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High resolution mapping of expression QTLs in heterogeneous stock mice in multiple tissues

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A proportion of the genetic variants underlying complex phenotypes do so through their effects on gene expression, so an important challenge in complex trait analysis is to discover the genetic basis for the variation in transcript abundance. So far, the potential of mapping both quantitative trait loci (QTLs) and expression quantitative trait loci (eQTLs) in rodents has been limited by the low mapping resolution inherent in crosses between inbred strains. We provide a megabase resolution map of thousands of eQTLs in hippocampus, lung, and liver samples from heterogeneous stock (HS) mice in which 843 QTLs have also been mapped at megabase resolution. We exploit dense mouse SNP data to show that artifacts due to allele-specific hybridization occur in ~30% of the cis-acting eQTLs and, by comparison with exon expression data, we show that alternative splicing of the 3' end of the genes accounts for ~1% of cis-acting eQTLs. Approximately one third of cis-acting eQTLs and one half of trans-acting eQTLs are tissue specific. We have created an important systems biology resource for the genetic analysis of complex traits in a key model organism.

[Supplemental material is available online at www.genome.org and at http://gscan.well.ox.ac.uk. The expression data from this study have been submitted to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) under accession nos. E-MTAB-86 and E-MTAB-88.]
collaborative cross (Valdar et al. 2006a). Due to improvements in high-throughput genotyping and methods of analysis, these populations can now be used in whole genome association studies.

3) Exclude artifactual explanations for eQTLs. Single-nucleotide polymorphisms (SNPs) can have a significant impact on microarray-based assays of transcript abundance (Alberts et al. 2007; Walter et al. 2007; Benovoy et al. 2008). Analysis of data from Affymetrix arrays on 30 recombinant mouse inbred lines (Bystrykh et al. 2005) indicated that almost half of the reported 100 most significant cis-eQTLs could be attributed to sequence diversity in probe regions (Alberts et al. 2007). The recently released high density SNP data for mouse inbred strains (Frazer et al. 2007; Yang et al. 2007) now make it possible to identify potential false cis-eQTLs.

Here we provide a comprehensive resource for integrating genetics and transcriptional profiling by mapping transcriptional abundance in hippocampus, liver, and lung in genetically heterogeneous stock (HS) mice, descended from eight inbred progenitor strains (AJ, AKR/J, BALB/cJ, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J, and LP/J) (Valdar et al. 2006b). The same animals have been used to map 843 phenotypic QTLs for 97 phenotypes, mapped into an average 95% confidence interval of 2.8 Mb (Valdar et al. 2006b). Many of these QTLs contain a small number of genes and will provide a powerful resource for exploring the relationship between variation in gene expression and quantitative phenotypic variation. All analyses are made available by our genome browser http://gscan.well.ox.ac.uk.

Results

We analyzed gene expression in hippocampus, liver, and lung using Illumina arrays with 47,430 oligonucleotides that assess expression of 21,288 Ensembl annotated transcripts, representing 19,687 genes. For the hippocampus we used 460 HS brains and also measured expression in the eight HS founders with both Illumina arrays and Affymetrix exon arrays. For the liver and lung a subset of 260 animals and the HS founders were tested with Illumina arrays only. The HS tissues were taken from animals that had been previously genotyped with 13,459 markers and phenotyped for 97 traits (Valdar et al. 2006b).

Genome scans were performed to map the eQTLs for all transcripts and tissues on to build 37 of the mouse genome. Because a transcript generally has more than one eQTL, and because different combinations of eQTLs may explain the variation in a transcript equally well, we mapped eQTLs using resample-based model averaging (described in the Supplemental Methods). We measured the robustness of the support for an eQTL by the resample-based model inclusion probability (RMIP), a generalization of the bootstrap posterior probability (BPP) we have described earlier (Valdar et al. 2006b). This measures the fraction of times the eQTL is included in a multiple-eQTL model in repeated subsamples of the data (Valdar et al. 2006b). From simulation of QTLs explaining 5% of the phenotypic variance, a detected eQTL that exceeds a RMIP threshold of 0.5 will be true in 85% of cases, and in 70% of cases for a threshold of 0.25 (Valdar et al. 2006b). At a RMIP threshold of 0.25, about one false positive eQTL occurs every four genome scans; no false positives are detected at a RMIP of 1.0. Unless otherwise stated, we consider only eQTLs with RMIPs $\geq 0.25$.

