Review

Control Selection for RNA Quantitation

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ABSTRACT

The study of mammalian gene expression is often carried out at the level of mRNA. In such analyses, one usually measures the amount of an mRNA of interest under different conditions such as stress, growth, development, cell and tissue localization or as part of an evaluation of the effects of gene transfection. A variety of techniques exist to measure gene expression and most commonly involve Northern hybridization analysis, ribonuclease protection or RT-PCR. Common to all of these assays is the inclusion of a so-called loading or internal control (i.e., analysis of an mRNA that does not change in relative abundance during the course of treatments). Here, we discuss the uses and pitfalls of the most popular of these controls, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and b-actin, with special emphasis on precautions associated with the use of GAPDH.

INTRODUCTION

Several controls for RNA quantitation have gained popularity over the years, as evidenced by their use in a representative and random sampling of recent papers from several highly cited journals. The studies selected were restricted to mammalian cells and included Northern hybridization analysis, ribonuclease protection and RT-PCR. The results are listed in Table 1 and indicate that quantitative measurement of transcripts encoding GAPDH and β-actin was most often used as a normalizing control. GAPDH is an important glycolytic enzyme that catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. β-actin is a major cytoplasmic structural protein.

In this laboratory, we have used most of the controls listed in Table 1, but similar to the general consensus, most often use GAPDH. Our use of GAPDH as a control for studies assessing the effect of the calcium stress agents, thapsigargin and cyclopiazonic acid (CPA), on hamster HA-1 cells is shown in Figure 1A. Here, a sharp, single and readily detected GAPDH band is observed. These characteristics of GAPDH mRNA expression, combined with its designation as a housekeeping gene, explain its popularity. As is often done, and as underscores the importance of using controls, the results of the mRNA levels of interest (*adapt78* and *grp78*) were normalized to GAPDH and plotted (Figure 1, B and C). Our conclusion regarding the effects of thapsigargin and CPA on these cells was based on these normalized ratios.

Despite the satisfactory use of GAPDH as a normalization control in this example, we have occasionally observed aberrant results during an analysis of cellular responses to certain stress or growth-arresting conditions. In these situations, the levels of GAPDH mRNA changed, even though equal loading of mRNA sample per lane of an electrophoresis gel was confirmed by several other criteria including spectrophotometric quantitation of loaded RNA, ethidium bromide staining, replicate assessments and the analysis of other transcripts listed in Table 1. For these particular studies, it was obvious that GAPDH was not an appropriate control. This led us to consider the probability that, despite its wide use, GAPDH is not always an ideal or even suitable normalizer.

GAPDH AS A CONTROL NORMALIZER

The prime requirement for any control transcript is maintenance of a consistent expression level under all experimental conditions. A comprehensive literature analysis, however, reveals that GAPDH mRNA levels modulate under many circumstances both in vitro and in vivo. In vitro, cultured vascular endothelial cells exposed to hypoxic conditions and transition metals upregulate a subset of stress proteins, including GAPDH (19). The transition metal manganese also induces GAPDH in astrocytes (20). In the glucose-responsive β-cell line INS-1, glucose induces GAPDH mRNA fourfold (33). GAPDH expression is induced by low doses of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ D_3) in BT-20 human breast carcinoma cells (12). Insulin stimulates GAPDH

expression in adipocytes and hepatoma cell lines (2). GAPDH gene expression also increased during programmed neuronal cell death (23). Heat shock induces GAPDH mRNA in BALB/c 3T3 cells at a time point that is considerably later than that observed for Hsp70 (30). Oxidative stress generated by diethyldithiocarbamate in aortic vessel rings increases the steady-state levels of GAPDH mRNA (24). Nakai et al. (30) observe a strong induction of GAPDH mRNA by an iron-chelating agent, namely α, α′ dipyridyl. Ultraviolet light decreases the level of GAPDH mRNA in human keratinocytes (16). The same cell type exhibits an elevation in the level of GAPDH mRNA following exposure to the potent dioxin compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (29). Platelet-derived growth factor increases the mRNA level of GAPDH in rat smooth muscle cells (32). GAPDH mRNA is elevated by interleukin-2 treatment of murine T lymphocyte (35). GAPDH levels are also increased by NAD^+ precursors in Jurkat cells (42) and by norepinephrine, triiodothyronine and insulin in differentiating brown adipocytes (3). The most documented modulator of GAPDH levels is probably cellular proliferation. An analysis of GAPDH mRNA levels in quiescent cell cultures that are stimulated to reenter the cell cycle following serum addition indicates apparent cell cycle-dependent regulation of the expression of this gene (28,34).

