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Sequence variants in the 5′ flanking region of the leptin gene are associated with obesity in women

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SUMMARY

Few mutations have been found in the human leptin gene and the relationship between leptin gene sequence variation and human overweight is uncertain. To determine whether sequence variation within the leptin gene and its regulatory elements contribute to extreme obesity, we screened ~ 3 kb of the 5′ flanking region and the three exons in 125 unrelated extremely obese (BMI $\geqslant$ 40 kg/m²) and 86 average weight women (BMI < 27 kg/m²). Within the protein coding regions only one heterozygous silent mutation was found (codon 102; AAC/AAT). Within the 5′ flanking region, six frequent sequence variants were detected ($q > 0.10$), and the allele frequencies of three of these variants differed between obese and average weight Caucasian women (+19, $\chi^2 = 4.46$, $p = 0.035$; −1823, $\chi^2 = 4.36$, $p = 0.037$; −2548, $\chi^2 = 5.73$, $p = 0.017$). Nine infrequent sequence variants were detected ($q < 0.05$) but they did not occur more often among obese women compared with those of average-weight. For extremely obese women, three polymorphisms (+19, −188, and −633) predicted the degree of obesity. Allelic variants may influence the regulation of the leptin gene and thereby influence body weight, particularly among extremely obese women. However, given the low variability in coding regions and the high variability in the 5′ flanking region, discerning the functional significance of each variant is likely to be difficult.

Introduction

Two families have been described with mutations in the protein coding regions of the leptin gene, and family members with these mutations recapitulate the phenotype of obese *Lep*^{ob} mice (Montague *et al.* 1997; Strobel *et al.* 1998). Most obese subjects have no missense or nonsense mutations within the leptin gene (Considine *et al.* 1995; Considine *et al.* 1996; Maffei *et al.* 1996; Niki *et al.* 1996; Carlsson *et al.* 1997; Echwald *et al.* 1997). These findings suggest individual sequence differences in protein coding regions are a rare cause of obesity. However, leptin is an important hormone in body weight regulation (Friedman & Halaas 1998), and a recent meta-analysis of mixed linkage results suggests the region of chromosome 7 containing the leptin gene does co-segregate with extreme obesity-related phenotypes (Allison & Heo, 1998).

Because variation within the protein coding regions of the leptin gene is rare, investigators have begun to characterize the 5′ flanking region and the promoter region (Oksanen *et al.* 1997; Shigemoto *et al.* 1997; Hager *et al.* 1998; Karvonen *et al.* 1998; Mammes *et al.* 1998). Several nucleotide sequence variants have been described, and we sought to establish whether these variants were associated with obesity, and to detect new variants.

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Materials and methods

Subjects

One hundred and twenty-five unrelated obese women (100 Caucasian and 25 African-American) were studied as a part of an ongoing study of the genetics of body weight (Price *et al*. 1998). In addition to being extremely obese themselves (BMI $\geqslant 40$), many of the women had a family history of obesity, with at least one first degree relative with a BMI $\geqslant 30$. Eighty-six average-weight control subjects (55 Caucasian, 31 African-American) were studied. The control subjects were women recruited through mental health protocols. The control sample (BMI < 27) was selected without regard for family history of obesity. All subjects gave informed consent and the protocol was approved by the Committee on Studies Involving Human Beings at the University of Pennsylvania.

DNA preparation and screening for sequence variants

DNA was extracted from blood samples using the high salt method (Lahiri & Nurnberger, 1991), and used as a template for PCR amplification. Overlapping sets of end-labelled primers were constructed using a previously published sequence (Gong *et al*. 1996) to amplify each exon, as well as the 5′ flanking region (primers and details of PCR conditions available upon request). After the DNA was denatured for 5 min, 3 μl of each PCR product was loaded onto a 0.5 × MDE gel (FMC) for single strand conformation polymorphism (SSCP) analysis. Samples were electrophoresed at 2 W constant power at room temperature for > 14 h. Selected samples with shifted bands were re-amplified in 100 μl volume, the resulting DNA purified using QIAquick PCR Purification Kit (QIAGEN), and diluted to 20 ng/μl. The Genetics Core Facility at the University of Pennsylvania sequenced this DNA with an ABI 377 automatic sequencer.

