Evidence for New Alleles in the Protein Kinase Adenosine Monophosphate-Activated γ_3-Subunit Gene Associated With Low Glycogen Content in Pig Skeletal Muscle and Improved Meat Quality

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ABSTRACT

Several quantitative trait loci (QTL) affecting muscle glycogen content and related traits were mapped to pig chromosome 15 using a three-generation intercross between Berkshire × Yorkshire pigs. On the basis of the QTL location the *PRKAG3* (protein kinase, AMP-activated, γ_3-subunit) gene was considered to be a good candidate for the observed effects. Differences in the *PRKAG3* gene sequences of the founder animals of the intercross were analyzed. The *RN*$^-$ mutation previously reported was not present in the cross but three missense substitutions and a polymorphic short interspersed element (SINE) were identified. To confirm the hypothesis that at least one of these mutations was associated with differences in meat quality, >1800 animals from several unrelated commercial lines were genotyped for the candidate substitutions and an association study was performed. The results demonstrate the presence of new economically important alleles of the *PRKAG3* gene affecting the glycogen content in the muscle and the resulting meat quality. Haplotype analysis was shown to resolve the effects of *PRKAG3* more clearly than analysis of individual polymorphisms. Because of their prevalence in the more common commercial breeds, the potential implications for the pig industry and consumers are considerably greater than the original discovery of the *RN*$^-$ mutation. Furthermore, these results illustrate that additional alleles of genes involved in major mutations may play a significant role in quantitative trait variation.

THE recent discovery (Milan *et al.* 2000) of a nonconserved substitution in the *PRKAG3* gene has explained the dominant mutation (denoted *RN*$^-$) that accounted for large differences in meat quality and processing yield in the Hampshire pig breed (Monin and Sellier 1985; LeRoy *et al.* 1990). This substitution (R200Q) in the *PRKAG3* gene caused a 70% increase in glycogen in muscle in *RN*$^-$ homozygous and heterozygous animals that then resulted in the observed lower muscle pH 24 hr after slaughter, in reduced water-holding capacity in the muscle, and in much lower yield of a cured cooked ham product. The 200Q allele is associated with all *RN*$^-$ animals and was present in a very high percentage of Hampshire pigs but not in pigs with an *rn*$^+$ phenotype or in other breeds (Milan *et al.* 2000 and this study).

Mammalian adenosine monophosphate (AMP)-activated protein kinase (AMPK) plays a key role in regulating energy homeostasis in eukaryotes (Hardie *et al.* 1998). It consists of a catalytic subunit (α) and two regulatory subunits (β and γ). Two isoforms have been identified for both the α- and β-subunit and there are three isoforms reported for the γ-subunit in several mammals (Gao *et al.* 1996; Stapleton *et al.* 1996, 1997; Milan *et al.* 2000). The γ_3-peptide, encoded by the *PRKAG3* gene, is one of three options for the γ regulatory subunit of AMPK. When eukaryotic cells are subjected to environmental or nutritional stress factors and the AMP/ATP ratio rises significantly, then the "AMPK cascade" is induced, initiating measures to conserve energy (Thornton *et al.* 1998) and to induce the ATP synthetic pathways (Hardie *et al.* 1998).

The identification of quantitative trait loci (QTL) for meat quality traits in the region of the *PRKAG3* gene in an *rn*$^+$ resource population (Malek *et al.* 2001) suggested that new allelic variation in this gene may be responsible for the observed effects. In this article we report the presence of new economically important alleles of the *PRKAG3* gene affecting the glycogen content in muscle and in general the meat quality traits of pigs that include ultimate pH and color measures and that are correlated with water-holding capacity, drip loss, tenderness, and cooking loss (Sellier 1998). Initial estimates of the allelic and haplotype effects and frequencies suggest that these alleles may have significant economic potential for the pig industry and ultimately for consumers in terms of improved pork quality.

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MATERIALS AND METHODS

Pedigree, linkage, and QTL mapping: We have generated an intercross between Berkshire and Yorkshire (B × Y) pig breeds, yielding 525 F_2 offspring, and used this pedigree to map QTL for meat quality (Malek *et al.* 2001) using an interval mapping method (Haley *et al.* 1994). In this cross, the Berkshire breed was chosen because it is regarded as having very good meat quality, particularly in terms of pH, color, water-holding capacity, and tenderness. The *PRKAG3* gene was mapped to the B × Y family linkage map using the CRI-MAP (version 2.4) mapping program (Green *et al.* 1990). The interval mapping method (Haley *et al.* 1994), including the *PRKAG3* site information, was used to map the QTL for meat quality for pig chromosome 15 (SSC 15; Figure 1). The QTL effects were estimated and represent the average Berkshire allelic effect compared to the average Yorkshire allelic effect.

Tissue samples and DNA/RNA isolation: Blood samples and phenotypes were collected and recorded on the F_0, F_1, and F_2 animals from the intercross family (Malek *et al.* 2001) together with blood samples and muscle tissue from the ham and loin area of several F_3 animals. We also obtained a large collection of blood samples from five different commercial lines of pigs (Landrace, Large White, Duroc, Duroc Synthetic, and Berkshire). Genomic DNA was isolated from whole blood by standard salting-out procedures and total RNA was extracted from ham and loin muscle tissue using the TRIzol reagent method according to the manufacturer's instructions (GIBCO/BRL, Rockville, MD).

PCR, reverse transcription-PCR, rapid amplification of cDNA ends, and polymorphism discovery: On the basis of the *PRKAG3* pig gene sequence available in GenBank (no. AF214521), we designed primers to amplify the entire coding regions of the *PRKAG3* gene. The PCR reactions were performed using 12.5 ng of porcine genomic DNA, 1.5 mm $MgCl_2$, 0.125 mm dNTP, 0.3 μm of each primer and 0.35 units *Taq* DNA polymerase (Promega, Madison, WI), and PCR buffer (10 mm Tris-HCl, 50 mm KCl, and 0.1% TritonX-100) in a 10-μl final volume. The reverse transcription of total RNA (3.5 μg) was performed by random hexanucleotide priming and Superscript II (GIBCO/BRL) according to the manufacturer's protocol (primers: Set A, forward 5′ ATGAGCTTCCTA GAGCAAGGAG 3′ and reverse 5′ CAGGTCTCAATCTTATG TTCTTC 3′; set B, forward 5′ CGTCCGAGCGGCACCTT TGT 3′ and reverse 5′ AAGGTTCCAAGGTTCTCAGGC 3′). 5′ rapid amplification of cDNA ends (RACE) experiments were performed using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions, followed by sequencing of the PCR products (gene-specific primers: outer 5′ CCCACGAAGCTCTGCTTCTT 3′ and inner 5′ TCCTTGCTCTAGGAAGCTCAT 3′). The amplicons were sequenced using dye terminators (PE Applied Biosystems, Foster City, CA) on an ABI 377 automated sequencer. We used Sequencer software (Gene Codes, version 4.0.5, Ann Arbor, MI) to assemble the sequences and to identify polymorphisms.

