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Corticosteroid Binding Globulin: A New Target for Cortisol-Driven Obesity

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We present data suggesting that corticosteroid-binding globulin (CBG) may be the causal gene of a previously identified quantitative trait locus (QTL) associated with cortisol levels, fat, and muscle content in a pig intercross. Because *Cbg* in human and mouse maps in the region orthologous to the pig region containing this QTL, we considered *Cbg* as an interesting positional candidate gene because CBG plays a major role in cortisol bioavailability. Firstly, we cloned pig *Cbg* from a bacterial artificial chromosome library and showed by fluorescent *in situ* hybridization and radiation hybrid mapping that it maps on 7q26 at the peak of the QTL interval. Secondly, we detected in a subset of the pig intercross progeny a highly significant genetic linkage between CBG plasma binding capacity values and the chromosome 7 markers flanking the cortisol-associated QTL. In this population, CBG capacity is correlated positively to fat and negatively to muscle content. Thirdly, CBG capacity was three times higher in Meishan compared with Large White parental breeds and a 7-fold difference was found in *Cbg* mRNA expression between the two breeds. Overall, the data accumulated in this study point to *Cbg* gene as a key regulator of cortisol levels and obesity susceptibility. (*Molecular Endocrinology* 18: 1687–1696, 2004)

CORTISOL, A GLUCOCORTICOID hormone, is involved in various important biological processes such as gluconeogenesis, lipid and protein metabolism, antiinflammatory action, and growth (1, 2). It is also a major component of the stress response. After exposure to stressful stimuli, cortisol is rapidly released from the adrenal glands to provide the energy necessary to the behavioral response to stress. By negative feedback control, cortisol levels go back to basal values when the stressful stimulus is controlled by the individual. If not, as in case of chronic stress, sustained high cortisol levels have deleterious effects on the organism (3, 4). Thus, abnormal cortisol levels and more generally the hypothalamic-pituitary-adrenal (HPA) axis are implicated in various pathologies such as obesity (5), constitutive sensitivity to inflammatory and autoimmune reactions (6) aging by the deleterious effects of glucocorticoid hormones on hippocampal neurons (7), or sensitization to drug addiction (8).

Genetic factors participate in the variability observed among individuals in HPA axis activity and reactivity as shown by twin studies (9–11) and by comparison of strains in mice and rats (12, 13).

In pigs, Mormède *et al.* (14) showed that the Meishan pig breed has plasma cortisol concentrations twice higher than a control breed derived from Landrace. In addition, Meishan pigs are obese and display a reduced growth rate that may be a consequence of their high cortisol levels. Further studies on the parental breeds confirmed that the Meishan pig has high plasma cortisol concentrations, twice higher than the Large White breed during the active phase of the diurnal cycle, *i.e.* between 0600 and 1200 h (15). Therefore, we considered the Meishan and Large White pigs as an interesting model to study HPA axis variability and its consequences on health.

We used a QTL genetic mapping analysis, *i.e.* a no-hypothesis-driven approach, to reveal genes influencing cortisol genetic variability and its relationships

Abbreviations: CBG, Corticosteroid-binding globulin; dNTP, deoxynucleotide triphosphate; FISH, fluorescent *in situ* hybridization; HPA, hypothalamic-pituitary-adrenal; P_c, chromosomal test significance level; P_g, genome-wide test probability; QTL, quantitative trait locus.

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with obesity in a Meishan × Large White F2 intercross. A total of 626 piglets (6 wk old) were exposed to a novel environment stress and blood samples were collected before and after the test. Plasma concentrations of cortisol and ACTH of these blood samples were measured to evaluate HPA axis activity and reactivity (16). All these animals were evaluated for carcass composition (17). The same animals were genotyped with 137 microsatellite markers covering the porcine genome. Genetic linkage analysis was performed for each chromosome using a multiple marker maximum likelihood procedure assuming a half-sibling family structure for F2 pigs. A strong QTL on chromosome 7 near the marker S0101 was found associated with basal and post-stress cortisol levels in this intercross explaining 20% of the variance in the F2 population (18). The same region was found to be linked, although more weakly, to several parameters of carcass composition (19).

Goureau *et al.* (20) have reported on the human and porcine correspondence of chromosome segments using bidirectional chromosome painting. The cortisol-associated QTL flanked by the markers S0101 and Sw764 was localized on the porcine 7q2.4–7q2.6 region. Among the genes localized onto the orthologous human region (Hsap14q), the gene encoding CBG and localized on Hsap14q32.1 (21) retained our attention. Indeed, 90% of plasma cortisol is bound to CBG, which is an α-glycoprotein synthesized from liver. Because only free cortisol is active, CBG has a major role in cortisol bioavailability. Thus, *Cbg* was a good functional candidate for our QTL associated to cortisol levels because it had a high probability to map in our QTL region.

Here we report on molecular genetics analysis revealing that corticosteroid binding globulin (*Cbg*) may be the causal gene of the QTL associated with plasma cortisol levels, fat deposition, and muscle content.

