

## HEPATOLOGY

# Sex-dependent expression of seven housekeeping genes in rat liver

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### Abstract

**Background and Aim:** Sexual differences in the transcript levels of various genes including the hepatic isoforms of cytochrome P450 have been extensively studied. Expression of these sexual dimorphic genes have been quantified by Northern blotting, nuclear run on assays and reverse transcriptase-polymerase chain reaction (RT-PCR) methods using numerous housekeeping genes to normalize results. Earlier reports apparently assumed that these internal controls were sex-independent. We have studied sex differences in the expression levels of seven different commonly used housekeeping genes.

**Method:** We have used quantitative and semiquantitative RT-PCR to monitor the levels of hepatic mRNAs in intact and hypophysectomized male and female rats.

**Results:** We have observed sex-dependent expression of the commonly used housekeeping genes tubulin, cyclophilin, tyrosine aminotransferase,  $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase, 18S and one unconventional housekeeping gene, that is, hypoxia inducing factor-1 $\alpha$ , in the livers of intact male and female rats. With the exception of glyceraldehyde-3-phosphate dehydrogenase and 18S which were female-predominant ( $P < 0.01$ ), the five other genes were found to be expressed at significantly ( $P < 0.01$ ) higher concentrations in the livers of intact male rats. Similar to findings in which hypophysectomy eliminates sexual dimorphisms in cytochromes P450 expression, of the five housekeeping genes examined, cyclophilin, tyrosine aminotransferase, glyceraldehyde-3-phosphate dehydrogenase,  $\beta$ -actin, and 18S, all lost their sex-dependent expression following pituitary ablation.

**Conclusion:** Our data suggest that expression levels of these commonly measured housekeeping genes (structural and metabolic) are not constant, but rather are directly or indirectly regulated by sex-dependent hormones, compromising their application as normalizing controls.

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**Key words:** housekeeping gene, liver, mRNA, reverse transcriptase-polymerase chain reaction (RT-PCR), sexual dimorphism.

## INTRODUCTION

The expression and sexually dimorphic regulation of numerous genes, including rat cytochrome P450 isoforms, have been extensively studied in several laboratories<sup>1–4</sup> including our own.<sup>5,6</sup> In these studies, as well as others, different housekeeping genes such as tyrosine aminotransferase (tat),<sup>1</sup> cyclophilin,<sup>7</sup> glyceraldehyde-3-phosphate dehydrogenase (g3dph),<sup>8</sup>  $\beta$ -actin,<sup>3</sup> 18S<sup>6</sup> and tubulin<sup>2,9</sup> were used to normalize sex-dependent levels of hepatic mRNA. However, no reports have mentioned any significant sex differences in expression of the housekeeping genes.

Although housekeeping genes are defined by their invariable presence constitutively in every cell, it does

not necessarily mean that their expression is unregulated.<sup>10</sup> Nevertheless, they are used as internal controls due to the assumption that their levels of expression remain constant from cell to cell, sample to sample, and between sexes.

Recently, using reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR methodologies, it was reported that the expression of various housekeeping genes are altered by hypoxia,<sup>11</sup> transplantation,<sup>12</sup> sex steroids,<sup>13</sup> fasting,<sup>14</sup> and diseases like liver cancer<sup>15</sup> and asthma.<sup>16</sup> From these observations, it can be inferred that the choice of a housekeeping gene has to be validated under the same conditions used in the study. This becomes crucial when the housekeeping gene is the sole means of normalizing data. Accordingly,

in this study, we examined the effects of sex on the expression of seven different housekeeping genes in intact and hypophysectomized male and female rat livers.

