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Original Articles

Selection of Normalizer Genes in Conducting Relative Gene Expression Analysis of Embryos

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BACKGROUND: In relative gene expression analysis, a reference gene for sample normalization is required for determining target expression changes among experimental treatment groups. Since some developmental toxicants secondarily cause general growth retardation and/or other general biological changes, commonly used housekeeping genes may not serve as accurate normalizers. **METHODS:** We conducted real-time polymerase chain reaction (PCR) with normalization to calculate relative target transcriptional change, using housekeeping and structure-specific expression genes as normalizers. Relative levels of *Hoxb1* expression were measured in cultured rodent embryos at 24 hr post retinoic acid (RA) administration. Transcriptional response was also evaluated using two novel compounds that produced posterior axial and growth defects in rat whole-embryo culture. Embryos treated with these compounds were evaluated for general biological processes, and their respective biological states were considered in the context of the relative gene expression change calculated with the housekeeping normalizers. **RESULTS:** Normalized RA-induced *Hoxb1* expression demonstrated that only some reference genes accurately quantitated the expected 1.5- to 2-fold increase in *Hoxb1* expression. Evaluation of the test compounds demonstrated that only normalization with the spatially-restricted hindbrain gene, *Krox-20*, calculated significant expression decreases of *T*-gene, a gene known to be functionally relevant in posterior axial development. Reduction in *T*-gene expression was confirmed qualitatively by whole-mount in situ hybridization. **CONCLUSIONS:** Prudent reference gene selection is important in evaluating relative gene expression in embryos. An experimental control design is proposed to facilitate the identification of normalizing genes that will accurately calculate relative gene expression change in treated embryos. *Birth Defects Research (Part A) 67:533–544, 2003.*

Key words: real-time PCR; reference gene; normalizer gene; relative expression PCR; quantitative gene expression; PCR analysis of embryos

INTRODUCTION

When relative change in gene expression analysis is performed with real-time or conventional polymerase chain reaction (PCR), the calculated level of target gene expression is normalized against a reference gene. Normalization is essential in studies that evaluate relative gene expression change among various treatment groups, because it enables mechanistically relevant gene expression changes to be distinguished from background expression variations. Normalization is particularly important in determining relative gene expression change in studies of treated embryos, as differences in the absolute copy number of target gene expression may be more reflective of general size differences between embryos in various treatment groups, and thus may not represent a true event of target expression change.

The traditional choice for a reference gene is a "housekeeping gene," a gene that is involved in endogenous cellular functions common to all cell types. However, when working with embryos, it can be difficult to determine which gene will serve as an optimal reference for normalizing relative gene expression changes. Unlike cell lines and single-organ tissues, the cells comprising the embryo have a vastly heterogeneous nature, which leads to greater variation in the endogenous biological processes, and greater variation in the sensitivity of the cells to administered compounds. Commonly used housekeeping reference genes, such as genes involved in protein translation, glycolysis, or cytoskeletal structure, may not serve as biologically accurate normalizers for conducting differential gene expression analysis of embryos. One reason why the use of these genes as normalizers is potentially problematic is that some developmental toxicants/teratogens may compromise embryonic growth secondarily to the initial insult that directly led to the morphological abnormalities. Furthermore, compounds that cause morpholog-

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ical abnormalities may also compromise the cytoskeletal structural integrity of various tissues.

The choice of genes to select as normalizers in relative gene expression studies is not limited to universally expressed housekeeping genes. The function of the normalizer is to represent background gene expression that is not linked to the biological response of the experimental treatment. In the context of comparative gene expression analysis of whole embryos, a reference gene can be one that is expressed in all embryos of the same gestational stage but is restricted to a relatively unaffected region of the embryo, or one that is expressed in a region that is affected by the compound but is not suspected to be directly involved in the teratogenic pathway.

Two objectives were addressed in this study. The first objective was to determine how the use of various normalizing genes can impact final calculations of relative gene expression change. A pilot study was performed to determine the optimal approach to accurately quantitate a known gene expression response to a teratogen. Previous reports using in situ hybridization or transgenic *Hoxb1*(Hox2.9)-LacZ reporter embryos demonstrated that *Hoxb1* expression becomes anteriorized and duplicated in the hindbrain, and increased in other structures following retinoic acid (RA) treatment, thus providing a documented standard of target gene expression change (Morris-Kay et al., 1991; Marshall et al., 1992). In this study, we repeated those investigations using cultured presomite rat embryos. Relative change in *Hoxb1* expression was evaluated qualitatively by whole-mount in situ hybridization and quantitated by real-time PCR using various genes as normalizers. The calculated relative gene expression change was then compared with the in situ hybridization results.

The second objective of this study was to develop an experimental control design for making a rational selection of accurate normalizer genes. This objective was addressed by examining relative gene expression change with various normalizer genes in embryos that were treated with two compounds with differential potency for inducing posterior somite/axis defects in rat whole-embryo culture, but had uncharacterized differential gene expression profiles. Biological activities that were representative of housekeeping gene function, including glycolysis, total protein, and cytoskeletal structural integrity, were evaluated in the embryos. Normalizer genes, including housekeeping genes and genes with spatially restricted expression patterns, were also evaluated in this study.

METHODS

Concepts and Calculations for Performing Relative Gene Expression Change by Real-Time PCR

Product detection by real-time PCR. Enhanced sensitivity in PCR product detection during real-time PCR amplification is achieved by the use of a fluorescent conjugated probe, which enables the product to be detected and measured throughout the course of the amplification cycles. The oligonucleotide probe is designed as the reverse complement of a small region of the cDNA sequence selected to be amplified. The probe, which is typically about 20–30 nucleotides in length, is designed to hybridize to the central region of the sequence. The probe is synthesized with two labels: the 5′ end is labeled with a fluorescent reporter dye, and the 3′ end is labeled with a quencher dye. When the probe is intact, the proximity of the quencher dye inhibits fluorescence from the reporter (Fig. 1). During PCR amplification, annealing of the probe to the target sequence produces a template that is cleaved by the 5′ nuclease activity of Taq DNA polymerase. This cleavage event releases the reporter dye from the probe and away from the quencher, resulting in increased reporter dye signal. This signal is detected by the real-time PCR system. As additional reporter dye molecules are cleaved from their respective probes with each cycle, an increase in fluorescence intensity results that is proportional to the amount of amplicon produced (Fig. 1).

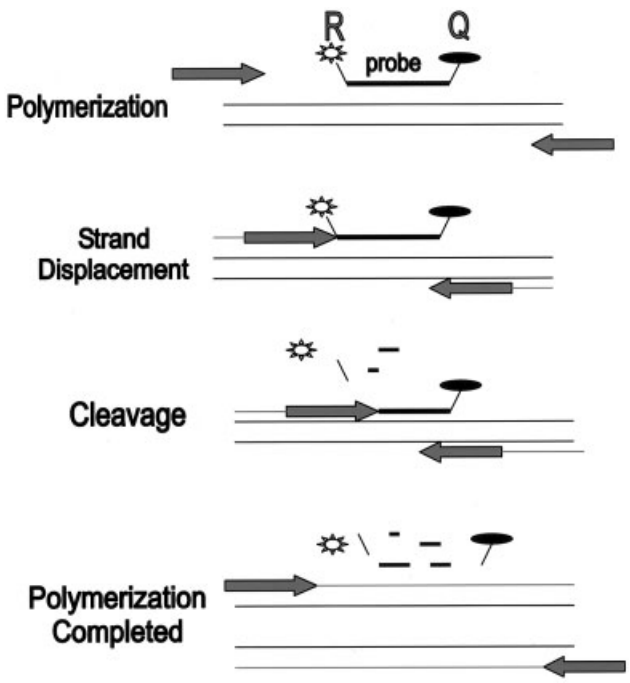
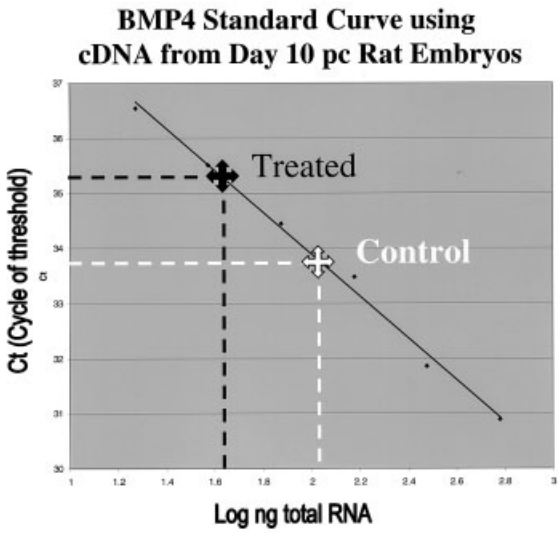


Figure 1. PCR amplification reaction in real-time PCR. Diagram of probe degradation and signal detection during one PCR cycle in real-time PCR. (Diagram adapted from Livak [1995]).