RESULTS

Pig Cbg Maps in the Locus Associated with Cortisol Levels

Because *Cbg* had been cloned in human, monkey, sheep, and mouse (22–25), we were able to align the various sequences available using the *multalin* program (26) and to design consensus oligonucleotide primers from exon 2 to obtain a PCR fragment of pig *Cbg*. After checking the sequence of the PCR fragment for high homology with *Cbg* from the other species, we used these primers to map pig *Cbg* using a panel of radiation hybrids (27). We found that pig *Cbg* maps between the markers S0101 and Sw764 (Fig. 1), as does the cortisol-associated QTL.

This chromosomal localization was confirmed by fluorescent *in situ* hybridization (FISH). First, we screened a porcine genomic BAC library by PCR with the primers amplifying pig *Cbg* exon 2. We obtained a 150-kb clone containing the totality of *Cbg* genomic sequence. We used this BAC clone as a probe to map pig *Cbg* by FISH on a metaphase chromosome spread and confirmed that pig *Cbg* maps on chromosome 7q26 (see the supplemental data published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

CBG Plasma Concentration Is Genetically Linked to the Cortisol Associated QTL

The binding capacity of CBG was measured in the plasma of 81 F2 pigs from the original cross, all offspring of a single F1 (no. 9110045) sire, which presented the highest contrast between the effect of its two QTL alleles. As expected, a high correlation was found between plasma CBG binding capacity and the level of cortisol ($r = 0.57$; $P < 0.01$). We evaluated genetic linkage between this new phenotypic measure and the pig chromosome 7 markers. A strong genetic linkage was detected exactly in the same area as for the cortisol QTL (Fig. 2). The maximum likelihood was even higher with CBG values ($P < 5.10^{-6}$) compared with cortisol ($P < 5.10^{-4}$), strengthening the implication of *Cbg* in this QTL. The estimated effect of Meishan minus Large White alleles at the likelihood peak for the animal no. 9110045 were: LnCBG, 0.358; LnCortisol basal, 0.444; and LnCortisol post stress, 0.218.

CBG Plasma Capacity and Cbg Locus Are Associated with Fat and Muscle Content Traits

Pearson correlation coefficients were calculated between CBG binding capacity values and parameters of carcass composition in the males of this F2 subpopulation (Table 1). Significant positive correlations were found between CBG levels and fat, whereas CBG was negatively correlated with muscle content. No significant correlation was found in the same samples between cortisol levels and carcass composition traits.

These data encouraged us to undertake additional genetic linkage analysis between carcass composition traits and the locus containing *Cbg*. A microsatellite marker named CBG-R was developed from the *Cbg* BAC clone and typed in the F2 progeny of F1 sire nos. 910045 and 910001, the latter being homozygous for the marker S0101. As reported previously (15), a QTL in the Cbg locus area (125–159 cM) was found for several leanness traits (muscle and loin weight). For fatness traits, a QTL at 60 cM was reported on chromosome 7. However, a second locus at 125–160 cM is also present and its significance *vs.* the 60 cM QTL varies among families. In particular, for two of the six pig families of the program (nos. 910045 and 910001), backfat weight and thickness show a strong linkage with the *Cbg* locus and none with the 60 cM region. Conversely, family from sire no. 910081 is strongly and uniquely linked to the region at 60 cM. As an example, genetic linkage analysis between the estimated carcass lean content, a trait combining fat and muscle percentage, and the chromosome 7 markers including

