Brief Genetics Report


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Obesity is one of the most serious threats to human health today. Although there is general agreement that environmental factors such as diet have largely caused the current obesity pandemic, the environmental changes have not affected all individuals equally. To model gene-by-environment interactions in a mouse model system, our group has generated an F16 advanced intercross line (AIL) from the SM/J and LG/J inbred strains. Half of our sample was fed a low-fat (15% energy from fat) diet while the other half was fed a high-fat (43% energy from fat) diet. The sample was assayed for a variety of obesity- and diabetes-related phenotypes such as growth rate, response to glucose challenge, organ and fat pad weights, and serum lipids and insulin. An examination in the F16 sample of eight adiposity quantitative trait loci previously identified in an F2 intercross of SM/J and LG/J mouse strains reveals locus-by-diet interactions for all previously mapped loci. Adip7, located on proximal chromosome 13, demonstrated the most interactions and therefore was selected for fine mapping with microsatellite markers. Three phenotypic traits, liver weight in male animals, serum insulin in male animals, and reproductive fat pad weight, show locus-by-diet interactions in the 127-kb region between markers D13Mit1 and D13Mit302. The phosphofructokinase (PFK) C (Pfkp) and the pitrilysin metalloprotease 1 (Pitrm1) genes are compelling positional candidate genes in this region that show coding sequence differences between the parental strains in functional domains. Diabetes 54:1863–1872, 2005

E stimates of the heritability of obesity in humans range as high as 70% based on twin studies (1). However, obesity in the developed world is increasing too rapidly to be caused by changes in genetic background (2). Moreover, some human genotypes are apparently resistant to an obesogenic environment and do not become obese (3). Genotype-by-environment interactions such as these have long been of concern to evolutionary biologists and have been commonly noted as important for evolutionary processes (4–6). Likewise, gene-by-diet interactions are known to influence obesity- and diabetes-related traits in humans (7–9).

The elucidation of the biochemistry of appetite control in mouse models generally depends on transgenic or knockout mice (7–8). Because of the complexity of the obese phenotype, it is unlikely that all gene-by-environment interactions will be discovered using these methods (9). Human studies examining gene-by-diet interactions rely on epidemiological data collected from large samples (10–12). While these and other studies make valuable contributions to our understanding of gene-by-diet interactions, large-scale human studies can be difficult due to unknown genetic lineages (13) as well as data collection biases (11).

With carefully controlled lineages and environments, samples derived from inbred mouse strains are amenable to powerful statistical dissection of complex genetic and environmental interactions (15). Crosses derived from the SM/J and LG/J mouse strains are particularly attractive for these types of studies. At 60 days of age, there is an ~24-g difference in weight between the two strains (16,17). Later experiments have demonstrated that this considerable weight difference is due to the many different genes of individually small effect (18). Furthermore, SM/J and LG/J respond differently to increased amounts of dietary fat (19,20).

To follow up on these earlier findings, we divided the 16th generation of an advanced intercross line (AIL) derived from SM/J and LG/J by diet, feeding half of the mice from each family a high-fat diet and the other half a low-fat diet. A quantitative genetic analysis of a number of obesity- and diabetes-related phenotypes, including growth rates, fat pad and organ weights, and serum levels of triglycerides, free fatty acids, cholesterol, and insulin,
demonstrated that most traits, including fat pad weights, liver weight, and serum insulin, were heritable and genetically correlated (T.H.E., unpublished observations). All of these traits show a low cross-environmental correlation, indicating that different genes or gene effects are influencing the traits on the two diets and that dietary responses for these phenotypes are themselves heritable and genetically correlated (T.H.E., unpublished observations). Genetic variation for obesity- and diabetes-related traits and their dietary responses were also encountered in a set of recombinant inbred strains formed from the same parental strains (21).

Cheverud et al. (22) have previously mapped eight adiposity loci in an F2 cross of SM/J and LG/J. The present study validates those QTLs in the F16 AIL and tests the current phenotypic trait suite for dietary response. One locus on chromosome 13 demonstrates a dietary response for a large number of traits. The region immediately around this locus has been fine mapped, and the results are presented here.

**RESEARCH DESIGN AND METHODS**

The sample used in this study is an F16 AIL (23) derived from SM/J and LG/J (WU:LG, SM-G14). The original SM/J (24) and LG/J (25) strains were selected before being killed (22). Therefore, QTLs that only mapped to one sex in the original study have been examined in both sexes in the present study.

Each animal was heterozygous at a given locus, according to the calculations previously derived equations (34) and our own software (35). Additive combination rates between a given locus and the flanking marker genotypes using the set developed for mouse gene mapping (26). Markers were chosen to map distances were calculated using the Mapmaker 3.0b program (27,28). Increased genetic recombination in an F16 generation results in an approximately eightfold expansion of chromosome map distances relative to an F2 generation when expressed in Haldane’s centimorgans (23), allowing for gene mapping on a much finer scale than is possible using F2 intercross design. Locations of microsatellite markers used in this study have been verified by examining the physical location of each marker in the Mouse Genome Database (www.ensembl.org). Genetic mapping data are available upon request.

