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Original article

A first genotyping assay of French cattle breeds based on a new allele of the *extension* gene encoding the melanocortin-1 receptor (Mc1r)

François ROUZAUD[a], Juliette MARTIN[a], Paul François GALLET[a], Didier DELOURME[a], Valérie GOULEMOT-LEGER[c], Yves AMIGUES[c], François MÉNISSIER[d], Hubert LEVÉZIEL[a,b], Raymond JULIEN[a], Ahmad OULMOUDEN[a,*]

[a] Unité de génétique moléculaire animale, UMR 1061 (Université/INRA), Université de Limoges, 87060 Limoges Cedex, France
[b] Former address Unité de génétique biochimique et cytogénétique, UR339, Institut national de la recherche agronomique, 78352 Jouy-en-Josas Cedex, France
[c] Laboratoire d'analyses génétiques pour les espèces animales (LABOGENA), Institut national de la recherche agronomique, 78352 Jouy-en-Josas Cedex, France
[d] Station de génétique quantitative et appliquée, UR337, Institut national de la recherche agronomique, 78352 Jouy-en-Josas Cedex, France

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Abstract – The seven transmembrane domain melanocortin-1 receptor (Mc1r) encoded by the coat color *extension* gene (E) plays a key role in the signaling pathway of melanin synthesis Upon the binding of agonist (melanocortin hormone, α-MSH) or antagonist (Agouti protein) ligands, the melanosomal synthesis of eumelanin and/or phaeomelanin pigments is stimulated or inhibited, respectively Different alleles of the *extension* gene were cloned from unrelated animals belonging to French cattle breeds and sequenced The wild type E allele was mainly present in Normande cattle, the dominant E^D allele in animals with black color (*i.e* Holstein), whereas the recessive e allele was identified in homozygous animals exhibiting a more or less strong red coat color (Blonde d'Aquitaine, Charolaise, Limousine and Salers). A new allele, named E^1, was found in either homozygous (E^1/E^1) or heterozygous (E^1/E) individuals in Aubrac and Gasconne breeds This allele displayed a 4 amino acid duplication (12 nucleotides) located within the third cytoplasmic loop of the receptor, a region known to interact with G proteins A first genotyping assay of the main French cattle breeds is described based on these four *extension* alleles

cattle / genotyping / coat color / *extension* / polymorphism

* Correspondence and reprints
E-mail oulmouden@unilim fr

Résumé – Premier essai de génotypage de races bovines françaises basé sur un nouvel allèle du gène *extension* codant pour le récepteur 1 aux mélanocortines (Mc1r). Le récepteur 1 aux mélanocortines (Mc1r), codé par le gène *extension*, présente sept domaines transmembranaires et joue un rôle clé dans la voie de signalisation de la biosynthèse des mélanines Lors de la liaison de l'agoniste (hormone « mélanotrope » α-MSH) ou de l'antagoniste (la protéine Agouti), la synthèse mélanosomale des pigments eumélaniques (brun / noir) et/ou phaéomélaniques (jaune / rouge) est respectivement stimulée ou inhibée À partir d'animaux non apparentés provenant de bovins de races françaises, différents allèles ont été clonés et séquencés L'allèle sauvage E est présent chez la Normande, l'allèle E^D chez les bovins à robe noire (par exemple Holstein), alors que l'allèle récessif e a été mis en évidence chez des animaux homozygotes présentant des couleurs de robe basées sur une présence plus ou moins prononcée de pigments phaéomélaniques (Blonde d'Aquitaine, Charolaise, Limousine et Salers) Un nouvel allèle E^1 a été découvert à la fois chez des individus homozygotes E^1/E^1 ou hétérozygotes E^1/E des races Aubrac et Gasconne. Il possède une duplication de quatre acides aminés (12 nucléotides) localisée dans la troisième boucle cytoplasmique, région du récepteur connue comme interagissant avec les protéines G Sur la base de ces quatre allèles du gène *extension*, un premier test de génotypage des principales races bovines françaises est décrit.

bovin / génotypage / couleur de la robe / *extension* / polymorphisme

1. INTRODUCTION

Mammalian pigmentation depends on relative amounts of black/brown eumelanin and yellow/red phaeomelanin [8]. Both melanins in skin and hair are produced in neural crest-derived melanocytes, owing to a biosynthetic pathway where tyrosinase is the rate limiting enzyme Eumelanin production requires a high level of tyrosinase activity, whereas a low level favors the synthesis of phaeomelanin [3,7]. In mice, it is well established that tyrosinase activity is regulated through α-MSH (α-Melanocyte Stimulating Hormone) and Mc1r (Melanocortin-1 receptor). A second gene, named *agouti*, is also involved in the regulation of pigment synthesis [2] and has been cloned in many species including the mouse [2], human [12], and cattle (Accession number: X99691). The *agouti* gene encodes a 131 amino acid protein which acts as a paracrine hormone to interrupt the Mc1r signaling pathway, thus inhibiting eumelanin synthesis [13].

