

Quantitative Trait Loci Associated with Elevated Thyroid-Stimulating Hormone in the Wistar-Kyoto Rat

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Thyroid hormones are essential for the regulation of developmental and physiological processes. The genetic factors underlying naturally occurring variability in mammalian thyroid function are, however, only partially understood. Genetic control of thyroid function can be studied with animal models such as the inbred Wistar-Kyoto (WKY) rat strain. Previous studies established that WKY rats have elevated TSH, slightly elevated total T_3 , and normal total T_4 levels compared with Wistar controls. The present study confirmed a persistent 24-h elevation of TSH in WKY rats compared with the Fisher 344 (F344) rat, another inbred strain. Acute T_3 challenge (25 $\mu\text{g}/100$ g body weight ip) suppressed serum TSH and T_4 levels in both strains. Quantitative trait locus analysis of elevated TSH

in a reciprocally bred WKY \times F344 F2 population identified one highly significant locus on chromosome 6 (LOD = 11.7, TSH-1) and one suggestive locus on chromosome 5 (LOD = 2.3, TSH-2). The confidence interval of TSH-1 contains the TSH receptor and type 2 deiodinase genes, and TSH-2 contains the type 1 deiodinase gene. The WKY alleles of each gene contain sequence alterations, but additional studies are indicated to identify the specific gene or genes responsible for altered regulation of the thyroid axis. These findings suggest that one or more genetic alterations within the TSH-1 locus significantly contribute to the altered thyroid function tests of the WKY rat. (*Endocrinology* 146: 870–878, 2005)

THYROID HORMONES REGULATE a variety of physiological processes, including nervous system development and adult metabolism, cardiac output, glucose balance, and immune and reproductive function. Most investigations of the genetics of hypothalamic-pituitary-thyroid (HPT) axis function focus on single-gene disorders, but recent studies examined the influence of quantitative genetic variation in normal populations. Genetic factors were found to account for one quarter to two thirds of the normal phenotypic variation in thyroid axis hormone measures (1), and genetic polymorphisms in several genes were associated with normal variation in HPT axis hormones (2). Quantitative traits such as these can be investigated with experimental crosses between inbred rodent strains that simulate an outbred population. Genetic techniques such as quantitative trait locus (QTL) analysis can be applied to such a cross to identify chromosomal regions contributing to the variance within a phenotype (3).

The inbred Wistar-Kyoto (WKY) rat strain was identified as hormonally and behaviorally responsive to stress (4). In-

vestigations of their thyroid axis function revealed that WKY rats have abnormally high serum TSH but normal serum total T_4 (tT_4) in comparison with Wistars, their outbred progenitor strain (5). The TSH elevation is persistent throughout the 24-h cycle in WKY rats, with an exaggerated peak at 1200 h (6). Additionally, a smaller elevation in serum tT_3 levels is often seen (5, 6). Despite the elevated tT_3 , mRNA levels of prepro-TRH (ppTRH) are normal in the WKY rat hypothalamus, as are mRNA levels of the prohormone convertases PC1 and PC2, which cleave ppTRH and are negatively regulated by T_3 (7, 8). Induction of chronic hyper- and hypothyroid states in WKY rats elicit appropriate hormonal responses, although TSH levels rose higher in response to hypothyroidism in WKY rats than in Wistar controls (5), a finding that is consistent with the elevated TSH in euthyroid WKY rats.

Although an increase in basal TSH might be expected to elevate T_3 levels, it is not obvious how it could be doing so without also increasing T_4 levels. Thus, we further characterized the WKY phenotype in comparison with a phenotypically and genotypically distinct strain, the inbred Fisher-344 (F344) rat (9). We analyzed the circadian rhythm of serum TSH, tT_3 , and tT_4 levels in WKY and F344 rats, and performed an acute T_3 challenge to gauge the responsiveness and kinetics of the WKY rats' TSH to T_3 suppression. Importantly, the WKY thyroid phenotype does not mimic any known monogenic thyroid axis disorder. Therefore, we hypothesized that the WKY dysregulation of TSH secretion is either polygenic or the consequence of a previously unrecognized modifier of HPT function. We took a genetic approach and employed QTL analysis, a powerful method that identifies

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Abbreviations: Chr6, Chromosome 6; cM, centimorgans; DIO1, type I deiodinase; tT_3 , free T_3 ; HPT, hypothalamic-pituitary-thyroid; OPA, opal; PORF-1, preoptic regulatory factor 1; ppTRH, prepro-TRH; QTL, quantitative trait locus; RTH, resistance to thyroid hormone; SeC, selenocysteine; SECIS, SeC insertion sequence; SNP, single-nucleotide polymorphism; TSHR, TSH receptor; tT_4 , total T_4 ; UTR, untranslated region; WKY, Wistar-Kyoto.

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genetic loci affecting a continuous trait of interest (10). WKY and F344 animals were reciprocally bred to produce 486 F2 intercross progeny. QTL analysis was performed on the F2 generation to identify genetic loci that significantly contribute to elevated TSH levels in WKY rats.

Materials and Methods

Animal husbandry

All animal experimentation was approved by the Northwestern University Animal Care and Use Committee. Animals were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 8–10 wk of age (males, 200–250 g; females, 150–200 g) and maintained in a 14:10-h light:dark cycle under constant ambient temperature (21 ± 1 C) with food and water available *ad libitum*. Animals newly obtained for use in experiments were allowed to rest for 2 wk after shipping and were handled during that period to reduce handling stress for the experiment.

Blood collection and RIA

In each experiment, the indicated amount of whole blood was collected into EDTA-coated tubes (2 mg/1.5-ml tube) and spun at 4 C, and serum was collected and stored at -80 C for later RIA. RIA for TSH was carried out as described in Rittenhouse and Redei (11). Standards and specific antiserum were obtained from Dr. Parlow at the National Hormone and Peptide Program (National Institute of Diabetes and Digestive and Kidney Diseases, Baltimore, MD). Rat TSH RP-3 (lot no. AFP5512B) was used for the iodination and standards. Assay sensitivity was 1.0 pg/tube with an intraassay coefficient of variation of 10.5%. RIAs for tT_3 , free T_3 (fT_3), tT_4 , and fT_4 were performed with ImmChem coated tubes from ICN Pharmaceuticals/MP Biomedicals (Costa Mesa, CA), using the protocols provided. Sensitivity limits and intraassay coefficients of variation were 6.7 ng/dl, 4.5% for tT_3 ; 0.76 μ g/dl, 8.1% for tT_4 ; 0.06 pg/ml, 3.6% for fT_3 ; and 0.045 ng/dl, 3.8% for fT_4 .

The 24-h secretion of HPT hormones

Adult male F344 and WKY rats ($n = 8$ and 6) were weighed and implanted with a jugular cannula as previously described (6). The 24-h bleed took place 2 d after the surgery. Blood samples were collected at 0800, 1100, 1400, 1700, 2000, 2200, 0030, 0300, 0600, and 0800 h. At each time point, 0.6 ml of blood was collected, and the same amount of donor blood was given back to each animal. A repeated-measures one-way ANOVA was used to determine the statistical significance in hormone levels across the 24-h period between WKY and F344 rats. A Bonferroni *post hoc* test was used to determine specific time points where statistical differences were seen.