For the +19 polymorphism, PCR products were labelled by inclusion of [^{33}P]dATP in the PCR reaction, and digested with 1.0 U MspA I (37 °C 3–6 h followed by 55 °C for > 3 h). The digested and undigested products were visualized on a 10% denaturing polyacrylamide gel. The gel was then dried and exposed to X-ray film for several days. After digestion with MspA I, DNA from persons homozygous for the G/G polymorphism had band sizes of 34 and 183 nucleotides, DNA from persons homozygous for the A/A genotype had band sizes of 217. DNA from persons heterozygous for this polymorphism had three bands of 217, 183 and 34 nucleotides. The −188 polymorphism was typed using a modification of a previously described method (Oksanen *et al*. 1997). All other variants were typed by SSCP, as above.

Statistical methods.

Subjects were grouped by allele or genotype, and the frequencies compared for obese and control groups using a χ^2 test. Because allele frequencies may differ between racial groups, all tests were conducted separately for Caucasians and African-Americans.

To determine how genotype might influence the degree of obesity, non-parametric one-way ANOVAs were conducted to determine whether genotype at a particular polymorphic site predicted phenotype within average-weight or obese groups. Because groups were selected for BMI and the phenotypes within each group were not normally distributed, a distribution-free test was used. Descriptive and other statistics were computed using SPSS 6.1.

To determine the degree of linkage disequilibrium between variant sites, allelic association (D) as computed for each combination of variant sites (where $q > 0.10$). Because D is constrained by allele frequencies that differ between marker combinations, D was expressed as a proportion of its maximal ($D/D_{\max}$) or minimum value ($D/D_{\min}$) (Lewontin, 1964). D was computed as follows: $(P_{ii}{*}P_{jj})-(P_{ij}{*}P_{ji})$ where P_{ij} is defined as the frequency of the observed haplotype carrying allele i at locus 1 and allele j at locus 2. We assessed the significance of linkage disequilibrium between alleles at two loci using a maximum likelihood method, as implemented by the EH program (Terwilliger

Table 1. *Description of sample characteristics for leptin variants for this study and previously published studies*

Study	Type	Country or ethnicity	Female /Male	BMI mean ± S.D. (range)	Age mean ± S.D. (range)
This study	Obese	Caucasian, N. Am	100/0	50.6 ± 9.1 (40.1–97.1)	39 ± 7 (23–53)
	Average	Caucasian, N. Am	55/0	21.8 ± 2.2 (17.2–26.1)	33 ± 14 (19–67)
	Obese	African-Amer	25/0	48.0 ± 9.0 (40.1–78.4)	36 ± 9 (18–54)
	Average	African-Amer	31/0	22.5 ± 3.0 (16.7–26.9)	30 ± 10 (19–63)
Shigemoto *et al.* 1997	Obese	Japanese	84#	Mean N/A (26–43.6)	N/A
Oksanen *et al.* 1997	Obese	Finnish	182/67	45.4 ± 5.6	46 ± 9 (18–60)
	Average	Finnish	80/61	22.3 ± 1.9	40 ± 11
Hager *et al.* 1998	Obese	French	291/104	47.4 ± 7.0	44 ± 12
	Average	French	67/54	23.0 ± 2.5	57 ± 11
	Mixed	French	72/0	32.0 ± 5.2 (24–49)	44 ± 10
Mammes *et al.* 1998	Obese	French	79/38	33.2 ± 5.4	42 ± 10
Karvonen *et al.* 1998	Obese	Finnish	112/29	34.7 ± 3.8	43 ± 8
	Average	Finnish	38/27	Mean N/A; BMI < 27	45–64

#Distribution of sex not available. N/A, not available or not applicable.

