Quantitative trait loci for insulin-like growth factor I, leptin, thyroxine, and corticosterone in genetically heterogeneous mice

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Although there is a growing body of knowledge about genes whose inactivation leads to radical alterations in hormone levels (1, 3, 7, 18), much less is known about the ways in which common polymorphic alleles may contribute to differences in circulating hormone levels, either at early or at later stages in life. Previous studies in rodents (2, 8, 27, 32, 45), swine (14), and humans (31, 34, 37, 42) have successfully identified quantitative trait loci (QTL) for a variety of hormones, but these studies have focused on the identification of QTL that were apparently age-independent. These results show that the genetic controls over late-life hormone levels are complex and dependent on effects of genes that act both early and late in the life course.

Episettes; UM-HET3 mice; late-life hormone levels; age-specific
In this study, 1,108 mice derived from a cross between CB6F1 females and C3D2F1 males were genotyped and sampled at the ages of 4, 15, and 17 mo to obtain blood or fecal material for the determination of IGF-I, leptin, T4, and CORT levels. This population is the genetic equivalent of a large sibship and thus allows us to identify QTL that have detectably large effects on hormone levels. By measuring T4, IGF-I, and leptin at both 4 and 15 mo, we were also able to determine whether the genetic effects noted might vary with age.

**MATERIAL AND METHODS**

**Mice.** The animals used in this study were of the UM-HET3 stock, bred at the University of Michigan as the offspring of (BALB/cJ × C57BL/6J)F1 (CB6F1) females and (C3H/HeJ × DBA/2J)F1 (C3D2F1) males; thus each mouse is genetically unique but shares ~50% of its genes, on average, with every other mouse in the group. A total of 1,108 (826 females, 282 males) virgin mice were used.

All mice were weaned at 3 to 4 wk of age, housed in same-sex cages, and given free access to food and water for the duration of the study. To ensure the specific pathogen-free status of the study population, groups of sentinel mice were exposed to spent bedding from the study population on a quarterly basis and were later evaluated serologically for the presence of specific viral and bacterial pathogens. The animals were also examined for pinworm. All test results were negative over the course of the study.

Mice were entered into the study in cohorts of ~30 per month with all mice housed in the Cancer Center and Geri- atrics Center Building at the University of Michigan Medical Center. For the determination of serum hormone levels, all mice were immunized with erythrocytes at the ages of 4 and 15 mo as part of another protocol and bled by tail venipuncture 2 wk after each immunization to assess antibody production as an index of their immunoresponsiveness. The remaining serum samples were used for the quantification of circulating T4, leptin, and IGF-I levels in this study. In addition, at 12 mo of age skin biopsy samples were obtained under brief Metofane anesthesia as part of another protocol, and 1–3 fecal pellets were collected from approximately one-third (n = 321 females) of the mice at 17 mo of age for the quantification of fecal corticosteroid levels for use in this study. Fecal samples were collected from each mouse for five consecutive days to control for the possibility of significant day-to-day variation in circulating CORT levels due to the influence of the estrus cycle (4). The mean 5-day fecal corticosteroid level for each mouse was used for the analysis.

**Hormone measurements.** Serum samples for hormone measurements were taken by tail venipuncture between the hours of 7 and 11 AM and stored at ~70°C for up to 3 yr prior to assay. Serum T4 levels were determined using a monoclo- nal solid-phase radioimmunoassay (RIA) kit (ICN Pharma- ceuticals, Costa Mesa, CA) run at one-quarter volume accord- ing to the manufacturer’s instructions. Each sample was assayed in duplicate and diluted up to 1:7 with phosphate- buffered saline (PBS) if necessary to achieve adequate sample volume. Serum IGF-I levels were quantified via a double- antibody RIA kit (Diagnostic Systems Laboratories, Webster, TX) run at one-quarter volume according to the manufacturer’s instructions. Prior to assay, 10 μl of serum from each individual was subjected to an acid-ethanol extraction procedure using the materials provided in the kit. All samples were assayed in duplicate. Serum leptin levels were quanti-

