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Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle

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Abstract. We have determined the entire myostatin coding sequence for 32 double-muscled cattle sampled from ten European cattle breeds. Seven DNA sequence polymorphisms were identified, of which five would be predicted to disrupt the function of the protein, one is a conservative amino acid substitution, and one a silent DNA sequence variant. Four additional DNA sequence polymorphisms were identified in myostatin intronic sequences. In all but two breeds, all double-muscled animals were either homozygous or compound heterozygotes for one of the five loss-of-function mutations. The absence of obvious loss-of-function mutations in the coding sequence of the two remaining breeds points either towards additional mutations in unexplored segments of the gene, or towards locus heterogeneity of double-muscling.

Introduction

The occurrence of individuals characterized by an inherited, exceptional muscularity—commonly referred to as double-muscling—has been reported in several cattle breeds (Culley 1807). The calving difficulties associated with double-muscling have led several breeding organizations to treat this condition as a genetic defect that needs to be eliminated. In specific economic contexts, however, the gains in feed conversion ratio, dressing out percentage, and meat quality (increased lean and tenderness) have outweighed the costs of dystocia, leading either to a systematic selection for double-muscled animals or their use in cross-breeding (Hanset 1991).

While the hereditary nature of this condition was recognized early on, the precise mode of inheritance has remained controversial. Contradictory inheritance models, including autosomal monogenic (dominant and recessive), oligogenic and polygenic, have been described (Ménissier 1982). It is at present unclear whether this reflects genuine genetic heterogeneity for this trait in different populations or whether it results from the difficulty to correctly classify animals for a trait that is in many respects a continuously distributed phenotype rather than a discrete affection status.

In the Belgian Blue Cattle Breed (BBB), segregation analysis performed both in experimental crosses and in the outbred population suggested an autosomal recessive inheritance (Hanset and Michaux, 1985a, & 1985b). This hypothesis was confirmed when the postulated *mh* locus (muscular hypertrophy) was unambiguously mapped in this breed to the centromeric tip of bovine Chromosome (Chr) 2 under a recessive model (Charlier et al., 1995). The same chromosomal region was subsequently shown to account for the recessive inheritance of the double-muscling condition in at least two other breeds: Asturiana (Dunner et al. 1997) and Maine-Anjou (Grobet et al. unpublished). Its role in the determinism of double-muscling in other populations, however, remained to be demonstrated.

Recently, it was shown that an 11-bp deletion (*nt821(del11)*) in the myostatin gene, resulting in a truncation of the bioactive carboxyterminal domain of the protein, was causing double-muscling in BBB (Grobet et al. 1997). The same *nt821(del11)* mutation was shown to be responsible for double-muscling in Asturiana as well. In the Maine-Anjou breed, however, in which the involvement of the same *mh* locus was demonstrated by linkage analysis, the *nt821(del11)* deletion proved to be absent among double-muscled animals (Grobet et al. 1997). This observation, therefore, pointed towards allelic heterogeneity for the myostatin gene. This presumption was confirmed with the recent identification of a distinct cysteine to tyrosine substitution (C313Y) in double-muscled Piedmontese animals (Kambadur et al. 1997).

To further clarify the issue of locus and allelic heterogeneity of double-muscled cattle, we have determined the entire coding sequence of the myostatin gene in double-muscled individuals from ten European cattle breeds. In so doing, we have identified a series of mutations that are predicted to disrupt the myostatin function, demonstrating and characterizing the allelic heterogeneity of the double-muscled condition in cattle.

Materials and methods

Pedigree material. In total, 32 animals with extreme muscular development were sampled from ten European beef cattle breeds in which double-muscled animals are acknowledged to occur at high to moderate frequency: (i) Belgium: Belgian Blue (4); (ii) France: Blonde d'Aquitaine (5), Charolais (2), Gasconne (2), Limousin (5), Maine-Anjou (4), Parthenaise (3); (iii) Spain: Asturiana (2), Rubia Gallega (2); (iv) Italy: Piedmontese (2). The determination of the double-muscled phenotype of the sampled animals was performed visually by experienced observers.

Four animals with conventional phenotype sampled from the Holstein-Friesian (2) and Jersey (2) dairy populations were analyzed as controls.