eQTLs were categorized as either cis or trans depending on whether an eQTL peak was either less or more than 2 Mb from the midpoint of its cognate transcript (in the HS, linkage disequilibrium measured by the mean $r^2$ falls to \(<0.5\) within 2 Mb and is \(<0.2\) within 8 Mb) (Valdar et al. 2006b). The relationship between the number of eQTLs detected and distance from the cognate transcript is shown in Supplemental Figure 1. The number of eQTLs detected depends on the RMIP threshold applied. In the hippocampus, at the most stringent RMIP value of 1.0, we detected 2732 cis-eQTLs and 205 trans-eQTLs; at the lower RMIP value of 0.25 there are 3961 cis-eQTLs and 4886 trans-eQTLs (Fig. 1).

It is important to appreciate that, while the RMIP measures the robustness of the eQTL detection, it is not a measure of the effect size of the eQTL. This is shown by the lack of correlation with the percentage of variance explained (which is itself highly correlated with the ANOVA \(-\log_{10}\) of the $P$-value $[\log P \approx 0.974];$ Fig. 2). The median effect size of all cis-eQTLs with RMIP $\geq 0.25$ is 20.83% (mean 26.35%) and 15.19% (mean 14.86%) for trans-eQTLs; 43 trans-eQTLs and 775 cis-eQTLs had effect sizes in excess of 50%.

Many large effect cis-eQTLs are due to SNPs within the target sequence

The large effect of many cis-eQTLs suggests that they may be due to highly penetrant mutations, which might in turn aid the discovery of the causative sequence variant. However, it is also possible that, as others have observed, sequence polymorphisms within the probe sequences are causing artifactual cis-eQTLs (Alberts et al. 2007; Walter et al. 2007). The extensive SNP data sets available for the mouse make it possible to investigate the contribution of SNPs to cis-eQTLs (Frazer et al. 2007; Yang et al. 2007).

In the hippocampus, 694 cis-eQTLs with RMIP $\geq 0.25$ contain an annotated SNP (17% of the total). There is a highly significant enrichment of SNPs in large effect cis-eQTLs: Figure 3 shows the distribution of logPs for cis-eQTLs in the hippocampus detected.
by probes containing annotated SNPs, compared to cis-eQTLs with no known SNP. The presence of SNPs correlates significantly with logP values ($P < 10^{-16}$). About 20% of cis-eQTLs with a logP between 10 and 20 (which constitute 8.3% of all cis-eQTLs) contain an annotated central SNP; more than half of cis-eQTLs with a logP $>50$ do so (2.9% of all cis-eQTLs). This suggests that many of the cis-eQTLs we have detected may be in part due to differences in hybridization efficiency.

An alternative explanation is that loci dense in SNPs are also dense in cis-eQTLs, and so a SNP within a probe reflects the presence of other SNPs nearby, some of which affect gene expression. However, Figure 3 shows that there is an important difference between eQTLs with SNPs in the center of the probe (and which are more likely to affect hybridization) and probes with SNPs in the terminal regions (within the last two base pairs at either end of the probe). A $\chi^2$ test in which eQTLs containing SNPs were classified by logP (low: logP $<10$; medium: $10 < $ logP $< 20$; high: logP $> 20$) and by SNP (central or terminal) was significant at $P < 4 \times 10^{-5}$. On average, while every central SNP adds 7.5 units (s.e. $\pm 0.5$) to the logP, every terminal SNP increases it by 0.4 (s.e. $\pm 1.4$; not significant). This suggests that SNPs are causing the variation in signal, because we would not expect probes with centrally located SNPs to be distributed differently across the genome from probes with terminally located SNPs.

We confirmed experimentally whether SNPs within probes were contributing to differential hybridization by performing quantitative PCR (qPCR). We chose 10 transcripts that contained two or more central annotated SNPs and for which mapping indicated the sole source of variation was attributable to a large cis-eQTL (percent variation $>50$). We designed primers to amplify the same exon as that detected by the Illumina array, using primers that hybridized to DNA without annotated SNPs. We then measured transcript abundance in the eight inbred progenitor strains by qPCR and by using Illumina arrays. The results are given in Table 1. Only two of the 10 transcripts show significant strain variation by qPCR.