fied with a double-antibody RIA kit (Linco Research, St. Charles, MO) according to the manufacturer’s instructions, except that all volumes were reduced by a factor of 4. Each sample was assayed in duplicate using dilutions up to 1:10.4 with PBS if necessary to achieve adequate sample volume. The inclusion of two pooled serum controls run in each assay (n = 13) indicated that the mean (±SD) intra-assay coefficients of variation (CV) were 6.95 ± 5.55%, 5.89 ± 4.75%, and 6.12 ± 4.53% for T4, IGF-I, and leptin, respectively, and that the interassay coefficient of CV was less than 25% for all. The lower limits of detection were 150 ng/ml, 0.2 ng/ml, and 0.625 μg/dl for IGF-I, leptin and T4, respectively.

Fecal corticosteroid levels were quantified with a double-antibody 125I-corticosterone RIA kit (ICN Biomedicals) after hormone extraction using an ethanol-based procedure as described in (23). This procedure has been validated previously for use in multiple species of rodent as a specific, precise, and accurate integrated measure of circulating corticosteroid concentrations (23). All samples were assayed in duplicate, and the assay was performed according to the manufacturer’s instructions at one-quarter volume. The 25 ng/ml standard provided with the kit was diluted 1:2 with steroid diluent (provided with the kit) to allow the inclusion of a 12.5 ng/ml standard (the lower limit of detection for this assay). Corticosteroid concentrations are presented as nanograms corticosteroids per gram dry weight of feces. As an indicator of the day-to-day variation in fecal CORT levels over the 5-day sampling period, the intra-individual CV of the 5-day mean fecal CORT measure was calculated for each mouse. The result of this analysis indicated that fecal CORT levels typically varied by ~36% within a five-day sampling period (mean ± SD: 35.95 ± 15.62%). In addition, the inclusion of two pooled fecal extracts in each assay (n = 20) indicated that the mean (±SD) intra-assay CVs were 3.73 ± 3.62% and 8.45 ± 7.31% for the low and high control, respectively. The interassay CV was 8.7% for the low control and 18.8% for the high control.

**Genotyping.** Genomic DNA was isolated from a 1-cm section of tail collected from 4-wk-old individuals using a phenol-extraction method. The DNA concentration, ability to sustain PCR amplification, and electrophoretic size distribution for each of the preparations were tested prior to simple-sequence length polymorphism (SSLP) genotyping using an ALFexpress automated sequence analyzer (Pharmacia, Piscataway, NJ) using previously described methods (28). Primer pairs were either purchased from Research Genetics or were synthesized locally (University of Michigan Molecular Biology Core Facility). Polymorphic loci were selected using data provided by the Mouse SSLP Database (White- head/MIT Center for Genome Research, Cambridge, MA) or the Mouse Genome Database (Jackson Laboratory, Bar Har- bor, ME). Chromosomal localization and marker order were calculated using the MapMaker QTX software package (Whitehead Institute, Cambridge, MA).

**Statistical analyses.** A single point genome-wide search was performed for each of the seven hormone measures to detect QTL that may be associated with each measure. To make the analysis consistent for all partially and fully infor- mative markers, four-way informative markers were split into two sets of bi-allelic markers that were informative for either the maternally or paternally transmitted alleles. One- way ANOVA models, with each hormone measure as the dependent variable and a bi-allelic marker as the indepen- dent variable, were used to perform a genome-wide search for all of the 164 bi-allelic markers. The statistical significance for each marker-trait combination was calculated empirically.
Table 1. Hormone levels in young adult and middle-aged UM-HET3 mice

