Linkage and Association Studies between the Melanocortin Receptors 4 and 5 Genes and Obesity-Related Phenotypes in the Québec Family Study

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ABSTRACT

Background: The *agouti* yellow mouse shows adult onset of moderate obesity and diabetes. A depressed basal lipolytic rate in adipocytes or a decreased adrenergic tone arising from antagonizing α-melanocyte-stimulating hormone (MSH) activation of melanocortin receptors (MCR) could be at the origin of the obesity phenotype.
Materials and Methods: MCR 4 and 5 (*MC4R, MC5R*) genes were studied in the Québec Family Study. Sequence variations were detected by Southern blot probing of restricted genomic DNA, and mRNA tissue expression was detected by RT-PCR. Subjects with a wide range of weight were used for single-point sib-pair linkage studies (maximum of 289 sibships from 124 nuclear families). Analysis of variance across genotypes in unrelated males (n = 143) and females (n = 156) was also undertaken. Body mass index (BMI), sum of six skinfolds (SF6), fat mass (FM), percent body fat (%FAT),

respiratory quotient (RQ), resting metabolic rate (RMR), fasting glucose and insulin, and glucose and insulin area during an oral glucose tolerance test were analyzed.
Results: *MC4R* showed polymorphism with NcoI, and *MC5R*, with PstI and PvuII, with a heterozygosity of 0.38, 0.10, and 0.20, respectively. Linkages were observed between *MC5R* and BMI (p = 0.001), SF6 (p = 0.005), FM (p = 0.001), and RMR (p = 0.002), whereas associations were observed in females between *MC5R* and BMI (p = 0.003), and between *MC4R* and FM (p = 0.002) and %FAT (p = 0.004). After correction for multiple tests, these p values are lowered by one tenth. *MC4R* and *MC5R* mRNAs have been detected in brain, adipose tissue, and skeletal muscle.
Conclusions: *MC4R* and *MC5R* exibit evidence of linkage or association with obesity phenotypes, but this evidence is strongest for *MC5R*.

INTRODUCTION

Obesity, which has become a prominent health problem in North American societies (1), has a polygenic and multifactorial origin (2). Animal

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models of obesity in rodents (3), such as those arising spontaneously or resulting from specific rodent crosses, could help to understand the causes of obesity. Among the single-gene mouse mutation models of obesity, the *agouti* yellow (A^Y) mouse shows adult onset of moderate obesity arising from excess fat in all depots, from hyperphagia, insulin resistance, glucose intolerance, and diabetes (4). *Agouti* mRNA has been shown to be expressed exclusively in the skin of

neonatal mice (5), and the ectopic expression of *agouti* produces the obesity phenotype (6). Moreover, it has been shown that *agouti*-specific expression in adipose tissue in mouse indicated obesity (7). The human homolog of *agouti*, renamed *agouti signaling protein* (*ASP*) gene (8), or *ASIP*, has been reported to be expressed in adipose tissue and testis (9), in heart, ovary, and, at a lower level, in liver and kidney (8). The *ASIP* gene has been located on human 20q11.2, but no significant linkage or association has been reported between markers in the proximity of the *ASIP* locus and obesity (10, 11).

In mice, it has been hypothesized that the effect of *agouti* could be mediated via the regulation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in skeletal muscle whereby an increase in $[Ca^{2+}]_i$ could lead to insulin resistance, hyperinsulinemia, and obesity (12,13). Other mechanisms that have been proposed to explain the obesity phenotype in these mice include a depressed basal lipolytic rate in adipocytes resulting from altered intracellular cAMP levels, or a decreased adrenergic tone that may trigger hyperphagia and increased efficiency of food utilization—both mechanisms arising from antagonizing α-melanocyte-stimulating hormone (MSH) activation of melanocortin receptors (6).

Five melanocortin receptors (MC1R to MC5R) have been identified so far in mouse and human. These receptors show various binding properties with their ligands, together with specific tissue expressions, the latter being less well characterized in humans. Agouti shifts the MSH functional curve of these receptors without affecting maximal activation, which suggests that agouti acts as a competitive antagonist inhibiting agonist binding (14). MC3R and MC5R are not antagonized by agouti in rodents (14), but human HEK-293 cells transfected with *MC3R* showed an increase in $[Ca^{2+}]_i$ in the presence of agouti (15). MC4R is clearly antagonized by human agouti (14), and it has been shown that the intracerebroventricular administration of an agonist of MC4R, as well as of MC3R, inhibits feeding in four mouse models of hyperphagia, including *agouti*; this shows that these receptors exert a tonic inhibitory effect on feeding behavior (16). Moreover, knockout mice for *MC4R* recapitulate several aspects of the *agouti* obesity syndrome, including hyperphagia, hyperinsulinemia, and late-onset obesity (17).

