ARTICLE
Journal of Cellular Biochemistry 110:118–128 (2010)

Journal of Cellular Biochemistry

Validation and Application of Normalization Factors for Gene Expression Studies in Rubella Virus-Infected Cell Lines With Quantitative Real-Time PCR

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ABSTRACT

Reference genes are generally employed in real-time quantitative PCR (RT-qPCR) experiments to normalize variability between different samples. The aim of this study was to identify and validate appropriate reference genes as internal controls for RT-qPCR experiments in rubella virus (RV)-infected Vero and MCF-7 cell lines using SYBR green fluorescence. The software programs geNorm and NormFinder and the $\Delta\Delta C_t$ calculation were used to determine the expression stability and thus reliability of nine suitable reference genes. HPRT1 and HUEL, and HUEL and TBP were identified to be most suitable for RT-qPCR analysis of RV-infected Vero and MCF-7 cells, respectively. These genes were used as normalizers for transcriptional activity of selected cellular genes. The results confirm previously published microarray and Northern blot data, particularly on the transcriptional activity of the cyclin-dependent kinase inhibitor p21 and the nuclear body protein SP100. Furthermore, the mRNA level of the mitochondrial protein p32 is increased in RV-infected cells. The effect on cellular gene transcription by RV-infection seems to be cell line-specific, but genes of central importance for viral life cycle appear to be altered to a similar degree. This study does not only provide an accurate and flexible tool for the quantitative analysis of gene expression patterns in RV-infected cell lines. It also indicates, that the suitability of a reference gene as normalizer of RT-qPCR data and the host-cell response to RV-infection are strictly cell-line specific. J. Cell. Biochem. 110: 118–128, 2010.

KEY WORDS: REFERENCE GENES; NORMALIZATION FACTORS; RUBELLA VIRUS; QUANTITATIVE REAL-TIME PCR; GENE EXPRESSION

The rubivirus rubella virus (RV) belongs to the family *Togaviridae*. Infection with RV is often subclinical and generally causes a mild exanthematous disease. However, severe damage and fetal malformation may occur after infection of seronegative women in early pregnancy. The molecular basis for RV teratogenesis remains largely unknown. However, the transcriptional activity of cellular genes appears to be different in RV-infected adult and fetal cells [Adamo et al., 2008]. The specific response could account for different manifestations of RV infection in the adult and fetal patient.

The methods of choice to analyze the transcription of cellular genes are real-time quantitative PCR (RT-qPCR), Northern blot, and DNA microarray analysis. While microarray analysis allows gene expression profiling of a high number of genes, precise quantification of gene expression levels can only be achieved by RT-qPCR [Huggett et al., 2005; Nygaard and Hovig, 2009]. For normalization of gene expression it is necessary to determine an endogenous reference gene that shows stable expression in virus-infected cells in comparison to the mock-infected control. Normalization against internal control genes allows for compensation of differences in sample preparation and processing as the reference gene and the gene of interest are subjected to the same experimental conditions [Vandesompele et al., 2002; Radonić et al., 2005]. So far, there is no universal reference gene that can be used for all possible experimental conditions.

The aim of this study was to quantify relative changes in the mRNA expression level of cellular genes encoding proteins such as the poly(A)-binding protein [Ilkow et al., 2008] and the mitochondrial protein p32 [Beatch et al., 2005] that are known to interact with RV, and to validate and precisely quantify microarray data that is available for RV [Mo et al., 2007; Adamo et al., 2008] using RT-qPCR. Several studies have investigated reference genes in virus-infected cells [Radonić et al., 2005; Bernasconi et al., 2006]. As far as we are aware, no such study on normalization factors has been

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Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Medical Faculty, University of Leipzig (Junior Research Grant).

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Received 14 October 2009; Accepted 6 January 2010 • DOI 10.1002/jcb.22518 • © 2010 Wiley-Liss, Inc.
Published online 9 March 2010 in Wiley InterScience (www.interscience.wiley.com).

performed for RV-infected cell lines and little is known about the expression of reference genes in RV-infected cell lines. Hence, the Microsoft excel-based applications NormFinder [Andersen et al., 2004] and geNorm [Vandesompele et al., 2002] and the $\Delta\Delta C_t$ calculation [Radonić et al., 2005] were used in this study to determine the most stable reference genes in RV-infected cell lines. Nine potential reference genes were chosen such that multiple cellular pathways are represented to reduce the likelihood of co-regulation of these genes by RV. Reference genes involved in translation were excluded as RV interacts with the poly(A)-binding protein [Ilkow et al., 2008]. Vero and MCF-7 cell lines were chosen for this study as they readily support RV infection. Besides, Vero is the most frequently used cell line for RV infection studies. Additionally, in contrast to the human cell line MCF-7, the primate cell line Vero is unable to induce an interferon response [Diaz et al., 1988] and could thus enable the identification of interferon-induced alterations in gene expression.

The use of the reference genes HPRT1, HUEL, and TBP in RT-qPCR reactions revealed that among the limited number of tested cellular genes the cyclin-dependent kinase inhibitor p21, the mitochondrial protein p32, and the nuclear body protein SP100 were significantly altered in their mRNA expression pattern in both cell lines. Only in MCF-7 cells the expression level of the tumor suppressor protein p53 and the adenine nucleotide carrier SLC25A4 was increased. These alterations appear to be cell-line specific and could at least be in part due to the induction of an interferon response. Additionally, this study includes the first description of an altered expression of p32 in general and of SLC25A4 in RV-infected cells of non-embryonic origin. Moreover, it provides the basis for a thorough evaluation of the importance of distinct cellular proteins and cellular pathways for RV replication and pathogenesis.

MATERIALS AND METHODS

CELL CULTURE AND VIRUS INFECTION

The epithelial cell line MCF-7 (human breast carcinoma; IAZ, Munich, Germany) was maintained in Eagle's minimum essential medium (EMEM; PAA, Cölbe, Germany) with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, and 10% fetal calf serum (FCS). The African green monkey (*Cercopithecus aethiops*) cell line Vero (ATCC number CCL-81) was propagated in Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 10% FCS. All cell lines were cultivated at 37°C under 5% CO_2 in a humidified incubator.

Control cells were mock infected with sterile medium excluding virus. In parallel, cell cultures were infected with RV, Therien strain at the indicated multiplicity of infection (moi). After adsorption for 2 h, the virus inoculum was substituted by maintenance medium. Viral titers were determined by standard plaque assay as described previously [Claus et al., 2006].

REFERENCE GENE SELECTION

Reference genes were chosen through a search of relevant literature employing RT-qPCR and a list of suitable reference genes published recently [Eisenberg and Levanon, 2003]. The candidate reference genes (Table I) include well-known reference genes such as β-actin and GAPDH and less known reference genes such as ECHS and HUEL. They were selected such that different cellular pathways are represented to reduce the possible occurrence of co-regulated genes. Oligonucleotides were designed based on human mRNA sequences and mRNA sequences available for *C. aethiops* using LC Probe Design® (Roche, Mannheim, Germany) or were chosen from the literature such that sense and antisense primer bind to different exons and intron-spanning amplicons are generated. Gene and primer specific data are listed in Table I.