Development of a primer set allowing for PCR amplification of the complete myostatin coding sequence from genomic DNA. In order to facilitate the study of the myostatin coding sequence from genomic DNA, we determined the sequences of the exon-intron boundaries of the bovine gene. In mice, the myostatin gene is known to be interrupted by two introns, respectively ≈1.5 and 2.4 kb long (McPherron & Lee, 1997). We designed two primer pairs, respectively in bovine exons 1 and 2, exons 2 and 3, that were predicted to flank the two introns, assuming conservation of gene organization between mouse and cattle (Fig. 1 and Table 1). With

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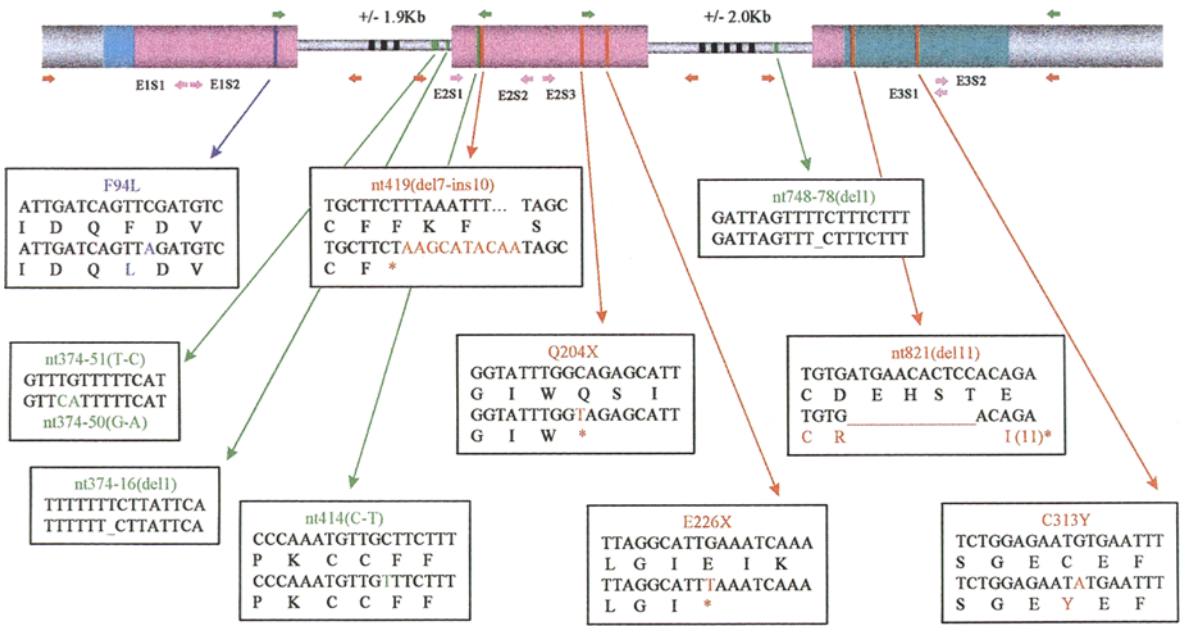

Fig. 1. Schematic representation of the bovine myostatin gene with position and definition of the identified DNA sequence polymorphisms. The gray boxes correspond to the untranslated leader and trailer sequences (large diameter), and the intronic sequences (small diameter) respectively. The blue, pink and green boxes correspond to the sequences coding for the leader peptide, N-terminal latency-associated peptide, and bioactive carboxyterminal domain of the protein respectively. Small green, red, and pink arrows point towards the positions of the primers used for intron amplification, exon amplification and sequencing, and exon sequencing respectively; the corresponding primer sequences are reported in Table 1. The positions of the identified DNA sequence polymorphisms are shown as green, blue, or red lines on the myostatin gene for silent, conservative, and disrupting mutations respectively. Each mutation is connected via an arrow with a box reporting the details of the corresponding DNA sequence and eventually encoded peptide sequence. In each box, the variant sequence is compared with the control Holstein-Friesian sequence, and differences are highlighted in color.

Table 1. Primers used for PCR amplification and cycle sequencing.