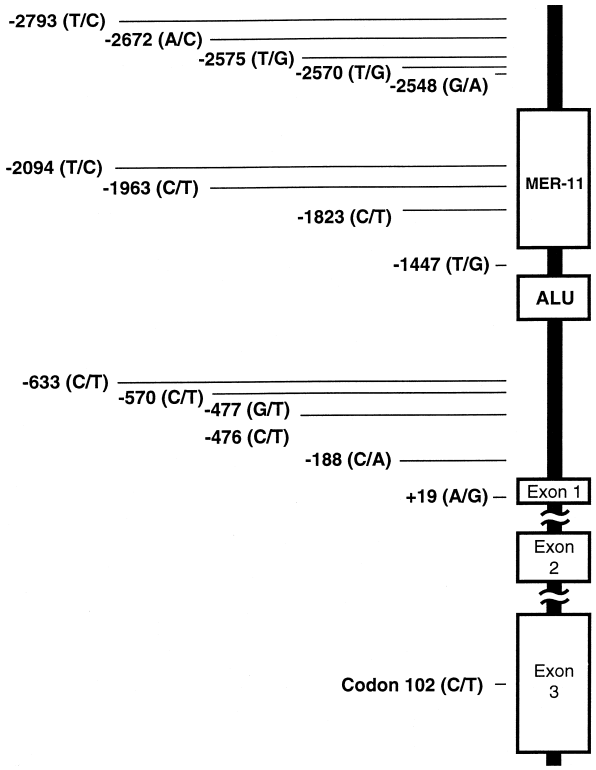

Fig. 1. The location of sequence variants for the 5′ flanking region of the leptin gene is shown. Exon 1 is untranslated. The previously published nucleotide sequence is given in parenthesis to the left, and the variant is given to the right.

& Ott, 1994). Using this method, the probability of the possible haplotypes given the genotype data is compared against the likelihood based on the assumption of independence between the two loci. Twice the log likelihood difference is tested using a χ^2 statistic with one degree of freedom.

RESULTS

Subjects

The mean BMI for the obese women was over 50 kg/m², and the mean BMI for the average-weight women was 22 kg/m² (Table 1). The obese were slightly older than the control women but the magnitude of the mean age difference was small relative to the difference in BMI. Mentally ill ($n = 31$) and mentally well controls ($n = 55$) did not differ in allele frequencies; therefore, all control subjects were pooled.

Variability in protein coding regions

Only one silent mutation in exon 3, at codon 102 (AAC/AAT; Asn) was detected among the extremely obese women.

Variability in the untranslated exon 1 and the 5′ flanking region

Fifteen sequence variants were detected within the 5′ flanking region (Fig. 1). Of these, six were common ($q > 0.10$) and located at position +19, −188, −633, −1823, −1963 and −2548. The

Table 2. *Allele frequencies for polymorphisms in the 5′ flanking and promoter region of the leptin gene between obese and average–weight groups for the current and previously published studies*

Location	Obese (frequency)	Average–weight (frequency)	Country or ethnicity	Study
+19*	G (0.71) A (0.29)	G (0.71) A (0.29)	Finnish	Karvonen *et al.* 1998
	G (0.67) A (0.33)	G (0.64) A (0.36)	French	Hager *et al.* 1998
	G (0.63) A (0.37)	G (0.75) A (0.25)	N. American	This study†
	G (0.62) A (0.38)	N/A	French	Mammes *et al.* 1998
	G (0.60)# A (0.40)	N/A	French	Hager *et al.* 1998
	G or A (1.0)[&]	N/A	Japanese	Shigemoto *et al.* 1997
−188	C (0.93) A (0.07)	C (0.94) A (0.06)	N. American	This study†
	C (0.94) A (0.06)	C (0.91) A (0.09)	Finnish	Oksanen *et al.* 1997
	C (0.94) A (0.06)	N/A	French	Mammes *et al.* 1998
−633	C (0.93) T (0.07)	N/A	French	Mammes *et al.* 1998
	C (0.94) T (0.06)	C (0.94) T (0.06)	N. American	This study†
−1387	G (0.49) A (0.51)	G (0.53) A (0.47)	German	Hinney *et al.* 1998
	G (0.59) A (0.41)	N/A	French	Mammes *et al.* 1998
−1823*	C (0.83) T (0.17)	C (0.92) T (0.08)	N. American	This study†
	C (0.86) T (0.14)	N/A	French	Mammes *et al.* 1998
−1887	C (0.91) T (0.09)	N/A	French	Mammes *et al.* 1998
−1963	T (0.91) C (0.09)	T (0.87) C (0.13)	N. American	This study†
−2437	T (0.99) G (0.01)	N/A	French	Mammes *et al.* 1998
−2548*	G (0.65) A (0.35)	G (0.49) A (0.51)	N. American	This study†
−2549	C (0.56) A (0.44)	N/A	French	Mammes *et al.* 1998

†Only Caucasian subjects were used to compute allele frequencies for the current study. #Subgroup of mixed lean and obese patients. [&]No variation was detected, and it is therefore unclear whether all subjects were A or G at +19 in this population. *Denotes $p < 0.05$. N/A, not available or not applicable.

