Microarray gene expression analysis of the Fob3b obesity QTL identifies positional candidate gene Sqle and perturbed cholesterol and glycolysis pathways

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Stylianou, Ioannis M., Michael Clinton, Peter D. Keightley, Clare Pritchard, Zuzanna Tymowska-Lalanne, Lutz Bünger, and Simon Horvat. Microarray gene expression analysis of the Fob3b obesity QTL identifies positional candidate gene Sqle and perturbed cholesterol and glycolysis pathways. Physiol Genomics 20: 224–232, 2005. First published December 14, 2004; doi:10.1152/physiolgenomics.00183.2004.—Obesity-related diseases are poised to become the primary cause of death in developed nations. While a number of monogenic causes of obesity have recently been identified, these are responsible for only a small proportion of human cases of obesity. Quantitative trait locus (QTL) studies using animal models have revealed hundreds of potential loci that affect obesity; however, few have been further analyzed beyond the original QTL scan. We previously mapped four QTL in an F2 between divergently selected Fat (F) and Lean (L) lines. A QTL of large effect on chromosome 15 (Fob3) was subsequently mapped to a higher resolution into two smaller-effect QTL (Fob3a and Fob3b) using crosses between the F-line and a congenic line containing L-line alleles at the Fob3b-QTL region. Here we report the gene expression characterization of Fob3b. Microarray expression analysis using the NIA-NIH 15K cDNA array set containing 14,938 mouse ESTs was employed to identify candidate genes and pathways that are differentially expressed between the F-line and a congenic line containing L-line alleles at the Fob3b QTL region. Several other cholesterol biosynthesis pathway genes unlinked to Fob3b were found to be differentially expressed, suggesting that a perturbation of this pathway could be in part responsible for the phenotypic difference between the F-line and Fob3b-line mice.

A polygenic mouse obesity model has been developed in Edinburgh by divergent selective breeding that has resulted in strains differing substantially in fat content (5, 19). The mice were selected for high-fat (Fat, F-line) or low-fat (Lean, L-line) content from a genetically highly variable base population for more than 60 generations (19), resulting in lines that differ more than fivefold in fat content and have 22% (F-line) or 4% (L-line) body fat. The lines have subsequently been inbred (5). These lines, originating from a classic selection experiment on fat content, therefore represent a resource for genetic dissection of QTL responsible for the observed selection response. The phenotypic difference most likely arose via selection of several loci of low-to-medium effect, rather than a few single-gene mutations of large effect, and so the genetics of obesity in these lines model the situation in humans, where a large proportion of obesity cases are thought to have a polygenic basis (21).

A genome-wide QTL analysis using the outbred F- and L-lines identified four QTL regions for fat content that were significant at a genome-wide level (8). Further research (6) indicated that variation in the Lep (chromosome Chr 4) and Lepr (Chr 6) genes had not contributed to the selection response. We have since further mapped and phenotypically characterized the Fob3b QTL on Chr 15, dissecting it into two smaller-effect QTL, Fob3a and Fob3b (22). In this study, we report the use of the NIA-NIH 15K cDNA array set to identify candidate genes and pathways that are differentially expressed between the parental F-line and a congenic line containing the Fob3b QTL region from the L-line (Fob3b-line). The additive effect of the QTL in the Fob3b-line peaks at 68 cM (P value = 0.006), having an absolute effect of 0.71% ± 0.26 SD on percentage of body fat (Fat%) (22).

Selection lines and microarrays have been used previously to identify candidate genes for quantitative traits (26). Similarly, the use of congenic strains and microarrays in rats has led to the successful identification of Cd36 as an insulin resistance gene (1). Here we report the application of congenic mouse lines derived from selection lines, combined with the application of high-throughput gene expression analysis. Candidate genes are identified by examining differences in gene expression for loci in the QTL region and by statistical tests of groups of genes in pathways to circumvent the problem of multiple statistical testing of expressed sequence tags (ESTs). This analysis highlighted the cholesterol biosynthesis and glycolysis gene pathways as differentially expressed between the F- and congenic Fob3b-lines.
**MATERIAL AND METHODS**

**Mouse Lines**

For a recent review on the original divergently selected F- and L-lines, see Bünger and Hill (5) and Bünger et al. (6). The generation of a congenic line encompassing the Fob3b QTL region has been described previously in Stylianou et al. (22), and it is named the Fob3b-D congenic line; here this line is referred to as Fob3b-line for simplicity. The Fob3b-line has L-line alleles between D15Mit184 and D15Mit107 on Chr15 (43.1 cm), and the rest of the genome is of the F-line background (~99.2%), introduced via seven generations of recurrent backcrossing.