TOTAL RNA PREPARATION

At 1, 2, and 3 days post-infection (dpi) or accordingly 2, 3, and 4 days after splitting, both infected and mock-infected cells were harvested for RNA extraction. Total cellular RNA was extracted from cell populations grown in 35 mm^2 culture dishes using TriFast™ reagent (PeqLab, Erlangen, Germany) according to manufacturer's instructions. The RNA was resuspended in nuclease-free water and its concentration was determined by UV spectrophotometry at 260 nm. Purity of RNA samples was determined by the A260/A280 (between 1.6 and 2.1) and the A260/A230 (>1.6) value and by analysis of the ribosomal RNA band integrity by conventional denaturing agarose RNA electrophoresis [Masek et al., 2005].

cDNA SYNTHESIS AND RT-qPCR

Samples were normalized to total RNA content before reference gene assessment and gene expression analysis. Equal amounts of RNA (1.25 μg per 20 μl reverse transcription reaction) were reverse transcribed at 50°C using 10 U AMV (Promega, Mannheim, Germany), 20 U RNase inhibitor (Fermentas, St. Leon-Rot, Germany) and 250 ng oligo$(dT)_{18}$ primer (Fermentas) according to manufacturer's instructions. All cDNA samples were diluted 1:10 in nuclease-free water and 5 μl of these dilutions were used as template in RT-qPCR reactions with the DyNAzyme™ II hot start DNA polymerase (NEB, Frankfurt am Main, Germany) in a total volume of 20 μl. RT-qPCR reactions were performed with SYBR green dye technique on a Light Cycler system (Roche). Two to three different RNA samples were collected at the indicated time points. The mean was used for data analysis.

The following standardized cycle conditions for RT-qPCR runs were applied: 95°C for 10 min to activate DyNAzyme™ II hot start DNA polymerase and for template denaturation, 40 cycles of denaturation at 95°C for 15 s, annealing of primers to template at appropriate temperature, and elongation as calculated according to manufacturer's instructions. Melting curve analysis of each sample was performed after every run by defined heating up to 95°C to assess the presence of unspecific PCR products. Specificity of amplicons was confirmed by sequencing analysis. For each biological replicate dual replicates were run. A minus RT control (RT−ve) was included in each assay run besides a negative water control reaction to assess for a possible contamination in the samples. RT −ve values are summarized in Table II and indicate whether contaminating DNA is present in the RT-qPCR reaction.

DATA ANALYSIS

Expression data of reference genes was validated by geNorm (version 3.5) [Vandesompele et al., 2002] and NormFinder software

TABLE I. Candidate Reference Genes and Cellular Genes Encoding RV Interaction Partners, and Viral Genes With Respective Data for Primer Sequences

Gene (accession number[a])	Gene name (cellular function)	Primer sequence (5′–3′)[b]	Reference
Candidate reference genes			
TBP (M55654)	TATA-box binding protein (transcription factor)	s ttcggagagttctgggattgta as tggactgttcttcactcttggc	Radonic [2005]
HPRT1 (NM_000194.1)	Hypoxanthine phospho-ribosyl transferase 1 (salvage pathway of purines)	s tgacactggcaaaacaatgca as ggtccttttcaccagcaagct	Cicinnati et al. [2008]
PPIA (NM_021130)	Peptidyl prolyl isomerase A (protein folding)	s catctgcactgccaagactgag as tgcaatccagctaggcatg	Radonic [2004]
HUEL (NM_006345)	Solute carrier family 30 (cellular replication)	s tcagacgacgaagtccccatgaag as tccttacgcaatttttctctctggc	Leong et al. [2005]
B2MG (NM_004048)	Beta-2-microglobulin (MHC class I molecules)	s gagtatgcctgccgtgtg as aatccaaatgcggcatct	Silver et al. [2006]
β-actin (NM_001101.2)	β-actin (cytosceletal protein)	s ctctcttccaaccttccttcc as cagactcgtcatactcctgctt	Yuan et al. [2007]
GAPDH (NM_002046)	Glyceraldehyde-3-phosphate (enzyme of glycolytic pathway)	s tgcaccaccaactgcttagc as ggcatggactgtggtcatgag	Vandesompele et al. [2002]
RPII (X74870)	RNA polymerase II (cellular transcription)	s gcaccacgtccaatgacat as gtgcggctgcttccataa	Radonic [2004]
ECHS (NM_004092)	Enoyl CoA hydratase (beta-oxidation)	s cgctgctgtcaatggctatg as cttggcgtcctgggctgag	Takahashi et al. [2007]
Genes possibly relevant for RV replication			
p32 (AF238300)	Mitochondrial/cell surface protein	s aaagttgccggggaaaaa as tcctcctcaccatcaaatgtt	–
p53 (NM_000546.4)	Tumor protein p53 (tumor suppressor protein)	s ccccagccaaagaagaaac as aacatctcgaagcgctcac	–
RB (NM_000321.2)	Retinoblastoma 1 (tumor suppressor protein)	s cagaataatcacactgcagcagata as cacgcgtagttgaacctttt	–
p21 (NM_000389)	Cyclin-dependent kinase inhibitor 1A (regulator of cell cycle)	s cgaagtcagttccttgtggag as catgggttctgacggacat	Kasahara et al. [2005]
PABP (NM_002568.3)	Poly(A)-binding protein (translation)	s gcacagccacaagttacaatg as ctcttgaggaggggcagat	–
SLC25A4 (NM_001151.2)	Solute carrier family 25 (mitochondrial carrier)	s tcgtagaatgatgatgcagtcc as cttggctccttcgtcttttg	–
SP100 (NM_003113)	Nuclear antigen (formation of nuclear bodies)	s aaagttgagtgccaagcccaag as tctaagggctcatcaacgtcagtg	Mo et al. [2007]
Target on RV genome			
RV, P150 (L78917)	Non-structural protein P150 (replicase)	s tgaccgcgcctatgtcaacc as gcccgtagacaaccacctcg	–

MHC, major histocompatibility.
[a]The database source is the NCBI reference sequence database (www.ncbi.nlm.nih.gov/RefSeq/).
[b]Oligonucleotides were in sense (s) or antisense (as) orientation.

[Andersen et al., 2004]. geNorm as an application tool for Microsoft Excel is freely available upon request from the programmers. C_t values were transformed into relative quantities for data analysis. geNorm calculates the reference gene stability factor M that is defined as the average pairwise variation of a certain gene in comparison to the remaining reference genes [Vandesompele et al., 2002]. Thus, the program provides the two most stable reference genes for normalization. The NormFinder application is also freely available on the internet (http://www.mdl.dk). The stability value of a certain reference gene is determined by NormFinder and takes into account the intra- and inter-group expression variation of a certain reference gene. A low stability value stands for a low variation and thus for a high-expression stability.