Calculations of relative gene expression change by real-time PCR. In a real-time PCR reaction, the reaction is characterized at the point in time during cycling when the PCR product is first detected (Heid et al., 1996; Gibson et al., 1996). This is different from traditional PCR methods in which the reaction is characterized by the amount of PCR product accumulated after a fixed number of cycles. Thus, in real-time PCR, the higher the starting copy number of nucleic acid target, the sooner a significant increase in fluorescence is observed above the baseline threshold. The parameter termed the "cycle of threshold" (Ct) is the fractional cycle number at which the cleaved probe's reporter fluorescence passes a fixed baseline threshold.

When the calculations are performed, a standard curve is generated by plotting the log value of the amount of total RNA inputed into the reaction versus the Ct (Fig. 2A). In studies using embryonic cDNA, standard curves are generated by making serial dilutions of defined amounts of RNA or cDNA collected from untreated embryos of the same gestational stage as the embryos evaluated in the study. In studies using whole embryos, the Ct values represent the levels of target gene expression in the defined amount of cDNA template used in generating the standard curve.

A.



B.

1): Log ng total input RNA (A): A: log ng total RNA= (ct-b)/m; b: intercept m: slope
2): Starting copy number of target or reference gene (B): B: ng RNA= 10^A
3): Normalized target gene expression (C): C=B(target gene) / B(reference gene)
4): Relative gene expression change (D): D=C(treated group) / C(control group)

Figure 2. Calculations of relative gene expression change using real-time PCR. An example of relative gene expression change of *BMP4* using standard curve methodology.

In this study, relative gene expression change following experimental treatments was calculated by the relative standard curve method (Gibson et al., 1996), which is defined by the following equation (Fig. 2B):

Log ng total input RNA (A): A: log ng total RNA
= (ct-b)/m; b: intercept m: slope

Starting copy number of target or reference gene
(B): B: ng RNA = 10^A

Normalized target gene expression (C):
C= B(target gene)/B(reference gene)

Relative gene expression change (D):
D= C(treated group)/C(control group)

This method is based on the principle that the quantity of target gene expression in the experimental groups is calculated as relative to the quantity of target expression calculated for the control group. Quantitation of the amount of target in control and experimental samples is accomplished by measuring the Ct and using the standard curve to determine the starting copy number (Fig. 2B). To determine relative gene expression change in control versus experimental treatment groups, the gene expression changes were first normalized in each group by dividing the target gene expression amount by the calculated amount of reference gene expression. Relative target gene expression change was determined by dividing the normalized experimental group value by the normalized control value (Fig. 2B).

Experimental Procedures

Real-time PCR. At the end of the culture periods, embryos intended for real-time PCR analysis were immediately vortexed in TriReagent containing microgel carrier (Molecular Research Center, Cincinnati, OH). Total RNA was extracted from cultured embryos according to the TriReagent protocol (Molecular Research Center). cDNA was synthesized using a SuperScript RT II synthesis kit (GIBCO, Gaithersburg, MD) with random hexamer primers. Briefly, real-time PCR was conducted as follows: First, 200 ng cDNA, or a respective serial dilution of cDNA for standard curve generation, was amplified with 200 nM primers and 100 nM FAM-labeled probe in TaqMan Super Mix (Perkin Elmer, Foster City, CA). Real-time PCR was undertaken using a 9700 Taqman System (Applied BioSystems, Foster City, CA). The amplification cycle conditions were (first cycle) 50°C for 2 min, denatured at 95°C for 10 min; and (remaining 40 cycles) 15 sec at 95°C and 1 min at 60°C. The results were analyzed using the relative standard curve method (Gibson et al., 1996). A standard curve was generated for each target gene using serial dilutions of cDNA generated from untreated embryos of the same stage as the experimental embryos at the end of their respective culture periods. Calculations were undertaken as described in the previous section. The sequences of the real-time PCR primers and probes are listed in Table 1.

Rat whole-embryo culture. Sprague Dawley IGS strain rats (Charles River, Raleigh, NC) were mated overnight and examined for the presence of a sperm plug the following morning, which was designated as postcoitus (p.c.) day one of pregnancy. At day 9 p.c., rats were euthanized by carbon dioxide asphyxiation and exsanguination, and the uterus was removed. Day 9 p.c. embryos were prepared for culture as described by New (1978) and Sadler (1985, 1993).

RA (Sigma, St. Louis, MO), SB245570, and SB236057 (internally supplied) were solubilized in DMF vehicle and

Table 1
Primer and Probe Sequence Data for Amplifying Target cDNA Templates in Real-Time PCR

Target gene	Forward primer sequence	Reverse primer sequence	Probe sequence
Hoxb1	5′-AGCCCCCATACGGAACTGAG-3′	5′-GAGAGTGCTGGGTTCTGACGA-3′	6FAM-CGCAACCTTTGCATCAGCCTACGAC-TAMRA
BMP4	5′-GTGGGCTGGAATGATTGGAT-3′	5′-TTGGTTGAGTTGAGGTGGTCC-3′	6FAM-ACCAGGCCTTCTACTGCCACGGG-TAMRA
Beta-actin	5′-CCAGCCATGTACGTAGCCATC-3′	5′-ACAGTGTGGGTGACCCCGT-3′	6FAM-TGCCTCTGGTCGTACCACTGGCATT-TAMRA
EF1	5′-TGGCCCCAAATTCTTGAAGT-3′	5′-CAACACACATGGGCTTGCC-3′	6FAM-TGATGCTGCCATTGTTGACATGGTCC-TAMRA
rRNA	5′-CGGCTACCACATCCAAGGAA-3′	5′-GCTGGAATTACCGCGGCT-3′	JOE-TGCTGGCACCAGACTTGCCCTC-TAMRA
BMP7	5′-GTGAGGGAGAGTGTGCCTTCC-3′	5′-GGGTACGGTGTCTGGGTTGA-3′	6FAM-TGAACGCCACCAACCATGCTATCG-TAMRA
Notch	5′-CCAACATCTCTGATTGGTCCG-3′	5′-GGAAAGGACTCCCAACAGAGC-3′	6FAM-CAGATCACCCACATTCCAGAGGCATTTAAGT-TAMRA
G3PDH	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′	JOE-CAGAAGACTGTGGATGGCCCCTC-TAMRA
T gene	5′-TGCTGCAGTCCCATGATAACTG-3′	5′-TATGACTCACAGGCAGCATGCT-3′	6FAM-AGCCTCGGAGTGCCTGGCCA-TAMRA
S100	5′-GGGCCTGCTTAACACTCGG-3′	5′-TTAGAAAGCATCAAGTCCGGC-3′	6FAM-AGGCTGCGAGCCGAGGACTGTG-TAMRA

dispensed into culture vials containing 1.75 ml of rat serum (WEC quality serum; Harland Labs, Indianapolis, IN) and 0.75 ml Tyrode's buffer (Sigma) at a final compound concentration of 1 μM for RA and 10 μM for the remaining test compounds.

At the end of culture, the embryos were stripped of their extraembryonic membranes and evaluated for morphological integrity according to previously described methods (Sadler, 1985, 1993), or were immediately placed in the appropriate buffers, fixatives, etc., for analysis. At least 10 embryos per vehicle or compound treatment were collected for each biological analysis and gene expression study. The biological analysis studies were performed two to three times, and the results of the relative gene expression analysis represent an average of three separate experiments.

Total protein analysis. Cultured embryos from control and treated groups were placed into hypotonic buffer, which contained 20 mM HEPES (pH 7.9), 20 mM NaF, 1 mM Na_3VO_4, 1 mM $NaPO_4$ (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, and 2% protease inhibitor cocktail (Sigma). The samples were vortexed, frozen, and thawed three times on dry ice to dissolve embryonic tissue. The protein lysate was diluted in water and mixed with protein dye reagent (1:5; Bio-Rad, Richmond, CA). Protein content was obtained by reading absorbance values at a wavelength of 595 nm and evaluating the sample optical density (OD) against a standard curve provided by the vendor (Bio-Rad).