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Age (mo)</th>
<th>Female</th>
<th>Males</th>
<th>n</th>
<th>Effect (SE)</th>
<th>Experiment-wise P-Value</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I, ng/ml</td>
<td>4</td>
<td>681(578–798)</td>
<td>725</td>
<td>798(685–919)</td>
<td>44(12)</td>
<td>0.05</td>
<td>C &gt; B6</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>4</td>
<td>3.1(2.1–4.4)</td>
<td>664</td>
<td>2.9(1.9–4.6)</td>
<td>0.01</td>
<td>C &gt; B6</td>
<td></td>
</tr>
<tr>
<td>T4, μg/dl</td>
<td>15</td>
<td>5.4(3.5–9.2)</td>
<td>645</td>
<td>5.0(3.2–8.8)</td>
<td>0.176</td>
<td>B6 &gt; C</td>
<td></td>
</tr>
<tr>
<td>CORT, ng/ml</td>
<td>17</td>
<td>71.1(51.9–93.2)</td>
<td>321</td>
<td>ND</td>
<td>0.01</td>
<td>C &gt; B6</td>
<td></td>
</tr>
</tbody>
</table>

Values are median, with interquartile range in parentheses; T4, thyroxine; CORT, corticosterone; ND, not done.

Detection of QTL. Genome-wide scans evaluated 87 SSLP markers known to discriminate among the four inbred progenitor strains. Of the 87 marker loci analyzed, 77 were informative for all of the progenitor strains, 10 were informative for either the maternal or paternal alleles only (8 maternal, 2 paternal), and 2 were X-linked. Considering maternally-inherited and paternally-inherited markers independently, the genotype data set included 164 bi-allelic markers. The average distance between markers in the scan is 15–20 cM, depending on how sex-specific recombination frequencies are treated, and excluding the region between the most distal marker and the telomere for each chromosome. About 5% of the genome lies more than 20 cM from a marker used in the survey.

Using a permutation method to derive experiment-wise significance criteria, we found 12 significant gene/trait associations, as shown in Table 2. IGF-I levels were affected by loci on chromosomes 1, 3, 8, 10, and 17; leptin levels were affected by a locus on chromosome 3; T4 levels were modulated by loci on chromosomes 4, 15, and 17; and fecal CORT levels were influenced by a locus on chromosome 1. Each marker was only associated with a single trait measured at a specific age with one exception: D4Mit155 was significantly associated with serum T4 levels in both 4- and 15-mo-old individuals. The marker D17Mit46 is also listed twice in Table 2, but it is the maternal allele that is associated with alterations in IGF-I levels (at 15 mo), and the paternal allele associated with differences in T4 levels in 4-mo-old mice. Four significant markers, i.e., D3Mit227, D3Mit86, D15Mit63, and D1Mit206, are not shown in Table 2, because they are each within 16 cM of another marker with a stronger level of association with the trait concerned and thus are unlikely to represent an independently segregating allele with functional effect.

There is evidence for sex-specific influences on many age-sensitive traits [e.g., bone geometry (32), obesity (49), behavior (43)]. We therefore conducted the ge-
nomi-wide QTL analysis twice: first with the data pooled across sex, and then a second time with sex included as an independent variable in the statistical model. Each of the markers listed in Table 2 remained significant at experiment-wise \( P < 0.05 \) in the sex-adjusted model, and therefore we report only the results obtained using the pooled dataset.

In addition to the statistically significant associations shown in Table 2, we have compiled a Supplementary Table listing each of the markers for which the genome scan provided suggestive evidence at a less conservative criterion of \( P < 0.20 \). This Supplementary Table can be viewed at http://www-personal.umich.edu/~millerr/Harper%20suppl%20table%201.htm, and is also available at the Physiological Genomics web site.¹

