Determination of variations in gene expression during fracture healing

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ABSTRACT – The genetic make-up and physiological state of a cell or tissue in an organism interact to determine the level at which specific genes are expressed. Identifying genes differentially expressed between 2 genetic or physiological states often gives insight into the molecular mechanisms controlled by the process in question. Various methods have been devised to identify differentially expressed genes and to quantify the expression of differentially regulated genes at the RNA or protein level. These methods are most accurate when the experimental samples are derived from highly controlled and reproducible sources, such as cultured cells. However, no simple in vitro models have been developed to study all biological processes and some are still best studied in the context of the whole organism. Using bone fracture healing as a model, we quantified the expression of 2 housekeeping and 2 regulatory genes during this complex biological process to determine the statistical parameters required to study differential gene expression in tissue samples derived from entire organisms. Our analysis shows that 5 samples in each group are needed to identify genes differentially expressed by a factor of 3 between 2 physiological or genetic states.

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Identification of genes differentially expressed between 2 different physiological or phenotypic states is commonly done to determine how normal biological processes, such as cell differentiation, occur or are controlled and to determine the etiology of mutation or disease. Differentially expressed genes are identified by screening subtracted complementary DNA (cDNA) libraries, by using other subtraction techniques, such as selective amplification via biotin- and restriction-mediated enrichment (SABRE) (Lavery et al. 1997, Schibler et al. 2001), by serial analysis of gene expression (SAGE) (Velculescu et al. 1995, Zhang et al. 1997), by differential display (Liang and Pardee 1992), and, more recently, by microarray analysis (Schena et al. 1995, 1996, DeRisi et al. 1997, Heller et al. 1997). Genes with an expression pattern on or off between 2 different physiological states are readily identified by the techniques listed. However, the more common scenario is that the expression level of a gene is modulated by the physiological state rather than being on or off. Unlike most subtractive techniques for identifying differentially regulated genes, SAGE and microarray analyses enable the investigator to identify genes whose expression has been modulated by the physiological state being examined.

Experimental determination of variation in gene expression becomes more complicated when the biological samples used for RNA isolation are not derived from a uniform source such as cultured cells. Tumor specimens are an ideal example of the inherent biological variation in certain biological samples. Even very specific types of tumors harvested from the same anatomical site can come from patients in different age and sex groups and have proportions of tumor cells that differ from other cell types in the specimen. To assess variations in gene expression in these non-uniform specimens, additional manipulations are often used like producing cell culture lines or laser capture techniques to create a more uniform cell population before analysis (DeRisi et al. 1996, Emmert-Buck et al. 1996, Kahn et al. 1998, Banks et al. 1999,

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Fend et al. 1999, Sgroi et al. 1999, Achary et al. 2000, Wolf et al. 2000). Another possible approach would be to increase the number of independent samples analyzed to identify significant variation in gene expression.

We have quantified gene expression during fracture healing in the rat to determine the inherent biological variation that occurs during this complex biological process. Fracture healing involves the proliferation and differentiation of osteoblasts and chondrocytes, the probable migration, proliferation, and differentiation of stem cells into osteoblasts and chondrocytes, and the function of osteoclasts and other inflammatory cells. The fracture healing process occurs in a particular spatial area called the callus and in a histologically-defined temporal pathway (Schenk 1992). Expression patterns for certain genes during fracture healing have been defined (Boden et al. 1989, Bolander 1992, Jingushi et al. 1992, Hiltunen et al. 1993, Sandberg et al. 1993, Nakase et al. 1994, Bostrom et al. 1995, Fujii et al. 1999, Ito et al. 1999). Thus, fracture healing is a good model in which to assess variations in gene expression during a complex biological process. We chose to examine these variations in rats 7 days after fracture since at this time in the fracture healing process, osteoblasts and chondrocytes are proliferating and differentiating and therefore substantial biological variation is occurring. We used the quantitative competitive reverse transcription polymerase chain reaction technique (QC-RT-PCR) (Becker-André and Hahlbrock 1989, Gilliland et al. 1990) to determine the variation inherent in the assay and between independent fracture callus specimens. A statistical power analysis was done to ascertain the number of independent samples required to identify significant 2-, 3-, 5-, and 10-fold differences in gene expression. Our analysis indicates that modest sample numbers (n = 5) can be used to identify genes with modulated expression levels between 2 complex biological states.

