The contributions of normal variation and genetic background to mammalian gene expression

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Abstract

**Background:** Qualitative and quantitative variability in gene expression represents the substrate for external conditions to exert selective pressures for natural selection. Current technologies allow for some forms of genetic variation, such as DNA mutations and polymorphisms, to be determined accurately on a comprehensive scale. Other components of variability, such as stochastic events in cellular transcriptional and translational processes, are less well characterized. Although potentially important, the relative contributions of genomic versus epigenetic and stochastic factors to variation in gene expression have not been quantified in mammalian species.

**Results:** In this study we compared microarray-based measures of hepatic transcript abundance levels within and between five different strains of *Mus musculus*. Within each strain 23% to 44% of all genes exhibited statistically significant differences in expression between genetically identical individuals (positive false discovery rate of 10%). Genes functionally associated with cell growth, cytokine activity, amine metabolism, and ubiquitination were enriched in this group. Genetic divergence between individuals of different strains also contributed to transcript abundance level differences, but to a lesser extent than intra-strain variation, with approximately 3% of all genes exhibiting inter-strain expression differences.

**Conclusion:** These results indicate that although DNA sequence fixes boundaries for gene expression variability, there remain considerable latitudes of expression within these genome-defined limits that have the potential to influence phenotypes. The extent of normal or expected natural variability in gene expression may provide an additional level of phenotypic opportunity for natural selection.

Background

Biological entities such as individual cells, organs, and entire organisms display phenotypes that are simultaneously dictated and constrained by the composition of nucleic acids comprising their genomes. Differences in DNA sequence between individuals within the same species may produce qualitative and quantitative alterations in gene expression that influence biochemical processes conferring disease susceptibility and the beneficial or adverse responses to pharmacological intervention [1,2]. Thus, a critical component of
biomedicine centers on establishing the cause, extent and result of gene expression variability with an aim toward establishing pathological associations. To this end, the development of technologies such as DNA microarrays have allowed for quantitative assessments of transcriptional activity for thousands of genes simultaneously [3]. Microarray-based methods have been used to measure transcriptional variance in a variety of organisms, including yeast [4], flies [5], fish [6], mice [7], and men [8]; usually in the context of assessing the contribution of gene expression to phenotypic attributes of age, sex, strain, or disease. While the major component of phenotypic diversity within species is thought to be provided by combinations of heritable variations in DNA, it is readily apparent that individuals sharing nearly identical genomes, such as inbred mouse strains and monozygotic twins, may exhibit strikingly different characteristics [9,10].

To assess the extent and nature of gene expression variability both within populations of genetically identical individuals and between genetically heterogeneous individuals, we selected five strains of commonly used laboratory mice; inbred 129, Balb/c, and FVB, and outbred CD1 and CFW, isolated RNA from the livers of three males from each strain, and quantified transcript abundance levels by comparative hybridizations to cDNA microarrays.

Results

Mice bred for more than 60 generations should fix the vast majority (potentially all) of genetic contribution to variation [11], and thus individual mice within each inbred strain are considered genetically identical. We studied the liver in view of its important contribution to a wide variety of metabolic processes as well as practical considerations involving sample quantities and the ease of tissue procurement. To account for technical inconsistencies and facilitate comparisons within and between strains, each array hybridization used a common reference consisting of RNA combined from the liver, testes, and kidney of all mice used in the experiments. Two replicate arrays were performed for each individual mouse liver sample with each of two different fluorescent dyes to control for potential dye bias, thereby generating 4 replicate arrays per mouse and a total of 60 arrays.

We anticipated that three major sources of measurable variation in transcript levels would be represented in this dataset. The first involves the technical inconsistencies in experimental procedures and was assessed by the four replicate arrays performed for each mouse sample. The second source of variation is represented predominantly by intrinsic and extrinsic non-genetic factors influencing gene expression. This variance component was measured through the determination of transcript levels between mice of the same strain with identical genomes. All mice were matched for age, and were provided consistent diets and living environments. The third source of gene expression variability was expected to be driven by differences in DNA sequence or genome structure between the different mouse strains. This inter-strain variability was measured by determining transcript abundance levels between mice of different strains.