## METHODS

All animals were housed in the University of Pennsylvania Laboratory Animal Resources Facility under the supervision of a certified laboratory animal medicine veterinarian. These animals were treated according to a protocol approved by the University's Institutional Animal Care and Use Committee. Adult intact and hypophysectomized male and female [CrI:CD(SD)BR] Sprague-Dawley rats were obtained from Charles River Laboratories, Wilmington, MA, USA. These animals were housed under condition of regulated temperature (20–23°C) and photoperiod (12 h of light/12 h of darkness; lights on at 08:00 h) to minimize inter-animal variation in light entrained<sup>17</sup> growth hormone secretory profiles. Some rats were hypophysectomized by the supplier at 8 weeks of age and maintained for 4–5 weeks in the animal care facility. The effectiveness of the surgery was verified by the lack of bodyweight gain during the observation period and absence of a pituitary or fragments at necropsy. Animals were killed at 14 weeks of age; livers were removed quickly, minced, plunged into liquid nitrogen and stored at –70°C for further processing.

Total hepatic RNA from frozen liver was extracted by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting RNA was reconstituted in diethylpyrocarbonate-treated water (DEPC-H<sub>2</sub>O). RNA was quantified spectrophotometrically at 260 nm and purity was confirmed by 260/280 ratio. Total RNA (20 µg) was separated on agarose gel containing 2.2 M formaldehyde. RNA integrity was assessed by the intensities of 28S and 18S rRNA bands visualized on ethidium bromide stained gels. This total RNA was stored in DEPC-H<sub>2</sub>O at –70°C.

cDNA synthesis was performed in a total volume of 20 µL. For the conversion of total RNA to cDNA, a 20 µL reaction mixture was prepared containing 1X reverse transcriptase (RT) buffer (Promega, Madison, WI, USA), 5 mM MgCl<sub>2</sub>, 1 mM of each dNTPs (Promega, USA), 1 unit of RNase inhibitor (Promega, USA), 2.5 units of MuLV reverse transcriptase (Promega, USA), 2.5 µM oligo d(T)<sub>16</sub>, or 2.5 µM random hexamers for 18S (Applied Biosystem, Foster City, CA, USA) and 1 or 2 µg of RNA. The reaction was incubated at 42°C for 1 h and stored at –20°C. Appropriate RT(–) controls included in this study confirmed the absence of genomic DNA.

For the PCR amplification of cDNA, a 100 µL reaction mixture was prepared containing 1X PCR buffer (Promega, USA), 2 mM MgCl<sub>2</sub> (Promega, USA), 2.5 units of Taq DNA polymerase (Promega, USA), 1 µL of cDNA and 150 nM of specific sets of primers. PCR cycles were performed with GeneAmp PCR System 9600 (Perkin-Elmer Life and Analytical Sciences,

Boston, MA, USA) thermocycler. Sequence of primers and cycling conditions for tubulin,<sup>18</sup> HIF-1 $\alpha$ ,<sup>18</sup> tat,<sup>19</sup> cyclophilin,<sup>20</sup> g3dph,<sup>21</sup>  $\beta$ -actin (Clontech, Palo Alto, CA, USA) and 18S (Ambion, Austin, TX, USA) have been reported earlier.

The quantitative real-time PCR reactions were carried out in a total 20 µL reaction mixture containing 2.5 µL of SYBR Green I (Molecular Probes, Eugene, OR, USA), 250 µM of dNTPs, 250 nM of each primers, 0.4 µL of DMSO, 0.6 µL of titanium Taq DNA polymerase (BD Biosciences, San Jose, CA, USA) and 1 µL of cDNA reverse transcription product. PCR cycling was performed in capillaries using the LightCycler rapid thermal cycler system (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. The final PCR products were subsequently melted by linear heating (0.2°C/s) to 95°C. The position and size of the melting peak provided an assessment of the generation of expected cDNAs, which were confirmed by agarose gel electrophoresis. For the estimation and extrapolation of copy numbers in the samples, a standard curve was generated from DNA samples containing known copy numbers of the gene under consideration. These samples were generated from the purified PCR products.

