

Gene expression in individual cells: analysis using global single cell reverse transcription polymerase chain reaction (GSC RT-PCR)

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Abstract

The determination of the gene expression pattern of single cells has important implications for many areas of cellular and developmental biology including lineage determination, identification of primitive stem cells and temporal gene expression patterns induced by changes in the cellular microenvironment. Global Single Cell Reverse Transcription-Polymerase Chain Reaction (GSC RT-PCR) enables the study of single cell gene expression patterns. Initial observations of significant heterogeneity among single cells derived from a population of cells prompted us to determine how much of this observed heterogeneity was due to the intrinsic variation within the method. In this paper we discuss the sensitivity of GSC RT-PCR for analysis of differences in gene expression between single cells and, in particular, detail the amount of variation generated by the method itself. We found that most of the intrinsic variation in the method occurred in the PCR step. The total variation induced by the method was in the range of 5 fold. While we have determined that there is a five fold methodological variation in GSC RT-PCR, any method which use its components (including generation of cDNAs for microarray analysis) is likely to be affected by such experimental variability, which could limit the interpretation of the resulting data. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Many aspects of both normal development and disease result from changes in the expression of multiple genes in individual cells [1]. It is widely accepted that cancer is a clonal disease (i.e., it arises from a single cell) and that tumor progression occurs

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as a result of molecular changes in individual cells [2] which confer a growth advantage. However, until recently most methods which attempted to study these molecular changes by necessity had to examine populations of cells. An obvious problem with using populations of cells for such studies is that only changes which have occurred in the majority of cells are likely to be identified and differences between individual cells will be difficult or impossible to detect.

To overcome the difficulties with ‘bulk’ methods, techniques have been developed to study gene expression at the single cell level. Disease processes in which multiple changes in gene expression have taken place (for example, colon cancer progression [3]) have been studied using *in situ* hybridization [4] and *in-situ* PCR [5]. Very few of these studies have demonstrated strict quantification. Furthermore, with the proliferation of new methods of measuring gene expression (i.e., cDNA microarrays¹ [6] and SAGE [7,8]) the issue of determining real variability in gene expression versus experimental variability has become increasingly important.

One method which allows the examination of relative differences in gene expression between cells is global single cell reverse transcription-polymerase chain reaction (GSC RT-PCR), a technique in which cDNA copies are made of the mRNA species within a single cell, including mRNA’s of low abundance [9]. At the same time the amounts of the cDNAs produced can be kept in relative proportion to the original mRNA starting material, allowing for studies of relative changes in gene expression. However, a compromise for the proportional representation of the cDNAs is that the length of the resultant cDNAs is shortened to approximately 300–700 bp at the extreme 3’ end. Groups using the GSC RT-PCR technique have documented quantitative changes in expression during haematopoietic development [9–11] and endochondral ossification [12] as well as phenotyping of mature osteoblasts [13].

We have been interested in using this technique to study genes involved in the process of metastasis [14], since this process involves individual cells

which may represent only a small fraction of cells within a much larger population of tumor cells. These metastatic cells have an altered phenotype but because they are only a very small fraction within the tumor population, bulk methods are not sensitive enough to detect altered expression of metastasis-related genes in these cells. Prior to initiating these studies we examined the sensitivity of GSC RT-PCR for studies with single cells. In this manuscript we describe experiments which determined the amount of variation generated by the method itself, by examining the expression of specific genes in single cells or in single-cell-equivalent aliquots obtained from groups of cells.