To identify genes whose transcript levels varied between genetically identical individuals, we first used an ANOVA model with a conservative assessment of significance [7]. This method yielded the following number of variable genes within each strain: 129, 37 genes; Balb/c, 36 genes; CD1, 26 genes; FVB, 21 genes; and CFW, 11 genes. Our previous study of liver gene expression in C57BL/6 mice identified 21 variable genes (0.8% of all genes assessed), indicating that the overall experimental results are quite consistent [7]. While this method identifies variable genes with high confidence, we concluded that the approach has a high rate of false negatives and is unduly restrictive when one is interested in assessing overall levels of variability rather than focusing on any particular gene product.

We next employed a less conservative strategy that involves controlling the positive false discovery rate (pFDR) [12]. We chose a level of acceptable false positives of 10% such that among the identified variable genes, about 10% probably do not actually vary. Separate analyses within each strain identified 554 (23%) genes exhibiting variability among individual 129 mice, 1,059 (44%) genes among Balb/c mice, 749 (31%) genes among CD1 mice, 610 (26%) genes among FVB mice, and 661 (28%) genes among the CFW mice (Table 1). In a joint analysis in which all strains were evaluated simultaneously, 1,876 genes (79%) varied within strain at a pFDR of 10% (see Materials and methods). Overall, mice in the Balb/c strain exhibited greater liver gene expression variability than mice from other strains. For a gene to be identified as variable, either the transcript level difference between individual mice is large, or the array variance - in this case the technical variability - is small. We specifically re-evaluated the array variability and did not identify a lower array variance in the Balb/c experiments.

Of the genes exhibiting variable expression levels within strains, 33 were variable within all 5 strains, and 154 were variable in 4 of the 5 strains (see Additional data file 2, supplemental Table 2). To determine how many genes are expected to be in common by chance if genes were chosen randomly, we undertook a simulation study with 50,000 datasets generated by randomly selecting groups of 554, 1,059, 749, 610, and 661 genes from the 5 strains for each data set and determined the number of genes represented in all 5 sets. The greatest number of genes in common, by chance alone, was 19, though typically fewer than 10 genes were found in common. This analysis indicates that the 33 genes identified in our study represent a highly significant level of overlap (p < 0.00002). Searches based on gene ontology (GO) classification indicated that genes associated with cell growth [GO:0008151], cytokine activity [GO:0005125], amine

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metabolism [GO:0009308], and the ubiquitin ligase complex [GO:0000151] were enriched among the genes with consistent intra-strain variation, when compared to the array as a whole. All genes showing significant inter-individual variability in our previous study of C57BL/6 mice [7] also varied in at least one strain analyzed in the current study. Moreover, genes previously found to exhibit substantial hepatic intra-strain variability, including Cish, Hhex, Cyp4a14, and Gadd45a, varied in at least three out of the five strains. Interestingly, four genes identified in the current study are involved in the ubiquitination process; Wsb1, Arih1, Cdc27, and Chordc1. This finding suggests the possibility that normal variability in protein degradation pathways could provide an additional level of global gene expression variability either through direct targeting of specific proteins or via a cascade of indirect effects influencing transcriptional regulation.