**Statistical analysis**

**Replication.** A previous study by Cheverud et al. (22) detected eight QTLs affecting adiposity in an F2 cross of SMJ and LGJ through interval mapping (29). Adiposity was defined for the purposes of this study as the weight of the reproductive fatpad divided by the total body weight of the animal at time of necropsy (22). Replication of the adiposity QTL detected in the original study was tested in the present experimental sample in a manner similar to that described by Vaughn et al. (30). Briefly, a regression analysis is performed (31) with adiposity as defined in the original study as the dependent variable and genotype at each marker tested as the independent variable. Markers were selected on the basis of proximity to the original QTL (22) as well as proximity to adiposity QTLs detected in the F2 AIL (L.M.C., unpublished data). A QTL is considered to have replicated if the probability of no genetic effect on adiposity at the locus examined is <0.05. Because the diet used in the original study (22) corresponds more closely to the low-fat diet used in the present study, only animals fed a low-fat diet have been considered for replication.

**Testing loci for interactions.** An initial test of previously identified loci affecting adiposity in a SMJ × LGJ F1 intercross (22) for gene-by-diet interactions has been performed using the following ANOVA model (33) at each microsatellite marker locus:

\[ Y_{ijkl} = \mu + \text{Genotype}_i + \text{Sex}_j + \text{Diet}_k + \text{Genotype}_i \times \text{Sex}_j \\
+ \text{Genotype}_i \times \text{Diet}_k + \text{Sex}_j \times \text{Diet}_k + \text{Genotype}_i \times \text{Sex}_j \times \text{Diet}_k + e_{ijkl} \]

where \( \mu \) is the mean and \( Y_{ijkl} \) is the measurement on the lth individual for genotype \( i \) and sex \( j \), raised on diet \( k \). The same traits and loci were also tested separately in males and females, using the following ANOVA model, in which all symbols are the same as above (33):

\[ Y_{ijkl} = \mu + \text{Genotype}_i + \text{Diet}_k + \text{Genotype}_i \times \text{Diet}_k + e_{ijkl} \]

**Fine mapping.** Following selection of a region for fine mapping, QTL detection was performed with minor modifications according to the interval mapping method detailed by Cheverud et al. (22). Briefly, the probability of an animal being homozygous for SMJ or LGJ was calculated from the recombination rates between a given locus and the flanking marker genotypes using previously derived equations (34) and our own software (35). Additive genotypic scores (\( X_n \)) were defined based on this probability: (−1) times the probability of an animal being homozygous SMJ at a given locus and (+1) times the probability of being homozygous LGJ at that locus. At observed marker loci, additive genotypic scores were then defined as (−1), (0), and (+1) for homozygous SMJ, heterozygous, and homozygous LGJ, respectively. Similarly, dominance scores (\( X_n \)) were defined as (+1) times the probability of an animal being heterozygous at a given locus, according to the calculations...
described above. At observed marker loci, dominance scores were defined as (+1) for heterozygous and (0) for homoyzgous individuals.

Phenotypes were then regressed on genotypes every 2 cm, as calculated in the F10 AIL (23), along the region of interest using the Set and Correlation feature of Systat 10.0. (36). Regression coefficients are the estimates of the additive (a) and dominance (d) genotypic values if a trait map to the tested locus (37). The QTL was defined as the locus at which the trait has the lowest probability of having occurred by chance, assuming no genotype-phenotype association. The probability was then transformed to a linear scale by taking the $\log_2$ conversion of the inverse of the lowest probability. This transformation converted the mapping results to a scale similar to that obtained using logarithm of odds (LOD) scores based on maximum likelihood analysis (28). All traits were calculated to account for the entire examined sample, as well as the following subsamples: females, males, animals fed the low-fat diet, animals fed the high-fat diet, female animals fed the low-fat diet, female animals fed the high-fat diet, male animals fed the low-fat diet, and male animals fed the high-fat diet.

The region of interest was also scanned for interactions between diet and additive and dominance scores, controlling for sex (when both sexes were analyzed simultaneously), diet, and the main effects of the genotype. A significant interaction effect indicated that the effect of genotype at a given locus was dependent on the diet of the animal.

After loci had been detected, confidence regions were better resolved by statistically accounting for flanking markers in the analysis according to the composite interval mapping method proposed by Zeng (38). Significance has been calculated to correct for multiple comparisons as described previously (22,39). Briefly, the number of effective markers is calculated to account for linkage according to the following equation:

$$M_{\text{eff}} = \frac{M}{1 - (1 - V_{\text{isolate}})}$$

where $M_{\text{eff}}$ is the number of effective markers, $M$ is the actual number of markers used in the study, and $V_{\text{isolate}}$ is the variance of the eigenvalues of a correlation matrix of the additive genotype scores of the markers used. The effective number of markers is then used to calculate a Bonferroni-corrected significance threshold that accounts for multiple comparisons (39). The Bonferroni-corrected 5% significance threshold for this study on mouse chromosome 13 is 0.009, and the 1% significance threshold is 0.002. When comparing the correlation matrix of the additive genotype scores of the markers used. The probability levels, significance thresholds are 2.06 and 2.76, respectively.

