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,
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-AR (5'-GCTATAGGTGCTGATCCAGA-3') and avian myeloblastosis virus–reverse transcriptase. We amplified 5' cDNA ends with MC4R-BR or MC4R-OR (5'-CGAGGAGAAGTGTGCATCCCA-3') and the search for genetic variants associated with obesity.

**SEX-ASSOCIATED MEASURES.** Potential cis-acting elements were identified using the TRANSFAC database and manual searches and are presented in reverse orientation with the prefix "^-".

**Cloning of 5' flanking MC4R luciferase reporter vectors.** Fragments of the 5' region of the human MC4R gene were obtained by restriction digestion from subjects. This region was amplified from human genomic DNA using primers MC4R-250 (5'-ACAGTGTCCCCCTGA-3') and MC4R-350 (5'-GCTATAGGTGCTGATCCAGA-3'). The PCR products were subcloned in pcDNA3.1/V5-His TOPO TA vector (Invitrogen). Cells were cotransfected with Firefly and Renilla luciferases. Forty-eight hours after transfection, cells were harvested and the luciferase activities were normalized to the activity of 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 366 children (aged 12.8 ± 0.8 years) from a previously described cohort (14). The protocol was approved by the Cochin Ethics Committee, and informed consent was obtained from subjects and parents.

**Mutation screening of the MC4R UTR.** A plasmid containing the Renilla luciferase gene in the plasmid pFOXLuc1 (a gift from Michael German, University of California San Francisco [UCSF]). 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 assayed 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.

**Cell transfection and luciferase assays.** Mouse hypothalamic GT1-7 cells were provided by Richard Weiner (UCSF). Neuro2A, HEK293, 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 assayed 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.

**DISCUSSION**

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.
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 (AAttGGTCA) 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

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Age (years)</th>
<th>Sex (% M/F)</th>
<th>BMI (kg/m²)</th>
<th>Mutation in MC4R</th>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>Coding region</td>
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<tr>
<td>Adults, U.S.</td>
<td>165</td>
<td>&gt;18</td>
<td>27/73</td>
<td>48.8 ± 8.06 (40–85.3)</td>
<td>R7H 1</td>
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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 GT1-7) (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 caused 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 mono-
genic 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 pheno-
types.

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