2. Methods and materials

2.1. GSC RT-PCR

Conditions for GSC RT-PCR have been described previously [9]. Briefly, individual cells were lysed in 4 μ l of 1st strand buffer, consisting of 0.4 U Human Placental RNase Inhibitors (Pharmacia, Montreal, PQ) and 0.1 U of InhibitAce (5’–3’ Research, Boulder, CO) in 0.25 mM dNTPs, 0.13 OD/ml oligo dT₂₀, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3.0 mM MgCl₂, and 0.5% NP-40, then heated to 65°C for 2 min and then allowed to cool at room temperature for 3 min. The mRNA was reverse transcribed with 2.0 U of AMV RTase (Boehringer Mannheim, Montreal, PQ) and 83.3 U of MMLV RTase (Gibco-BRL, Burlington, ON) in 50 mM Tris–HCl pH 8.3, 75 mM KCL and 3 mM MgCl₂ for 15 min at 37°C and the enzymes were then denatured by heating for 10 min at 65°C. The cDNAs were polyadenylated at the 3’ end with 10 U of terminal deoxynucleotidyl transferase (Gibco-BRL, Burlington, ON) in the supplied 5X TdT buffer (200 mM potassium cacodylate, pH 7.2, 4 mM CoCl₂, 0.4 mM DTT, supplemented with 200 μ M dATP) at 37°C for 30 min and the enzyme denatured by heating to 65°C for 10 min. The tailed cDNAs were then ready to be used immediately in a PCR reaction using a single primer. Oligonucleotides for PCR were obtained from Oligos Etc. (Wilsonville, OR) and each batch was optimized for MgCl₂ and primer concentration before use. The PCR primer contains polycloning sites 5’ to the oligo

¹ <http://cmgm.stanford.edu/pbrown/>;
<http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/>.

dT for use in subcloning cDNAs of interest. The PCR primer sequence is 5'-ATG TCG TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC (T₂₄)-3'. PCR was performed using a Perkin-Elmer 480 thermal cycler with the following cycles: 25 cycles of 1 min 94°C, 2 min 42°C, 6 min 72°C, followed by 25 cycles of 1 min 94°C, 2 min 42°C, 2 min 72°C. In most experiments only the inside wells of the thermal cycler were used. Analysis of amplification of samples in different wells, in this region of the thermal cycler, did not reveal systematic differences.

2.2. Single cell PCR

For single cell RT-PCR reactions, cells from tissue culture were trypsinized to give a single cell suspension, centrifuged, and resuspended to 5×10^3 cells/ml in α -Minimal Essential Media (α -MEM) containing 10% fetal bovine serum (FBS) (PA Biologicals, Sydney, Australia). An aliquot of 0.5 μ l was placed in each well of a 60-well Terasaki plate (NUNC, Burlington, ON) and then examined under a microscope. Wells containing a single cell were marked. Cells were lysed directly in the well and then the contents transferred to 0.6 ml thin-walled PCR tubes (Rose Scientific, Edmonton) for RT-PCR as described above.

2.3. Aliquot PCR

For aliquot RT-PCR reactions, cells were trypsinized to give a single cell suspension, centrifuged and resuspended to 5×10^4 cells/ml in α -MEM. An aliquot of 1.0 μ l (containing 50 cells) was lysed in 25 μ l of 1st comp buffer to give a final concentration of 2 cells/ μ l. This was then further diluted in 1st comp buffer to give the equivalent of 1 cell in 4.5 μ l of 1st comp buffer. This volume (4.5 μ l) was then transferred to individual PCR tubes for RT-PCR as described above.

2.4. Quantitation of PCR products

Five to ten microlitres of PCR product were loaded into each well of a 1.7% agarose gel, electrophoresed until the bromophenol blue dye had migrated about 2 in. into the gel. The gel was then denatured for 30 min in denaturing buffer (3 M

NaCl, 1 M NaOH), rinsed with water, and incubated in 1 M NH₄OAc for a further 30 min. The DNA in the gel was then transferred to Zetaprobe nylon membrane (Biorad, Burlington, ON) overnight in 20X SSC, UV cross-linked, and probed with ³²P-labelled probes. Following transfer the gels were examined under a UV light to determine that all of the DNA was transferred to the agarose gels. The murine partial cDNA probes used were L32 (K02060), which codes for a ribosomal protein, GAPDH (M17701, glyceraldehyde-3 phosphate dehydrogenase), uPAR (U12235, urokinase plasminogen activator receptor), and nm23-H1 (X75598, gift of Dr. Ann Chambers). The former two probes are often used as housekeeping genes and are discussed further in the Analysis of Data section. The latter two probes were selected because there are reports in the literature that changes in the expression of these genes are correlated to enhanced metastatic ability. Probes were labelled with ³²P-dCTP (Dupont, Pointe-Claire, PQ) using an oligo labelling kit (Pharmacia, Montreal, PQ). The blots were stripped between probes by boiling in 0.1% SDS for 15 min. Each blot was probed separately, with all of the aforementioned probes. Quantitation of the probes bound to the membranes was obtained using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA) on blots scanned using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.5. Analysis of data