**Mouse Tissue Collection**

Mice were fed ad libitum Rat and Mouse No. 3 diet [Special Diet Services, Essex, UK; digestible crude (dc) oil, 3.8%; dc protein, 20.2%; starches, 33.9%; sugars, 4.4%; digestible energy, 13.8 MJ/kg] (Special Diets Services, Essex, UK; digestible crude (dc) oil, 3.8%; dc protein, 20.2%; starches, 33.9%; sugars, 4.4%; digestible energy, 13.8 MJ/kg)) from weaning onward and maintained with controlled lighting (12:12 h light-dark cycle) at a temperature of 21 ± 2°C. Mice were weaned at 21 days of age and kept in sex-matched groups in plastic cages. All procedures were authorized and approved by the Home Office (UK) under the Animals (Scientific Procedures) Act 1986. Only female mice from the three lines (F-, L-, and Fob3b-lines) were used in this study. From weaning onward, mice were maintained on the same rack, and cages were randomized in their position on the rack after each cage change to minimize localized environmental effects.

At 98 days of age (±1 day), mice were transferred into individually labeled cages with water but no food for 3 h at 0800. If >10 mice were to be dissected in 1 day, the first 10 would be isolated at 0800 and next 10 at 0900, thus maintaining a 3-h isolation period. For each line, a total of 10 mice from 3 litters were dissected, and the order in which litters were dissected was randomized. These procedures attempted to standardize gene expression differences that might arise between mice because of prekill eating. Each mouse was transported individually from the stock room to the dissecting room and then processed, typically within 5 min. Mice were cervically dislocated and decapitated, and tissues were removed, one at a time. All tissues were transferred to 2-ml Eppendorf tubes, immediately snap-frozen in liquid nitrogen, and then stored at −80°C. All nondisposable dissecting instruments were autoclaved before use, and all disposable instruments used were sterile.

**RNA Isolation**

RNA extraction was carried out using TRIzol reagent following the manufacturer’s guidelines (Invitrogen, Paisley, UK). Briefly, samples were homogenized, using a Polytron homogenizer (PT100, Kinematically Polytron), for 30 s to 2 min depending on tissue type and volume. This was carried out in “batches” appropriate to the downstream pairwise comparisons. RNA samples were quantified by measuring absorbance at 260 and 280 nm (GeneQuant; Amersham Biosciences). Total RNA (10 μg) was loaded onto 1% denaturing formamide agarose gels, and integrity of RNA was examined by ethidium bromide staining. Gels were capillary transferred onto nylon membrane and fixed by UV light. Each Northern blot membrane was prerattled in 50 mM sodium phosphate (pH 7.2) and prehybridized for 1 h at 65°C in 20 ml of 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 100 mg/ml denatured salmon sperm DNA, and 25 μg/ml denatured rRNA. Hybridization was carried out in glass bottles rotating in a Biometra O/V5 (Goettingen, Germany) hybridization oven. Washed membranes were exposed overnight at −80°C to Kodak BIOMAX-MS film (Amersham Biosciences). Where signals were weak, exposure time was extended accordingly.

**Data Analysis**

The raw data files have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/?acc=GSE1558; series GSE1558, platform GPL1341–1342, arrays GSM26714–26733).

The significance of differential expression in liver and BAT between the F- and Fob3b-line was assessed for all 14,938 ESTs by use of a mixed model procedure coded in SAS (SAS Institute, Cary, NC). This was done for all four experiments, that is, liver cDNAs on the first and second array sets and BAT cDNAs on the first and second array sets. The mixed model is adapted from Kerr and Churchill (10) and is processed in two steps, based on the procedures described by Wolfinger et al. (27). The full model is given in Eq. 1:

\[ Y_{adgips} = \mu + A_s + D_d + P_p + A_D_d + A_D_l + L_L_g + A_G_g + D_G_g + P_G_p + S_A_S + \varepsilon_{adgips} \]  

where \( Y_{adgips} \) is the normalized log2 background-corrected measure-ment for any EST g; \( \mu \) represents the average background-corrected signal across all the factors in the experiment; D is a fixed dye effect (d: Cy3, Cy5); P is a random pool effect (p: 1 or 2); A is a random effect for any array a; I is a random effect for any grid (i: 1–48) on an
array; AD is the dye-by-array interaction; and ADI is the dye-by-array-by-grid interaction. Gene (G) specific interactions include the line (L)-by-gene interaction (LG), where L is the fixed line effect (F: F-line or Fob3b-line); the array-by-gene interaction (AG); the dye-by-gene interaction (DG); the pool-by-gene interaction (PG); and the spot-by-array-by-gene interaction (SAG). e is the stochastic error. Equation 1 is broken down into two components. The first component is a global normalization to remove array, dye, pool, and grid effects using an analysis over all genes (Eq. 2).

\[ Y_{\text{adip}} = \mu + A + D + P + AD + ADI + e_{\text{adip}} \] (2)