Table 3. *Genotype frequencies for polymorphisms within the 5′ flanking region and untranslated exon 1 of the leptin gene for extremely obese Caucasian women (BMI ⩾ 40) and average-weight Caucasian women (BMI ⩽ 27)*

Location	Genotype	Obese (%)	Average (%)	χ^2	p-value
+19	G G	32 (35.2)	28 (56.0)	5.76	0.056#
	A G	50 (54.9)	19 (38.0)		
	A A	9 (9.9)	3 (6.0)		
−188	C C	85 (86.7)	48 (87.3)	0.01	0.925
	C A	13 (13.3)	7 (12.7)		
−633	C C	87 (87.0)	46 (88.5)	0.07	0.796
	C T	13 (13.0)	6 (11.5)		
−1823	C C	70 (72.2)	46 (85.2)	3.58	0.167
	C T	21 (21.6)	7 (13.0)		
	T T	6 (6.2)	1 (1.8)		
−1963	C C	82 (84.5)	45 (83.3)	3.30	0.192
	C T	12 (12.4)	4 (7.4)		
	T T	3 (3.1)	5 (9.3)		
−2548	G G	42 (42.4)	11 (21.2)	6.94	0.031*
	G A	45 (45.5)	31 (59.6)		
	A A	12 (12.1)	10 (19.2)		

Only sequence variants with an allele frequency greater than $q > 0.10$ are shown. Percentages of subjects with each genotype are given in parentheses. #$0.05 < p < 0.10$; *denotes $p < 0.05$.

allele frequencies differed between obese and average-weight groups for three of the common polymorphic sites (+19, $\chi^2 = 4.46$, $p = 0.035$; −1823, $\chi^2 = 4.36$, $p = 0.037$; −2548, $\chi^2 = 5.73$, $p = 0.017$; Table 2). The allele frequencies in Table 2 and the genotype frequencies in Table 3 were computed using only Caucasian subjects. The small number of African-American women precluded statistical comparisons between obese and control groups.

Differences between the obese and average-weight groups in allele frequency were in agree-

Table 4. *Genotype is related to phenotype among obese Caucasian women*

Location	Genotype	N	BMI	S.D.	*F*-ratio
+19	G G	32	48.9	8.4	5.78*
	A G	50	52.6	10.1	
	A A	9	48.7	8.1	
−188	C C	85	51.6	9.5	6.56**
	C A	13	45.3	3.3	
−633	C C	87	51.4	9.4	6.44**
	C T	13	45.3	3.3	

No individuals were homozygous for the −188 or −633 polymorphism and therefore these rows are not included in the table. *Denotes $p < 0.05$; **denotes $p < 0.01$.

ment with differences in genotype frequency. Of the three polymorphisms that differed in allele frequency between Caucasian obese and average–weight groups, two differed in genotype frequency (+19, −2548; Table 3). The genotype frequency for the third variant (−1823) differed marginally between obese and average-weight groups ($p = 0.17$). All genotypes were in Hardy–Weinberg equilibrium. Therefore, differences in genotype frequencies between groups reflect differing allele frequencies.

Nine rare sequence variants were detected ($q < 0.05$), −476, −477, −570, −1447, −2094, −2570, −2575, −2672, −2793. These rare variants were *not* observed more commonly among the obese Caucasian women relative to average-weight Caucasian women, but it is difficult to draw conclusions about group differences for low frequency alleles. Therefore, statistical comparisons were not made.

Racial differences in allele frequencies

The effects of race were examined by comparing genotype frequencies within the average-weight groups. Most sequence variants were equally common between the African-American and Caucasian groups, with two exceptions: −2793 (more common in African-Americans; $\chi^2 = 12.7$, $p = 0.00036$) and −2548 (more common in Caucasians, $\chi^2 = 19.7$, $p = 0.00005$). Three sequence variants were detected only once within the entire sample. An African-American woman had two adjacent sequence variants at −476 and −477 (BMI = 26.9). Another African-American woman had a sequence variant at −570 (BMI = 47.7).