Mc1r is a G protein-coupled receptor of 45 kDa with seven transmembrane domains, encoded by the *extension* gene [4, 15, 19] Melanotrophic ligands, such as α-MSH, resulting from the cleavage of proopiomelanocortin (POMC), allow an increase in intracellular concentration of cAMP and favor eumelanin rather than phaeomelanin synthesis [5].

Several mutations of the *extension* gene have been described that induce various coat colors in many species including the mouse [16], cattle [11], horse [14], sheep [18] and pig [10]. Mc1r mutations causing a constitutive activation of the signaling pathway have been shown to induce eumelanin synthesis whereas those causing a loss of function induce phaeomelanin synthesis [16].

Three *extension* alleles have been identified in Norwegian and Icelandic cattle [11]. The wild type E allele produces a variety of colors. The dominant

Table I. Primers used in this study

Name	Sequence
T7	5′ TAATACGACTCACTATAGGGA 3′
U-19	5′ GTTTTCCCAGTCACGACGT 3′
MC1 5	5′ CCATGAGTTGAGCAGGACCCTCAG 3′
MC1 3	5′ CCTCTTTGTCAAGGGACTGCCCTG 3′
Ia*	5′ CCTGGCTGTGTCTGACCTGCTGGTG 3′
IIa	5′ GATGAATGGGGCGCTGCCTCTTCTG 3′

* This primer is coupled to 6-FAM (6-carboxyfluorescein).

E^D allele with a point mutation gives black color whereas a frameshift mutation producing a prematurely terminated receptor in homozygous e/e animals induces a red coat color.

In this study, we report the molecular characteristics of a new fourth *extension* allele named E^1 found in French cattle breeds. We also report the achievement of a genotyping assay on eight French cattle breeds.

2. MATERIALS AND METHODS

2.1. Materials

Animals unrelated up to the third generation, and belonging to eight different French breeds were examined in this study. Their coat colors were fawn to brown (Aubrac), light-colored (Blonde d'Aquitaine), creamy white (Charolaise), grey (Gasconne), black with white spotting (Holstein), red (Limousine), brown and white (Normande) or dark mahogany (Salers).

2.2. DNA isolation

Ten mL of fresh blood sample were mixed with 25 mL of cold lysis solution (150 mM NH_4Cl, 10 mM KCl, 0.1 mM EDTA, pH 7) and stored on ice for 2 h before centrifugation (5 min, 5 000 × g) at 4 °C. The pellet was washed twice with 10 mL of saline solution (140 mM NaCl, 0.5 mM KCl, 0.25 mM Tris-Cl, pH 7 4) and resuspended in 9 mL TE (10 mM Tris-Cl, 0.1 mM EDTA, pH 8), then incubated 1 h at 50 °C with 50 μL proteinase K (20 mg · mL^{-1}), 0.5 mL 0.5 M EDTA, pH 8, and SDS 0.5 mL 10% (w:v). Then 4.3 mL of NaCl saturated solution was added, after centrifugation for 15 min at 20 000 × g. DNA was recovered by ethanol precipitation.

2.3. PCR amplification and molecular cloning of the *extension* coding sequence

The complete *MC1R* coding region was amplified using MC1.5 and MC1.3 primers (Tab. I) designed on the basis of the bovine sequence [19]. PCR reactions were carried out using a DNA thermal cycler (Perkin Elmer 9600). Each

assay contained, in a total volume of 25 μL, 100 ng of genomic DNA, 10 pmol of each primer, 100 μM of each dNTP, 2.5 μL of 10X buffer containing 15 mM $MgCl_2$ (supplied with *Taq* Polymerase; Pharmacia) and 1 U *Taq* Polymerase. Genomic DNA was denatured for 3 min at 94 °C, and PCR was run for 34 cycles at 94 °C for 10 s, 61 °C for 30 s, and 72 °C for 40 s. Amplified DNA was resolved in 1% (w:v) agarose gel and purified using the QiaQuick kit (QIAGEN). The DNA fragment was then cloned into a T-vector (pMOS*Blue*, Amersham) and sequenced using T7 and U-19 primers (Tab. I) with an ABI Prism 310 DNA Genetic Analyzer (Perkin Elmer).