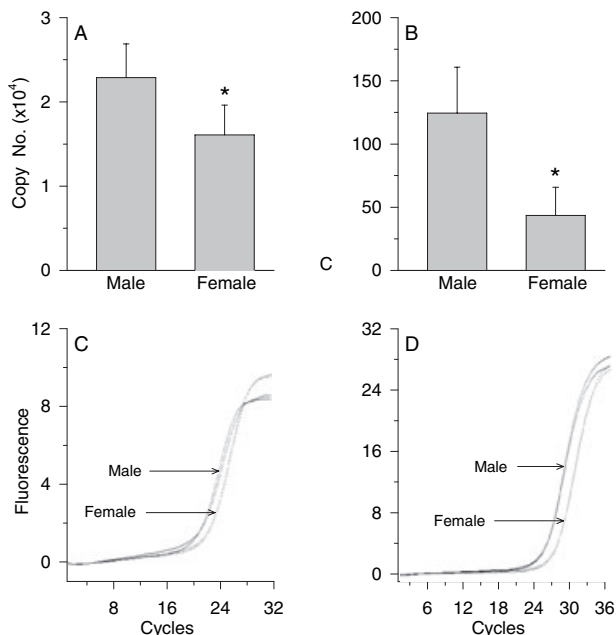
The final PCR products were electrophoretically separated on 1.5% agarose gel run with 0.5X TBE for 90 min at a constant current of 80v. These electrophoresed gels were stained for 20 min with 0.5X TBE containing 1 µg/mL ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). The final PCR product was quantified with an Alpha Innotech FluorChem 8800 (Alpha Innotech, San Leandro, CA, USA) gel documentation system using a UV lamp.

Statistical analysis of data was performed using Student's *t*-test.

## RESULTS

A structural protein like tubulin is commonly used as a housekeeping gene in quantitative RT-PCR.<sup>18</sup> As such, it is assumed that basal expression levels of tubulin mRNA are constant and do not change with the experimental conditions nor should they vary between sexes. On the contrary, our data indicate that the copy number of tubulin mRNA were significantly ( $P < 0.01$ ) higher in intact male liver than female liver (Fig. 1a). This is clearly shown by the lower numbers of cycles in intact male livers (Fig. 1c). The Ct values (mean  $\pm$  SD) for hepatic tubulin were  $20.47 \pm 0.38$  and  $21.25 \pm 0.36$  ( $P < 0.02$ ) for males and females, respectively. The slope of the standard curve, a surrogate marker of amplification efficiency, was –3.92.

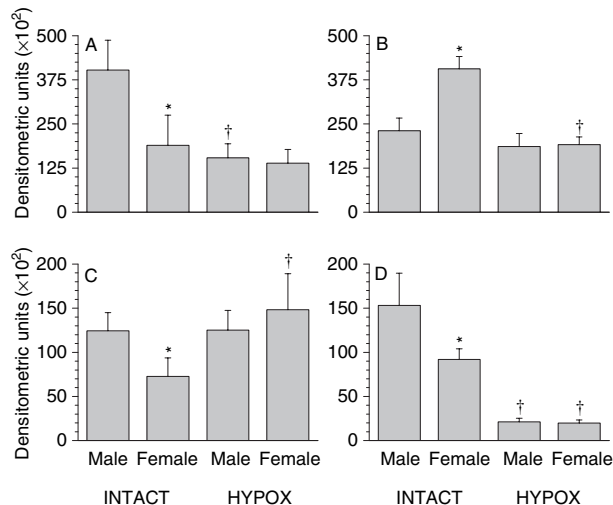
Having observed sex-dependent expression of tubulin mRNA, we examined five other commonly used housekeeping genes; g3dph, tat,  $\beta$ -actin, cyclophilin and 18S in male and female liver by a semiquantitative RT-PCR method. Our results demonstrate that the expression of tat (Fig. 2a), cyclophilin (Fig. 2c) and  $\beta$ -actin (Fig. 2d) mRNA were significantly ( $P < 0.01$ ) higher in the livers of intact males than females; whereas the sexual dimor-



