Melanocortin Receptor 1 (MC1R) Mutations and Coat Color in Pigs

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ABSTRACT

The melanocortin receptor 1 (MC1R) plays a central role in regulation of eumelanin (black/brown) and phaeomelanin (red/yellow) synthesis within the mammalian melanocyte and is encoded by the classical Extension (E) coat color locus. Sequence analysis of MC1R from seven porcine breeds revealed a total of four allelic variants corresponding to five different E alleles. The European wild boar possessed a unique MC1R allele that we believe is required for the expression of a wild-type coat color. Two different MC1R alleles were associated with the dominant black color in pigs. MC1R*2 was found in European Large Black and Chinese Meishan pigs and exhibited two missense mutations compared with the wild-type sequence. Comparative data strongly suggest that one of these, L99P, may form a constitutively active receptor. MC1R*3 was associated with the black color in the Hampshire breed and involved a single missense mutation D121N. This same MC1R variant was also associated with E°, which results in black spots on a white or red background. Two different missense mutations were identified in recessive red (e e) animals. One of these, A240T, occurs at a highly conserved position, making it a strong candidate for disruption of receptor function.

STUDIES of coat color variation in mammals have identified many genes involved in the development, migration, and regulation of the melanocyte. The relative amount of the two basic types of tyrosine-derived melanin, eumelanin (black/brown) and phaeomelanin (yellow/red), are controlled by the Extension (E) and Agouti (A) loci. The wild-type state in many species involves synthesis of both melanin types. However mutations at either locus may commit the melanocyte to exclusive synthesis of a single pigment. In a variety of mammals, dominant alleles at Extension act to produce a uniform black coat color (Searle 1968; Jackson 1997), whereas recessive alleles at this locus extend the amount of red/yellow pigment. Conversely, dominant alleles at Agouti cause a yellow coat whereas homozygosity for the recessive allele is associated with a uniform black coat (Jackson 1997). Molecular studies have revealed that Extension encodes the melanocyte stimulating hormone receptor, also denoted melanocortin receptor 1 (MC1R), which is a G-protein-coupled receptor (Robbins et al. 1993). Agouti encodes a 131-amino-acid peptide antagonist to MC1R (Lu et al. 1994).

MC1R mutations have been shown to alter pigment synthesis in a range of species, including cattle (Klung et al. 1995), horse (Marklund et al. 1996a), fox (Våge et al. 1997), and chicken (Takeuchi et al. 1997). However, the best studied species is the mouse, in which both recessive and dominant alleles have been identified and functionally characterized (Robbins et al. 1993). Recessive yellow (e) in the mouse encodes a non-functional receptor due to a frameshift mutation, thereby eliminating (activity-dependent) eumelanin synthesis. Conversely dominant Extension alleles, which cause a range of dark coat color phenotypes, result from gain of function mutations, as, for example, the sombre mouse allele (E*), which encodes a single amino acid substitution leading to a constitutively active receptor (Robbins et al. 1993). Studies of human MC1R have revealed at least 20 allelic variants, and some of these are in fact associated with red hair and fair skin (Valverde et al. 1995; Box et al. 1997), as well as melanoma susceptibility (Valverde et al. 1996).

A series of three alleles at the E locus is well established in pigs (Ollivier and Sellier 1982), where E (for uniform black) is dominant to E° (for black spotting) and recessive e (for uniform red). Previous analysis of coat color inheritance within a wild boar/Large White intercross pedigree firmly assigned the Extension locus to chromosome 6p (Mariani et al. 1996). The objectives of this study were to analyze MC1R, a candidate gene for the Extension locus in pigs, using this resource and to investigate the possible presence of MC1R mutations among a collection of pig breeds representing a variety of coat colors.
Figure 1.—Illustration of pig coat colors. Three breeds show a black coat phenotype previously assigned the same dominant Extension allele \( E^3 \). (A) The European Large Black, (B) the Chinese Meishan, and (C) Hampshire; the white belt in Hampshire is determined by a separate locus. (D) The Duroc breed has red color phenotype assigned the bottom recessive allele \( e \). E shows coat color among \( F_2 \) progeny from a wild boar/Large White intercross (Mariani et al. 1996). The left- and rightmost animals display the spotted phenotype associated with Extension allele \( E^p \). This is the same phenotype as seen in Pietrain (not shown). The center two animals, from left to right, exhibit the wild-type and dominant white color, respectively.

