Peptidylprolyl Isomerase A (PPIA) as a Preferred Internal Control Over GAPDH and β-Actin in Quantitative RNA Analyses


ABSTRACT

A good internal control is critical in all quantitative analyses of gene expression. Levels of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and peptidylprolyl isomerase A (PPIA) were analyzed in 78 samples (data obtained from our laboratory and from a publicly available database at http://www.ncbi.nlm.nih.gov/SAGE/). These libraries included cell lines and tissues from brain, breast, colon, kidney, ovary, pancreas, prostate, skin, and vascular origin. The level of PPIA mRNA is the most constant among the three genes. Hence, our study suggests that PPIA is a better internal control than β-actin and GAPDH.

INTRODUCTION

Quantitative gene expression assays have been widely used to investigate many cellular and pathological events. Assessment of mRNA is typically referenced to an internal control gene such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. However, it has been shown that both GAPDH and β-actin are regulated by internal factors, such as the cell cycle (9), and by external factors, such as growth factors (3,15), cytokines (16), serum (8,17), stress (5,23,25), vitamins (2,12), and other chemical compounds such as dioxins (10) and neuroendocrine peptide (26). In addition, GAPDH has proven to be an inappropriate internal standard for studies involving comparisons between certain tissues and corresponding cell lines (13) or between normal and tumor samples (1,4,7). Thus, the choice of an internal standard with consistent expression levels under all experimental conditions is crucial for accurate quantitative data analysis.

We have performed serial analysis of gene expression (SAGE) on human prostate samples (22) to identify genes that are regulated by dihydrotestosterone (DHT) and to find genes that are differentially expressed between normal prostate and tumor tissues. We identified approximately 800 genes that were differentially expressed, including GAPDH and β-actin. In search of an alternative internal control, we identified the peptidylprolyl isomerase A (PPIA) gene (also known as cyclophilin A). Here we provide evidence that shows PPIA as a better internal control than β-actin and GAPDH.

MATERIALS AND METHODS

Samples

Prostate cell lines LNCaP, PC3, and DU145 and tissues were obtained, maintained, and treated as described previously (21,22).

SAGE Data Analysis

SAGE data for human prostate were generated and analyzed as described previously (20,22). SAGE data for other tissue types were obtained from the National Center for Biotechnology Institute (NCBI) SAGE database (http://www.ncbi.nlm.nih.gov/SAGE/). Only libraries larger than 10,000 transcripts were included in our analysis.

External Control RNA

pLacZ(A)28 contains a fragment of the lacZ gene (2181 bp) and a poly(A) tail (28 bases) under the control of the T7 promoter. pLacZ(A)28 was linearized by restriction digestion with NotI and then purified using Wizard® (Promega, Madison, WI, USA). Transcription in vitro was performed with the Megascript™ High-Yield Transcription kit (Ambion, Austin, TX, USA) using 1 µg linearized plasmid DNA. Template DNA was removed by treatment with 2 U DNase I at 37°C for 15 min, and RNA was purified using TRIzol®.

Reverse Transcription

cDNA was reverse-transcribed from 1 µg total RNA from each prostate sample in the presence of 5 × 10⁶ molecules of LacZ transcribed in vitro with the SUPERSCRIPT™ II reverse transcriptase, as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA).

Quantitative PCR

Real-time quantitative PCR was performed on an iCycler™ (Bio-Rad Lab-
oratories, Hercules, CA, USA). All primers were designed using the Oligo Express™ program (Molecular Biology Insights, Cascade, CO, USA) as previously described (21). Primers used were: lacZ control forward 5'-GC-TTTTGCTACCTGGAGAGAC-3' and reverse 5'-GAAACGCCGCCAGTATTTAG-3'; PPIA forward 5'-CAAAATGCTGGACCCACACA-3' and reverse 5'-TGCCATCCAACCTCAGTC-3'; GAPDH forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAA-GATGGTGATGGGATTC-3'; and β-actin forward 5'-TGAGGCCCAGAGCAAGAGA-3' and reverse 5'-TCGTCCAGTTGGTGACGAT-3'.

