Brief Genetics Report

The Human *MC4R* Promoter

Characterization and Role in Obesity

Cecile Lubrano-Berthelier,[1] Martha Cavazos,[1] Catherine Le Stunff,[2] Kurt Haas,[1] Astrid Shapiro,[1] Sumei Zhang,[1] Pierre Bougneres,[2] and Christian Vaisse[1]

Heterozygous mutations in the coding sequence of the serpentine melanocortin 4 receptor (MC4R) are the most frequent genetic cause of severe human obesity. Since haploinsufficiency has been proposed as a causal mechanism of obesity associated with these mutations, reduction in gene transcription caused by mutations in the transcriptionally essential regions of the *MC4R* promoter may also be a cause of severe obesity in humans. To test this hypothesis we defined the minimal promoter region of the human MC4R and evaluated the extent of genetic variation in this region compared with the coding region in two cohorts of severely obese subjects. 5′RACE followed by functional promoter analysis in multiple cell lines indicates that an 80-bp region is essential for the transcriptional activity of the *MC4R* promoter. Systematic screening of 431 obese children and adults for mutations in the coding sequence and the minimal core promoter of *MC4R* reveals that genetic variation in the transcriptionally essential region of the *MC4R* promoter is not a significant cause of severe obesity in humans. *Diabetes* 52:2996–3000, 2003

Obesity is a complex multifactorial disease caused by the interaction of genetic and environmental factors (1,2). Heterozygous mutations in the coding region of the melanocortin 4 receptor (*MC4R*) gene are the cause of 1–6% of severe early-onset obesity cases (3–10). MC4R is a seven transmembrane G-protein–coupled receptor (GPCR) encoded by a single exon gene localized on chromosome 18q22. *MC4R* is expressed in the paraventricular nucleus of the hypothalamus, and its activation results in the inhibition of food intake. In mice, homozygous deletion of *Mc4r* results in an obese phenotype (11). In humans, several recent studies reported the screening of 2,600 subjects leading to the discovery of 46 different *MC4R* mutations associated with obesity, most of them leading to a functional alteration of the receptor (6,7,9,10).

Theoretically, *MC4R* mutations in humans could cause obesity through haploinsufficiency, a dominant-negative effect, or a combination of both (12,13). Since some heterozygous human obesity–causing *MC4R* alleles are null or early nonsense mutations, and since heterozygous deletion of the *Mc4r* in mice leads to an intermediate increase in weight between wild-type mice and mice with homozygous deletion of *Mc4r* (11), haploinsufficiency at the *MC4R* locus seems sufficient to cause obesity. Under this assumption, reduced gene transcription caused by mutations in essential regions of the *MC4R* promoter could also be a cause of obesity in humans. The minimal promoter of the human *MC4R* has not been delineated,