Figure 2. Relationship between the RMIP and effect size (expressed as percentage of the variation in transcript level [%var]) for cis- and trans-eQTLs.

Figure 3. Effect of sequence variants on cis-eQTL detection. The frequency (vertical axis) of logP (negative logarithm of the $P$-value) scores (horizontal axis) for cis-acting eQTLs is shown. Data for cis-eQTLs with no annotated variant (solid line) are compared with data for cis-eQTLs with terminal variants (a SNP in the first two or last base pairs of the probe sequence) and with central variants (defined as any SNP that is not a terminal variant).

We estimated the fraction of unannotated SNPs in probe sets by sequencing 45 probes (without annotated SNPs), randomly selected from three ranges of logPs. We found six central SNPs in 17 probes with logPs $>20$, three in 12 probes with cis-eQTLs with logPs between 10 and 20 and no variants in 16 probes with cis-eQTLs with logPs $<10$. Logistic regression shows the relationship between logP and the presence of a novel SNP is significant ($P < 0.05$) and that every increase of 10 logP units ups the log odds of a SNP by 0.13 ($\pm 0.07$). Applying this model to the logPs of the remaining unannotated cis-eQTLs, we estimate that a further 16% (95% confidence interval: 9%–31%) would contain SNPs. Including both known and unknown SNPs, ~30% of cis-eQTLs are expected to contain a SNP.

Since some of the cis-eQTLs at transcripts containing annotated SNPs reflect true variation in transcript abundance, we incorporated all cis-eQTL results into our web-based interface to the phenotypic QTLs (http://gscan.well.ox.ac.uk). We indicate whether the probe contains an annotated SNP or not by a difference in color (red for SNPs, black for no known SNPs).

Few cis-eQTLs are due to splicing variation

The existence of many large effect cis-eQTLs, where some alleles correspond to very low or no expression and other alleles produce high levels of expression, was surprising. For example, in the hippocampus, after leaving out cis-eQTLs with annotated SNPs there are 288 with effects $>50$, including 20 with effects $>80%$. Since we measured gene expression using 3‘-based expression arrays, there is a possibility that some of the large effect cis-eQTLs may be attributable to allele-specific alternative splicing of the 3‘ end of the genes (Kwan et al. 2008), while the variation in expression of other parts of the genes are low.

To estimate the contribution of splicing variation to all cis-eQTLs (not just the largest), we took advantage of the derivation of the HS animals from inbred progenitor strains. We first obtained
Table 1. Comparison between microarray-based assays of transcript variation in inbred strains and quantitative PCR analyses for 10 large effect cis-eQTLs with annotated SNPs

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Gene</th>
<th>cis-logP</th>
<th>Array logP</th>
<th>qPCR logP</th>
<th>Annotated SNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>scl0075423.2_59-S</td>
<td>At5a</td>
<td>214.72</td>
<td>2.695</td>
<td>0.130</td>
<td>2</td>
</tr>
<tr>
<td>scl013590.4_108-S</td>
<td>Pyc2</td>
<td>87.02</td>
<td>2.057</td>
<td>2.495</td>
<td>2</td>
</tr>
<tr>
<td>scl22226.9_81-S</td>
<td>Nud6</td>
<td>188.97</td>
<td>5.370</td>
<td>0.004</td>
<td>2</td>
</tr>
<tr>
<td>scl20141.21_29-S</td>
<td>Pygb</td>
<td>76.66</td>
<td>7.495</td>
<td>0.126</td>
<td>2</td>
</tr>
<tr>
<td>scl18572.5_283-S</td>
<td>Rbbp9</td>
<td>75.89</td>
<td>2.858</td>
<td>0.381</td>
<td>3</td>
</tr>
<tr>
<td>scl15961.15_3-S</td>
<td>Uap1</td>
<td>141.1</td>
<td>4.433</td>
<td>0.056</td>
<td>4</td>
</tr>
<tr>
<td>scl15766.4_27-S</td>
<td>Nenf</td>
<td>58.89</td>
<td>2.936</td>
<td>0.017</td>
<td>2</td>
</tr>
<tr>
<td>scl23602.11_383-S</td>
<td>N eccentric2p</td>
<td>100.66</td>
<td>5.767</td>
<td>2.195</td>
<td>3</td>
</tr>
<tr>
<td>scl22909_7_136-S</td>
<td>Them4</td>
<td>82.46</td>
<td>2.332</td>
<td>0.425</td>
<td>2</td>
</tr>
</tbody>
</table>

