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 ΔΔCt 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. © 2010 Wiley-Liss, Inc.

KEY WORDS: REFERENCE GENES; NORMALIZATION FACTORS; RUBELLA VIRUS; QUANTITATIVE REAL–TIME PCR; GENE EXPRESSION
performed for RV-infected cell lines and little is known about the expression of reference genes in RV-infected cell lines. The Microsoft excel-based applications NormFinder [Andersen et al., 2004] and geNorm [Vandesompele et al., 2002] and the DD act calculation [Radonic 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, Cölbe, Germany) with 10% FCS. 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.

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 mm² 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) ratio 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
which was designated as 1 simultaneously in the corresponding mock-infected cell culture, cultures was calculated relative to the expression of each gene at the reference gene. A low stability value stands for a low variation and account the intra- and inter-group expression variation of a certain reference gene is determined by NormFinder and takes into available on the internet (http://www.mdl.dk). The stability value of genes for normalization. The NormFinder application is also freely 2002]. Thus, the program provides the two most stable reference comparison to the remaining reference genes [Vandesompele et al., 2002].

| Candidate reference genes | Gene name (cellular function) | Primer sequence (5'–3') | Reference |
|---------------------------|------------------------------|-------------------------|-----------|
| TBP (M55654)              | TATA-box binding protein [transcription factor] | s tggagagtctgagggattga | Radonic [2005] |
| HPRT1 (NM_000194.1)      | Hypoxanthine phosphoribosyl transferase | s tgcagctgacaaaga | Cincinetti et al. [2008] |
| PPIA (NM_021130)          | Peptidyl prolyl isomerase A [protein folding] | s ctgctgacagag | Radonic [2004] |
| HUEL (NM_006345)          | Solute carrier family 30 [cellular replication] | s ttcatagcagc | Leong et al. [2005] |
| B2MG (NM_004048)          | Beta-2-microglobulin (MHC class I molecules) | s gatgtgctctgt | Silver et al. [2006] |
| β-actin (NM_001101.2)     | β-actin [cytoskeletal protein] | s actcttctaggct | Yuan et al. [2007] |
| GAPDH (NM_002046)         | Glyceraldehyde-3-phosphate [enzyme of glycolytic pathway] | s ggtggagggctag | Vandesompele et al. [2002] |
| RPII (X74870)             | RNA polymerase II [cellular transcription] | s gggctgggaggct | Radonic [2004] |
| ECHS (NM_004092)          | EnolCoA hydratase (beta-oxidation) | s gcttcaaaccttca | Takahashi et al. [2007] |

**Results**

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 × 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 × 10³ to 1 × 10⁴ was used to assess copy number of RV genomic RNA in the respective sample.

**RESULTS**

**ANALYSIS OF GENE EXPRESSION OF EACH CANDIDATE REFERENCE GENE**

Variations in RNA transcription levels can be assessed through comparison of the Ct values obtained for each gene sample. The Ct 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 Ct 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 Ct value below 30 indicates a high...
RNA transcription level [Radonić et al., 2005]. The nine reference genes under investigation displayed a Ct value range from 16 to 31, which groups them among genes with high RNA transcription (Fig. 1). The average Ct value of each reference gene is given in Table II. The highest expression level with the lowest Ct value of 16 cycles was displayed by PPIA, while the lowest expression level with the highest Ct 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 (Ct) values. RNA transcription of selected reference genes in Vero (four experiments) and MCF-7 (two experiments) cells displayed as RT-qPCR cycle threshold numbers (Ct 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.](image-url)

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 | Ct | 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 | 612 | 95 | 82 | 28 | – | – |
| 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.
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

geneNorm 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 ($V_{2}/3$) reference gene did not increase threshold value. Thus two reference genes should be adequate for future applications.