The variation in gene expression between single cells was studied primarily using the two housekeeping genes, L32 (a ribosomal protein, which has been demonstrated to be highly and stably expressed throughout the cell cycle and during differentiation [15]) and GAPDH. These two genes were chosen because they are commonly used in studies of gene expression as the loading control to genes of interest. All of the gene expression data in this paper are presented in the format of 'normalized' data, to allow direct comparison of results from a large number of samples that were hybridized with probes made and labelled at different times during the course of these studies. The data were normalized for each blot by dividing the individual PhosphorImager value obtained for each sample within the given blot by the

average sum above background of all of the samples on that blot. This normalization changes the mean gene expression level (on any given blot) to a value of 1, however, it does not affect the relative variance of the individual samples on that blot. It eliminates the effect of different levels of labelling of the different probes and differences in their relative hybridization efficiency from blot to blot.

3. Results

Differences in gene expression between single cells were studied both in proliferating tumour cells (KHT-LP1, a murine fibrosarcoma [16]) and in stationary phase, early passage, normal rat embryo fibroblasts (REF), which arrest in G_0/G_1 of the cell cycle and do not cycle once they reach confluence [17]. Fig. 1 shows a representative phosphorimage for amplified samples of individual cells or single cell aliquots probed with L32 and GAPDH for these two cell lines. Fig. 2A and B present the analyzed data for the expression of L32 and GAPDH in single cells from both cell lines. These results demonstrate substantial heterogeneity between individual cells in the detected levels of gene expression. Some of the heterogeneity seen for KHT cells might have arisen because the cells are in different parts of the cell cycle, however, it is apparent that even in the sta-

tionary phase REF cells there is a large amount of heterogeneity between individual cells.

The large variation in the gene expression pattern of the single cells prompted us to ask whether the variation was due to real differences in gene expression between individual cells, or whether it arose from the experimental procedure. To analyze the amount of variance associated with the experimental procedure we drew individual samples from pooled lysates of populations of 50 cells. We found, as illustrated in Fig. 3, that there is a substantial amount of variation between the individual pooled samples. The data in Fig. 3 present the results from various experiments each of which drew upon a different pooled group, however there is substantial variation even between samples derived from the same pooled group (data not shown).

We were interested in differences in gene expression between the single cells but the results from the pooled sample experiments suggested that at least some of the observed variation was due to intrinsic heterogeneity within the technique itself. Initially we examined the last part of the procedure; the loading, blotting and probing of the different samples. To determine the amount of variance generated by this step, 10 individual samples derived from a pooled lysate of 50 KHT-LP1 cells, were divided into four aliquots and each was loaded onto one of four different agarose gels, the samples were electrophoresed and each gel was transferred to nylon membrane from a continuous roll, blocked and then probed for L32. Separately prepared and labelled L32 probes were used for the different blots. These nylon blots, probed with different probes, displayed variation in the raw data acquired from the Phosphorimager (data not shown). However, after normalization of the data, each blot had a normal distribution and the variances between the different blots were not significantly different. While there was some variation between the samples (as seen in Figs. 2 and 3), there was very little variation between the same sample loaded on different blots (data not shown). The range of values for the four replicates from the ten samples was a factor of 1.1 to 1.48 with the mean ($\pm$ SE) range being $1.25 (\pm 0.03)$. These results suggest that the loading, transferring and probing steps contribute minimally to the observed heterogeneity.