Acute T_3 challenge

Adult male WKY and F344 rats ($n = 25$ and 26) were weighed and injected with T_3 (25 μ g/100 g body weight) or saline *ip* between 1100 and 1500 h, and 4–8 ml of trunk blood was collected 3 or 24 h after injection. Data were analyzed for significance by two-way ANOVA with Tukey honestly significantly different *post hoc* tests performed for each pair of groups.

QTL analysis: comparison strain selection, animal breeding, and phenotyping

Hormonal studies of the adrenal system in WKY indicated that the F344 strain could be an appropriately phenotypically different strain (9). The Rat Genome Database (<http://rgd.mcw.edu>) reported a global polymorphism rate of 68% and a useful polymorphism rate (>2 bp) of 54% between WKY and F344. Preliminary studies (unpublished; Table 1 is representative) of the thyroid axis found that the F344 strain had TSH values low enough to be a good comparison strain with WKY animals.

Parental WKY and F344 animals were bred reciprocally (WKY females mated with F344 males and vice versa), pairing one male with two females, to generate 121 F1 animals. Dominance was calculated (Posidente, B., personal communication) using *t* tests: a two-tailed significant difference between the F1 value and the midpoint between the

parental values indicates that one of the alleles is dominant; another *t* test comparing F1 and the dominant parent means determines whether the dominance is partial or complete. Sister-brother breeding of both lineages (WKY mother and F344 mother) of F1s generated 486 F2 generation animals. Pups were weaned at 24 d of age, separated by sex and housed three to five animals per cage. At weaning, 5-mm tail samples were collected for isolation of genomic DNA.

Serum was collected from F2 generation animals at 14 wk of age using the tail-cut method (12, 13). Samples were collected between 1300 and 1500 h, when TSH levels are highest (Fig. 1A). Within 2 min of removal from the cage, animals were placed into a restraining bag, tails were nicked, and blood samples were collected on ice.

QTL analysis: genotyping

Genomic DNA was extracted from tail samples of F2 animals by phenol-chloroform extraction. For the initial genome scan, 108 simple-sequence-length polymorphism markers polymorphic by at least 2 bp between WKY and F344 strains were chosen with the Genome Scanner tool at the Rat Genetic Database, Medical College of Wisconsin (Milwaukee, WI) (<http://rgd.mcw.edu>), and purchased from Research Genetics (Huntsville, AL). Markers were spaced an average of 16 centimorgans (cM) (range, 2–27 cM) apart. For the later fine-mapping genome scans, additional markers were chosen to closely flank the region identified by the first genome scan. Markers with interstrain differences under 12 bp were amplified in PCR of 5 μ l and visualized by overnight autoradiography on 6% polyacrylamide gels, as previously described (14). Markers with interstrain differences larger than 12 bp were amplified in PCR of 20 μ l (2 μ l DNA, 50 ng/ μ l; 2 μ l 10 \times PCR buffer; 1.6 μ l dNTP, 1.25 mM; 1.2 μ l $MgCl_2$, 25 mM; 2 μ l BSA, 10 mg/ml; 10.7 μ l

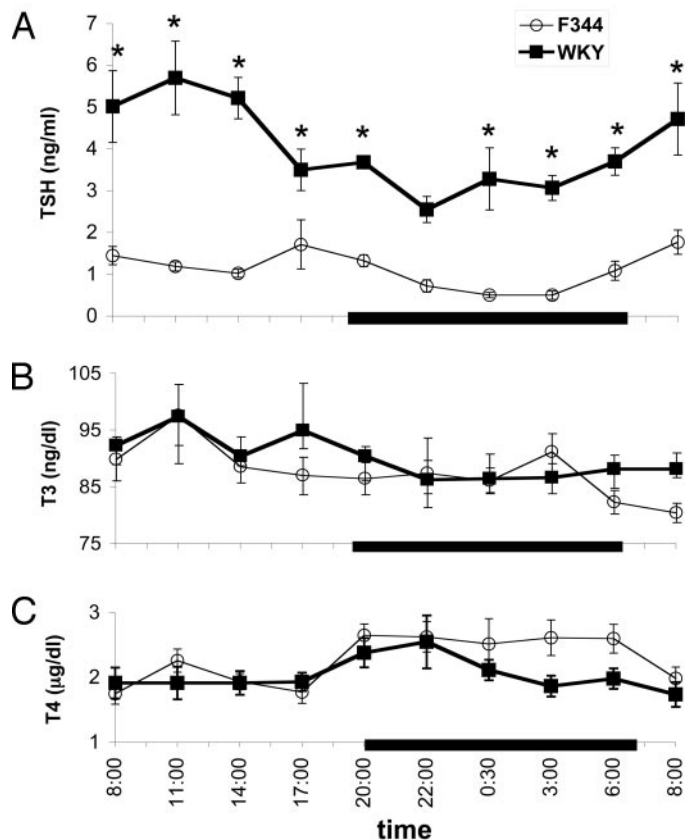


FIG. 1. Twenty-four-hour profiles of serum TSH (ng/ml; A), tT_3 levels (ng/dl; B), and tT_4 levels (μ g/dl; C) in male WKY and F344 rats. All values are means \pm SEM of five to eight animals per strain at each time point. The dark bar along the x-axis represents the dark phase (2000–0600 h CDT). *, $P < 0.05$ by Bonferroni *post hoc* test, WKY vs. F344.

ddH₂O; 0.2 μ l forward primer, 100 μ M; 0.2 μ l reverse primer, 100 μ M; and 0.5 U *Taq* polymerase, as used in the acrylamide procedure) and visualized on agarose gels (NuSieve or GenePure, ISC Bioexpress, Kaysville, UT) using ethidium bromide staining (12–17 bp difference, 4%; 18 bp or greater, 3%).

QTL analysis: genome scans

Standard genome scans controlling for sex and lineage differences were performed using the pseudomarker 0.91 software package (15). TSH values were log-transformed [$\log(x + 1)$] to minimize skew. Significance thresholds were established using permutation analysis (16). QTLs considered significant exceeded the 0.05 genome-wide adjusted threshold (LOD = 4.0), and suggestive QTLs exceeded or approached the 0.10 genome-wide adjusted threshold (LOD = 2.8) (17). Because the covariates of sex and lineage had a large effect on other traits studied in this cross (18), scans were also performed using sex and lineage as additive or interacting covariates. Complete data files and analysis scripts for the analyses carried out here are available at <http://www.jax.org/research/churchill>.

A pairwise search strategy was employed to search for epistatic interactions between QTL (19, 20). Significant interaction was determined by requiring a joint LOD greater than 11 and significance level of the interaction component alone at $P < 0.001$.

