W. Harding, R.E. Handschumacher, D.W. Speicher, Isolation and amino acid sequence of cyclophilin, J. Biol. Chem. 261 (1986) 8547–8555.

[10] M. Jakubowski, M. Blum, J. Roberts, Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain, Endocrinology 128 (1991) 2702–2708.

[11] R.D. Kornberg, Structure of chromatin, Annu. Rev. Biochem. 46 (1977) 931–954.

[12] R. Kumar, R.W. Joyner, Expression of protein phosphatases during postnatal development of rabbit heart, Mol. Cell. Biochem. 245 (2003) 91–98.

[13] V.A. McKusick, G.F. Neufeld, in: J.B. Wyngaarden, D.D. Fredrickson, J.L. Goldstein, M.S. Brown (Eds.), The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 1983, pp. 751–777.

[14] R.K. Murray, Biochemical properties of cancer cells, in: I.F. Tannock, R.P. Hill (Eds.), The Basic Science of Oncology, Pergamon Press, New York, 1987, pp. 176–191.

[15] C. Robert, S. McGraw, L. Massicotte, M. Pravetoni, F. Gandolfi, M.A. Sirard, Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos, Biol. Reprod. 67 (2002) 1465–1472.

[16] T.D. Schmittgen, B.A. Zakrajsek, Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR, J. Biochem. Biophys. Methods 46 (2000) 69–81.

[17] E. Spanakis, Problems related to the interpretation of autoradiographic data on gene expression using common constitutive transcripts as controls, Nucleic Acids Res. 21 (1993) 3809–3819.

[18] B.K. Steele, C. Meyers, M.A. Ozbun, Variable expression of some "housekeeping" genes during human keratinocyte differentiation, Anal. Biochem. 307 (2002) 341–347.

[19] T. Suzuki, P.J. Higgins, D.R. Crawford, Control selection for RNA quantitation, BioTechniques 29 (2000) 332–337.

[20] A. Szabo, C.M. Perou, M. Karaca, L. Perreard, J.F. Quackenbush, P.S. Bernard, Statistical modeling for selecting housekeeper genes, Genome Biol. 5 (2004) R59.

[21] O. Thellin, W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout, E. Heinen, Housekeeping genes as internal standards: use and limits, J. Biotechnol. 75 (1999) 291–295.

[22] V.M. Vehaskari, J.M. Hempe, J. Manning, D.H. Aviles, M.C. Carmichael, Developmental regulation of ENaC subunit mRNA levels in rat kidney, Am. J. Physiol. 274 (1998) C1661–C1666.

[23] H. Zhong, J.W. Simons, Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia, Biochem. Biophys. Res. Commun. 259 (1999) 523–526.