It is possible polymorphic alleles might modify more than one of the tested hormones but that only one of the associations is sufficiently strong to reach the experiment-wise significance threshold. To examine this question, further analysis was performed to determine whether any of the QTL listed in Table 2 had an influence on the level of the other hormones measured in this study. We used a Bonferroni-corrected \( P \) value, \( P = 0.004 \), as our criterion for rejecting the null hypothesis that the alleles listed in Table 2 had no effect on any of the other hormones. The results of this post hoc analysis are presented in Table 3, which also includes the original associations from Table 2 for completeness. Overall, these results suggest that some of the chromosomal segments containing significant QTL show associations with the production or metabolism of multiple hormones or of the same hormone at more than one age. It is of particular interest to note that the B6 allele on chromosome 8 is associated with high IGF-I and low \( T_4 \) levels, whereas the B6 allele on chromosome 15 is associated with low levels of both of these hormones, suggesting that the two alleles may affect different aspects of the hormonal response. Further work would be needed to determine whether the associations noted in Table 4 reflect the actions of single QTL or of multiple QTL linked to one another on the indicated chromosomes, and to investigate the extent to which changes in one hormone linked to another on the indicated chromosomes, and to investigate the extent to which changes in one hormone might bring out secondary, compensatory alterations in the levels of other hormones in our test battery.

Tests for gene-gene interactions. For each of the hormones for which two or more QTL reached the experiment-wise significance criterion (Table 2), we explored the question of whether the genetic effects were additive or were instead conditional on inheritance at the other influential loci. We used two different methods. In the first, less formal approach, the sum of the individual effects for each of the significant markers was compared with the pooled genetic effect, i.e., to the difference in hormone levels between the two composite genotypes that differed the most for the hormone measure in question. The top row of Table 4, for example, shows these statistics for the three QTL with effects on IGF-I levels at 4 mo of age. Each of these three loci has an individual effect (shown in Table 2), and the sum of these effects is 149 ng/ml. The three loci define eight possible genotypic combinations, and the difference in mean IGF-I levels between the two groups with highest and lowest levels comes to 145 ng/ml, a value similar to the sum of the individual effects. We thus see no evidence for non-additivity in this or indeed in any of the other hormones evaluated in this way, as illustrated in Table 4.

In the second approach, a full-factorial ANOVA was performed that used all of the significant markers as factors in the model, and the \( P \) value of the interaction term, \( p(I) \), that involved all of the significant markers was determined. A nonsignificant interaction term is interpreted as no genetic interaction, or epistatic effect, for the markers tested. The results of these analyses are also presented in Table 4 and indicate that there was no significant evidence for epistasis for any of the hormones tested, with the possible exception of serum IGF-I levels in 15-mo-old mice. In this case, the second-order interaction between \( D8Mit51 \) and \( D3Mit25 \) was significant at \( P = 0.05 \). However, it is possible this finding is due simply to chance. Neither the third-order interaction, nor the two remaining second-order interactions, are significant at \( P = 0.10 \), and

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¹The Supplementary Material for this article (a table listing each of the markers for which the genome scan provided suggestive evidence at a less conservative criterion of \( P < 0.20 \)) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00063.2003/DC1.
Fig. 1. Additive genetic effects of the loci linked to D8Mit51, D17Mit46, and D3Mit25 on serum insulin-like growth factor I (IGF-I, ng/ml) levels in 15-mo-old UM-HET3 mice shown for each of the eight possible genotypic allelic combinations at these three loci. In each plot, the circle represents the median; the vertical lines show the 25th and 75th percentiles, and the density trace shows the distribution of the values for each genotype. C, BALB/cJ mice; B6, C57BL/6J mice.

Table 4. Tests for additivity and epistatic genetic effects on serum hormone levels

<table>
<thead>
<tr>
<th>Hormone (Age, mo)</th>
<th>Markers</th>
<th>Sum of Individual Effects</th>
<th>Pooled Effect</th>
<th>p(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I (4)</td>
<td>D1Mit206, D10Mit230, D17Mit185</td>
<td>149</td>
<td>145</td>
<td>0.22</td>
</tr>
<tr>
<td>IGF-I (15)</td>
<td>D3Mit25, D8Mit51, D17Mit46</td>
<td>173</td>
<td>187</td>
<td>0.05</td>
</tr>
<tr>
<td>Leptin (15)</td>
<td>D11Mit289, D13Mit64</td>
<td>2.6</td>
<td>2.6</td>
<td>0.64</td>
</tr>
<tr>
<td>T4 (4)</td>
<td>D4Mit155, D17Mit46</td>
<td>1</td>
<td>0.9</td>
<td>0.42</td>
</tr>
<tr>
<td>T4 (15)</td>
<td>D4Mit155, D15Mit100</td>
<td>0.9</td>
<td>0.9</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Units for each of the hormone measures are as follows: IGF-I, ng/ml; leptin, ng/ml; T4, μg/dl; and CORT, ng/ml.

