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TECHNIQUES

# Developmental Expression Profiles of *Xenopus laevis* Reference Genes

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**Cell differentiation depends mainly on specific mRNA expression. To quantify the expression of a particular gene, the normalisation with respect to the expression of a reference gene is carried out. This is based on the assumption that the expression of the reference gene is constant during development, in different cells or tissues or after treatment. *Xenopus laevis* studies have frequently used eEF-1 alpha, GAPDH, ODC, L8, and H4 as reference genes. The aim of this work was to examine, by real-time RT-PCR, the expression profiles of the above-mentioned five reference genes during early development of *X. laevis*. It is shown that their expression profiles vary greatly during *X. laevis* development. The developmental changes of mRNA expression can thus significantly compromise the relative mRNA quantification based on these reference genes, when different developmental stages are to be compared. The normalisation against total RNA is recommended instead. *Developmental Dynamics 235:754–758, 2006.* © 2006 Wiley-Liss, Inc.**

**Key words:** *Xenopus*; real-time PCR; normalisation; housekeeping genes

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## INTRODUCTION

Cell properties depend mainly on the nucleic acid and protein content. The mRNA molecules represent a connection between DNA and protein. The spatial and temporal gene expression changes are a key mechanism in cell differentiation. Two different types of *Xenopus* mRNAs can be distinguished: (1) the maternal mRNAs, which occur in the oocyte before fertilization and originate from the female, and (2) the zygotic mRNAs, newly synthesized mainly after the midblastula transition (MBT). The correct spatial and temporal expression of both types of mRNAs is necessary for the first developmental processes such as the main body axis formation, gastrulation, germ layers induction, and others. The mRNA expression has been broadly studied by common methods such as Northern blotting, RNase protection assays, in situ hybridization, reverse transcription-polymerase chain reaction (RT-PCR), and microarray analysis. In the last decade, a new highly sensitive and specific method for RNA/DNA quantification, the real-time RT-PCR (qPCR), was introduced. The sensitivity, dynamic range, linearity of the measurements, and robustness make the qPCR a method of choice for the quantitative analysis of mRNA expression. For these reasons, it is also frequently used as an independent validation tool for the verification of microarray expression data (Giulietti et al., 2001).

The quantification can be carried out by two different approaches. The absolute method determines the number of mRNA copies in the sample from a calibration curve obtained from samples of cDNA complementary to mRNA of known concentrations. The relative approach compares copies of the target mRNA with those of a reference gene. The metabolic, structural, and ribosomal RNA genes, such as those coding for beta-actin, GAPDH, beta-tubulin, 18S rRNA, and so on, belong among the most popular reference genes (Suzuki et al., 2000). The normalisation against a reference gene requires a constant mRNA expression of the reference gene, which would not vary during the cell cycle, in different cell types, or during development. Moreover, the expression level of the

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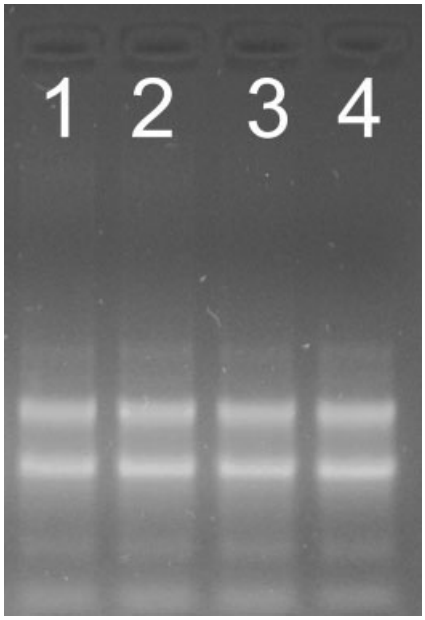


**Fig. 1.** One percent ethidium bromide agarose gel electrophoresis of 1 μg of total RNA from stage 10.5 (line 1 and line 2) and stage 13 (line 3 and line 4).