2.4. Studies of *extension* alleles frequency by PCR-RFLP

PCR amplifications were carried out in a volume of 50 μL using, in standard buffer conditions, Ia primer coupled to 6-FAM and IIa primer (Tab. I). The reaction mixture contained 200 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTP, 5 μL of 10X buffer containing 15 mM $MgCl_2$, and 2 U *Taq* Polymerase (Pharmacia). DNA was denatured for 5 min at 94 °C, and PCR was run for 36 cycles at 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s. PCR products were purified in a 1.2% (w:v) agarose gel electrophoresis, then eluted using the QiaQuick kit (QIAGEN) with 30 μL of water. Five microliters of eluted DNA were used for direct size determination of PCR products The remaining 25 μL of the eluted DNA were submitted to a simultaneous digestion by 1 U *Bsr*FI and 1 U *Aci*I. The digested products were precipitated by ethanol, washed and resuspended in 10 μL of water. Five microliters were then used for analysis. DNA fragments, digested or not, were analyzed using the Genescan Program of the ABI Prism 310 Genetic Analyzer (Perkin Elmer), after a denaturation step at 95 °C for 3 min in 20 μL of deionized formamide.

3. RESULTS

3.1. *MC1R* alleles

The *MC1R* coding region from several animals belonging to eight different breeds was amplified and sequenced (Tab. II). Analysis of the nucleotide sequences revealed four distinct alleles (Fig. 1). The wild type E allele coding sequence is 954 bp long and encodes a full length Mc1r of 317 amino acids (Fig. 2A). The E^D allele results from one single base substitution (T296C) leading to an amino acid change from leucine to proline in position 99 (Figs. 1b and 2A). The e allele has a deletion of guanine in position 310, leading to the frameshift and a predicted protein of 155 amino acids with only three putative transmembrane domains (Fig. 2B). The E^1 allele was characterized by a 12 bp duplicated sequence in position 669. This new bovine allele led to a four amino acid (gly-ile-ala-arg) duplication within the third intracellularloop of the receptor (Figs. 1c and 2A).

3.2. RFLP analysis

The Ia and IIa primers used for PCR-RFLP analysis allowed the amplification of a 460/461 bp fragment for e, E, or E^D alleles (Figs. 3A1 and 3A2), and 473 bp for the E^1 allele (Figs. 3A1 and 3A3).

Table II. Genotypes and alleles frequency of the *extension* locus for eight French cattle breeds.

		Genotype					Allele			
Breed	Σ	e/e	E/E	E/E^1	E^1/E^1	E^D/E^D	e	E	E^D	E^1
Aubrac	68	–	7	18	43	–	–	0 24	–	0 76
Blonde d'Aquitaine	27	27	–	–	–	–	1	–	–	–
Charolaise	59	59	–	–	–	–	1	–	–	–
Gasconne	64	–	18	23	23	–	–	0 46	–	0 54
Holstein	87	–	–	–	–	87	–	–	1	–
Limousine	42	42	–	–	–	–	1	–	–	–
Normande	75	–	75	–	–	–	–	1	–	–
Salers	56	56	–	–	–	–	1	–	–	–
Σ	478	184	100	41	66	87				