Genotype is related to phenotype within the obese group

Within the group of extremely obese Caucasian women, genotype was related to the degree of obesity for several polymorphic sites (Table 4). The variants at −188 and −633 were associated with *reduced* BMI and the original sequence was associated with *elevated* BMI. For the +19 polymorphism, obese heterozygous individuals had a higher BMI than did the homozygous individuals of either genotype. Within the group of average-weight Caucasian group, leptin genotypes did not predict phenotype (all $p > 0.10$) although there was a marginal effect of the +19 polymorphism ($\chi^2_{(2)} = 4.4$, $p = 0.13$).

Evidence for linkage disequilibrium

There were strong allelic associations, consistent with linkage disequilibrium over the ∼ 3 kb region (Table 5). The two locations with the largest difference in allele frequencies between obese and average-weight groups (+19 and −2548) were in strong linkage disequilibrium with each other and were associated with most, but not all, adjacent markers. In the interval between the outer markers, there was a strong positive association among the variable sites with less common alleles (−188, −633 and −1963). This was true for both the obese and average-weight groups. The strength and pattern of linkage disequilibrium among variant sites suggests a complex historical process.

DISCUSSION

Mutations of the leptin gene do not occur frequently within the protein coding regions in obese individuals. We found no functional mutations within the protein coding regions of the leptin gene among extremely obese women, in agreement with previous studies. Several silent mutations (C to T codon 102, current study; A to G codon 25 (Shigemoto *et al.* 1997); A to G codon

Table 5. *Allelic association between polymorphisms within average-weight and obese groups*

Locus	+19	−188	−633	−1823	−1963	−2548
+19		*−0.998*	*−0.998*	*+0.105*	*−0.226*	*−1.000*
		(*0.055*)	(*0.052*)	(*0.026*)	(*0.597*)	(*0.000*)
−188	−0.996		*+1.000*	*+0.100*	*+0.556*	*−0.996*
	(0.233)		(*0.000*)	(*0.603*)	(*0.000*)	(*0.075*)
−633	−0.964	#		*+0.075*	*+0.556*	*−0.996*
	(0.427)			(*0.708*)	(*0.000*)	(*0.069*)
−1823	+0.342	−0.337	−1.000		*−0.454*	*−0.006*
	(0.196)	(0.862)	(0.315)		(*0.062*)	(*1.000*)
−1963	−1.000	+1.000	+1.000	+0.056		*+0.181*
	(0.021)	(0.000)	(0.000)	(0.699)		(*0.413*)
−2548	−1.000	−1.000	−0.999	−0.748	−0.325	
	(0.000)	(0.036)	(0.064)	(0.022)	(0.237	

Values obtained from obese Caucasian women are given above the diagonal and are *italicized* ($n = 100$); values below the diagonal are from average–weight Caucasian women ($n = 55$). Because D is constrained by allele frequencies that differ between marker combinations, D was expressed as the proportion of its maximal (D/D_{max}) or minimal (D/D_{min}) value. D/D_{min} was computed when D was negative and $D/D_{max}$was computed when D was positive. The positive or negative sign of the top value indicates the direction of D. The lower value is the p-value associated with the difference between the expected and observed haplotype frequency as calculated by the EH algorithm. #For this combination, no average–weight individuals were heterozygous for −633, and thus D could not be computed.

48 (Karvonen *et al*. 1998), and missense mutations (Val^{94}Met (Considine *et al*. 1996); Phe^{17}Leu and Val^{110}Met (Echwald *et al*. 1997); Val^{110}Met (Karvonen *et al*. 1998)) have been described.

In contrast to the protein coding regions, the 5′ flanking region is highly polymorphic. Fifteen variants were detected with a range of frequencies, over approximately a 3 kb region. The allele frequencies of these single nucleotide polymorphisms ranged from 0.5% to 50%; six were common (> 10%) and nine were rare (5%). In some cases, the nucleotide in the original sequence was less frequent in the population than the nucleotide discovered subsequently (e.g. +19 and −1963).

Four previous studies examined the nucleotide sequence of the untranslated exon 1 of the leptin gene, and three of those studies reported a polymorphism at +19 (Table 2). The allele frequency of this polymorphism varies between racial groups, with the frequency of the A allele lowest in the Finnish population (29%) and highest in an obese French population (40%). In obese Japanese patients, no sequence variants were found in this exon using SSCP.