RESULTS

Replication. Eight previously detected adiposity QTLs were reexamined in this present experimental sample using both an expanded set of traits as well as a low- and high-fat diet. The fat content of the low-fat diet used in this study corresponds most closely to the diet used in the study that identified the loci under examination (22). Because of this, there is an inherent bias against finding QTLs in animals fed a high-fat diet and a bias toward finding QTLs in animals fed a low-fat diet in this selection of genomic regions. Of the original eight QTLs affecting adiposity, five (Adip2, Adip3, Adip5, Adip6, Adip7, and Adip8) replicated with a probability of ≤5% or less whereas two (Adip1 and Adip4) did not. Adip7 was nearly significant in the low-fat sample, with a probability of 0.09. Although Adip7 did not replicate in the low-fat sample for adiposity as defined in the original study, it was significant for the weight of the reproductive fat pad in male animals ($P = 0.02$). When the high-fat sample was examined, Adip7 replicated in female animals ($P = 0.01$). The two loci that did not replicate may have been due to tested markers missing the region of interest (see Discussion). Of the QTLs that did replicate, two (Adip3 and Adip6) did so with a probability <0.01. QTLs that replicated are indi-
cM, which corresponds to 10.75 cM in F2 units (23). On the
nation data in MapMaker 3.0b calculates a distance of 86
to the methods outlined above. Analysis of the recombi-
D13Mit115

Adip 8
18
D18Mit51
0.027
Free fatty acids(m)
D18Mit209
0.027
None

TABLE 2
QTL replication and locus-by-diet interactions

<table>
<thead>
<tr>
<th>Adiposity QTL</th>
<th>Chromosome</th>
<th>Markers tested</th>
<th>Replication probability</th>
<th>Traits significantly affected by locus-by-diet interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adip 1</td>
<td>1</td>
<td>D1Mit78</td>
<td>0.139</td>
<td>10-week AUC(f), 20-week AUC(m), mesenteric/total fat(m)</td>
</tr>
<tr>
<td>Adip 2</td>
<td>6</td>
<td>D6Mit14</td>
<td>0.726</td>
<td>Renal fat(m), mesenteric fat, inguinal fat(m), triglycerides(m), total fat(m), renal/body(m), inguinal/body(m), reproductive/total(m), inguinal/total(m), adiposity(m)</td>
</tr>
<tr>
<td>Adip 3</td>
<td>7</td>
<td>D6Mit58</td>
<td>0.023</td>
<td>Postweaning growth, adult growth(m)</td>
</tr>
<tr>
<td>Adip 4</td>
<td>8</td>
<td>D7Mit148</td>
<td>0.004</td>
<td>20-week AUC, adult growth, inguinal/body(m), mesenteric/body(m), adiposity(m)</td>
</tr>
<tr>
<td>Adip 5</td>
<td>9</td>
<td>D7Mit17</td>
<td>0.107</td>
<td>None</td>
</tr>
<tr>
<td>Adip 6</td>
<td>12</td>
<td>D9Mit18</td>
<td>0.004</td>
<td>Free fatty acids(f), spleen(m), mesenteric/total(m)</td>
</tr>
<tr>
<td>Adip 7</td>
<td>13</td>
<td>D9Mit19</td>
<td>0.095</td>
<td>None</td>
</tr>
<tr>
<td>Adip 8</td>
<td>18</td>
<td>D13Mit115</td>
<td>0.027</td>
<td>None</td>
</tr>
</tbody>
</table>

Adiposity QTLs are taken from Cheverud et al. (22). Chromosomal location and markers chosen to test for replication and interactions are indicated. All QTLs with the exception of Adip1 and Adip4 replicated for at least one of the associated markers tested. All loci replicated in the sample were fed a low-fat diet with the exception of Adip1, which only replicated in female animals fed a high-fat diet. Traits that are significantly affected by diet-by-genotype interactions for each locus are shown in the right-hand column. Sex-specific effects are indicated by (f) for female-specific effects or (m) for male-specific effects.

cated in boldface in Table 2, and the probability associated with replication is indicated.

**QTL-by-diet interactions.** Because of the increased re-
combination in the F16 AIL, two markers have been used for many of the QTLs to provide more complete coverage of the confidence region observed in the F2 generation. Traits showing a significant locus-by-diet interaction are shown in Table 2. All of the QTLs with the exception of Adip6 show a significant locus-by-diet interaction for at least one of the phenotypic traits examined. Of the loci examined, Adip7 demonstrated a significant locus-by-diet interaction for the largest number of traits and so this locus on the proximal end of chromosome 13 was selected for further analysis by fine mapping.

**Fine mapping.** The following markers were chosen to saturate the Adip7 region: D13Mit1, D13Mit302, D13Mit300, D13Mit80, D13Mit172, D13Mit134, D13Mit207, D13Mit57, D13Mit115, D13Mit135, D13Mit163, and D13Mit84 according to the methods outlined above. Analysis of the recombination data in MapMaker 3.0b calculates a distance of 86 cM, which corresponds to 10.75 cM in F2 units (23). On the Ensembl physical map, these markers cover the distance from 6.2 to 25.1 Mbp from the centromere of chromosome 13. Three of the markers initially scored, D13Mit300, D13Mit172, and D13Mit57, were uninformative due to lack of recombination with adjacent markers (D13Mit302, D13Mit80, and D13Mit207, respectively) and so were dropped from the analysis.