The residuals from step 1 (\(R_{\text{adip}}\)) are obtained by subtracting the fitted values for the effects from the \(Y_{\text{adip}}\) values. The next component of Eq. 1 is an EST-specific ANOVA (Eq. 3) using the residuals obtained from Eq. 2, allowing a different variance for each gene. All the effects here are indexed by g and hence are EST-specific effects (as opposed to the global effects modeled in Eq. 2).

\[ R_{\text{adg}} = L_g + A_g + D_g + P_g + SA_g + e_{\text{adg}} \] (3)

SA interaction is introduced to account for the double spotting of each EST on each array; \(e_{\text{adg}}\) is the stochastic error for each EST. Because the goal is to assess the EST-specific line (L) effect, the estimates of primary interest are those of the \(L_g\) effects. Differences between these effects are tested by use of mixed model-based t-tests of all possible pairwise comparisons within an EST.

Filtering for Significant Differentially Expressed ESTs

ESTs were ranked by \(P\) values calculated from the mixed model analysis. For each array, 7,680 independent tests were carried out (1 slide set at a time), so the \(\alpha\) level was corrected with the Bonferroni adjustment to generate a conservative experiment-wide threshold, hence, 0.05/7,680, which reduces the \(\alpha\) for each test to 0.00000651 (6.51 \times 10^{-6}).

This experiment-wide threshold is extremely stringent and may result in many false negatives. To circumvent this problem, we employed Mann-Whitney U-tests to perform pairwise comparisons of the \(P\) values of groups of genes. The null hypothesis is that the sums of the ranks of the groups do not differ (allowing for differences in sample sizes). The pairs of exclusive sets of ESTs tested were within or outside specific pathways or within or outside the QTL 95% confidence interval (CI).

Real-Time Quantitative PCR

Real-time quantitative PCR (Q-PCR) was performed to confirm known genes identified as differentially expressed from the array analysis. Design of primers was carried out by use of Primer3 (16) software, available online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, using sequences that were selected from the Mouse Genome Sequencing Consortium Mouse Assembly (build 30). The rodent mispriming library was chosen as a constraint for improved primer design. Additionally, RNA samples were DNase treated with DNase-free (Ambion) according to the manufacturer’s instructions.

The First-Strand cDNA synthesis kit (Amersham Biosciences) was used for first-strand cDNA synthesis, using RNA pooled from five individual mice. SYBR Green Q-PCR was performed using a kit (Applied Biosystems, CA, USA) on an ABI Prism 7700 Sequence Detector (PerkinElmer) with accompanied software (Sequence Detector v1.6.3). Reactions were carried out on 96-well MicroAmp optical plates and caps (Applied Biosystems). For each reaction, four identical (systematic) replicates were carried out. For each gene examined, identical reactions were processed on the same 96-well plate with primers for the mouse \(\beta\)-actin gene to normalize for variation in loading between samples (\(\Delta\Delta C_t\)). For each gene, dilution comparisons were performed between the gene under investigation and the control (\(\beta\)-actin) to establish the working concentration.

RESULTS

In the microarray experiment, we compared expression profiles between the F- and congenic Fob3b-lines in liver and BAT for 14,938 mouse ESTs. We first analyzed ESTs on Chr 15, in particular ESTs that map to the Fob3b QTL region to identify positional candidate genes that show differential expression between the F- and Fob3b-lines. We next sought to identify perturbed pathways containing genes that map within or outside the Fob3b region. Finally, the expression differences of candidates identified as being differentially expressed in the microarray analysis were verified by the Northern or Q-PCR assays.

Analysis of Chr 15 and the Fob3b QTL Interval

Across the two slides, 145 different ESTs (67 on slide set 1 and 78 on slide set 2) mapping to the Fob3b 95% CI were spotted on the arrays (Fig. 1). The \(P\) values of these ESTs were compared with those outside the QTL region (of each slide set) using the Mann-Whitney U-test. The analysis shows little evidence that the ESTs in the 95% CI of the Fob3b QTL region.

Fig. 1. Differential expression of expressed sequence tags (ESTs) between the Fat (F) line and Fob3b-line in brown adipose tissue (BAT; solid diamonds) and liver (shaded triangles) on mouse chromosome (Chr) 15. ESTs are shown with map position on Chr 15.
Table 1. Mann-Whitney U-tests for subsets of ESTs in the QTL region and differentially expressed pathways