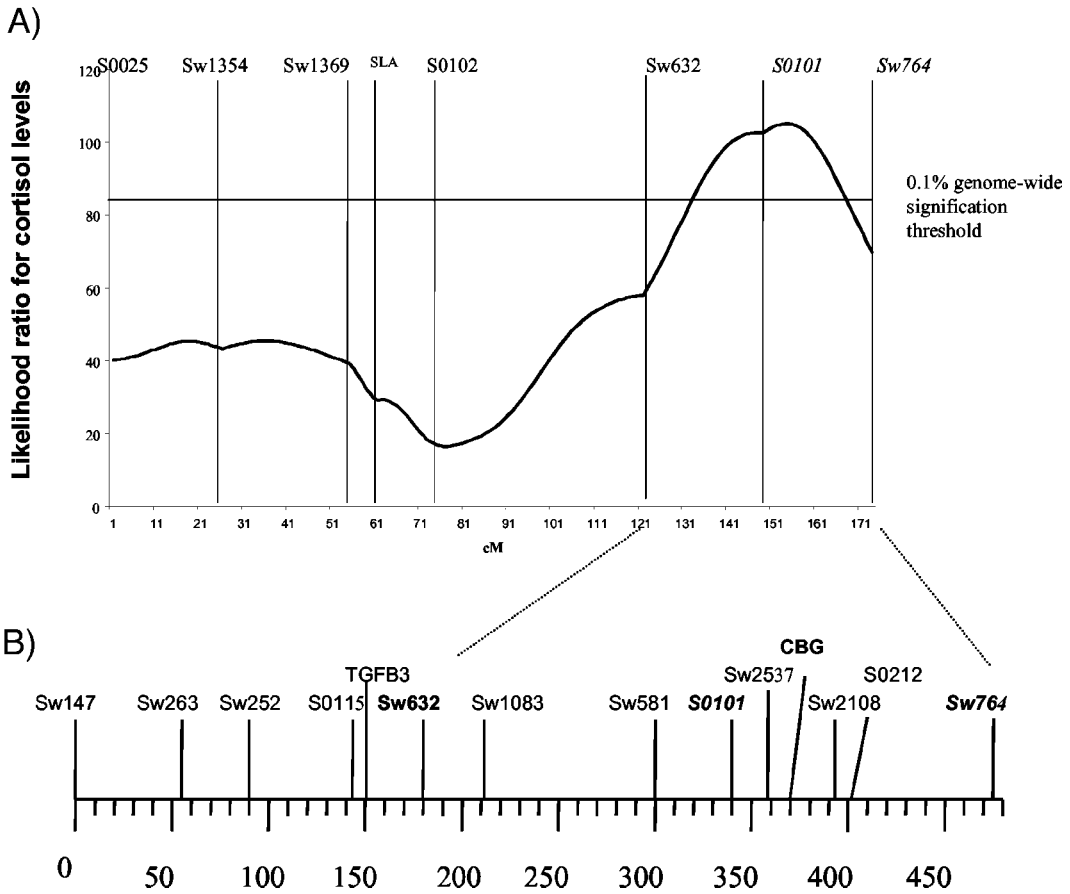

Fig. 1. Localization of Pig *Cbg* by Radiation Hybrids Mapping in the Cortisol Associated QTL Interval

The QTL associated with cortisol levels (18) is shown in A (evolution of maximum likelihood ratio test along Sscr 7 for plasma cortisol concentrations). Detailed RH map of the QTL interval is shown in B (framework radiation hybrid map of swine chromosome 7 distances are in cR_{7000}).

CBG-R is presented in Fig. 3. Linkage analysis for all other carcass composition traits can be found in the supplemental data.

Meishan Pigs Have Higher CBG Binding Activity and mRNA Expression than Large White

We compared the binding capacity and affinity constant of CBG between Large White and Meishan parental breeds by radio-binding studies. We detected no sex difference within breeds. Maximal binding capacity was on average three times higher in Meishan compared with Large White pigs. The dissociation constant was 40% higher in Meishan (Table 2). To determine how these CBG parameters affect the free concentrations of cortisol in each breed we measured cortisol concentrations in the same plasma samples and then we calculated the free cortisol concentration using the equation as described in Sodergard *et al.* (28). These cortisol values obtained from blood collected during the day on pigs fed *ad libitum* were above basal cortisol concentrations measured in previous work (15). There were no sex differences be-

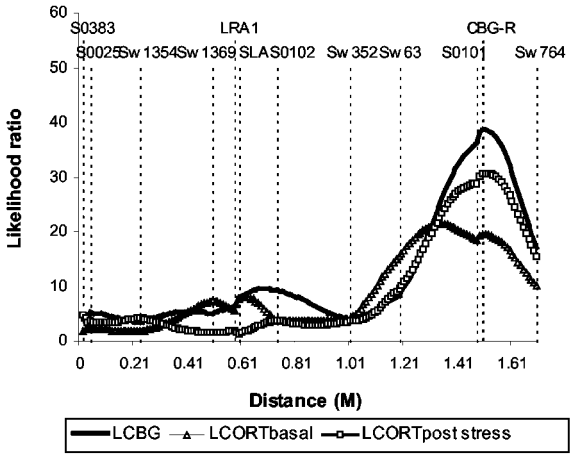

Fig. 2. Genetic Linkage Analysis of CBG Plasma Concentrations on 81 F2 Pigs

LCBG, Log-transformed CBG capacity values; LCORT post stress, Log-transformed post-stress cortisol levels; LCORT basal, Log-transformed basal cortisol levels.

Table 1. Pearson Correlation Coefficients between Carcass Composition Traits, Plasma CBG Capacity, and Total Plasma Control

	CBG	Cortisol (Basal)	Cortisol (Post Stress)
Backfat weight	r = 0.39	r = −0.18	r = 0.11
	P = 0.014	*P* = 0.28	*P* = 0.50
% (Ham + loin)	r = −0.40	r = 0.18	r = −0.11
	P = 0.012	*P* = 0.27	*P* = 0.52
% (Back + leaf fat)	r = 0.42	r = −0.17	r = 0.12
	P = 0.008	*P* = 0.29	*P* = 0.45
Estimated muscle content	r = −0.43	r = 0.16	r = −0.13
	P = 0.0064	*P* = 0.33	*P* = 0.41

Each cell of the table contains the Pearson correlation (r) coefficient and the probability (*P*). The number of observations is n = 39 in all cases.

tween breeds, but there was a 2-fold difference of total cortisol as found in previous work and 1.85-fold variation in free cortisol concentration.