We have uncovered new restriction fragment-length polymorphisms (RFLP) for the melanocortin receptors 4 and 5, and we report here on linkage and association of these receptors with obesity-related phenotypes in subjects from the Québec Family Study (QFS). Adiposity, energy metabolism, and insulin and glucose homeostasis–related variables were analyzed. We also examined the mRNA tissue expression in humans of the MCR family genes.

MATERIALS AND METHODS

Subjects and Phenotypes

The QFS cohort has been previously described (18). Normal-weight, overweight, and obese subjects from Caucasian nuclear families of French descent from the greater Québec City area were tested for a battery of morphometric and physiologic variables. At the same time, blood samples were obtained for various assays and permanent lymphoblastoid cell lines were established for the extraction of DNA. Adiposity variables included the body mass index (BMI = weight in kg divided by height in squared meters) and the percent body fat (%FAT), which was estimated from body density measurements obtained by underwater weighing (19) using the equation of Siri (20) with estimation of the residual pulmonary volume (21). Fat mass (FM) and fat-free mass (FFM) were obtained from percent body fat and body weight. Subcutaneous fat was estimated by the sum of six skinfold thicknesses (SF6 = abdominal, subscapular, suprailiac, medial calf, triceps, and biceps). Respiratory quotient (RQ) and resting metabolic rate (RMR) were assessed in a fasted state by indirect calorimetry over a 30-min period using an open-circuit system with a ventilated hood, with data from the last 10 min being used for the calculation (22). Fasting plasma insulin (INSULIN) and glucose (GLUCOSE) levels were measured, and the total area under the curve during a 75 g oral glucose tolerance test (OGTT) for insulin (INSULIN AREA) and glucose (GLUCOSE AREA) was computed from the plasma levels determined at each 15 min for the first hour following the load and every 30 min for the next 2 hr, as reported earlier (23). Insulin was measured by radioimmunoassay with polyethylene glycol precipitation (24), and glucose was measured enzymatically (25).

DNA Polymorphism Analysis

Genomic DNA was prepared from permanent lymphoblastoid cells by the proteinase K and
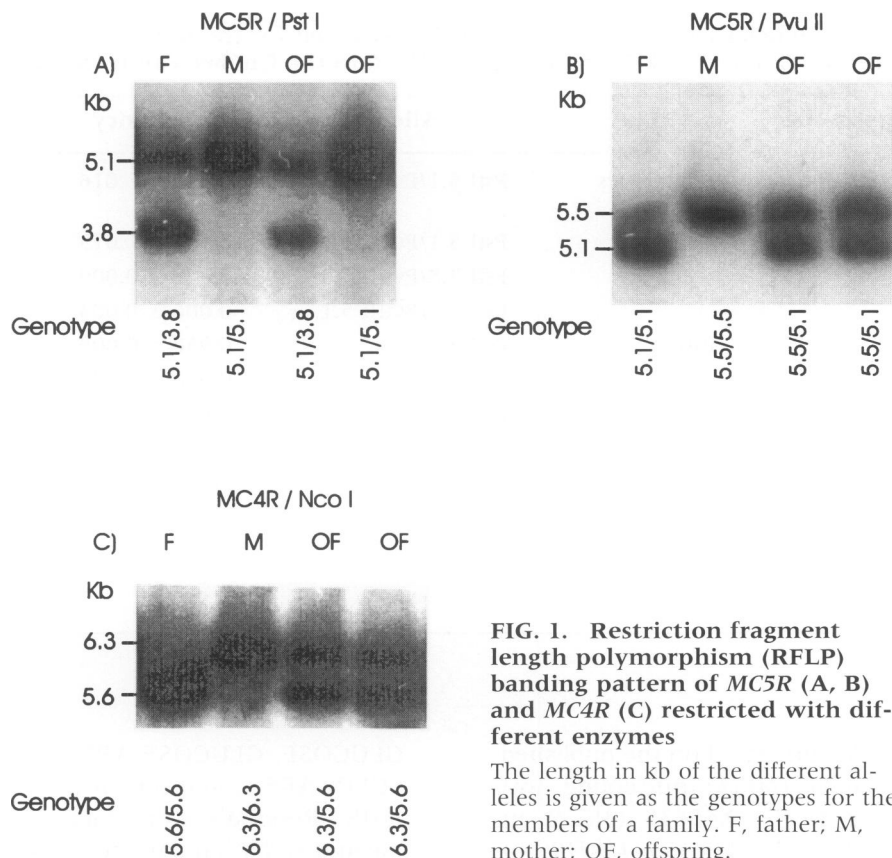
FIG. 1. Restriction fragment length polymorphism (RFLP) banding pattern of *MC5R* (A, B) and *MC4R* (C) restricted with different enzymes