Sequencing and analysis of candidate genes

Markers were designed with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to cover the target region with approximately 100-bp overlap between amplicons and at either end and were ordered from Integrated DNA Technologies (Coralville, IA). PCRs were performed with Platinum PCR Supermix from Invitrogen (Carlsbad, CA), and products were purified with the QIAquick PCR purification kit from QIAGEN (Valencia, CA) and sequenced in both directions by ACGT Inc. (Wheeling, IL). Sequences were aligned using the Sequencher program from GeneCodes (Ann Arbor, MI) and compared with published sequences (accession nos.: TSHR, M34842; DIO2, U53505; DIO3, NM_017210) obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) or genomic sequences (Rat Genome Sequences Con-

sortium version 3.1, July 2003) obtained from University of California, Santa Cruz (<http://genome.ucsc.edu>).

Results

Diurnal secretion of TSH, tT_4 , and tT_3 in WKY and F344 rats

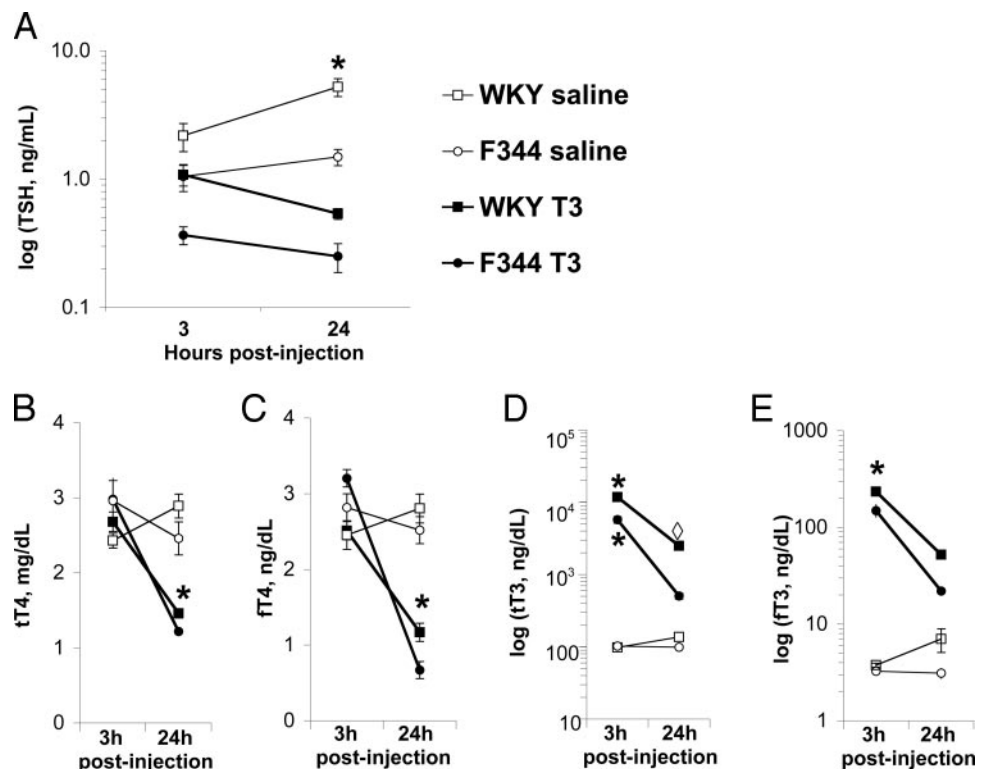
The diurnal secretion of serum TSH, tT_3 , and tT_4 were measured in freely moving WKY and F344 males 2 d after jugular cannulation. Blood was taken every 2–3 h for a 24-h period, and repeated-measures ANOVAs were performed on TSH, tT_3 , and tT_4 levels. TSH levels (Fig. 1A) were higher in WKY than F344 rats (strain, $F_{9,108} = 104.8$; $P < 0.001$) and had a circadian rhythm (time, $F_{9,108} = 5.7$; $P < 0.001$) that differed between the strains (strain \times time, $F_{9,108} = 2.9$; $P = 0.005$). Levels of TSH were significantly higher in WKY relative to F344 rats at each time point sampled except at 2200 h (Bonferroni multiple comparisons), similar to a previous study comparing the WKY to the Wistar strain (6).

WKY rats showed a trend toward higher levels of tT_3 relative to F344 rats (Fig. 1B), consistent with some earlier findings (5). Total T_3 was subject to a circadian rhythm (time, $F_{9,70} = 3.8$; $P = 0.001$); the difference between the strains' rhythms approached significance (strain \times time, $F_{9,70} = 2.0$; $P = 0.06$). There was a detectable circadian rhythm in tT_4 secretion (time, $F_{9,104} = 3.4$; $P = 0.001$), but there were no significant differences in serum tT_4 levels between WKY and F344 rats (Fig. 1C).

T_3 -induced suppression of TSH and tT_4 in WKY and F344 rats

Male WKY and F344 animals were acutely challenged with supraphysiological doses of T_3 and responses of TSH, tT_3 ,

FIG. 2. Hormonal responses to acute T_3 challenge in male WKY and F344 rats. Values are means \pm SEM of five to seven animals per data point, and group comparisons were done with the Tukey *post hoc* test. A, Serum TSH (ng/ml); *, $P < 0.001$, WKY controls at 24 h post injection have TSH significantly higher than all other groups. B, Serum total T_4 (tT_4 ; μ g/dl); and C, Free T_4 (fT_4 ; ng/dl). 24 h post- T_3 treatment, WKY and F344 tT_4 and fT_4 levels are not different, but do significantly differ from all other groups (*, $P < 0.001$). D, Serum total T_3 (ng/dl) and E) free T_3 (fT_3 ; ng/dL). At 3 h post-treatment, T_3 -treated WKY and F344 significantly differ from each other and from all other groups (*, $P < 0.01$); at 24 h, T_3 -injected WKY total T_3 levels significantly differ from all except the corresponding treatment group (224 $P < 0.05$, $P = 0.061$ between F344 and WKY tT_3 24 h), but free T_3 levels in both strains are not significantly different after 24 h.



tT_{4r} , fT_{3r} , and fT_4 were assayed at 3 h (1500–1700) and 24 h (1200–1500) after challenge. Results (Fig. 2) were analyzed by three-way ANOVA.

At both time points, TSH (Fig. 2A) was higher in WKY than in F344 rats (strain, $F_{7,34} = 30.0$; $P < 0.001$) and lower in T_3 -treated animals compared with controls (treatment, $F_{7,34} = 51.7$; $P < 0.001$). There was an effect of time, likely because of circadian rhythmicity, on TSH levels (time, $F_{7,34} = 7.0$; $P = 0.012$) that was stronger in WKY animals (strain \times time, $F_{7,34} = 4.172$; $P = 0.049$). The midday TSH peak seen in the circadian rhythm study was also seen in control WKY animals as illustrated in Fig. 1A (strain \times time \times treatment, $F_{7,34} = 8.1$; $P = 0.008$).