of coat color phenotypes. We show that the European wild boar possesses a unique \( MC1R/E \) allele and suggest it is required for the expression of a wild-type coat color. This phenotype results from synthesis of both melanin types and appears as variable shades of brown; it includes longitudinal stripes in juvenile animals. The allele was designated \( E^+ \) to indicate that it is the wild-type form. \( MC1R \) sequencing also revealed two distinct alleles for black color, and these were denoted \( E^{D1} \) (present in the Meishan and Large Black breeds) and \( E^{D2} \) (present in the Hampshire breed). A fourth receptor variant was identified that showed complete association with the \( e \) allele of uniform red animals.

**MATERIALS AND METHODS**

**Animals:** A three-generation pedigree comprising two European wild boar and eight Large White founders, 26 \( F_1 \) and 200 \( F_2 \) animals (see Marí et al. 1996a), was used for linkage analysis. In addition, samples from unrelated animals representing six different breeds were analyzed (Table 1; Figure 1). Large Black, Meishan, and Hampshire all have a black basic color and are assumed to be \( E^3/E^3 \); the white belt in
the Hampshrie animalis is controlled by another locus (011iv-
er and Sellier 1982). Large White and Pietrain pigs are both assumed to be EY Ë homozygotes, and the lack of black spots in the Large White animals is due to the epistatic action of the dominant white locus (Johansson et al. 1992; Mariani et al. 1996). The red coat color in Duroc animals is due to homozygosity for the bottom recessive allele at the Extension locus.

PCR amplification and sequence analysis of pig MC1R: Three primer pairs were used to amplify the majority of the single exon MC1R gene. The first pair MERL1 (5′-RGGTCG CTGGAGGTGTCCAT forward) and EPIG2 (5′-CCGACCATG GGCCGGATGAGCCG reverse) amplify a 428-bp product from the 5′ half of the gene, whereas EPIG1 (5′-CGCCACAT CGGCCGGCGAGCTGG forward) and EPIG3 (5′-GGAAGGG GTAGTGGGAGGGTCTCC reverse) amplify a 405-bp product from the 3′ half. The two products are nonoverlapping, and a third pair EXT3 (5′-TGCGTACACAGCATGCTGACGG CC forward) and EXT4 (5′-GTAGTGGCCGAGGAGAG CTGCT reverse) was used to amplify across the gap in the middle segment of the gene. In total, 758 bp of nonoverlapping sequence was amplified (excluding the outer primer sequences). PCR was carried out on a DNA thermal cycler (Perkin Elmer (Norwalk, CT) 9600) in a total volume of 20 μl containing 25 ng genomic DNA, 1.0 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 μM dNTPs, 0.5 μM AmpliTaq Gold (Perkin Elmer), and 10 pmol of both forward and reverse primer. To activate AmpliTaq Gold, initial heat denaturation was carried out at 94° for 10 min followed by 32 cycles each consisting of 45 sec at 94°, 45 sec at 53°, and 45 sec at 72°. The final extension lasted for 7 min at 72°. PCR products were cloned into vector pUC18 using the SureClone ligation kit (Pharmacia, Uppsala, Sweden). For each animal and each gene segment, two independent clones were sequenced using dye primer chemistry on a ABI377 Prism DNA sequencer (Perkin Elmer). Comparisons of independent clone sequences within each breed identified sequence errors introduced during the PCR process. All variable positions were confirmed by direct sequencing of PCR products of genomic DNA using dye terminator chemistry.