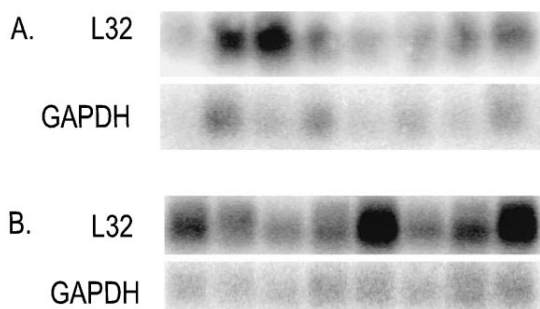


Fig. 1. PhosphorImage of L32 and GAPDH from single KHT-LP1 cells and aliquots of REF cells. Individual KHT-LP1 murine fibrosarcoma cells (A) and aliquots (single cell equivalents) of REF cells (B) were subjected to GSC RT-PCR and probed individually for L32 and GAPDH. The membranes were imaged with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the individual bands were quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

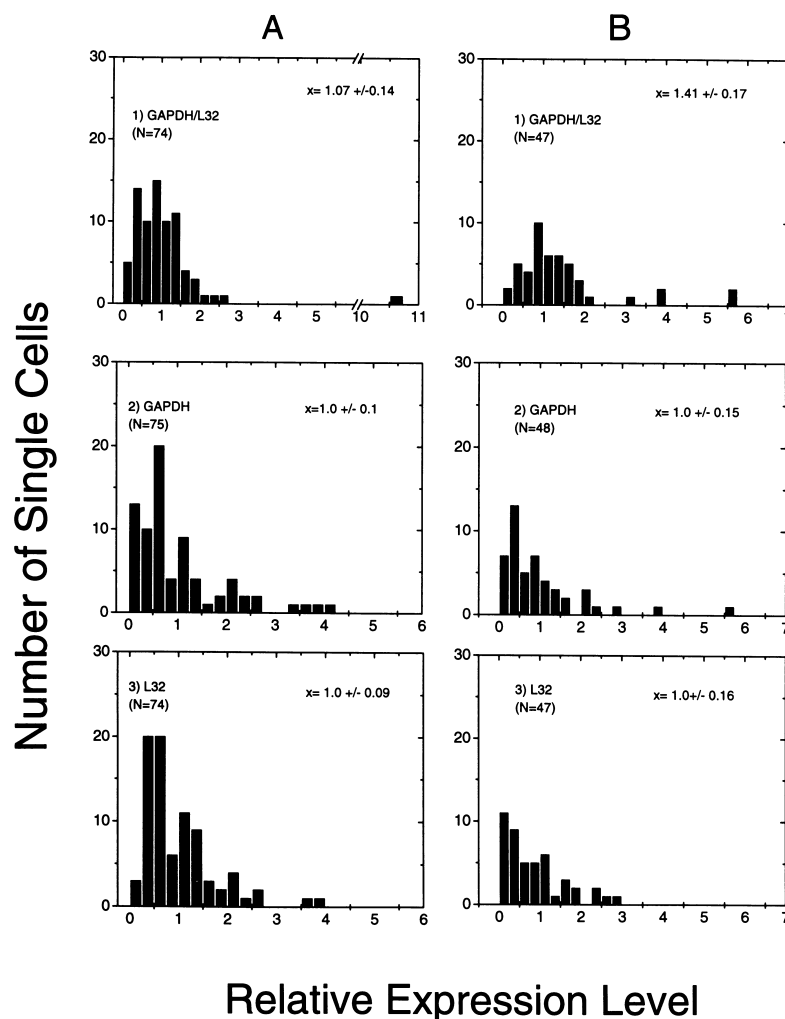


Fig. 2. Gene expression in single cells. Individual KHT-LP1 murine fibrosarcoma cells (A) or Rat Embryo Fibroblasts (B) underwent GSC RT-PCR and were probed for L32 and GAPDH. Panel 1 displays the relative expression of GAPDH as a ratio to L32 relative expression. Panels 2 and 3 give the individual relative gene expression of GAPDH and L32 respectively. See the methods section for discussion of the method of determining the relative expression level ($\bar{x}$ = mean $\pm$ standard error, N = number of samples).