Genotyping and PCR-restriction fragment length polymorphism analysis: The region flanking each analyzed missense mutation was amplified using the same pair of primers for the T30N and G52S substitutions (forward 5′ ATGAGCTTCC TAGAGCAAGGAG 3′ and reverse 5′ GGCTGCATGATGTTAT GTGCCT 3′) and a different pair for I199V (forward 5′ GGAG CAAATGTGCAGACAAG 3′ and reverse 5′ CCCACGAAGCT CTGCTTCTT 3′). After digestion with *Bsa*HI (for I199V), *Hph*I (for G52S), and *Sty*I (for T30N) restriction enzymes, the digested PCR products were separated on 4% NuSieve agarose (FMC, Rockland, ME) gels and stained with ethidium bromide. For the short interspersed element (SINE) polymorphism, PCR amplification (primers: forward 5′ GAAACTCTT CTCCCCACAGAC 3′ and reverse 5′ GGCTGCATGATGTTA TGTGCCT 3′) was followed by separation of the products on a 1% agarose (AMRESCO, Solon, OH) gel. After genotyping for these polymorphisms, all the animals with haplotype 2 (Table 6) were also genotyped for the R200Q substitution to increase the chance of finding the RN^- or 200Q allele (see Milan *et al.* 2000). Two homozygotes for the 200Q allele and four carriers were found and these were removed from further statistical analyses so that the RN^- mutation did not affect our analysis of the other substitutions. For the R200Q substitution we used the same primers as for the I199V mutation and the digestion was performed with the *Bsr*BI restriction enzyme. As a final check, a random sample of ~100 animals with different haplotypes was also scored for the R200Q substitution, but none of the animals carried the 200Q allele.

Phenotypic trait measurement: Phenotypic measures for the B × Y family were made using typical industry techniques (Malek *et al.* 2001) and included pH, color, and glycolytic potential. For the pigs from five commercial lines, data were collected at a commercial packing plant and individual meat color (loin and ham reflectance—lower values preferred) and individual loin and ham pH 24 hr after harvest (higher values preferred) were obtained. For the packing plant data no measures of glycogen or glycolytic potential were obtained. The measures of color and pH phenotypic traits are common industry measures of meat quality that are indirectly correlated with glycogen and glycolytic potential.

Statistical analysis: *Berkshire × Yorkshire F_2 population analysis:* Associations between the *PRKAG3* I199V substitution and glycogen, lactate, glycolytic potential, and meat quality traits in the B × Y F_2 population were tested using general linear model procedures (SAS procedure GLM; SAS Institute, Cary, NC) with a model that included dam, slaughter date, sex, and I199V genotype. Least-squares (LS) means for all three genotypes were obtained for the I199V substitution.

Commercial lines analyses: The associations between the *PRKAG3* polymorphisms and meat quality traits were tested using mixed-model procedures (SAS procedure MIXED; SAS Institute) with a model that always included sire as a random effect and slaughter date and marker genotype(s) as fixed effects. Line was added as a fixed effect for across-line analyses. Sex and farm were not included because all traits were measured on females only and no more than one farm was represented on each slaughter date. While males were not used in this portion of the analysis, our results in the B × Y suggest no sex-by-genotype effect. A full model including a separate genotype effect for each of the three substitution sites was fitted across the five commercial lines. Nonsignificant genotype effects were removed by backward elimination (P to remove >0.10) to identify which substitutions were associated with effects on the meat quality traits.

LS means for the three genotype classes were obtained within the commercial lines for each of the substitutions analyzed individually. No line-by-genotype interactions were found and therefore, to improve the reliability of the estimates of the allele effects, the data from five lines were pooled for an across-line analysis.

The combined effects of the three substitutions were estimated as haplotype substitution effects. Contrasts between haplotypes were estimated from a model including sire (random), slaughter day, and one variable for each haplotype with values −1, 0, and 1 corresponding to the animal having 0, 1, or 2 copies of the haplotype in question. The haplotype substitution effects were presented as deviations from the mean of the haplotypes and reflect the differences from the worst to the best haplotype. The number of animals used in association analyses varied on the basis of the trait measured and are listed in Tables 3–5.