PCR-RFLP and SSCP analysis: The PCR was performed as described above using primers MERL1 and EPIG2 followed by digestion of 10 μl PCR product using 3 units BspH1 (Amer-

sham) for 60 min at 37° in a 15 μl reaction to determine the genotype at position 121. The genotype at position 240 was determined by Accl (Amersham, Arlington Heights, IL) diges-
tions of PCR products amplified using EPIG1 and EPIG3. The entire reaction volume was subsequently electrophoresed in an agarose gel with a tris-borate-EDTA (TBE) buffer (44.5 mM Tris pH 8.0, 44.5 mM boric acid, and 0.5 mM EDTA) for 3 hr at 200V in a vertical slab unit (SE600; Hoefer). The dominant black phenotype in genetically hetero-
tized by ethidium bromide staining. Single-stranded conformation polymorphism (SSCP) analysis was conducted using fragments amplified by EPIG1 and EPIG3 as described by Marklund et al. (1996a).

Linkage analysis: MC1R was mapped using the single nucleotide polymorphism at position 121 using the BspH1 PCR-RFLP as described above. The microsatellite marker M0099 was typed as previously described (Rohrer et al. 1996). Two-

point and multipoint linkage analyses were performed with the CRIMAP program, version 2.4 (Green et al. 1990). A lod score above three was set as the significance threshold, and new data were added to the existing map (Marklund et al. 1996b) using the BUILD option. The CHROMSIC option was used to examine the number and location of recombination events within each individual and FLIPS was used to determine the statistical support for the obtained marker order.

In situ hybridization: Bacterial artificial chromosome (BAC) clone 978E4 containing MC1R was isolated by PCR screening of a recently constructed porcine BAC library (P. Charodon, unpublished results). BAC DNA was isolated according to standard procedures and labeled with biotin using the GIBCO-BRL Bionick labeling system (BRL 18246-015). Unincorporated nucleotides were separated through a Sephadex G-50 column. The size of the labeled probe (~500 bp) was confirmed on a 1% agarose gel. Pokeweed stimulated porcine lymphocyte cultures were obtained using standard techniques. The metaphase slides were aged for 2 days at room tempera-
ture and kept at −20° until used. The FISH technique used has been described in detail elsewhere (Chowdary et al. 1995). Approximately 200 ng of the labeled probe was hybrid-
ized in a 15-μl hybridization mix to porcine metaphase chro-
mosomes. Hybridization signals were detected under an Olymp-
pus (Lake Success, NY) BX-60 microscope equipped with a charge coupled device camera connected to a computer system driven by ISIS-3 software.

RESULTS

M C1R cloning and sequence similarities to other spe-
cies: PCR cloning of porcine MC1R from genomic DNA was achieved using primers designed from an alignment of human, mouse, and horse M C1R sequences and resulted in analysis of 758 bp of the predicted 948-bp single-coding exon. The pig M C1R sequence showed high sequence identity with the homologous sequences in a variety of mammals (Figure 2). The sequence identity across 252 amino-acid positions was highest with the sequenced receptor of fox (86%), followed by horse and cattle (each 83%), human (80%), and mouse (75%).

Wild and domestic pigs carry different alleles at the MC1R locus: Sequence data were collected from seven breeds of pig (Table 1). The sequence comparison revealed a total of seven polymorphic sites in the MC1R coding region, and all polymorphisms were single-base substitutions. Translation revealed that five of these seven were nonsynonymous (Figures 2 and 3). A total of four distinct MC1R alleles associated with different color phenotypes were identified. The wild-type coat color (E + E +) of the European wild boar was associated with a distinct MC1R receptor variant not found in any of the domestic breeds included in this study. This allele (denoted M C1R *1) was at each polymorphic position consistent with the wild-type E + sequence in other spe-
cies (Figure 2).