Results shown are negative logarithms of the P-value for the ANOVA for the Illumina microarray analysis of the inbred strains (Array logP) and for the qPCR analyses of the inbred strains (qPCR logP). The logP of the cis-eQTLs found in the HS is shown (cis-logP), and the last column gives the number of annotated SNPs.

a conservative estimate of the extent (though not the frequency) of isoform variation in the HS from that measured in the eight progenitors in three replicate experiments. Using Affymetrix exon arrays consisting of 216,129 probe sets, (each probe set representing a single exon, and in total representing 15,620 unique genes) we looked for exons with patterns of strain-specific expression in the hippocampus that differed significantly from the overall pattern of strain expression for the gene. After removing 23,499 probe sets with annotated SNPs (Frazer et al. 2007), there was evidence for isoform variation at 1985 genes at a false discovery rate of 0.05.

Since a proportion of the isoform variation detected by the array is likely to be due to unannotated polymorphisms in the probe-target sequences (Benovoy et al. 2008), we looked for differences in the hybridization of individual oligonucleotides within a probe set that do not follow the overall pattern of strain differences for that probe set (this is equivalent to looking for an interaction between oligonucleotide and strain in an ANOVA of the probe set). However, a significant interaction can be due to anything that differentially affects hybridization of one oligonucleotide in the probe set, not just a SNP, so this is a conservative way of assessing the contribution of sequence variants.

We identified 234 genes in which the probe sets’ ANOVA interaction test was not significant (P-value of >0.05) and that were also predicted to show splicing variation between strains. These splice isoforms could not be explained as artifacts of annotated SNPs. Out of the 3225 cis-eQTLs in the hippocampus with RMIP >0.25 and without annotated SNPs, 47 intersected with this set. However, the Illumina probe, used to detect the cis-eQTL, overlapped the exon probe set in only 20 of those cases, meaning that in 27 cases the alternative splicing event could not be causing the expression difference detected by the Illumina assay. This limits the fraction of alternative splicing events causing expression eQTLs to 20/3225, or 0.6%.

To test these cases we designed specific primers for the 20 candidate splicing variants coinciding with eQTLs. Using quantitative real-time PCR we detected a significant interaction between exon and strain in eight cases. In two genes (Gna13 and Tbc1d24) we observed two products in the melt curves from RNA isolated from different inbred strains, suggesting that there was an additional intron not annotated in Ensemble, an interpretation supported by finding different PCR product sizes when we used primers to amplify the spanning exons of predicted splice isoforms (Fig. 4).

In summary, our results indicate that large variation in alternative splicing may explain only a minority of cis-eQTLs that we have detected (e.g., Gna13 and Tbc1d24, Fig. 4). However, our data also show that large variation in alternative splicing, which could contribute to the phenotypic QTL, could be missed by 3’-based expression arrays (e.g., Soat1 and Zscan21; Fig. 4).

Tissue specificity of eQTLs

We compared gene expression in the hippocampus, liver, and lung in 260 mice, excluding the additional 200 hippocampus samples. The number of transcripts with expression levels significantly above negative control probes differed between the three tissues: 12,966 for liver, 19,243 for lung, and 20,743 for hippocampus. These numbers were not reflected in the proportion of heritable transcripts in the three tissues: 4198 transcripts had heritabilities >10% in liver, 7990 in lung, and 2661 in the hippocampus. Thus, while there are many more detectable transcripts in the hippocampus, heritable variation is most common in the lung.

As expected from tissue differences in transcript heritability, there is only modest correlation between the eQTLs in different tissues. Figure 1 shows the number of eQTLs detected at RMIPs from 0.1 to 1 after removing probes with annotated SNPs. In order to provide estimates of tissue specificity in eQTL detection, in Table 2 we show the number of eQTLs (without annotated SNPs) detected at all RMIP thresholds from 0.1 to 1. For a given RMIP threshold, an eQTL is said to be tissue specific if the RMIP exceeds that threshold in one tissue and is zero in other tissues. By this definition approximately one third of cis-acting eQTLs and one half of trans-acting eQTLs are tissue specific. For cis-eQTLs that are expressed in two tissues, the mean difference between the effect size (expressed as percentage of the variation explained) is 3.3% (standard deviation of 15.7).