We next sought to assess the gene expression variability between different mouse strains. For this analysis we used an ANOVA model in which we considered the F ratio of mouse (between strain) to mouse (within strain) effect, where the significance of the F statistic is determined again by the pFDR (see Materials and methods). Using a pFDR of 10%, the analyses of individual mice identified 66 transcripts out of 2,382 (2.8%) that exhibited greater inter- than intra-strain variability (Figure 1a). Several transcripts exhibited substantial inter-strain variability, and we confirmed the microarray results for four of these genes by quantitative PCR (Figure 1b). Apolipoprotein A-IV (ApoA4) message levels varied 5.7-fold between mice of the 129 strain and mice of the FVB strain, a finding that confirms previous studies demonstrating a high level of expression variability for this gene [13,14]. ApoA4 is synthesized in the liver and intestine, and is a mediator of plasma lipid transport. Human studies have identified polymorphisms in the ApoA4 gene that associate with ApoA4 plasma levels, inter-individual variability in cholesterol levels, and risk of coronary heart disease [15]. The regulation of ApoA4 expression involves both transcriptional and post-transcriptional processes influenced by genetic variation in the gene itself [14]. Other genes exhibiting high inter-strain differences in expression levels encode proteins modulating cellular oxidative stress responses. These include NADPH oxidase 4 (Nox4), cytochrome p450 4a14 (CYP4a14), glutathione S transferase pi (Gstpi), peroxiredoxin 4, and ferritin light chain (Ftnl) (Figure 1a). Our results are consistent with previous studies that have demonstrated substantial mouse strain differences in basal iron status, ferritin levels and the potential for modulating oxidative hepatic stress. Immunoquantitation of total liver ferritin levels in four mouse strains determined a three- to fourfold difference between the SWR and C57BL/6 strains, with Balb/c and DBA/2 strains having levels between these extremes [16]. Our results identified the highest levels of Ftnl expression in CD1 mice, a strain not examined in the previous report. This study and our previous work each determined that stress-response genes exhibit substantial individual or within-strain variability [7]. Thus, these inter-strain measurements represent a level of consistency superimposed on the underlying gene expression variability. The specific reason for the high representation of the stress-response genes in these experiments has not been determined. A rapid physiological response to the process of CO2-induced death could be contributory. If so, then these results indicate a robust strain-dependent physiological difference in the response to sacrifice. Alternatively, these gene expression patterns might reflect fundamental differences between strains relating to the generation and control of oxidative stress that could correlate with differences in lifespan and disease susceptibility.

To determine if the intra- and inter-strain gene expression measurements were reproducible, we examined selected genes in 3 additional mice from each strain, all males between 68 and 72 days old. To ensure that inconsistencies in RNA isolation and cDNA synthesis procedures were not contributing to variance, we resected the livers from the additional mice, divided each liver into four sections, and separately isolated RNA from each portion. We used quantitative RT-PCR to measure transcript levels for three genes that varied between strains but were stable within strain (Cth, ApoA4, Dnase2a), two genes that varied within strain (Cish and Socs2), and one gene that was stable both within and between strains (S16). The results demonstrated highly reproducible intra-individual measurements in the RNA samples isolated from the same mouse (standard deviation ± 0.4-fold), a result that indicates minimal technical variation associated with RNA preparations (Figure 2). Concordant with the results

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**Table 1**

**Variable genes within strains**

|                | 129     | Balb/c  | CD1     | FVB     | CFW     |
|----------------|---------|---------|---------|---------|---------|
| Total variable genes* | 554(23%) | 1,059(44%) | 749(31%) | 610(26%) | 661(28%) |
| >1.5-fold difference | 240(10%) | 371(16%) | 384(16%) | 255(11%) | 280(12%) |
| >2.0-fold difference | 67(2.8%) | 68(2.9%) | 61(2.6%) | 64(2.7%) | 74(3.1%) |
| >3.0-fold difference | 13(0.5%) | 11(0.5%) | 7(0.3%) | 8(0.3%) | 19(0.8%) |