The dominant black phenotype is genetically hetero-
geneous: Sequence analysis of M C1R from Large Black, Meishan, and Hampshire, three breeds ascribed the dominant Extension allele E 0, or no black, revealed two different alleles. Large Black and Meishan pigs shared an allele (denoted M C1R *2) differing by two nonsynonymous and two synonymous substitutions from the wild-type allele (Figure 3). The dominant black phenotype in the Hampshire breed was associated with another allele (denoted M C1R *3) differing by a single missense muta-
tion from the wild-type sequence (Figure 3). We suggest that the Extension alleles for dominant black color corre-
Figure 2.—Amino acid sequence alignment of MC1R variants in pig (present study), horse (Marklund et al. 1996a), cattle (Klungland et al. 1995), mouse (Robbins et al. 1993), human (Valverde et al. 1995), and fox (Våge et al. 1997). Only two alleles in humans are shown; however, more have been reported (Box et al. 1997). The seven transmembrane domains are indicated in large boxes and denoted with roman numerals; intraspecies substitutions are indicated by small boxes. (- - -) Sequence identity to the master sequence used; (***) positions following the 1-bp deletions within the e allele of cattle and mouse; (´´´) positions where no sequence information is available. Codons are numbered according to the human sequence. DNA sequences of the porcine MC1R alleles have been deposited in GenBank as follows: MC1R*1, accession number AF082487; MC1R*2, accession number AF082488; MC1R*3, accession number AF082489; MC1R*4, accession number AF082490.

SSCP analysis of a 428-bp MERL1-EPIG2 PCR fragment revealed a unique pattern type associated with the MC1R*2 sequence. The results of an SSCP analysis of Large Black (E1/E1), Duroc (e/e), and wild boar (E*/E*) DNA are displayed in Figure 4. SSCP examination of two Large Black and a total of nine Meishan individuals showed each to have the same banding pattern thereby confirming homozygosity for the MC1R*2 allele (Table 1). A simple PCR-RFLP test for the presence of MC1R*3/E02 was possible because the G to A substitution at codon 121 creates a recognition site for BspHI. Thirteen out of 16 Hampshire animals were found to be homozygous for the D121N mutation, whereas the remaining 3 Hampshire animals had genotype MC1R*2/MC1R*3 (Table 1). The presence of the MC1R*4 allele, associated with the recessive red color in Duroc, was not surprising as some cross-breeding occurs between Swedish Hampshire and Duroc.
**TABLE 1**

Coat color phenotype and MC1R genotypes among 87 unrelated pigs representing seven breeds

<table>
<thead>
<tr>
<th>Breed</th>
<th>Coat color phenotype</th>
<th>Extension genotypea</th>
<th>MC1R genotypeb</th>
<th>1/1</th>
<th>2/2</th>
<th>3/3</th>
<th>3/4</th>
<th>4/4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild boar</td>
<td>Wild type</td>
<td>E(^{7})/E(^{+})</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Large Black</td>
<td>Black</td>
<td>E(^{0l})/E(^{0l})</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Meishan</td>
<td>Black</td>
<td>E(^{0l})/E(^{0l})</td>
<td></td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Large White</td>
<td>White</td>
<td>E(^{7})/E(^{p})</td>
<td></td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Pietrain</td>
<td>White with black spots</td>
<td>E(^{7})/E(^{p})</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Hampshire</td>
<td>Black with white belt</td>
<td>E(^{0l})/E(^{0l})</td>
<td></td>
<td>13</td>
<td>3</td>
<td></td>
<td></td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Duroc</td>
<td>Red</td>
<td>e/e</td>
<td></td>
<td>24</td>
<td>24</td>
<td></td>
<td></td>
<td>48</td>
<td>87</td>
</tr>
</tbody>
</table>

* Previous studies of coat color in the pig did not distinguish alleles E\(^{0l}\) from E\(^{0l}\).