the sum of the estimated and pooled genetic effects are virtually identical (173 vs. 187 ng/ml). Figure 1 illustrates this additivity for serum IGF-I levels in 15-mo-old mice. For each of the three significant markers, the median concentration of serum IGF-I is shown with the effect of the B6 or C allele at each of the three loci. At D3Mit25 and D8Mit51 inheritance of the B6 allele is associated with an increase in serum IGF-I, whereas at D17Mit46 inheritance of the C allele has the same effect. For mice that inherit the B6 allele at D3Mit25 and D8Mit51 and the C allele at D17Mit46, their serum IGF-I concentration is 187 ng/ml greater than those who inherited the opposite alleles at these loci (Fig. 1).

We also determined what proportion of the variance observed for each of the seven hormone measures was attributable to the genetic markers found to be statistically significant for each hormone. These results indicate that the proportion of attributable variance ranges from 2 to 8%, depending upon the measure. The net effect, calculated as the difference in mean hormone level between the highest and lowest genotypic group, was ~15–20% of the mean level for IGF-I, leptin, and T4.

Age-related effects on hormone measures. Circulating levels of three of the hormones were measured at two ages (4 and 15 mo) in most of the mice. This allowed us to test whether the effect of a given allele on circulating IGF-I, leptin, or T4 levels varies with age. For a more systematic exploration of this issue, we performed a repeated measures ANOVA for each of the 10 markers with an experiment-wise $P < 0.05$, as well as for the two leptin QTL for which experiment-wise $P < 0.10$. For most of the markers the [age × genotype] interaction term failed to reach statistical significance, providing no evidence for an age-specific effect. There were five instances, however, in which the strength of the genetic effect was indeed age sensitive. In four of five of these cases, the effect of the genetic polymorphism is more dramatic at the older age than it is in young mice. In particular, for IGF-I the markers D3Mit25 and D17Mit46, and for leptin the markers D11Mit289 and D13Mit64, all had a significant effect on circulating hormone levels in 15- vs. 4-mo-old mice. On the other hand, the marker D10Mit230 had a greater effect on circulating IGF-I levels in 4- vs. 15-mo-old mice. Figure 2 shows two examples of situations in which the effect of an allele is equally apparent at both ages tested (IGF-I, D8Mit51; leptin, D3Mit127) and two others for which the allele effect is apparent only at the older age (IGF-I, D3Mit25; leptin, D11Mit289). In this example, for IGF-I $p(I) = 0.40$ for the marker D8Mit51, whereas $p(I) = 0.01$ for the marker D3Mit25. This suggests that the effect of D3Mit25 on circulating IGF-I levels changes with age but that D8Mit51 does not; a suggestion that is clearly borne out by the illustration of these effects in Fig. 2.

DISCUSSION

Using a population derived from a four-way breeding scheme, we have demonstrated 12 significant QTL that regulate serum IGF-I, leptin, T4, and/or fecal CORT levels in mice using a genome-wide scan. Ten of these QTL were associated with serum IGF-I, leptin, and T4 levels at one of two ages (4 and 15 mo), one QTL was associated with the serum T4 level in both 4- and 15-mo-old mice, and one QTL was associated with fecal CORT levels in 17-mo-old female mice (the only age at which CORT was tested). In addition, we found suggestive evidence for nine other QTL (experiment-wise $0.05 < P < 0.20$) with effects on serum IGF-I, leptin, and T4. Moreover, for each of the QTL with an experiment-wise $P < 0.10$, post hoc analyses revealed that the genetic effects of each of these loci was additive and that they explained ~2–8% of the variation in hormone levels at each of the ages tested.
Previous studies have also identified QTL for serum IGF-I (8, 45) and leptin (8) in mice. There was, however, little overlap between the QTL identified in the previous studies and the QTL identified here. For example, the chromosome 14 QTL for serum leptin identified by Brockman and colleagues (8) was documented ample, the chromosome 14 QTL for serum leptin identified by Brockman and colleagues (8) was documented and are often clustered in proximity to other QTL proximately the same region as a suggestive QTL on chromosome 15 identified in this study.