Total lactate measurement. Cultured embryos from control and treated groups were placed in lactate reagent containing lactate oxidase, peroxidase, and chromogen precursors (Sigma Diagnostics, St. Louis, MO) and dissociated by pipetting. The lactate reagent was reconstituted with deionized water. The samples were incubated for 5–10 min and read for absorbance at a wavelength of 540 nm. The lactate concentration was obtained using the formula: [absorbance test/absorbance standard] $\times$ 40.

Total pyruvate measurement. Cultured embryos from control and treated groups were dissociated by gentle pipetting. Then 30 μl of dispersed embryonic fluid was placed in 70 μl 10% perchloric acid, precipitated for 5 min, and centrifuged. The supernatant was incubated with TRIZMA base solution (Sigma Diagnostics) in an NADH vial. The absorbance at the beginning and end of reaction was recorded at a wavelength of 340 nm. The pyruvate concentration was calculated as: [initial absorbance – final absorbance] $\times$ 0.723. The ratio of lactate/pyruvate was used as an indicator of the overall metablic state of an embryo (Bozimowski et al., 1985).

Immunofluorescence. Cultured embryos from DMF vehicle control and tool compound–treated groups were fixed in 4% paraformaldehyde for 4 hr at room temperature and transferred to PBS buffered with 0.1% bovine gelatin (225 bloom), 0.2% saponin, and 0.02% sodium azide. They were then incubated overnight at 4°C in a 1:40 dilution of Alexa 568 furo phalloidin (Molecular Probes, Eugene, OR), followed by overnight washing in PBS. Images were obtained with an LSM 510 laser scanning confocal microscope (Zeiss, Zurich, Switzerland) at an excitation wavelength of 568.

In situ hybridization. The *Hoxb1* and *T*-gene probe templates were generated by PCR.

Hoxb1. A 478-bp-long DNA fragment was amplified from rat day 10 embryonic cDNA, using forward primer 5′-TCT CCG CAC AAA CTT CAC CAC-3′ and reverse primer 5′-CCC CAG CCA TCA ATC ATC C-3′.

T-*gene.* A 314-bp-long DNA fragment was amplified from rat day 10 embryonic cDNA, using forward primer 5′-GCC TGC AGT ACC GAG TGG ACC-3′ and reverse primer 5′-GCT CTG GTT TGC CCC CAG GTA-3′. The transcription promoter site was introduced to the *T*-gene fragment by a second PCR using primers designed with a T7 promoter sequence added at the 5′ end of the forward primer, and a SP6 promoter sequence added to the 5′ end of the reverse primer.

In vitro transcription was performed according to the manufacturer's instructions provided by Roche, using the purified PCR product as the template and a DIG RNA labeling kit (Roche, Indianapolis, IN). Whole-mount in situ hybridization was performed using methods described by Henrique et al. (1995) and Nigel A. Brown (personal communication).

Because the purpose of the in situ hybridization experiments was to obtain a semiquantitative evaluation of *Hoxb1* or *T*-gene expression, for each experiment all embryos were processed for in situ hybridization using the same batch and concentration of *Hoxb1* or *T*-gene riboprobe. All embryos were processed and hybridized at the same time using the same lots of buffers, hybridization solutions, and detection reagents. In addition, the time allotted for signal detection was identical for all embryos, with the colorimetric reaction being terminated approximately 24 hr after the addition of bromochloroindolylyl phosphate/nitroblue tetrazolium (BCIP/NBT).

The in situ hybridization conditions are briefly described as follows: Following standard dehydration/rehydration processing in a graded methanol series, the embryos were fixed in 4% paraformaldehyde for 4 hr at room temperature, and then permeabilized with 10 μg/ml proteinase K

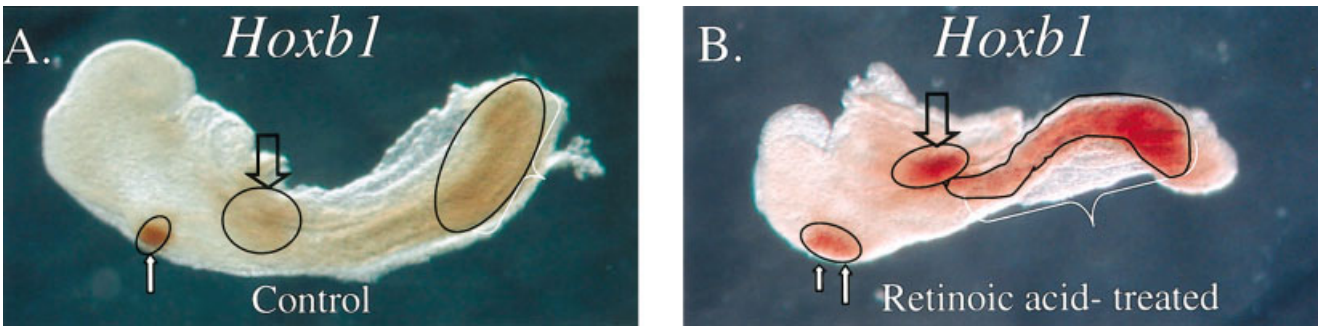


Figure 3. *Hoxb1* expression in control (**A**) and RA-treated (**B**) rat embryos analyzed by whole-mount in situ hybridization. Presomite (early day 9 p.c.) rat embryos were cultured in the presence of 1 μM RA or 0.04% DMF vehicle. Vehicle control embryos exhibited normal *Hoxb1* expression patterns, including *Hoxb1* expression, in a single rhombomere pair in the hindbrain (white arrow), the foregut region (open arrow), and the primitive streak and paraxial mesoderm (bracket). *Hoxb1* was evaluated in a semiquantitative fashion by obtaining pixel intensities in the described anatomical regions (outlined in black in A and B). RA-treated embryos exhibited anteriorized *Hoxb1* expression in the hindbrain, with the appearance of at least two rhombomere pairs exhibiting expression (white arrows). In addition, treated embryos exhibited increased expression of *Hoxb1* in the foregut (open arrow) and along the posterior axis, as well as in the streak region (bracket). Outlined regions represent designated parameters of *Hoxb1* expression in the respective anatomical regions that were quantitated for pixel intensity by Image Quant analysis.

for 20 min, followed by 20-min postfixation in 4% paraformaldehyde plus 0.1% glutaraldehyde in PBS with 0.05% Tween 20 (PBT) (Sigma). The embryos were prehybridized at 65°C for 4 hr and then hybridized with 100 ng/ml DIG-labeled riboprobe at 65°C overnight. The riboprobe was detected with an anti-DIG antibody conjugated with alkaline phosphatase, using BCIP/NBT as chromagens (Roche, IN). The embryos were then rinsed with PBT and photographed.

Digital photographs of embryos hybridized with *Hoxb1* were taken at the same magnification. These images were imported into an Image Quant (Molecular Dynamics) densitometer. Because the compound-treated embryos generally had a pinker background staining than the vehicle or untreated embryos, regions of *Hoxb1* expression in the hindbrain rhombomere(s), foregut, and paraxial mesoderm/somites were defined using the Image Quant software to specifically highlight and measure expression in these anatomical regions. Relative pixel intensity was quantitated for the respective *Hoxb1* expression domain, and three regions were then added together to provide a total expression intensity representing *Hoxb1* expression in one side of the embryo.

Statistical analysis. Statistical analysis was undertaken using Student's two-tailed *t*-test, and significant difference against the vehicle group was established at a 99% or 95% confidence level. Statistical programs supplied with PrismGraph software, San Diego, CA, were utilized in this study.

RESULTS

Pilot Study: Quantitation of *Hoxb1* Expression in Neurulating Embryos Following Treatment With RA

To determine whether certain reference genes may be optimal for determining relative gene expression changes in embryonic studies, we initially worked with RA, a teratogen that has been demonstrated to induce changes in expression of specific *Hox* genes (Marshall et al., 1992). In the previous study, transgenic mice were generated with a *Hoxb1* (Hox2.9) promoter driving expression of the lacZ reporter gene. Transgenic mouse embryos treated with RA on days 7.25 or 7.5 postcoitum (p.c.) resulted in increased *Hoxb1* expression, with anteriorization and duplication of the domain of *Hoxb1* expression in the hindbrain rhombomeres by 24 hr post RA administration.

Our early day 9 p.c. Sprague Dawley rat embryos are gestationally equivalent to day 7.5 p.c. mouse embryos, both groups being at the headfold/late presomite stage (Rugh, 1968; Kaufman, 1992). In our study, using rat embryos, we repeated the experiment reported by Marshall et al. (1992) and cultured day 9 p.c. presomite embryos with 1 μM RA for 24 hr. At 24 hr, the embryos were pooled into control and RA-treated groups. They were then processed as whole-mount for *Hoxb1* in situ hybridization, or the RNA was isolated and cDNA was synthesized for real-time PCR analysis.