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Slide</th>
<th>N1</th>
<th>N2</th>
<th>U-Test</th>
<th>P Value (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>QTL region</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Liver</td>
<td>1</td>
<td>7.545</td>
<td>67</td>
<td>271.656</td>
<td>0.14</td>
</tr>
<tr>
<td>BAT</td>
<td>1</td>
<td>7.545</td>
<td>67</td>
<td>273.242</td>
<td>0.12</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>7.248</td>
<td>78</td>
<td>324.478</td>
<td>0.044</td>
</tr>
<tr>
<td>BAT</td>
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<td>7.248</td>
<td>78</td>
<td>288.440</td>
<td>0.92</td>
</tr>
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<td><strong>Cholesterol biosynthesis pathway</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
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<td>7.602</td>
<td>10</td>
<td>66.298</td>
<td>0.000004</td>
</tr>
<tr>
<td>BAT</td>
<td>1</td>
<td>7.602</td>
<td>10</td>
<td>56.486</td>
<td>0.0064</td>
</tr>
<tr>
<td>Liver</td>
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<td>7.524</td>
<td>2</td>
<td>8.268</td>
<td>0.76</td>
</tr>
<tr>
<td>BAT</td>
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<td>7.524</td>
<td>2</td>
<td>11.091</td>
<td>0.24</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>7.607</td>
<td>6</td>
<td>24.778</td>
<td>0.28</td>
</tr>
<tr>
<td>BAT</td>
<td>1</td>
<td>7.605</td>
<td>6</td>
<td>48.196</td>
<td>0.00012</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>7.322</td>
<td>4</td>
<td>24.357</td>
<td>0.017</td>
</tr>
<tr>
<td>BAT</td>
<td>2</td>
<td>7.322</td>
<td>4</td>
<td>19.498</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Glucoregenes pathway</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
<td>7.606</td>
<td>7</td>
<td>28.006</td>
<td>0.37</td>
</tr>
<tr>
<td>BAT</td>
<td>1</td>
<td>7.604</td>
<td>7</td>
<td>44.680</td>
<td>0.058</td>
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<tr>
<td>Liver</td>
<td>2</td>
<td>7.321</td>
<td>5</td>
<td>26.003</td>
<td>0.1</td>
</tr>
<tr>
<td>BAT</td>
<td>2</td>
<td>7.321</td>
<td>5</td>
<td>26.334</td>
<td>0.089</td>
</tr>
</tbody>
</table>

N1, no. of expressed sequence tags (ESTs) not involved in the test set; N2, no. of ESTs in the test set; U-test, Mann-Whitney U-test statistic; BAT, brown adipose tissue; QTL, quantitative trait locus.

have P values differing from all other ESTs; there is only weak evidence in liver: slide set 2 (P = 0.044; Table 1).

ESTs mapping to Chr 15 were ranked according to their evidence in liver: slide set 2 and known genes were examined for their involvement in known pathways at the Kyoto Encyclopaedia of Genes and Genomes (www.genome.ad.jp/kegg).

**Characterized genes.** Two ESTs corresponding to characterized Chr 15 genes were identified as potential candidates for further investigation. BG074498 corresponds to squalene transferase (Tst) and is downregulated in BAT of the F-line, relative to the Fob3b-line. BG077950 corresponds to squalene epoxidase (Sqle), ranks highly in both tissues, and is upregulated in the F-line (Table 2, top, and Fig. 1). Tst maps just distal to the Fob3b 95% CI, whereas Sqle maps within this interval, which, along with its differential expression and its involvement in the metabolic pathway for cholesterol biosynthesis, makes Sqle a strong positional candidate for Fob3b.

**Uncharacterized genes.** Four differentially expressed Chr 15 ESTs, BG081526, BG068787, BG063349, and BG066896, corresponding to uncharacterized genes were identified as having low P values. The highest ranking is BG081526; it has very low P values in both tissues examined (Table 2, bottom, and Fig. 1). BG066896 shows a low P value in BAT and has a sequence that maps 1,500 bp from BG081526, suggesting that it may be part of the same gene. BG081526 maps close to the proximal end of the Fob3b 95% CI (Fig. 1). Given that both BG063349 (low P value in BAT) and BG068787 (low P value in liver) map outside the Fob3b QTL region, these ESTs from unknown genes have not been further investigated within this study.

Analysis of Sqle and the Cholesterol Biosynthesis Pathway

Given that Sqle was found to be differentially expressed, mapping to the Fob3b region, and is involved in the cholesterol biosynthesis pathway, other genes of this pathway were examined. Of the 13 possible enzymes involved in converting hydroxymethylglutaryl-coenzyme A into cholesterol, 10 had ESTs that were spotted on the arrays, and 8 were upregulated in the F-line (P < 0.05, uncorrected) as summarized in Table 3.