To the best of our knowledge this study is the first to identify any QTL for either T4 or CORT in mice, although two QTL for serum cortisol levels have been reported for humans (42). Interestingly, both of these human QTL are on human chromosome 1q and map to a region that is orthologous to that containing the locus D1Mit105 in mice, linked in our study to mouse CORT levels (http://www.informatics.jax.org/reports/homologymap/mouse_human.shtml).

For several of these loci, likely candidate genes can readily be identified. In addition to the linkage of D10Mit230 to the mouse Igf1 locus mentioned earlier, we note that the IGF-I QTL linked to D17Mit46 is in close proximity to the IGF-II receptor (Igf2r) gene. Furthermore, the markers D4Mit155 and D15Mit100 are included in regions on each of the two chromosomes that contain genes integral to the metabolic control of thyroid hormones: type I iodothyronine deiodinase (Dio1) on chromosome 4; and the thyroglobulin releasing hormone receptor (Trhr) and thyroglobulin (Tgn) on chromosome 15. Each of the chromosomal regions identified by this low-resolution study, however, may well contain additional genes with direct or indirect effects on hormone levels, and additional work will be needed to determine the specific locus (or loci) responsible for the effects documented in Table 2.

Each of the 12 loci with an experiment-wise P ≤ 0.10 was tested formally to see whether we could exclude the hypothesis of age independence. In five cases we were able to reject the null hypothesis, and found that in four of these five cases the genetic effect was stronger in older than in young animals. For example, the loci at D3Mit25 and D17Mit46 had only negligible
effects on the serum level of IGF-I in 4-mo-old mice but a very large effect in 15-mo individuals. However, the opposite relationship was observed for the locus D10Mit230, which had an appreciable effect on serum IGF-I levels in 4-mo-old mice but little effect in the 15-mo-old individuals. Thus five of the QTL seem to have an age-sensitive effect, and four of these only manifest their effect later in life. It will be interesting to learn whether these affect different metabolic pathways than do loci whose effect on hormone levels is established in young adults and unvarying thereafter.

In a previous study, mice generated using an identical breeding scheme indicated that there were multiple QTL for the regulation of T-cell subset distributions in this stock and that the effects of these loci could either be stable or age specific (29), similar to what we observed in this study. Interestingly, the QTL at D13Mit57 identified by Jackson and colleagues (29) for the regulation of CD4 levels in 18-mo-old mice and the QTL at D13Mit64 identified in this study for the regulation of serum leptin in 15-mo-old mice are only 20 cM apart on chromosome 13 and only exhibit their effects in middle-aged mice (15 and 18-mo-old). This suggests that the regions of chromosome 13 containing and/or flanked by these two loci may harbor genes that are capable of modulating the pace of age-related changes across multiple physiological systems. This segment of chromosome 13 bears a host of genes known to affect proliferative, regenerative, and cell death pathways. It will be of considerable interest to follow-up on these findings to identify other potential loci with significant age-related effects on multiple systems.

We thank Emily Gray and Dana Knutzen for technical assistance with the genotyping, Gretchen Buehner and Maggie Vergera for technical assistance and support in this study for the regulation of serum leptin in 15-mo-old individuals. Thus it was of considerable interest to follow-up on these findings to identify other potential loci with significant age-related effects on multiple systems.

**DISCLOSURES**

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