Representative control and RA-treated day 10 p.c. embryos hybridized for *Hoxb1* expression are presented in Figure 3. In untreated or vehicle-treated embryos, *Hoxb1* expression was localized to a pair of hindbrain rhombomeres (small circle and white arrow), and diffuse *Hoxb1* expression was also apparent in the foregut region below the heart (open arrow, medium circle) and in the primitive streak/paraxial mesoderm region (bracket, outlined region) (Fig. 3A). Embryos treated with RA appeared to have increased *Hoxb1* expression (Fig. 3B). Increased *Hoxb1* expression was demonstrated by a duplication of *Hoxb1* expression in the hindbrain rhombomeres (Fig. 3B), in agreement with previous reports (Morris-Kay et al., 1991; Marshall et al., 1992). In addition, more intense *Hoxb1* expression was observed in the foregut region, and expression in the primitive streak region appeared extended along the posterior axis (Fig. 3B). Relative pixel intensity in the respective *Hoxb1* expression domains was quantitated by densitometric analysis, for which pixel intensity was acquired by measuring *Hoxb1* expression in the designated anatomical domains (illustrated in Fig. 3). The pixel intensities of the three expression domains were added together to represent *Hoxb1* expression on one side of the embryo. Table 2 summarizes the densitometric analysis of relative *Hoxb1* expression in the control and RA-treated embryos

Table 2
Relative *Hoxb1* Expression by Wholemount In Situ Hybridization Measured by Densitometric Analysis of Size Matched Control Vs. RA-Treated Embryos As Well As Measured by Total Group Control Vs. RA-Treated Embryos

	Embryo number			
	1	2	3	Total average ± SEM
Pixel intensity of *Hoxb1* expression on the right side of the embryo, dimethyl formamide (DMF) treated vehicle control	135.5	37.3	53.6	75.3 ± 30
Pixel intensity of *Hoxb1* expression on the right side of the embryo, retinoic acid (RA) treated	176.3	77.7	111.9	121.6 ± 29
Ratio RA/DMF	1.30	2.07	2.09	
Mean ratio of RA/DMF ± SEM of size matched control vs. treated		1.82 ± 0.26		
Mean ratio of RA/DMF of grouped control vs. treated embryos				1.61

that were processed for in situ hybridization. Densitometric expression ratios, which were calculated by rudimentary normalization based upon comparison of size-matched embryos, demonstrated a 1.8-fold increase in *Hoxb1* expression following RA treatment. Without size-matched normalization, the densitometric ratio calculated a 1.6-fold increase in *Hoxb1* expression following RA treatment.

Table 3
Relative mRNA Levels for *Hoxb1* Calculated Without and With Normalization Using the Relative Standard Curve Method

Experimental treatment	Experiment 1	Experiment 2	Experiment 3
A: ng *Hoxb1* following retinoic acid treatment	630.5	1566.4	484.0
B: ng *Hoxb1* following vehicle treatment	311.5	1407.2	285.9
A/B = C: Relative expression change in *Hoxb1* expression following retinoic acid treatment	2.0	1.1	1.7
D: ng BMP7 following retinoic acid treatment	40.78	25.63	109.2
E: ng BMP7 following vehicle treatment	40.3	39.83	135.1
B/E = F: Relative BMP7 to *Hoxb1* levels following vehicle treatment	7.73	35.3	0.47
A/D = G: Relative BMP7 to *Hoxb1* levels following retinoic acid treatment	15.46	61.1	0.23
G/F = H: Relative *Hoxb1* expression change following retinoic acid treatment using BMP7 as a normalizer	2.0	1.7	2.1

Real-time PCR was performed using 1 μl of cDNA per reaction, and relative changes in *Hoxb1* expression were calculated using various reference genes as normalizers. Two categories of reference genes were used: 1) housekeeping genes (*18SRNA*, *β-actin*, *EF-1*, and *G3PDH*) and 2) restricted expression genes (*BMP4*, *BMP7*, *T*-gene, *Notch-1*, *Krox-20*, and *S100-β*).

Table 3 illustrates the value of normalization in measuring target mRNA expression response. Table 3 summarizes the calculated amounts of *Hoxb1* mRNA from a 1-μl aliquot of control and RA cDNA pools using the relative standard curve method (Perkin Elmer Applied Biosystems, 1997) (Fig. 2). In each experiment, a 20-μl cDNA stock was generated from 1 μg total RNA isolated from a pool of 10 embryos per treatment group. Relative amounts of *Hoxb1* mRNA was measured from a real-time PCR amplification using a 1-μl aliquot of cDNA stock generated for each treatment group. The results demonstrate that the baseline levels of *Hoxb1* mRNA varied according to the cDNA pool generated from each experiment. Without normalization, the calculated levels of *Hoxb1* mRNA suggested that RA treatment induced a range of expression responses, dependent upon the experimental runs. Expression responses ranged from a minimal response (1.1-fold expression increase) to the expected 1.5- to 2-fold increase in expression. However, as with *Hoxb1*, baseline amounts of reference gene mRNA also varied per experiment, and when the relative *Hoxb1* levels were normalized against the relative ng levels of a reference gene (e.g., *BMP7*), the relative expression response to RA became more consistent among the experimental runs. In this example, normalization with *BMP7* calculated 1.7- to 2.1-fold increases in *Hoxb1* expression in response to RA treatment.

A summary of relative *Hoxb1* levels calculated by normalization with various reference genes is presented in Figure 4. The reference gene used in normalization impacted final calculations of *Hoxb1* expression change. In this study, only half of the reference genes calculated *Hoxb1* expression to be increased by the expected 1.5- to 2-fold range (Fig. 4). Normalization using the housekeeping genes *18SRNA*, *β-actin* and *EF-1* produced similar results, indicating that *Hoxb1* expression increased 1.5- to 2-fold in response to RA. However, normalization using the glycolysis gene, *G3PDH*, demonstated *Hoxb1* change to be about 30–40% higher than that of the other three housekeeping genes. Normalization with genes that exhibited

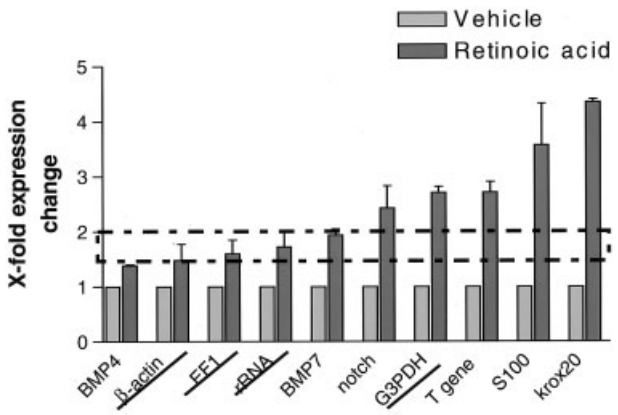


Figure 4. Relative *Hoxb1* mRNA expression change at 24 hr following RA treatment, calculated by normalization with various reference genes. The outlined area (dashed box) denotes the expected range of *Hoxb1* expression increase based upon previously reported responses determined by qualitative analysis (Morris-Kay et al., 1991; Morris et al., 1992), as well as results obtained from the rat in situ hybridization studies presented in Figure 3 and Table 1.

restricted expression in the embryo produced varying results, with an increase in *Hoxb1* expression ranging from 1.4- to 4-fold. Normalization using *BMP4* and *BMP7* calculated a 1.4- or 2-fold increase in *Hoxb1* expression, respectively. In contrast, normalization using *S100* and *Krox-20* resulted in the most significant deviations, such that normalization calculated 3- to 4-fold increases in *Hoxb1* expression, respectively (Fig. 4). Furthermore, the latter data confirmed the expectation that normalization with *Krox-20* would skew the *Hoxb1* results, since previous studies have demonstrated *Krox-20* to be decreased following RA treatment (Morris-Kay et al., 1991).