A comparison of the ranks of the P values of the 10 cholesterol biosynthesis genes with the remaining genes in the

Table 3. Genes involved in the lipid sterol biosynthesis pathway assayed on the array (with P value) and not present on the array (no P value)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>GenBank Accession No.</th>
<th>Chr</th>
<th>Tissue</th>
<th>Log2 Ratio</th>
<th>SE</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fdf1l</strong></td>
<td>BG082232</td>
<td>BAT</td>
<td></td>
<td>-1.2</td>
<td>0.16</td>
<td>0.000021</td>
</tr>
<tr>
<td><strong>Lss</strong></td>
<td>BG079196</td>
<td>liver</td>
<td>2</td>
<td>-2.52</td>
<td>0.58</td>
<td>0.0005</td>
</tr>
<tr>
<td><strong>Sqle</strong></td>
<td>BG079795</td>
<td>BAT</td>
<td></td>
<td>-1.12</td>
<td>0.26</td>
<td>0.00055</td>
</tr>
<tr>
<td><strong>Hmgcr</strong></td>
<td>BG078816</td>
<td>BAT</td>
<td></td>
<td>-1.82</td>
<td>0.57</td>
<td>0.00057</td>
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<tr>
<td><strong>Sc5d</strong></td>
<td>BG079164</td>
<td>BAT</td>
<td></td>
<td>-2.74</td>
<td>0.95</td>
<td>0.011</td>
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<tr>
<td><strong>Cyp7b1</strong></td>
<td>BG081383</td>
<td>BAT</td>
<td></td>
<td>0.82</td>
<td>0.3</td>
<td>0.023</td>
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<tr>
<td><strong>Idi1</strong></td>
<td>BG079899</td>
<td>BAT</td>
<td></td>
<td>-1.61</td>
<td>0.74</td>
<td>0.044</td>
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<tr>
<td><strong>Mvd</strong></td>
<td>BG079503</td>
<td>BAT</td>
<td></td>
<td>-1.05</td>
<td>0.48</td>
<td>0.046</td>
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<td><strong>Ggps1</strong></td>
<td>BG066451</td>
<td>BAT</td>
<td></td>
<td>0.61</td>
<td>0.78</td>
<td>0.45</td>
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<td><strong>Dhcr7</strong></td>
<td>BG063343</td>
<td>BAT</td>
<td></td>
<td>-1.25</td>
<td>0.65</td>
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<td><strong>Mvk</strong></td>
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<td>BAT</td>
<td></td>
<td>-0.60</td>
<td>0.49</td>
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<td><strong>Pmvk</strong></td>
<td></td>
<td>BAT</td>
<td></td>
<td>0.5</td>
<td></td>
<td>X</td>
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</table>

**Fdf1l**, farnesyl diphasate farnesyl transferase; Lss, lanoster synthase (oxidosqualene cyclase); Sqle, squalene epoxidase; Hmgcr, hydroxymethylglutaryl-coenzyme A reductase; Sc5d, sterol-C5-desaturase; Cyp7b1, cytochrome P-450, family 7, subfamily b, polypeptide 1; Idi1, isopentenyl-diphosphate-Δ-1-isomerase; Mvd, mevalonate (diphasate) decarboxylase; Ggps1, geranylgeranyl-diphasate synthase-1; Dhcr7, 7-dehydrocholesterol reductase; Mvk, mevalonate kinase; Pmvk, phosphomvalonate kinase; Ehp, emopamil.

*Genes not significant in either BAT or liver.
gluconeogenesis. We carried out a Mann-Whitney
U-test (Table 1), which corrects for multiple comparisons.

Table 4. Genes assayed on the arrays belonging to the
glycolysis/gluconeogenesis pathway

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>GenBank Accession No.</th>
<th>Chr</th>
<th>Tissue</th>
<th>Log2 Ratio</th>
<th>SE</th>
<th>P Value</th>
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<tr>
<td>Eno1*</td>
<td>BG078409</td>
<td>2</td>
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<td>-0.97</td>
<td>0.11</td>
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<td></td>
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<td></td>
<td>liver</td>
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<td>0.08</td>
<td>0.37</td>
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<td>Gapd*</td>
<td>BG063729</td>
<td>8</td>
<td>BAT</td>
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<td>0.31</td>
<td>0.0021</td>
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<td>Gpi</td>
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<td>Pfk</td>
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<td>BAT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>liver</td>
<td>0.53</td>
<td>0.22</td>
<td>0.026</td>
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Glycolysis-specific genes

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Gluconeogenesis-specific genes

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Genes in bold are significant glycolysis genes (P < 0.05). *Unknown
locus; **Known locus.

Other Chr 15 Candidates

Previously, we reported a search for candidate genes based on
the flanking markers of the 95% CIs for the Fob3b QTL.

Q-PCR Analysis

Of the two potential candidate pathways identified, we
focused on the cholesterol biosynthesis pathway. The reasons
for this are that Sgce, a cholesterol biosynthesis enzyme, maps
within the Fob3b QTL region (Fig. 1), and that several other
pathway genes have small P values and large fold changes
(Table 3) and are all upregulated in the F-line. Consequently,
Q-PCR was performed on Sgce and Lss (which has both a small
P value and a large fold change). Additionally, analysis was
performed on the gene sterol regulatory-binding factor-2
(Srebf2), since no EST for this gene was spotted on the arrays,
and the gene is a transcriptional regulator of cholesterol bio-
synthesis genes (13, 17) that maps to Chr 15 (83.7 Mb), close
to Fob3b. The Q-PCR results confirm the differential expres-
sion of both Lss and Sgce, with almost identical fold changes as
those observed in the array analysis. In addition, a significant
differential expression of Srebf2 was detected (see Fig. 3A).