The mRNA expression of cellular genes in RV-infected cell cultures was calculated relative to the expression of each gene at the same time point in the corresponding mock-infected cell culture, which was designated as 1× or 100%. The statistical significance of the expression pattern of each cellular gene was determined by ANOVA (analysis of variance, microsoft excel) program. A *P*-value ≤ 0.05 was regarded as significant.

A Robo503 (a kind gift of Dr. Frey, Georgia State University, Atlanta, USA) plasmid standard curve based on plasmid DNA copies/μl in the range of 1×10^9 to 1×10^1 was used to assess copy number of RV genomic RNA in the respective sample.

MIQE GUIDELINES

RT-qPCR experiments are in agreement with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [Bustin et al., 2009]. The MIQE checklist (http://www.rdml.org/miqe) is provided as supplementary data.

RESULTS

ANALYSIS OF GENE EXPRESSION OF EACH CANDIDATE REFERENCE GENE

Variations in RNA transcription levels can be assessed through comparison of the C_t values obtained for each gene sample. The C_t value is defined as the number of cycles required for the fluorescence signal to reach a certain threshold of detection and thus directly correlates with the amount of template. The range of C_t values for each reference gene is illustrated by a boxplot in Figure 1. This boxplot highlights the variation in expression level of RV (moi 1 and 10) and mock-infected Vero and RV (moi 2)- and mock-infected MCF-7 cells. A median C_t value below 30 indicates a high

TABLE II. Respective Reference Genes and Cellular Genes Encoding RV Interaction Partners and Viral Genes With Respective Data for RT-qPCR

Gene	Amplicon size (bp)	E_{Slope} (%)[a]	E_{LinReg} (%)[b]	C_t	C_t of RT −ve[c]	P (bp)[c]
Candidate reference genes						
TBP	226	86	84	25	–	–
HPRT1	94	90	88	20	–	–
PPIA	325	96	73	20	>36	+(602)
HUEL	104	94	86	24	–	–
B2MG	92	89	73	20	>36	–
β-actin	317	82	78	18	>36	+(506, 296)
GAPDH	86	95	89	19	31	+(67)
RPII	632	95	82	28	–	+(609)
ECHS	200	89	85	22	>36	–
Genes possibly relevant for RV replication						
p32	77	n.d.	90	19	>36	–
p53	77	n.d.	89	25	33	–
RB	76	n.d.	87	24	>36	–
p21	67	n.d.	87	27	>36	–
PABP	96	n.d.	70	22	28	–
SLC25A4	100	n.d.	87	21	–	+(994)
SP100	110	n.d.	82	19	–	+(1129)
Target on RV genome						
P150	90	n.d.	84	variable	>36	–

n.d., not determined; C_t, cycle threshold; RT −ve, minus RT control.
[a]10-fold serial dilutions of cDNA obtained from Vero cells were plotted against dilution factors. RT-qPCR efficiencies (E) were calculated by the following equation (Rasmussen, 2001): $E = 10^{(-1/slope)}$. Only C_t values < 40 were included.
[b]PCR efficiency was calculated based on the starting point of the exponential phase of amplification using LinReg PCR program.
[c]The C_t values of RT −ve samples appear to be due to retropseudogenes (P) that lead to amplification of contaminating genomic DNA and could thus possibly interfere with RT-qPCR results. If present, melting peaks for the RT−ve samples were distinguishable from the specific amplicon. The possible amplification of retropseudogenes with the primer sequences used in this study was determined by BLAT search [Kent, 2002]. –, no retropseudogenes; +, retropseudogenes present, yielding amplicons of the indicated length.

RNA transcription level [Radonić et al., 2005]. The nine reference genes under investigation displayed a C_t value range from 16 to 31, which groups them among genes with high RNA transcription (Fig. 1). The average C_t value of each reference gene is given in Table II. The highest expression level with the lowest C_t value of 16 cycles was displayed by PPIA, while the lowest expression level with the highest C_t value of 31 was exhibited by RPII. The lowest transcription range, as an indicator of constant RNA transcription, was observed for HPRT1 and HUEL in RV-infected Vero cells (range = 1 and 1.27, respectively), and for HUEL and HPRT1 in

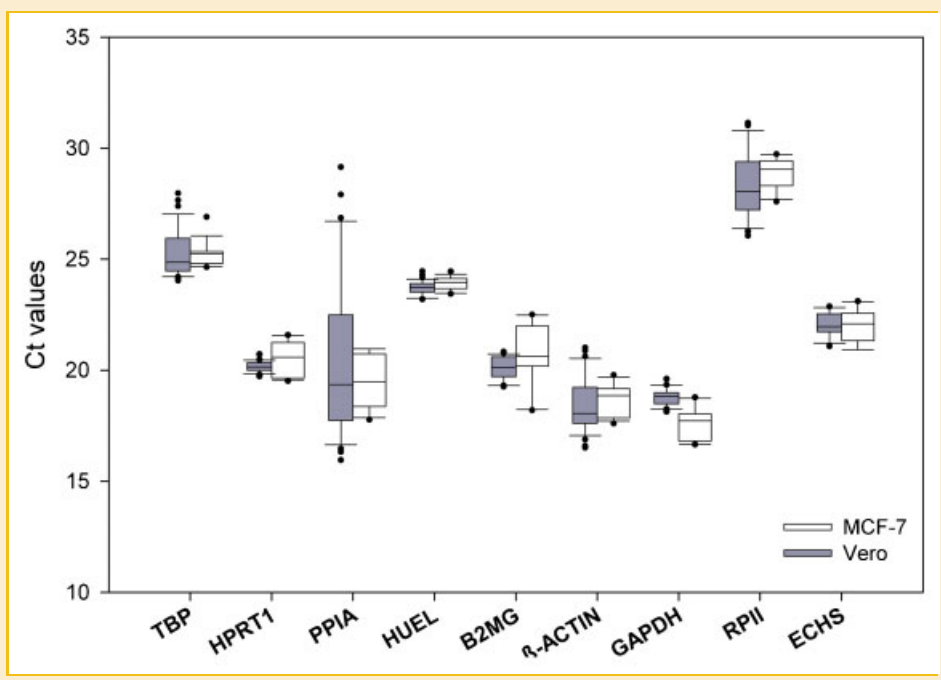

Fig. 1. Stretch of RT-qPCR cycle threshold (C_t) values. RNA transcription of selected reference genes in Vero (four experiments) and MCF-7 (two experiments) cells displayed as RT-qPCR cycle threshold numbers (C_t values) over all samples. The median is indicated by a line in each box, which in turn represents the 25th and 75th percentile. Whiskers indicate the 10/90 percentile ranges, circles represent potential outliers.

RV-infected MCF-7 cells (range = 1.01 and 2.07, respectively). The highest RNA transcription range, which is indicative for a variable gene expression, was seen in RV-infected Vero cells for PPIA (range = 13.2).

