The sequence of Myostatin in double-muscled pigs

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Introduction

Myostatin, a TGF-β family member, is an essential factor for growth and development of the muscle mass. This protein functions as a negative regulator of muscle growth in mice and is related with the so-called double-muscling phenotype in cattle. Hereby a series of mutations render the gene inactive. In some breeds of cattle, as Belgian Blue and Piedmontese, this phenotype occurs at a very high frequency (Grobet *et al.*, 1997, Mc Pherron *et al.*, 1997, Bellinge *et al.*, 2005).
One particular breed of pigs, the Piétrain pig, also shows a heavily-muscled phenotype. The similarity of muscular phenotypes between the double-muscled cattle and Piétrain pigs suggested that myostatin is also a candidate gene for muscular hypertrophy in pigs.

Materials en methods

Animals: Pigs of different breeds and different herds were used. All pigs had a number according to breed (capital letter) and herd (subscript): 6 Piétrain (P1 - P6), 19 extremely-muscled Piétrain (P_D1 - P_D11,P_L1 - P_L4, P_v1 - P_v4), 4 Landrace (LR1 - LR4), 7 Large White (LW1 – LW7) and 2 pigs of a synthetic line based on Large White and Landrace (S1, S2) were used. The Large White pigs, the Landrace pigs and the pigs of the synthetic line were used as control animals.

"Published sequence": In order to design the first primers, a sequence of the entire porcine myostatin gene was assembled, based on the partial sequences with EMBL accession number AY527152, AJ133580, AF019623, AJ237662, AJ237920. This sequence had a length of 6289 bp. In this paper this sequence will be referred to as "published sequence".

Sequencing of the myostatin gene: DNA was extracted from blood or sperm and amplified by a PCR on the Peltier Thermal Cycler-200 (MJ Research). If the sequence to be amplified was less than 1000 bp long, RedTaq™ DNA polymerase (Sigma) was used, if the sequence was more than 1 kb, the 'Expand Long Template PCR' kit was used. In total 14 different PCR primer pairs were designed. The cycle sequencing reactions were done with the BigDye® Terminator v3.1 Ready Reaction Mix (Applied biosystems) and the sequence analysis was done by gelsequencing (ABI Prism® 377 DNA sequencer) and capillary sequencing (ABI Prism® 3700 sequencer).

Analysis of the obtained sequence: The obtained myostatin sequence of the Piétrain pigs was compared to the myostatin sequences of the control animals and with the "published sequence". Also the myostatin sequences of the control animals were compared to the "published sequence". Comparison of these sequences was done with the ChromasPro software package of Technelysium. Assembly of the sequences obtained by the different primer pairs was done with the DNAMAN version 5.0 software package of Lynnon Corporation.
Transcription binding sites were found with MatInspector (http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.pl?s=da0a26177d26d35deb04c6fe8138503d).

Results

Assembled sequence: The sequences obtained with the different primer pairs were assembled with DNAMAN version 5.0 and had a length of 6123 bp. Compared to the "published sequence" it started at bp 140 and ended at bp 6263. So, the promoter sequence and the 3' UTR are not complete. The three exons have a length of respectively 373, 374 and 381 bp and the two introns have a length of respectively 1809 bp and 1978 bp.

Comparison of the obtained myostatin sequence and the "published sequence" (Figure 1): In the promoter sequence, two polymorphisms were found. These polymorphisms will be discussed later in the comparison of the obtained myostatin sequence among breeds and animals.
Also in the intronic sequence, polymorphisms were found when compared with the "published sequence". In intron 1, three polymorphisms were found. In intron 2, seven polymorphism were found. In both introns, one of the polymorphisms is an insertion, which means that both introns differ one basepair with the introns in the "published sequence".
In the coding sequence no polymorphisms were found.

