Myostatin Mutations Cause Double Muscling in Cattle

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Introduction

The differences between humans and farm animals are obvious, from body structure to diet to reproduction. Unlike humans, swine have litters of young; cattle are ruminants; and sheep are woolly. Despite such significant differences, growth and development of mammalian species is very similar. The biological controls that govern the establishment of body axes, induction and morphogenesis of tissues, and organogenesis are conserved from flies to man. Generally, the same genes and gene products regulate the same processes in a mouse as in a pig or cow, or human. Recently, it has been determined that mammalian species are highly similar even at the genomic level, with the same genes clustered in groups on chromosomes, a phenomenon called *conserved synteny*. This syntenic conservation is a consequence of the common evolution of mammals until relatively recent times (on an evolutionary time scale). As a result of the fundamental similarity among mammals, it has become clear in the last two or three decades that research being conducted in the biomedical fields can have direct impact on production agriculture. Although nonhuman species are thought of by the biomedical community as models for the study of human disease, from an agricultural perspective humans and mice can be considered model species for livestock. Since the level of financial support afforded to biomedical research is in staggering excess to that of animal agriculture, the success or failure of livestock genomic research is likely to be inevitably tied to progress in studies of humans and mice whose goal is not at all directed towards livestock.

Numerous examples of the application of molecular genetic studies in mice and/or humans to livestock have already been reported. For example, human genetic and biochemical studies formed the basis by which it was determined that the malignant hyperthermia (halothane susceptibility) observed in swine is due to a genetic defect in a calcium release channel gene known as the ryanodine receptor (Fujii et al., 1991). This discovery has resulted in the ability of the swine production industry to more effectively manage their herds with regards to this heritable trait. A more recent example is the discovery of a mutation in the fibroblast growth factor 3 receptor (FGFR3) gene in sheep with spider lamb syndrome, a finding which was based on studies of mice having targeted disruptions of this gene (Beever et al., 1998).

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The *mh* Locus Lies On BTA2

The discovery of the gene that causes double muscling in cattle represents another striking example of the direct benefit human biomedical studies can have on livestock research. Double muscling is a phenotype recognized as early as the 19th century, characterized by a visible, generalized muscular hypertrophy (*mh*). Today a number of breeds exist that have been intensively selected for the phenotype to the point that the frequency of the *mh* allele is high. Some breeds, especially the Belgian Blue, have been the subject of genetic evaluation over the past 25 or more years. The first steps in characterizing the trait involved carcass-composition studies to quantify the impact on muscle, fat, bone, and other production traits such as fertility. A number of studies, reviewed by Arthur (1995), demonstrated that animals of the double-muscled phenotype had substantially more muscle, generated by hyperplasia of the muscle fibers (that is, a higher number of fibers, as opposed to increased size of the individual fibers). The animals also had significantly less fat and bone than unaffected animals, and a notable reduction in the size of internal organs. However, the production advantages of the phenotype come at a price: there appear to be substantial effects on fertility, calving ease, and stress tolerance. Although the carcass and reproductive effects are seen in all double-muscled breeds, the degree to which both the desirable and problematic traits are presented shows some variation between breeds, suggesting that genetic background can influence their manifestation.

Early genetic studies of the double muscled syndrome suggested either a polygenic or recessive mode of transmission. The advent of genetic markers, as the livestock genome maps were developed in the 1990's, supported more accurate analysis that demonstrated an autosomal recessive mode of transmission of the phenotype in Belgian Blue. The analysis utilized a Belgian Blue/Holstein backcross population specifically designed to map genes affecting the muscular hypertrophy. It strongly suggested

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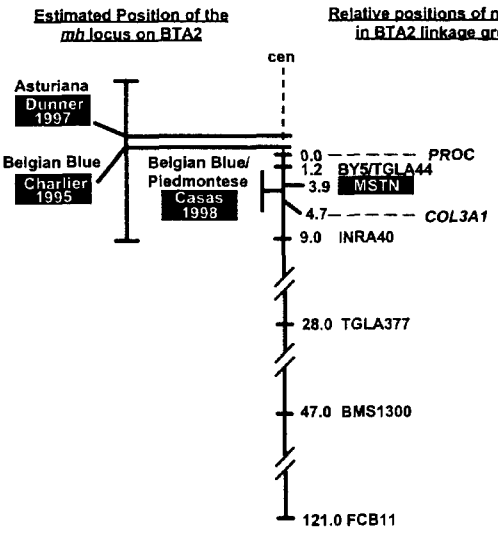
that a single locus was involved which mapped to the centromeric end of bovine chromosome 2 (BTA2; Charlier et al., 1995). Subsequently, the same map position was found for the Piedmontese and Asturiana breeds (Dunner et al., 1997; Casas et al., 1998), suggesting the same locus is responsible in all three breeds (Figure 1). In combination with historical evidence, these results were interpreted to suggest a common origin of the BTA2 *mh* allele in double-muscled breeds, and that a mutation event in a single common ancestor is the source of all double muscling. The mapping of the *mh* locus also revealed a weakness of the livestock maps: the number of polymorphic genetic markers in the vicinity of *mh* was quite low, and the predicted position of the locus lay outside the linkage map (that is, it was not flanked on both sides by known genetic markers). This lack of flanking markers limited the resolution of the mapping studies, highlighting the need for further improvement of the livestock genome maps to increase marker density and genome coverage.