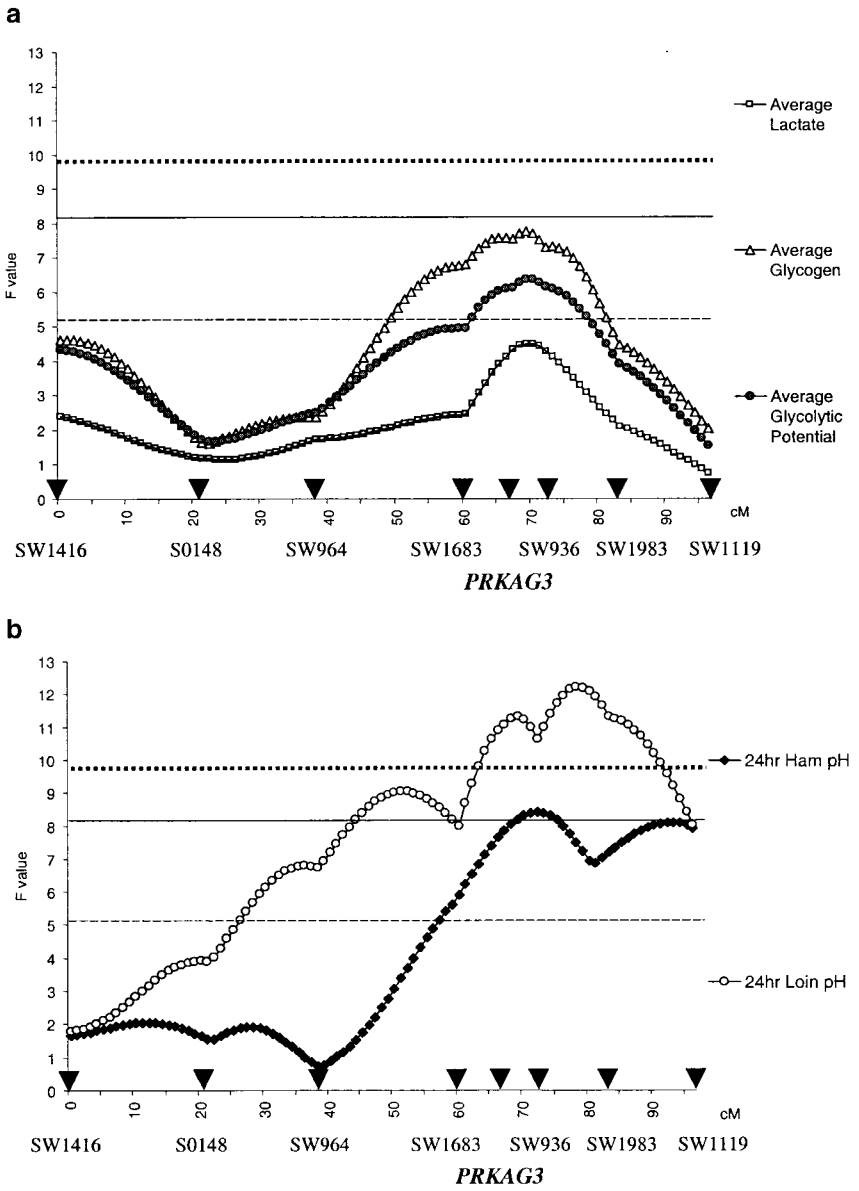

FIGURE 1.—*F*-ratio curves for evidence of QTL associated with meat quality for SSC 15. The *x*-axis indicates the relative position on the linkage map. The *y*-axis represents the *F*-ratio. Arrows on the *x*-axis indicate the position where a marker was present. (- - -) 5% chromosome-wise significance; (—) 5% genome-wise significance; (· · ·) 1% genome-wise significance. (a) Average glycogen, average lactate, and average glycolytic potential traits. (b) pH traits.

RESULTS

Marker development and linkage mapping: Several significant QTL were detected on SSC15 (MALEK *et al.* 2001) in the region where the *PRKAG3* gene was located (MILAN *et al.* 2000) between the markers SW1683 and SW1983 (Figure 1). These included QTL for average glycogen content and glycolytic potential that have been reported (MILAN *et al.* 2000) to be affected by the *PRKAG3* 200Q allele, as well as the traits 24-hr ham and loin pH and 24-hr loin Hunter *L* values (light reflectance). The favorable allele at this QTL, which, interestingly, has an additive effect (the RN^- mutation is dominant), was derived predominantly from the Berkshire breed (generally regarded as having very good meat quality), as expected (Table 1). The *PRKAG3* gene was the unique candidate gene in this area, based on the recent development of the bacterial artificial chromosome contig in the porcine *RN* region (MILAN *et al.* 2000), the high degree of linkage order conservation of the porcine map in this area with the human transcript map (JEON *et al.* 2001), and the recently developed human genome map (LANDER *et al.* 2001). We first tested the founder animals, two Berkshire sires and nine Yorkshire dams, for the published RN^- substitution (R200Q). All the founder animals had the rn^+ allele (200R). By sequencing the entire coding region of the *PRKAG3* gene in B × Y family founders and in four F_3 individuals with extreme values for meat quality, we identified three missense mutations: the T30N and the I199V substitutions previously described (MILAN *et al.* 2000) and a new missense mutation (G52S). Another nonsynonymous substitution (P53L) found by MILAN *et al.* (2000) was not found to be segregating in the founders of the B × Y family where they were all 53P. Due to the lack of information on the 5′ untranslated region, we used RACE to find the complete 5′ flanking sequence

TABLE 1

Evidence for significant QTL at the 5% chromosome-wise level for various meat quality traits for pig chromosome 15

Trait	*F*-value[a]	Location (cM)	Additive		Dominance		Variance[c] (%)
			Effect[b]	SE	Effect	SE	
Ave. glycogen[d]	7.74	69	−0.70	0.21	0.65	0.031	3.52
Ave. lactate[d]	4.50	69	−2.24	0.79	−1.10	1.16	2.00
Ave. glyoytic potential[d]	6.37	69	−3.63	1.02	0.18	1.50	4.69
24 hr ham pH	8.42[e]	72	0.05	0.01	−0.02	0.02	4.00
24 hr loin pH	12.21[f]	78	0.05	0.01	−0.01	0.02	5.61

[a] Chromosome-wise *F*-statistic threshold at the 5% level, as determined by permutation test, was 5.02.
[b] Additive (*a*) and dominance (*d*) QTL effects correspond to genotype values of $+a$, d, and $-a$, respectively, for individuals having inherited two Berkshire alleles, heterozygotes, and individuals with two Yorkshire alleles. Positive additive effects indicate that Berkshire alleles increased the trait, and negative effects indicate that the Berkshire alleles decreased it. Dominance effects are relative to the mean of the two homozygotes.
[c] Percentage of variance is the genetic variance at the QTL based on estimated additive and dominance effects and allele frequencies of ½, as a percentage of the residual variance in the F_2.
[d] Units of measure, μmol/g.
[e] Significant at the 5% genome-wise level ($F > 8.22$).
[f] Significant at the 1% genome-wise level ($F > 9.96$).

and gene organization in that region. An intronic SINE polymorphism was discovered starting 79 bp upstream of the start codon but this was present in only three Yorkshire grandams. On the basis of the differences in allele frequency of each site between the founders of the intercross family, we considered the G52S and I199V substitutions as the most likely candidates for the meat quality QTL reported previously. Using the I199V substitution we mapped the *PRKAG3* gene in the B × Y linkage map to a position below the broad peak(s) of the QTL for glycogen, lactate, and glycolytic potential and 24-hr pH (Figure 1). After adding the *PRKAG3* I199V information, the map length and marker order on SSC 15 was the same as in Malek *et al.* (2001). Reanalysis of the QTL including *PRKAG3* I199V (Figure 1) caused small changes in the *F* value and the location of the QTL peaks on SSC 15 (from 0 to 3 cM) when compared with the results of Malek *et al.* (2001).