Experimental Design Paradigm for Rational Selection of Reference Genes in Relative Gene Expression Analysis

Characterization of the general biological responses following treatment. In studies using compounds with previously characterized gene expression response, one can design control studies to identify optimal reference genes for use in relative gene expression analysis. Typically, however, investigators use compounds with uncharacterized gene expression changes. In this study, we evaluated two structurally-similar test compounds that had the same pharmacological activity but differed in potency for inducing posterior somite/axis defects in rat whole-embryo culture (Table 4). Compound treatments did not produce morphologically discernable malformations at 24 hr, but were morphologically distinguishable at 48 hr. However, gene expression analysis was conducted at the 24-hr time point with the intent to examine a more direct effect of the compound exposure on transcriptional expression. Figure 5 presents the final manifestation of toxicant exposure following treatment on day 9 p.c. with 10 μM of compound or culture for 48 hr. Vehicle controls exhibited normal day 11 p.c. morphology (Fig. 5A and D). SB245570-treated embryos exhibited normal caudal extension; however, somites posterior to the forelimb bud were frequently rounded and had less defined borders (Fig. 5B and E). The SB236057-treated embryos were reduced in overall size, and also exhibited shortened extension of the caudal axis and loss of posterior somite formation (Fig. 5C and F). The posterior region of the SB236057-treated embryos was relatively translucent compared to control embryos, suggesting that there may also be edema and/or a loss of cellularity in this region.

To ascertain whether there is an optimal reference gene for conducting normalization calculations in relative gene expression analysis, we observed how certain housekeeping biological processes were affected following treatment with the two compounds. At 24 hr post compound administration, cultured embryos were collected and measured for total protein content and metabolic state, and evaluated for cytoskeletal integrity.

In comparison with the vehicle control group, total protein content was decreased in embryos treated with SB236057, but remained unchanged following SB245570 treatment (Fig. 6A).

To determine whether the metabolic state of the embryos was affected by the treatments, lactate and pyruvate production was measured, and the ratio of lactate/pyruvate (L/P) was used as an indicator of the overall redox state of the embryos (Bozimowski et al., 1985). Elevated ratios indicated an increased glycolytic rate. Only mild (20–25%) increases in L/P ratios were noted for embryos treated with SB245570 compared to vehicle controls. A marked increase in metabolic rates was demonstrated with the SB236057-treated embryos, such that the L/P ratios increased by 300% ($p < 0.05$) (Fig. 6B).

The general integrity of the actin filaments' cytoskeletal organization was assessed by staining the cultured embryos as whole-mounts with Alexa-labeled phalloidin, and viewing them by confocal microscopy. Phalloidin specifically binds to actin filaments, and is a useful marker for

Table 4
Relative Growth and Morphology of Rat Embryos Following Administration of 10 μM Test Compound on Day 9 pc and Cultured for 48 Hours*

Compound	Number of embryos	Reduction in overall size	Shortened caudal extension	Mild posterior somite dysplasia (somites 15–27): rounded somites and/or less defined borders	Missing and/or disorganized somites
Vehicle (DMF)	13	8%	0%	0%	0%
SB-245570	10	0%	0%	80%	0%
SB-236057	11	64%	45%	9%	82%

*Size and morphology was evaluated on day 11 pc, a developmental stage when compound-induced effects were morphologically discernable.

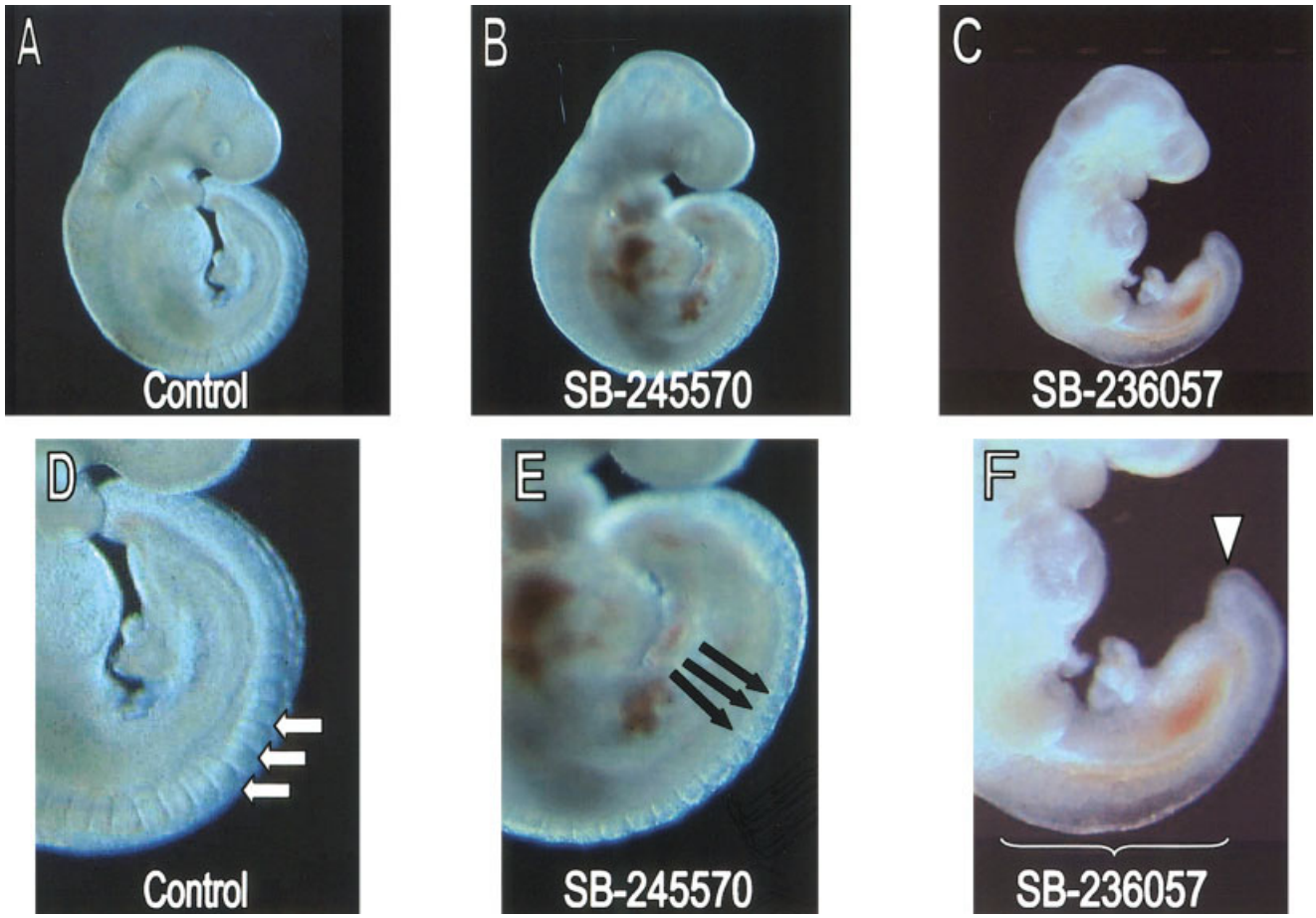


Figure 5. Representative external morphology of the vehicle control versus compound-treated embryos at 48 hr post compound administration. Vehicle controls (**A** and **D**) exhibited normal morphology, including well defined posterior somite formation (D, white arrows). SB245570-treated embryos (**B** and **E**) exhibited mild posterior somite dysplasia (loss of defined somite borders) (E, black arrows). SB236057-treated embryos were reduced in overall size (**C**) and also exhibited shortened extension of the caudal axis (**F**, white arrowhead) and loss of posterior somite formation (F, bracket). The posterior region was also relatively translucent compared to control embryos, suggesting that there may have been edema and/or a loss of cellularity in this region.

analyzing cytoskeletal integrity (Wieland and Faulstich, 1978). In normal states, actin filaments concentrate near the cell membranes; thus, single cells and various embryonic structures become clearly demarcated by the Alexa-conjugated phalloidin. The actin filament organization appeared to be normal in the vehicle- and SB245570-treated embryos. However, in the SB236057-treated embryos, actin organization appeared to be disrupted in cell populations along the posterior axis, including cells in the somites, and in mesenchymal cells surrounding somites (Fig. 6C). In affected cells, actin filaments appeared to be dispersed throughout the cell (Fig. 6C wide arrows) or in concentrated "clumps" within the cell (Fig. 6C, narrow arrow), rather than concentrated along the membrane.