In vivo, triiodothyronine increases steady-state levels of GAPDH mRNA in mouse skeletal muscle (36). Pancreatic GAPDH mRNA levels increase

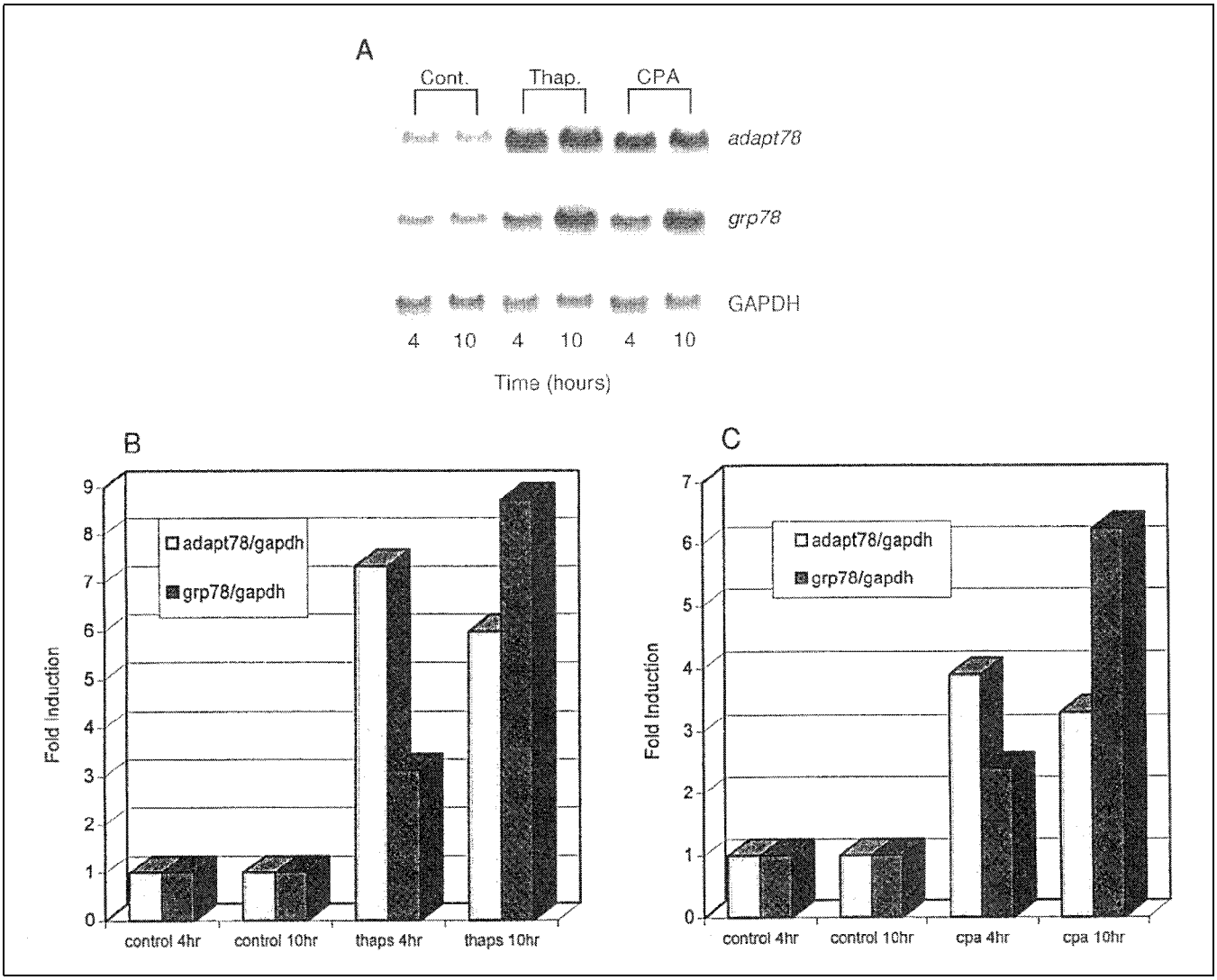

Figure 1. Example of the use of GAPDH as a loading control: the effect of inhibitors of the endoplasmic reticulum calcium-ATPase on the levels of *adapt78* and *grp78* mRNAs in HA-1 hamster cells. HA-1 hamster cells were exposed to 300 nM thapsigargin (Thap.) and 100 µg/mL CPA, both inhibitors of the endoplasmic reticulum calcium-ATPase, for the times indicated. Total RNA was then extracted, electrophoresed, blotted and probed with radiolabeled cDNA to *adapt78*, *grp78* and GAPDH as described (10,26). (A) Northern blot results. (B) Densitometric analysis of *adapt78*/*grp78* induction by thapsigargin. The *adapt78* signal was divided by the corresponding GAPDH signal, and the resulting values for untreated cells 4 and 10 h after experiment initiation were arbitrarily set at a value of 1. Fold inductions were then calculated by comparing the *adapt78*/GAPDH ratio for thapsigargin with the same time control values. (C) Densitometric analysis of *adapt78*/*grp78* induction by CPA. Results are representative of three experiments (reproduced from reference 26).

following acute pancreatic induction in rats using caerulein injection (6). In pregnant ewes, the level of GAPDH mRNA and protein is increased in uterine artery endothelial cells (5). Food-deprived rats exhibit a decrease in the level of GAPDH mRNA but not 18S rRNA (41). Intramuscular injection of the immunosuppressant rapamycin in rat increases the levels of GAPDH mRNA in the liver (15). GAPDH expression was also stimulated in the fat of diabetic rats treated with insulin (2) and in rat brain after oral adminstration of imipramine (38). Finally, the spleen of tumor-implanted mice contained significantly elevated GAPDH levels (1).

While these data represent acute modulations of GAPDH, constitutive differences in cellular GAPDH expression have been reported, most notably in transformed cells in which GAPDH is characteristically elevated. Higher levels of GAPDH have been reported in cancer of the lung, pancreas, prostate, cervix and Hodgkin's lymphoma as compared with nontransformed cells (25). Elevated GAPDH also occurs in