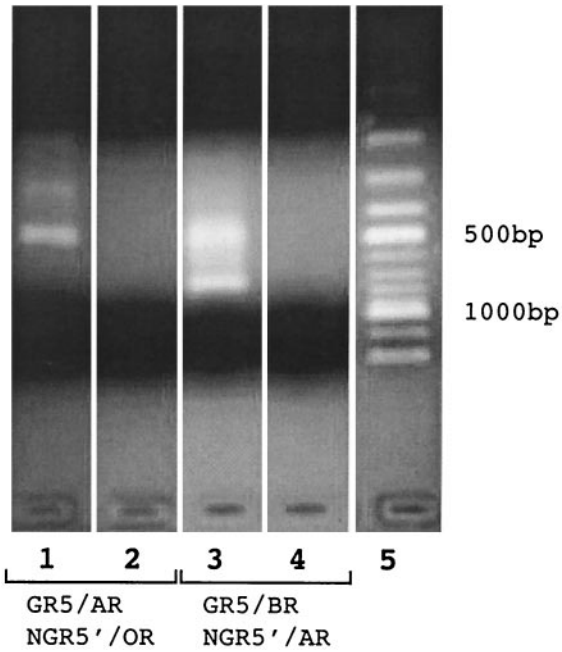

FIG. 1. Determination of the human MC4R transcription start site. *Lanes 1* and *3*: cDNA from human embryonic brain. *Lanes 2* and *4*: PCR negative control. *Lane 5*: DNA ladder. *Lanes 1* and *2*: The first PCR was performed using the GeneRacer 5′ primer and MC4R-AR and the nested PCR with nested generacer 5′ primer and MC4R-OR. *Lanes 3* and *4*: The first PCR was performed using the GeneRacer 5′ primer and MC4R-BR and the nested PCR with nested GeneRacer 5′ primer and MC4R-AR.

From the [1]Department of Medicine, Division of Endocrinology and the Diabetes Center, University of California San Francisco, San Francisco, California; and the [2]Department of Pediatric Endocrinology, Saint Vincent de Paul Hospital, University Rene Descartes, Paris, France.

Address correspondence and reprint requests to Dr. Christian Vaisse, Diabetes Center, University of California San Francisco, 513 Parnassus Ave., Box 0573, San Francisco, CA 94143-0573. E-mail: vaisse@medicine.ucsf.edu.

Received for publication 16 July 2003 and accepted in revised form 21 August 2003.

5′RACE, 5′ rapid amplification of cDNA ends; GPCR, G-protein–coupled receptor; MC4R, melanocortin 4 receptor; SNP, single nucleotide polymorphism; UCSF, University of California San Francisco; UTR, untranslated region.

FIG. 2. DNA sequence alignment of human and mouse MC4R promoter regions. The major transcription start sites are indicated by a "plain" arrow and defined as +1. Minor transcription start sites are indicated only for the human sequence with "open" arrows. The translation initiation codon ATG is indicated in lower case. Conserved regions between human and mouse are indicated in bold. The minimal promoter region determined in this study is indicated in italics. Potential *cis*-acting elements were identified using the TRANSFAC database and manual searches and are indicated as underlined. Sequences present in reverse orientation are designated with the prefix "r".

and the search for genetic variants associated with obesity at the *MC4R* locus has so far been limited to coding mutations.

In this study, we report the characterization of the minimal promoter of the *MC4R* gene and the search for sequence variants in the *MC4R* promoter associated with human obesity.

RESEARCH DESIGN AND METHODS

Human fetal brain RNA preparation and 5′RACE. Transcriptional start site determination by 5′ rapid amplification of cDNA ends (5′RACE) was performed on total human fetal brain RNA using the GeneRacer kit (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using specific primer MC4R-BR (5′ TCCACTGCAATTGAAAGCAG 3′) and avian myeloblastosis virus–reverse transcriptase. We amplified 5′ cDNA ends with MC4R-BR or MC4R-AR primer (5′ CATGGGTGAATGCAGATTCT 3′), followed by a nested PCR with MC4R-AR or MC4R-OR (5′ GCAGGAGAAGTGTGCATCCCA 3′). The PCR products were subcloned in pcDNA3.1/V5-His TOPO TA vector (Invitrogen) and analyzed by enzymatic digestion using a frequent cutter restriction enzyme (HphI) to eliminate duplicate clones. We then systematically sequenced 2 clones presenting with the same restriction map (16 clones sequenced).

Alignment of human and mouse proximal promoter. Alignments of mouse and human 5′untranslated regions (UTRs) and regions 500 bp upstream of the main transcription start site were performed and analyzed with MacVector software (Accelrys, San Diego, CA), TRANSFAC (the Transcription Factor Database), and manual searches.

Cloning of 5′ flanking *MC4R* luciferase reporter vectors. Fragments of the 5′ region of the human MC4R gene were obtained by restriction digestion of a bacterial artificial chromosome clone isolated from a human genomic bacterial artificial chromosome library (Research Genetics; Invitrogen). Fragments were ligated upstream of the Firefly luciferase gene in the plasmid pFOXLuc1 (a gift from Michael German, University of California San Francisco [UCSF]).