F_2 association study: Using an association analysis, we found significant effects of all three of the substitutions (T30N, G52S, and I199V) on average glycogen and lactate content and also on glycolytic potential in the F_2 B × Y population (data shown only for I199V substitution; Table 2). The most significant effects were revealed for I199V substitution for most of the traits analyzed, including glycogen and lactate content and glycolytic

TABLE 2

Association results between the genotypes at the I199V substitution site of the *PRKAG3* gene and meat quality traits in Berkshire × Yorkshire F_2 animals

Traits	I199V		
	II	IV	VV
Ave. glycogen	8.01 $(0.31)_{a,b}$**	9.10 $(0.24)_{a}$**	9.37 $(0.33)_{b}$**
Ave. lactate	84.83 $(1.17)_{a}$***	86.83 $(0.91)_{b}$*	90.54 $(1.27)_{a,b}$***,*
Ave. glycolytic potential	100.84 $(1.50)_{a,b}$*,****	105.02 $(1.17)_{a,c}$*	109.28 $(1.64)_{b,c}$***,*
Packing plant ham pH	5.91 $(0.02)_{a}$**	5.89 (0.02)	5.84 $(0.02)_{a}$**
Packing plant loin pH	5.80 $(0.02)_{a,b}$**,****	5.75 $(0.01)_{a,c}$**,*	5.71 $(0.02)_{b,c}$***,*
Lab loin pH	5.86 $(0.02)_{a,b}$**,****	5.80 $(0.01)_{a,c}$**,*	5.77 $(0.02)_{b,c}$***,*
Lab loin Minolta	21.54 $(0.29)_{a}$**	22.11 (0.22)	22.76 $(0.31)_{a}$**
Packing plant loin Hunter	44.17 $(0.41)_{a}$*	45.07 (0.32)	45.49 $(0.45)_{a}$*
Lab loin Hunter	46.56 $(0.30)_{a}$*	47.07 (0.23)	47.70 $(0.33)_{a}$*

The following traits were not significant at $P < 0.05$: Lab ham Minolta, Lab ham Hunter, and packing plant loin Minolta. Least-squares means were estimated for each trait and are presented with standard errors of the estimates in parentheses. The numbers of animals in each genotypic class are $n = 131$ (II), 260–265 (IV), and 111–113 (VV). Estimates with the same subscript are significantly different. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

TABLE 3

Genotypic frequencies for the T30N, G52S, and I199V substitutions in the *PRKAG3* gene in five commercial pig breeds

Single nucleotide polymorphism	Genotype	Landrace	Large White	Berkshire	Duroc	Duroc Synthetic
T30N	TT	0.27	0.69	0.89	0.34	0.37
	TN	0.50	0.29	0.11	0.46	0.48
	NN	0.23	0.02	0.00	0.20	0.15
	n	556	404	103	298	627
G52S	SS	0.07	0.19	0.00	0.02	0.03
	SG	0.42	0.49	0.10	0.25	0.24
	GG	0.51	0.32	0.90	0.73	0.73
	n	560	409	91	257	649
I199V	II	0.02	0.07	0.74	0.17	0.14
	IV	0.23	0.31	0.25	0.44	0.47
	VV	0.75	0.62	0.01	0.39	0.39
	n	569	375	89	260	578

potential measures, but also for some of the meat quality traits associated with these measures. From the F_2 data, the 30T, 52G, and 199I alleles were favorable in terms of meat quality. Given the large expected linkage disequilibrium in the intercross, it was necessary to investigate and confirm the effects of these mutations in several outcross commercial lines of pigs to determine whether this gene is likely to be involved directly in the observed variation in meat quality.

Analysis of commercial populations: The genotypic frequencies for the analyzed substitutions are presented in Table 3. For all three substitution sites, the Berkshire line had a higher frequency for the genotypes associated with low glycogen content (and higher meat quality) in skeletal muscle on the basis of the B × Y F_2 data. The other commercial populations have lower frequencies of the favorable alleles with this being particularly marked for the I199V substitution when compared with the Berkshire population.

The *PRKAG3* mutations and their associations with meat quality were tested for each of the five commercial lines and also across all of the lines. Backward elimination of substitution sites, in the across-line analysis, kept I199V in the model for all six traits; G52S for ham pH, loin pH, loin Minolta L, and loin Minolta b; and T30N was kept for ham Minolta L, loin Minolta L, and ham Minolta b.

Because each of the substitutions showed distinct associations with at least three of the traits, the effects of each substitution were estimated independently. Least-squares estimates of the genotype means across lines (Table 4) and within lines (Table 5) showed significant effects between the analyzed substitutions and measures of meat quality, suggesting that several additional (new) rn^+ alleles may exist.

The association study revealed that the largest effects across the lines (Table 4) and also within the lines (Table 5, data shown only for I199V) were obtained with the I199V substitution for all the traits analyzed. For this substitution the associations were highly significant ($P < 0.0005$) for all of the meat quality traits used in this study when analyzed across lines. Significant associations with at least one of the traits were revealed for the same substitution within each of the individual lines, with highly significant effects for ham Minolta b in Duroc and Duroc Synthetic and for loin pH in Duroc Synthetic. These two breeds (Duroc Synthetic and Duroc) have the best frequency distribution for association analysis with a sufficient number of animals for each genotype (Table 5). In the across-line analysis and most of the individual line results, the effects were in the same direction for all traits with allele 199I being the favorable allele for high meat quality.

Significant effects, but smaller when compared to the I199V, were revealed for the T30N substitution in five of the traits when analyzed across lines (Table 4). Within-line analyses of T30N revealed effects almost exclusively in Duroc and Duroc Synthetic populations (data not shown). In most of the situations, the effects were in the same direction, the allele 30T being associated with a better meat quality.

For the G52S substitution, significant ($P < 0.05$) effects were identified for only two of the traits (ham pH and loin Minolta L) in across-line analysis, and a different allele was identified as favorable for those traits. Within-line analysis revealed significant associations for loin Minolta color scores for just the Duroc Synthetic population (data not shown).