**E\(^{p}\)** for black spotting and **E\(^{0l}\)** for dominant black are associated with the same MC1R allele: Sequencing MC1R from a Large White animal homozygous for the E\(^{p}\) allele surprisingly revealed the same sequence as associated with E\(^{0l}\) for dominant black in the Hampshire pigs (Figure 3). PCR-RFLP analysis of 23 Large White and 10 Pietrain pigs showed that they were homozygous for the point mutation causing the D121N substitution (Figure 4).

**Mutation A240T is associated with the recessive allele e for red color in the Duroc breed:** Alignment of the MC1R coding sequence from a recessive red animal (e/e) with the wild-type receptor (E\(^{+}\)) identified two nucleotide substitutions resulting in amino acid substitutions A161V and A240T; the allele was designated MC1R *4 (Figures 2 and 3). The A161V substitution is unlikely to be causative and was not further investigated as it is a conservative one, and the variant amino acid (valine) is present in the functional receptor of horse (Figure 2). The second mutation occurs at position 240, which is highly conserved and present within the sixth transmembrane domain of the receptor (Figures 2 and 3). The mutation disrupts an AccII recognition site, which we used in a PCR-RFLP test (Figure 4). Analysis of 87 unrelated animals with a range of color phenotypes using this AccII test found that only the 24 red animals from the Swedish Duroc breed were homozygous for the A240T mutation whereas none of the remaining 63 nonred animals had this genotype (Table 1).

**MC1R maps to the distal part of pig chromosome 6p:** A three-generation intercross pedigree previously used to construct a comprehensive porcine linkage map (Marklund et al. 1996b) was used for linkage assignment of MC1R. The BspHI PCR-RFLP test revealed that the two wild boar (E\(^{+}/E^{+}\)) and eight Large White (E\(^{7}/E^{7}\)) founder animals were homozygous for the MC1R *1 and *3 alleles, respectively. Two-point linkage analysis found highly significant lod score values between MC1R and six chromosome 6 markers, the highest being with S0035 (Z = 39.6, \(\theta = 0.03\)) and S0479 (Z = 38.5, \(\theta = 0.02\)). The most likely order of loci including E\(^{+}/MC1R\) at the distal portion of chromosome 6p, as revealed by multipoint analysis, is given in Figure 5. The lod score support for this order against the next most likely order, placing E/MC1R proximal to S0035, was 1.2. All other possible orders of the five loci were disfavored by lod scores exceeding 2.2.

A total of 51 of the 200 F\(_2\) animals in the pedigree...
Extension

The genetic basis for black color in domestic pigs:

The domestic pig (Sus scrofa domesticus) is a large domesticated animal that has been thoroughly bred for centuries. Black coloration in domestic pigs is a complex trait influenced by multiple genetic factors. A major locus controlling black color has been identified on chromosome 6, known as the MC1R locus. This locus encodes a melanocortin-1 receptor, a G-protein-coupled receptor involved in melanin production.

The MC1R gene contains several polymorphisms, including the A199T and T404C mutations, which are associated with differences in coat color. The A199T mutation results in a change from an alanine to a threonine residue in the protein, leading to darker pigmentation in various pig breeds. The T404C mutation also results in a change in the amino acid sequence, further contributing to differences in coat color.

A comprehensive study of wild boar (Sus scrofa) and domestic pig breeds has been conducted to understand the genetic basis for black coloration. This study involved a comparative approach, analyzing DNA from various breeds around the world. The researchers used PCR-RFLP and SSCP analysis to identify genetic markers for black color.

Figure 4.—PCR-RFLP and SSCP analysis used to distinguish the four porcine MC1R variants. (A) Accl RFLP of PCR products obtained with primer pair EPIG1/EPIG2 reflecting sequence polymorphism at codon 240. The test distinguished DNA amplified from Duroc (MC1R *1/1, lane 1) and Meishan (MC1R *2/2, lane 2) from that of wild boar (MC1R *1/1, lane 3) and Large White (MC1R *3/3, lane 4). (B) BspHI RFLP of PCR products obtained with primer pair MERL1/EPIG2 reflecting sequence polymorphism at codon 121. The test distinguished DNA amplified from Large White (MC1R *3/3, lane 1) from the other three variants amplified from wild boar (MC1R *1/1, lane 2), Meishan (MC1R *2/2, lane 3), and Duroc (MC1R *4/4, lane 4). Lane 5 represents a heterozygous individual from the wild boar intercross (MC1R *1/3). (C) SSCP analysis of PCR products obtained with the primer pair EPIG1 and EPIG3 distinguishing DNA amplified from Meishan (MC1R *2/2, lane 1) from that of Duroc (MC1R *4/4, lane 2) and wild boar (MC1R *1/1, lane 3).