OPTIMAL NUMBER OF REFERENCE GENES

geNorm determines the optimal number of reference genes for normalization by calculating pairwise variation (V_n/V_{n+1}) between sequential normalization factors (NF_n and NF_{n+1}). A threshold of 0.15 is recommended for pairwise variation [Vandesompele et al., 2002]. Below this threshold it is not necessary to include additional reference genes in relative gene expression calculations. Calculation of pairwise variation V showed that the inclusion of two reference genes is sufficient for data normalization of RV (moi 1 and 10)-infected Vero and RV (moi 2)-infected MCF-7 cells as the threshold in each case was below 0.15 (Fig. 2). The addition of a third (V2/3) reference gene did not increase threshold value. Thus two reference genes should be adequate for future applications.

EXPRESSION STABILITY OF REFERENCE GENES

geNorm algorithm was used to calculate the degree of variation of each candidate reference gene, which is given as expression stability M. The expression stability M represents an average pairwise variation of a reference gene with all other reference genes. Unstable reference genes are gradually excluded with the two most stable genes leading the ranking. Genes with higher M values are usually associated with a greater variation in gene expression and should thus be excluded from normalization. All reference genes included in this study have a low stability value M and arrange below the default limit of M = 1.5 [Vandesompele et al., 2002] (Fig. 3).

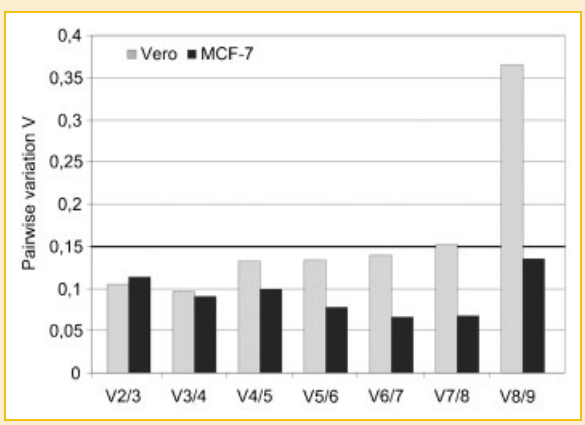

Fig. 2. Determination of the optimal number of reference genes. Pairwise variation was applied by geNorm algorithm to determine the optimal number of reference genes for normalization after successive inclusion of less stable genes. On the left-most side is the pairwise variation upon enlargement of the number of reference genes from two to three (V2/3). Inclusion of less stable genes results in the next data point. Inclusion of a third gene has no significant effect on normalization factors. The threshold value of 0.15 is indicated. The results of four experiments for RV-infected Vero, and of two experiments for RV-infected MCF-7 are shown.

NormFinder was applied to complement geNorm findings. NormFinder software gives out the expression of each gene as stability value [Vandesompele et al., 2002]. Genes with a lower stability value are usually associated with high expression stability. For comparison, corresponding data by geNorm and NormFinder are both included in Figure 3. For RV-infected Vero (moi 1 and 10) cells geNorm and NormFinder give out the same top four reference genes, but differ in their order (Fig. 3A). geNorm has identified HUEL, GAPDH, and HPRT1 as the three most stable reference genes. However, geNorm (HUEL, TBP, β-actin) and NormFinder (ECHS, HUEL, PPIA) differ on the three most stable reference genes for RV-infected MCF-7 cells (moi 2) (Fig. 3B). However, HUEL was either the best or one of the two best ranked genes by geNorm, NormFinder, and $\Delta\Delta C_t$ calculation. Therefore, the expression pattern of the remaining eight reference genes was normalized against HUEL and calculated for mock-infected and RV-infected MCF-7 cells for dpi 1 and 3 (expression at dpi 1 for mock-infected cultures was set as 100%). Figure 3C indicates that ECHS as suggested by NormFinder is less well suited for normalization of RV-infected MCF-7 cells as TBP, which was suggested by geNorm. TBP shows a lower degree of variation in its expression than ECHS (Fig. 3C). Additionally, geNorm is the most widely used software for identification of suitable normalization factors [Vandesompele et al., 2009] and includes differences in PCR efficiencies in its calculations and selects the two most stable reference genes. Hence, results by geNorm were favored over those by NormFinder.

EFFECT OF THE INITIAL INFECTIOUS DOSE ON REFERENCE GENE EXPRESSION

A suitable reference gene should show no variable expression between cells infected with a low and high moi of RV. As in future experiments it is intended to determine the gene expression pattern of a target gene in RV-infected cells over time of incubation and with different mois, it was necessary to analyze reference gene expression at 1 and 3 dpi after application of a high moi (moi 10) and a low moi (moi 1) separately. Table III summarizes gene stability results for RV-infected Vero cells obtained by geNorm and NormFinder software. Both algorithms gave similar results and ranked the reference genes almost identical between the different mois. Thus, it appears that different mois of RV have only a slight influence on reference gene expression. geNorm suggests HPRT1 and HUEL for normalization of RV (moi 10)-infected Vero cells, whereas for moi 1 and the average of both mois together HUEL and GAPDH were suggested (Table III).

Although GAPDH was one of the two best reference genes for RV (moi 1 and average of samples for moi 1 and 10)-infected Vero cells (Fig. 3), GAPDH should not be used as normalization factor for RV-infected Vero cells for two reasons. First, GAPDH gave a distinct signal in RT −ve reactions. Although the C_t value of the GAPDH RT −ve was much higher than the C_t value of the positive sample, it was lower than the C_t value of the RT −ve of the remaining reference genes (Table II). This is probably caused by amplification of a retropseudogene due to contaminating genomic DNA. Performing a primer sequence search by BLAT, the BLAST-like alignment tool [Kent, 2002], several retropseudogenes for GAPDH were detectable, one with binding sites for sense and antisense