Intron1-5′	5′-GAAGACGATGACTACCACGCCAGGACG-3′	Intron1-3′	5′-CTAGTTTATTGTATTGTATCTTAGAGC-3′
Intron2-5′	5′-AGACTCCTACAACAGTGTTTGT-3′	Intron2-3′	5′-ATACTCWAGGCCTAYAGCCTGTGGT-3′
Exon1-5′	5′-ATTCACTGGTGTGGCAAGTTGTCTCTCAGA-3′	Exon1-3′	5′-CCCTCCTCCTTACATACAAGCCAGCAG-3′
Exon2-5′	5′-GTTCATAGATTGATATGGAGGTGTTCG-3′	Exon2-3′	5′-ATAAGCACAGGAAACTGGTAGTTATT-3′
Exon3-5′	5′-GAAATGTGACATAAGCAAAATGATTAG-3′	Exon3-3′	5′-ATACTCWAGGCCTAYAGCCTGTGGT-3′
Exon1-Seq1	5′-TTGAGGATGTAGTGTTTTCC-3′	Exon1-Seq2	5′-GCCATAAAAATCCAAATCCTCAG-3′
Exon2-Seq1	5′-CATTTATAGCTGATCTTCTAACGCAAG-3′	Exon2-Seq2	5′-TGTCGCAGGAGTCTTGACAGGCCTCAG-3′
Exon2-Seq3	5′-GTACAAGGTATACTGGAATCCGATCTC-3′		
Exon3-Seq1	5′-AGCAGGGGCCGGCTGAACCTCTGGG-3′	Exon3-Seq2	5′-CCCCAGAGGTTCAGCCGGCCCCTGC-3′

these primer sets, two PCR products, respectively 2 kb and 3.5 kb long, were generated from a YAC clone (179A3) containing the bovine myostatin gene (Grobet et al., 1997). The PCR products were purified with QiaQuick PCR Purification kit (Qiagen) and partially sequenced with Dye terminator Cycle Sequencing Ready Reaction (Perkin Elmer) and an ABI373 automatic sequencer Alignment with the bovine cDNA sequence identified the four predicted exon-intron boundaries. Based on the available exonic and intronic sequences of the bovine myostatin gene, we subsequently designed three primer pairs that jointly allow for convenient amplification of the entire coding sequence from genomic DNA. The position of the corresponding primers is shown in Fig. 1, and the corresponding sequences are reported in Table 1.

Sequence determination of myostatin alleles. After PCR amplification of the entire coding sequence from genomic DNA in the three described fragments, these were purified with QiaQuick PCR Purification kit (Qiagen) and sequenced with Dye terminator Cycle Sequencing Ready Reaction (Perkin Elmer) and an ABI373 automatic sequencer, using the primers used for amplification as well as a series of nested primers (Fig. 1 and Table 1). Chromat files produced with the ABI373 sequencer were analyzed with the Polyphred application (D. Nickerson, personal communication), which is part of a series of sequence analysis programs including Phred (B. Ewing, & P Green, unpublished). Phrap (P. Green, unpublished) and Consed (D. Gordon, unpublished).

Results

Identification of an allelic series of loss-of-function mutations in the bovine myostatin gene. The coding sequence of the four control Holstein-Friesian and Jersey individuals was identical to the previously described wild-type allele (Grobet et al. 1997), indicating that we were indeed more than likely amplifying the genuine myostatin coding sequence and not dealing with a non-functional pseudogene.

Among the 32 double-muscled animals, on the contrary, we identified seven DNA sequence variants within the coding region, as summarized in Fig. 1.

In addition to the previously described *nt821(del11)* mutation in the third exon, deleting 11 base pairs at position 821 after the initiation codon and revealing a premature stop codon, we identified four new mutations that would be predicted to disrupt the myostatin function. An insertion/deletion at position 419 counting from the initiation codon, replacing 7 base pairs (bp) with an apparently unrelated stretch of 10 bp, reveals a premature stop codon in the N-terminal latency-associated peptide at amino acid position 140. This mutation is referred to as *nt419(del7-ins10)*. Two bp substitutions in the second exon, a C → T transition at