Animals and methods

Production of closed, mid-diaphyseal fractures

Retired breeder, male Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY), housed in individual cages, and given food and water ad libitum. All animal procedures were approved by the Animal Use and Care Committee of the New Jersey Medical School.

Closed mid-diaphyseal femur fractures were created in 10 rats (7–9 months old) by the method of Bonnarens and Einhorn (1984). The animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (8 mg/kg). The right hind limb was shaved and scrubbed with betadine. A medial para-patellar incision was made to expose the patella which was dislocated laterally to expose the femoral condyles. An entry point was made in the femur through the intercondylar notch by using a 1.2 mm diameter needle as a hand-held drill. A stainless steel rod (1.1 mm diameter) was inserted into the medullary canal of the femur and the rod was secured in the proximal end of the femur by tamping. Excess rod was cut as closely as possible to the femoral condyles with wire cutters. The dislocated patella was reduced and the incision was sutured in two layers. Finally, a mid-diaphyseal fracture was created using a 3-point bending device (Bonnarens and Einhorn 1984). Postoperative radiographs were taken to determine the quality of the fracture. The animals were permitted to ambulate freely immediately after surgery and were killed 7 days later. The fractured femurs were resected and the calluses carefully cleansed of soft tissue without disturbing the callus. All parts of the bone not associated with the callus were removed and the latter was flash-frozen in liquid nitrogen and stored at –80 °C.

Preparation of total RNA in the fracture callus

Total RNA was purified from individual calluses as follows. A frozen callus was weighed and then pulverized to a fine powder. TRIzol (Life Technologies, Bethesda, MD) was added to the callus powder and the mixture homogenized to insure complete disruption of the small bone fragments and enhance the RNA yield. RNA was isolated using the RNeasy Kit (Qiagen, Santa Clara, CA). RNA concentrations were determined by UV spectrophotometry and quality was assessed by agarose gel electrophoresis. RNA yields varied from 200–850 μg per callus. RNA from 2 of the 10 fracture calluses was degraded and not used in our analysis.

Table 1. List of oligodeoxynucleotides used in this study

cDNA	Primer	Sequence	Amplicon size (bp) cDNA	Amplicon size (bp) Competitor	Temp. °C
GAPDH	RH15	5'-GAG CTG AAC GGG AAG CTC ACT GG-3'	311	261	60
	RH16	5'-TCC ACC ACC CTG TTG CTG TAG CC-3'			
	POC397	5'-GAG CTG AAC GGG AAG CTC ACT GGG CCG CCT GGA GAA ACC TGC C-3'			
EF-1α	POC395	5'-GGA ATG GTG ACA ACA TGC TG-3'	348	301	60
	POC396	5'-CG TTG AAG CCT ACA TTG TCC-3'			
Bmp4	POC358	5'-CCC AAG CAT CAC CCA CAG CGC-3'	361	288	52.4
	POC359	5'-GGA CTG CCT GAT TTC AGC GGC-3'			
	POC398	5'-CCC AAG CAT CAC CCA CAG CGC GGA TCG TGG CCC CAC CAG GC-3'			
TGFβ1	POC411	5'-CAA ACG TCG AGG TGA CCT GG-3'	432	349	55
	RH8	5'-CGC ACG ATC ATG TTG GAC AAC-3'			
	POC412	5'-CAA ACG TCG AGG TGA CCT GGC ACC GGA GAG CCC TGG ATA CCA A-3'			

Reverse transcriptase reactions

cDNA was prepared from 5 μg total RNA in a 100 μL reverse transcriptase (RT) reaction according to the manufacturer's recommendations (RETROscript, Ambion Inc., Austin, TX). Total RNA was mixed with oligo(dT)$_{18}$ and dNTPs, heated to 65 °C for 3 minutes, and cooled on ice. The reaction was allowed to proceed for 1 hour at 42 °C and was stopped by a 10-minute incubation at 92 °C.