To investigate this further, we performed a real-time quantitative RT-PCR to estimate *Cbg* mRNA expression in the liver of animals from the two breeds. As depicted on Fig. 4, normalized *Cbg* mRNA expression was much higher in Meishan (30.16 ± 9.26) than Large White pig liver (3.89 ± 1.6).

Sequence Analysis of Pig Cbg

From the BAC clone (383F4), we identified the genomic organization and sequence of pig *Cbg* cDNA that had not been cloned before (GenBank accession no. AF324155). As in the other species, pig *Cbg* contains five exons with the ATG codon in exon 2. At the amino acid level, we found 66% and 80% homology between pig CBG and respectively human and sheep CBG. The nucleotide sequence was used to derive oligonucleotides to amplify the five exons of *Cbg* and bits of introns in the 12 founder pigs and the six F1 sires of the program. On the 1837 bp screened on 24 different chromosomes, 17 polymorphisms were detected, among which four led to amino acid substitutions (Table 3). In the Large White breed, four haplotypes were defined with a major one (haplotype 1) found seven times over the 12 chromosomes analyzed. In the Meishan breed, six haplotypes could be inferred from the 10 polymorphic sites. Haplotype 5, 6, and 7 differ only by two or three silent polymorphisms in exon 5 and thus are very similar and the most frequent ones. There is no amino acid substitution present in every haplotype of one breed and absent in the other breed. In other words, none of the four amino acid substitutions could explain globally the difference in CBG capacity or affinity between the two pig breeds. However, we looked at the effect of the S15I and I265V mutations on CBG capacity and affinity using an *in vitro* transfection assay of plasmids containing the cDNA variants, considering that the causal mutation may be different between families and because S15 and I265 are highly conserved across the eight mammalian species in which *Cbg* has been cloned. Moreover, S15I and I265V mutations are present in the pig family of F1 sire nos. 910045 and 910001, respectively, both of them showing the highest effect on cortisol and fat traits as mentioned above. No significant effect was detected for any of these two amino acid mutations (shown in the supplemental data).

DISCUSSION

In this paper we provide evidence in favor of the hypothesis that *Cbg* is the causal gene of a QTL associated with cortisol levels and carcass composition in the pig.

CBG is a well-conserved α-glycoprotein in vertebrate species, synthesized by liver and secreted in blood, where it binds cortisol and progesterone with a high affinity ($K_A \sim 10\ nM^{-1}$). The primary role of CBG is to regulate the bioavailability and metabolic clearance of cortisol because only the free hormone is active. Recent studies provide evidence for a larger spectrum of action of CBG. From its molecular structure, CBG belongs to the serine protease inhibitors and substrates (SERPINS) superfamily, and indeed CBG is a substrate of the serine-protease elastase which cleaves CBG near its steroid binding site resulting in the local release of cortisol at sites of inflammation (29, 30). Other evidence comes from the discovery of CBG membrane receptors that would capture the CBG-cortisol complex at specific sites and transport it into the cell where it will then be dissociated (31). Finally CBG may have an intrinsic biological activity as suggested by *in vitro* studies showing that after the binding of the CBG-cortisol complex to its receptors, cAMP increases within the cell (for a recent review see Ref. 32). Furthermore, in many species, more than 68% of CBG remains in the cortisol-free state under physiological conditions, supporting the hypothesis that CBG may act as a proper hormone (33).

In this study, we obtained a genomic clone of pig *Cbg* and demonstrated that it maps on chromosome 7q26 very close to marker S0101, at the peak of the maximum likelihood curve obtained by genetic map-