*At a pFDR of 10%
### Table 1

| Gene                                | HUGO Accession | Q-val | Δ fold |
|-------------------------------------|----------------|-------|--------|
| Cystathione gamma-lyase*            | AI427530       | 0.00  | 3.2    |
| Deoxyribonuclease II*               | AI666549       | 0.07  | 5.1    |
| Deleted in polyposis 1              | AI324241       | 0.07  | 1.6    |
| Apolipoprotein A4*                  | AI326922       | 0.07  | 4.2    |
| AMP deaminase 2                     | AI450899       | 0.07  | 4.0    |
| Ferritin light chain 1              | AI449517       | 0.07  | 1.8    |
| NADPH oxidase 4                     | AI452077       | 0.07  | 2.4    |
| Aldo-keto reductase*                | AI451194       | 0.07  | 1.5    |
| Cytochrome P450, 4a14               | AI885721       | 0.07  | 3.6    |
| Insulin induced gene 2              | AI893426       | 0.07  | 3.5    |
| T-complex testis expressed 1        | AI413228       | 0.07  | 2.1    |
| Immune associated nucleotide 1      | AI465254       | 0.07  | 2.7    |
| Membrane-spanning 4-dom A1         | AI413394       | 0.08  | 2.0    |
| Four jointed box 1                  | AI465262       | 0.08  | 2.2    |
| Neurogranin                         | AI327212       | 0.08  | 2.8    |
| Stromal cell derived factor 1       | AI326818       | 0.08  | 1.3    |
| SAM decarboxylase 1                 | AI528734       | 0.08  | 2.5    |
| HLA class II antigen E beta        | AI324640       | 0.08  | 2.1    |
| Acylphosphatase 2                   | AI323599       | 0.08  | 2.2    |
| Elov of long chain fatty acids 6    | AI327338       | 0.08  | 2.2    |
| Cyclin D1                           | AI894115       | 0.08  | 4.1    |
| Cyclin D1                           | AI894115       | 0.08  | 4.1    |

*These genes were confirmed by quantitative PCR.

### Figure 1

(a) Heatmap showing gene expression levels across different mouse strains.

(b) Bar charts showing fold differences for Dnase2a and Apoa4.

(c) Bar charts showing fold differences for Pfk2 and other genes.

Legend: Balb, CD1, FVB, CFW.
from the original mice, Cth exhibited a low level of intra-

strain variability, but was expressed approximately fourfold

lower in the FVB and CFW strains (Figure 2a). Measurements

of ApoA4 and Dnase2a expression were also highly concord-

ant with the original results (see Additional data file 2, sup-

plemental Figure 1a,b). CisH and Secs2 expression again

exhibited substantial within-strain variability, with measure-

ments differing by up to 50-fold between Balb/c mice (Figure

2b; Additional data file 2, supplemental Figure 1c), while S16

expression remained quite stable across individuals and

strains (Figure 2c).

We anticipated that transcripts expressed differentially

between mouse strains would primarily reflect heritable dif-

ferences in strain genomes. As such, mice sharing a common

ancestry might be expected to exhibit similar variability in

gene expression compared to more distantly related mice.

Supporting this concept are studies in humans showing less

variability in lymphocyte transcript levels between identical
ts twins relative to siblings, and siblings relative to unrelated

individuals [8]. We performed hierarchical clustering on the

subset of genes exhibiting significant inter-strain differences

and arranged the different mouse strains according to their

similarities in expression profiles (Figure 3). Clustering based

on the entire list of genes, either unweighted or weighted by

F-values, produced similar results (data not shown). Compar-

ing this expression-based dendrogram with the known phylo-

genetic relationship of these strains supports a genetic basis

for a component of the expression variability [17].