Competitor template production

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), bone morphogenetic protein-4 (Bmp4) and transforming growth factor-β1 (TGFβ1) cDNAs were amplified from fracture callus RT reactions by polymerase chain reaction (PCR) using Vent(exo-) polymerase (New England Biolabs) and oligodeoxynucleotide primers listed in Table 1: RH15 and RH16 for GAPDH, POC358 and POC359 for Bmp4 and POC411 and RH8 for TGFβ1. In a subsequent PCR reaction, the PCR products from the GAPDH, Bmp4, and TGFβ1 cDNAs were used as templates to generate a shortened competitor template for each mRNA species (Welle et al. 2000). Truncation primers (POC397, POC398, and POC412) were designed that would hybridize to a position on the PCR amplified cDNA products of GAPDH, Bmp4, and TGFβ1 about 50 base pairs (bp) from the 5' end. The truncation primers also contained the sequence of the original amplifying primers (RH15, POC358, and POC411) at their 5' ends. Thus by using the truncation primers and the original 3' end primers (RH16, POC359, and RH8), a competitor template for each cDNA was made by PCR amplification that was about 50 bp shorter than the cDNA PCR product, but which had the same 5' and 3' sequences. In contrast, the elongation factor-1α (EF-1α) competitor was made by PCR amplification of a cloned rat EF-1α cDNA containing a 40 bp internal deletion as template (Dostal et al. 1994) and primers POC395 and POC396. PCR amplified competitor was purified by agarose gel electrophoresis and quantified fluorometrically using a Hoechst dye binding assay (Labarca and Paigen 1980) and a Turner Designs TD360 fluorometer. PCR annealing temperatures for the primer sets are listed in Table 1.

Preliminary experiments were done to determine the correct range of competitor amounts to be used in the QC-RT-PCR reactions. From these data, stock solutions of serially diluted competitor templates were made and stored at –20 °C.

Quantitative competitive reverse transcription polymerase chain reactions (QC-RT-PCR)

QC-RT-PCR was done as previously described (Gilliland, et al. 1990) with the following modifications. We used 25 μL PCR reactions, which were composed of 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 (Thermol Pol Buffer, New England Biolabs), 200 μM dNTPs, 1 μM of each primer, 2 units of Vent (exo-) polymerase (New England

Biolabs), 2.5 μL of a diluted RT reaction, and varying amounts of competitor template. RT reactions were diluted 1:8-fold with water. Thus each QC-RT-PCR reaction contained the equivalent cDNA from 15.6 ng of total RNA. The primers used for QC-RT-PCR were RH15 and RH16 for GAPDH, POC395 and POC396 for EF-1α, POC358 and POC359 for Bmp4, and POC411 and RH8 for TGFβ1 (Table 1). Following a 5-minute denaturing step at 95 °C, amplification was done for 30 cycles (95 °C for 30 seconds, annealing temperature for 30 seconds, 72 °C for 30 seconds) followed by a final 3-minute polymerization step. PCR was performed in an MJ Research PTC100 or PTC200 thermal cycler.

The PCR products were separated in 6% polyacrylamide gels. Gels were scanned using a Molecular Dynamics Fluoroimager (Sunnyvale, CA).