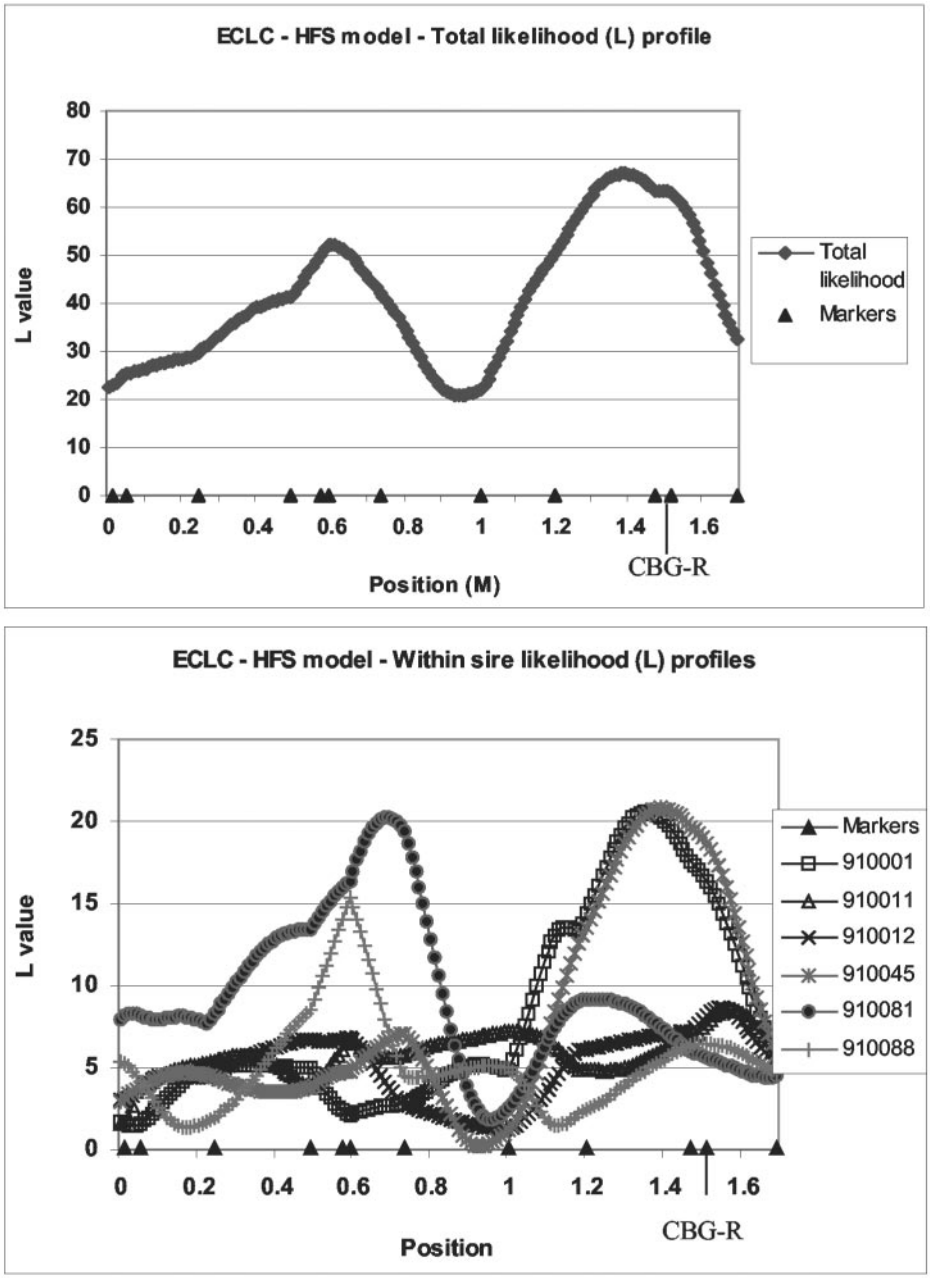

Fig. 3. QTL Mapping for Muscle Content

ECLC, Estimated carcass lean content; HFS model, half full-sibling model for interval mapping analysis. Markers are the same as in Fig. 2.

ping analysis for cortisol values. Moreover, when we calculated the genetic linkage between chromosome 7 markers and CBG binding capacity in the F2 population in which the cortisol QTL had been detected, we obtained a maximum likelihood curve of the same shape and in the same area as for cortisol values. This result was not unexpected because cortisol levels and CBG binding capacities were highly correlated. However, the fact that CBG values show a stronger linkage to marker S0101 than cortisol values strengthens the hypothesis of its implication in the QTL. As expected, biochemical properties of CBG are different between the two breeds. The 2-fold increase in binding capacity seems to overcome the 40% drop in affinity in the Meishan breed because free cortisol concentration is still higher in this breed compared with the Large White breed in our experiment. However, this may vary during the nycthemeral rhythm in particular when total cortisol levels are low. For instance, at a basal total cortisol concentration of 55 nM as detected previously for both breeds at night (15), the calculated free cortisol concentration is four times higher in Large White

Table 2. Binding Capacity (B_{max}) and Dissociation Constant (K_D) of CBG for Cortisol and Total ([F] total) and Free ([F] free) Concentrations of Cortisol in Large White and Meishan Breeds

	Large White (n = 31)	Meishan (n = 31)	*P*
CBG B_{max} (nM)	22.8 + 1.45	68.29 + 3.44	<0.005
K_D (nM)	0.72 + 0.06	1.02 + 0.06	<0.001
[F] total (nM)	138.25 + 10.15	281.02 + 12.86	<0.001
[F] free (nM)	32.8 + 0.50	60.6 + 0.65	<0.001

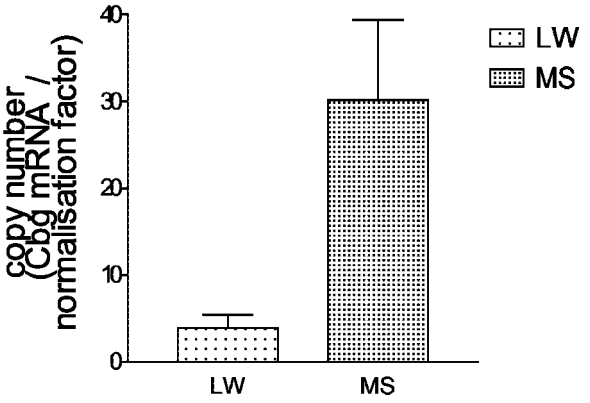

Fig. 4. Real-Time Quantitative RT-PCR of *Cbg* mRNA Expression in Liver of Large White (LW) and Meishan (MS) Breeds

Cbg mRNA expression was normalized using the expression of three housekeeping genes (HSK) as normalization factor.