There were no strain differences in tT_4 levels (Fig. 2B; $F_{7,34} = 0.4$; $P = 0.544$) or fT_4 levels (Fig. 2C; $F_{7,39} = 0.7$; $P = 0.417$). T_3 suppressed tT_4 and fT_4 levels equally in both strains (treatment \times strain: total, $F_{7,34} = 0.6$, $P = 0.432$; free, $F_{7,39} = 0.02$, $P = 0.896$). This effect was not evident at 3 h but was clearly demonstrable at 24 h after injection (treatment \times time: total, $F_{7,34} = 39.6$, $P < 0.001$; free, $F_{7,39} = 68.8$, $P < 0.001$).

Post-injection T_3 levels in WKY rats

Predictably, injection of T_3 raised total (Fig. 2D) and free (Fig. 2E) T_3 levels in both strains immediately (treatment: total, $F_{7,34} = 292.9$, $P < 0.001$; free, $F_{7,39} = 102.9$, $P < 0.001$), but the increase in T_3 in the WKY rat was much higher. After 24 h, and despite the injection of identical doses of T_3 per gram body weight, tT_3 and fT_3 levels were higher in WKY compared with F344 rats (time: total, $F_{7,34} = 153.2$, $P < 0.001$; free, $F_{7,39} = 49.7$, $P < 0.001$) (strain \times treatment: total, $F_{7,34} = 41.5$, $P < 0.001$; free, $F_{7,39} = 6.5$, $P = 0.015$). The rate of change in tT_3 between 3 and 24 h after injection differed between strains, with F344 rats showing a 10-fold decrease and WKY rats showing only a 5-fold decrease.

TSH levels in a WKY \times F344 F2 intercross

Although TSH and tT_3 were significantly different between the strains in both sexes, TSH was chosen as the phenotype to be analyzed by QTL because the differences are larger than those in tT_3 levels. Male and female WKY and F344 animals were purchased from Harlan (Indianapolis, IN) to serve as the QTL parent generation, and TSH was measured by RIA in each generation. Reciprocal breeding (F344 mother \times WKY father and WKY mother \times F344 father) was used to create strain-specific lineages.

As in previous studies (5, 6), parental generation WKY rats had higher TSH levels than F344 animals, both as a group and when analyzed by sex. Three-way ANOVA (sex, lineage, and generation) was performed upon all animals: WKY and F344 parents, F1 males and females with WKY or F344 mothers, and F2 males and females with WKY or F344 grandmothers. Table 1 gives, for each generation, the number of male and female animals, their TSH levels, and differences between groups as calculated by Tukey *post hoc* test. There were significant main effects of all three factors and of all interactions except sex \times lineage (generation \times lineage \times sex, $F_{11,575} = 4.8$; $P = 0.009$). As expected, and in all generations including the parental strains, males had higher TSH levels than females of their group (sex, $F_{11,575} = 190.3$; $P < 0.001$).

TABLE 1. Summary of TSH values for parent, F1, and F2 generations

Strain/cross	Male		Female	
	Mean TSH (ng/ml \pm SD)	n	Mean TSH (ng/ml \pm SD)	n
F344	2.23 \pm 0.62 ^{a,b}	16	1.13 \pm 0.07 ^a	15
WKY	5.28 \pm 1.16 ^c	16	3.00 \pm 0.21	15
F1	2.88 \pm 0.81 ^c	44	1.36 \pm 0.09	36
F mother	2.78 \pm 0.23	18	1.07 \pm 0.13	11
W mother	2.95 \pm 0.14	26	1.49 \pm 0.10	25
F2	2.11 \pm 1.05 ^c	237	1.22 \pm 0.04	208
F grandmother	2.10 \pm 1.16	120	1.11 \pm 0.06	103
W grandmother	2.12 \pm 0.94	117	1.33 \pm 0.06	105

^a $P < 0.001$ between WKY and F344 strain.

^b $P < 0.05$, ^c $P < 0.001$ between males and females of that strain or generation.

WKY and F344 animals were reciprocally bred to produce 121 F1 animals. F1 males and females still showed the sex difference and had low TSH levels; dominance calculations (see *Materials and Methods*) revealed that the F344 phenotype was partially dominant (F1 *vs.* midpoint: $t = 3.19$, $P = 0.003$; F1 *vs.* F344: $t = -2.09$, $P = 0.041$). F1 animals were brother-sister mated to produce 486 F2 generation animals. The sex difference and dominance of the F344 phenotype persisted in the F2 generation.

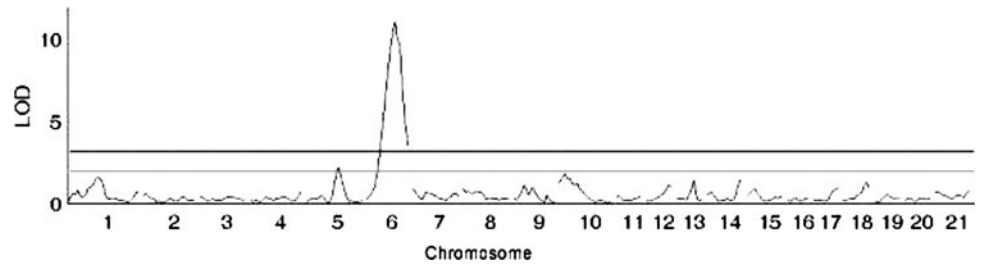
Genome scan of the F2 generation

Basal serum TSH values of adult F2 progeny were log-transformed to reduce skew, and all analyses are reported on the transformed trait. Genome scans were run with sex and lineage as additive covariates as described previously (21) to account for the observed differences in these groups of rats. We found no evidence for QTL \times covariate interaction for either sex or lineage (18, 21). Initial whole genome scans identified one highly significant locus ($P < 0.0001$) with LOD score of 11.125, located at 55 cM on chromosome 6 (chr6) (Fig. 3). One suggestive locus (peak LOD 2.25) was identified on chr5 at 65 cM. Additional markers flanking the chr6 region were genotyped (Fig. 4), which revealed that the locus is centered near D6Rat103 with a peak LOD of 13.5 and a 95% confidence interval of approximately 10 cM. Pairwise genome scans identified no epistatic interactions.

The F2 population was divided by sex, lineage, and genotype at the chr6 locus (homozygous F344, FF; homozygous WKY, WW, or FW, heterozygous) to create allele effect plots (Fig. 5). The WKY allele had an additive, dose-dependent effect on TSH levels of F2 females from a F344 grandmaternal lineage, with each WKY allele increasing TSH levels ($P = 0.003$). However, in F2 females from a WKY lineage, there was no significant effect of genotype; all had TSH levels comparable to those of a F344-lineage F2 female with two WKY alleles ($P = 0.45$). In both lineages, TSH levels were higher in WW homozygote males than in either the FF homozygotes or FW heterozygotes.