Comparison of the obtained myostatin sequence among breeds and animals (Figure 1): When we compared the obtained sequences among animals and breeds, no polymorphisms were found in the coding sequence or the introns.
In the promoter region however, two polymorphisms were found.
In Figure 2 the three observed situations are shown:

a) Homozygous A on position 1 and homozygous G on position 2:
   This situation was observed only in the Piétrain pigs
b) Homozygous G on position 1 and homozygous A on position 2:
   This situations was observed only in the control group and in the "published sequence". None of the Piétrain pigs showed this genotype
c) Heterozygous G/A on position 1 and heterozygous A/G on position 2:
   This genotype was observed in both control animals as Piétrain pigs.

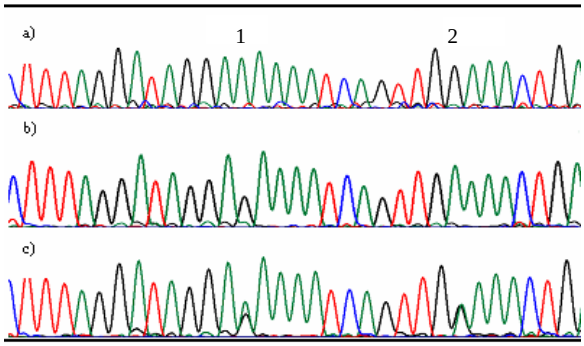

Figure 2: Illustration of the polymorphisms in the promotor region (position 1 and 2)
a) animal P6, Piétrain, b) animal LW7, Large White, c) animal LW2, Large White.
(Red=T, green=A, black=G, blue=C)

Discussion

Polymorphisms in the introns: The polymorphisms we found are not located in the splice donor site, nor the splice acceptor site, nor the branch site of intron 1 and 2. So these polymorphisms are expected to have no effect on the splicing of the primary mRNA. The pigs that were used to assemble the sequences we used for the introns of our "published sequence" (EMBL accession number AJ237662 and AJ237920) are unknown so it's possible that the polymorphisms we found are due to differences in breed.

Polymorphisms in the promoter sequence: When analysing the results it was found that none of the Piétrain pigs, nor normally muscled nor extremely muscled, are homozygous G on position 1 and homozygous A on position 2. On the other hand, it shows that only Piétrain pigs are homozygous A on position 1 and homozygous G on position 2. In order to check if the polymorphisms were located in a conserved region, promoter sequences of pig (EMBL accession number AY527152), mouse (EMBL accession number AY204900), human (EMBL accession number AC073120), cattle (EMBL accession number AJ310751), sheep (EMBL accession number AY918121) and goat (EMBL accession number AY827576) were aligned with EMMA. It shows that in this region the promoter sequence is well conserved, especially among ruminants. In the pig a deletion of 11 nt in comparison to the promoter sequence of