QC-RT-PCR data analysis

Gel scan data were analyzed using ImageQuant version 5.0 software to determine the relative amounts of cDNA PCR product and competitor PCR product. The ratio of cDNA PCR product to competitor PCR product from the gel scan data was plotted versus initial competitor amounts (number of template molecules) in the PCR reaction. An equation for the plotted data was obtained using a least squares fit of $y = cx^b$ where y equals the ratio of cDNA to competitor PCR product and x equals the initial competitor template amount. An x value was then obtained for each data set at a y value of 1 since at this ratio of cDNA to competitor PCR product, the amount of cDNA specific for the target mRNA should be equal to the amount of initial competitor template. Calculated mRNA values per μg of total RNA were normalized to the calculated amount of GAPDH mRNA per μg of total RNA from the QC-RT-PCR results.

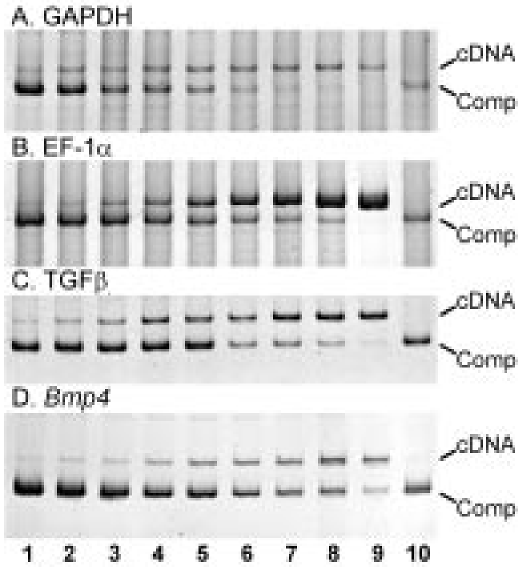
Results

We determined the amounts of mRNA for GAPDH, EF-1α, Bmp4, and TGFβ1 in total RNA prepared from rat femur fracture calluses at 7 days after fracture. Since GAPDH and EF-1α mRNAs are transcribed from housekeeping genes, these two mRNAs were used as internal controls for these experiments. In contrast, Bmp4 and TGFβ1 are known to be differentially expressed during healing of the fracture (Bolander 1992, Bourque et al. 1993, Nakase et al. 1994, Bostrom, et al. 1995) and were therefore used to assess variation in gene expression between fracture callus specimens.

QC-RT-PCR was done to determine the amount of GAPDH, EF-1α, Bmp4, and TGFβ1 mRNA present in 1 μg of total RNA prepared independently from 8 rat femur fracture calluses. The same sets of competitor template dilutions were used to quantify the amount of all 4 mRNAs from each fracture callus. The QC-RT-PCR reactions were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide and the fluorescence in each band was determined using a Molecular Dynamic Fluorimager (Figure). The amount of cDNA in each PCR reaction was constant, but the amount of competitor template varied incrementally 2-fold between reactions. Since the amount of initial competitor template in each PCR reaction was known, the point at which the PCR products from the cDNA template and competitor template were the same was used to determine the initial amount of mRNA present in the total RNA aliquot as described in Material and Methods.

To determine the variation in gene expression between RNA preparations, the amounts of EF-1α, Bmp4, and TGFβ1 mRNA were normalized to the amount of GAPDH mRNA measured in a given RNA sample. This normalization should reduce the variability associated with errors in RNA purity, RNA quantification, or in the amount of total RNA initially used in the cDNA reaction. The GAPDH normalized values for EF-1α, Bmp4, and TGFβ1 from each RNA preparation were then used to calculate a mean and standard deviation (Table 2). We found that the coefficient of variation (one standard deviation as a percentage) for normalized EF-1α was 21%. In contrast, the normalized Bmp4 and TGFβ1 coefficients of variation were 47% and 42%, respectively. Thus, as expected, the amount of variation in gene expression detected from the 2 biologically regulated genes was greater than that for a housekeeping gene.