Cell transfection and luciferase assays. Mouse hypothalamic GT1-7 cells were provided by Richard Weiner (UCSF). Neuro2A, HEK293, and GT1-7 cells were transfected using Effectene reagent (Qiagen, Valencia, CA), and NIH3T3 cells were transfected with Lipofectamine Reagent (Invitrogen). Cells were cotransfected with *MC4R* promoter constructs and a plasmid containing the Renilla luciferase to assess for transfection efficiency. Forty-eight hours after transfection, cells were harvested and Firefly and Renilla luciferases were assessed using the Stop & Glo kit (Promega, Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity, and results were expressed relative to the result obtained with the promoterless vector.

Human subjects and phenotypes. Two cohorts were screened for mutations in the *MC4R* coding sequence and minimal promoter. The first group was comprised of 266 children (aged 12.8 ± 0.8 years) from a previously described cohort (14). The protocol was approved by Cochin Ethics Committee, and informed consent was obtained from subjects and parents.

The second population was comprised of 165 unrelated, severely obese adults (BMI 40–85.3 kg/m^2 [median 48.8]). This study was approved by the UCSF Committee on Human Research, and informed consent was obtained from subjects.

Mutation screening of the *MC4R* minimal promoter and 5′UTR. Mutation screening was performed by direct sequencing of the coding region (9), the 5′UTR, and the minimal promoter of *MC4R*. This region was amplified from lymphocyte-derived genomic DNA using primers MC4R-250 (5′ GCTATAGGT ACCCTTGAAAACTTAAAAAGG GA 3′) and MC4R PromRev1 (5′ GCTATACT CGAGAATTCCAGTGTCCCCC TGA 3′). Sequencing was performed on a ABI3700 automated DNA Sequencer (Applied Biosystems, Foster City, CA) and analyzed using Sequencher software (Gene Codes, Ann Arbor, MI).