(9.6 nM) compared with Meishan (2.2 nM) pigs due to the lower CBG binding capacity in Large White pigs. This may explain the higher total cortisol secretion in Meishan compared with Large White pigs because negative feedback control will be increased in the latter during the night preceding the daily surge of cortisol secretion. The overall effect of elevated CBG in the Meishan breed is thus difficult to ascertain, but the fact that the Meishan breed displays signs of hypercorticism (high fat deposits, low muscle content, and a reduced growth) favors the hypothesis that CBG properties in the Meishan lead to increased total and free cortisol concentrations as a global effect.

Most interestingly, we provide evidence suggesting that *Cbg* gene may be a regulator of fat accumulation and muscle content. Indeed, plasma CBG capacity was found correlated to carcass composition traits in the 39 males tested, positively with fat deposition and negatively with muscle content. The correlation found here suggests that the effect of CBG is strong at least in this subpopulation of F2. No correlation was detected between total cortisol and these carcass composition traits; this may be due to less environmental influences on CBG compared with cortisol values. These data are corroborated by the overlap of the cortisol associated QTL with QTL related to carcass composition traits at the *Cbg* locus. These results fit well with the acknowledged metabolic role of cortisol on fat deposition and protein catabolism in muscles (34) and show that CBG is a better predictor of carcass composition than cortisol levels. Another line of arguments suggests that CBG is indeed involved in the obesity phenotype: QTL mapping analyses in mouse and rat have pointed to the *Cbg* locus for obesity-related traits. In a backcross between the mice strains SPRET/Pt and C57BL/6, a QTL for body fat percentage was found around the marker D12Mit27 that is 1 cM from *Cbg* (35). In rats, a QTL associated with fat weight was detected in a backcross between rats OLETF (a model of type II diabetes) and Brown Norway near markers D6Mit4-D6Mit9 where rat *Cbg* maps (36). Furthermore, patients with CBG deficiency or low-affinity CBG are obese or overweight (37, 38). Although few CBG-deficient patients have been reported, this is in accordance with recent data showing that low CBG levels are associated with fat accumulation and insulin resistance in a human healthy population (39). Similarly, in the Zucker rat, lower levels of CBG binding capacity were found in the obese rats compared with the lean controls (40). In our model, a high CBG capacity is associated with elevated total cortisol levels, high fat deposition, and low muscle content. The *obese* chicken strain is another example of an animal model in which obesity is associated with high levels of CBG (41). Thus, depending on the animal model, elevated or decreased CBG is associated with obesity. Because CBG immunoreactivity and corticosterone binding activity have been detected in rat adipocytes (40, 42), it has been suggested that CBG acts as a barrier to glucocorticoid action in adipose tissue. Thus, deficiency or lower affinity of CBG will lead to increased cortisol influence on adipocytes. Indeed, increased proliferation and enhanced differentiation were found in cultured preadipocytes from a patient totally deficient in CBG compared with controls (43). Then, how can we explain that high CBG levels are associated with obesity in Meishan pigs or *Obese* chicken? it may be that the bigger pool of cortisol-CBG complex circulating in the bloodstream of these individuals can be more readily dissociated near adipose tissue for example by local free fatty acid concentrations. Free fatty acids have been shown to be potent modulators of steroid-protein interaction, reducing corticosterone-CBG binding in immature rats and increasing it in adult rats (44). In this case, CBG acts as a cortisol reservoir, similarly to what occurs at sites of inflammation (30). Therefore, for both deficiency or excess CBG capacity, fat accumulation may be the consequence of a higher local bioavailability in

Table 3. Haplotype Analysis of the Six Large White and Six Meishan Parental Animals

Breed	#	Haplotype																	Occur /12
		e	x	o	n	2	e	x	o	n	3	i	n	3	ex4	e	x	5	
Large White	1	G	C	C	A	G	C	T	A	G	C	T	C	C	A	T	C	C	7
	2	G	C	C	A	A	C	T	A	G	C	T	C	C	A	T	C	C	2
	3	G	C	C	A	A	C	T	A	G	C	T	C	C	G	T	C	C	1
	4	G	T	T	A	G	C	C	G	C	C	T	C	T	G	T	C	C	2
Meishan	5	G	C	C	G	G	T	C	G	C	T	C	T	C	G	C	T	G	3
	6	G	C	C	G	G	T	C	G	C	T	C	T	C	G	T	C		1
	7	G	C	C	G	G	T	C	G	C	T	C	T	C	G	T	C	C	4
	8	G	C	C	G	G	C	C	G	G	C	C	C	T	G	T	C	C	2
	9	G	C	C	G	G	C	C	G	G	C	C	T	C	G	C	T	G	1
	10	T	C	C	A	G	C	C	G	G	C	C	T	C	G	T	C	G	1
		*					*		*						*				
position		133	134	539	620	626	859	866	882	890	960	i+38	i+46	i-58	1008	1220	1437	1446	
		S15I					T257M		I265V						G307R				

*, Mutations leading to amino acid change.

free cortisol. Alternatively, it cannot be ruled out that CBG may act as a proper hormone as hypothesized by various authors (32, 33).