Confirmation of differential expression of the candidate
genes was firstly performed with pooled RNA. The F-, L-, and
congenic Fob3b-lines used are inbred, so averaging over pools
of data set, using the Mann-Whitney U-test (Table 1), provides
strong evidence that the ESTs in this pathway tend to show
lower P values (for both tissues; liver slide set 1, P =
0.0000040; BAT slide set 1, P = 0.0064). P values for the
second slide set are nonsignificant, presumably because there
are only two cholesterol biosynthesis genes in that set, provid-
ing little power (Table 1).

Analysis of Enolase 1: the Glycolysis/Gluconeogenesis
Pathway

An EST belonging to a characterized gene, Enolase 1
(Eno1), on Chr 2 had a very low P value, ranking as the 14th
most significant EST (P = 0.0000068, uncorrected). This gene
is involved in the glycolysis/gluconeogenesis pathway. Exam-
ination of ESTs matching the genes for enzymes in the glyco-
lisis and gluconeogenesis pathways revealed several points.
First, the two gluconeogenesis-specific genes spotted on the
arrays are not differentially expressed (P > 0.05; Table 4),
suggesting that gluconeogenesis might not be affected by
Fob3b. Second, 7 of the 10 genes coding for enzymes involved
in glycolysis are spotted on the array, and of these 6 are
differentially expressed in BAT (P < 0.05, uncorrected).
However, there is no differential expression in liver, the site
of gluconeogenesis. We carried out a Mann-Whitney U-test
to compare the ranks of the P values for glycolysis and gluco-

Fig. 2. Summary of the Fob3b quantitative trait locus (QTL) region and
positions (in Mb) of potential candidates and genetic markers. At left, thick
black line indicates F-line alleles, thin black line indicates Lean (L) line alleles,
and hatched area is unknown genotype in the congenic Fob3b-line. *Markers
that have not yet been mapped by the Mouse Genome Sequencing Consortium.
averses their environmental effects, and our statistical and methodological approach limits environmental variation. Therefore, the use of pools for confirmation of the array candidates in RNA-limiting tissues such as BAT and white adipose tissue (WAT) is robust. However, RNA from liver tissue was not limiting, and so further confirmation was performed using unpooled RNA from individual mice. RT-PCR was performed on four individuals for each line, each with three technical replicates for the genes shown. Q-PCR analysis indicated that BG081526 and BG066896 belong to the same transcription unit (data not shown). Hybridization of Northern blots with the BG081526 EST clone as a probe indicates that the transcript is ~8 kb in size, with a possible 7-kb-long alternative transcript seen in WAT (Fig. 4). Of 16 different tissues examined, expression was observed in all (except the pancreas), with varying degrees of abundance. Generally, BG081526 is downregulated in all L-line and Fob3b-line tissues compared with the F-line tissues, with the lowest amount of expression in L-line tissues. Time series Northern analysis at time points of 28, 49, and 98 days of age indicates an early upregulation of this gene in the F-line relative to both the Fob3b-line and the L-line, which is particularly noticeable in WAT (Fig. 4). Sequence analysis reveals two protein domains of unknown function (DEP domains), leading to the preliminary gene name for the BG081526-associated gene of Depdc6 (Dep domain containing 6). BLAST analysis (nucleotide and translated amino acid sequence) of the BG081526 sequence was performed against the genomic sequences of various species. Depdc6 appears to be a highly conserved gene, with orthologs in the human and rat genomes having 88 and 95% nucleotide similarity, respectively. There is also evidence of homologous sequences in species as diverse as fugu, zebrafish, and fruitfly, but not in the worm Caenorhabditis elegans.

**DISCUSSION**

The final goal of a QTL study is to identify the molecular basis for the QTL effect(s). In the present study, we focused on the Fob3b QTL by performing a microarray-based expression experiment with the aim of identifying possible positional candidates and metabolic pathways that may be involved in the Fob3b QTL. Microarray analysis enabled examination of the expression profiles of a large number of genes, providing data on 67% of all known transcripts in the Fob3b region and elsewhere in the genome. Although a large proportion of the genes in this region are represented on the arrays, the genes that are not represented may also be as important for the QTL effect. Our search for pathways may point to genes that are involved, but not represented on the arrays. Of the 14,938 different ESTs spotted on the arrays, a number of differentially expressed candidates were identified. Differentially expressed positional candidate genes as well as two perturbed metabolic pathways have been uncovered, providing essential information to speed up future positional cloning efforts of this QTL.