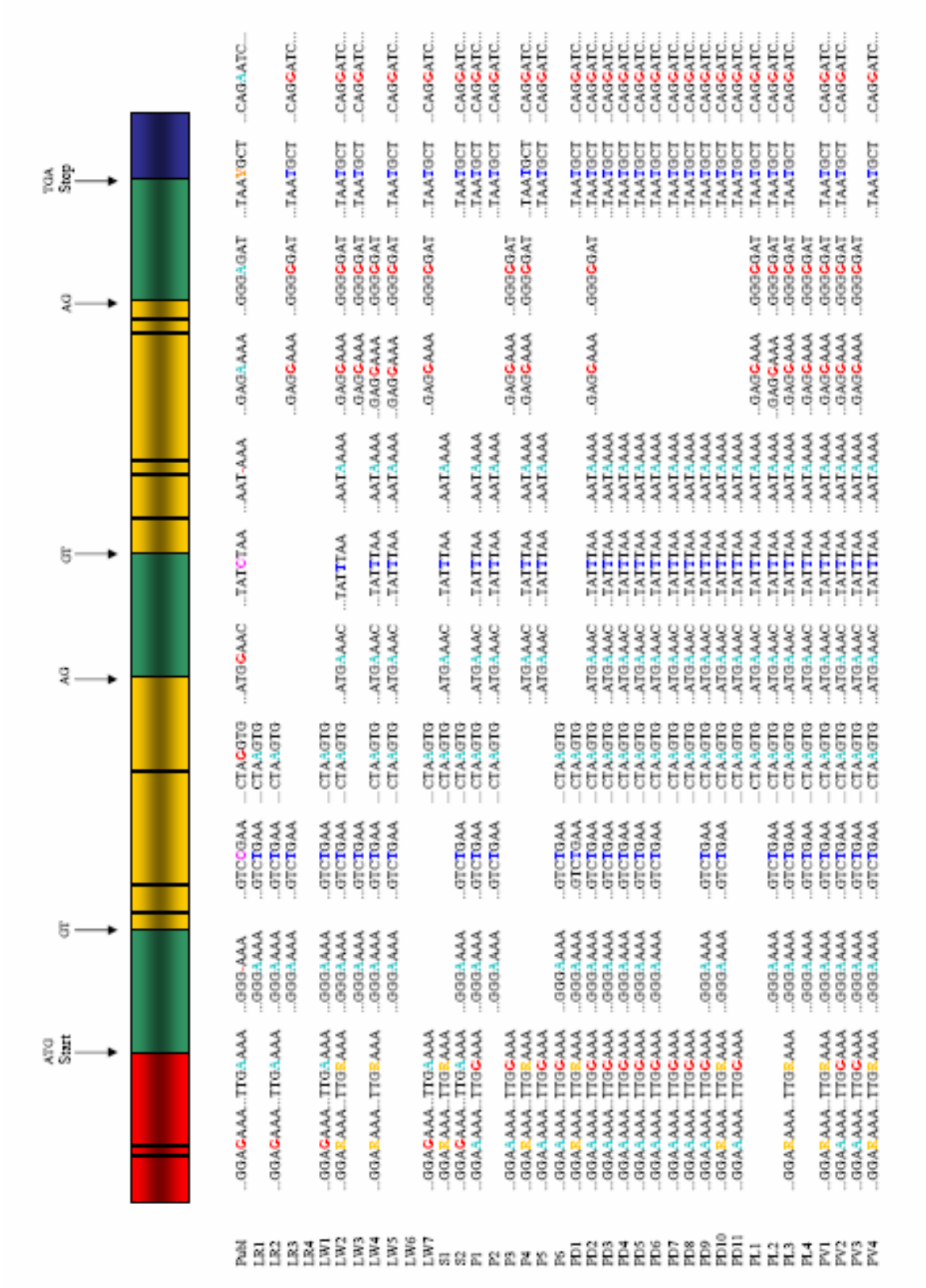

Figure 1: Overview of all polymorphisms found in the porcine myostatin gene and the occurrence in the individual pigs. Publ: "published sequence", LR: landrace, LW: Large white, P: Piétrain, PL, PN, PV: extremely muscled Piétrains. Promoter region (red), exons (green), introns (yellow), 3' UTR (blue).

these animals was found. When the promoter sequence around the mutations was checked for transcription factor binding sites (MatInspector) we found a myocyte enhancer factor-3 (MEF-3) binding site on position 446 to 458, when there was an A on position 2. This MEF-3 binding site has not been found before in human or bovine myostatin (Ma *et al.,* 2001; Spiller *et al.*, 2003). Here we confirm that this MEF-3 binding site in the promoter of myostatin is unique for the pig, and due to the 11 nt deletion previously discussed. MEF-3 binding sites were found though in other muscle-specific regulatory regions of the myogenin, aldolase A and troponine C gene (Parmacek *et al.*, 1991; Hidaka *et al.*, 1993; Salminen *et al.*, 1996; Spitz *et al.*, 1998).

Perspectives

In this experiment the myostatin sequence of Piétrain pigs was compared to those of Large White and Landrace pigs. Both these breeds that were used as a control group are also kept for meat production and are thus maybe not the best breeds to compare with. In the future it is of interest to compare the obtained sequence of the Piétrain with older breeds as Meishan and Wild Boar. These breeds lack the selection for meat production and may provide new polymorphisms.
It is also of interest to perform an association study in order to investigate if MEF-3 is a transcription factor that binds myostatin. Currently this research is in progress.

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