In the five commercial populations we tested, we found just four haplotypes (Table 6). The Berkshire population is the least polymorphic, having haplotype 3 (30T-52G-199I) at a high frequency (0.87). In Large White, haplotype 2 (30T-52S-199V) is the most frequent and haplotype 1 (30N-52G-199V) has the highest fre-

TABLE 4

Association results between the genotypes at T30N, G52S, and I199V substitution sites of the *PRKAG3* gene and meat quality traits across five commercial pig breeds

	T30N			G52S			I199V		
Traits	TT	TN	NN	SS	SG	GG	II	IV	VV
Ham pH	5.76 $(0.01)_{a,b}$***	5.71 $(0.01)_{a,c}$****	5.69 $(0.01)_{b,c}$****	5.77 $(0.02)_{a}$*	5.74 (0.01)	5.74 $(0.01)_{a}$*	5.81 $(0.01)_{a}$***	5.74 $(0.01)_{a}$***	5.71 $(0.01)_{a}$***
n	461	506	176	76	378	659	128	376	559
Loin pH	5.74 $(0.01)_{a,b}$******	5.73 $(0.01)_{a,c}$**	5.70 $(0.01)_{b,c}$*****	5.74 (0.01)	5.73 (0.01)	5.73 (0.01)	5.78 $(0.01)_{a}$***	5.74 $(0.01)_{a}$***	5.71 $(0.01)_{a}$***
n	772	788	258	135	620	1054	199	614	922
Ham Minolta L	45.9 $(0.25)_{a,b}$******	46.6 $(0.26)_{a}$**	47.2 $(0.36)_{b}$***	45.5 (0.49)	46.2 (0.29)	46.5 (0.25)	44.9 $(0.38)_{a,b}$***	46.5 $(0.27)_{a}$***	46.9 $(0.26)_{b}$***
n	462	509	176	76	379	662	128	376	561
Loin Minolta L	44.8 $(0.17)_{a}$*	44.8 $(0.18)_{b}$*	45.3 $(0.24)_{a,b}$*	45.6 $(0.30)_{a,b}$*	45.0 $(0.19)_{a}$*	44.9 $(0.16)_{b}$*	44.2 $(0.26)_{a}$***	44.7 $(0.18)_{b}$**	45.2 $(0.18)_{a,b}$******
n	774	790	260	135	622	1060	200	615	925
Ham Minolta b	4.18 $(0.10)_{a,b}$******	4.49 $(0.10)_{a,c}$****	4.79 $(0.14)_{b,c}$****	4.05 (0.19)	4.28 (0.11)	4.40 (0.10)	3.63 $(0.15)_{a}$***	4.31 $(0.10)_{a}$***	4.70 $(0.10)_{a}$***
n	459	504	175	75	376	656	128	373	554
Loin Minolta b	3.34 (0.05)	3.43 (0.06)	3.49 (0.08)	3.45 (0.10)	3.42 (0.06)	3.34 (0.05)	3.15 $(0.08)_{a}$***	3.31 $(0.06)_{b}$**	3.49 $(0.06)_{a,b}$******
n	765	777	256	131	610	1050	198	609	906

Least-squares means were estimated for each substitution site individually and are presented with standard errors of the estimates in parentheses. Significant differences (within a trait and substitution site) between the genotype classes are indicated with the same subscript letter. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

TABLE 5

Association results between the genotypes at the I199V substitution site of the *PRKAG3* gene and meat quality traits within five commercial pig breeds

	Ham pH			Ham Minolta L			Ham Minolta b		
Genotype	II	IV	VV	II	IV	VV	II	IV	VV
Landrace	5.82 (0.05)$_{a,b}$***	5.72 (0.01)$_{a,c}$*	5.68 (0.01)$_{b,c}$***	44.3 (1.6)$_{a}$*	47.2 (0.48)	47.8 (0.34)$_{a}$*	3.74 (0.62)	4.27 (0.18)	4.57 (0.13)
n	6	74	242	6	74	242	6	74	238
Large White	5.75 (0.05)	5.70 (0.02)	5.67 (0.02)	44.4 (1.3)	46.3 (0.61)	45.8 (0.53)	3.23 (0.48)$_{a,b}$***	4.38 (0.22)$_{a}$*	4.65 (0.19)$_{b}$**
n	9	56	109	9	56	111	9	55	109
Berkshire	5.91 (0.03)	5.89 (0.06)	5.69 (0.15)	42.4 (0.65)	44.8 (1.1)	45.0 (3.0)	3.21 (0.26)	4.34 (0.50)	3.20 (1.3)
n	28	10	1	28	10	1	28	10	1
Duroc	5.77 (0.03)$_{a,b}$***	5.69 (0.02)$_{a}$*	5.66 (0.02)$_{b}$**	46.0 (0.73)$_{a,b}$*	47.9 (0.46)$_{a}$*	47.9 (0.53)$_{b}$*	3.29 (0.29)$_{a,b}$***	4.48 (0.18)$_{a}$***	4.58 (0.21)$_{b}$***
n	33	88	78	33	88	78	33	87	78
Duroc Synthetic	5.74 (0.02)$_{a}$*	5.70 (0.01)	5.67 (0.01)$_{a}$*	47.3 (0.60)$_{a}$*	48.0 (0.39)	49.0 (0.41)$_{a}$*	4.33 (0.23)$_{a}$**	4.42 (0.15)$_{b}$***	5.16 (0.16)$_{a,b}$*****
n	52	148	129	52	148	129	52	147	128
	Loin pH			Loin Minolta L			Loin Minolta b		
Genotype	II	IV	VV	II	IV	VV	II	IV	VV
Landrace	5.76 (0.04)$_{a}$*	5.71 (0.01)	5.69 (0.01)$_{a}$*	41.5 (0.95)$_{a,b}$*	44.2 (0.31)$_{a}$*	44.2 (0.23)$_{b}$*	2.52 (0.31)	2.98 (0.09)	3.04 (0.06)
n	11	129	398	11	129	399	9	129	387
Large White	5.73 (0.03)$_{a}$*	5.69 (0.01)	5.66 (0.01)$_{a}$*	44.7 (0.71)	44.6 (0.36)	44.9 (0.30)	3.31 (0.23)	3.05 (0.12)	3.20 (0.10)
n	22	110	224	22	110	224	22	105	217
Berkshire	5.88 (0.02)$_{a}$*	5.80 (0.03)$_{a}$*	5.70 (0.13)	44.4 (0.46)	45.6 (0.86)	44.4 (3.2)	3.49 (0.17)	3.81 (0.29)	2.55 (1.1)
n	55	20	1	56	20	1	56	20	1
Duroc	5.75 (0.02)	5.74 (0.01)	5.71 (0.02)	44.8 (0.63)	45.1 (0.39)	45.8 (0.45)	3.15 (0.20)	3.36 (0.12)	3.50 (0.14)
n	36	104	90	36	103	90	36	103	90
Duroc Synthetic	5.76 (0.02)$_{a,b}$*****	5.72 (0.01)$_{a,c}$***	5.68 (0.01)$_{b,c}$*****	45.6 (0.41)$_{a}$*	45.7 (0.24)$_{b}$**	46.8 (0.26)$_{a,b}$***	3.36 (0.13)$_{a}$**	3.52 (0.08)$_{b}$**	3.83 (0.08)$_{a,b}$**
n	75	251	209	75	253	211	75	252	211

Least-squares means were estimated for the I199V substitution site individually and are presented with standard errors of the estimates in parentheses. Significant differences (within a breed and trait) between the genotype classes are indicated with the same subscript letter. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.