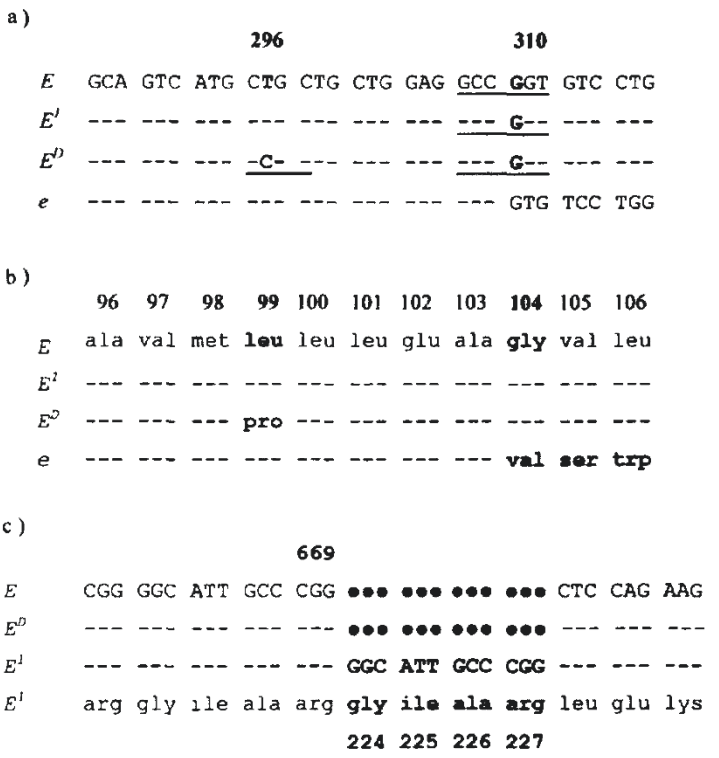

Figure 1. *MC1R* alleles found in French cattle breeds. Partial base (a) and amino acid (b) sequences for e and E^D alleles. The e allele bears a G310 deletion leading to a frameshift at amino acid position 104 and to a premature stop codon at position 156 (not shown) Allele E^D presents a T296C substitution; from it, results a leu to pro change at position 99. (c) Partial bases and amino acid sequences for the E^1 allele. A duplication of 12 nucleotides beginning at position 669 characterized allele E^1 and gave an insertion of four amino acids at positions 224 to 227. Sequences for *Aci*I (CCGC) and *Bsr*FI ($^{G/A}$CCGG$^{T/C}$) enzymes are underlined. Hyphens represent identity with the reference E allele Points represent gaps in the nucleic sequences

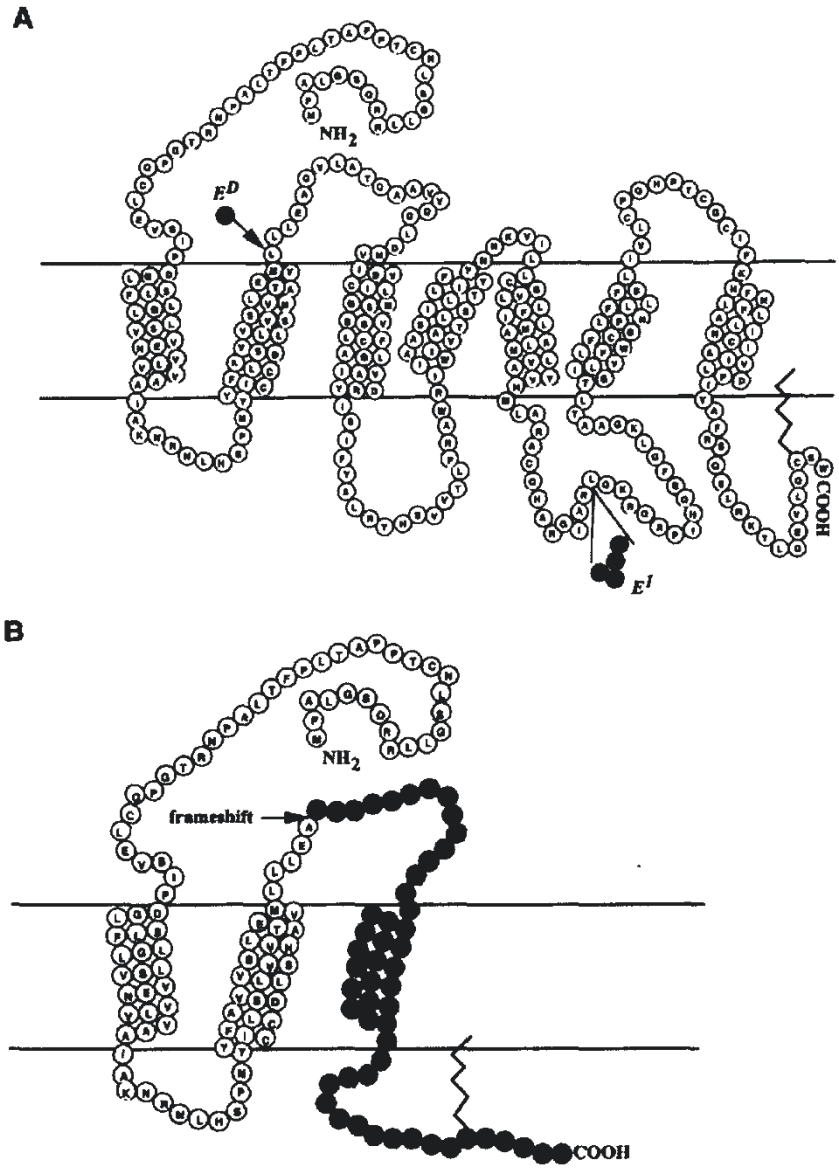

Figure 2. Secondary structure prediction of the Mc1r receptor variants Amino acid changes are indicated using the amino acid sequence of the E allele as reference. A: E, E^{D}, and E^{1} alleles. B· e allele. (○) conserved amino acid residues; (●) amino-acids resulting from base deletion, substitution or addition. Secondary structure was deduced with the Predict Protein program (EMBL).