reference gene should be similar to that of the target gene. However, several recent studies have shown that, in vertebrate systems, the mRNA expression of reference genes differed among tissues, various cells, and after treatment (Zhang, 2003; Bas et al., 2004; de Kok et al., 2004; Bustin and Nolan, 2004; Vandesompele et al., 2002; Brunner et al., 2004; Radonic et al., 2004). To date, the most reliable method for normalisation appears to relate the mRNA data to the total RNA content of the sample preparation subjected to the reverse transcription reaction (Bustin, 2002). The most important step in the normalisation to total RNA is thus a precise determination of RNA concentration and the quality of RNA preparation (Fig. 1). *Xenopus laevis* mRNA expression studies have almost always used the genes coding for elongation factor eEF-1 alpha, ornithine decarboxylase (ODC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), L8-ribosomal protein, and H4-histone protein as reference genes (Chang, 2004; Veldholen et al., 2002; Wardle and Smith, 2004). The aim of this study was to determine, by qPCR and normalisation to total RNA content, the mRNA expression profiles of the above-mentioned five reference genes, and, of two other genes, coding for N-tubulin and Xbra, in the course of *X. laevis* early development. The profiles are stage- and gene-specific and allow to determine stages at which the normalisation with the reference genes appears to be plausible and when it would give more or less biased results.

## RESULTS

The concentration of total RNA per embryo in the course of early *Xenopus* embryogenesis stays essentially constant up to at least stage 32, and then a slow and continuous increase is observed so that the concentration of RNA at stage 44 is about two-and-half times higher than that at earlier stages (Fig. 2). On the contrary, as shown in Figure 3, the expression levels of all studied genes greatly varied during *X. laevis* development and among genes themselves. As expected, the levels of all tested mRNAs fluctuated less until the MBT stage, after which significant increases in expression and fluctuation were observed at various later stages of the development.

The level of elongation factor eEF-1 alpha mRNA changed only slightly by stage 9. Neither loss nor increase of the mRNA was apparent during this period. However, its concentration began to rise sharply after stage 10.5 and reached the maximum level at stage 26, which was about 80 times higher than at stage 1. This profile correlates nicely with the results that Krieg et al. (1989) obtained by a different method and is consistent with the idea that the MBT stage serves as an activation switch of the transcription of the *X. laevis* embryonic eEF-1 alpha gene.

The GAPDH mRNA kept an approximately constant level up to stage 24 with only slight, about twofold, fluctuations. Then, it rose steeply, about 10 times, and this elevated level was maintained until stage 44, the last stage we measured. The expression profile of L8 mRNA was similar to that of GAPDH except for the decrease at stages 33/34 and 37/38, followed by a steep increase at stage 44. The expression of the ODC mRNA, recently the most frequently used "gene" for normalisation, displayed only slight, about two- to three-fold fluctuations, both up and down, during the whole examined developmental period, except for stages 11.5 and 13 (tenfold decrease), and for stage 44 (tenfold increase).

The expression of the H4 gene shows a different profile when compared with other reference genes. The highest H4 mRNA level was detected in the egg. After stage 4 post-fertiliza-