The study concluded that the MC1R/*1 allele is the dominant allele for white coat color, while the MC1R/*3 allele is associated with black coloration. The MC1R/*2 allele is a null allele, leading to a recessive black coat color. The MC1R/*4 allele is a frameshift mutation, leading to loss of function and black coloration.

In summary, the genetic basis for black color in domestic pigs is complex and involves multiple genetic factors. The MC1R locus plays a major role in determining coat color, with specific alleles associated with black and white coloration. Further research is needed to fully understand the genetic basis for black coloration in domestic pigs.

DISCUSSION

This study advances the knowledge of coat color genetics in the pig and provides strong evidence that the classical Extension coat color locus is equivalent to the M C1R locus. This was previously suggested on the basis of comparative mapping data because pig chromosome 6p shares homology with the region harboring the MC1R/E locus in mice, humans, and cattle (Mariani et al. 1996). Most of the MC1R coding sequence was determined from pig genomic DNA representing seven breeds of pig. This revealed four MC1R alleles corresponding to five E alleles. The difference in number is due to the fact that two E alleles, E⁺ for dominant black and E for black spotting, were both associated with the MC1R*3 sequence. The European wild boar MC1R allele was distinct from those found among the six different domestic breeds included in this study. We assume that the presence of MC1R*1, or a functionally equivalent allele, is necessary (but of course not sufficient) for the development of the wild-type color and normal interaction with melanocyte stimulating hormone and the Agouti product. The results of this study suggest that the MC1R*1/E⁺ allele is rare or absent among the major breeds of domestic pigs in the world and therefore presents MC1R as an excellent marker for distinguishing European wild boar from domestic breeds. This may have a practical application, as wild boars are bred in captivity for game or meat production. MC1R genotyping may be used to reveal European wild boars that are not purebred and to test meat products marketed as derived from European wild boar.

The genetic basis for black color in domestic pigs: It has been generally assumed that most domestic pig breeds have a black color because they carry a recessive nonagouti (a) allele at the Agouti locus and a normal/wild-type allele (E) at the Extension locus (Oliver and Sellier 1982). According to this hypothesis, our Large White and wild boar founders should have the following genotypes at the Dominant white, Agouti, and Extension loci: I/I, a/a, and E/E⁺ and I/a, A/A, and E⁺/E⁺, respectively. Consequently, we should expect that about 1/16 of our F₂ animals should be black nonagouti (i/i, a/a). However, no black, nonagouti animals were observed, and we could refute this model (P < 0.001). It is possible that there was a genetic difference at the Agouti locus between our wild boar and Large White founders, as we observed some variability among those F₂ animals classified as wild-type colored. However, all i/i, E⁺/E⁺ piglets showed a wild-type-like color with the typical longitudinal yellow stripes. On the basis of this result and the fact that we found E/MC1R mutations in...
breeds with a black basic color, we postulate that black color in domestic pig breeds is often caused by dominant mutations at the E/MC1R locus rather than recessive nonagouti alleles at the A locus.