Since the ratio of any 2 housekeeping genes should be constant, we suspected that the 21% coefficient of variation found for the normalized EF-1α mRNA amounts represented a variation



Representative polyacrylamide gels of QC-RT-PCR reaction products. PCR reaction products were separated on 6% polyacrylamide gels, stained with ethidium bromide, and scanned with a Molecular Dynamics Fluorimager. Here are shown representative Fluorimager scans for GAPDH (panel A), EF-1α (panel B), TGFβ1 (panel C), and Bmp4 (panel D) quantification. PCR reaction products from a constant amount of cDNA (equivalent to 15.625 ng of total RNA) and serial 2-fold dilutions of the appropriate competitor template were separated in lanes 1-8. PCR reaction products from a cDNA only amplification reaction are shown in lane 9. PCR reaction products from a competitor template only amplification reaction are shown in lane 10. Panel A. The initial competitor template amount for lane 1 was about 3.5×10^6 templates (0.5 pg) and for lane 10 874,000 molecules. Panel B. The initial competitor template amount in lane 1 was about 3×10^6 molecules (0.5 pg) and in lane 10 757,800 molecules. Panel C. The initial competitor template amount in lane 1 was about 200,000 molecules (0.039 pg) and for lane 10 was 25,530 molecules. Panel D. The initial competitor template amount in lane 1 was about 200,000 molecules (0.03125 pg) and in lane 10 24,750 molecules. The cDNA only reaction for Bmp4 often produced an aberrant PCR product with the same apparent molecular weight as the Bmp4 competitor template PCR product (Panel D, lane 9). No steps were taken to correct for this aberrant PCR product when quantifying the amounts of Bmp4 cDNA versus Bmp4 competitor template PCR products from the Fluorimager data. Thus, our analysis for Bmp4 probably underestimates the true amount of Bmp4 mRNA present, but would not affect the derived coefficients of variation.

inherent in the assay and was not due to some biological effect. To test this assumption, one fracture callus RNA preparation (rat #26) was assayed an additional 5 times for GAPDH, EF-1α, and Bmp4 mRNA amounts using independent reverse transcriptase reaction products. If our assumption was correct, then we would have expected to find a similar 21% coefficient of variation between the GAPDH normalized levels of EF-1α and Bmp4 from the independent assays. The normalized EF-1α and Bmp4 coefficients of variation for the repeated measures were 24% and 17%, respectively (Table 3).

Using the empirically determined coefficient of variation for the biologically regulated mRNAs, we performed a statistical power analysis to define the minimum independent sample number required to identify a gene of which the expression is modulated by some physiological event. We used the data obtained when quantifying Bmp4 mRNA (Table 2) since this biologically-regulated mRNA showed the most variation in our assays. The statistical power analysis was done using the PASS v6.0 software package (NCSS, Kaysville, UT). The results of this analysis are summarized in Table 4. An often used rule-of-thumb for microarray analyses is that any modulation in gene expression that is less than 3-fold is not significant. On the basis of our statistical power analysis, we found that to detect a 3-fold difference in mRNA levels between a control and experimental set of samples would require 5 samples in each set.

On the other hand, we did a statistical power analysis using the 24% coefficient of variation which we had found for the repeated measures of normalized EF-1α mRNA levels (Table 3). Since this 24% coefficient of variation more accurately represents experimental error associated with these assays, the 24% coefficient of variation may be more useful for analyzing gene expression variation in less complex systems, such as cell culture models or purer tissue samples, such as those obtained from laser capture. This power analysis showed that a control and experimental set would require only 2 samples to detect a statistically significant 3-fold difference in expression levels (Table 5).

Discussion

The purpose of our study was to determine the variability in quantifying specific gene expression levels in tissue samples obtained from various ani-

Table 2. QC-RT-PCR quantitation of specific mRNAs in various fracture callus specimens