The length in kb of the different alleles is given as the genotypes for the members of a family. F, father; M, mother; OF, offspring.

phenol/chloroform technique. DNA was dialyzed four times against TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 6 hr at 4°C and ethanol precipitated (26). From 40 restriction enzymes tested, three—PstI, PvuII, and NcoI—showed a polymorphism with a frequency greater than 5% of variation. Five micrograms of DNA was digested 18 to 20 hr with 50 U of restriction enzyme PstI and PvuII for *MC5R*, and NcoI for *MC4R*, according to conditions recommended by the manufacturer (New England Biolabs, Mississauga, Ontario, Canada). Generated DNA fragments were separated on a 1.2% agarose gel by electrophoresis in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and transferred by alkali blotting in 0.25 M NaOH, 1.5 M NaCl (27) to nylon membrane (Hybond N+, Amersham). DNA on the resulting Southern blot was then hybridized with the following probes. Complete coding sequences from *MC4R* (28) and *MC5R* (29,30) were used as probes. cDNAs were labeled to a specific activity of $\geq 1 \times 10^9$ cpm/μg by random priming (T7 QuickPrime Kit, Pharmacia Biotech, Baie D'Urfé, Québec, Canada) with [α-^{32}P] dCTP (NEN DuPont Canada, Inc). Blot

prehybridization was carried out in a hybridization oven (Robbins Scientific Corporation, Sunnyvale, CA) at 65°C for a minimum of 4 hr in a solution containing 1 mM EDTA, 0.25 M NaH_2PO_4 (pH 7.4), 7% sodium dodecylsulfate (SDS), and 100 μg/ml sonicated salmon sperm DNA. The membranes were then hybridized in fresh buffer containing the appropriate denatured labeled probe for 18 hr at 65°C. They were then washed for 15 min three times in 0.1× SSPE (20 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM EDTA, pH 7.7) and 0.1% SDS at 65°C. Autoradiography was performed using Kodak XAR-5 films with intensifying screens for 3 to 5 days at −70°C. The size of the fragments was estimated using lambda DNA digested with *Hin*dIII and *Eco*RI as standards.

mRNA Tissue Distribution

Poly A$^+$ RNA of different tissues was obtained from Clontech. A first-strand cDNA was synthesized from 1 μg of Poly A$^+$ RNA using a cDNA cycle kit (Invitrogen). PCR primers used for reverse transcription–polymerase chain reaction

TABLE 1. Allele frequencies (±SE) and heterozygosity observed for the four RFLPs among the melanocortin receptor 5 and 4, and the haplotype of *MC5R* in the Québec Family Study

Gene	Chromosome	RFLP	Allele	Frequency	Heterozygosity
MC5R	18p11.2[a]	Haplotypes PstI/PvuII	PstI 5.1/PvuII 5.5	0.842 ± 0.016	0.28
			PstI 5.1/PvuII 5.1	0.103 ± 0.013	
			PstI 3.8/PvuII 5.5	0.049 ± 0.009	
			PstI 3.8/PvuII 5.1	0.006 ± 0.003	
		PstI	PstI 5.1	0.950 ± 0.009	0.10
			PstI 3.8	0.050 ± 0.009	
		PvuII	PvuII 5.5	0.89 ± 0.01	0.20
			PvuII 5.1	0.11 ± 0.01	
MC4R	18q21.3-q22[b]	NcoI	NcoI 6.3	0.74 ± 0.02	0.38
			NcoI 5.6	0.26 ± 0.02	

[a]ref. 35; [b]ref. 28.

(RT-PCR) of mRNA were based on the published sequences of X65634 (*MC1R*; nucleotide [nt] 463–487; nt 1398–1421), X65635 (*MC2R*; nt 687–716; nt 1562–1595), L06155 (*MC3R*; nt 1–31; nt 1059–1083), L08603 (*MC4R*; nt 1–27; nt 972–999), and L27080 (*MC5R*; nt 430–451; nt 976–1000; nested: nt 457–480; nt 906–931). For *MC1R* to *MC4R*, PCR was performed in a 50-μl reaction containing 5 units of Ampli*Taq* DNA polymerase (Perkin Elmer), 2 mM $MgCl_2$, 0.2 mM dNTPs, 10% glycerol, 20 pmole of primers, and one-fiftieth of the first-strand cDNA reaction as a template. The PCR amplification was performed for 35 cycles of 95°C, 30 sec; 60°C, 30 sec; 72°C, 1 min, and one-fifth of the reaction was analyzed on a 1% agarose gel. For *MC5R*, the same PCR protocol was applied except that one-tenth of the first-strand cDNA synthesis reaction was used as a template and a second PCR was performed using a set of nested internal primers for 20 cycles under the same amplification conditions.