To determine the relative quantity of PPIA in each sample, the amount of PPIA was normalized to that of lacZ. The normalized amount of PPIA is a unitless number that was used to compare the relative amount in different samples. To make this comparison, PC3 was designated as the calibrator (given the value of 1). The relative values were obtained by dividing the normalized value of a sample with that of PC3. The standard deviation of the quotient was calculated from the standard deviation of PPIA and lacZ values using the following formula for the coefficients of variation:

\[
CV = \sqrt{\left(\frac{\sigma_1}{\bar{X}_1}\right)^2 + \left(\frac{\sigma_2}{\bar{X}_2}\right)^2},
\]

where \(CV = S/\bar{X}\), \(S\) is the standard deviation and \(\bar{X}\) is the mean value. Therefore, the standard deviation of a quotient = \((CV)(\bar{X})\). Relative amounts of GAPDH and β-actin were calculated using the same formulae.

### Statistical Analysis

Expression data for each of the three candidate genes were recorded as one observation per sample per organ. We assumed that the distribution of expression is the same for each gene in all tissue types for a given organ. First, each gene was analyzed separately. A one-way analysis of variance (ANOVA) was used to test differences in the mean gene expression among the organs (11). Levene’s test was used to test the homogeneity of variances across organs. When variances were found to differ across organs, Welch’s variance-weighted ANOVA (24) was used. The Kruskal-Wallis test (6) was used to corroborate the results of parametric tests. Whenever the null hypothesis of equality of means across all organs was rejected, plots of organ-specific means were examined to identify subgroups of organs for which the mean levels were similar. We then tested the equality of mean expressions in the subgroups. One-sided Pitman tests (14) for equality of variance for paired data were used to assess the variability of expression

![Figure 2. Comparison of expression pattern of β-actin (A), GAPDH (B), and PPIA (C) in different tissue types. The column on the right demonstrates the distribution of the transcripts.](image)
across organs. This analysis was performed separately for each organ because the visual inspection of the plot of expression data for each candidate gene indicated that organ-specific expression variances vary greatly from one organ to another (confirmed by Levene’s test).

RESULTS AND DISCUSSION

An ideal gene serving as an internal control would be one whose expression remains constant under any conditions in all tissue types. However, it is unlikely that such a gene would exist since biological systems are dynamic and constantly responding to their environment. Therefore, the most appropriate internal control would be one that has the least variation in its expression under various experimental conditions and in different tissue types. With our previous SAGE data, we performed a thorough search for genes that were expressed at a similar level in the absence or presence of androgen and in normal or tumor tissue. We identified 77 genes that were not differentially expressed ($P > 0.5$). Among those genes, we selected those that were expressed at a level similar to that of $\beta$-actin and GAPDH. With these criteria, we identified the gene PPIA.

To confirm the steady level of PPIA expression, we performed quantitative PCR analysis and compared the variability in the expression of PPIA to that of $\beta$-actin and GAPDH in prostate sam-

<table>
<thead>
<tr>
<th>Organ</th>
<th>No. of Tissues</th>
<th>$\beta$-actin mean (± SEM)</th>
<th>GAPDH mean (± SEM)</th>
<th>PPIA mean (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>brain</td>
<td>23</td>
<td>2059.48 ± 372.91</td>
<td>2564.96 ± 534.83</td>
<td>1456.00 ± 303.60</td>
</tr>
<tr>
<td>breast</td>
<td>16</td>
<td>845.63 ± 184.53</td>
<td>2196.81 ± 570.46</td>
<td>3053.00 ± 426.66</td>
</tr>
<tr>
<td>colon</td>
<td>8</td>
<td>2181.82 ± 361.78</td>
<td>1192.63 ± 181.5</td>
<td>934.38 ± 144.03</td>
</tr>
<tr>
<td>ovary</td>
<td>11</td>
<td>2193.00 ± 450.80</td>
<td>3058.27 ± 614.41</td>
<td>1827.00 ± 349.16</td>
</tr>
<tr>
<td>pancreas</td>
<td>10</td>
<td>1298.10 ± 368.78</td>
<td>1084.90 ± 408.46</td>
<td>1817.00 ± 229.97</td>
</tr>
<tr>
<td>prostate</td>
<td>14a</td>
<td>1103.75 ± 201.68</td>
<td>1345.71 ± 268.27</td>
<td>1795.57 ± 158.49</td>
</tr>
</tbody>
</table>

$^a$There were only 12 non-zero values for $\beta$-actin.
$A$, $B$, $C$ Means with same superscript do not differ significantly from each other.