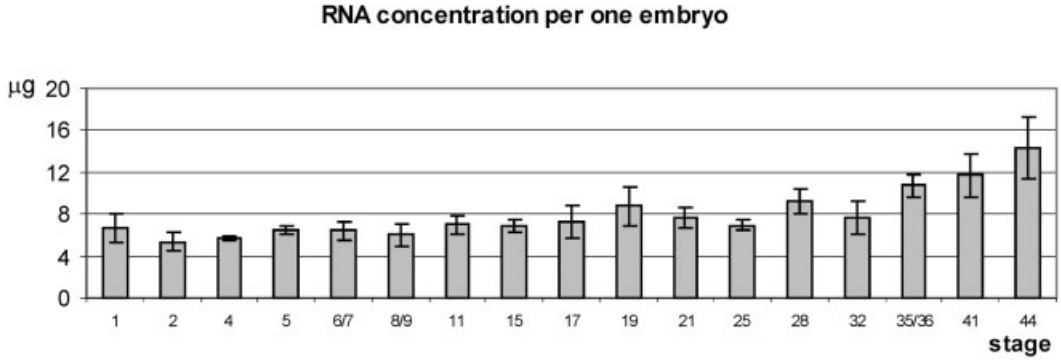


**Fig. 2.** Total RNA amount in *Xenopus laevis* early developmental stages. RNA was extracted from eggs/embryos and diluted to 50 μl as described in the Experimental Procedures section. Average RNA concentrations (columns) per embryo and standard deviations (bars) from 6 sets of the five eggs/embryos from stages indicated on the horizontal axes are shown.

tion, it gradually, about 5-fold, decreased with a minimum at stage 11. Subsequently, it recovered again, but the H4 mRNA level never exceeded that one detected in the egg.

Two non-reference genes, one coding for Xbra and the other for N-tubulin, were chosen to compare the reliability of our real-time PCR procedure with the already published data (Saka and Smith 2004). The *Xbra* gene belongs among the genes important for early *Xenopus* development. The *Xbra* mRNA expression profile presented here (Fig. 4a) had a similar shape to that described previously for stages 1 to 24 (Saka and Smith 2004). The N-tubulin gene transcription has been known to be closely related to the differentiation of the neural tissue. No expression of N-tubulin mRNA was detected until stage 13. Then, from stage 15, the level of the N-tubulin mRNA continuously rose, peaking around stage 28, the onset of its expression correlating with the onset of neurulation (Fig. 4b). Subsequently, the N-tubulin mRNA levels declined ~4 fold by stage 44. qPCR reactions with mRNA of stage 15 and later stages generated only one specific product with the right sequence and size as confirmed by sequencing and melting curve analyses. These two results prove the reliability of our real-time PCR procedure.

## DISCUSSION

Various reference genes in various vertebrate organisms have been used to normalise mRNA expression in their tissues. In this work, the developmental profiles of mRNA expression of *X. laevis* reference genes most frequently used for relative mRNA quantification were determined. The results show fluctuations of at least an order of magnitude in the expression of the reference genes in the course of *X. laevis* early development, and different genes show different profiles. This restricts their use as reference genes to only particular developmental stages depending on the type of the reference gene. None of these genes is particularly well-suited as a reference gene throughout the developmental period most frequently studied in *Xenopus* experiments. The ODC mRNA level appears to be suitable as a refer-