Statistical Analyses

Before linkage and association analyses, the different phenotypic variables were adjusted using sex-specific regression procedures, and residuals from the regressions were used for the analyses. Insulin variables were log transformed. All variables were adjusted for age and age^2, with further adjustment made for fat mass of RQ, RMR,

GLUCOSE, GLUCOSE AREA, INSULIN, and INSULIN AREA, and for fat-free mass of RQ and RMR. Residuals were then standardized to a mean 0 and a standard deviation of 1. The single locus sib-pair linkage analysis was performed using the SIBPAL version 2.6 software from the S.A.G.E. package (31) with the *t* statistic and the degree of freedom adjusted for the nonindependence of the sib-pairs. Association studies were undertaken by comparing mean values of the residuals across genotypes for the unrelated subjects from the parental generation using analysis of variance (ANOVA). The adjustments of the variables and the ANOVA to test for the association were performed with the SAS package (version 6.08) for PC. Probability values (*p*) of the linkage and association tests were adjusted for multiple tests using the Bonferroni correction in which the adjusted *p* value $p^* = 1 - (1 - p)^{\text{number of traits}}$ (31).

RESULTS

The three different RFLPs observed among the two MCR genes are illustrated in Figure 1. *MC5R* showed two alleles with PstI (alleles 5.1 and 3.8 kb) and two with PvuII (alleles 5.5 and 5.1 kb) restriction enzymes. *MC4R* showed polymorphism when restricted with NcoI with two generated fragments of 6.3 and 5.6 kb, respectively. For all three polymorphisms, it is likely that the polymorphic sites are outside the coding se-

TABLE 2. Number of subjects (N), mean and range by generation and gender for each variable in the Québec Family Study

Variables	Parent						Adult Offspring					
	Male			Female			Male			Female		
	N	Mean	Range	N	Mean	Range	N	Mean	Range	N	Mean	Range
Age (years)	143	54.8	37–73	156	52.8	36–68	143	27.7	18–60	154	27.9	18–61
BMI (kg/m²)	141	27.9	19–47	154	27.0	19–56	143	25.7	17–45	153	25.4	17–65
SF6 (mm)	141	97.8	24–287	151	143.9	48–401	141	91.5	26–320	153	125.9	39–448
FM (kg)	121	22.4	5–50	120	25.9	9–59	138	16.9	2–57	148	21.1	3–109
%FAT	121	26.9	8–43	120	37.5	18–54	138	19.9	4–42	148	29.3	6–60
FFM (kg)	121	58.1	41–82	120	41.1	29–66	138	61.2	42–83	148	45.3	32–72
RQ	131	0.81	0.7–1.0	143	0.80	0.6–0.9	138	0.80	0.7–0.9	147	0.80	0.7–0.9
RMR (kj)	131	1.13	0.7–1.8	143	0.89	0.6–1.7	138	1.19	0.9–1.9	147	0.96	0.6–1.7
Glucose (mmol/L)	142	5.5	4–14	154	5.2	3–20	143	4.9	4–13	154	4.6	4–6
Glucose area (mmol/ L min 10⁻³)	105	1.4	0.8–3.3	114	1.2	0.8–2.4	103	1.1	0.6–2.0	113	1.1	0.7–1.7
Insulin (pmol/L)	115	73.6	<1–276	129	62.2	1–588	107	68.1	9–260	121	70.8	4–350
Insulin area (pmol/ L min 10⁻³)	104	74.7	13–233	113	71.3	20–314	101	73.6	14–418	111	78.0	21–502

See text for the abbreviations.