a human breast carcinoma cell line and a variety of tumorigenic and highly malignant mouse 10 T1/2 cell lines (4,12). The levels of GAPDH mRNA in human hepatocellular carcinoma tissues is significantly higher than in adjacent non-hepatocellular carcinoma tissues and normal liver tissues (18). Such constitutive elevations of GAPDH probably relate, at least to some extent, to the elevated glycolytic activity characteristic of tumor cells (25) as well as with the above association of GAPDH levels with proliferation because cancers usually represent a more rapidly dividing cell type.

The usefulness of GAPDH as a control appears to be further compromised by a growing list of activities now associated with the protein, including reported functions in endocytosis, translational control, nuclear tRNA export, DNA replication, DNA repair, apoptosis and, of course, glycolysis (37). Measurement of GAPDH transcript abundance was originally selected as a normalizer for other expressions because it encodes for a protein with a housekeeping function; that is, it is a glycolytic intermediate and is therefore expected to be present in all cells and exhibit minimal modulation. Other potential activities for GAPDH were not appreciated at this time. The newly uncovered non-glycolytic activities are more likely to be susceptible to modulation and may explain many of the variations cited above in GAPDH mRNA and protein levels. For example, there is evidence that at least one non-glycolytic activity of GAPDH leads to modulation because a nuclear GAPDH, which would not be involved in glycolysis, is elevated in hypoxic endothelial cells (19).

Table 1. Relative Use of Different Loading Controls

Control	Number of uses	Relative frequency of use*
GAPDH	148	33%
β-actin	146	32
18S rRNA	63	14
28S rRNA	51	11
36B4	8	2
tubulin	3	1
other	33	7

The relative frequency of use of different loading controls in Northern hybridization analysis, ribonuclease protection or RT-PCR was determined by examining 1999 issues of each of the following journals: *Cell, Science, Nature, Proceedings of the National Academy of Sciences, Journal of Biological Chemistry* and *Molecular and Cellular Biology*.

*Defined as the number of uses of that control divided by the number of uses of all loading controls.