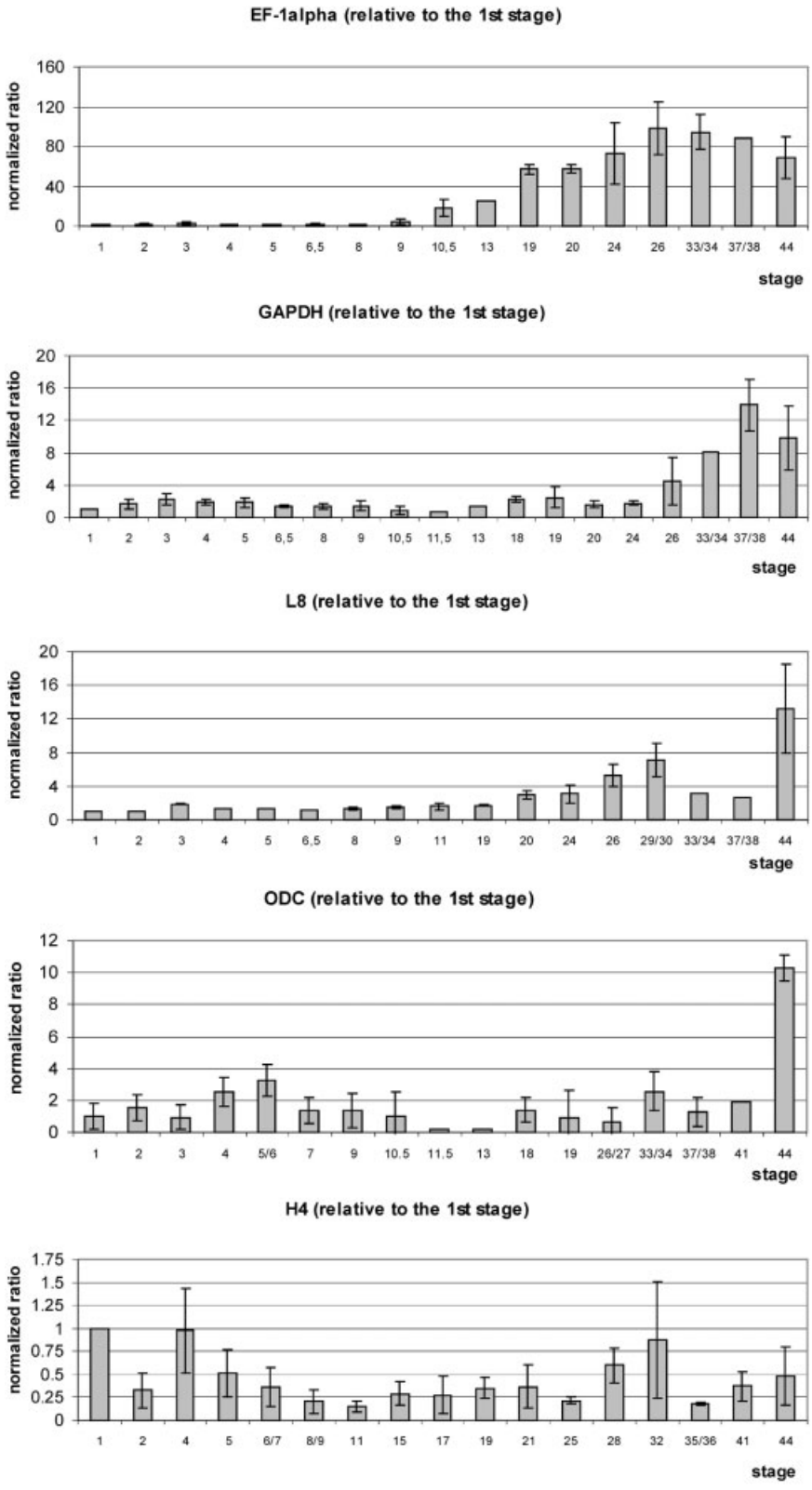


**Fig. 3.** The mRNA expression profiles of reference genes coding for (**a**) eEF-1 alpha, (**b**) GAPDH, (**c**) L8, (**d**) ODC, (**e**) H4 normalised to total RNA at stage 1 and expressed in arbitrary units. The numbers on the vertical axis represent the ratio between the average amount of copies of mRNA in a particular developmental stage and stage one normalised to the same amount of input RNA. For details see the Experimental Procedures section.