To determine if the observed variation between samples was being generated within either the RT or the PCR step of the protocol, cells from tissue culture were prepared as for aliquot PCR and lysed as described above. The lysate was then divided into two groups, in one group, single cell equivalents were aliquoted into 0.6 ml PCR tubes then reverse-transcribed, polyadenylated and subjected to PCR (single cell surrogate-RT). In the second group, the lysate was reverse transcribed and polyadenylated

before being aliquoted into single cell equivalents for PCR (bulk-RT). Fig. 4 shows the results of this experiment which suggest that there is less variation in the samples from the bulk-RT than from the single cell surrogate-RT, however the variances of the two groups are not significantly different (F -test, $p > 0.05$).

The data from both groups of figures in Fig. 4 were plotted as a ratio of the two housekeeping genes (Fig. 5). When the data is plotted in this

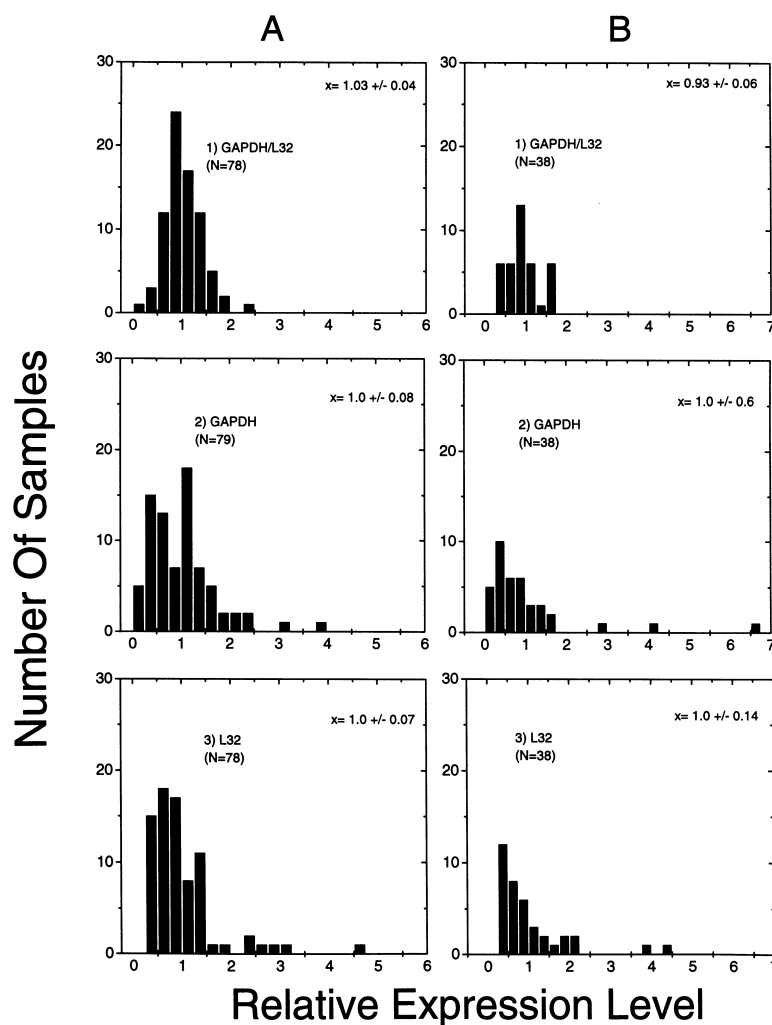


Fig. 3. Gene expression in KHT and REF cell aliquots. Aliquots were created by lysing 50 KHT-LP1(A) or REF(B) cells which were then reverse transcribed and tailed. These samples were then fractionated into single cell equivalent samples and amplified by polymerase chain reaction, individually. The amplified samples were then probed for L32 and GAPDH. Panel 1 displays the relative expression of GAPDH as a ratio to L32 relative expression. Panels 2 and 3 give the individual relative gene expression of GAPDH and L32 respectively ($x = \text{mean} \pm \text{standard error}$, $N = \text{number of samples}$).

manner, the highest and lowest values are from bulk-RT samples. Furthermore, the overall amount of variation is reduced, and is closely centred around one, suggesting that under certain conditions either housekeeping gene may be acceptable for use as a housekeeping control for GSC RT-PCR. The range of values is approximately a factor of 5 (relative expression of between 0.5 and 2.5), although the

majority of the samples lie in a range which is a factor of about 2. Again there is no significant difference in the variance (F -test, $p > 0.05$).