An ongoing issue related to microarray analysis is the criteria used to determine significance of differentially expressed genes. Under our analysis, each EST was examined in two tissues (liver and BAT), leading to 30,000 tests. Thirteen ESTs were found to meet the conservative Bonferroni experiment-wide 0.05 significance threshold; however, all of these are from uncharacterized genes. And 2,491 tests were significant at the nominal 0.05 threshold, a figure far higher than the ~1,500 expected under the null hypothesis (note, however, that this does not represent 2,491 different ESTs, since some were significant in both tissues). However, by grouping the ESTs into pathways and comparing
the ranks of their \( P \) values with a nonparametric test, we were able to show significant differential expression for these groups of genes, even though at the single gene level the \( P \) values do not meet the Bonferroni experiment-wide significance threshold.

Chow-based diets have been shown previously (11) to contain inconsistent proportions of fatty acids among batches. Although we have used a robust statistical analysis and have been able to replicate our results in multiple biological replicates, these potential inconsistencies could affect the gene expression of the metabolic genes in the pathways discussed here. Future analyses can examine the impact of different diets, and batches can be analyzed by gas chromatography for consistency of fatty acids.

**Sqle and the Cholesterol Biosynthesis Pathway Genes**

When all the genes known to be involved in the cholesterol biosynthesis pathway were examined, 8 of 10 that were spotted on the arrays were significant at the nominal level (\( P < 0.01 \)).

Given that *Sqle*, which maps within the Fob3b region, is also significantly differentially expressed at the nominal level, the evidence for this pathway being associated with this QTL is compelling. Interestingly, not all the genes were significantly differentially expressed in both tissues, which may indicate that the *Fob3b* action is different for some genes in this pathway in the two examined tissues.

*Sqle* catalyses the first oxygenation step in sterol biosynthesis and is thought to strongly influence the flux through the cholesterol biosynthesis pathway. Differences in cholesterol biosynthesis may be associated with the *Fob3b* obesity QTL in several ways. For instance, the causative genomic alteration(s) resulting in the *Fob3b* QTL effect (*Fob3b* allele for simplicity) may act to increase cholesterol biosynthesis in the F-line, resulting in a higher level of cholesterol deposition in adipose cells and increased adiposity (2, 12). Alternatively, the causative *Fob3b* allele may be acting on increased cholesterol biosynthesis in the F-line, which then acts as an activator of fatty acid synthesis that in turn results

### Table 5. Raw \( C_T \) values for real-time PCR performed on 4 congenic Fob3b-line individuals (1–4) and 4 F-line individuals (5–8), each with 3 technical replicates

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<th>Lss</th>
<th>Sqle</th>
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| F-line     |                     |        |     |      |                 |            |                                               |                    |                    |                    |
| 5          | 1                   | 24.28  | 23.86| 21.61| 20.69           | 5          | 3.79                                          | 3.24              | 0.73              |
|            | 2                   | 23.60  | 23.63| 20.94| 20.42           |            |                                               |                    |                    |                    |
|            | 3                   | 24.86  | 23.60| 21.00| 20.26           |            |                                               |                    |                    |                    |
| 6          | 1                   | 23.54  | 22.55| 19.48| 19.77           | 6          | 3.76                                          | 2.61              | −0.39             |
|            | 2                   | 23.83  | 22.62| 19.18| 19.75           |            |                                               |                    |                    |                    |
|            | 3                   | 23.37  | 22.12| 19.63| 19.95           |            |                                               |                    |                    |                    |
| 7          | 1                   | 30.10  | 28.70| 25.09| 24.91           | 7          | 4.90                                          | 2.40              | 0.58              |
|            | 2                   | 29.15  | 28.60| 25.54| 24.80           |            |                                               |                    |                    |                    |
|            | 3                   | 29.39  | 23.85| 25.05| 24.23           |            |                                               |                    |                    |                    |
| 8          | 1                   | 24.26  | 23.40| 20.14| 19.87           | 8          | 3.48                                          | 3.20              | 0.38              |
|            | 2                   | 23.76  | 23.17| 20.58| 20.10           |            |                                               |                    |                    |                    |
|            | 3                   | 22.53  | 23.15| 20.55| 20.15           |            |                                               |                    |                    |                    |

| Mean       | 3.98                | 2.86          | 0.32 |
| SD         | 0.63                | 0.42          | 0.50 |
| \( n \)    | 4                   | 4             | 4    |
| SE         | 0.31                | 0.21          | 0.25 |

| Mean \( \Delta C_T \) | 0.76                | 1.05          | 1.42 |
| Fold difference (F-line:Fob3b-line) | 1.69                | 2.07          | 2.67 |
| \( P \) value       | 0.070               | 0.0019        | 0.0087 |

| F-line, Fat line; \( C_T \), critical threshold; \( \Delta C_T \), difference in \( C_T \) relative to \( \beta\)-actin; \( \Delta \Delta C_T \), difference in \( \Delta C_T \) between strains. |
in increased obesity (3, 14). Finally, the target of the causative Fob3b allele that leads to obesity may be entirely independent of the cholesterol biosynthesis pathway, and yet the expression of the cholesterol biosynthesis genes are perturbed because they share a common transcription factor. Such a scenario may be demonstrated by the Srebf2 transcription factor, which has been shown to act on the cholesterol biosynthesis genes as well as on fatty acid synthase (Fasn) and stearoyl-coenzyme A desaturase 2 (Scd2), each of which have been shown to give rise to obesity independently (23).