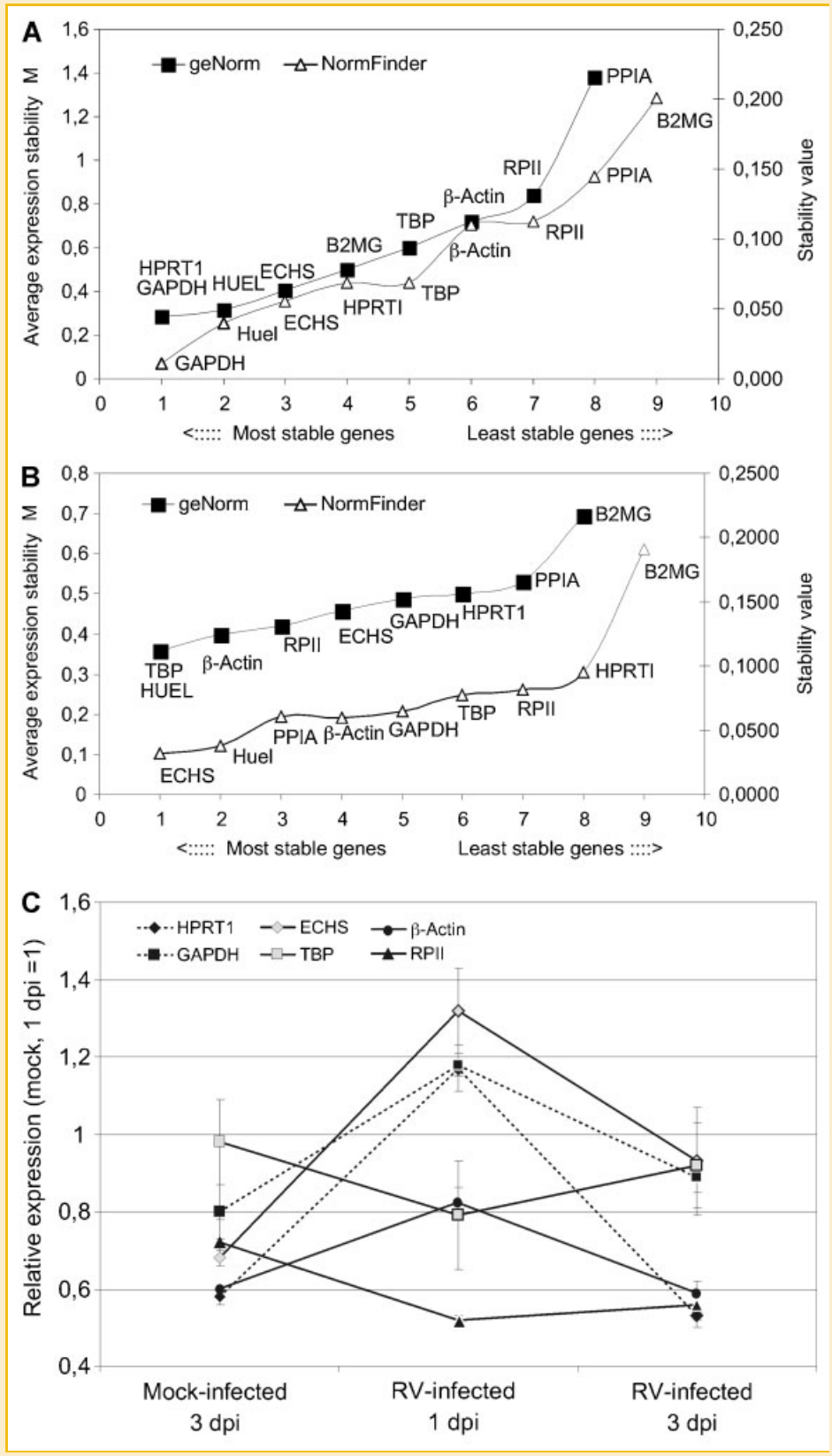

Fig. 3. Ranking of candidate reference genes. geNorm (points and M-value) and NormFinder (boxes and stability value) algorithm group reference genes in order of decreasing expression stability in RV-infected Vero (A) and MCF-7 cells (B). Expression stability is given for paired cell samples (mock- and RV-infected Vero, n = 24; mock- and RV-infected MCF-7, n = 16). High expression stability is represented by a low stability value and a low M-value. C: Analysis of variation of reference gene expression in mock- and RV-infected MCF-7 cells. Data for B2MG and PPIA was omitted for better resolution.

TABLE III. Ranking of Genes by Expression Stability M Calculated by geNorm (Stable Genes Have Lower M Values) and by Stability Value as Analyzed by NormFinder (the Lower the Stability Value Better the Reference Gene)

moi 1		moi 10		moi 1 and 10	
geNorm (M-value)	NormFinder (stability value)	geNorm (M-value)	NormFinder (stability value)	geNorm (M-value)	NormFinder (stability value)
Selected reference genes ranked in order of decreasing expression stability[a]					
HUEL/GAPDH (0.205)	GAPDH (0.011) HUEL (0.040)	HUEL/HPRTI (0.204)	GAPDH (0.011) HUEL (0.039)	HUEL/GAPDH (0.203)	GAPDH (0.011) HUEL (0.039)
HPRTI (0.226)	ECHS (0.053)	GAPDH (0.220)	ECHS (0.058)	HPRTI (0.219)	ECHS (0.055)
TBP (0.331)	TBP (0.068)	ECHS (0.312)	HPRT1 (0.061)	ECHS (0.316)	HPRT1 (0.069)
ECHS (0.390)	HPRT1 (0.077)	TBP (0.423)	TBP (0.070)	TBP (0.402)	TBP (0.069)
RPII (0.553)	β-actin (0.104)	β-actin (0.575)	β-actin (0.117)	β-actin (0.559)	β-actin (0.110)
β-actin (0.621)	RPII (0.106)	RPII (0.642)	RPII (0.118)	RPII (0.559)	RPII (0.112)
B2MG (0.699)	PPIA (0.152)	B2MG (0.706)	PPIA (0.137)	B2MG (0.697)	PPIA (0.145)
PPIA (0.893)	B2MG (0.209)	PPIA (0.959)	B2MG (0.193)	PPIA (0.915)	B2MG (0.201)

[a]Data are given for paired cell samples (mock- and RV-infected, n = 24). The two most stable reference genes identified by geNorm can not be grouped any further as geNorm algorithm is intended to identify a pair of most stable reference genes.

primer possibly yielding a PCR product of 67 bp in length. This product would not be discriminatable from the specific amplicon of 86 bp (Table II). Second, HPRT1 and HUEL were ranked as the two most stable reference genes for RV (moi 10)-infected Vero cells, suggesting that both genes remain stable even after application of a high moi of RV.

TBP and HUEL should be used as normalizers for RV-infected MCF-7 cells, and HUEL and HPRT1 should be used for normalization of RV-infected Vero cells. The use of two reference genes was considered to be advantageous over the use of only one reference gene as it enables compensation of minimal differences of their expression pattern [Vandesompele et al., 2009].

APPLICATION OF THE $\Delta\Delta C_t$ METHOD

Virus-induced alterations in the expression pattern of a respective target gene are generally assessed by relative quantification in relation to the uninfected control population. Hence, it was necessary to determine reference genes that remain unchanged between infected and mock-infected cell cultures over time of incubation. As it is not possible to compare dependent samples by NormFinder and geNorm, the $\Delta\Delta C_t$ method was applied. $\Delta\Delta C_t$ values were calculated by the following equation, which was modified from an earlier report [Radonić et al., 2005]. Calculation of ΔC_t for comparison of uninfected (uinf) and infected (inf) cell lines at 1 and 3 dpi was as follows:

$$\Delta C_t(\text{inf}) = C_t(\text{inf 1 dpi}) - C_t(\text{inf 3 dpi}) \text{ and}$$
$$\Delta C_t(\text{uinf}) = C_t(\text{uinf 1 dpi}) - C_t(\text{uinf 3 dpi})$$

Based on this calculation the $\Delta\Delta C_t$ value was determined: $\Delta\Delta C_t = \Delta C_t(\text{inf}) - \Delta C_t(\text{uinf})$.