Although most of this work has been performed using housekeeping genes, we were also interested in examining the expression of genes for which we expected the mRNA to be in lower abundance. We therefore analyzed the expression of the *nm23* gene,

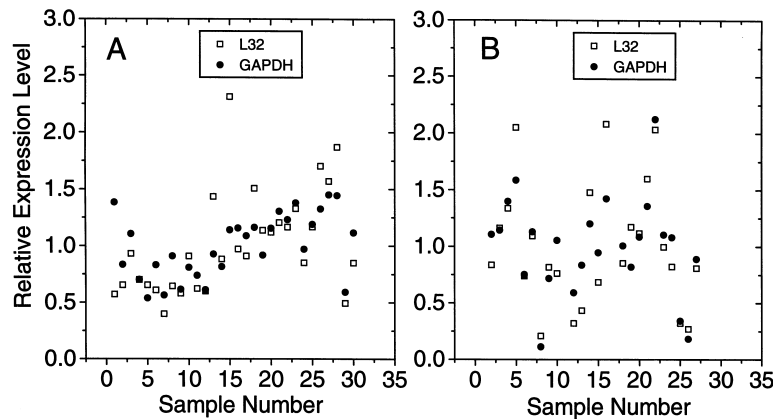


Fig. 4. Variation in the expression levels of L32 and GAPDH in both the bulk RT method and the single cell surrogate RT method. Relative expression level for L32 or GAPDH is expressed as the sum above background for an individual sample divided by the mean sum above background for all samples in either (A) or (B). In both panels the mean expression levels for both genes is 1.0, due to the normalization. However, in Panel A the SEM for L32 and GAPDH are 0.08 and 0.05 respectively, in Panel B they are 0.11 and 0.09. These differences are not statistically significant.

which codes for a nucleoside diphosphate kinase that is associated with low tumor metastatic potential [18], and uPAR, which codes for the urokinase plasminogen activator receptor [19], in individual KHT cells. Fig. 6 demonstrates that the expression pattern of *nm23* and uPAR is similar to that of the house-keeping gene L32. There is no statistical difference

in the variance between the expression patterns for the three genes (*F*-test, $p > 0.05$).

4. Discussion

The interpretation of gene expression at the single cell level using the GSC RT-PCR technique relies on information relating to the variation introduced by the method. Our initial observations (Fig. 2) from studying single cells derived from the same initial populations, suggested that there was a large amount of variation in the gene expression of 'housekeeping' genes. To verify that the observed variation in the gene expression was real, we performed experiments where we used pooled starting material. Although there is less variation in the distributions of both L32 and GAPDH in both the REF and KHT aliquots (Fig. 3) it is clear that there is a significant amount of variation that can be attributed to the GSC RT-PCR method. The loading of the samples onto the agarose gel, electrophoresis, transfer of the cDNA libraries to nylon membrane, and the probing of the membranes with radioactive probes (data not shown) was only a minor source of variation. When we examined the RT and PCR steps by comparing the Bulk RT and the Single Cell Surrogate-RT (Fig. 4), the spread in the Single Cell Surrogate-RT data was similar to that