QTLs detected are shown in Fig. 1. Horizontal lines represent the LOD values for the trait listed over the 1-LOD drop confidence region. The peaks of these curves represent the most likely location for the QTL mapped. As can be seen in Fig. 1A, when animals fed both diets are considered together, QTLs for seven traits clustered in two regions along the chromosome are significant. The postweaning growth period among female animals occupies a large confidence region, from midway between D13Mit11 and D13Mit302 to midway between D13Mit134 and D13Mit207 with an LOD score of 2.2. This region overlaps with the QTL for 10-week AUC, which is highly significant with an LOD score of 4.1. The confidence interval for the QTL for 10-week AUC also occupies a much smaller area of the region mapped, ~1.5 F2 cM around D13Mit134.

All other significant QTLs occupy the region from D13Mit207 to D13Mit163, the most distal marker considered in this study. These traits include the inguinal fat pad in male animals, heart weight, 20-week AUC in male animals, and spleen weight. A QTL for postweaning growth phase is also in this region. This differs from the more proximal QTL for the same trait in that this QTL is for both sexes considered together, rather than only female animals.

**Locus-by-diet interactions.** As can be seen from a comparison of Fig. 1B and Fig. 1C, the pattern of QTLs detected differs dramatically based on which diet is considered. The four QTLs detected in animals fed a high-fat diet fall into two groups along the region of interest, as shown in Fig. 1B. The confidence interval for liver weight in male animals occupies a region running from D13Mit1, the most proximal marker considered, to midway between D13Mit302 and D13Mit134. The LOD score for this trait is significant with an LOD of 2.3. The other traits detected, 20-week AUC in male animals, serum triglyceride levels in male animals, and mesenteric fat pad–to–body weight
ratio in female animals all occupy a relatively small region near the distal end of the region of interest, around D13Mit163. The significance region for these traits extends beyond the region fine mapped in the present study. This region in the high-fat diet subsample corresponds to the distal region containing five significant traits when both diets are considered together. One trait, 20-week AUC in male animals, is significant when both diets are analyzed together as well as in the high-fat sample. Because the LOD score for this trait is higher in the sample of male animals fed a high-fat diet than in the total male sample, and because the QTL for this trait is not significant when male animals fed a low-fat diet are considered, the effect seen in the total sample of male animals derives from male animals fed a high-fat diet. None of the traits detected in the sample of animals fed a high-fat diet are significant at a 1% level.

In contrast to the relatively small number of traits detected in the sample of animals fed a high-fat diet, QTLs representing 10 traits spanning the entire region of interest were detected in the sample of animals fed a low-fat diet, as shown in Fig. 1C. As previously noted, the study design is biased to detect traits in animals fed a low-fat diet because that is how the locations for further study were chosen, which may account for the greater number of QTLs detected in the low-fat–fed sample. Of special interest is the QTL for liver weight in male animals, which overlaps with the QTL for the same trait detected in the sample of animals fed a high-fat diet (Fig. 1D). However, the QTL for this trait is not significant when both diets are considered together (see Fig. 1A), indicating that this region has opposite effects depending on which dietary treatment is considered.

A comparison of the QTL for the postweaning growth phase in female animals fed a low-fat diet versus the same trait for all female animals considered together indicates that the effect detected with both diets is due almost entirely to the low-fat–fed sample. The QTL for postweaning growth phase among female animals is highly significant in this sample, with a high LOD score of 4.0. The confidence region for this QTL, from slightly distal of D13Mit302 to slightly distal of D13Mit134, overlaps the confidence region for the same trait in Fig. 1A. Because the QTL for this trait is not significant for the subsample of female animals fed a high-fat diet, and because the LOD score of the subsample of female animals fed a low-fat diet is higher than that of the total female sample, the effect seen in the total female sample is due to the subsample of female animals fed a low-fat diet. A similar pattern is true of the QTL for heart weight, which is more strongly significant when only the subsample of animals fed a low-fat diet is considered (LOD 2.5) than when the total sample of animals is considered (LOD 2.3) and is not significant in the subsample of animals fed a high-fat diet.

Figure 1D shows QTLs for gene-by-diet interactions. Rather than mapping a trait per se, this analysis considered the difference in effect between animals fed a low-fat diet versus animals fed a high-fat diet, as described in the statistical analysis above. These are QTLs for dietary response. A total of six significant dietary response QTLs have been detected in this region. The three most proximal QTLs, occupying the region from D13Mit1 to midway between D13Mit134 and D13Mit207, are also the traits of this set with the highest LOD scores. The QTL for serum insulin in male animals, with an upper LOD score >3.5, is highly significant when the interaction between genotype and diet is considered. The same trait is significant when the subsample of male animals fed a low-fat diet is...
considered, but not at the same level (LOD 2.6), and the QTL for this trait is not significant in either the subsample of male animals fed a high-fat diet or the total sample of male animals.