To assess the effects of pooling on the ability to characterize

inter-strain variation in liver gene expression, we performed

a separate microarray analysis using RNA samples combined

from the three mice of each strain. Four replicate arrays were

hybridized for each of the five pooled strain samples with two

replicates in each dye orientation. We identified 374 genes

(about 15% of all genes included in the analysis) that varied

significantly between strains using a pFDR of 10%. Of the 66

genes exhibiting significant inter-strain variability deter-

mined from analyses of individual mice, 60 were evaluable in

this experiment and 41 (68%) were also determined to be var-

iable in the analysis of pooled samples. These results indicate

that a large portion of genes presumed to be variable between

mouse strains and representing potential genetic determin-

ants of quantitative phenotypic traits are actually quite noisy

among individuals. This conclusion is easily visualized by

plotting the transcript levels for individual mice and for

pooled mice (Figure 4a). Genes, such as Cystathione gamma-

lyase (Cth), that vary significantly in both the individual and

pooled analyses showed relatively steady expression within

each strain (Figure 4b), while genes that vary significantly

only in the pooled analysis, such as CisH, tend to have high

intra-strain variance (Figure 4c). This result emphasizes that

for many genes the intra-strain or within-genotype variation

is large, and a single pool of a small number of mice will not

accurately reflect the population mean for the most variable

genes.

Discussion

Comprehensive studies of gene expression in model organ-

isms such as Saccharomyces and Drosophila have delineated

the contributions of age, sex, and genotype to corresponding

variations in transcript levels. However, the size constraints

of these species necessitates the use of sample pools

composed of hundreds to millions of discrete organisms, an

approach that eliminates the ability to assess variability at

the level of the individual. In contrast, assessing the relationships

between the genome and gene expression variability in

humans is hampered by the inability to precisely control the

multitude of environmental influences that profoundly influ-

ence gene expression in qualitative and quantitative ways. In

this context, the mouse represents a useful model system

highly suited for establishing that component of variability

that is independent of diversity directly encoded in the

genome. Measurements of intra-strain gene expression levels

reflect the allowable latitudes of gene expression in any single

individual in a fixed environment at a given point in time. The

inter-strain measurements reflect the additional contribution

of heterogeneity at the level of the genome.

Based on the analyses of transcript levels in individual mice,

we found the greatest contribution to overall gene expression

variability occurred among genetically identical individuals:

23% to 44% of all genes exhibited measurable variation,

depending on strain (see Additional data file 2, supplemental

Figure 3). Substantially less variance was attributable to

genome differences between strains (about 2.8%). Few stud-

ies assessing natural gene expression variability in mamma-

lian species that might provide a context for these findings

References

[1] C.S. Pritchard et al., “An identification of inter-strain variation in liver gene expression in the C57BL/6J, C57BL/10ScN, C57BL/6ScN, and Balb/c mouse strains,” Genome Biology 2006, Volume 7, Issue 3, Article R26.
have been reported. Analyses of transcript levels in skeletal muscle between five mouse strains found greater inter-strain than intra-strain differences. [18]. This suggests that muscle tissue exhibits a narrow range of normal variation relative to liver. However, the study design in which two mice per strain and two microarrays per mouse were compared provides substantially less statistical power to detect differences within strain. Interestingly, concordant with the findings reported here, Balb/c mice demonstrated the greatest level of intra-strain variation. A comparative analysis of mRNA abundance levels in the hippocampus of mice from 8 mouse strains identified more than 200 genes with significant strain differences using very stringent statistical criteria [19]. The experimental design involved tissue pooled from six mice of each strain, rather than individual mice. This pooling strategy was apparently based in part on the results of a prior microarray study indicating that transcript levels of genes expressed in the hippocampus of genetically identical mice were quite similar with only about 0.1% of all transcripts called differentially expressed [20]. It is possible that there is lower inter-individual variability in hippocampus than in liver. However, this previous study directly compared only pairs of mice in a head-to-head fashion, and the criteria for differential expression were based on a 1.7-fold change in abundance level, and not on statistical criteria.