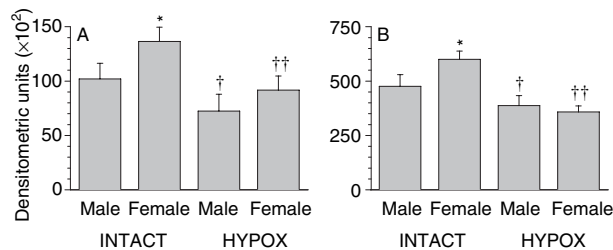
**Figure 1** Expression of tubulin and HIF-1 $\alpha$  mRNA in intact male and female rat livers as determined by the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). (a) Differences in the number of copies of hepatic tubulin mRNA in intact male and female rats. (b) Differences in the copy numbers of hepatic HIF-1 $\alpha$  mRNA in intact male and female rats. Actual fluorescence curves (two livers of each sex) representing the cycle numbers for mRNA of tubulin (c) and HIF-1 $\alpha$  (d). Values presented are means  $\pm$  SD of at least five animals for each sex. \* $P < 0.01$ .

phic expression of g3dph was reversed (Fig. 2b), that is  $F > M$ . Similarly, 18S, likely the most commonly measured housekeeping gene used to normalize results, also exhibited greater expression levels in females (Fig. 3a). In order to identify any possible poly-A bias resulting from the use of oligo-dT primers that could lead to erroneous quantitation, we measured 18S expression using random hexamer primers (Fig. 3b). Expectedly, while the magnitude of the PCR products were greater with the random hexamer primers, we observed the same sexual dimorphic expression,  $F > M$ .

Having found a pattern of sex-dependent expression for the six housekeeping genes tubulin, cyclophilin, tat, g3dph,  $\beta$ -actin, and 18S, we measured mRNA levels of the unconventional housekeeping gene hypoxia inducing factor-1 $\alpha$  (HIF-1 $\alpha$ ). There were two reasons for the selection of HIF-1 $\alpha$ ; (i) HIF-1 $\alpha$  mRNA is constitutively expressed;<sup>22</sup> and (ii) HIF-1 $\alpha$  mRNA can be induced by hypoxia, but remains unchanged under normoxic conditions.<sup>23,24</sup> These two characteristics of HIF-1 $\alpha$  indicate that it is a fairly unique gene in which to test for sexual dimorphic expression. Using quantitative RT-PCR methods, we observed from the difference in cycle numbers that HIF-1 $\alpha$  mRNA was significantly ( $P < 0.01$ ) higher in intact male livers as compared to that of female livers (Fig. 1b,d). The Ct values (mean  $\pm$  SD) for hepatic HIF-1 $\alpha$  were  $21.07 \pm 0.56$  and



**Figure 2** Expression of tyrosine aminotransferase, glyceraldehyde-3-phosphate dehydrogenase, cyclophilin, and  $\beta$ -actin mRNAs in livers of intact and hypophysectomized (HYPOX) male and female rats using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). (a) Expression of hepatic tyrosine aminotransferase mRNA. (b) Expression of hepatic glyceraldehyde-3-phosphate dehydrogenase mRNA. (c) Expression of hepatic cyclophilin mRNA. (d) Expression of hepatic  $\beta$ -actin mRNA. Values presented are means  $\pm$  SD of at least five livers from each group. \* $P < 0.01$ ; compares males to females of the same treatment. <sup>†</sup> $P < 0.01$ ; compares intact to HYPOX of the same sex.



**Figure 3** Expression of 18S RNA in livers of intact and hypophysectomized (HYPOX) male and female rats using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). (a) Values determined using oligo-dT primers of cDNA synthesis. (b) Values determined using random hexamer primers. Values presented are means  $\pm$  SD of at least five livers from each group. \* $P < 0.01$ ; compares males to females of the same treatment, <sup>†</sup> $P < 0.05$ , <sup>††</sup> $P < 0.01$ ; compares intact to HYPOX of the same sex.