We have not found a functional mutation in the coding region of pig *Cbg* gene that could explain the difference in CBG expression or affinity between the two breeds. The S15I substitution lies in the signal peptide domain of the CBG precursor; thus, it could have had an effect on CBG maximal binding capacity by an increased secretion rate. This was not confirmed in the *in vitro* transfection assay and did not fit with the large difference in Cbg mRNA expression observed. The binding site of CBG for cortisol has not been clearly defined but may be located on the Cys249 (45). The substitutions T257M and I265V are close to this site. However, T257M is found equally frequently in both breeds and is not well conserved in evolution. Conversely I265V is well conserved and present only in haplotype 4 of the Large White breed, but we did not detect a dissociation constant difference in the *in vitro* transfection assay. The G307R mutation does not lie in a known domain of the protein and is present in both breeds. Therefore, the lower cortisol affinity in Meishan remains enigmatic and may be of artifactual origin. Concerning the CBG expression differences, extensive analysis of the promoter, intronic, and intergenic regions of *Cbg* gene is now required.

The high circulating cortisol levels of the Meishan pig could result from many biological mechanisms involved in cortisol production, bioavailability, and clearance. The fact that our QTL genetic mapping analysis points to CBG as a major factor at the origin of high cortisol levels emphasizes even more the importance of this protein in the regulation of the HPA axis and to its pathophysiological outcomes. In particular, our results show that *Cbg* may be a better predictor and an interesting new target for understanding obesity susceptibility.

MATERIALS AND METHODS

Radiation Hybrid Mapping

Reactions were performed in independent duplicates on IMpRH panel (27). PCR products were analyzed on 2% agarose gels in 1× TBE buffer after staining with ethidium bromide. A third amplification was carried out on clones for which discordant results were obtained. Vectors of amplification results were submitted to IMpRH database accessible at http://imprh.toulouse.inra.fr (46).

FISH Mapping

Metaphase chromosomes were obtained from cultures of peripheral blood lymphocytes cultures. To identify chromosomes, metaphase spreads were G-banded using G-T-G banding technique before hybridization, and pictures of the best metaphases were taken using a video printer as described earlier (47).

In situ hybridization experiments were performed according to Ref. 47 with some of the published modifications (48).

QTL Mapping

Data were first checked for the normality of distributions. The three traits (CBG capacity, basal, and post-stress levels of cortisol) had log-normal distributions and data were transformed into their logarithmic scores before analysis. Details about animals and carcass composition traits can be found in Ref. 19. QTL mapping was performed using multipoint maximum likelihood techniques. A test statistic defined as the ratio of likelihoods under the hypotheses of one (H1) *vs.* no (H0) QTL linked to the set of markers considered was computed at each position (each centimorgan) along the chromosome. The chromosome 7 marker map used was that

computed from the genotypes of more than 1100 pigs by Bidanel *et al.* (17). Under H1 hypothesis, a QTL with a gene substitution effect for each sire and dam was fitted to the data. Further details on likelihood computation procedures can be found in (17). Estimates of average substitution effects were computed at the position with the highest likelihood ratio.

Chromosome-wide significance thresholds were determined empirically by simulating the data assuming a polygenic infinitesimal model and a normal distribution of performance traits. A total of 50,000 simulations was performed for each trait. Chromosomal test significance level (P_c) corresponding to a genome-wide test probability (P_g) was obtained using the Bonferroni correction, *i.e.* as a solution to: $P_g = 1 - (1 - P_c)^{19}$, which gives $P_c = 0.0027$ and 0.000054, respectively, for significant ($P_g = 0.05$) and highly significant levels ($P_g = 0.001$) (49).

BAC Library Screening and Development of Microsatellite Marker CBG-R

BAC clones were isolated by three-dimensional PCR-based screening of a porcine BAC library as described previously (50). BAC 383F4 containing the pig CBG sequence was recovered using a primer pair designed from the human exon 2 CBG sequence (forward, ACACCTGTCTTCTCTGGCTG; reverse, ACAGGCTGAAGGCAAAGTC). PCRs were run for 35 cycles of 30 sec at 94 C, 30 sec at 56 C, and 30 sec at 72 C, in a 20-μl reaction volume containing 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 8 pM of each primers, 2 U *Taq* DNA polymerase and reaction buffer (PerkinElmer Applied Biosystems, Foster City, CA).