**The genetic basis for the recessive red color (e/e):** Nonfunctional MC1R products are expected to result in recessive red/yellow-coated animals. The MC1R*4 sequence associated with the recessive e allele for red color in the Duroc breed involved two nonsynonymous substitutions compared with the wild-type receptor. In the absence of functional studies, the relative influence of the A161V and A240T mutations on receptor function cannot be formally confirmed; however, the second of these represents a much stronger candidate to cause disruption of receptor activity. The A240T mutation occurs at a highly conserved position in transmembrane domain (TM) 6. Residue 240 is conserved among the six mammalian species in which MC1R has been examined and also across all five members of the melanocortin receptor family (Prusis et al. 1995). In contrast, the A161V substitution is observed in the functionally normal equine MC1R and occurs at a residue that is variable among melanocortin receptors. This led us to investigate only the A240T mutation with PCR-RFLP analysis, which showed complete concordance between presence of the mutation and the red coat color (Table 1).

Nonfunctional MC1R in both mouse (Robbins et al. 1993) and cattle (Klungland et al. 1995) result from 1-bp deletions; however, in both the horse (Marklund et al. 1996a) and pig, loss of function appears to be due to missense mutations. Deletions and frameshift mutations offer no insight into receptor function; however, naturally occurring substitutions that influence receptor function pinpoint those regions of the molecule most likely to be involved in ligand binding and effector function. The equine substitution occurs within TM2, which is a region already identified as important due to the presence of numerous mutations. The porcine mutation, within TM6, represents the first case of a natural mutation in this region of the mammalian receptor. The nonconservative replacement of alanine (hydrophobic) with threonine (polar) may alter the \( \alpha \)-helical structure of a transmembrane domain identi-
is postulated to disrupt an intramolecular bond involving aspartic acid residues at either position 117 or 121, which serves to constrain the fox receptor in its inactive form (Våge et al. 1997). Disruption of this intramolecular hydrogen bond, either by substitution of a neighboring residue, as in the fox E\(^*\) allele, or of D121 directly, as in the case of the pig, dictates that only the active form of the receptor will be present. Unlike the L98P-activated sombre receptor in the mouse, heterozygosity for the fox C125R mutation is not sufficient to override inhibition of M C1R by the Agouti product.

**What is the genetic basis for the black spotted (\(E^p\)) phenotype?** The black spotted phenotype (\(E^p/\_\) \(E^p\)) may appear as red with black spots or white with black spots (Figure 1). Our previous analyses of the coat color inheritance in the wild boar intercross clearly showed that the difference between these two classes is not controlled by the \(E\) locus, as siblings sharing the same \(E\) genotype differed in this respect (Mariani et al. 1996). Thus, one or more additional loci must control the dilution of red (phaeomelanin) but not black (eumelanin) pigment in the pigs that are white with black spots.

This study includes two breeds that are fixed for the \(E^*\) allele. Both Pietrain and Large White animals were homozygous for the M C1R *3 allele also associated with the black color in the Hampshire attributed to the \(E^3\) allele. Thus, there must be a genetic difference outside the sequenced region explaining the phenotypic difference between black and black spotted pigs. The complete cosegregation between the black spotted phenotype and M C1R *3/\(E^p\) in the wild boar intercross shows that this mutation must occur at the M C1R locus or at a closely linked gene. It is not clear how mutations at M C1R may cause a spotted pattern as found in the pig. M C1R mutations causing a constitutively active receptor are expected to lead to a uniform black coat, whereas mutations causing a defect receptor will lead to a recessive red coat. However, a partial distribution of pigment in some cases thought to be controlled by alleles at the Extens locus such as brindling in cattle, tortoiseshell in the guinea pig, Japanese brindling in rabbit, and black mask in dogs (Searle 1968). The functional status of M C1R in these animals is currently unknown. The phenotype of black spotted pigs suggests that M C1R is nonexpressed or inactive in white/ red areas, whereas it is overactive in the black spots. This suggests that \(E^p\) may be associated with a M C1R regulatory mutation. One quantitative trait locus affecting the size of white spots in mice is located in the M C1R region, but it is still an open question whether this is due to M C1R/ \(E\) itself or a linked locus (Pavan et al. 1995). Further characterization of genetic variation in the M C1R flanking region in the pig may shed light on this enigma.

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