Overall, we found that the expression of most hepatic genes in mice housed in standard 'steady-state' laboratory vivarium conditions is similar between individuals of the same or different strain. However, the transcript levels of a sizeable minority varied substantially. The proportion of genes exhibiting significantly variable expression between individual fish (18%) [6], yeast strains (24%) [4], and fly genotypes (25%) [5] is similar to that observed here between individual mice (23% to 44%). Analyses of gene expression in human tissues have also shown considerable variability between individuals. Importantly, substantial contributions to this variation cannot be attributed to genotypic differences between subjects [8,21,22]. Comparisons of transcript and protein levels between humans and non-human primates identified significantly greater variation among the human subjects than
between humans and chimpanzees [23], a finding further supporting the conclusion that a sizeable component of transcript abundance measurements reflects non-genomic variation.

There are several possible contributors to the gene expression variability observed in genetically identical individuals. Technical factors include subclinical disease states, unrecognized differences in environments and diet, or heterogeneity in the cell-type compositions of the analyzed tissues. We attempted to precisely control environmental and handling effects during the design of this study, and we did not observe any histological differences in the cellular composition of livers within or between strains. The ideal experiment would assess temporal variation in tissue transcript levels within an individual mouse, but in the case of liver gene expression these measurements would be confounded by changes resulting from repeated tissue biopsies. Importantly, our analyses of separate liver samples acquired from the same mouse yielded highly concordant transcript measurements.

One component of inter-individual variability could be represented by stochastic events or noise. Recently, gene expression measurements at the level of the single cell have provided direct experimental evidence of quantifiable contributions of stochastic biochemical noise to phenotypic variation in isogenic populations [24,25]. The end-result of this component of variability has long been appreciated through studies of developmental processes that revealed requirements for feed-back amplifications of initial asymmetrical noise for cell fate determination [26].

A second potential contributor to individual differences in gene expression centers on epigenetic regulation. Methylation of cytosine residues in the CpG islands of gene promoters and the covalent modifications of histones represent two important epigenetic modifications that influence gene transcription. Recent studies emphasize the importance of these regulatory mechanisms for dictating phenotypes in individuals with minimal divergence in genome sequence. A provocative report by Rakyan et al. [27] determined that the penetrance of the highly variable kinkya-tail phenotype found in the well-studied Axin-fused (AxinFv) mouse strain correlated with the differential methylation of a retrotransposon within AxinFv. Importantly, the methylation state of the retrotranspon was inherited transgenerationally after both maternal and paternal transmission, and was influenced by strain background. Striking differences in DNA methylation and histone acetylation have been observed in identical twins with increasing ‘epigenetic drift’ associated with advanced age [28]. Similar age-related epigenetic shifts have been reported in mice [29]. In the studies reported here, we found several genes that exhibited high variability in more than one strain, suggesting that certain genomic loci may be prone to imprecise regulatory control.

Conclusion

In the context of complex multicellular organisms, the end-result of phenotypic diversity in the setting of a fixed genome has long been appreciated. Toxicology studies have repeatedly shown differing susceptibilities to drug effects, such as carcinogen-induced tumor promotion within isogenic mouse strains [30]. Genetically identical animals aged under tightly controlled environments exhibit wide ranges in lifespans [31]. Indeed, the seeming incongruity between genetic homogeneity and phenotypic variability was recognized more than 40 years ago [32]. Importantly, the magnitude of gene expression variability measured in this study suggests either a tolerance for wide abundance ranges of certain transcripts, or potentially an organismal advantage for maintaining a state of gene expression variability offering an additional level of phenotypic opportunity for natural selection.