The QTL for liver weight in male animals is not significant when all male animals are considered together, but, as noted above, is significant when either dietary subsample is considered separately. This indicates that this region of the genome has opposite effects on liver weight in male animals, depending on which diet the animal is fed. This is also shown in Fig. 1D. With an LOD score of 3.5, the QTL for the interaction effect on liver weight in male animals is more significant than in either dietary subsample.

The QTL representing the ratio of the weight of the mesenteric fat pad in female animals when considered as a percentage of the total amount of fat removed from the animal shows significant locus-by-diet interaction, corresponding to the region in the low-fat subsample with reproductive fat pad-to-body weight ratio. Liver weight among female animals also shows a significant interaction effect in this region. Finally, serum triglycerides show a significant interaction effect in the distal part of the region of interest.

Figure 2 shows the effect of genotype on dietary response traits with the highest LOD scores: serum insulin levels in male animals, liver weight in male animals, and reproductive fat pad weight. In all cases, the effects of genotype and diet are examined at the locus with the highest LOD score for each trait. As shown in Fig. 2A, an animal that is SM homozygous at $D13Mit1 + 1.5$ F2 cM averages ~1.95 ng/ml serum insulin at time of necropsy if fed a low-fat diet since weaning. When an animal with the same genotype at this locus has been fed a high-fat diet, it averages 8.34 ng/ml of serum insulin at time of necropsy, presumably following a well-established pattern of increased serum insulin in response to decreased insulin sensitivity after prolonged exposure to a high-fat diet.

Animals that are heterozygous at this locus and have been fed a low-fat diet have slightly higher levels of serum insulin (2.23 ng/ml) than SM/J homozygotes at this locus that have been fed a low-fat diet, while $D13Mit1 + 1.5$cM heterozygotes fed a high-fat diet have somewhat lower levels of serum insulin (7.55 ng/ml) than comparably fed SM/J homozygotes. When animals that are LG/J homozygous at this locus are considered, the difference between the two diets almost disappears: animals that are SM homozygous at $D13Mit1 + 1.5$ F2 cM averages ~1.32 g, versus only 0.67 g for male animals that are SM/J homozygous at this locus. The diet-based difference in age of necropsy is 1.32 g, versus only 0.67 g for male animals that are SM/J homozygous at this locus. In summary, the difference in serum insulin levels between animals fed the low-fat versus the high-fat diet is highly contextual, depending strongly on the underlying genotype at $D13Mit1 + 1.5$. The diet-based difference varies from 6.39 ng/ml for SM/J homozygotes at this locus, to 5.32 ng/ml for heterozygotes, and down to 1.10 ng/ml for LG/J homozygotes. This is a locus-by-diet interaction and indicates that the effect of a genotype at this locus on the serum insulin level of the animals is strongly dependent on the diet the animal consumes.

A similar phenomenon occurs with liver weight at nearly the same genomic position: the difference in liver weight between two male animals that are both homozygous SM/J at $D13Mit1 + 1.5$ F2 cM but fed a low-fat versus high-fat diet is 1.32 g, versus only 0.67 g for male animals that are homozygous LG/J at this locus but fed a low-fat versus high-fat diet. Figure 2C shows the interaction of locus $D13Mit1 + 2.75$cM with diet in affecting the weight of the reproductive fat pad. The overall pattern is similar to the above two cases in that there is a greater response to dietary fat in animals that are homozygous SM/J at this locus than in animals that are homozygous LG/J.

**Genomic region.** The focus of this study is on possible gene-by-diet interactions, as opposed to genetic effects on both diets or on a high- or low-fat diet separately. The highest LOD peaks for gene-by-diet interactions occur between $D13Mit1$ and $D13Mit134$, as shown in Fig. 1D. Table 3 shows the known and putative transcripts located between these markers according to Build 32 (1 April 2004) of the Ensembl database. Microsatellite markers used to fine map the interaction effects are shown in bold italics, with F2 cM distances indicated.

The region of interest for the locus-by-diet interaction fine mapping corresponds to the short arm of human
chromosome 10. The actual LOD peaks all are immediately distal to D13Mit302. A search of this region yields two known genes, either or both of which are positional candidate genes that may contribute to the genotype-by-diet differences observed in this study. The first is a pitrilysin (Pitrm1; Ensembl ID ENSMUSG00000021193; GenBank AF513714 and NM_145131) and the second is 6-phosphofructokinase isozyme C (Pfkpc; Ensembl ID ENSMUSG00000021196; GenBank AF123533).

The Pfkp gene codes for a 6-PFK that catalyzes the transfer of a phosphate residue from ATP to fructose-1-P to form fructose-1,6-P2 (42). Enzymes in the PFK family transfer of a phosphate residue from ATP to fructose-1-P.