Figure 1. Partial map of the BTA2 linkage group showing the predicted intervals containing the *mh* locus (left side) and the relative positions of genetic markers (right side) including MSTN. The breeds used in each of the three *mh* mapping studies are ahown above the reference for each study, and the confidence intervals for each study are represented by vertical bars extending along the linkage group. The positions of microsatellite markers as well as the genes COL3A1, PROC, and MSTN are shown. Italicized markers were produced from comparative mapping studies (see text). Orientation is indicated by "cen", the centromere.



The conserved synteny between mammals was used to overcome the limitations of the livestock genetic maps. Since it has been established that genes are grouped together similarly in cattle and humans, genes likely to lie near the centromere of BTA2 could be selected from the well-developed maps of mouse and human in a process known as comparative mapping. The first task was to identify the region of the human genome that corresponds to the centromeric portion of BTA2. This was accomplished through steps of increasing resolution, until very specific regions could be targeted for comparative mapping. At the lowest resolution, a technique called ZOO-FISH provides a coarse comparison between the genomes of species. In this technique, single human chromosomes that have been separated by flow sorting are used to generate chromosome-specific probes, which are then hybridized to metaphase spreads of the bovine genome. There is sufficient conservation between species at the DNA sequence level to allow these human probes to selectively hybridize to their bovine counterparts. Probes made from human chromosome 2 (HSA2) were shown by ZOO-FISH to hybridize to the majority of BTA2, including the centromeric end, and to BTA11 (Solinas-Toldo et al., Genomics 27, 489-496 1995; Chowdhary et al., Mamm Gen 7, 297-302, 1996), suggesting that some portion of HSA2 is likely to carry the gene(s) whose bovine counterpart(s) underlie the *mh* locus.

The resolution of the ZOO-FISH experiments was insufficient to accurately select genes from the human map that are likely to lie near the centromere of BTA2. Resolution of the comparative map was therefore increased by mapping in cattle specific, individual genes known to lie on HSA2. This was accomplished by developing polymorphic genetic markers for each gene, and placing them onto the bovine physical and linkage maps (reviewed in Sonstegard et al., 1997). These mapping studies were conducted in specialized reference populations designed to maximize heterozygosity of markers, rather than in the double-muscled mapping families, in order to more accurately place the gene markers on the bovine map. The results (Figure 2) demonstrate the value of integrating physical and linkage maps, and of developing a dense comparative map between livestock species and the more developed human and mouse maps.

A benefit of the comparative approach was the generation of new markers in the vicinity of the *mh* locus. These markers significantly increased the resolution of mapping the gene when they were applied in the double muscled mapping families (Figure 1, italicized markers). Re- analysis with the new marker data demonstrated that the *mh* locus was flanked by polymorphic genetic markers, allowing the conclusion that *mh* lies in a small interval between the genes for alpha collagen type III (COL3A1) and protein C (PROC) on the cattle map. However, this exciting result underscored a significant difficulty in directly using comparative mapping data to identify genes from the human map that lie in an interval containing a trait locus (called positional candidate genes). The difficulty is that COL3A1 and PROC on the human map are separated by a very large interval (Figure 2), suggesting that *mh* lies very close to a breakpoint in conserved gene order between the two species. Some genes lying close to

PROC on HSA2 were found to map to an entirely different chromosome (BTA11); others to BTA2, but distant from the centromere. Similarly, genes lying fairly close to COL3A1 on the human map were found to map to BTA2, but not near the centromere. It was therefore unclear whether to pick positional candidate genes from the human map in the vicinity of COL3A1 or of PROC, and it became apparent that any positional candidate chosen from the human map must be mapped in cattle to verify its position. The shuffling of gene order within syntenic groups is likely to complicate future mapping efforts for other loci as well, suggesting that any positional candidate genes should in general be mapped in cattle before assessment as candidates. It should be noted that the genes chosen for aligning the human and bovine comparative maps (Figure 2) were chosen with position on the human map as the principal criteria. Only after initial definition of the interval containing the locus was their significant effort to identify genes of functional significance relative to the double muscled phenotype.