Comparative evaluation of relative *G3PDH* expression changes normalized with various reference genes. Based on the finding that SB236057 administration affected various housekeeping processes in the treated embryos, an initial relative gene expression analysis was undertaken to determine whether transcriptional expression change in the housekeeping gene, *G3PDH*, was affected in embryos treated with the various tool compounds. *G3PDH* was selected for this analysis because of its role in glycolysis, and because we had identified marked increases in the metabolic rate in embryos treated with SB236057. We suspected that SB236057-treated embryos would exhibit a change in *G3PDH* expression, which may reflect the observed changes in the metabolic rate. In this study, embryos cultured in the presence of each test compound were harvested for RNA at 24 hr post compound administration on day 10 p.c. Real-time PCR was used to quantitate transcriptional expression of *G3PDH* where its relative expression changes were compared following normalization against several reference genes. The reference genes selected for this analysis included the four housekeeping genes. In addition, two spatially restricted genes were selected. The gene encoding the serotonin uptake protein, S100-β, which is expressed in the notochord and headprocess (Augustine et al., 1995), was selected as a representative reference gene. *S100-β* was expressed in a region suspected to be affected by the more potent compounds but not suspected to be affected by the test compounds, although there was no preconceived mechanistic basis for involvement of that particular gene. *Krox-20* was selected because its expression is restricted to specific hindbrain

rhombomeres, a region that is not morphologically affected by the compounds.

Normalized *G3PDH* levels are presented in Figure 7. The results of this analysis demonstrated that the choice of reference gene used in the normalization calculations could alter the resulting trend in target gene expression change. In this analysis, normalization with the restricted expression genes, *Krox-20* and *S100-β*, calculated decreased *G3PDH* expression in the compound treatment groups. In contrast, normalization with the housekeeping genes resulted in calculating *G3PDH* transcription expression to be greater than or relatively equal to the vehicle control levels, regardless of the tool compound used in treatment.

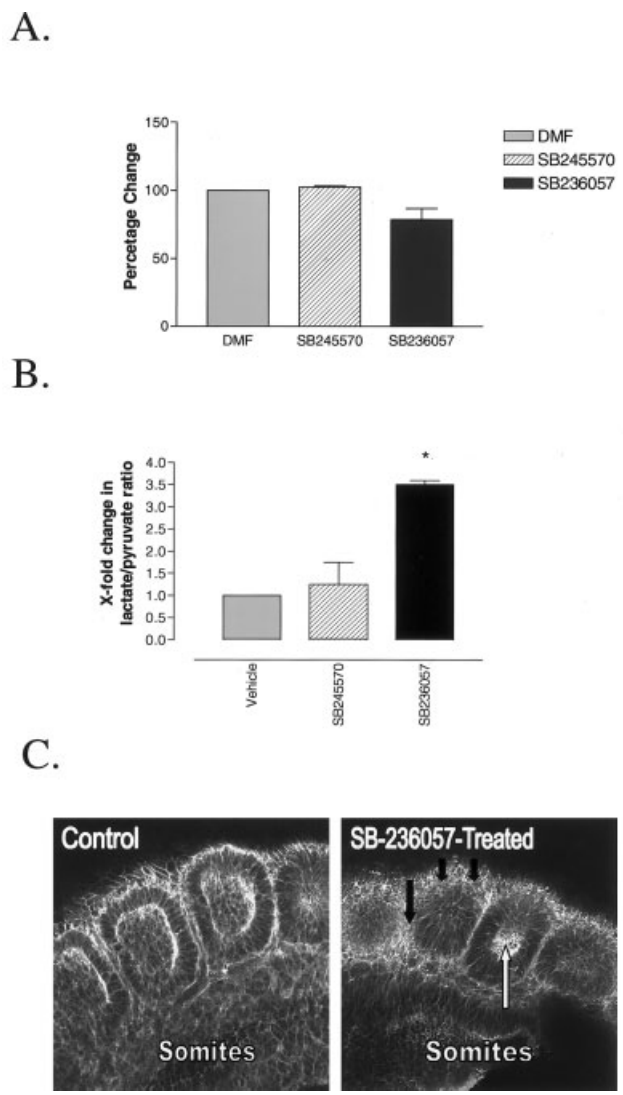


Figure 6. Effects of test compounds SB245570 and SB236057 on housekeeping biological processes. **A:** Effect of the compounds on total protein content of cultured rat embryos at 24 hr post compound administration. **B:** Effect of the compounds on metabolic states of cultured rat embryos at 24 hr post compound administration. The glycolytic rate was determined by measuring the concentrations of lactate and pyruvate. The ratio of lactate/pyruvate was used as an indicator of the overall redox state of the embryos. The asterisk indicates statistically significant difference from the control value, $p \leq 0.05$. **C:** Effect of SB236057 on cytoskeletal actin organization of cultured rat embryos at 24 hr post compound administration. Optical section of embryos stained with Alexa-labeled phalloidin. White arrow: loss of cytoskeletal organization in interior posterior somite cells. Black arrows: loss of cytostructural organization in mesenchymal cells surrounding the somites.

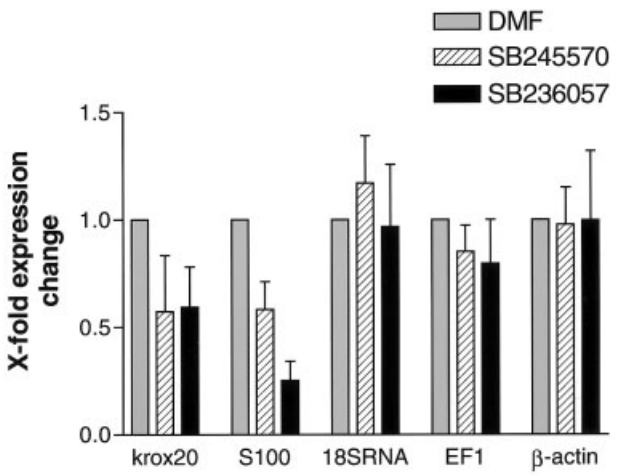


Figure 7. Relative expression change of G3PDH determined by normalization against several reference genes at 24 hr post tool-compound administration.

Comparative evaluation of relative target gene expression changes normalized with various reference genes. In this study, using the embryonic cDNA produced from the respective treatment groups, real-time PCR with normalization was used to quantitate three genes (*BMP4*, *Hoxd11*, and *T*-gene) as representative target genes involved in early axial skeletal development. The four housekeeping genes and the restricted expression genes (*Krox-20* and *S100-β*) were used as normalizers in the relative gene expression change calculations. Because the test compounds differed in their potency for inducing posterior somite/axis defects, a difference in gene expression was anticipated for at least some of the genes evaluated.

The normalized expression changes are presented in Figure 8. Variations in trends in target gene expression change resulted when treatment groups were normalized against the different reference genes. The variations were such that opposing trends in gene expression change would be interpreted depending on the reference gene used in the normalization calculations. For instance, there were notable variations in target gene expression normalized with the housekeeping reference genes. Normalization with *EF1-α* and *β-actin* generally followed the same trend in target expression change; however, normalization with *18S* ribosomal and *G3PDH* presented different trends in expression change. Normalization with *S100-β* produced trends in *Hoxd11* and *BMP4* gene expression similar to those obtained by normalization with *18SRNA*, but produced different trends for *T*-gene expression. Normalization with *Krox-20* presented trends in target gene expression that were unique in the context of the other normalizers, and the target expression changes appeared to be realistic in the context of the relative teratogenic potency of the test compounds. For instance, normalization with *Krox-20* calculated a downward gradient in *BMP4*