TABLE 3
Positional candidate genes located between D13Mit1 and D13Mit134

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start</th>
<th>Stop</th>
</tr>
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<tbody>
<tr>
<td>D13Mit1</td>
<td>6,217,175</td>
<td></td>
</tr>
<tr>
<td>D13Mit302</td>
<td>6,344,138</td>
<td></td>
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<tr>
<td>Pitrm1</td>
<td>6,360,469</td>
<td>6,382,544</td>
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<tr>
<td>Pfkp</td>
<td>6,392,256</td>
<td>6,460,617</td>
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<tr>
<td>Tub2</td>
<td>6,649,117</td>
<td>6,651,880</td>
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<tr>
<td>D13Mit80</td>
<td>(D13Mit1 + 3.25 cM)</td>
<td>8,858,870</td>
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<tr>
<td>3110035P10Rik</td>
<td>8,905,602</td>
<td>8,971,735</td>
</tr>
<tr>
<td>Id1</td>
<td>8,986,339</td>
<td>8,991,445</td>
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<td>4833405L16Rik</td>
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<td>9,081,660</td>
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<td>9,145,600</td>
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<td>Gtpbp4</td>
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<td>A1256361</td>
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<td>2900024P20Rik</td>
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<td>221002622Rik</td>
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<td>Chrm3</td>
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<td>D13Mit134</td>
<td>(D13Mit1 + 5.25 cM)</td>
<td>12,186,106</td>
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</table>

All information presented in this table is from Build 32 (1 April 2004) Ensembl database (www.ensembl.org). Microsatellite markers used in fine mapping are indicated in bold italics, with the distance from D13Mit1 shown in F2 cM immediately below. Genes and putative genes are indicated in italics. While the 5% confidence region for the interaction effects shown in Fig. 1 lies between D13Mit1 and D13Mit302, the LOD peaks for these effects are closest to D13Mit302.

TABLE 4
Pfkp gene

<table>
<thead>
<tr>
<th>Position</th>
<th>129</th>
<th>201</th>
<th>782</th>
<th>849</th>
<th>874</th>
<th>1050</th>
<th>1281</th>
<th>1704</th>
<th>2028</th>
<th>2031</th>
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Position represents a base pair position from the 5’ end of the Pfkp gene. Sequence data from C57BL/6J mouse are from Ensembl Mouse Build 32. Codon signifies which codon the respective position represents, and AA signifies resulting amino acid change, if any. Prob signifies the log_{10} odds of amino acid changes derived from the BLOSUM62 matrix. SIFT signifies probabilities of amino acid changes having a significant deleterious effect. *Statistical significance at the 5% level. Amino acid changes between LG/J and SM/J mice are highlighted in bold.

In comparison with the C57BL/6J allele, the SM/J allele shows no difference in amino acid composition and differs by only one silent transition at position 129. The LG/J allele differs from the C57BL/6J allele at 14 nucleotides, 9 of which are silent mutations, with the remaining 5 mutational differences resulting in five putative amino acid changes. Associated log_{10} odds of specific amino acid changes derived from the BLOSUM62 matrix are shown in Table 4 along with the probability of tolerated functional effects at those locations derived using the SIFT algorithm (40). Translational probabilities <0.05 are considered to have a significantly deleterious effect.

Briefly, in order of least to most likely amino acid changes in this protein are the replacement of valine with methionine at amino acid 292 (change at nucleotide 874), arginine with glutamine at position 261 (nucleotide 782), serine with proline at position 777 (nucleotide 2329), glutamic acid with glycine at position 782 (nucleotide 2345), and glutamic acid with aspartic acid at position 694 (nucleotide 2080). The valine-to-methionine replacement in the LG/J mouse strain is predicted to have a significantly deleterious effect (P = 0.03), although change in these residues represents a relatively small structural change from medium to large nonpolar amino acid. An analysis of 112 sequences deposited in GenBank indicated that amino acid mutations at this position are very rare across taxa and across Pfkp gene paralogues. Only the zebrafish had the amino acid threonine at this position, while all other vertebrates including all human and nonhuman primates had valine. The arginine-to-glutamine replacement (P = 0.10) does not appear to be significantly deleterious; however, this replacement represents a relatively large structural change due to a replacement of a medium-large polar amino acid by a medium-small polar amino acid. This mutation is also rare, with only the zebrafish (alanine), chicken (glycine), and rabbit (glutamine) deviating from the common amino acid (arginine) found in all other vertebrates including all human and nonhuman primates. Together with the valine-to-methionine replacement (P = 0.03), the arginine-to-glutamine change (P = 0.10) is likely to have the greatest functional effect.

Functionally divergent changes also include the glutamic acid-to-glycine (P = 0.27) and serine-to-proline (P = 0.15) transitions that result in small polar to small nonpolar and small nonpolar to small polar changes, respectively. The close proximity of these amino acid

remaining 10 sites are silent, i.e., at degenerate positions.
TABLE 5

Pitrm1 gene

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Position represents a base pair position from the 5′ end of the Pf kp gene. Sequence data from C57BL/6J mouse are from Ensembl Mouse Build 32. Codon signifies which codon the respective position represents, and AA signifies resulting amino acid change, if any. Prob signifies the log_{10} odds of amino acid changes derived from the BLOSUM62 matrix. SIFT signifies probabilities of amino acid changes having a significant deleterious effect. *Statistical significance at the 5% level. Amino acid changes between LG/J and SM/J mice are highlighted in bold. Amino acid changes between C57BL and LG/J + SM/J mice are in bold italics; these mutational changes result in no differences between LG/J and SM/J mice. Mutations at site 631 and 632 both contribute to the change from valine to threonine.

residues and the parallel but opposite changes in their polarities may result in functional compensation. Additionally, these amino acids are located at the COOH-terminus of the protein and are therefore less likely to be of functional significance. This hypothesis is supported by an observation of large variation in amino acid composition at the COOH-terminus among vertebrate taxa. The glutamic acid to aspartic acid replacement is expected to result in amino acid changes between C57BL and LG/J deleterious effect. *Statistical significance at the 5% level. Amino acid changes between LG/J and SM/J mice are highlighted in bold. Amino acid changes between C57BL and LG/J + SM/J mice are in bold italics; these mutational changes result in no differences between LG/J and SM/J mice. Mutations at site 631 and 632 both contribute to the change from valine to threonine.