A high $\Delta\Delta C_t$ value represents significant changes in RNA transcription of the respective gene and indicates that the gene under surveillance is not constantly expressed between infected and mock-infected cells. A positive $\Delta\Delta C_t$ value indicates downregulation of the RNA transcription of the respective gene, whereas a negative $\Delta\Delta C_t$ value suggests upregulation of the RNA transcription of the respective gene. The $\Delta\Delta C_t$ values for the nine reference genes applied in this study are shown in Figure 4 as the mean with standard deviation of two (RV-infected MCF-7, moi 2) and four (RV-infected Vero cells, moi 1 and 10) independent biological samples run in duplicate in RT-qPCR reactions. The $\Delta\Delta C_t$ value of PPIA in RV-infected Vero and MCF-7 cells shows a high standard deviation indicative of a high variation in RNA transcription. geNorm and NormFinder also ranked PPIA as a reference gene with low stability (Fig. 3). On the contrary, HPRT1 and HUEL were approved by $\Delta\Delta C_t$ equation as stable reference genes and show almost no variation between infected and mock-infected Vero and MCF-7 cells. Thus, data by geNorm and NormFinder agree with $\Delta\Delta C_t$ values and $\Delta\Delta C_t$ calculation confirms HUEL and HPRT1 as stable and suitable reference genes in RV-infected Vero and TBP and HUEL in RV-infected MCF-7 cells.

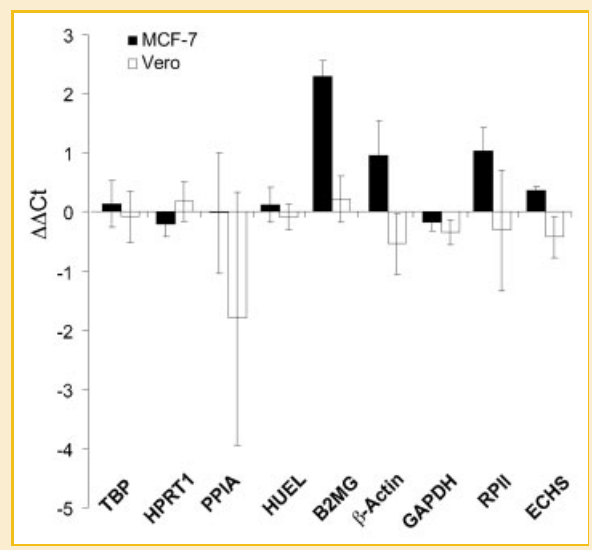

Fig. 4. $\Delta\Delta C_t$ of reference genes in Vero and MCF-7 cell lines. The $\Delta\Delta C_t$ was calculated from the changes of ΔC_t (three independent experiments) in mock-infected and RV-infected cell lines between 1 and 3 dpi. A positive $\Delta\Delta C_t$ value indicates downregulation of gene expression and a negative $\Delta\Delta C_t$ value suggests upregulation of gene expression. Error bars indicate standard error of the mean. The results of four experiments for RV-infected Vero and of two experiments for RV-infected MCF-7 are shown.

GENE EXPRESSION PATTERN OF CELLULAR INTERACTION PARTNERS

The mRNA expression pattern of selected host genes in RV-infected Vero and MCF-7 cells was determined by relative quantification using RT-qPCR with HPRT1 and HUEL, and HUEL and TBP as normalizers, respectively. geNorm software was used to calculate changes in gene expression pattern, which are illustrated with the corresponding *P*-values in Figure 5.

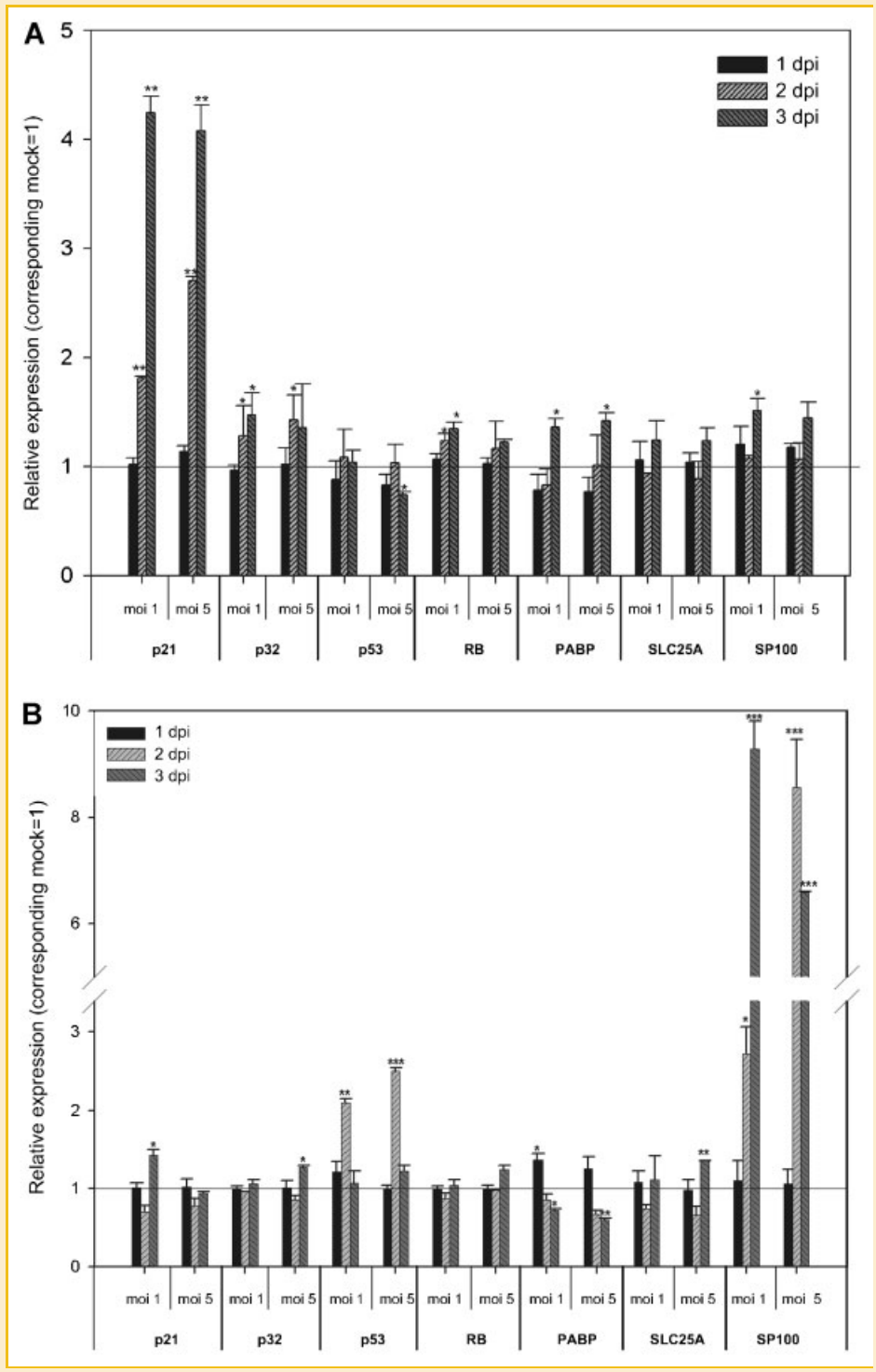

Fig. 5. Alteration of expression of selected cellular genes in RV-infected cells. The expression pattern of the indicated genes in RV-infected Vero (A) and MCF-7 cells (B) was determined relative to the corresponding mock sample. Error bars indicate standard error of the mean. The results of four experiments for RV-infected Vero, and of two experiments for RV-infected MCF7 cells are shown. Level of significance: $^{*}P < 0.05$ and $^{**}P < 0.01$ and $^{***}P < 0.001$.