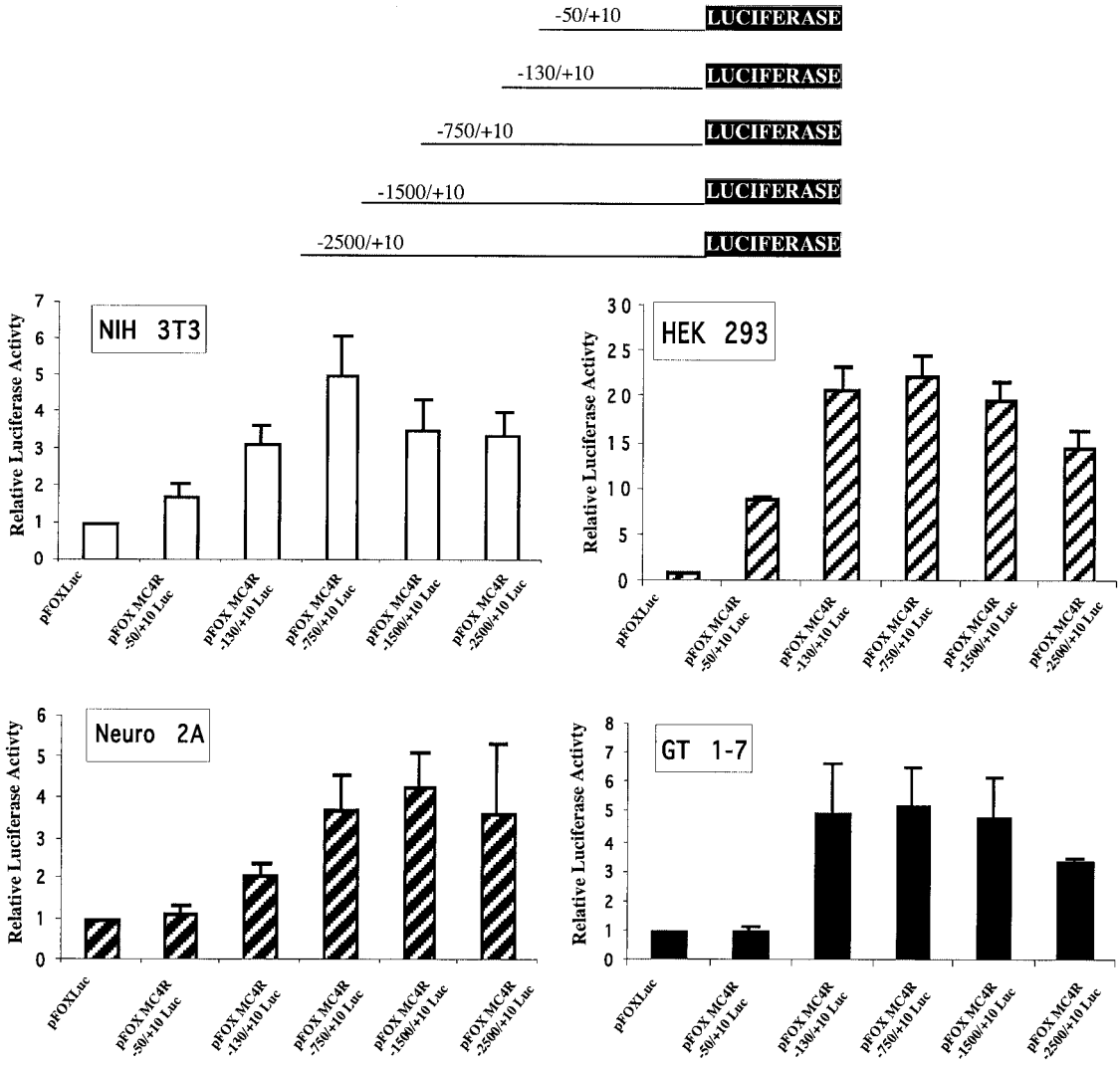

FIG. 3. Transcriptional activity of the human MC4R promoter in cell lines. Promoter fragments containing sequences extending from −2,500 to −50 (5′end) to +10 bp of the major transcription site (3′end) were ligated upstream of the firefly luciferase gene and transfected into four cell lines, mouse NIH 3T3, human HEK 293, mouse Neuro 2A, and rat GT1-7. Reporter gene activity is expressed relative to the promoterless luciferase vector in the same cell type. Transfections were performed in triplicate on at least two independent occasions, and data are shown as means ± SD.

RESULTS

Determination of the human *MC4R* transcriptional start site. We determined transcriptional start sites of the *MC4R* gene from human fetal brain RNA by 5′RACE (Fig. 1). The nucleotide sequences of all the clones were colinear with the genomic sequence in the 5′ end of the coding region of human *MC4R*, indicating that no introns were present in the 5′UTR. Analysis of human fetal brain by 5′RACE indicates that the *MC4R* gene possesses two major transcriptional start sites, at 426 and 139 bp upstream of the translation initiation codon, and multiple minor transcription start sites, ranging from 213 to 366 bp (Fig. 2). We designated the transcription start site of the longest transcript as nucleotide "+1". A TATA-like box and classical CAAT sequence (in an antisense orientation) were found at 33 and 94 bp upstream of this major transcription start site, respectively.

In silico analysis of the proximal human *MC4R* promoter region reveals the presence of E-boxes, cAMP response element binding protein, GATA and signal transducers and activators of transcription sites, homeoboxes, and a response element for the neural zinc finger family of transcription factors (Fig. 2).