The Pitrm1 gene plays a critical role in energy production in mitochondria. It is involved in the processing and degradation of NH₄-terminus presequence peptides of other nuclear-encoded mitochondrial genes as they enter the mitochondrial matrix through the lipid membrane. Failure to degrade these peptides is toxic for the mitochondria. Together with ATP-dependent and other metal-dependent proteases, Pitrm1 functions as a quality control gene of the mitochondrial inner membrane. Through its quality control role, it regulates mitochondrial functionality and efficiency in energy production.

We also examined sequence variation in the Pitrm1 gene (Table 5). The LG/J mice differ from SM/J mice at nine nucleotide positions, three of which result in amino acid changes. Both LG/J and SM/J differ from C57BL/6J mice at an additional six nucleotide positions, all of which result in amino acid changes. However, two of those positions are adjacent and part of the same putative amino acid; thus, the six nucleotide differences result in only five amino acid changes. The change from valine to methionine at position 462 (nucleotide 1384) has a low probability and most likely results in a strong functional effect (P = 0.01). All sequences deposited in GenBank contain valine at amino acid position 462, but only the LG/J mice contain methionine at this position. The two other amino acid changes observed between LG/J and SM/J mice represent relatively common changes and would appear to have nonsignificant functional effects. At amino acid position 546 (nucleotide 1637), threonine in SM/J mouse is replaced by lysine in LG/J mouse with nonsignificant functional effect (P = 0.21), while at position 583 (nucleotide 1747) a functionally nonsignificant (P = 0.47) transition from isoleucine to valine is observed.

**Discussion**

This study utilizes an F₁₀ AIL segregated by diet to map gene-by-diet interactions in previously identified adiposity QTLs. Of the previously identified adiposity QTLs, five of the original eight replicated at the 5% level or better. Two of the previously identified adiposity QTLs did not replicate. However, the lack of replication may be explained by discrepancies in the genetic maps used for each study. The 2001 study relied on recombinational mapping to determine marker position (22), and markers were chosen for the genome-wide scan on the basis of F₁₀ recombinational data. Subsequent examination of the Ensembl physical database (www.ensembl.org) reveals that the marker chosen to replicate Adip1, D1Mit178, is actually centromeric of its original apparent map position. Examination of other experimental samples derived from the same parental cross by our group has replicated Adip1 (data not shown). Similarly, the markers chosen to replicate Adip4, D8Mit56 and D8Mit324, are telomeric of the original QTL position (data not shown).

Seven of the eight previously detected QTLs (Adip1, 2, 3, 4, 5, 7, and 8) demonstrated locus-by-diet interactions for a large series of obesity- and diabetes-related traits. These include a variety of fat pads, responses to glucose challenge, organ weights, and serum plasma levels of insulin, triglycerides, and free fatty acids. These collective results indicate that adiposity QTLs also affect dietary responses, in that the LG/J and SM/J alleles are differentially sensitive to a high-fat diet. Most often (60% of the time) SM/J homozygotes at these QTLs are more responsive to a high-fat diet than LG/J homozygotes. This is especially true for Adip3, -5, -7 and -8. In contrast, LG/J homozygotes at Adip2 and -4 are more sensitive to dietary fat than SM/J homozygotes.

When animals fed both a low- and high-fat diet are considered together, a number of additional phenotypes map to Adip7, the adiposity QTL on chromosome 13. These include postweaning growth in female animals, 10-week AUC, and 20-week AUC in male animals. However, more striking are the differences between the QTLs detected among animals fed a low-fat diet and those fed a high-fat diet. In some cases, such as with postweaning growth among female animals and 20-week AUC among male animals, it becomes apparent that the overall effect is due only to half of the sample assayed: one sex is affected while the other is not. Other phenotypes, such as serum triglycerides in male animals fed a high-fat diet and renal fat pad weight in male animals fed a low-fat diet, are observable only in one dietary subsample, but not the
other. The LOD peaks in the subsample fed a high-fat diet for 20-week AUC in males, serum triglycerides in males, and mesenteric fat pad–to–body weight ratio in females all occur distally of the region fine mapped in this study, and further analysis will be needed to determine the location of the actual peaks for these traits. Perhaps most intriguingly, the QTL for liver weight among male animals is significant in both dietary subsamples considered separately, but not together.

This mystery is resolved when the difference between traits on different diets, or dietary responses, are mapped rather than the traits themselves. The interaction between locus and diet affecting liver weight among male animals, for example, has an LOD of 3.5, which is higher than the main effect in either dietary subsample. The QTL for serum insulin in male animals is not significant in animals fed a high-fat diet, is significant at the 5% level in animals fed a low-fat diet, but is significant well above the 1% level when considered as an interaction.