Tissues differ in the number of eQTLs: most were detected in the lung, which at an RMIP of ≥0.1 contains >11,000 compared with 8770 in the hippocampus and 8400 in the liver (Fig. 1). However, this summary obscures a difference in the prevalence of cis- and trans-eQTLs. At RMIPs between 0.1 and 0.7 every tissue has an excess of trans-eQTLs, but the ratio of cis to trans varies significantly between tissues: In the hippocampus trans-eQTLs are twice as common as cis-, and more than five times more common in the liver (the lung is intermediate). cis-eQTLs that contain SNPs (not included in Table 2) are less tissue specific than cis-eQTLwithout SNPs, suggesting that tissue-specific gene expression has been underreported. Table 3 shows that eQTLs not containing SNPs are three times more likely to be found in a single tissue rather than in all three, while the numbers of eQTLs with SNPs are approximately equal, whether they occur in one or all three tissues (ratio 0.85).

Mapping resolution

The mean 95% confidence intervals for cis-eQTLs is 2.45 Mb (s.d. 1.50) and for trans-eQTLs 3.75 (s.d. 1.31). Figure 5 shows the relationship between resolution and logP: For every increase in 10 logP units resolution decreases by ~0.76 Mb for cis-eQTLs and by ~0.70 Mb for trans-eQTLs. The mean number of genes in the cis-eQTL and trans-eQTLs 95% intervals is 40.7 and 47.1, respectively. In Supplemental Table 1 we provide a list of genes that lie within eQTLs containing five or fewer genes.
Discussion

We have provided a high resolution map of thousands of expression QTLs in HS mice; on average cis-eQTLs are mapped into 2.45 Mb 95% confidence intervals and trans-eQTLs into 3.75 Mb intervals. We observed a remarkable degree of tissue specificity: Approximately one third of cis-acting eQTLs and one half of trans-acting eQTLs are tissue specific (Table 2). We find that trans-eQTLs outnumber cis-eQTLs.

To interpret our results, it is important to appreciate the way we analyze the HS mice, a structured population which contains animals of differing degrees of genetic relatedness. We report evidence for the existence of an eQTL using a resample-based model inclusion probability, the RMIP. The ANOVA logPs from the association analysis, which in other designs would provide a robust measure of statistical significance, do not distinguish ghost peaks (that arise from genotype correlations) from real peaks. If we relied solely on logPs and single locus associations, we would detect many apparently large effect trans-eQTLs that were in fact ghosts of the true cis-effects.

For phenotypes where there is a single true peak, the RMIP is more likely to be correlated with the logP value (and effect size). We found more single large effect (high logP) cis-than trans-eQTL and as a result RMIPs close or equal to one are more common among the cis-eQTLs. This explains the pattern in Figure 1, where there are fewer cis-eQTLs than trans-eQTLs with RMIPs $<0.6$.

Our results raise a number of concerns about using eQTL data. The first concerns the validity of cis-eQTLs. We were able to use high density SNP data for mouse inbred strains (Frazer et al. 2007; Yang et al. 2007) to identify potential false cis-eQTLs. We found that, among cis-eQTLs with a logP $>20$, $>40\%$ contain a known polymorphism in the target sequence. Furthermore, from sequencing we estimated the frequency of unannotated SNPs and conclude that overall $>30\%$ of cis-eQTLs contain a SNP.

For a number of reasons, it is unlikely that the increased variation in expression for probes containing SNPs can be attributed to additional nearby SNPs lying within functional elements (Guha-Thakurta et al. 2006). Critically, we find that SNPs lying within terminal regions of the probes do not have a significant effect on logPs, while SNPs lying within central regions of the oligonucleotide do; furthermore, the more variants within a probe, the higher the logP (Fig. 3). Testing large effect eQTLs that contain annotated SNPs failed to confirm expression variation in eight out of 10 cases. We also found that cis-eQTLs containing SNPs are less tissue specific than cis-eQTLs without SNPs, suggesting that tissue-specific gene expression has been underreported. cis-eQTLs that occur in multiple tissues and contain SNPs are extremely likely to be due, at least in part, to hybridization artifacts.