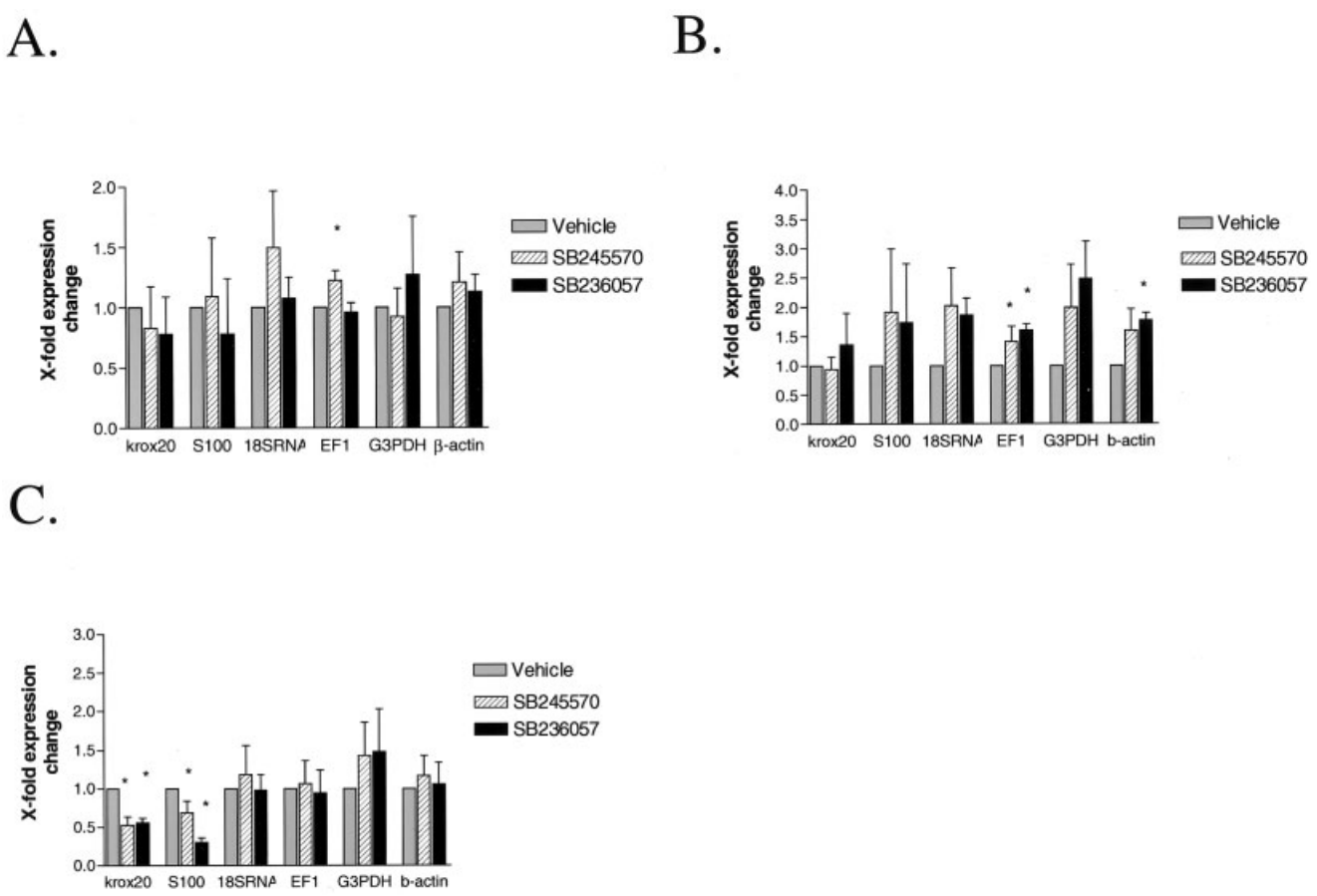


Figure 8. Relative expression change of three genes involved in posterior axis formation, as determined by normalization against several reference genes at 24 hr post test-compound administration. **A:** Relative expression change of *BMP4*. **B:** Relative expression change of *Hoxd11*. **C:** Relative expression change of *T*-gene. Asterisks indicate significant change in target gene expression compared to vehicle controls ($p \leq 0.05$).

expression. Furthermore, *T*-gene expression appeared to be repressed in embryos following treatment with both tool compounds.

Confirmation of target gene expression change determined by whole-mount in situ hybridization. Whole-mount in situ hybridization was conducted on treated embryos to qualitatively confirm relative change in target gene expression. The *T*-gene was selected as the target gene because the degree of relative expression change calculated with each normalizer was expected to be qualitatively distinguishable, and in situ localization would confirm whether *T*-gene was underexpressed, overexpressed, or not affected in the respective treatment groups. Embryos were collected from culture at 24 hr post compound administration and processed for in situ hybridization. Vehicle control embryos exhibited normal expression patterns of *T*-gene at day 10 p.c., with the hybridization signal located in the posterior notochord and primitive streak region (Fig. 9A and B) (Herrmann, 1991). Relative to expression in the vehicle controls, *T*-gene expression appeared to be reduced in embryos treated with both test compounds (Fig. 9C and D), confirming the relative gene expression results achieved when *Krox-20* was used as the normalizer.

DISCUSSION

The selection of an appropriate reference gene for use as normalizers in relative gene expression analysis is integral to the validity of the results. Therefore, it is important to select a normalizing gene that represents the background biology of the tissue or organism, in the sense that its transcriptional activity is not directly tied to the pathological consequences of the toxicant/teratogen. In relative gene expression studies of whole embryos, the selection of an accurate normalizer is challenging because of the heterogeneous nature of the cellular populations, and the embryo's dynamic metabolic changes and rapid growth. In this study, we evaluated and compared relative gene expression change in neurulating rat embryos following treatment with the well-characterized teratogen RA, and two structurally-similar test compounds that had the same pharmacological activity but differed in potency for inducing posterior somite/axis defects in rat whole-embryo culture. Relative gene expression analysis was conducted using real-time PCR methodology normalized with either housekeeping genes or genes with restricted expression patterns.

The first objective was to determine how the use of various normalizing genes can impact final calculations of

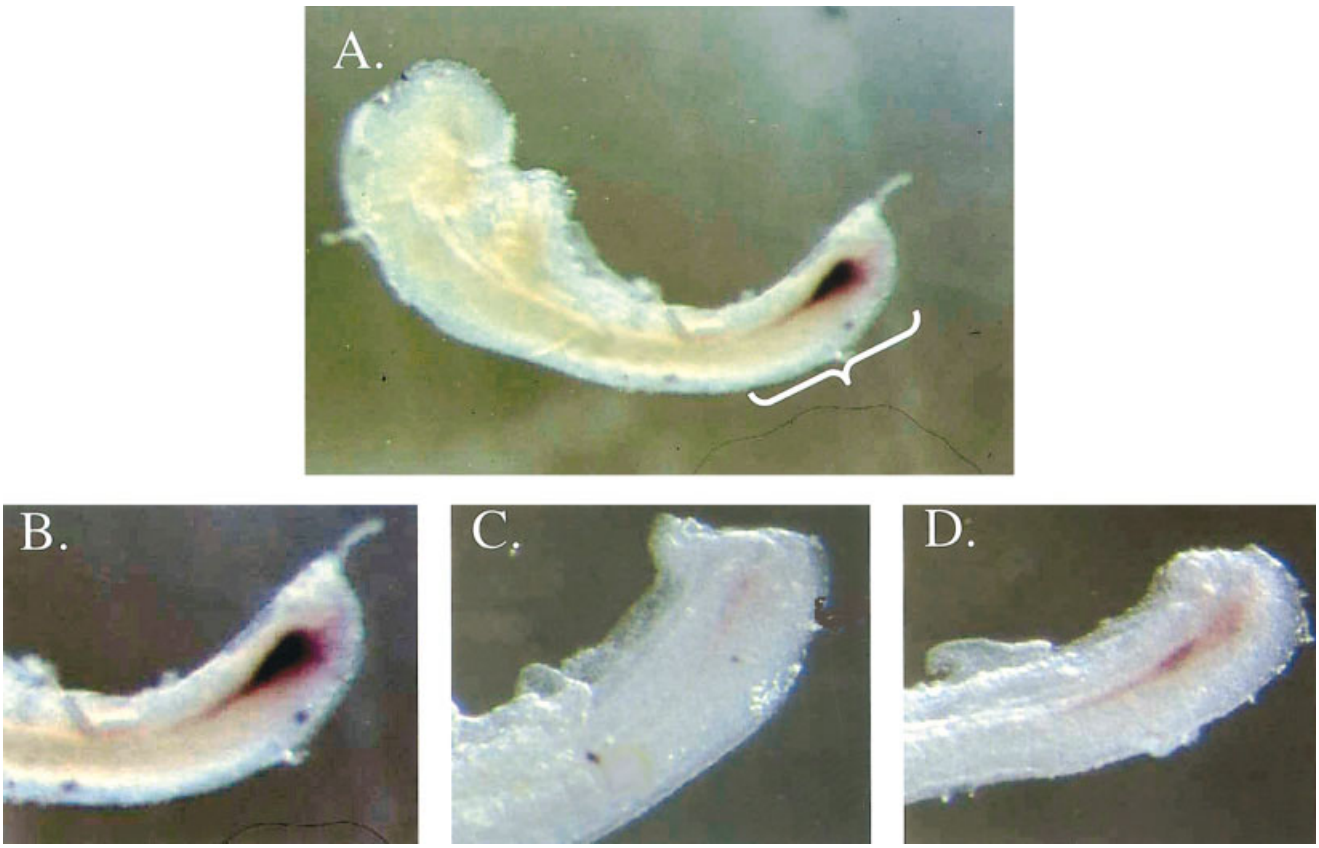


Figure 9. Confirmation of relative *T*-gene expression change at 24 hr post compound administration by whole-mount in situ hybridization. **A:** Vehicle control embryo exhibiting normal levels of *T*-gene expression. Bracket: *T*-gene expression is expressed in the day 10 p.c. rat primitive streak and posterior notochord. **B:** Closer view of *T*-gene expression in a vehicle-treated embryo. **C:** Representative *T*-gene expression in SB245570-treated embryos. **D:** Representative *T*-gene expression in SB236057-treated embryos.

relative target expression change. In situ hybridization of *Hoxb1* expression in presomite rat embryos cultured with RA confirmed previous studies of presomite mouse embryos, which found that RA treatment resulted in increased *Hoxb1* expression with apparent duplication of *Hoxb1* expression in the hindbrain rhombomeres (Morris-Kay et al., 1991; Marshall et al., 1992). Densitometric analysis of the hybridized specimens estimated a 1.6- to 1.8-fold increase in *Hoxb1* expression in the RA-treated embryos without or with, respectively, the use of size-matched normalization.