Development of Microsatellite Marker CBG-R

The 383F4 BAC clone was digested using Sau3A enzyme and subcloned in pGEM vector. After screening with a (CA)10 probe, a subclone containing a microsatellite was selected and sequenced. Two primers were defined (CBGR1/132 5′-TTTGCTATGCTAGGTTCATGGTT-3′ and CBGR1/5 5′-AGGGTAAAGGGTCATGAGGTACA-3′) to amplify CBGR marker in the following conditions: 35 cycles of 30 sec at 94 C, 30 sec at 58 C, and 30 sec at 72 C, in a 25-μl reaction volume containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.25 μM of primers, 50 ng of DNA.

Sequencing

Sequencing reactions were performed using the Prism AmpliTaq FS diChloroRhodamine Dye Terminators kit (ABI, Foster City, CA) on a PerkinElmer 9700 thermocycler, and analyzed on a 3700 automatic sequencer (ABI). Sequences were obtained from PCR, RT-PCR fragments or directly from the BAC clone 383F4. For the haplotype analysis, sequences were obtained from PCR products covering all exons. The sequence of oligonucleotides pairs used for these PCRs were: exon 1, forward 5′-ATTAACCAGCAGGGAAGCTG, reverse 5′-GCAGTCATGGTTTCGTTTTG-3′; exon 2, forward 5′-CCCTGTATGCCTGTCTCCTC-3′, reverse 5′-CCTGCTCCAAGAACAAGTCC-3′; exon 3, forward 5′-GTCAAGGTGCCCATGATGTTCC-3′, reverse 5′-GCCAGGTGCACCCCTTTCC-3′; exon 4, forward 5′-CCTCACTAAAATATCTAACCAGCA-3′, reverse 5′-ACCTACCTTGGATCTTCG-3′; exon 5, forward 5′-TCTGCAATTTGACGAGAAGG-3′, reverse 5′-CCTAGGACAACGATCGAACC-3′. All 12 F0 and six F1 animals were tested.

CBG Binding Assay

Blood samples were collected in evacuated heparinized tubes from 6- to 8-wk-old piglets (15 males and 16 females in each breed Large White and Meishan) fed *ad libitum*. Tubes were kept on ice until centrifugation and plasma aliquots were frozen at −80 C until analysis.

The binding capacity of CBG and its affinity for cortisol were measured at 4 C by a solid phase binding assay using Concanavalin A-Sepharose (51). The equilibrium association constant and the binding capacity of CBG for cortisol were calculated by Scatchard analysis using "bound" as the quantity of cortisol specifically bound to the glycoproteins adsorbed to the gel and "free" as the concentration of cortisol in the aqueous phase.

Cortisol RIA

Plasma concentrations of cortisol were quantified by RIA, as previously described (15). The intra- and interassay coefficients of variation were 7.6% and 12.5%, respectively. Because the low-affinity binding of cortisol to albumin was not measured experimentally in this study, we used the value measured by Barnett *et al.* (52), *i.e.* 2.531.

Real-Time RT-PCR

Liver total RNA from three pigs of each breed was extracted with the Trizol kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative RT-PCR was performed using a Rotor-Gene 2000 (Corbett Research, Sydney, Australia) as described previously (53). Triplicate PCRs were assembled in 0.1 ml strip tubes containing cDNA from 10 ng of total RNA, 0.2 μl 50× Titanium *Taq* DNA polymerase, 1× Titanium *Taq* PCR Buffer (CLONTECH Laboratories, Inc., Palo Alto, CA), 1 mM dNTP, 100 mM each of the appropriate primer, 0.5× Sybr Green I (Molecular Probes, Eugene, OR). Preliminary results showed that the RPL19, β-microglobulin and β-actin housekeeping genes had the most stable gene expression in pig liver within our experimental conditions. The RT-PCR expression of the target gene is thus presented as a ratio, normalized using the *genorm* software (54) and the expression of the above-mentioned housekeeping genes. Primers pairs used were: CBG: 154 bp, GenBank accession no. AF324155, forward 5′-CCAGAATGCCCTGCCGAAGAT-3′, reverse 5′-GATGAAGGGCCGGTTGAAG-3′; RPL19: 165 bp, accession no. AF435591, forward 5′-AAATCGCCAACGCCAACTC-3′, reverse 5′-TGGCAGTACCCTTCCGCTTAC-3′; HPRT1: 267 bp, accession no. AF143818, forward 5′-CCTAATCATTATGCCGAGGAT-3′, reverse 5′-ATCGCCCGTTGACTGG-3′; β-actin 158 bp, accession no. U07786, forward 5′-CCACACGGTGCCCATCTACGA-3′, reverse 5′-TGATGTCCCGCACGATCTC-3′; β-microglobulin 221 bp, accession no. L13854, forward 5′-ACGGAAAGCCAAATTACCTGA-3′, reverse 5′-CTTGGGCTTATCGAGAGTCA-3′.

Statistics

Correlation matrices and Student's *t* tests were performed using *Statistica* version 5 software.

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