We find that splicing variation is not an important contributor to cis-eQTLs identified by 3′-based expression arrays, but we note that possible important variation in splicing patterns might be missed. Less than 1% of the cis-eQTLs in the hippocampus coincided with a gene predicted to have isoform differences between strains. Furthermore, where the Illumina and Affymetrix exon array probe sets interrogated the same exon, we validated less than half of the predicted isoforms. While we cannot rule out the possibility that alternative splicing occurs in some of those
The identification of genes at 95% of QTLs in the HS. Available at http://gscan.well.ox.ac.uk, could in principle lead to confidence intervals (Valdar et al. 2006b). Our results, freely phenotypic QTLs that contain at least one eQTL within their 95% that accelerate gene discovery are needed. We have identified 799 genes have been robustly identified (Flint et al. 2005), methods traits.

Consequently the two loci must coincide and, among the many genes that lie within the confidence interval of the phenotypic QTL, those involved in the phenotype should have transcripts whose abundance is in part under the control of a cis-eQTL. Since there are >2000 known QTLs in mice, but less than a hundred genes have been robustly identified (Flint et al. 2005), methods that accelerate gene discovery are needed. We have identified 799 phenotypic QTLs that contain at least one eQTL within their 95% confidence intervals (Valdar et al. 2006b). Our results, freely available at http://gscan.well.ox.ac.uk, could in principle lead to the identification of genes at 95% of QTLs in the HS.

By combining analysis of networks of gene expression covariation (Chen et al. 2008) and haplotype structure (Yalcin et al. 2005), our data will be useful for determining the extent to which, through their control over gene expression, eQTLs may reveal the presence of genes involved in quantitative phenotypes (Bystryk et al. 2005; Mehrabian et al. 2005; Schadt et al. 2005). In its simplest formulation, the causal link between sequence variant, gene expression, and phenotype arises because the sequence variant is responsible for both a cis-eQTL and the QTL contributing to a quantitative phenotype. The two loci must coincide and, among the many genes that lie within the confidence interval of the phenotypic QTL, those involved in the phenotype should have transcripts whose abundance is in part under the control of a cis-eQTL. Since there are >2000 known QTLs in mice, but less than a hundred genes have been robustly identified (Flint et al. 2005), methods that accelerate gene discovery are needed. We have identified 799 phenotypic QTLs that contain at least one eQTL within their 95% confidence intervals (Valdar et al. 2006b). Our results, freely available at http://gscan.well.ox.ac.uk, could in principle lead to the identification of genes at 95% of QTLs in the HS.

Methods

Tissue preparation and RNA extraction

Tissue from HS animals was snap-frozen in liquid nitrogen. Whole brains were cut in half sagittally and the left hemisphere soaked into RNAlater-Ice (Ambion) for 20 h at −20°C. The hippocampus was dissected for RNA extraction by using TissueLyser and RNeasy Lipid Tissue Kit (Qiagen). Tissue samples (25 mg for liver and lung down to a few milligrams for hippocampus) were homogenized using 5-mm stainless steel beads (Qiagen) on a Tissue Lyser (Retech MM300 Mixer Mill) for 10 min at 25 Hz. Total RNA was then extracted using the RNeasy 96 Universal Tissue Kit (Qiagen) for liver and lung and the RNeasy Lipid Tissue Kit (Qiagen) for hippocampus according to the manufacturer’s instructions. RNA quantity and integrity were assessed using a NanoDrop ND-1000 Spectrophotometer and an Agilent 2100 Bioanalyzer. Total RNA samples with RNA Integrity Number >9 were used for messenger transcription was carried out at 37°C for 14 h during which biotinylated UTPs (75 mM Biotin-16-UTP, Ambion) were used for RNA labeling. Messenger RNA quantity and size were determined in the same way as total RNA using a NanoDrop ND-1000 Spectrophotometer and an Agilent 2100 Bioanalyzer. Size ranged from 250 to 5500 nucleotides (nt) with a peak centered at 1000–1500 nt.