In the current study, there were significant differences in allele frequency between obese and average weight groups for the +19 polymorphism, with the AG genotype associated with a higher body mass index among obese women. Among obese French subjects, those homozygous for the G allele had low leptin levels, but BMI was similar among subjects grouped by genotype. In the Finnish population, there was no relationship between genotype at +19 and any indices of obesity, nor were there differences in allele frequency between obese and average-weight groups. The interpretation of these discrepant results is uncertain. Differences in other clinical characteristics of the patients across studies may be responsible for the lack of agreement, though subjects were similar in age and degree of obesity (Table 1). Alternatively, the +19 variant may be in linkage disequilibrium with other variants that have a direct effect on phenotype, but the degree of disequilibrium may vary among populations. For the current sample, the +19 site is in strong linkage disequilibrium with most other variable sites within this region (Table 5).

The variants located at −188 and −633 are equally frequent in Finnish, French and American populations (Table 2). The frequencies of these variants do not differ between obese and average-weight groups in the populations studied thus far.

The variant at −1823 occurs with the same frequency in obese American and obese French

populations, but is less common in average-weight American populations (Table 2). Allele frequencies for this location are not available for average-weight French subjects, but given the observed differences in obese and average-weight American groups, further analysis of this variant is warranted.

The allele frequency at −2548 differs considerably between obese and average-weight subjects in our study but this variant has not been detected or described in other populations. French subjects are polymorphic for the adjacent nucleotide (−2549). Both of these polymorphic sites have high frequency alleles. In the French population, the A allele of −2549 was associated with higher leptin levels for obese patients and obese patients homozygous for this allele lost less weight during caloric restriction compared to subjects homozygous for the C allele. In the current study, the largest differences in allele frequency between obese and average-weight groups occurred at −2548. We have ruled out insensitive SSCP detection methods as explanations for this discrepancy and we have verified our observation through direct sequencing (11 subjects).

Three of the polymorphisms described in the current study are located in the MER-11 repetitive sequence (Kaplan *et al*. 1991) (−2094, −1963, and −1823). Although this type of DNA is usually considered to lack transcriptional function, a 60 bp placenta-specific enhancer is contained within this particular MER sequence (Bi *et al*. 1997). Moreover, the variant at −1823 occurs more frequently in obese subjects, and therefore this nucleotide change may have some functional significance. None of the remaining variants described thus far are contained within motifs with known transcriptional function when the sequence is evaluated using TESS and the TRANSFAC v3.3 database (Heinemeyer *et al*. 1998) (http://www.cbil.upenn.edu/cgi–bin/tess/tess33).

Within the obese but not average–weight groups, genotypes at +19, −188 and −633 were associated with increased BMI. Several lines of evidence support the hypothesis that these variants do not lead directly to the phenotypic differences, but rather reflect linkage disequilibrium with functional variants. First, the three variants associated with the phenotype within the obese group are in close proximity (∼ 600 bp) and are in very strong linkage disequilibrium with each other and with other variants (Table 5). The more common alleles are associated with increased BMI, consistent with the possibility that the functional variant arose on a common haplotype containing these high–frequency alleles. None of these polymorphisms appear to have similar effects on obesity in the other population groups examined (Table 1 and 2). Finally, the −188 polymorphism is unlikely to have direct effects because it does not alter leptin expression in a reporter gene system (Oksanen *et al*. 1998).

Another explanation would account for the observation that alleles at +19, −188 and −633 predict body weight among extremely obese subjects but not average-weight controls. These variants may directly influence leptin transcription only when the leptin levels are high, i.e. when a subject's fat mass is large. This possibility would explain the limitation of the association between phenotype and genotype to extremely obese subjects. It would also be congruent with the observation that linkage to the leptin gene is most consistently observed for extremely obese sibling pairs (Clement *et al*. 1996; Reed *et al*. 1996).

Because the variability of the human genome is more abundant than anticipated and is becoming increasingly well described, discerning functional from inconsequential variability is a central task. For example, there was an unexpectedly high level of variability in the lipoprotein lipase gene and adjacent regions (Nickerson *et al*. 1998) and our results for the leptin gene show a similar level of variability. A high frequency of single nucleotide polymorphisms within and adjacent to candidate genes may be the rule rather than the exception. Whatever difficulties may lie ahead in determining which variants influence gene expression or function, the description of sequence differ-

ences within the regulatory regions of the leptin gene is the first step to understanding how variation at the nucleotide level may influence body weight.

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