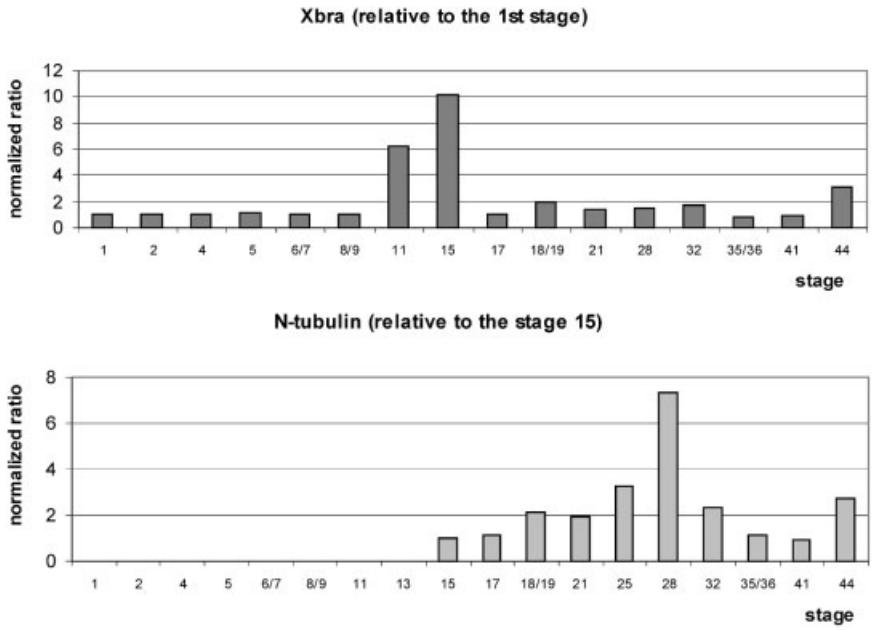


**Fig. 4.** The mRNA expression profiles of a non-reference gene *Xbra* (**a**) and a gene coding for N-tubulin (**b**) normalised to total RNA at stage 1 (*Xbra*) or stage 15 (N-tubulin) and expressed in arbitrary units. The expression of gene *Xbra* begins at stage 1 and the expression of N-tubulin is linked with neurogenesis and does not begin before the stage 15. The numbers on the vertical axis represent the ratio between the average amount of copies of mRNA in a particular developmental stage and stage one (*Xbra*) or stage 15 (N-tubulin) normalised to the same amount of input RNA. For details see the Experimental Procedures section.

ence throughout stages 1–10.5 and 18–41. The H4 mRNA level displays the smallest fluctuations over the entire developmental period; about fivefold change was detected throughout stages 1 to 44. The normalisation using the L8 and GAPDH mRNA appears to be suitable up to stage 19 whereas the normalisation using the eEF-1 alfa mRNA level should not exceed stage 9. Thus, the normalisation against total RNA according to Bustin (2002) seems to represent for *X. laevis* mRNA measurements a more practicable, reliable, convenient, and versatile method of choice than the normalisation against any one of the five reference genes described above. To find a true reference gene with constant and stable transcription in each tissue type and cell would be extremely useful. However, to date, such a gene has not been identified. As shown here, normalisation against any one of the examined five reference genes should be used only for the same *Xenopus* developmental stage or for a few carefully selected stages. The differential and stage-related expression of the reference genes should be taken into consideration in all relative mRNA quantification assays.

## EXPERIMENTAL PROCEDURES

### RNA Extraction and cDNA Preparation

*X. laevis* embryos were staged according to Nieuwkoop and Faber (1967). Two sets of 5 embryos from stages indicated on the horizontal axis in Figures 2–4 were collected from three independent in vitro fertilizations (three different females) and frozen immediately (Fig. 5). RNA from each sample was extracted using Trizol reagent (Invitrogen) according to instructions of the manufacturer and its concentration was determined by UV spectrophotometry at $A_{260}$. Each sample was measured three times and the average value was determined (Fig. 2). The quality of total RNA was analysed by 1% ethidium bromide agarose gel electrophoresis (Fig. 1).

cDNA was synthesized using 1 μg of total RNA and 10 pmol 25-dT oligo, the mixture was incubated at 72°C for 10 min, then 100 U MMLV reverse transcriptase (Promega), 12 U RNasin (Promega), 5 nmol dNTP were added to a total volume of 10 μl and incubation continued at 37°C for 70 min. The reactions were subsequently diluted to 50 μl and frozen.