For both serum insulin and liver weight, the locus-by-diet interaction effect is caused by a difference in magnitude of effect: SM/J homozygous animals respond more strongly to higher levels of dietary fat than do LG/J homozygous animals. In both cases, heterozygous animals fall somewhere between the homozygotes, although SM/J is partially dominant to LG/J with respect to dietary response. Reproductive fat pad weight also shows a difference of magnitude of response to diet dependent on genotype, although it also seems to show some degree of overdominance, since the degree of difference between the two diets is greatest among animals that are heterozygous at the locus examined. Serum triglyceride levels in male animals also show a gene-by-diet interaction at the distal end of the region examined, and a distal extension of fine-mapping efforts will be necessary to elucidate the locus for this effect.

Liver weight in male animals and serum insulin levels in male animals showed the strongest gene-by-diet interactions as measured by LOD interaction scores in Fig. 1D. The peaks for both of these traits occur between D13Mit1 and D13Mit134, and a preliminary gene scan was performed in this region, which comprises ~6 million bp. Although identification of the genes involved in the different dietary responses is an ongoing process, two promising potential candidate genes were located in the dietary response QTL support interval on chromosome 13: the Pfkp gene and the Pitrm1 gene.

The Pfkp gene plays a crucial role in metabolism as the rate-limiting and most commonly regulated step in glycolysis. The PFK family of enzymes (EC 2.7.1.11) catalyzes one of the rate-limiting steps in the formation of pyruvate in mammalian cells: the phosphorylation of fructose-6-phosphate to fructose 1,6-bisphosphate in an MgATP-dependent manner (43). Different isoforms of PFK form both homo- and heterodimers, allowing for a range of regulatory function (43). In fact, there is some evidence that the human form of the C isozyme identified in this study plays a primarily regulatory role in ascites tumors by regulating the activity of other PFK isozymes (44). The Pfkp gene has five amino acid differences between the SM/J and LG/J alleles, two of which have potentially important functional effects.

The valine-to-methionine replacement at amino acid position 292 has been identified as the most likely functionally deleterious mutation. The arginine-to-glutamine replacement at amino acid position 261 has a lower probability of functional effect but structurally presents a relatively large change because of the replacement of a medium-large polar amino acid by a medium-small polar amino acid. Both of these positions lie in highly conserved regions and are mutations that might potentially cause secondary and tertiary conformational changes, affecting the specificity of regulatory binding sites. Unfortunately, no three-dimensional structure for eukaryotic PFK enzymes exists, so there is no way, at present, to examine this possibility with certainty.

We can speculate on the possible role of PFK in response to a high-fat diet. Given the contextual relationship between genotype, diet, and the phenotype of increased fat pad/liver weight/hyperinsulinemia, the mice with SM/J genotypes likely manifest accelerated deposition of fat in adipose tissue (reproductive fat depot) when challenged with a high-fat diet. Increased adiposity can alter the secretion of adipokines, such as adiponectin (45), leading to insulin resistance. In an effort to maintain normal circulating glucose levels, the pancreatic β-cell compensates for insulin resistance by secreting more insulin, resulting in hyperinsulinemia. Insulin resistance is commonly associated with fatty liver, perhaps related to enhanced delivery of free fatty acids from fat stores to the liver (46), thus the effect on liver weight. While the mechanisms by which these candidate genes affect dietary response are speculative, there is previous evidence supporting a potential role for PFK in mediating weight gain in response to high-fat feeding in rodents (47).

Pitrm1 is related to the insulin-degrading enzyme and other peptide processing enzymes. It removes presequence mitochondrial targeting peptides, which serve an important role in moving peptides from the cytosol into the mitochondrial matrix. If these targeting peptides are not removed, they can be toxic to the mitochondrion and thus could affect mitochondrial activity. It has recently been suggested that impaired mitochondrial activity may play an important role in insulin resistance and diabetes (48–51). The Pitrm1 alleles found in the SM/J and LG/J mice show functionally important differences at five amino acids when compared with C57BL/6J mice. However, there are only three amino acid differences observed between the SM/J and LG/J alleles. Of these three mutations, the amino acid change from valine to methionine in LG/J mice at amino acid 462 is the most likely to have a functional effect (P = 0.01). The amino acid methionine is clearly the aberrant residue, since human sequences also contain valine at position 462.

Pitrm1 is probably required for the normal processing of peptides by mitochondria, and defects in its function could disrupt mitochondrial function. Dysfunctional mitochondria would be less able to carry out the metabolism of fatty acids, leading to lipid accumulation in the setting of a high-fat diet.

Further research will be required to better define the physiological roles of both of these two positional candidate genes and to determine whether the amino acid differences detected between the LG/J and SM/J alleles are
indeed responsible for the observed QTL effect. These future studies will be based not on gene mapping but on functional evaluation of protein levels and activities associated with the variant alleles. In addition, other genes within or near the confidence region on proximal chromosome 13 will be similarly sequenced and analyzed.

ACKNOWLEDGMENTS

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