TABLE 3. Single locus sib-pair linkage results between melanocortin receptors 4 and 5 genes and obesity, and diabetes-related variables in the Québec Family Study

Variables	MC5R/PstI			MC5R/PvuII			MC4R/NcoI		
	N	t	p	N	t	p	N	t	p
BMI	255	−0.423	0.34	289	−3.041	**0.001*****	258	−0.037	0.49
SF6	253	−0.103	0.46	287	−2.634	**0.005***	256	0.166	0.57
FM	217	−0.306	0.38	242	−3.145	**0.001*****	213	0.116	0.55
%FAT	217	−0.362	0.36	242	−2.184	**0.02**	213	0.195	0.58
FFM	217	−0.955	0.17	242	−2.423	**0.008**	213	−0.443	0.33
RMR	214	0.169	0.57	239	−2.928	**0.002****	210	−1.087	0.14
RQ	214	−0.180	0.43	239	−0.042	0.48	210	−1.816	**0.04**
Glucose	217	0.888	0.81	242	−0.107	0.46	213	0.586	0.72
Glucose area	170	0.268	0.61	191	−0.458	0.32	160	2.232	0.99
Insulin	174	1.027	0.85	195	1.171	0.88	164	−0.208	0.42
Insulin area	163	−0.584	0.28	183	−0.375	0.35	153	−0.724	0.24

Significant results at $p \leq 0.05$ are highlighted in bold, and those remaining significant after the Bonferroni correction for multiple tests are indicated with asterisks. N, number of sib-pairs; t, t statistic from the linkage regression; p, probability values. See text for variables abbreviations.

$*p = 0.05$; $**p = 0.02$; $***p = 0.01$.

quences. For instance, these genes are intronless and these sites have not been reported in the published sequences, except for a PstI site in MC5R (28,30,32). Furthermore, if the sites were located within the coding sequences, the generated restricted fragments (0.4 kb for MC5R PvuII and 0.7 kb for MC4R NcoI) should be detected by the probes. On the other hand, these polymorphisms arise probably from a gain of a restriction site since the most frequent allele showed the larger DNA fragment. But an ancestral type has to be inferred from the study of other species to confirm these gains. Finally, because of the presence of the PstI site at 20 bp of the 3' end of the coding sequence, the polymorphic PstI site in MC5R has to be located upstream or 5' of the coding region, otherwise, the 3.6 kb allele could not be detected by the probe.

Observed allele frequencies for the three RFLPs and for the haplotype combining the two RFLPs from the MC5R gene are presented in Table 1. Relatively low variation was observed for MC5R polymorphisms (frequencies of 95% and 89% for the most frequent alleles), which is slightly raised by using MC5R haplotypes (frequency of 84% for the most frequent haplotype). Accordingly, the heterozygosity level for these polymorphisms was increased from 0.10 and

0.20 for PstI and PvuII individual RFLPs to 0.28 in the MC5R haplotypes. MC4R showed a slightly higher degree of variation, with the frequency of the most frequent alleles reaching 0.74 and showing a heterozygosity of 0.38. All polymorphisms and the MC5R haplotypes are in Hardy-Weinberg equilibrium, and MC5R RFLPs are not in linkage disequilibrium ($\chi^2 = 0.44$).

The mean and range of the different phenotypic variables are shown in Table 2 for each of the sex-by-generation groups. Subjects ranged in age from 18 to 73 years with a BMI from 17 to 65 kg/m². Significant correlations ($p \leq 0.05$) were observed between all variables and age except in males with INSULIN ($p = 0.15$) and RQ ($p = 0.44$), and in females with INSULIN ($p = 0.06$), INSULIN AREA ($p = 0.68$), and RQ ($p = 0.56$). FM and FFM contributed significantly to the variance of RMR, whereas adjustment for fat-free mass was not significant for RQ in both males ($p = 0.15$) and females ($p = 0.07$). In spite of these occasional nonsignificant correlations and because of borderline significance level for some variables, residuals were used for all analyses.

Table 3 shows the results of the single locus sib-pair linkage analysis between the phenotypic variables and the three polymorphisms observed

TABLE 4. Analysis of variance (ANOVA) across genotypes for the two polymorphisms PstI and PvuII of the *MC5R* gene in the Québec Family Study

Variables	Sex	*MC5R* RFLP Genotypes									ANOVA
		PstI 5.1/PstI 5.1			PstI 5.1/PstI 3.8			PstI 3.8/PstI 3.8			
		N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	
BMI	Males	105	28.1	0.5	19	29.0	1.1	—	—	—	$p = 0.71$
(kg/m^2)	Females	130	26.5	0.5	10	32.2	1.9	—	—	—	$p = 0.003^*$
SF6 (mm)	Males	105	99.8	3.8	19	106.8	9.0	—	—	—	$p = 0.74$
	Females	128	140.5	4.9	9	179.1	18.5	—	—	—	$p = 0.03$

		PvuII 5.5/PvuII 5.5			PvuII 5.5/PvuII 5.1			PvuII 5.1/PvuII 5.1			
%FAT	Males	106	27.8	0.7	22	24.3	1.4	1	26.9	—	$p = 0.04$
	Females	92	37.2	0.8	22	37.6	1.6	—	—	—	$p = 0.79$
Glucose	Males	106	5.5	0.1	29	5.7	0.3	1	5.7	—	$p = 0.98$
(mmol/L)	Females	120	5.0	0.2	28	5.9	0.3	—	—	—	$p = 0.03$