The highest level of transcription induction (1.5-fold in Vero and up to ninefold in MCF-7) was seen for SP100 upon infection with RV (moi 1). The expression pattern of the genes encoding the tumor suppressor proteins p53 and retinoblastoma protein (RB) was also investigated. While the mRNA of p53 was found to be decreased by 0.3-fold in Vero cells at 3 dpi (moi 5), its expression level increased by 2.5-fold in RV-infected MCF-7 cells (moi 5). Vero cells (moi 5) showed a 1.3-fold increase in the expression of RB, but the mRNA level of RB remained unchanged in MCF-7 cells. In contrast to the MCF-7 cell line, Vero cells are deficient in the type 1 interferon response. This could provide an explanation for the difference in the mRNA level of p53, RB, and SP100 in both cell lines.

Besides SP100, only p21 and the mitochondrial protein p32 showed an increase in its expression level in both cell lines, Vero and MCF-7. The highest mRNA level of p21 and p32 was reached at 3 dpi. The p21 protein was induced about fourfold in Vero cells (moi 1 and 5), but only 1.4-fold in MCF-7 cells (moi 1). The increase in the mRNA level of p32 was similar in both cell lines – about 1.5-fold in Vero (moi 1 and 5) and 1.3-fold in MCF-7 cells (moi 5).

The mRNA expression level of the PABP gene was increased by 1.4-fold (moi 1 and 5) in Vero cells, whereas it was decreased in RV-infected MCF-7 cells by about 0.4-fold (moi 1 and 5). The mRNA level of SLC25A4 also differed between MCF-7 and Vero cells. The expression of SLC25A4 remained unchanged in Vero cells, but was increased by 1.4-fold in MCF-7 cells at 3 dpi (moi 5).

mRNA levels were also determined at 6 h post-infection. However, none of the examined cellular genes showed an altered expression pattern at this time point (data not shown). It appears that the applied moi (moi 1 and moi 5) has no strong influence on the mRNA expression of the genes examined in this study. Generally, the tendency of altered expression (up or down-regulation) was comparable between the applied mois. Only the change of transcription of p53 in RV-infected Vero cells and of p32 in RV-infected MCF-7 cells appears to be influenced by the applied moi of RV. The former is decreased by 0.3-fold only at moi 5, whereas the latter is increased by 1.4-fold only at moi 1.

QUANTIFICATION OF RV-SPECIFIC RNA

The amount of RV-specific RNA was determined for RV-infected Vero cells (moi 1 and moi 5) using RT-qPCR. For this purpose primers were chosen to amplify a 87 bp region from the non-structural protein P150 of RV. Primer sequences are given in Table I. The infectious cDNA clone of RV, Robo 503 was used to establish a standard curve and to characterize RT-qPCR conditions. The sensitivity of the RT-qPCR was estimated to be 10–30 copies of plasmid DNA per reaction. PCR efficiency was 84% as determined by LinReg software (Table II). The RT −ve samples were characterized by a C_t value ≥ 36. Figure 6 summarizes the estimates of genomic RNA copies (per 1.25 μg of total RNA) on day 1, 2, and 3 for RV after infection with moi 1 and moi 5. The copy number of genomic RNA determined for both mois was plotted against viral titer given as plaque forming units, PFU/ml (Fig. 6). There appears to be a linear relation between these two parameters. However, there is no significant difference between moi 1 and moi 5 over time of incubation, neither on the level of RNA copy number nor on the level of virus titer. RV titer and RNA copy number were also determined for 6 h post-infection. The RNA copy number in RV-infected Vero cells was 8 and 22 copies per 1.25 μg of total RNA at 6 h post-infection for moi 1 and 5, respectively. Viral titer was 9 ± 3 and 24 ± 2 PFU/ml at 6 h post-infection for moi 1 and 5, respectively. Data for 6 h post-infection were not included in Figure 6 as no significant change in host gene expression was detected at this time point and to obtain a better resolution on the y-axis. However, the data obtained for 6 h post-infection reflects feasibility of the experimental system. Similar results were obtained for RV-infected MCF-7 cells (data not shown).

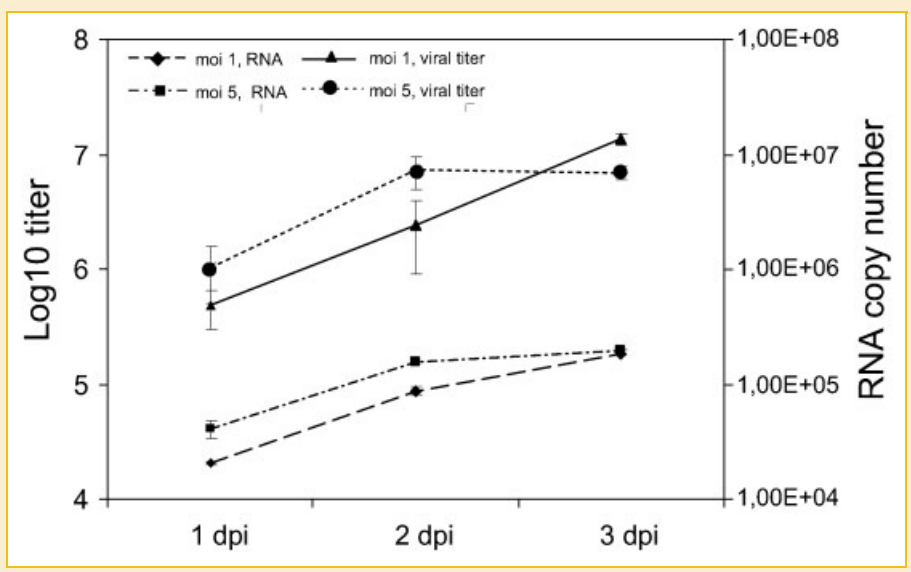

Fig. 6. Quantification of RV genome content in comparison to viral titer. Copy numbers of genomic RNA were plotted against viral titers. Mean RV genomic RNA copies per sample were determined on day 1, 2, and 3 post-infection (dpi). Virus titer was determined by standard plaque assay. Error bars indicate standard error of the mean. Results of four experiments are shown.