Comparative genomic analysis of the mouse (15) and human *MC4R* promoter regions indicates that only the signal transducers and activators of transcription site, neural zinc finger response element, and CAAT box are conserved between species. In addition, this comparative analysis reveals the presence of a 30-bp sequence starting with the putative CAAT box that is 100% conserved between human and mouse (Fig. 2). This sequence is also present in the putative *MC4R* promoter of the rat and pig and is highly specific for the *MC4R* promoter, since BLAST analysis does not reveal the presence of another similar sequence in all four genomes. This sequence contains a potential binding site (AttGGTCA) for monomeric nuclear receptors, such as thyroid receptor or SF1 (steroidogenic factor 1). However, we did not observe any effect of SF1 on MC4R promoter activity (data not shown).

TABLE 1
Population characteristics and genetic variations in *MC4R* coding sequence and 5′ flanking region

Population	*n*	Age (years)	Sex (% M/F)	BMI (kg/m^2)	Mutation in *MC4R*			
					Coding region	*n*	Promoter	*n*
Children, France	266	12.8 ± 0.2 (4–17.2)	37 63	29.51 ± 033 (>85th) (20.19–49.95)	R7H	1	−178 A/C	15
					R18L	1	−360 G/T	1
					Q156P	1		
					506-ΔCAT	1		
					1103V	3		
					T112M	1		
Adults, U.S.	165	>18	27 73	48.8 ± 8.06 (40–85.3)	Y35STOP/ D37V	1	−178A/C	9
					631ΔCTCT	1	−176A/G	1
					R236C	1		
					I269N	1		
					P299S	1		
					Q307STOP	1		
					I251L	2		

Data are means ± SD.

Characterization of the essential promoter region for *MC4R* gene transcription. A series of progressive 5′ deletions of the human *MC4R* promoter, each extending to +10 bp of 5′UTR, were tested for transcriptional activity in nonneuronal cell lines (NIH 3T3 and HEK293) and in neuronal cell lines (Neuro 2A and GT-1-7) (Fig. 3). GT1-7 cells express the *MC4R* (16). A 2.5-kb fragment of human *MC4R* promoter stimulates transcription in all of the cell lines, with the highest relative level of expression in HEK 293 cells (Fig. 3). Serial deletions from 2.5 kb to −130 bp did not diminish the promoter activity in any of the cell lines (Fig. 3), while deletion of 80 additional base pairs abolished the transcriptional activity of the promoter in NIH 3T3, Neuro 2A, and GT1-7 cells and reduced it in HEK 293 cells. Therefore, we defined the presence of a minimal core promoter between −130 and −50 bp. However, this minimal promoter does not recapitulate tissue specificity because we observed a basal transcription activity in all cell lines tested. This result suggests that the regulation of the *MC4R* gene expression involves at least an additional repressor elsewhere in the sequence. Because they would lead to an increase in *MC4R* transcription, mutations in such tissue-specific repressors are unlikely to be a cause of severe obesity in humans.

Are *MC4R* promoter mutations implicated in human obesity? To determine whether mutations in essential regulatory regions of the *MC4R* are a significant cause of severe obesity, we screened two populations of severely obese subjects for mutations in the coding region and in the minimal *MC4R* promoter defined above (Table 1). The frequency of MC4R mutations, other than known polymorphisms, was 3.6% (95% CI 0.78–6.49) in the adult population and 1.5% (0.04–2.97) in the child population. Both results are consistent with previous results obtained in this laboratory and with the literature. We detected three variants in the 5′UTR of *MC4R*. The −178 A/C single nucleotide polymorphism (SNP) was present at a frequency of 5.57% (3.4–7.73) and has been found previously at the same frequency in nonobese control populations (17). Two rare variants (A/G and G/T at 176 and 360 bp upstream of the ATG, respectively) were found in two different subjects. In contrast, no genetic variation was observed in the minimal promoter of *MC4R* in the 431 obese subjects of this cohort.