Only variables showing significant results at $p \leq 0.05$ (in bold) are reported. Result significant after the Bonferroni correction for multiple tests is indicated with an asterisk.
$^*p = 0.03$.

among the two MCR genes. The number of sib-pairs used for the linkage analysis, including those from the parental generation, varied according to the combination of variable and marker analyzed. A minimum of 153 sibships from 80 nuclear families with an average sibship size of 1.9 (range 1 to 10) for INSULIN AREA versus MC4R/NcoI, and a maximum of 289 sibships from 124 nuclear families with an average sibship size of 2.3 (range 1 to 15) for BMI versus MC5R/PvuII, were used. Linkages were observed mainly for *MC5R*. MC5R/PvuII polymorphism showed linkages with all adiposity-related variables such as BMI, SF6, FM, and %FAT, with p values ranging from 0.001 to 0.02, and with FFM ($p = 0.008$) and RMR ($p = 0.002$). MC5R/PstI polymorphism showed no linkage. MC4R/NcoI showed only weak linkage with RQ ($p = 0.04$). After Bonferroni correction for multiple traits tested—here, 11 traits by marker—linkages remained significant between MC5R/PvuII and BMI (corrected p value $p^* = 0.01$), SF6 ($p^* = 0.05$), FM ($p^* = 0.01$), and RMR ($p^* = 0.02$). Linkage analysis using *MC5R* haplotypes (data not shown) gave positive linkages for almost the same variables as when the individual RFLPs

were used (BMI: $p = 0.004$; SF6: $p = 0.02$; FM: $p = 0.005$; %FAT: $p = 0.02$; FFM: $p = 0.009$), except for the linkage with RMR, which was no longer significant ($p = 0.10$). Significant results, after Bonferroni correction, were observed for BMI ($p^* = 0.04$) and FM ($p^* = 0.05$). None of the three polymorphisms showed linkage with variables related to glucose and insulin metabolism (GLUCOSE, GLUCOSE AREA, INSULIN, INSULIN AREA).

Tables 4 and 5 present results of the analysis of variance across genotypes, performed on residuals. Adjusted mean values are reported only for phenotypes showing evidence of an association. MC5R/PstI (Table 4) showed significant association with BMI ($p = 0.003$; $p^* = 0.03$) and suggestive association with SF6 ($p = 0.03$) in females, but not in males. In these cases, heterozygotes PstI 5.1/PstI 3.8 showed higher mean values than those of the homozygotes for the frequent allele PstI 5.1 (BMI: 32.6 ± 3.3 versus 26.5 ± 0.5; SF6: 184.3 ± 31.8 versus 140.1 ± 4.6). Unfortunately, no PstI 3.8 homozygote was available for this analysis. MC5R/PvuII showed no significant associations, but comparison of carriers of the less-frequent PvuII 5.1 allele and

TABLE 5.   Analysis of variance (ANOVA) across genotypes for the melanocortin receptor 4 (*MC4R*) NcoI RFLP in the Québec Family Study

| Variables | Sex | NcoI 6.3/NcoI 6.3 | | | NcoI 6.3/NcoI 5.6 | | | NcoI 5.6/NcoI 5.6 | | | ANOVA |
		N	Mean	SE	N	Mean	SE	N	Mean	SE	
FM (kg)	Males	62	21.4	1.2	40	24.4	1.4	9	23.1	3.1	$p = 0.29$
	Females	60	23.0	1.3	40	30.3	1.6	3	28.0	5.7	$p = \mathbf{0.002}$**
%FAT	Males	62	26.3	0.8	40	28.8	1.0	9	26.3	2.2	$p = 0.17$
	Females	60	35.4	0.9	40	40.2	1.2	3	41.4	4.3	$p = \mathbf{0.004}$*
FFM (kg)	Males	62	57.2	0.9	40	58.1	1.2	9	62.8	2.4	$p = 0.14$
	Females	60	40.4	0.7	40	43.0	0.9	3	38.4	3.2	$p = \mathbf{0.05}$

Only variables showing significant results at $p \leq 0.05$ (in bold) are reported. Results remaining significant after the Bonferroni correction for multiple tests are indicated with asterisks.
*$p = 0.04$; **$p = 0.02$.

noncarriers suggested associations in males for BMI ($p = 0.04$), SF6 ($p = 0.05$), FM ($p = 0.04$), and %FAT ($p = 0.02$), with carriers showing the lowest mean for all these variables (BMI: 26.3 ± 0.7 versus 28.5 ± 0.5; SF6: 86.3 ± 5.4 versus 102.9 ± 4.2; FM: 19.1 ± 1.5 versus 23.5 ± 1.0; %FAT: 24.2 ± 1.4 versus 27.8 ± 0.7). No evidence of association was found for MC5R haplotypes (data not shown).