TABLE 6

Haplotype frequencies for the T30N, G52S, and I199V substitutions in the *PRKAG3* gene in five commercial pig breeds

Commercial lines	n	Haplotype[a] frequency				N[b]					
		1	2	3	4	Ham pH	Loin pH	Ham min L	Loin min L	Ham min b	Loin min b
Landrace	518	0.48	0.29	0.13	0.10	271	488	284	489	281	475
Large White	337	0.17	0.45	0.22	0.16	151	319	153	319	150	308
Berkshire	83	0.05	0.05	0.87	0.03	37	71	37	72	37	72
Duroc	234	0.43	0.15	0.38	0.04	184	216	184	215	183	215
Duroc Synthetic	511	0.39	0.17	0.37	0.07	299	472	299	474	297	474

[a] Haplotype 1, 30N-52G-199V; haplotype 2, 30T-52S-199V; haplotype 3, 30T-52G-199I; haplotype 4, 30T-52G-199V.

[b] *N*, number of animals used in the haplotype association analyses.

quency in Landrace, Duroc, and Duroc Synthetic populations. Haplotype 4 (30T-52G-199V) has the lowest frequency in all the populations.

The haplotype substitution effects for each line and across lines were calculated as the deviation from the average of the four haplotypes (Figure 2). Across- and within-line analyses showed bigger differences between haplotypes for ham pH and color measurements than for traits of the loin. For ham pH, across- and within-line analyses showed haplotype 3 having the highest effect, which was significantly different from each of the other haplotypes in the across-line analysis ($P < 0.0005$) and from at least one other haplotype in each individual line analysis ($P < 0.05$). Haplotype 2 was the next best for most of the traits and lines with haplotypes 1 and 4 tending to be the worst with respect to meat quality. This hierarchy is not evident in the Berkshire population, where significant differences are seen only with haplotype 4, which has the lowest value corresponding to the across-line result. The nonsignificant results in Berkshire are likely to be due in part to the low level of polymorphism in this breed and the concomitant very low number of observations for haplotypes 1 and 4. The estimate for haplotype 4 in the Duroc Synthetic population appears to be different from that in the other lines [especially for ham pH, where it is significantly higher than haplotype 2 ($P < 0.05$) and haplotype 1 ($P < 0.01$)], but the frequency of haplotype 4 in this population was very low (0.07). The synthetic nature of this line (although its inception was six generations ago) also provides the opportunity for extended linkage disequilibrium to be present, increasing the chance for linked loci to contribute to the haplotype substitution effects.

The haplotype results for Minolta scores were in line with the pH results. Haplotype 3 was generally found to have the favorable effect (lower color scores). There are a few exceptions in the results from individual lines and these may be the result of sampling. The only significant deviation is with haplotype 2, which is associated with a lower Minolta b score in Berkshire ($P < 0.05$). In the across-line analysis haplotype 2 was second to haplotype 3 in most cases.

DISCUSSION

The results reported in this work provide important evidence in favor of the presence of new alleles of the *PRKAG3* gene affecting meat quality traits. This conclusion is based on three points: (1) the known effect of *PRKAG3* alleles rn^+ and RN^- on meat quality; (2) observation of several QTL for related meat quality traits discovered on SSC15 in the region where *PRKAG3* is located in the B × Y family (these QTL were discovered in the pig cross where the original R200Q substitution was not segregating); and (3) results presented here on the association between the *PRKAG3* substitutions and glycogen and lactate content, glycolytic potential, and meat quality traits in the B × Y F_2 population and association with meat quality traits in several unrelated commercial pig lines.

Association analyses of the individual substitutions revealed that, of the three studied here, the I199V substitution showed the most significant and largest differences in meat quality traits. For example, B × Y F_2 analysis showed significant differences between the I199V genotype classes for glycolytic potential, but also in glycogen and lactate content (Table 2). Important effects were also revealed for most of the meat quality traits analyzed. Allele 199I was found to be associated with a lower level of glycogen, lactate, and glycolytic potential, with higher ham and loin pH and with better color scores. This marker was sufficiently informative in B × Y F_2 to provide good estimates of the allele effects.

In the analyses of the commercial populations, the I199V substitution is associated with significant differences in LS means between the homozygous classes up to 0.14 in the Landrace line and 0.10 across the lines for ham pH (Tables 4 and 5). For one of the meat color measures, Ham Minolta L, significant LS means differences were found between homozygous genotypes up to 3.5 units of reflectance (in Landrace) and 2.0