Materials and methods

Animal work and RNA preparation

Mice were purchased from Charles River Laboratories (Wilmington, MA, USA), maintained in a barrier facility and cared for in accordance with an approved Animal Care and Use Committee (IACUC) protocol. All mice were between 68 and 73 days old and were housed in identical environments with the same diet (Harlan Teklad 8664), constant temperature (20 to 22°C), and consistent light and dark cycles (controlled photoperiod of 12 hour light/12 hour dark). Water was provided ad libitum. Three male mice were sacrificed from each of the following strains (nomenclature in italics is used throughout this paper): 129S4 (129), Balb/cAnNcrlBr (Balb/c), Crl:CD-1®(ICR)Br (Crl), FVB/NcrlBr (FVB), and Crl:CFW®(SW)Br (CFW); CFW is sometimes referred to as ‘Swiss Webster’. Each mouse was brought individually into a separate room for sacrifice and killed in a CO₂ chamber. The liver, left kidney, and left testis were removed from each mouse and immediately snap-frozen in liquid nitrogen. Care was taken to ensure that the minimum amount of time elapsed from the sacrifice of the first mouse to the last. Total
RNA was extracted from the tissue using the TRIzol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. For an RNA reference standard, equal quantities of total RNA were combined from all three organs of all the mice. This same reference RNA was used on every array to standardize comparisons between arrays. For

**Figure 4**
Comparison of transcript levels exhibiting inter-strain variability determined by analyses of individual samples and pooled samples. (a) Examples of genes that demonstrated significant inter-strain variance after pooling the RNA from three mice of each strain. The relative expression values are shown for the three mice individually (1, 2, 3) and for the three mice pooled (P). Note that the individual and pooled results are data from independent hybridizations. Q-values are listed for both the individual mouse experiment (q1) and the pooled mouse experiment (q2), and values less than 0.1 are shaded gray to indicate statistical significance. Genes with low intra-strain variability (stable within strain) were statistically significant in both the individual and pooled experiments, while genes that had large intra-strain variability (noisy within strain) were significant only in the pooled experiment. Asterisks denote genes that were verified by quantitative PCR. (b) Genes such as Cth were relatively stable within strain. (c) Genes such as Cish were relatively noisy within strain. Error bars indicate standard deviations of four microarray experiments.

*These genes were confirmed by quantitative real-time RT-PCR.
confirmation studies, 3 additional male mice of each strain, ages 68 to 72 days, were processed in a similar manner except that the liver was divided into 4 sections before snap-freezing.

**Microarray construction, probe generation, and data collection**
Each microarray comprised 5,285 mouse cDNAs obtained from the Research Genetics’ sequence-verified set of IMAGE clones (Research Genetics, Invitrogen Corporation, Carlsbad, CA, USA). All cDNA clones used for array construction were sequence verified and annotated accordingly. Clone inserts were amplified by PCR, purified, verified by gel electrophoresis and spotted onto polylysine-coated glass microscope slides using a GeneMachines (San Carlos, CA, USA) robotic spotter as described previously [7]. cDNA probes were generated from 50 µg of total RNA in a reaction volume of 30 µl containing oligo(dT) primer/0.2 mM amino acid-dUTP (Sigma-Aldrich, St Louis, MO, USA)/0.3 mM dTTP/0.5 mM each dATP, dGTP, and dCTP/380 units of Superscript II reverse transcriptase (Life Technologies). The purified cDNA was combined with either Cy3 or Cy5 monoreactive fluorochedamines (Amersham Pharmacia) that covalently couple to the cDNA-incorporated aminoallyl linker in the presence of 50 mM NaHCO₃ (pH 9.0). The experimental and reference probes were combined and competitively hybridized to microarrays under a coverslip in a volume of 24 µl for 16 h at 63°C. Slides were washed in graded sodium chloride/sodium citrate buffer (SSC, 1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) and spun dry. Array images were collected for the Cy3 and Cy5 emissions using a GenePix 4000A fluorescent scanner (Axon Instruments, Foster City, CA, USA). The image data were extracted and analyzed using GENEPIX 3.0 microarray analysis software (Axon Instruments).