MC4R/NcoI (Table 5) showed significant associations with FM (p* = 0.02) and %FAT (p* = 0.04) in females, with the lowest mean values observed for FM in the most frequent homozygote NcoI 6.3 (23.1 ± 1.1 versus 30.1 ± 1.8 and 27.7 ± 4.3), and the highest mean value for %FAT in the less frequent homozygote NcoI 5.6 (42.2 ± 5.3 versus 39.8 ± 1.2 and 35.6 ± 0.9). When carriers of the NcoI 5.6 allele are compared with noncarriers, significant associations are observed for FM ($p = 0.0008$; p* = 0.009) and for %FAT ($p = 0.004$; p* = 0.04), with carriers showing higher mean values in both cases (FM: 29.9 ± 1.7 versus 23.1 ± 1.1; %FAT: 40.0 ± 1.2 versus 35.6 ± 0.9).

The tissue distribution of the various MCR mRNAs by RT-PCR was also explored. For *MC5R*, which exhibits significant linkage with BMI, SF6, FM, and RMR, mRNA was found in adipose and brain tissues, as well as in pancreas, lung, heart and testes (Figure 2). However, this mRNA was hard to detect, as reamplification was necessary to obtain a clear signal.

In Figure 3, the distribution of the other four

MCRs is illustrated. All these receptor mRNAs were also identified in adipose tissue, in heart and testes. MC1R was expressed in pancreas, kidney, and placenta, as was MC2R, but at a lower level. Additionally, *MC2R* was expressed in skeletal muscle. *MC4R*, which showed association with FM and %FAT, showed a strong expression in brain and adipose tissue, with a detectable amount in skeletal muscle and liver.

DISCUSSION

After correction for multiple tests, *MC5R* showed significant linkages with BMI, SF6, FM, and RMR, whereas suggestive linkages were also observed with %FAT and FFM. Similarly, significant associations were observed with BMI, whereas suggestive results were found for the other linked adiposity-related variables SF6, FM, and %FAT. No association was observed with RMR and FFM. These results suggest that variation at the *MC5R* gene could influence adiposity in humans, although it is possible that another gene in linkage disequilibrium with *MC5R* could be at the origin of the observed linkages and associations. Interestingly, and according to the RFLP used, the associations appear to be sex-specific. For instance, in males, carriers of the less-frequent PvuII 5.1 allele had lower mean values for BMI, SF6, FM, and %FAT, whereas in females, carriers of the less-frequent PstI 3.8 allele exhibited higher mean values for BMI and
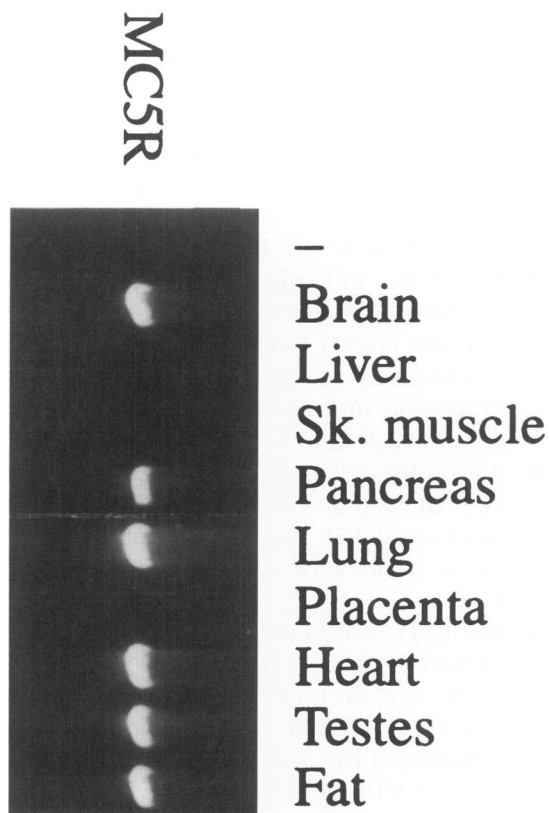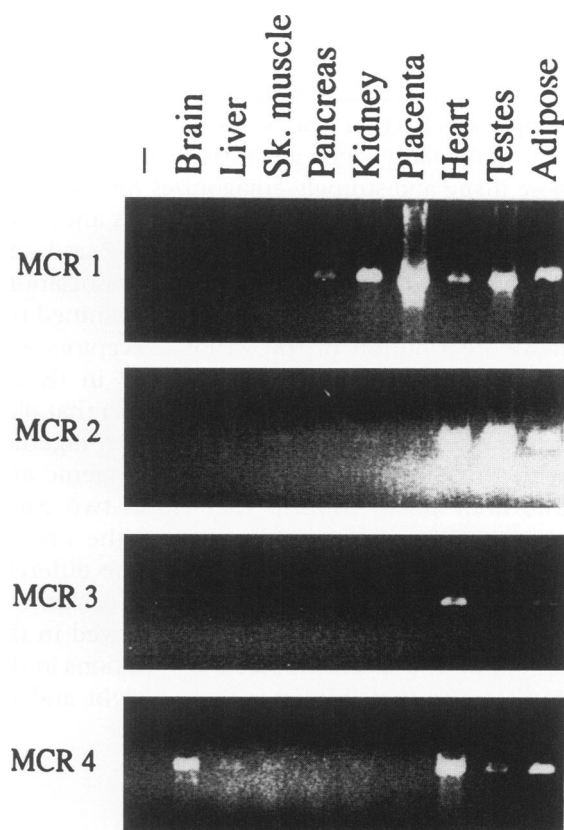
FIG. 2. Melanocortin receptor 5 (*MC5R*) mRNA distribution in nine different human tissues