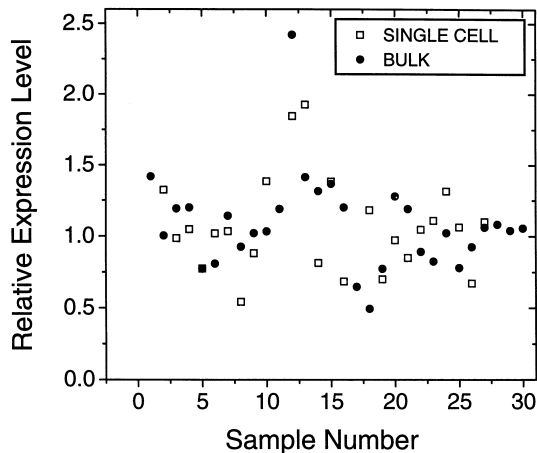


Fig. 5. The Ratio of GAPDH to L32 in both bulk RT method and the single cell surrogate RT method. Relative expression values for the data shown in Fig. 3 plotted as a ratio (GAPDH/L32) for each sample.

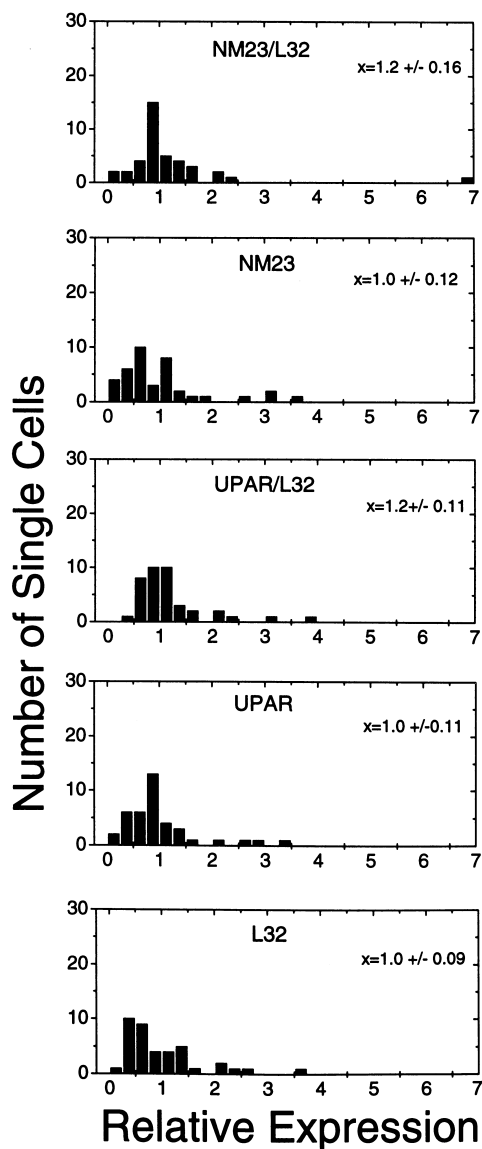


Fig. 6. Variation in the expression level of the non-housekeeping genes *nm23* and *uPAR* as compared to the housekeeping gene *L32*. Single KHT-LP1 cells were subjected to GSC RT-PCR and probed for their expression of *L32*, *nm23* and *uPAR*. The variation in expression of the *nm23* gene and the *uPAR* gene is similar to the variation in the housekeeping gene ($\bar{x}$ = mean $\pm$ standard error).

of the Bulk-RT data (F -test, $p > 0.05$). This implies that the RT step on individual samples does not add significantly to the variation between the samples.

We therefore conclude that the tailing or the PCR step introduces the variation. It is unlikely that the