$23.20 \pm 0.78$  ( $P < 0.01$ ) for males and females, respectively. The slope of the standard curve was  $-4.36$ .

Our observation of sexually dimorphic expression levels of seven housekeeping genes implicated the involvement of sex-dependent hormones. Because sex-dependent growth hormone is the sole regulator of hepatic P450 isoforms,<sup>1,5</sup> we chose to examine expression levels of representative housekeeping genes in hypophysectomized rats of both sexes sensitive to the possibility that they too might be regulated by growth

hormone. Additionally, even if the housekeeping genes were growth hormone-independent, hypophysectomy was an effective method of eliminating all sexually dimorphic hormone secretions from the thyroid, adrenals, gonads as well as other pituitary hormones, that is, LH, FSH and prolactin. The amplification conditions for the mRNA from hypophysectomized rats were similar to those used for intact rats. In brief, hypophysectomy eliminated sexual differences in hepatic *tat*, *g3dph*,  $\beta$ -actin, cyclophilin and 18S levels (Figs 2,3). The sex expressing the lower concentration of *tat* (female), and *g3dph* (male), in intact animals was unaffected by hypophysectomy. Rather, mRNA levels of the housekeeping gene in the sex expressing the higher intact levels declined following hypophysectomy to that of the opposite sex. However, in spite of sex differences in  $\beta$ -actin and 18S, expression levels declined in both sexes following pituitary ablation. Cyclophilin in the livers of female rats appeared to be the only housekeeping gene repressed by the pituitary; concentrations increasing ( $P < 0.01$ ) following surgical ablation to that of intact or unchanged hypophysectomized male levels (Fig. 2c).

Thus, the loss of sexual differences in expression of the housekeeping genes by hypophysectomy suggest some degree of sex-dependent regulation.

## DISCUSSION

We report, likely for the first time, our observation that hepatic mRNA levels of seven commonly measured housekeeping genes; cyclophilin, tubulin, *g3dph*, *tat*,  $\beta$ -actin, 18S and HIF-1 $\alpha$ , are constitutively sexually dimorphic. Elimination of sex differences in expression levels of the genes following hypophysectomy demonstrates hormonal regulation, although the particular hormone(s) involved have not been identified.

For several reasons the wide application of these housekeeping genes in biochemical research has proven to be highly beneficial. That is, because transcriptional levels of the genes are measured in numerous laboratories, adopting the same primers and synthetic conditions allows one to compare the effectiveness of their own expression system with that of other groups. More importantly, the expression levels of housekeeping genes are considered to be invariable, and thus serve as controls or constants to compare or standardize the activities of those genes altered by the experimental conditions. However, not a single housekeeping gene has been accorded the designation as the universal or gold standard. Accordingly, several xenobiotics have reported to alter the expression levels of tubulin mRNA in livers.<sup>25</sup> Using both quantitative and semiquantitative RT-PCR methods, *g3dph* mRNA concentrations were found to be changed by carcinogenesis,<sup>26–28</sup> fasting<sup>14</sup> and administration of sex hormones.<sup>13</sup> The expression levels of both *g3dph*<sup>11</sup> and cyclophilin<sup>29</sup> are responsive to hypoxia. Finally, 18S and  $\beta$ -actin mRNA expression has been reported to increase in cell cultures exposed to serum<sup>30</sup> or matrigel treatment,<sup>31</sup> as well as from tissues obtained from asthmatics<sup>16</sup> and transplantation patients.<sup>12</sup> Considering the above reports and the

present findings that the expressions levels of commonly used hepatic housekeeping genes are sexually dimorphic, the constancy of any selected control gene should first be validated under the experimental conditions studied.

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