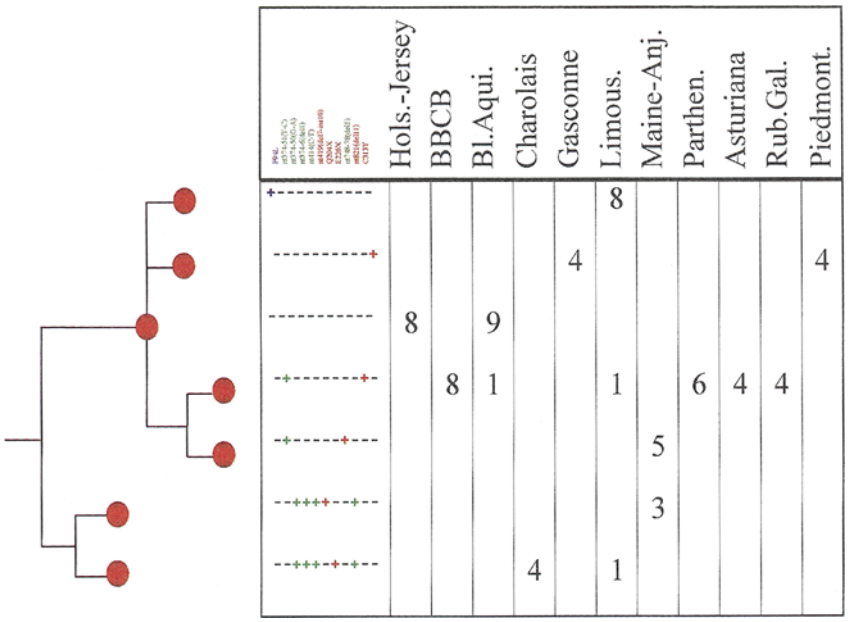

Fig. 2. Distribution of myostatin haplotypes by breed. The order and color of the myostatin mutations in the description of the observed haplotypes correspond to Fig. 1. All analyzed animals were double-muscled except for the two Holstein-Friesian and two Jerseys used as controls (column 1). A maximum parsimony cladogram connecting the different haplotypes is shown adjacent to the table. The rooting of the tree was based on the ovine myostatin sequence (data not shown).

nucleotide position 610 and a G → T transversion at nucleotide position 676, each yield a premature stop codon in the same N-terminal latency-associated peptide at amino-acid positions 204 and 226 respectively. These mutations are called *Q204X* and *E226X* respectively. Finally, a G → A transition at nucleotide position 938 results in the substitution of a cysteine by a tyrosine. This mutation is referred to as *C313Y*. This cysteine is the fifth of nine highly conserved cysteine residues typical of the members of the TGF-β superfamily and shared in particular by TGF-β1, -β2 and -β3, and inhibin-βA and -βB (McPherron and Lee 1996). It is thought to be involved in an intramolecular disulfide bridge stabilizing the three-dimensional conformation of the bioactive carboxyterminal peptide. Its substitution is, therefore, likely to affect the structure and function of the protein. This *C313Y* has recently also been described by Kambadur and associates (1997).

We also identified a conservative phenylalanine-to-leucine substitution at amino acid position 94 in the first exon, owing to a C → A transversion at nucleotide position 282 of the myostatin gene. Given the conservative nature of the amino acid substitution, its location in the less conserved N-terminal latency-associated peptide, and as this mutation was observed at the homozygous condition in animals that were not showing any sign of exceptional muscular development, we predict that this mutation does not interfere drastically with the myostatic function of the encoded protein, if at all. This mutation is referred to as *F94L*. Note that the murine protein is characterized by a tyrosine at the corresponding amino acid position.

Finally, we identified a silent C → T transition at the third position of the 138th cytosine codon in the second exon, referred to as *nt414(C-T)*.

In addition to these DNA sequence polymorphisms detected in the coding region of the myostatin gene, we identified four DNA sequence variants in intronic sequences that are probably neutral polymorphisms and that have been assigned the following symbols: *nt374-51(T-C)*, *nt374-50(G-A)*, *nt374-16(del1)* in intron 1, and *nt748-78(del1)* in intron 2 (Fig. 1).

Distribution of myostatin haplotypes in different cattle breeds. Figure 2 reports the observed myostatin haplotypes as well as their distribution in the analyzed sample sorted by breed.

For the majority of the studied breeds, the analyzed double-muscled animals were homozygous for one of the five described mutations predicted to disrupt the myostatin function or compound heterozygotes for two distinct of these mutations. This is compatible with the hypothesis that the double-muscled condition has a recessive mode of inheritance in all these breeds.

Only in Limousin and Blonde d'Aquitaine could we not demonstrate clear evidence for the role of myostatin loss-of-function mutations in the determinism of the observed muscular hypertrophy. Most Limousin animals were homozygous for the conservative *F94L* substitution which is unlikely to cause the muscular hypertrophy characterizing these animals for the reasons cited above. Two Limousin animals proved to be heterozygous for this mutation, the other allele being either the *nt821(del11)* or *Q204X* mutation. All Blonde d'Aquitaine animals but one were homozygous wild-type, the latter carrying the *nt821(del11)* mutation. These data indicate either that the myostatin gene is not involved in the double-muscled condition characterizing these two breeds, or that there are additional myostatin mutations outside of the coding region. It should be noted that the double-muscling condition is often considered to be less pronounced in Limousin animals than in other breeds; this may point towards a phenocopy with a distinct determinism in this breed.