Rat [a] No.	GAPDH [b] (× 10^6)	EF-1α [b] (× 10^6)	Bmp4 [b] (× 10^3)	TGFβ1 [b] (× 10^3)	EF-1α/ GAPDH	Bmp4/ GAPDH (× 10^{-4})	TGFβ1/ GAPDH (× 10^{-3})
1	39	16	43	606	0.409	11	15
2	112	27	153	1,010	0.245	13	8
3	93	26	129	542	0.281	13	5
11	16	6	28	229	0.418	17	13
16	98	23	56	419	0.234	5	4
25	36	10	88	247	0.291	24	6
26	101	28	52	635	0.283	5	6
44	136	40	129	1,492	0.293	9	10
Mean	79	22	85	647	0.307	12	9
SD	40	10	43	394	0.065	5	3
CV [c]	50	45	52	61	21	47	42

[a] Indicates rat from which fracture callus total RNA was isolated.
[b] mRNA molecules per μg of total RNA
[c] Coefficient of variation (%)

Table 3. Repeated measurements of specific mRNAs from a single RNA preparation

cDNA preparation (Rat #26)	Bmp4 [a] (× 10^3)	EF-1α [a] (× 10^6)	GAPDH [a] (× 10^6)	Bmp4/ GAPDH (× 10^{-4})	EF-1α/ GAPDH
Test 1	52	28	101	5.126	0.283
Test 2	124	51	165	7.517	0.310
Test 3	96	46	208	4.648	0.222
Test 4	117	76	200	5.881	0.382
Test 5	77	45	143	5.429	0.315
Test 6	47	16	96	4.921	0.170
Mean	86	44	152	5.587	0.281
SD	29	18	43	0.946	0.068
CV [b]	35	43	29	17	24

[a] mRNA molecules per μg of total RNA
[b] Coefficient of variation (%)

Table 4. Statistical power analysis for sample numbers used to determine significant variation in mRNA amounts between complex biological samples, based on the variation detected in normalized Bmp4 mRNA levels (47% coefficient of variation)

Difference in detected mRNA amounts	Required number of complex samples	Alpha error	Beta error	Statistical power
2-fold	9	0.05	0.18	0.82
3-fold	5	0.05	0.14	0.86
5-fold	3	0.05	0.17	0.83
10-fold	3	0.05	0.09	0.91

Table 5. Statistical power analysis for sample numbers used to determine significant variation in mRNA amounts between 2 simple biological samples, based on the variation detected in repeated measurements of normalized EF-1α mRNA levels (24% coefficient of variation)

Difference in detected mRNA amounts	Required number of complex samples	Alpha error	Beta error	Statistical power
2-fold	3	0.05	0.13	0.87
3-fold	2	0.05	0.05	0.95
5-fold	2	0.05	0.01	0.99
10-fold	2	0.05	< 0.01	> 0.99

mals to estimate the sizes of experimental groups. Our group, and others, are interested in determining the gene expression pathways that control endochondral ossification during healing of fractures and fetal development. However, simple cell culture systems to study endochondral ossification do not exist and therefore our studies require the use of specimens obtained from animals, which increases the variations in the experiments. Therefore, it can be difficult to design experiments that make use of in vivo models and that have ample statistical power. Variations associated with quantifying gene expression would not only be inherent in the experimental manipulations needed to perform the quantification assay, but would also be affected by the nature of the sample being assayed. For simple biological samples, such as cultured cells, little sample-to-sample variation would be expected. On the other hand, tissue samples harvested from various animals could be expected to show increased variations in gene expression on the basis of age, gender, physiological, and genetic parameters as well as differences associated with collecting each tissue sample. We assessed this variability in fracture healing as a model biological process since gene expression is known to be temporally regulated during fracture healing and because a fracture callus specimen contains many different cell types which increase complexity of samples (Bolander 1992, Schenk 1992, Bostrom, et al. 1995). Two genes known to be regulated during fracture healing (Bmp4 and TGF-β1) and two housekeeping genes (GAPDH and EF-1α) were quantified to assess variability.