The deletion of guanine at position 310 in the e allele induced the removal of the *Bsr*FI restriction endonuclease site (Fig. 1a), leading to a 91 bp fragment after both *Bsr*FI and *Aci*I digestions (Fig. 3B3). The E^{D} allele identification resulted from the substitution of a thymine by cytosine and the appearance of a new restriction site for *Aci*I (Fig. 1a) giving a 59 bp digested fragment

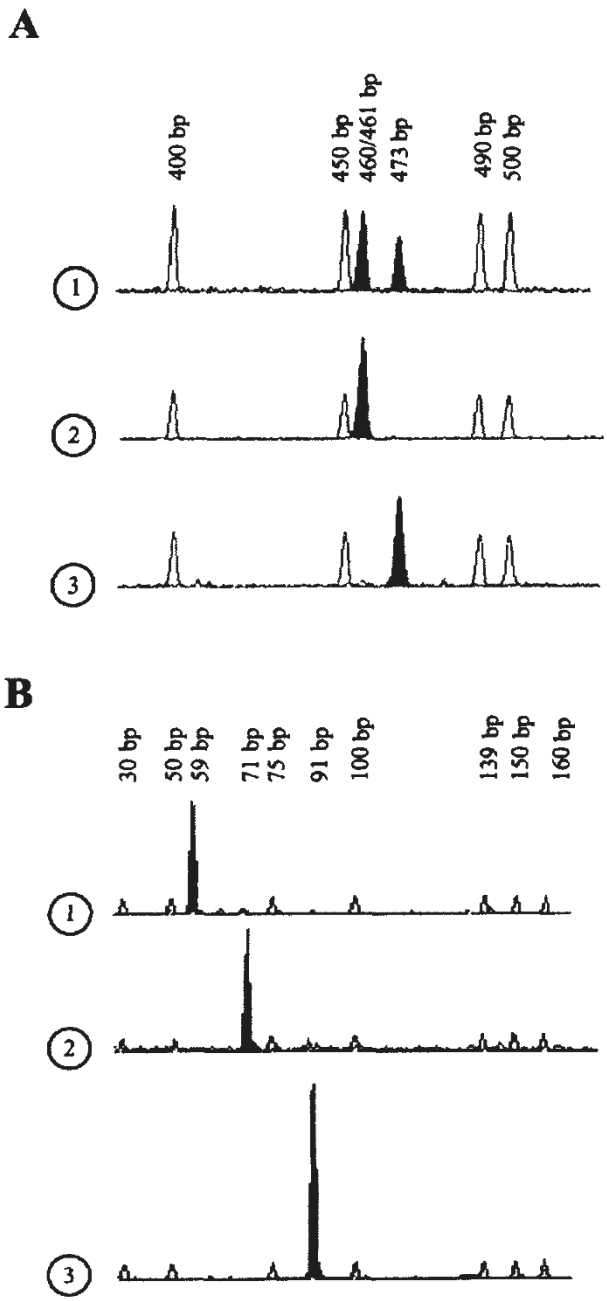

Figure 3. Molecular characterization of gene *extension* alleles. The partial coding sequence of the *extension* was amplified using Ia and IIa primers and sizes of fragments were analyzed before (A) or after (B) restriction enzyme treatment. (A): Analysis of amplified DNA before enzyme treatment. A1: Presence of the *e*, *E* or E^D alleles (460/461 bp) and the E^1 allele (473 bp); A2: Presence of the *e*, *E* or E^D alleles (460/461 bp) A3: Presence of the E^1 allele (473 bp). (B): Analysis of amplified DNA after cleavage by *Act*I and *Bsr*FI enzymes. B1: Presence of the E^D allele (59 bp). B2: Presence of the *E* or E^1 alleles (71 bp). B3: Presence of the *e* allele (91 bp)

(Fig. 3B1). Neither the *Bsr*FI nor *Aci*I digestions allowed the distinction between the E and E^1 alleles (Figs. 1a and 3B2). However, the analysis of the size of the undigested amplified products (461 bp and 473 bp respectively) allowed their characterization (Fig. 3A1).