To assess the contribution of these covariate-QTL interactions, the interaction terms (chr6 \times sex and chr6 \times lineage) were included in a multiple regression with both loci and both covariates. The individual terms for the chr6 \times sex and chr6 \times lineage interactions were not significant in the re-

FIG. 3. Whole genome scan of all F2 generation animals for log(TSH) with chromosomal location on the x-axis and LOD score on the y-axis. The upper line indicates the significant (LOD = 4.0) and the lower line indicates the suggestive (LOD = 2.8) threshold.



gression model (both at $P = 0.07$). When only significant components are included, the multiple regression model (Table 2) accounts for 37% of the total variance. (Table values are based on adjusted, type III, sums of squares, and the total variance explained will differ slightly from the sums of individual contributions.)

Locus analysis

Marker positions in cM units were obtained from the Rat Genome Database (<http://rgd.mcw.edu>; SHRSP \times BN cross) and were used in interval mapping and locus analysis. Using these distances, the confidence interval of the chr6 locus was 54–64 cM. The Rat Genome Database was used to identify flanking markers for this region to use in locus analysis with genomic databases; D6Rat18 (53.4 cM) and D6Rat119 (64.6 cM) were chosen. The region between these two markers is 18.1 Mb in length (<http://genome.ucsc.edu>, July 2003 freeze of rat genome) and contains 45 known genes. It represents most of a 25-Mb cluster of 61 known genes that ranges from 53–69 cM and is flanked by gene-free gaps on either side (11 Mb proximal and 8 Mb distal). Allowing that there may be some uncertainty in the confidence interval, we took this larger cluster with more natural boundaries as our locus, which we named TSH-1.

The suggestive chr5 QTL has a peak near marker D5Rat157 (64 cM) and a confidence interval that spans a region from 54–82 cM, corresponding to 53 Mb between flanking markers D5Rat149 and D5Rat93. The broad confidence region is typical of suggestive QTLs and contains 88 known genes. We named this QTL TSH-2.

Sequence alterations in *prima facie* candidate genes

The TSH receptor (TSHR) and the enzyme type II iodothyronine deiodinase (DIO2) are located in the TSH-1 locus. Type I deiodinase (DIO1) is located in the TSH-2 locus. These three genes are directly involved in TSH regulation and T_3 availability, making them obvious candidate genes for a syndrome of inappropriate TSH secretion. The type III deiodinase enzyme (DIO3) is unmapped in the rat but is located 15–20 Mb downstream of the homologous location of TSH-1 in the human and mouse genomes. Because DIO3 could have a direct role in metabolism of T_3 , which may influence the elevated TSH levels, DIO3 was also considered as a candidate gene.

TSHR

The coding region of the TSHR cDNA was directly sequenced in three WKY and three F344 animals. No insertions

FIG. 4. Fine-map scan of additional markers on chr6 for log(TSH). Centimorgans are on the x-axis, and triangles represent markers. LOD y-axis is on the left, and the solid line plots results of interval mapping of chr6. Posterior probability density is plotted along the right y-axis and is represented with a dotted line. The gray bar represents the confidence interval of the locus. PPD, Posterior probability density.

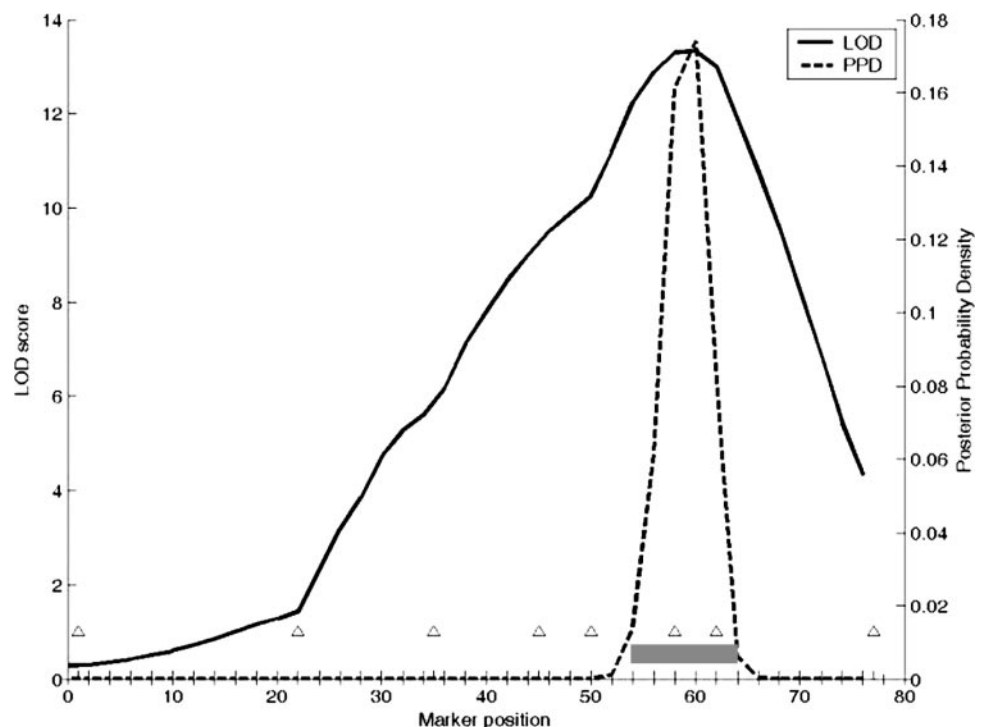
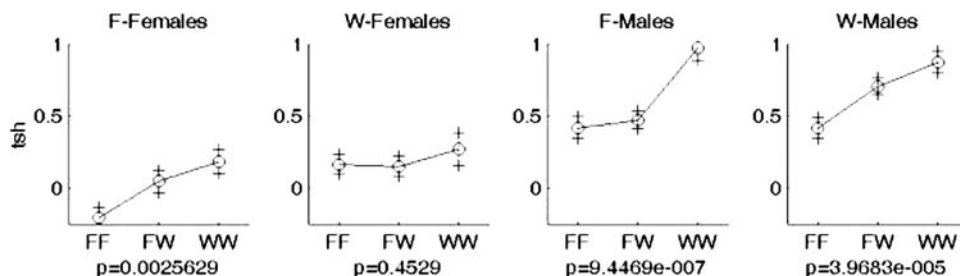


FIG. 5. Allele effect plots for the chr6 locus affecting $\log(\text{TSH})$, with F2 population separated by lineage and sex. F and W stand for F344 or WKY lineage or allele depending on context. Genotypes are along the x-axis (FF, F344 homozygote; WW, WKY homozygote; FW, heterozygote), and $\log(\text{TSH})$ values are along the y-axis with *P* values reported below each graph.



or deletions were found, but 7 bp substitutions were identified in the WKY sequence compared with the F344 and the published sequence derived from the Fisher rat (Table 3). Three of these substitutions are nonsynonymous, resulting in amino acid substitutions in the coding region of the TSHR. One of these, a G→A transition at nucleotide 1637 resulting in a change from arginine to histidine at amino acid 528, is located within the second intracellular loop and has been found in a human family with TSH abnormalities (22). Two patients heterozygous for R528H had histories of adult-onset hyperthyroidism, and although functional expression of the mutant receptor did not result in increased constitutive activity, stimulated cAMP formation and [¹²⁵I]TSH binding were reduced.