Although the analyzed sample is very limited so far, our data indicate that some mutations, such as the *nt821del(11)* and *C313Y*, are shared by several breeds, which points towards gene migration between the corresponding populations, while others seem to be confined to specific breeds. Moreover, while some breeds (the Belgian Blue breed in particular) seem to be essentially genetically homogeneous, others show clear evidence for allelic heterogeneity (for example, Maine-Anjou).

Figure 2 also depicts a preliminary maximum parsimony cladogram predicting the evolutionary relationships between the identified haplotypes. The tree was rooted based on the observation that the ovine myostatin sequence exhibited the *nt374-50(G-A)*, *nt374-16(del1)* and *nt748-78(del1)* variants. It can be seen from this figure that the *nt419(del7-ins10)* and *Q204X mutations*, encountered respectively in the French breeds Maine-Anjou and Charolais/Limousin, appear to have occurred in the same "ancestral" myostatin haplotype characterized by the *nt374-50(G-A)*, *nt374-16(del1)*, *nt748-78(del1)*, and *nt414(C-T)* neutral variants (the former three being shared with the ovine haplotype). Like-

wise, the *E226X* and *nt821(del11)* mutations seem to have occurred in a common, more recently evolved haplotype, characterized by the *nt374-51(T-C)* variant.

Discussion

We demonstrate in this work that the double-muscling phenotype is genetically heterogeneous in cattle, involving at least five different mutations in the bovine myostatin gene.

The observation of allelic heterogeneity contradicts the classical view that a single *mh* mutation spread through the European continent in the beginning of the 19th century with the dissemination of the Shorthorn breed from the British Isles (Ménissier, 1984). Two of the mutations, at least, are shared by more than one breed, indicating some degree of gene migration, but definitely not from a single origin.

While the observed level of allelic heterogeneity might reflect a particularly high mutation rate for this gene, we are inclined to believe that it results from the long-standing history of artificial selection for meaty animals, which must have favored such mutations and maintained them in the corresponding populations. In the BBB, evidence has been gathered in favor of a modest but significant superiority of muscular development in heterozygous (*nt821(del11)*/+) over homozygous (+/+) normal individuals, reflecting some degree of haploinsufficiency (Hanset and Michaux, 1985a, 1985b). A similar tendency was apparent from the weight and carcass analysis of myostatin knock-out mice (McPherron et al. 1997). Even a modest expression in heterozygotes may considerably have increased the selective advantage of the bovine myostatin loss-of-function alleles and therefore their population frequency.

The observation that in at least eight of the ten studied breeds double-muscling involves five independent myostatin mutations indicates that the number of genes susceptible to affect muscular development in a comparable way is likely to be limited in cattle. It is tempting, therefore, to anticipate that myostatin similarly plays a key role in other species as well. To the best of our knowledge, however, no evidence is available to date of muscular hypertrophies involving this gene in livestock species other than cattle. The exceptional muscularity characterizing callipyge sheep involves a gene located on sheep Chr 18, which is orthologous to bovine Chr 21 (Cockett et al. 1994, 1996). The meatiness typical of Piétrain pigs is determined, at least to some degree, by the *CRC* or a tightly linked gene on pig Chr 6 (Fuji et al. 1991). The *CRC* gene has been mapped to Chr BTA18q23-24 in cattle. In contradiction to double-muscling in cattle, which from an histological point of view is a hyperplasia, the enhanced muscular development of callipyge sheep and Piétrain pigs involves a true hypertrophy of the muscle fibers, that is, an increase in their individual diameter.

In mice, in addition to the in vitro generated myostatin knock-out mice (McPherron and Lee 1997), the compact mutation could be due to a naturally occurring mutation at the myostatin gene. The compact locus has indeed been mapped to the *D1Mit375–D1Mit21* interval on mouse Chr 1 known to be orthologous to *HSA2q31-32* and *BTA2q12-22* (Varga et al. 1997).

From an applied point of view, the characterization of a panel of mutations in the myostatin gene associated with double-muscling will contribute to the establishment of a diagnostic screening system allowing for marker-assisted selection for or against this condition in cattle. The observed high level of genetic heterogeneity of double-muscling forces one to carefully evaluate the relevance of the already identified mutations in each population and eventually to seek other causative mutations.

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