ACTIN AS A CONTROL NORMALIZER

The above results clearly indicate a need for caution when using GAPDH as a control. Because β-actin is the other most frequently used control, it was important to assess its modulation as well. Compared with GAPDH, our literature search found significantly fewer examples of β-actin modulation when it was used as a control for RNA quantitation. In vitro, β-actin mRNA levels increase following hypoxia (44) and decrease in human uroepithelial cell lines in response to bacterial but not cytokine exposure (21). In both of these studies, even greater changes in GAPDH mRNA levels were observed. β-actin mRNA levels decrease in Syrian hamster embryo cells exposed to ionizing radiation (40). As for GAPDH, β-actin mRNA levels also increase following the exposure of low serum-cultured mammalian cells to individual growth factors or high serum (8,13,27).

In vivo, β-actin mRNA increases significantly in the pancreas following a supramaximal dose of cerulein, an agent that leads to an acute interstitial pancreatitis (43), and increases in liver from rats with vitamin B6 deficiency (31). Again, GAPDH mRNA was also found to increase in these model systems. Additionally, β-actin mRNA levels were found to increase in the adrenal glands of hypophysectomized rats exposed to adrenocorticotropin (22), to increase in rat myocardium following abdominal aortic banding (7) and to increase in the granulosa cells of rats injected with gonadotrophin (11).

SELECTION CONSIDERATIONS AND CONCLUSIONS

Clearly, GAPDH and β-actin are not perfect RNA quantitation controls because of their modulation under a variety of conditions, although this variation is much more prevalent for GAPDH. In general, therefore, β-actin is probably the superior control choice. However, selection strongly depends on the system being studied and other circumstances; both β-actin and GAPDH can be useful, perhaps in combination, when used with caution as outlined below. Other such considerations include relative steady-state actin levels, actin mRNA size, drawbacks associated with the use of rRNA controls and comparisons between different cell or tissue types. Indeed, constitutive steady-state β-actin mRNA levels are high in many cells, and, as such, transcriptional modulators may only have minimal effect on abundance. This was demonstrated by Crawford and Cerutti (9) in mouse epidermal JB6 cells exposed to active oxygen. Thus, β-actin may still be a

useful control despite modulation of β-actin gene transcription by the agent or condition of interest.

On the other hand, the size of β-actin mRNA is similar to 18S rRNA, which sometimes leads to an aberrantly shaped band or even multiple bands on total RNA Northern blots. As shown in Table 1, 18S and 28S rRNAs are also popular controls but have their own drawbacks as well. 18S and 28S are ribosomal, not messenger, RNAs; they are therefore not always representative of the overall cellular mRNA population. Significant changes in the levels of particular mRNAs may not be accompanied by the same change, or even any change, in rRNAs. Since various conditions lead to the modulation of GAPDH and β-actin mRNA levels (as outlined above), similar caution should be used for rRNA and other controls. Finally, investigators may want to compare constitutive mRNA expression across tissues. For these studies, there is evidence that GAPDH is superior to β-actin (14).

While none of the controls appears to be ideal, there are several choices for the best candidate. A specific control can be selected for each experiment. First, the RNAs from this experiment are quantified and then analyzed for expression of the most likely control(s), and one that exhibits no modulation is chosen. The chosen mRNA species should be proportional to the amount of loaded or input RNA. Second, more than one control can be used. Similar expression for different controls would lend credibility to their use and to the accuracy of the normalized data. This approach has been used by a number of investigators (e.g., 44). Third, levels of the mRNA of interest can be simply normalized to the RNA loaded (μg) per lane and repeated multiple times. Any variable control results would not be expected in replicate experiments. This approach is limited by the amount of rRNA present in the sample because, as with rRNA probes, normalization would be to an RNA type that does not necessarily represent total polyA$^+$ mRNA levels.

Another option is to normalize to overall polyadenylation as an indicator of total polyA$^+$ RNA rather than any one specific control gene. Total mRNA can be analyzed using a poly(T) probe (17,39). This approach, however, runs the risk of misestimations under certain conditions where variable polyadenylation of transcripts occurs, such as S-phase expression as compared with G1-phase expressed genes. Finally, it should be noted that the identification of conditions that modulate GAPDH or β-actin does not necessarily eliminate the use of those controls in other experimental systems. For example, neither glucose deprivation nor heat shock, both listed in Table 1 as stresses that can induce GAPDH, modulate GAPDH in vascular endothelial cells (19).

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