<table>
<thead>
<tr>
<th>Organ</th>
<th>No. of tissues</th>
<th>$\beta$-actin SD</th>
<th>GAPDH SD</th>
<th>PPIA SD</th>
<th>$P_{\text{PPIA vs. } \beta \text{-actin}}$</th>
<th>$P_{\text{PPIA vs. GAPDH}}$</th>
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</thead>
<tbody>
<tr>
<td>brain</td>
<td>23</td>
<td>1788.39</td>
<td>1587.51</td>
<td>1025.29</td>
<td>0.004</td>
<td>0.007</td>
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<tr>
<td>breast</td>
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<td>738.13</td>
<td>2281.84</td>
<td>1706.65</td>
<td>0.001$^b$</td>
<td>0.13</td>
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<tr>
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<td>8</td>
<td>1023.26</td>
<td>513.36</td>
<td>407.37</td>
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<td>0.26</td>
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<td>2037.77</td>
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<td>1003.77</td>
<td>593.02</td>
<td>0.22</td>
<td>0.04</td>
</tr>
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</table>

$^a$There were only 12 matched pairs for the PPIA versus $\beta$-actin comparison.
$^b$Alternative hypothesis: PPIA variance is greater than $\beta$-actin variance.
samples PC3, DU145, LNCaP (with and without DHT treatment), and tumor and normal tissues. A slight difference in expression patterns was observed for GAPDH, β-actin, and PPIA in LNCaP cells treated with or without DHT (Figure 1). Although these differences may not be statistically significant, PPIA appeared to show the least variability. This suggested that PPIA is a better internal control under these conditions than GAPDH or β-actin. In tumor versus normal tissues, GAPDH appeared to be more constant than PPIA.

To determine the expression pattern of these three genes in other tissue types and experimental conditions, we analyzed 78 SAGE libraries. Figure 2 shows a comparison of the expression distribution of PPIA to that of GAPDH and β-actin. Since only two observations of expression were available for skin, vascular, and renal tissue, we restricted our analysis to brain, breast, colon, ovary, pancreas, and prostate. Welch’s ANOVA procedure was used for all models since organ-specific expression variances were found to be significantly inhomogeneous for PPIA ($P = 0.03$) and GAPDH ($P = 0.008$) and marginally so for β-actin ($P = 0.09$).

The mean expression of PPIA among brain, ovary, pancreas, and prostate tissues was similar ($P = 0.23$) (Table 1). Interestingly, the mean expression was much higher ($P = 0.004$) in breast tissue and much lower ($P = 0.03$) in colon tissue than the other four organs. These results were corroborated by nonparametric tests. In the case of β-actin, its mean expressions appeared to be closer in tissues of the brain, colon, and ovary. The mean expressions in prostate and pancreas tissues were similar but approximately 50% less than that of brain, colon, and ovary. The mean expression in the breast was the lowest. Significant differences in GAPDH expression were detected when the means for all six organs were compared ($P = 0.005$). In comparison with β-actin, PPIA had significantly lower variability of expression levels in brain and colon tissues (Table 2). Although the difference was not statistically significant, PPIA variance was lower in ovarian, pancreatic, and prostate tissue. Note that the variance of PPIA was significantly higher than that of β-actin in breast tissue ($P = 0.001$). The variability of PPIA expression was significantly lower than GAPDH in brain, ovarian, and prostate tissue. In fact, the PPIA expression variance was lower than that of GAPDH for all six organs (Table 2). These results indicated that PPIA expression is more consistent than that of β-actin and GAPDH in a majority of organs analyzed.

The use of the PPIA gene as an internal control has been cited in a few papers (21). Other molecules such as 18S rRNA, 28S rRNA, 36B4, HPRT, and
tubulin have also been used as controls (18,19). However, rRNA molecules are unsuitable as internal standards for any analysis in which mRNA is the source reagent. HPRT, on the other hand, has been excluded from our study, as the gene is not highly expressed in many SAGE libraries derived from cell lines and tissues. In addition, like HPRT, tubulin is not widely used as an internal control. To date, no systematic study of the expression of these housekeeping genes has been reported. To our knowledge, this report is the first comprehensive study that compares the expression levels of GAPDH, β-actin, and PPIA in a large sample setting. The results of our study suggest that PPIA is a better internal control than GAPDH or β-actin in most circumstances. We recommend the use of PPIA as an internal control in RNA quantitative studies.

REFERENCES


21. Welch, B.L. 1938. The significance of the difference between two means when the population variances are unequal. Biometrika 29:350-362.


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Received 13 June 2001; accepted 10 December 2001.

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