### Real-Time RT-PCR

Sequences of primers for *X. laevis* elongation factor eEF-1alpha, GAPDH, and N-tubulin cDNA amplification were designed according to XMMR (http://www.xenbase.org/WWW/Marker_pages/primers.html). Primers for ODC cDNA amplification were the same as designed by Heasman et al. (2000), primers for *Xbra* cDNA amplification were identical with those designed by Sun (1999), and primers for L8 and H4 cDNA amplification were designed by using the Beacon Designer 2.00 program (Premier Biosoft International). L8- Forward primer: TCCGTGGTGTGGCTATGAATCC; Reverse primer: GACGACCAGTACGACGAGCAG, H4- Forward primer: GACGCTGTCACCTACACCGAG; Reverse primer: CGCCGAAGCCGTAGAGAGTG. The experiments were performed according to the scheme given in Figure 5 (Stahlberg et al., 2004). The real-time RT-PCR mixture, in the final volume of 25 μl, contained 2 μl of cDNA, 50-fold diluted SYBRGreen solution (Molecular Probes), 0.5 mM forward and reverse primer and 1U Taq polymerase (Promega). The reactions were measured in iCycler (Bio-Rad) with cycling conditions: 95°C for 5 min, 40 cycles at 95°C for 15 sec, and 60°C for 60 sec. Serially diluted PCR fragments (standards), identical with those amplified in the real-time PCR experiment, were prepared to obtain calibration curves. Reaction efficiencies determined from calibration curves for each set of primers were between 85 and 100%. The Cts (threshold cycles) of the samples and standards were analyzed with Microsoft Excel program and copies of PCR products from particular stages of development were determined from calibration curves. The average deviation between Cts in parallel experiments did not exceed about 4.3% for all tested genes and stages. The expression profiles were derived from three independent *X. laevis* serial experiments. Specificity of every amplification reaction was verified by melting curve analysis and gel electrophoresis. The numbers on the vertical axis in Figures 3 and 4 represent the ratio between the average amount of copies of mRNA in a particular developmental stage and stage one normalised to the same amount of input RNA. The concen-

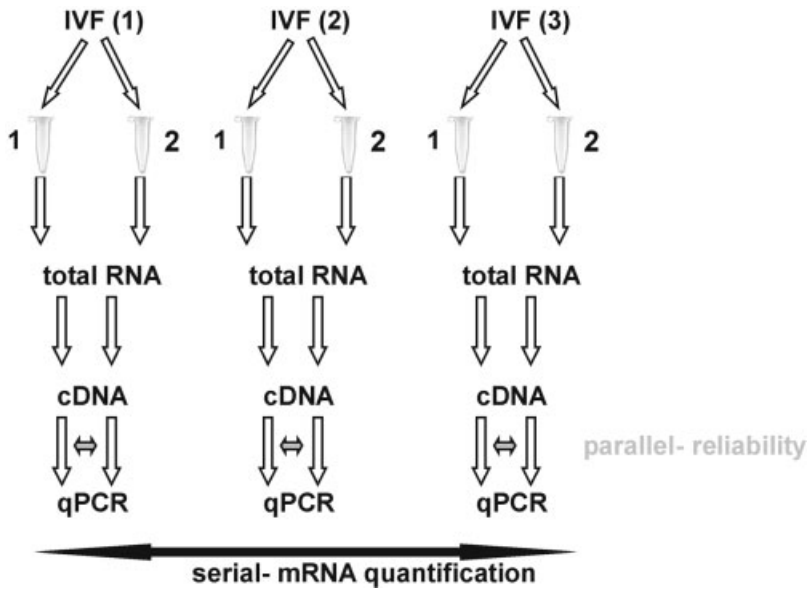


**Fig. 5.** A scheme of experiment: two samples (5 embryos) from three independent IVFs (in vitro fertilizations) were collected. The parallel samples served for the evaluation of the reproducibility of the procedure from the stage of RNA isolation to the qPCR measurement. The serial analysis shows variations between different IVF.

tration of every mRNA in stage one samples is arbitrarily taken as equal to 1. Standard deviation between results from serial experiments was determined and it is shown by error bars in Figure 3.

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