A second transition, A→G at nucleotide 191 that leads to a glutamine to arginine substitution at amino acid 46, is a recently identified nonsynonymous, single-nucleotide polymorphism (SNP) in the rat (<http://www.ncbi.nlm.nih.gov/SNP/>, ID no. 8164121). The altered amino acid is located in the extracellular region in an area thought to be a target for stimulating antibodies and directly adjacent to an eight-amino-acid sequence crucial for high-affinity TSH binding (23). The remaining variation, A→G at nucleotide 2236 resulting in threonine to alanine at amino acid 728, has not been reported in the human, rat, or mouse TSHR sequences. This amino acid is located within the intracellular C terminus, in which no activating or inactivating mutations have been found in human thyroid disorders (24); however, a nonsynonymous SNP in this region (Asp727Glu) was found to lower serum TSH in humans (2).

DIO1 and DIO2

In the rat, DIO1 is the primary deiodinase in peripheral tissues, and it converts T₄ into T₃ and rT₃ at equal rates. DIO2, the primary brain deiodinase, produces predominantly T₃ and is responsible for 25% of plasma T₃ and 75% of brain tissue T₃ (25). The coding regions and intron/exon junctions of the WKY DIO1 and DIO2 genes were sequenced from genomic DNA and found to be identical to published sequences.

TABLE 2. Multiple regression model of the TSH trait

Source	F	p (F)	% Variance	LOD	Locus name
Sex	148.67	00	22.06		
Lineage	9.03	0.003	1.34		
Chr5 (65 cM)	5.21	0.006	1.55	2.27	TSH-2
Chr6 (55 cM)	28.28	2.8×10^{-12}	8.39	11.74	TSH-1
Full model: components		00	37	41.63	

DIO1 and DIO2 3' untranslated region (UTR) and preoptic regulatory factor 1 (PORF-1)

All three deiodinase enzymes have a selenocysteine (SeC) residue in or near their functional site, without which they show no deiodinase activity (25). The SeC residue is coded for by UGA, the opal (OPA) stop codon. A stem-loop structure named the SeC insertion sequence (SECIS) element must be present in the mRNA at least 60 bp downstream from the target codon, and the appropriate *trans*-activating cofactors must also be present, for the OPA to code SeC. For this reason, and because mutations in noncoding mRNA can have functional effects (26), we sequenced the noncoding regions of the DIO1 and DIO2 mRNAs from genomic WKY DNA to look for alterations that might affect gene expression or function.

Although no rat mRNA containing the DIO2 coding region and a SECIS element has been described, complete mRNAs have been found in other species, and analysis of the genomic region downstream of the DIO2 gene found a SECIS element approximately 4.6 kb from the end of the coding region (<http://genome.unl.edu/SECISearch.html>) (27). No base pair substitutions were found in 1 kb of the promoter, the 5' UTR, or the coding region of DIO2, but nine were found in the 4.9 kb of the 3' UTR (Fig. 6). Three are known SNPs in the rat (dbSNP ID nos. 8164518, 8156256, and 8157932; http://www.ensembl.org/Rattus_norvegicus), four are identical to the mouse sequence at that nucleotide, and the remaining two are novel in the WKY rat. One of those alters an amino acid in the coding region of the PORF-1 gene, a putative transcription factor located between the coding region and SECIS element of the predicted 3' UTR of DIO2 (28, 29). PORF-1 has been hypothesized to regulate DIO2 itself, and it contains two OPA codons that could be translated as SeC by the SECIS element 1.1 kb downstream (29). This nonsynonymous SNP is adjacent to a leucine zipper motif in PORF-1 and may alter its DNA binding. The second novel substitution is noncoding and is located between PORF-1 and the SECIS element.

Three novel point alterations (in comparison with both published genomic sequence and mouse homologous se-

TABLE 3. WKY and F344 sequence variations in the TSHR coding region

Altered nucleotide	Amino acid position or change	Strains with alteration ^a
111C→G	19	F344, WKY
191A→G	Q46R	WKY ^b
1095A→G	347	F344, WKY
1331C→T	P426L	F344, WKY
1617T→C	521	WKY
1637G→A	R528H	WKY
1809T→C	585	WKY
1830T→C	592	WKY
2019T→C	655	WKY
2032G→A		
2033G→A	G660V	F344, WKY
2034G→A		
2035G→A	K661I	F344, WKY
2236A→G	T728A	WKY

^a In comparison with the published FRTL-5/Fisher rat strain sequence.

^b SNP rs8164121, identified in SD × BN cross.

quence) were found in the 0.85-kb DIO1 3' UTR: two between the stop codon and the SECIS element and one 10 bp downstream of the SECIS element (data not shown). Structural prediction of the secondary mRNA structure of the SECIS element (27) showed that the alteration nearest to it, a C→T transition, would create a non-Watson-Crick base pair (U-U) that could increase the stability of the nearest mRNA stem structure.

DIO3

DIO3 is found in brain and skin and is the most prevalent deiodinase in the placenta. It catalyzes the deiodination of T₄ to rT₃ and of rT₃ to T₂, thereby deactivating the active hormone (25). Because WKY rats have slightly elevated T₃ and show persistently elevated T₃ after T₃ injection relative to F344 animals (Fig. 2), DIO3 function may be impaired in this strain. However, sequencing of the entire DIO3 (which consists of one exon) from genomic DNA revealed no alterations in coding or noncoding regions in comparison with the published sequence.

Discussion

Using QTL analysis of a segregating F2 generation of a reciprocal WKY × F344 cross, one locus, TSH-1, was identified on chr6. This single locus accounts for over 8% of the

variance in F2 TSH levels and is highly significant with a LOD score of 11.7. QTL analysis usually identifies multiple genetic loci of smaller effect across a number of chromosomes; hence, the strength and singularity of the TSH-1 locus is remarkable. In comparison, the other locus influencing the trait, TSH-2, accounts for only 1.5% of the variance and is of suggestive significance (LOD = 2.3). Lineage, or parental origin, accounts for a similar low proportion of the variance. Sex accounts for over 20% of the variance but does so in a directly additive fashion, likely attributable to well-characterized general effects of gonadal steroid milieu on the HPT axis. Within the confidence interval of TSH-1, over 50 genes are mapped and over 100 cDNA SNPs identified. We focused our initial investigation on three obvious thyroid-related candidates within the region: the *TSHR*, *DIO2*, and *DIO3* genes. The remaining deiodinase, *DIO1*, maps to the TSH-2 locus and was also considered a candidate gene.

A well-known cause of increased TSH with normal T₄ is TSH resistance (30), such as that caused by partially inactivating human TSHR mutations. Compared with the published rat sequence, the TSHR contains three sequence alterations in its coding region, two of which are specific to the WKY strain (Table 3). Related amino acid changes reported in humans (2, 22) indicate that these TSHR alterations may partially inactivate the WKY TSHR and therefore could account for the elevated TSH component of the phenotype. Functional studies thoroughly characterizing the individual and composite effects of the three WKY polymorphisms are underway.