Q-PCR on two genes from the cholesterol biosynthesis pathway, Sgle and Lss, confirmed the results obtained from the array analysis, in that Sgle and Lss were both upregulated in the F-line relative to Fob3b-line (and the L-line). Confirmation was performed using the original pooled samples used for array analysis, and on liver samples from four individual mice for each line. The transcription factor Srebf2 that was absent from the arrays, yet maps to Chr 15, was also investigated for its known regulator properties of the genes involved in cholesterol biosynthesis (13, 17). Differential expression of Srebf2 was observed, indicating that Srebf2 may be responsible for the gene expression differences observed in Sgle, Lss, and the accompanying cholesterol biosynthesis pathway genes.

Additional Differentially Expressed Candidates and Genes

Enol1 was identified as the most significant differentially expressed characterized gene, upregulated in BAT tissue of the F-line relative to the congenic Fob3b-line. Enol1 is involved in the glycolysis pathway. When other genes in the same pathway were examined, it was determined that four additional glycolysis genes (Gpi, Pfk, AldoA, and Tpi) were significant at the nominal level (P < 0.05), bringing the total to six of a possible eight that were spotted on the arrays. None of the eight genes was significantly different in liver tissue, indicating that this may be a pathway that is perturbed in a tissue-specific manner. The individual P values for each of the four genes, Gpi, Pfk, AldoA, and Tpi, are below the genome-wide significance threshold, so each would have been disregarded with the use of stringent selection thresholds; however, when considered together by a nonparametric test as part of the glycolysis pathway, the evidence for their differential expression is strengthened. Increased expression of the glycolysis pathway genes in the F-line could lead to a greater rate of production of the end product, acetyl-CoA, which is a precursor for both cholesterol biosynthesis and fatty acid biosynthesis.

In addition to identifying candidate pathways, a few uncharacterized ESTs were also identified and some verified with follow-up expression studies. We chose the BG081526 EST mapping close to the Fob3b interval on Chr 15 as the most promising uncharacterized candidate to confirm and further characterize, since it was highly differentially expressed in both liver and BAT.

Differential expression of the BG081526 EST, indicated by the array analysis, was confirmed by Northern analysis and shows that the BG081526 EST belongs to an 8-kb transcript whose gene is currently named Depdc6 (DEP domain containing 6). Depdc6 is upregulated in all F-line tissues relative to Fob3b- and L-line tissues, and the differences are apparent from an early stage (28 days). The reported 2 kb of sequence are entirely unique, having no similarity to any gene family, and are highly conserved, while 6 kb of the cDNA sequence remain unidentified. Full gene characterization and further analysis of Depdc6 are ongoing. Although Depdc6 may not be the prime candidate for Fob3b given its map position, it may be interesting to determine why this gene of unknown function is so highly differentially expressed in our lines; it may be that it is a downstream target of the causal Fob3b allele.

Given that, of all the differentially expressed cholesterol biosynthesis pathway genes, Sgle maps within the Fob3b QTL region, this makes Sgle a strong candidate for the Fob3b QTL. It is therefore possible that a genomic alteration of Sgle in the F-line results in an allele that is responsible for the QTL effect. However, given that the confidence interval of Fob3b is still relatively large (26 Mb), it could also be that other linked genes are causal for the Fob3b QTL effect and that expression of Sgle is somehow influenced by the action of this linked QTL. In addition, existence of multiple smaller-effect QTL (7) within the currently mapped Fob3b region resulting in a “cumulative” Fob3b effect cannot be ruled out. Further high-resolution genetic mapping studies combined with detailed expression (RNA and protein) analysis of cholesterol biosynthesis pathway, determination of Sgle polymorphisms between the lines, and possibly transgenic studies (i.e., allele replacement) are needed to finally identify the molecular cause of the Fob3b effect.

![Fig. 4. Time series Northern analysis of the unknown Chr 15 candidate Depdc6 on brown (BAT) and white adipose tissue (WAT). Each lane is loaded with 10 μg of RNA pooled from 5 individual females. F, F-line; Fob3b, congenic Fob3b-line; L, L-line. Bottom panels indicate the ethidium bromide staining for each gel. Probe used was derived from the IMAGE clone H2066B12 (GenBank accession nos.: BG081526 and BG068497).](http://physiolgenomics.physiology.org/)
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