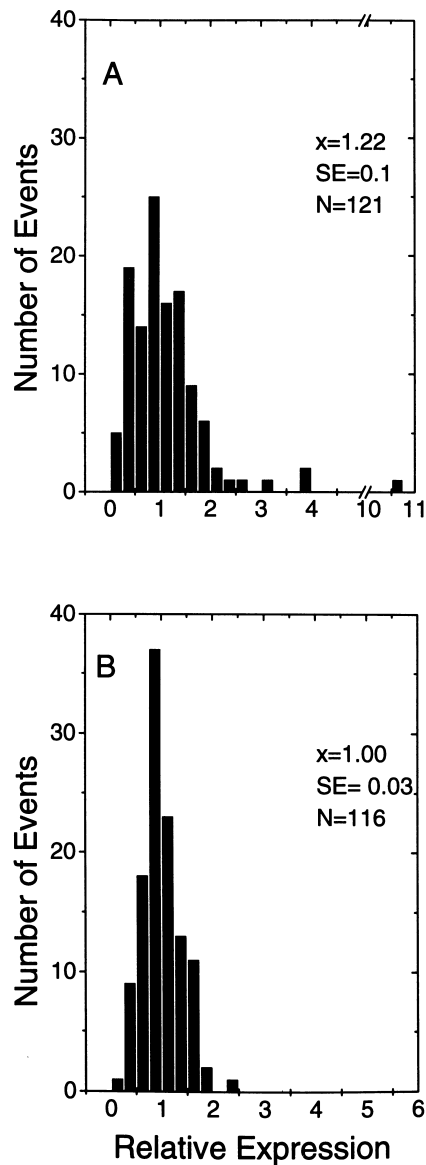


Fig. 7. The ratio of GAPDH to *L32* in all single cell and aliquot samples. The data in this figure is combined from both Fig. 2 and Fig. 3, representing all of the data on GAPDH and *L32* expression of single cells (A) and aliquots (B) from both KHT-LP1 cells and REF cells. There is a significant difference in the variance of the distribution between the single cells and the aliquots (F -test, $p < 0.05$) ($\bar{x}$ = mean $\pm$ standard error, N = number of samples).

variation is a result of the large number of cycles used to amplify cDNA from single cells, since two separate groups [9,11], using this method have demonstrated that the relative abundance of many genes is maintained over 50 cycles of PCR.

One of the purposes of the study was to investigate whether there were differences in gene expression between individual cells. To address this question we collected all the data from the single cells and aliquots, regardless of origin (KHT or REF), and plotted it as a pair of frequency histograms (Fig. 7). Since the data was normalized for differences between the different blots, this allows for the comparison of the data between the cell lines of different origin. The variance is statistically greater in the single cells than in the aliquots (F -test, $p < 0.05$), which is consistent with our original expectation. As seen in Fig. 5 the variance introduced by the GSC RT-PCR method is in the range of 0.5 to 2.5 (i.e., 5-fold). There are clearly some single cell samples which fall out of this range. These may represent real variation in gene expression levels of individual cells but, they represent only a small fraction of all the measurements made.

One might expect that there would be less variation in expression of housekeeping genes than in non-housekeeping genes. Consequently the variation in expression of two non-housekeeping genes, *nm23* and *uPAR* was analysed (Fig. 6). The range of expression of these genes was of similar magnitude to that seen for the housekeeping genes (*GAPDH* and *L32*) suggesting that it was largely a result of variation introduced by the method and that these genes did not show greater variation in expression than housekeeping genes.

Based on our results with the GSC RT-PCR, it is clear that the lower threshold for detecting real differences is in the 5 fold range, which is similar to the value that DeRisi et al. [20] have used as a cut off for determining significant differences in gene expression between samples assayed using cDNA microarray chip technology. Other groups have examined the expression of individual genes using quantitative RT-PCR [21–23]. While this method is extremely accurate for quantifying the changes in a single gene, these methods cannot, currently, be used to quantitate multi-gene expression patterns. The advantage that GSC RT-PCR has over other methods of study-

ing gene expression is that it generates a cDNA library which can be used in conjunction with both membrane and chip based gene arrays to study expression patterns of a wide range of genes. With the constant stream of new genetic information (both full length cDNAs and ESTs) that is/and will be available, one can return to the original cDNA libraries derived from the single cells and examine them for the presence of these new genes.

In this paper we restricted ourselves to discussing the variation associated with GSC RT-PCR, however, any method which uses these components is likely to be affected by such experimental variability, which could affect the interpretation of the resulting data. There are many examples of genes that are so highly overexpressed in comparisons between two different cells that it is obvious that the background variation is of little importance. However, many differences are likely to be of a smaller magnitude. With the imminent widespread use of DNA microarrays for identification of genetic differences between cells [24], it is critical to carefully examine the amount of variation within the applied methods to estimate the real differences in gene expression.

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