The three selenodeiodinases are of central importance in thyroid hormone metabolism. Two of them are mapped in the rat, *DIO2* to TSH-1 and *DIO1* to TSH-2. Although no coding region variants that alter deiodinase function have yet been described (2, 31, 32), mutagenesis of nonessential residues near the SECIS element strongly affects SeC incorporation (33). No alterations were found in the coding or noncoding regions of DIO3, but DIO2 contains alterations within its 3' UTR. Any of these could potentially affect enzyme translation or activity, either through proximity to the SECIS element (33) or by altering mRNA structure, stability, or folding (2, 34). Furthermore, one of the alterations in the WKY DIO2 3' UTR lies within the coding region of the putative transcription factor PORF-1, a gene embedded within the DIO2 mRNA between the DIO2 coding region and the SECIS element (29). The three 3' UTR alterations found

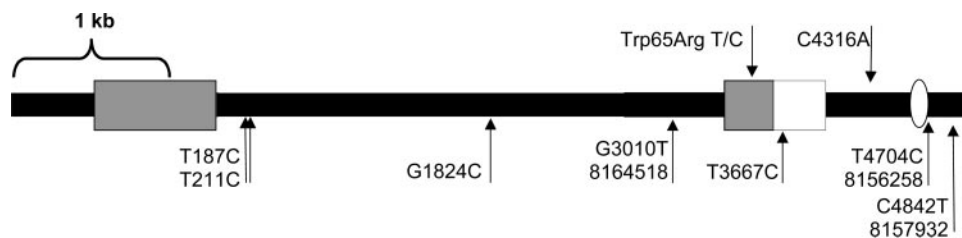


FIG. 6. Sequence variation map of DIO2 cDNA. The nucleotide change and the distance relative to the TAG stop codon of DIO2 are indicated. Arrows above the map represent variations that are novel in the WKY strain compared with published rat and mouse sequences. The arrows below the map represent known sequence variations. Those labeled with only the position and nucleotide change are identical to the mouse sequence, and those known as SNPs in the rat are labeled with their SNP identifiers. The leftmost gray box represents the DIO2 coding region, the rightmost gray box the PORF-1 coding region, and the white box the PORF-1 3' UTR. Black lines correspond to intronic sequences of DIO2 or the 3' UTR. The white oval represents the SECIS element.

TABLE 4. Genetic disorders of the HPT axis compared with the WKY rat

Alteration	tT ₃	tT ₄	TSH	Ref.
Human				
T ₃ resistance	↑	↑	↑ to ↑ ↑ ↑	35
Reduced TSH bioactivity	Normal or ↓	Normal or ↓	↑	36
TSH resistance (caused by partially inactive TSHR)	Normal to ↓	Normal to ↓	↑	30
TSHR coding SNPs	Normal	Normal	Normal or ↓	2, 22
DIO1 3' UTR SNPs	Altered ratios		Normal	2
Mouse				
TRH knockout (reduced TSH bioactivity)	ND	↓	↑	37
DIO2 knockout	Normal	↑	↑	39
Rat, WKY				
	Normal or ↑	Normal	↑	5, 6

ND, Not determined.

in the *DIO1* gene of the WKY rat may also be of significance, because 3' UTR SNPs in the human *DIO1* gene are associated with altered plasma hormone ratios (2). We are currently investigating whether these *DIO1* and *DIO2* alterations contribute to the WKY phenotype. Although they may play a role in the unusual T₄/T₃ level pattern, it is not clear whether they could also be affecting TSH levels.

Based on the result of this study, genes outside the TSH-1 and TSH-2 loci are unlikely to play a major role in the pathogenesis of the elevated TSH level found in the WKY rat. This, and the comparison with other genetic disorders in the HPT axis (Table 4) illustrate that it has a distinct etiology. For example, resistance to thyroid hormone (RTH) (reviewed in Ref. 35) is caused, in most instances, by dominant negative mutations in the TR β gene on rat chromosome 15, which is located within neither locus. The hormonal profile of RTH is distinct and characterized by elevated TSH, T₄, and T₃ levels. In contrast, the WKY rat has an elevated TSH, but the T₄ levels are normal. It is conceivable that the discrepant TSH and T₄ levels could result from reduced TSH bioactivity (36), such as that caused by absence of TRH (37) or TSH β subunit mutations (38). However, similar to the exclusion of RTH, the genetic linkage of the elevated TSH to chr6 and -5 rules out a monogenic cause linked to other chromosomes (TSH β on chr2, ppTRH on chr4, and TRH receptors on chr7 and -19). However, idiopathic and/or multigenic cases of TSH resistance exist, and 28 families with TSH resistance in the absence of TSHR mutations have been identified to date (30). The unidentified genetic causes in those families may, at least in part, reside within our loci.

In conclusion, using QTL analysis, we have successfully identified two genetic loci linked to the elevated TSH in the inbred WKY rat strain. The presence of the *TSHR*, *DIO2*, *DIO1*, and possibly *DIO3* genes within the loci, together with the fact that three of these genes harbor alterations unique to the WKY rat, suggest that they are valid candidates involved in the pathogenesis of the abnormal phenotype. Alterations similar to those found in this study have been shown to affect function of these genes in other studies (2, 22, 33). It is important to recognize that none of the single gene alterations in the TSHR or the deiodinases could plausibly explain both components of the WKY phenotype, *i.e.* the TSH elevation that is not associated with an increase in T₄ or the mild and transient T₃ elevation that is independent of T₄. It is possible that alterations in an unidentified gene within these

loci may be contributing to the phenotype. Alternatively, the alterations identified here may act as a haplotype with each other or in combination with unknown alterations.