We found a QC-RT-PCR assay variation of about 24% (Tables 2 and 3). The assay variation between samples was 21% (Table 2) on the basis of the EF-1α/GAPDH ratios and was 24% for EF-1α and 17% for Bmp4 using repeated measures (Table 3). The assay variation could be accounted for at several different steps including simple pipetting differences at the reverse transcriptase and PCR reaction steps. Yue et al. (2001) reported a 12% coefficient of variation between independent fluorescent-tagged cDNA preparations made with reverse transcriptase and an identical mRNA preparation, but Welle et al. (2000) reported a < 10% coefficient of variation associated with the RT reaction step during QC-RT-PCR. Similarly, Anderson et al. (1985) found an approximate overall 15% coefficient of variation when measuring the coomassie blue staining intensities of specific mouse liver proteins from the same extract separated by 2-D gel electrophoresis as measured in 10 independent gels. In a similar analysis using 2-D gels to separate rat liver proteins, Steiner et al. (1995) found an approximate 20% variation in protein amounts from 10 2-D gels of the same protein extract. Considering that the independent cDNA preparations we used underwent additional manipulations (PCR) before quantification by polyacrylamide gel electrophoresis, the approximate 24% coefficient of variation we found between two different housekeeping gene mRNA amounts is not an unexpected finding.

Data from other studies support our assumption that the 24% coefficient of variation found in our repeated measures analysis (Table 3) is a useful measure of variation in simple biological systems. Zhao et al. (1995) reported a 20% coefficient of variation when quantifying matrix Gla protein mRNA by QC-RT-PCR after normalization to GAPDH mRNA levels, although only 3 independent RNA samples from cultured normal rat kidney cells were measured. David and Crerar (1986) found a 21% coefficient of variation in muscle glycogen phosphorylase protein levels taken from skeletal muscle samples of 5 to 7 rats and that muscle glycogen phosphorylase mRNA levels, as measured by Northern blot analysis, showed a 27% coefficient of variation from 3 separate skeletal muscle RNA preparations. All of these experimental analyses are consistent with an approximate 24% coefficient of variation when measuring the expression level of any one specific gene in a simple biological sample.

In contrast, the regulated expression of Bmp4 and TGF-β1 showed higher levels of variation between animals with coefficients of variation of 47% and 42%, respectively. These observations suggest that about half of the inter-animal Bmp4 and TGF-β1 gene expression variation is due to assay variation, as discussed above, and that the other half is due to specimen-to-specimen variation. This is an expected result given that Bmp4 and TGF-β1 expression is temporally regulated during fracture healing (Bourque et al. 1993, Bostrom et al. 1995) and due to any variation associated with specimen

(fracture callus) collection from each animal, such as the relative proportions of fracture callus tissue to bone, bone marrow, and other tissue types.

We used the data to estimate required sample numbers to identify 2-, 3-, 5-, and 10-fold differences in gene expression between two sets of samples (Tables 4 and 5). We used the repeated measures coefficient of variation (24%) as an indication of the variability that would be associated with performing a QC-RT-PCR experiment on RNA samples derived from simple, highly reproducible, biological samples. Using this assumption, we found that only 2 samples in each experimental group are required to identify a 3-fold difference in gene expression and that only 3 samples in each group would be needed to identify a 2-fold difference. On the basis of similar assumptions that the 47% coefficient of variation determined for Bmp4 expression between specimens represents assay variability and sample-to-sample variability associated with more complex biological samples, we calculated the experimental group sizes required to identify 2-, 3-, 5-, and 10-fold differences in gene expression (Table 4). Surprisingly, we found that only 5 samples in each group would be needed to identify a 3-fold difference in gene expression; although 9 samples would be required in each group to detect a smaller 2-fold difference in expression.

These calculations indicate that a modest increase in sample size, from 2 to 5, can be used to circumvent labor intensive (primary cell cultures) or specialized techniques (laser capture) to analyze gene expression in complex biological systems, such as during bone fracture healing. The techniques for generating cDNAs to identify differentially expressed genes by subtractive methodologies or by microarrays are very similar to those used in this QC-RT-PCR analysis. Consequently, the statistical analysis of variation performed on our QC-RT-PCR data may be generally applicable to other gene expression quantification methods.

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