DISCUSSION

Most studies employ only one reference gene with GAPDH, 18S rRNA, and β-actin being most commonly used as normalization factors [Fu et al., 2009]. However, several studies have shown that the expression of these ''historical'' reference genes commonly used for normalization of RNA samples run by Northern blot or conventional RT PCR assays can vary considerably between different cell lines and under different experimental conditions [Glare et al., 2002; Radonić et al., 2005; Huggett et al., 2005]. Normalization represents one of the most difficult problems associated with RT-qPCR. Careful validation of suitable reference genes is time-consuming but of importance for reliable RT-qPCR data [Huggett et al., 2005; de Jonge et al., 2007].

Previous studies on RT-qPCR analysis of virus-infected cell lines provide a preset list of potential reference genes. They include PPIA, TBP, and GAPDH for human herpesvirus 1 and cytomegalovirus [Watson et al., 2007], and TBP, PPIA for SARS-coronavirus, yellow fever virus, human herpesvirus-6, and cytomegalovirus [Radonić et al., 2005]. In this study, we have conducted a thorough validation of nine putative reference genes for normalization of RV-infected cell lines: β-actin, B2MG, ECHS, GAPDH, HUEL, HPRT1, PPIA, RPII, and TBP. 18S rRNA was not included in this study as oligo(dT) primers were used for reverse transcription and 18S rRNA lacks a poly(A) tail.

Applying geNorm and NormFinder algorithms and $\Delta\Delta C_t$ calculation, HUEL and HPRT1, and HUEL and TBP were identified as suitable reference genes for RV-infected Vero and MCF-7 cells, respectively. All three algorithms agreed on the top four reference genes for RV-infected Vero cells, but differed for MCF-7 cells. Other studies have also shown that ranking differences between geNorm and NormFinder can occur [Skovgaard et al., 2007]. However, the least variation of reference gene expression in mock- and RV-infected cell lines over time of incubation was associated with the first-choice reference genes suggested by geNorm (Fig. 3C). This is supported by calculating the expression values of two representative genes (p53 and SP100) for mock- and RV-infected MCF-7 cells through normalization against HUEL, TBP, and ECHS. The resulting data revealed, that normalization against either HUEL or TBP, or the combination of both resulted in almost identical expression values, while ECHS or ECHS together with HUEL revealed large differences (3- to 14-fold) in calculated gene expression (data not shown). The optimal reference gene must be carefully evaluated and considered within the experimental context. So far no thorough study of reference gene expression has been performed for RV. However, HPRT1 and GAPDH were used as reference genes for RT-qPCR analysis of RV-infected human embryo fibroblasts and the adult lung fibroblast cell line Hs888Lu [Adamo et al., 2008]. The results presented in this study suggest that normalization factors should be determined for each cell line separately. However, HUEL could represent a suitable reference gene for most RV-infected cell lines.

Previously published microarray and RT-qPCR data showed an increase in the expression level of SP100 in RV-infected ECV304 [Mo et al., 2007], which was confirmed in this study in RV-infected Vero and especially in RV-infected MCF-7 cells. The expression of the SP100 gene is inducible by interferon alpha and beta, but also by interferon gamma [Grötzinger et al., 1996]. This explains why the induction of SP100 is higher in RV-infected MCF-7 and ECV304 than in Vero cells, which lack a type I interferon inducibility [Diaz et al., 1988]. We were also able to confirm Northern blot data for the increased transcription level of p21 in RV-infected Vero cells [Mo et al., 2007]. However, a discrepancy in the expression level of p53 and RB was detected. Mo et al. have reported a constant transcription level for p53 and RB in RV-infected Vero cells whereas our data reveals a 0.3-fold decrease in p53 and a 1.3-fold increase in the RB mRNA level at 3 dpi. RT-qPCR is more sensitive than Northern blot data and allows for precise quantification. Hence it could be possible that the slight, but significant change in p53 and RB transcription could be missed by Northern blot analysis. The 2.5-fold increase in the p53 mRNA level in RV-infected MCF-7 is probably due to induction of a type I interferon response, which has been shown to upregulate p53 transcription [Takaoka et al., 2003].

The RT-qPCR analysis of RV-infected Vero and MCF-7 cells revealed a significant difference in the mRNA level of PABP between the two cell lines over time of incubation. While at 3 dpi the mRNA level of PABP was decreased in MCF-7 cells, it was increased in Vero cells. However, the mRNA level of PABP was significantly increased in RV-infected MCF-7 cells at 1 dpi. Ilkow et al. have recently shown, that the protein levels of PABP were increased by about 100% in both, Vero and the human lung carcinoma cell line A549 at 2 dpi [Ilkow et al., 2008]. This is in agreement with the results presented in this paper. We hypothesize in accordance with the data presented by Ilkow et al. that the PABP protein level in Vero cells is increased rather by a post-translational mechanism. In MCF-7 cells upregulation of PABP transcription appears to be involved in increasing PABP mRNA levels.

The importance of mitochondrial functions and especially of the p32 protein for RV replication has been shown previously [Beatch et al., 2005]. The p32 protein is a multifunctional and multi-compartmental protein, which participates in the antiviral response of the host cell. Although several viral proteins interact with p32 and modulate its function, mRNA and protein level of p32 were reported to be constant between mock- and virus-infected cell populations [Xu et al., 2009]. However, the mRNA level of p32 is increased in both, RV-infected Vero and MCF-7 cells. The influence of RV on p32 transcription appears to be quite unique among viruses. Data presented in this paper highlight p32 as an important cellular interactor of RV. However, Western blot analysis of RV-infected Vero cells revealed no significant difference in the protein level of p32 (data not shown).

The cell-specific response to RV infection as shown in this study is in agreement with microarray data that is available for RV-infected ECV304 cells [Mo et al., 2007] and adult lung fibroblasts Hs88Lu and primary fetal fibroblasts HEF [Adamo et al., 2008]. Moreover, gene chip analysis of RV-infected adult and fetal fibroblasts showed that roughly 90% of the genes that were up- or downregulated were cell-type specific [Adamo et al., 2008]. Differences in the cellular response to bovine viral diarrhea virus infection were also observed in MDBK and BFM cells by microarray data analysis [Yamane et al., 2009].

In conclusion, a thorough and detailed evaluation of reference genes for normalization in RV-infected Vero (HUEL and HPRT1) and MCF-7 (TBP and HUEL) cells was provided. This enables the flexible and precise analysis of the change in transcription of cellular genes induced by RV with RT-qPCR analysis. While microarray data provides a general survey of gene expression by a high-throughput screen, the RT-qPCR format illustrated in this study can be easily adopted to the analysis of different cell lines and different experimental conditions and thus extends microarray data. It was shown by this study that the cellular response to a virus infection varies remarkably between different cell lines. However, transcription of genes that are of general importance to RV appears to be ubiquitously altered.

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