3.3. Genotyping assay of the French cattle breeds

The genotyping assay revealed (Tab. II) that Blonde d'Aquitaine, Charolaise, Limousine and Salers breeds were exclusively composed of homozygous e/e individuals. The Holstein breed was composed of homozygous E^D/E^D individuals. Aubrac and Gasconne animals were either homozygous E/E or E^1/E^1 and heterozygous E/E^1. Among a combination of ten potential genotypes, only five were observed, corresponding to e/e, E/E, E/E^1, E^1/E^1, and E^D/E^D (Tab. II). The e allele was fixed in the breeds with phaeomelanic hair color. The E^D allele was fixed in Holstein cattle. The wild type E allele frequency was fixed in Normande, 0.24 for Aubrac and 0.46 for Gasconne. The new E^1 allele had a frequency of 0.54 in the Gasconne and 0.76 in the Aubrac (Tab. II).

4. DISCUSSION

Mammalian coat color results from interactions of several genes including those implicated in neural crest-derived melanocyte development (*i.e. c-kit*), melanogenesis regulation (*i.e. agouti, extension*), and those directly involved in the melanin biosynthesis pathway (*i.e. tyr, trp1, trp2*) [9].

We analyzed the coding sequence of the *extension* gene in French cattle breeds to elucidate the relationships between French bovine coat colors and the mutations affecting the coding sequence of this gene. In cattle, three *extension* alleles have already been described [11]. The E allele is 954 bp long and encodes a full length Mc1r of 317 amino acids (Fig. 2), which seems to allow a phenotypic expression of both Agouti and α-MSH [1]. The protein encoded by the E allele is a seven transmembrane domain G protein-coupled receptor with three intracellular loops and a COOH-tail anchored to the melanocyte membrane by a putative cystein palmitoylated in position 315 (Fig. 2A).

The dominant E^D allele is characterized by a single nucleotide mutation (T296C) leading to the L99P substitution (Figs. 1 and 2A). This receptor has not yet been pharmacologically characterized but is considered as the mouse E^{so} allele analogue [16] which gives a constitutively activated receptor.

The recessive e allele is characterized by a deletion of guanine at position 310 (Fig 1a), leading to a frameshift and a premature stop codon. The translation product of the e allele is a truncated protein with only three putative transmembrane domains (Fig. 2B). Such a putative receptor might not be able to bind the α-MSH hormone [17], but can bind the Agouti protein, although it has been shown not to be functional in the mouse [16].

The fourth allele, named E^1, was distinguished by a duplication of 12 nucleotides (GGCATTGCCCGG) at position 669 of the coding sequence (Fig. 1c). The translated protein presented a duplication of four amino acids (GIAR from

224 to 227, Fig. 2B). Few mutations have been described in this part of the receptor. In the pig, a A240T mutation observed in the 6th transmembrane domain, is correlated with a red coat color [10]. In the bovine receptor encoded by the E^1 allele, a four amino acid duplication was located in the third intracellular loop (Fig. 2A), thought to interact with the G protein [6]. These additional amino acids could induce a modification of the third loop conformation, and consequently a change of interactions with the corresponding G protein, and in the signal transmission resulting in the Agouti and/or α-MSH binding. The attenuated coat color from the animals carrying this E^1 allele in Aubrac and Gasconne breeds could be correlated with these molecular changes.

Allele frequency analysis showed that breeds with phaeomelanic hair color carry only the e allele (Charolaise, Blonde d'Aquitaine, Limousine, and Salers). Normande and Holstein breeds were characterized by the wild type E and dominant E^D alleles, respectively. The new E^1 allele had an equal representativeness with the E allele in Gasconne breed, whereas it seemed to be predominant in the Aubrac breed.

Molecular characterization of the four *extension* alleles allowed us to identify the individual allelic combination. Polymorphisms of the coding sequence of the *extension* gave genetic markers specific for some cattle breeds such as the Holstein (E^D/E^D) or groups of breeds such as Blonde d'Aquitaine, Charolaise, Limousine and Salers (e/e).

Klungland *et al.* [11] reported that in Norwegian cattle, all e/e animals are red. Surprisingly, in French cattle breeds, e/e animals with various coat colors were found. The four breeds carrying the e mutation had hair phenotypes that encompass a large range of color intensity from the creamy white colored Charolaise to the dark mahogany of the Salers. If the truncated receptor retains a partial function, it can be hypothesized that products of *agouti* and/or other related genes could modify the phaeomelanic phenotype.

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