Arrays hybridization, washing, and scanning

For the Illumina arrays, 1.5 mg of labeled messenger RNA was hybridized to Illumina Sentrix mouse-6 expression beadchips. After 17-h hybridization at 55°C, beadchips were washed according to recommended protocols from Illumina. FluoroLink Cy3-labeled Streptavidin (Amersham Biosciences) was used to detect expression signals. The BeadStation 500 G system was then used to scan the beadchips. For the exon arrays a GeneChip Fluidics Station 450/250 was used to wash and stain the Mouse Exon 1.0 ST Array. Quality control was carried out using Affymetrix Expression Console Software Version 1.0.

Microarray expression data handling

The Illumina Mouse WG-6 v1 BeadArray contains 47,429 unique probe sequences. These were mapped to Build 37 (mm9) using the alignment tool BLAT (Kent 2002) and perfect and unique matches were retained. This resulted in 30,029 probe sequences matched to a genomic location pertaining to 19,688 and 21,289 unique Ensembl gene and transcript identifiers, respectively.

Data generated from scanning were imported into Illumina BeadStudio version 3.0 to generate background subtracted signal values for each bead type. Positive signals were defined to be probes with signals in excess of the mean of the negative control probes +2 standard deviations, giving the thresholds of hippocampus 3.58, liver 3.77, lung 3.92. Processed data were then exported to the R statistical computing language, transformed, and normalized using the BioConductor package, vsn (Huber et al. 2002), and analyzed for differential expression using the limma

| Table 2. Tissue specificity of cis-eQTLs at different RMIP thresholds, for 260 HS mice assayed for gene expression in hippocampus, liver, and lung |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| **Cis** | **Tissue** | **0.1** | **0.2** | **0.3** | **0.4** | **0.5** | **0.6** | **0.7** | **0.8** | **0.9** | **1** |
| Hippocampus | 1085 | 1086 | 1084 | 1077 | 1070 | 1050 | 1024 | 973 | 906 | 693 |
| Liver | 419 | 419 | 417 | 417 | 415 | 405 | 365 | 325 | 290 | 184 |
| Lung | 817 | 813 | 810 | 802 | 789 | 767 | 734 | 682 | 615 | 459 |
| Hippocampus liver | 96 | 94 | 94 | 92 | 92 | 83 | 79 | 79 | 61 | 30 |
| Hippocampus lung | 536 | 534 | 531 | 531 | 528 | 517 | 491 | 456 | 405 | 159 |
| Liver lung | 134 | 134 | 133 | 128 | 124 | 121 | 109 | 86 | 70 | 56 |
| Hippocampus liver lung | 303 | 303 | 302 | 299 | 294 | 286 | 277 | 268 | 237 | 209 |

The numbers are counts of the cis-eQTL found only in the listed tissues (e.g., “liver” means only found in liver, “liver lung” means only found in liver and lung, and not in hippocampus).

Table 3. Tissue specificity of cis-eQTLs according to the presence or absence of annotated SNPs

<table>
<thead>
<tr>
<th><strong>No. of tissues</strong></th>
<th><strong>cis-eQTLs with SNPs</strong></th>
<th><strong>cis-eQTLs without SNPs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>511</td>
<td>3054</td>
</tr>
<tr>
<td>2</td>
<td>532</td>
<td>1828</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>1011</td>
</tr>
</tbody>
</table>

Numbers in the first column refer to the number of tissues in which a cis-eQTL is found. cis-eQTLs that contain SNPs are less tissue-specific than cis-eQTLs without SNPs.
Candidate exons for alternative splicing were detected by testing for significant differences in probe set signal between different strains after controlling for the gene-level differences. This was done with ANOVA to compare between two linear models that predict the observed expression level of each probe set by the gene level or by both the gene level and the strain type. An estimated local false discovery rate value (as implemented in R in the “fdrtool” package) was assigned to each probe set, expressing the probability of not being differentially expressed.

To test for SNP effects, ANOVA was performed separately within each probe set, modeling the intensity $y_{iks}$ observed in replicate $r$, oligonucleotide $k$ ($k = 1 \ldots 4$) and strain $s$ as $y_{iks} = \mu + \alpha_s + \beta_k + \gamma_i + \theta_{ks} + \epsilon_{iks}$. Evidence for a hybridization artifact affecting a particular combination of oligonucleotide probe and strain is indicated by the contribution of the interaction term $\theta_{ks}$ being significant in an ANOVA.

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