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References

- Samollow PB, Perez G, Kammerer CM, Finegold D, Zwartjes PW, Havill LM, Comuzzie AG, Mahaney MC, Goring HH, Blangero J, Foley TP, Barnada MM 2004 Genetic and environmental influences on thyroid hormone variation in Mexican Americans. *J Clin Endocrinol Metab* 89:3276–3284
- Peeters RP, van Toor H, Klootwijk W, de Rijke YB, Kuiper GG, Uitterlinden AG, Visser TJ 2003 Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects. *J Clin Endocrinol Metab* 88:2880–2888
- Harper JM, Galecki AT, Burke DT, Pinkosky SL, Miller RA 2003 Quantitative trait loci for insulin-like growth factor I, leptin, thyroxine, and corticosterone in genetically heterogeneous mice. *Physiol Genomics* 15:44–51
- Pare W, Redei E 1995 A biobehavioral profile of an ulcer susceptible rat strain. In: Szabo S, Tache Y, eds. *Neuroendocrinology of gastrointestinal ulceration*. New York: Plenum Press; 201–208
- Redei EE, Solberg LC, Kluczynski JM, Pare WP 2001 Paradoxical hormonal and behavioral responses to hypothyroid and hyperthyroid states in the Wistar Kyoto rat. *Neuropsychopharmacology* 24:632–639
- Solberg LC, Losee-Olsen S, Turek FW, Redei E 2001 Altered hormonal secretions and circadian rhythm of activity in the Wistar Kyoto rat, a putative animal model of depression. *Am J Physiol* 281:R786–R794
- Suzuki S, Solberg LC, Redei EE, Handa RJ 2001 Prepro-thyrotropin releasing hormone 178–199 immunoreactivity is altered in the hypothalamus of the Wistar-Kyoto strain of rat. *Brain Res* 913:224–233
- Li QL, Jansen E, Brent GA, Naqvi S, Wilber JF, Friedman TC 2000 Interactions between the prohormone convertase 2 promoter and the thyroid hormone receptor. *Endocrinology* 141:3256–3266
- Redei E, Pare WP, Aird F, Kluczynski J 1994 Strain differences in hypothalamic-pituitary-adrenal activity and stress ulcer. *Am J Physiol* 266:R353–R360
- Abiola O, Angel JM, Avner P, Bachmanov AA, Belknap JK, Bennett B, Blankenhorn EP, Blizard DA, Bolivar V, Brockmann GA, Buck KJ, Bureau JF, Casley WL, Chesler EJ, Cheverud JM, Churchill GA, Cook M, Crabbe JC, Crusio WE, Darvasi A, de Haan G, Dermant P, Doerge RW, Elliot RW, Farber CR, *et al.* 2003 The nature and identification of quantitative trait loci: a community's view. *Nat Rev Genet* 4:911–916
- Rittenhouse PA, Redei E 1997 Thyroxine administration prevents streptococcal cell wall-induced inflammatory responses. *Endocrinology* 138:1434–1439
- De Souza EB, Van Loon GR 1989 Rate-sensitive glucocorticoid feedback inhibition of adrenocorticotropin and B-endorphin/B-lipotropin secretion in rats. *Endocrinology* 125:2927–2934
- Jones MT, Brush FR, Neame RL 1972 Characteristics of fast feedback control of corticotrophin release by corticosteroids. *J Endocrinol* 55:489–497

14. Shimomura K, Low-Zeddies SS, King DP, Steeves TD, Whiteley A, Kushla J, Zemenides PD, Lin A, Vitaterna MH, Churchill GA, Takahashi JS 2001 Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. *Genome Res* 11:959–980
15. Broman KW, Wu H, Sen S, Churchill GA 2003 R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 19:889–890
16. Churchill GA, Doerge RW 1994 Empirical threshold values for quantitative trait mapping. *Genetics* 138:963–971
17. Lander E, Kruglyak L 1995 Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* 11:241–247
18. Ahmadiyeh N, Churchill GA, Shimomura K, Solberg LC, Takahashi JS, Redei EE 2003 X-linked and lineage-dependent inheritance of coping responses to stress. *Mamm Genome* 14:748–757
19. Sugiyama F, Churchill GA, Li R, Libby LJ, Carver T, Yagami K, John SW, Paigen B 2002 QTL associated with blood pressure, heart rate, and heart weight in CBA/CaJ and BALB/c mice. *Physiol Genom* 10:5–12
20. Sen S, Churchill GA 2001 A statistical framework for quantitative trait mapping. *Genetics* 159:371–387
21. Solberg LC, Baum AE, Ahmadiyeh N, Churchill GA, Shimomura K, Li R, Turek FW, Takahashi JS, Redei EE 2004 Sex- and lineage-specific inheritance of depressive-like behavior in the rat. *Mamm Genome* 15:648–662
22. Gruters A, Schoneberg T, Biebermann H, Krude H, Krohn HP, Dralle H, Gudermann T 1998 Severe congenital hyperthyroidism caused by a germ-line neo mutation in the extracellular portion of the thyrotropin receptor. *J Clin Endocrinol Metab* 83:1431–1436
23. Wonerow P, Neumann S, Gudermann T, Paschke R 2001 Thyrotropin receptor mutations as a tool to understand thyrotropin receptor action. *J Mol Med* 79:707–721
24. Kopp P 2001 The TSH receptor and its role in thyroid disease. *Cell Mol Life Sci* 58:1301–1322
25. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 23:38–89
26. Mendell JT, Dietz HC 2001 When the message goes awry: disease-producing mutations that influence mRNA content and performance. *Cell* 107:411–414
27. Lescure A, Gautheret D, Carbon P, Krol A 1999 Novel selenoproteins identified in silico and in vivo by using a conserved RNA structural motif. *J Biol Chem* 274:38147–38154
28. Nowak FV 1990 Cloning of two hypothalamic cDNAs encoding tissue-specific transcripts in the preoptic area and testis. *Mol Endocrinol* 4:1205–1210
29. Nowak FV 2003 Expression and characterization of the preoptic regulatory factor-1 and -2 peptides. *Regul Pept* 115:179–185
30. Refetoff S 2003 Resistance to thyrotropin. *J Endocrinol Invest* 26:770–779
31. Hamann A, Disque C, Munzberg H, Tafel J, Hinney A, Mayer H, Hebebrand J, Ziegler R 2001 Mutational screening of the human type II deiodinase gene. *Horm Metab Res* 33:508–509
32. Mentuccia D, Proietti-Pannunzi L, Tanner K, Bacci V, Pollin TI, Poehlman ET, Shuldiner AR, Celi FS 2002 Association between a novel variant of the human type 2 deiodinase gene Thr92Ala and insulin resistance: evidence of interaction with the Trp64Arg variant of the β -3-adrenergic receptor. *Diabetes* 51:880–883
33. Martin 3rd GW, Harney JW, Berry MJ 1998 Functionality of mutations at conserved nucleotides in eukaryotic SECIS elements is determined by the identity of a single nonconserved nucleotide. *RNA* 4:65–73
34. Gereben B, Kollar A, Harney JW, Larsen PR 2002 The mRNA structure has potent regulatory effects on type 2 iodothyronine deiodinase expression. *Mol Endocrinol* 16:1667–1679
35. Refetoff S, Weiss RE, Usala SJ 1993 The syndromes of resistance to thyroid hormone. *Endocr Rev* 14:348–399
36. Persani L 1998 Hypothalamic thyrotropin-releasing hormone and thyrotropin biological activity. *Thyroid* 8:941–946
37. Yamada M, Saga Y, Shibusawa N, Hirato J, Murakami M, Iwasaki T, Hashimoto K, Satoh T, Wakabayashi K, Taketo MM, Mori M 1997 Tertiary hypothyroidism and hyperglycemia in mice with targeted disruption of the thyrotropin-releasing hormone gene. *Proc Natl Acad Sci USA* 94:10862–10867
38. Medeiros-Neto G, Herodotou DT, Rajan S, Kommareddi S, de Lacerda L, Sandrini R, Boguszewski MC, Hollenberg AN, Radovick S, Wondisford FE 1996 A circulating, biologically inactive thyrotropin caused by a mutation in the β -subunit gene. *J Clin Invest* 97:1250–1256
39. Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA 2001 Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T₄. *Mol Endocrinol* 15:2137–2148

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