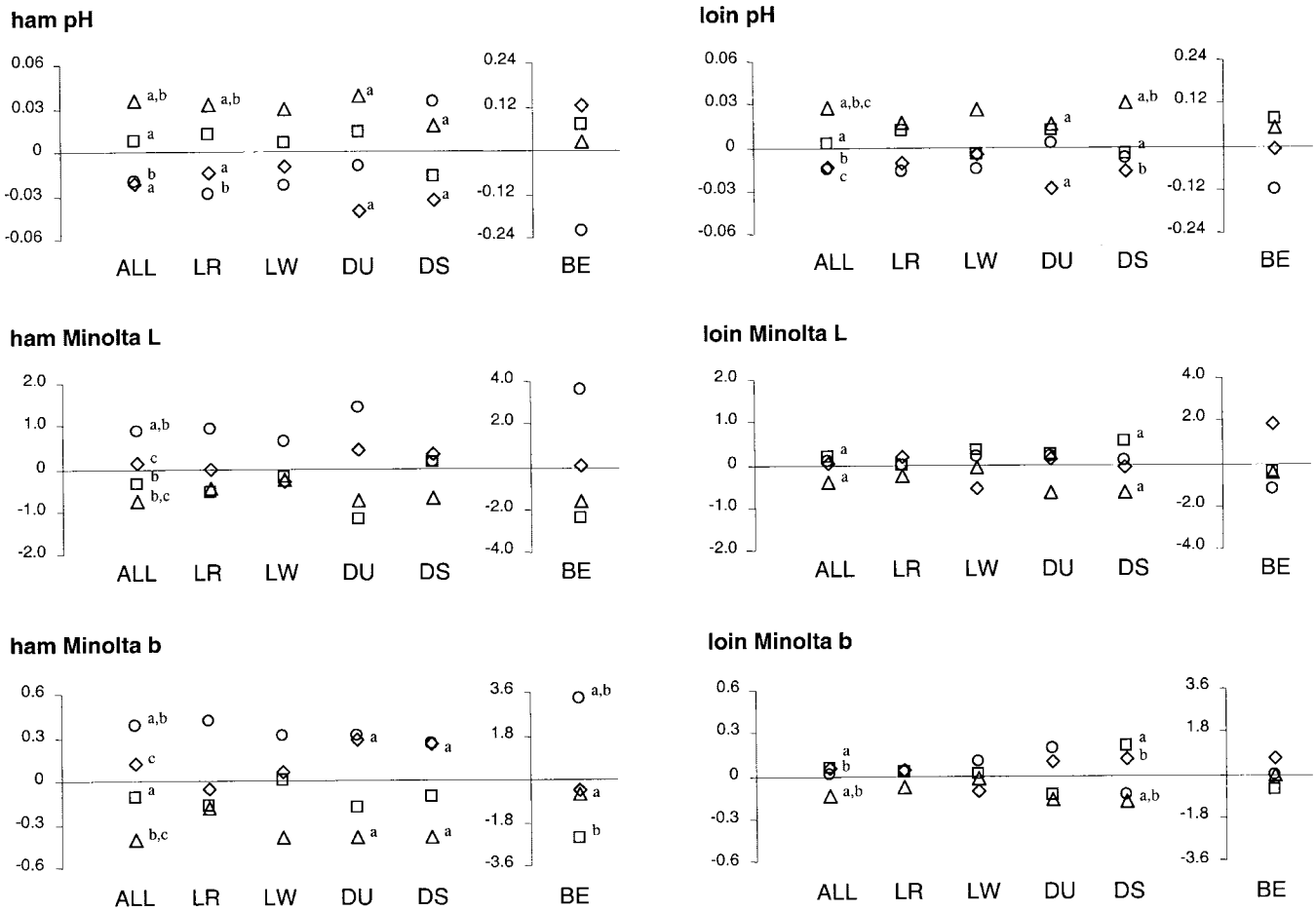

FIGURE 2.—Haplotype substitution effects of *PRKAG3* on pH and color scores in the ham and loin. Haplotype substitution effects are estimated across five lines (ALL) and within each line (◇, haplotype 1; □, haplotype 2; △, haplotype 3; ○, haplotype 4). Lines are based on Landrace (LR), Large White (LW), or Duroc (DU); a Duroc-based synthetic line (DS); and a Berkshire-based line (BE). A separate scale is used for the BE line. Estimates within a column that have the same superscript are significantly different at $P < 0.0005$ for the across-line estimates and at $P < 0.005$ for the within-line estimates.

across the lines. These effects are in the range of 0.5 to 1 phenotypic standard deviation. Important differences were also revealed for the other traits and breeds. Effects of this magnitude in traits important for overall meat quality are of great interest to the animal breeding industry.

Besides I199V, large effects were also estimated from single substitution analysis of T30N. However, only modest effects of the T30N substitution remained if I199V was also included in the analysis. Strong linkage disequilibrium between sites 30 and 199 is considered to be in large part responsible for the effects being detected for site 30. Small effects, which were mostly nonsignificant, were observed for the single-site analysis of G52S.

Haplotype analysis helped to dissect the effects of the nonsynonymous substitutions and provided additional evidence for an effect at position 199 as well as position 52. Haplotype 3, which is the only haplotype containing 199I, was the most favorable haplotype with respect to pH and meat color measurements. In most of the situations tested, haplotype 2, which is the only haplotype containing 52S, showed an intermediate value, especially for ham quality traits where the differences in effects were more significant and greater than in other traits. Values for haplotypes 1 and 4 are close together at the bottom of the range and in most cases not significantly different from each other.

The observation that the values of these two haplotypes (1 and 4) are relatively similar for most estimates leads us to the conclusion that the T30N substitution is making only a marginal contribution to meat quality variation. In across-line, Landrace, and Large White analyses, where the frequency of haplotype 4 is >0.10, we find a favorable effect of haplotype 1 on ham Minolta scores (this haplotype being associated with the 30N variant) when compared with haplotype 4. In the other populations, differences between these haplotype effects are poorly estimated due to very low frequency of haplotype 4.

The difference between haplotype 4 and haplotype 2 is only at the G52S site. The effects of haplotype 4 and 2 are significantly different for pH and Minolta L

scores in both ham and loin in the across-line analysis and for several traits of the individual lines, most notably the Large White. Haplotype 2 (which contains 52S and encodes a serine) is favorable over haplotype 4 (which contains 52G and encodes a glycine). This is the opposite of what was found in the B × Y study where 52G was predicted to be the favorable allele. Strong linkage disequilibrium with the I199V site, due to the limited number of founders of the F_2, may have masked the true effect of the G52S substitution in this population. Interestingly, the individual analysis of G52S did not show any effect for most traits and lines. That analysis compares haplotype 2 with the other three combined. From Figure 2 it can be seen that a mixture of the other three haplotypes, depending on haplotype frequencies, can result in a mean value close to that of haplotype 2 so that a difference would not be detected when G52S is analyzed individually, which points out the value of haplotype-based analysis.

The 30T variant, present in haplotype 4, was found to be favorable for meat quality on the basis of single-site analysis, because it was associated with significant effects in the Duroc and Duroc Synthetic lines for most of the traits. In these two populations haplotype 3 has a moderate frequency (Table 6) and contains both the 30T and the favorable 199I variant. Thus, the 199I variant contributes to the higher effects for the 30T site variant due to linkage disequilibrium.