Relative expression analysis by real-time PCR demonstrated that without normalization, calculated *Hoxb1* expression change ranged from a minimal response (1.1-fold increase) to the expected 1.5- to 2-fold increase in response to RA. Normalization was found to equilibrate *Hoxb1* expression responses among the separate experiments; however, this effectiveness was dependent upon the reference gene selected for normalization. The current results demonstrate that only a portion of the housekeeping normalizers calculated the expected 1.5- to 2-fold increase in *Hoxb1* expression. In this study, *Krox-20* was used as a reference gene. This was expected to alter the normalization calculations, based upon previous findings that *Krox-20* expression is directly inhibited by RA (Morris-Kay et al., 1991). Our results confirmed that hypothesis: normalization with *Krox-20* resulted in a much higher outcome than the expected value. Although *S100-β* has not been reported to be directly responsive to RA treatment, its expression is localized in the headprocess/notochord. This structure expresses genes, such as *sonic hedgehog*, that are sensitive to RA treatment. As with nomalization with *Krox-20*, normalization of *Hoxb1* expression with *S100-β* resulted in a significantly higher outcome than the expected value. This suggests that inaccurate normalization calculations can result from the use of normalization genes that are expressed in affected structures but are not necessarily directly responsive to the teratogen.

The second objective of this study was to develop an experimental control design strategy for rationally selecting normalizer genes to accurately calculate relative gene expression changes. This objective was addressed by examining relative gene expression change with various normalizer genes in embryos that were treated with two compounds that were observed to differ in their potency for inducing posterior somite/axis defects, but had uncharacterized gene expression profiles.

For each compound treatment, we characterized the embryos' biological activities that were representative of certain housekeeping gene functions. We found that SB236057 altered all of the biological activities evaluated, and inferred that a number of housekeeping biological processes were affected in embryos treated with the more embryotoxic test compound. This information was used to make an initial selection of candidate housekeeping references

for use as normalizers. Based on our findings that increased metabolic rates were observed in embryos treated with SB245570 and SB236057, we measured relative *G3PDH* expression change calculated by normalization with the other housekeeping genes, as well as restricted-expression genes. We found that most of the housekeeping genes calculated *G3PDH* expression to be relatively unchanged by the test compounds, whereas normalization with *Krox-20* and *S100-β* calculated decreased *G3PDH* expression.

We continued this study by measuring relative changes in expression of three genes involved in axial development, using the various housekeeping and restricted expression genes as normalizers. This study models a typical study an investigator would undertake in characterizing novel gene expression response to a teratogen/toxicant. *BMP4*, *Hoxd11*, and *T*-gene represented candidate targets of which respective transcriptional expression might be altered by compounds inducing posterior axis defects. However, confirmed response was unknown because the transcriptional effects of these compounds had not yet been characterized. Normalization with the various reference genes calculated considerable fluctuation in relative gene expression response of the axial targets to the test compounds. Normalization with *Krox-20* calculated no statistically significant changes in expression of *BMP4* or *Hoxd11*, but it did calculate significant repression of *T*-gene expression following treatment with both compounds. We proceeded by qualitatively assessing *T*-gene expression by whole-mount in situ hybridization. We observed an obvious reduction in *T*-gene expression in embryos treated with both of the test compounds, confirming the validity of the relative target expression change calculated by normalization with *Krox-20*. In this study of novel test compounds, we found that selecting a reference gene with expression restricted to an unaffected region of the embryo provided a basis for relative gene expression by PCR that was consistent with in situ hybridization data.

Taken together, the gene expression outcomes demonstrate that in certain cases, such as in the case of embryotoxic test compounds, housekeeping genes may not be accurate normalizers. In other cases (for example, the RA study), certain housekeeping genes did serve as accurate normalizers; however, other genes were not as accurate. Thus, the choice of an optimal reference gene will vary according to the experimental treatment, and therefore it is prudent to incorporate an appropriate control design that will provide an experimentally substantiated rationale for choosing the normalizer used in quantitating relative gene expression change. On the basis of the current results, we suggest the following preliminary experiments to facilitate the selection of an optimal reference gene for normalization in relative gene expression studies.

1. In the context of the RA study described in this report, if target gene expression change has been previously demonstrated qualitatively with the intended treatment, repeat the experiment and normalize the target gene expression gene with several test reference genes. Select the reference gene that calculates gene expression change that most accurately reflects the qualitative response reported in the published studies.

2. When working with a compound for which the gene expression response has not been previously characterized, carry out preliminary studies to determine the relative biological state of various housekeeping processes.

3. If housekeeping processes appear to be affected, select a respective target gene involved in the housekeeping process that would be expected to exhibit expression change, and measure its relative expression change normalized with housekeeping genes involved in biological processes that appear to be unaffected, as well as restricted-expression genes. Based upon our results, we do not recommend selecting genes as normalizers that exhibit expression in regions/structures suspected to be a site of insult by the treatment.

4. Relative target expression change calculated by PCR approaches should be qualitatively confirmed by in situ hybridization. Qualitative confirmation is particularly important in cases in which the developmental toxicants have been identified to alter housekeeping processes.

We conclude that careful reference gene selection is particularly important in evaluating relative gene expression change in experimentally treated embryos. As was demonstrated in this study, the administration of embryotoxic test compounds to embryos can produce morphological abnormalities, as well as alter general housekeeping processes. The experimental control design described in this work should aid the investigator in the rational selection of genes that will serve as accurate normalizers in relative gene expression studies using PCR-based methodology.

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REFERENCES

Augustine KA, Liu ET, Sadler TW. 1995. Interactions of Wnt-1 and Wnt-3a are essential for neural tube patterning. Teratology 51:107–119.

Bozimowski D, Artiss JD, Zak B. 1985. Sensitive determination of cerebrospinal fluid pyruvate, lactate and glucose concentrations. Clin Chim Acta 153:63–69.

Gibson U, Heid CA, Williams PM. 1996. A novel method for real time quantitive RT-PCR. Genome Res 6:995–1001.

Heid CA, Stevens J, Livak KJ, Williams PM. 1996. Real time quantitative PCR. Genome Res 8:549–556.

Henrique D, Adam J, Myat A, Chitnis A, Lewis J, Ish-Horowicz D. 1995. Expression of a Delta homologue in prospective neurons in the chick. Nature 375:787–790.

Herrmann BG. 1991. Expression pattern of the Brachyury gene in whole-mount Twis/Twis mutant embryos. Development 113(Suppl):913–917.

Kaufman MH. 1992. The atlas of mouse development. San Diego: Academic Press, Inc. pp. 1–17, 371.

Marshall H, Nonchev S, Sham MH, et al. 1992. Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature 360:737–741.

Morris-Kay GM, Murphy P, Hill RE, Davidson DR. 1991. Effects of retinoic acid excess on expression of Hox-2.9 and *Krox-20* and on morphological segmentation in the hindbrain of mouse embryos. EMBO J 10:2985–2995.

New DAT. 1978. Whole-embryo culture and the study of mammalian embryos during organogenesis. Biol Rev 53:81–122.

Perkin Elmer Applied Biosystems. 1997. Relative quantitation of gene expression. User Bulletin #2. Foster City, CA: Perkin Elmer Applied Biosystems. pp. 1–35.

Rugh R. 1968. The mouse. Oxford, UK: Oxford University Press. pp. 102–138.

Sadler TW. 1985. Mammalian embryos in culture: a new approach to investigating normal and abnormal developmental mechanisms. Prog Clin Biol Res 171:227–240.

Sadler TW, Kavlok RJ, Daston GP. 1993. Whole embryo culture: organogenesis of rodent embryos in vitro. In: Methods in toxicology. Vol. 1A. San Diego: Academic Press, Inc. p 418–430.

Wieland T, Faulstich H. 1978. Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous Amanita mushrooms. CRC Crit Rev Biochem 5:185–260.