FIG. 3. Melanocortin receptors 1 to 4 (*MC1R*, *MC2R*, *MC3R*, *MC4R*) mRNAs distribution in nine different human tissues

SF6. These results suggest that different alleles may have a similar effect, depending on the gender of the individual, with allele MC5R/PstI 3.8 and allele MC5R/PvuII 5.5 favoring fat accretion in females and males, respectively. It is also obvious that BMI and SF6 mean values are higher when subjects are classified according to the carrier status of the PstI polymorphism than when classified by that of PvuII, which probably reflects the gender difference effect of these alleles.

Human MC5R, as a receptor for MSH and adrenocorticotropin (ACTH), is expressed in the brain and adipose tissue, which are relevant tissues for the control of adiposity. While agouti is a weak antagonist of melanocortin peptide binding to MC5R, it is unable to stimulate $[Ca^{+2}]_i$ by this receptor. Recently it has been reported that *MC5R* is also expressed in rodent adipose tissues (33), as we report here in humans. An effect of *MC5R* on adiposity-related variables in humans could be expected directly at the level of the adipose tissue by a differentially modulated increase in $[Ca^{+2}]_i$ according to the expressed *MC5R* alleles. It could also be hypothesized that variation in *MC5R* could modulate adrenergic

tone in brain in conjunction with other molecules, such as the recently reported agouti-related transcript or ART protein, which is expressed in the hypothalamus of both human and mouse and which showed a 10-fold increased expression in ob/ob and db/db mice (34). Additional studies in other cohorts including more subjects homozygotes for *MC5R* PvuII 5.1 and PstI 3.8 alleles would help to confirm and strengthen these interpretations.

MC4R showed significant associations with FM and %FAT in females, with significantly higher values being observed for carriers of the less-frequent NcoI 5.6 allele. In contrast to *MC5R*, only these two adiposity-related variables showed associations, without linkage, with *MC4R*, which suggests a weaker effect of MC4R on adiposity in humans. MC4R has not been observed in rodent adipose tissue (33), but our data show a clear expression of *MC4R* both in brain and adipose tissue in humans (Fig. 3). Recent data showed that the knockout of this gene in mouse recapitulates several aspects of *agouti* obesity syndromes (17) and that intracerebroventricular administration of a specific agonist of

MC4R inhibits feeding behavior in mouse models of hyperphagia (16). These data argue in favor of a central effect of agouti on MC4R, but a peripheral effect on adipose tissue cannot be ruled out, particularly since agouti is expressed in adipose tissue and strongly antagonizes MC4R (14).

In conclusion, significant linkages and associations were observed between *MC5R* and adiposity-related variables, whereas associations were observed for *MC4R*. We have examined the tissue distribution of the various receptors and have found that they are expressed in tissues (mainly brain, adipose, skeletal muscle) that play a role in energy balance. These results, together with those obtained recently in transgenic and knockout mice, confirm that these two MCR genes are important candidates for the obesity phenotype expression in humans. The different MCR alleles showing linkage and association in humans need to be further characterized in the search for the specific molecular variations in the MCR genes that influence body weight and fat deposition.

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