**Data analysis**
For each array spot, the intensity levels of the two fluorophores were obtained by subtracting median background intensity from median foreground intensity. A gene was only considered expressed if the fluorescence intensity of the corresponding spot was at least six foreground pixels greater than four standard deviations above background on every array. For each gene, the logarithm base 2 ratios (referred to henceforth as log₂ ratios) of the two channels were calculated to quantify to relative expression levels between the experimental and reference samples. To allow for inter-array comparisons, each array was normalized to remove systemic sources of variation. This normalization was accomplished by means of a print-tip-specific intensity-based normalization method. [33]. A scatter-plot smoother, which uses robust locally linear fits, was applied to capture the dependence of the log ratios on overall log-spot intensities. The log ratios were normalized by subtracting the fitted values based on the print-tip-specific scatter-plot smoother from the log ratios of experimental and control channels. Examination of the spread of the normalized log ratios via boxplots indicated no systemic variation due to any experimental variable such as different batches of arrays or RNA preparations. Therefore, no scale adjustment was performed on the arrays before combining data across samples.

The expression of genes that vary among mice within each strain was evaluated using an ANOVA model (Pritchard et al. [7]). Here, an F-value with degrees of freedom 2 and 8 was used to assess the variability of mouse variance within each strain.

To identify genes that varied among strains of mice, a nested mixed effects ANOVA model was used. Specifically, the model is written as:

\[ y = \text{overall mean} + \text{dye} + \text{strain} + \text{mouse within strain} \]

where \( y \) is the normalized log₂ ratio and the mouse within strain is a random effect. Treating the mouse as a random effect basically assumes that the three mice have been randomly selected from a ‘infinite’ mouse population of that strain and its observed effect for a particular mouse is an observation of a random variable. Specifically, the F test statistic is:

\[ F = \frac{\text{msStrain}}{\text{msMouse(Strain)}} \]

where

\[ \text{msStrain} = \frac{12 \sum \sum (\bar{Y}_{ij} - \bar{Y})^{2}}{4} \]

\[ \text{msMouse(Strain)} = \frac{4 \sum \sum (\bar{Y}_{ij} - \bar{Y}_{j})^{2}}{10} \]

where \( ij \) indexes the \( i \)th mouse for the \( j \)strain, and \( \bar{Y}_{ij} \), \( \bar{Y}_{j} \), and \( \bar{Y} \) are the means of normalized ratios for the \( i \)th mouse in the \( j \)th strain, all mice in \( j \)th strain, and over all strains, respectively. An F-value with degrees of freedom 4 and 10 for each gene is used to assess how variable the gene is among strains. An ANOVA table for this analysis is provided in Additional data file 2 (supplemental Table 1). To examine a possible dependence of statistical significance and signal intensity, we plotted the F-values versus the log₂ (intensity_{Cy3} / intensity_{Cy5}). There was no dependence on intensity for the significant genes either within strain or between strains (see Additional data file 2, supplemental Figure 2). The significance of these F-values was determined through estimating the pFDR, which is the proportion of falsely rejected hypotheses among the rejected hypotheses for pre-selected critical values [12]. As the overall goal here is to assess how genes vary among and within strains, it is natural to control the proportion of falsely rejected hypotheses among the rejected
Quantitative RT-PCR

Quantitative PCR was performed using SYBR GREEN as a reporter as previously described [7]. Total RNA from each mouse liver was treated with DnaseI, purified using a Rneasy Minikit (Qiagen, Valencia, CA, USA), and 20 µg was used to generate cDNA for PCR reactions. Primers to ribosomal protein S16 were used to normalize for cDNA loading. The normalized log ratios, F-values, q-values, and mean squares for the 2,382 genes assessed in the unpooled analysis are included in Additional data file 1. In addition, information about the microarray used in this study and the unprocessed gpr files may be obtained through the ArrayExpress website at the European Bioinformatics Institute [34]. The accession number is: A-MEXP-320.

Acknowledgements

We thank Barbara Trask and Catherine Peichel for critical reviews of this work and for helpful suggestions. We thank the microarray facility at the Fred Hutchinson Cancer Research Center. This work was supported by NIH grant DK65204, CA84294 and CA85859. CP was supported by a Poncin Scholarship and a Molecular Training Program in Cancer Research Fellowship (T32 CA09437).

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