EXPRESSION STABILITY OF REFERENCE GENES
geneNorm 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 geneNorm 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 ($V_{2}/3$). 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.

geneNorm was applied to complement geneNorm 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 geneNorm and NormFinder are both included in Figure 3. For RV-infected Vero (moi 1 and 10) cells geneNorm and NormFinder give out the same top four reference genes, but differ in their order (Fig. 3A). geneNorm has identified HUEL, GAPDH, and HPRT1 as the three most stable reference genes. However, geneNorm (HUEL, TBP, $\beta$-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 geneNorm, 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 geneNorm. TBP shows a lower degree of variation in its expression than ECHS (Fig. 3C). Additionally, geneNorm 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 geneNorm 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 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 geneNorm 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. geneNorm 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 $\Rightarrow$ ve reactions. Although the $C_{t}$ value of the GAPDH RT $\Rightarrow$ ve was much higher than the $C_{t}$ value of the positive sample, it was lower than the $C_{t}$ value of the RT $\Rightarrow$ 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  | NormFinder | geNorm       | NormFinder | geNorm       | NormFinder |
| (M-value) | (stability value) | (M-value) | (stability value) | (M-value) | (stability value) |
| HUEL/GAPDH (0.205) | GAPDH (0.011) | HUEL/GAPDH (0.204) | GAPDH (0.011) | HUEL/GAPDH (0.203) | GAPDH (0.011) |
| HUEL (0.040) | HUEL (0.039) | HUEL (0.039) | HUEL (0.039) | HUEL (0.039) | HUEL (0.039) |
| HPRT1 (0.226) | ECHS (0.053) | HPRT1 (0.219) | ECHS (0.058) | ECHS (0.058) | ECHS (0.058) |
| TBP (0.131) | TBP (0.068) | TBP (0.107) | TBP (0.070) | TBP (0.070) | TBP (0.070) |
| ECHS (0.390) | HPRT1 (0.077) | ECHS (0.312) | ECHS (0.112) | ECHS (0.112) | ECHS (0.112) |
| RPII (0.553) | β-actin (0.104) | β-actin (0.575) | β-actin (0.117) | β-actin (0.117) | β-actin (0.117) |
| β-actin (0.621) | RPII (0.106) | β-actin (0.642) | RPII (0.118) | RPII (0.118) | RPII (0.118) |
| B2MG (0.699) | PPIA (0.152) | PPIA (0.137) | B2MG (0.697) | B2MG (0.697) | B2MG (0.697) |
| PPIA (0.893) | B2MG (0.209) | PPIA (0.959) | PPIA (0.915) | PPIA (0.915) | PPIA (0.915) |
| PPIA (0.117) | PPIA (0.575) | PPIA (0.110) | PPIA (0.110) | PPIA (0.110) | PPIA (0.110) |

aData are given for paired cell samples (mock- and RV-infected, n = 24). The two most stable reference genes identified by geNorm cannot 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 ΔΔCt 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 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.
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 (3 dpi).

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 Cᵢ 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 moi 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).
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 ΔΔCt 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 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].

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 [Beach 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 diarrhoea virus infection were also observed in MEBK 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 (TBPA 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.

REFERENCES

Adamo MP, Zapata M, Frey TK. 2008. Analysis of gene expression in fetal and adult cells infected with rubella virus. Virology 370:1–11.

Andersen CL, Jensen JL, Ørntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250.

Beath MD, Everitt JC, Law LJ, Hohman TC. 2005. Interactions between rubella virus capsid and host protein p32 are important for virus replication. J Virol 79:10807–10820.

Bernasconi M, Berger C, Sigrist JA, Bonanomi A, Soek J, Niggli FK, Nadal D. 2006. Quantitative profiling of housekeeping and Epstein-Barr virus gene expression in Burkitt lymphoma cell lines using an oligonucleotide microarray. Virol J 3:43.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622.

Cincinati VR, Shen Q, Sotiropoulos GC, Radtke A, Gerken G, Beckehaun S. 2008. Validation of putative reference genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR. BMC Cancer 8:350.

Claus C, Hofmann J, Uberla K, Liebert UG. 2006. Rubella virus pseudotypes and a cell-cell fusion assay as tools for functional analysis of the rubella virus transmembrane protein in Burkitt lymphoma cell lines. J Virol 80:4657–4665.

Diaz MO, Ziemin S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.

Diaz MO, Ziemien S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.

Diaz MO, Ziemien S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.

Diaz MO, Ziemien S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.

Diaz MO, Ziemien S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.

Diaz MO, Ziemien S, Le Beau MM, Pitha P, Smith SD, Chilcote RR, Rowley JD, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9):e898.