We conclude that the joint analyses of substitutions and the haplotype analyses demonstrate the presence of three nonsynonymous substitutions in the *PRKAG3* gene with different size effects on meat quality measurements in pigs. This interesting model of "one gene-several polymorphisms-diverse phenotypes" is based on distinguishable additive effects of a complex phenotypic trait and can serve as a model for future studies with other traits. The presence of multiple alleles as a consequence of consecutive mutations in a gene under selection has also been proposed recently in pigs (Jeon *et al.* 1999; Nezer *et al.* 1999)

The I199V substitution is in a cystathionine β-synthase (CBS) domain, a very conserved region in genes of this family (Milan *et al.* 2000). The role of the CBS domain is still unclear but it has been suggested that it is involved in cytoplasmic targeting (Ponting 1997), protein-protein interaction (Bateman 1997), and/or regulation (Bateman 1997) of protein activity. There are four CBS domains in the *PRKAG3* gene (Milan *et al.* 2000) and the I199V substitution is located in the first and most conserved domain. Alignment between the CBS domain and the γ_3-peptide obtained using Pfam software revealed that the preferred amino acid at this position is isoleucine (result not shown). Interestingly, in this study, allele 199I (coding isoleucine at the site 199) was found to be associated with better meat quality in commercial populations and the B × Y F_2 family and also with lower levels of glycogen, lactate, and glycolytic potential in the latter one.

Milan *et al.* (2000) show that the 200Q variant (*RN*$^-$) is always found with 199V. However, 199V is found with both 200R and 200Q and 199I is always found with 200R. As only three nucleotides separate these substitution sites, the probability of recombination between them is extremely small. For this reason we can consider R200Q to be the most recent substitution, a hypothesis also supported by the presence of this mutation in only the Hampshire pig breed. Both of the haplotypes 199V-200R and 199I-200R could be ancestral, because each has been identified in most of the breeds analyzed to date (Milan *et al.* 2000), including wild boar and several species of the suborder Suisformes (D. Ciobanu, G. Plastow and M. Rothschild, unpublished results).

The 199V-200R haplotype is associated with higher glycogen content and lower postmortem ham/loin pH when compared with the 199I-200R haplotype (B × Y F_2 data). The substitution at codon 199 presumably leads to an effect on glucose metabolism and therefore an increase in the muscle glycogen content. The third haplotype 199V-200Q confers the RN$^-$ phenotype. The associated effect 199V-200Q on glycogen content is greater than the effect of other haplotypes and the 199V-200Q haplotype is dominant over the others. For these reasons we suggest that the RN$^-$ phenotype could be a combined effect of the 199V-200Q haplotype rather than solely a result of the R200Q substitution. This effect could be caused by the modification of the CBS domain by these substitutions.

The exact functions of the β and γ regulatory subunits of the AMPK are still unclear. However, it is known that both are essential for kinase activity (Hardie and Carling 1997). *In vitro* experiments show that the β-subunit may have an important role in the formation of the heterotrimeric structure of AMPK, as β interacts with both the γ- and α-subunits, which do not interact directly with each other (Woods *et al.* 1996). Recent evidence suggests that the allosteric AMP-binding site may involve both the γ- and α-subunits of the AMPK complex (Cheung *et al.* 2000). Cheung *et al.* (2000) proposed an elegant model in which, in the absence of AMP, the heterotrimeric complex may be predominantly inactive without interaction between the γ- and α-subunits. In this situation, phosphorylation of the Thr^{172} site in the α-subunit and interaction with substrates are blocked by the autoinhibitory region of the α-subunit. In the active form of AMPK the interaction between the α autoinhibitory region and one or more of the γ CBS domains prevents the autoinhibition, and AMP binds on both subunits to stabilize the assembly (Cheung *et al.* 2000). The alignment information, the proposed model of the regulation of the AMPK complex, and the presence of the R200Q site nearby support the hypothesis of a possible role of the I199V substitution in the activity of AMPK. Even though the molecular

structure of the AMPK complex has not been resolved yet, we hypothesize that the amino acid change may also influence the structure and activity of the enzyme, resulting in the observed effect of the G52S substitution.

Although the γ_3-subunit is highly expressed in skeletal muscle, AMPK activity appears to be associated more with γ_1- and γ_2-isoforms (Cheung *et al.* 2000). However, in a mechanism not yet understood, the R200Q substitution (or I199V-R200Q combination) in the *PRKAG3* gene causes important differences in AMPK activity in Hampshire pigs (Milan *et al.* 2000), which suggests that the γ_3-isoform has an important role in glucose metabolism in skeletal muscle. Detailed functional studies of the different subunit combinations will be necessary to resolve the situation. The role of AMPK in glucose metabolism makes physiological sense on the basis of comparisons with the related SNF1 complex from yeast. Also, several studies show that AMPK participates in glycogen metabolism by inactivating glycogen synthase (Poulter *et al.* 1988; Carling and Hardie 1989; Zhang *et al.* 1993), by activating the nitric oxide synthase (Fryer *et al.* 2000), and by increasing the translocation of the glucose transporter 4 to the plasma membranes (Hayashi *et al.* 1998; Bergeron *et al.* 1999; Holmes *et al.* 1999; Kurth-Kraczek *et al.* 1999).

While the effects of the substitutions reported here on the measures of meat quality are of lesser magnitude than those of the dominant *RN*$^-$ mutation, they are of importance both biologically and economically. In particular, these alleles are segregating in all of the commercial lines and breeds analyzed to date in contrast to the *RN*$^-$ mutation, which is associated only with the Hampshire breed and has limited use in most pork production programs. The results reported here for *PRKAG3* also suggest that geneticists should look for additional mutations with an economic impact in genes known to cause more drastic effects both within and between species. This notion is supported by reports of major effects associated with other genes outside the target species or breed, *e.g.*, large effects of *MC4R* mutations in mice (Huszar *et al.* 1997), humans (Yeo *et al.* 1998) and, to a lesser extent, in pigs (Kim *et al.* 2000).

The identification of novel genes with biochemical significance in animal species will also provide useful information for human biomedical targets. This knowledge is enhanced when new and interesting alleles are discovered. In the case of *PRKAG3*, it has been suggested (Milan *et al.* 2000) that this gene and other AMPK-related genes in humans are interesting candidates for human type II diabetes on the basis of their function and QTL locations. For this reason, the effect of these new alleles may provide new insights about potential factors affecting glucose metabolism and should be considered in further investigations of this disease.

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