DISCUSSION

We have used comparative genomic tools and classical promoter studies to begin to elucidate the essential regulatory mechanisms underlying human *MC4R* gene expression. The major transcriptional site, according to our 5′RACE result, is located 426 bp upstream of the start of translation, where sequence analysis indicates the presence of a consensus CAP site. The DNA sequence upstream of this site contains a CAAT box (in an antisense orientation) and a nontypical TATA box. This is in contrast to the mouse *Mc4r* promoter that lacks a classic TATA box (15). In addition to the mouse *Mc4r* promoter, the 5′ flanking regions have been described for two melanocortin receptor subtypes: the human MC1R (18) and human and mouse Mc2r (19,20). Neither of these contain a TATA box nor a CAAT box. While several studies reported that most GPCR promoters are TATA-less and GC-rich (21,22), it appears that the human *MC4R* promoter does not share these characteristics.

Cross-species comparison reveals the presence of an identical 30-bp sequence following the putative CAAT box in the human, mouse, rat, and pig *MC4R* promoter. Our results confirm that this sequence is part of the minimal region that is necessary for basal *MC4R* promoter activity. Further characterization of *trans*-acting factors interacting with this *cis* element may lead to the discovery of novel candidate genes for human obesity.

While mutations in the *MC4R* gene are well established as an infrequent cause of obesity in humans (3–10), it is plausible that common SNPs in regulatory regions of *MC4R* could also contribute to obesity. Indeed, among multiple studies searching for linkage to obesity phenotypes, some have provided suggestive evidence of linkage between BMI (23) and body fat (24,25) on chromosome 18q21, flanking the *MC4R* region. These reports suggest that in addition to the rare severe obesity–causing variants

in the *MC4R* coding region, common genetic variants/SNPs in the regulatory regions of the *MC4R* gene may be contributing to the risk of developing obesity, whereas in our study, the frequencies of mutations in the coding sequence of *MC4R* and of common genetic variants in the 5′UTR were concordant with previous reports, our results indicate that genetic variants in the essential/minimal region of the *MC4R* promoter are not a common monogenic cause of human obesity. Further epidemiologic studies are necessary to rule out any effect of the common SNPs found in the promoter on obesity-related phenotypes.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health RO1 DK60540 and an American Diabetes Association Career Development Award (to C.V.). This study was carried out in part in the General Clinical Research Center, Moffitt/Mount Zion Hospital, UCSF, with funds provided by the National Center for Research Resources, M01 RR-00079, U.S. Public Health Service.

We thank Drs. John Kane, Raphael Merriman, James Ostroff, and Marco Patti and Karen Bagatelos, MSN, FNP, for their help in implementing the clinical study at UCSF.

REFERENCES

1. Comuzzie AG, Allison DB: The search for human obesity genes. *Science* 280:1374–1377, 1998
2. Hill JO, Peters JC: Environmental contributions to the obesity epidemic. *Science* 280:1371–1374, 1998
3. Vaisse C, Clement K, Guy-Grand B, Froguel P: A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat Genet* 20:113–114, 1998
4. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S: A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat Genet* 20:111–112, 1998
5. Hinney A, Schmidt A, Nottebom K, Heibult O, Becker I, Ziegler A, Gerber G, Sina M, Gorg T, Mayer H, Siegfried W, Fichter M, Remschmidt H, Hebebrand J: Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. *J Clin Endocrinol Metab* 84:1483–1486, 1999
6. Gu W, Tu Z, Kleyn PW, Kissebah A, Duprat L, Lee J, Chin W, Maruti S, Deng N, Fisher SL, Franco LS, Burn P, Yagaloff KA, Nathan J, Heymsfield S, Albu J, Pi-Sunyer FX, Allison DB: Identification and functional analysis of novel human melanocortin-4 receptor variants. *Diabetes* 48:635–639, 1999
7. Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel P: Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest* 106:253–262, 2000
8. Dubern B, Clement K, Pelloux V, Froguel P, Girardet J, Guy-Grand B, Tounian P: Mutational analysis of melanocortin-4 receptor, agouti-related protein, and alpha-melanocyte-stimulating hormone genes in severely obese children. *J Pediatr* 139:204–209, 2001
9. Lubrano-Berthelier C, Durand E, Dubern B, Shapiro A, Dazin P, Weill J, Ferron C, Froguel P, Vaisse C: Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. *Hum Mol Genet* 12:145–153, 2003
10. Farooqi IS, Keogh JM, Yeo GS, Lank EJ, Cheetham T, O'Rahilly S: Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. *N Engl J Med* 348:1085–1095, 2003
11. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141, 1997
12. Ho G, MacKenzie RG: Functional characterization of mutations in melanocortin-4 receptor associated wih human obesity. *J Biol Chem* 274: 35816–35822, 1999
13. Yeo GS, Lank EJ, Farooqi IS, Keogh J, Challis BG, O'Rahilly S: Mutations in the human melanocortin-4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms. *Hum Mol Genet* 12:561–574, 2003
14. Le Stunff C, Fallin D, Schork NJ, Bougneres P: The insulin gene VNTR is associated with fasting insulin levels and development of juvenile obesity. *Nat Genet* 26:444–446, 2000
15. Dumont LM, Wu CS, Aschkenasi CJ, Elmquist JK, Lowell BB, Mountjoy KG: Mouse melanocortin-4 receptor gene 5′-flanking region imparts cell specific expression in vitro. *Mol Cell Endocrinol* 184:173–185, 2001
16. Khong K, Kurtz SE, Sykes RL, Cone RD: Expression of functional melanocortin-4 receptor in the hypothalamic GT1–1 cell line. *Neuroendocrinology* 74:193–201, 2001
17. Jacobson P, Ukkola O, Rankinen T, Snyder EE, Leon AS, Rao DC, Skinner JS, Wilmore JH, Lonn L, Cowan GS Jr, Sjostrom L, Bouchard C: Melanocortin 4 receptor sequence variations are seldom a cause of human obesity: the Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis cohort. *J Clin Endocrinol Metab* 87:4442–4446, 2002
18. Moro O, Ideta R, Ifuku O: Characterization of the promoter region of the human melanocortin-1 receptor (MC1R) gene. *Biochem Biophys Res Commun* 262:452–460, 1999
19. Naville D, Barjhoux L, Jaillard C, Lebrethon MC, Saez JM, Begeot M: Characterization of the transcription start site of the ACTH receptor gene: presence of an intronic sequence in the 5′-flanking region. *Mol Cell Endocrinol* 106:131–135, 1994
20. Cammas FM, Pullinger GD, Barker S, Clark AJ: The mouse adrenocorticotropin receptor gene: cloning and characterization of its promoter and evidence for a role for the orphan nuclear receptor steroidogenic factor 1. *Mol Endocrinol* 11:867–876, 1997
21. Minowa T, Minowa MT, Mouradian MM: Analysis of the promoter region of the rat D2 dopamine receptor gene. *Biochemistry* 31:8389–8396, 1992
22. Zhu QS, Chen K, Shih JC: Characterization of the human 5-HT2A receptor gene promoter. *J Neurosci* 15:4885–4895, 1995
23. Ohman M, Oksanen L, Kaprio J, Koskenvuo M, Mustajoki P, Rissanen A, Salmi J, Kontula K, Peltonen L: Genome-wide scan of obesity in Finnish sibpairs reveals linkage to chromosome Xq24. *J Clin Endocrinol Metab* 85:3183–3190, 2000
24. Chagnon YC, Chen WJ, Perusse L, Chagnon M, Nadeau A, Wilkison WO, Bouchard C: Linkage and association studies between the melanocortin receptors 4 and 5 genes and obesity-related phenotypes in the Quebec Family Study. *Mol Med* 3:663–673, 1997
25. Norman RA, Tataranni PA, Pratley R, Thompson DB, Hanson RL, Prochazka M, Baier L, Ehm MG, Sakul H, Foroud T, Garvey WT, Burns D, Knowler WC, Bennett PH, Bogardus C, Ravussin E: Autosomal genomic scan for loci linked to obesity and energy metabolism in Pima Indians. *Am J Hum Genet* 62:659–668, 1998