Discovery And Mapping Of MSTN

In light of the results of the comparative mapping analysis between the bovine and human genomes, the approach taken to identify the gene underlying double muscling was the same in several laboratories. Genes lying near COL3A1 and/or PROC on the human and mouse maps were selected for mapping on the basis of their suitability as candidates; for example, genes previously known to play a role in myogenesis or cell proliferation. Success of this approach is entirely dependent on the hope that the gene has previously been cloned, characterized, and mapped in these other species. A number of genes known to affect development lie near both COL3A1 and PROC, but no clear phenotype similar to double muscling had yet been seen in human disease or mouse development. Thus it appeared that the gene of interest either had not yet been identified in these species, or that a particular allele in cattle existed that had no corollary with any recognized human phenotype or mouse study. Then, in May of 1997, a breakthrough in mouse research provided a new candidate gene. A group at Johns Hopkins University was screening for new members of the transforming growth factor beta (TGFb) superfamily of genes, and assessing their biological role by producing "knockout" mice that have targeted disruption of each gene. Mutation of some of these important growth and development factors (GDFs) are lethal; but one of these genes, GDF-8, had a very striking, nonlethal effect in mice homozygous for disruption of the signaling domain of the protein (shown in Figure 3a). These mice

Figure 2. Comparative mapping in cattle of genes found on the q arm of human chromosome 2 (HSA2q). Ideograms of R-banded chromosomes are shown, and the physical assignments of each gene are indicated by vertical lines. Most of the genes mapped to bovine chromosome 2 (BTA2) with the remainder mapping to chromosome 11 (BTA11). Rearrangements in relative gene order and breaks in conserved synteny are apparent. This figure is adapted from Sonstegard et al, 1997.

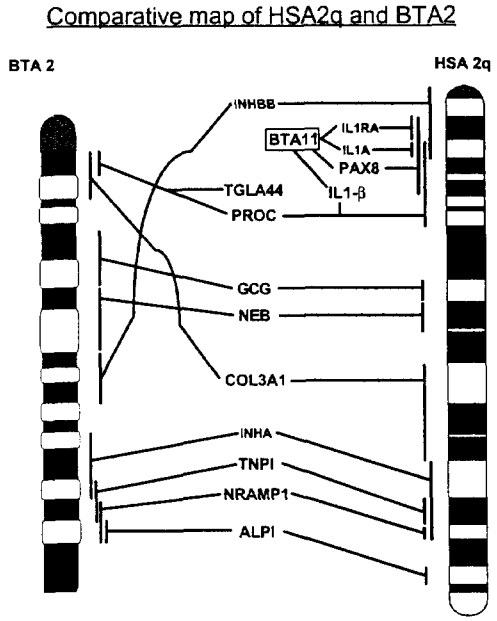
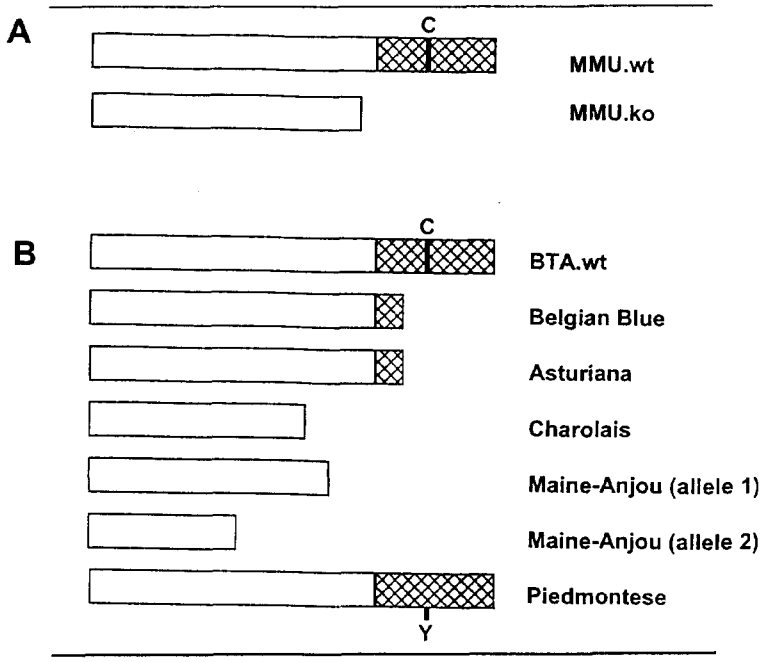


Figure 3. Schematic representation of mutations in the murine (A) and bovine (B) myostatin proteins. White boxes represent the pro-region of the peptide; stippled boxes represent the mature peptide (signaling portion) remaining after proteolytic processing. The wild-type proteins are shown for both mice and cattle (MMU.wt and BTA.wt). Underneath are shown the portions of the protein produced in each mutant strain or breed. MMU.ko, MSTN knockout mice. The heavy line indicates the position of a conserved cysteine (C) residue necessary for function, that is altered to tyrosine (Y) in the Piedmontese breed.



veloped extremely large muscles, reminiscent of the double-muscle phenotype. The magnitude of the effect was much larger in mice than in cattle, with muscles in the -/- mice 2-3 *times* the normal size. Other aspects of the knockout mice also differed from the double muscle syndrome, such as the GDF-8 knockout mice showed no effect on organ size. However, the important feature was that the muscle increase resulted from hyperplasia, making it the first mouse model to mimic this fundamental aspect of the *mh* allele. The gene was renamed myostatin (MSTN) to reflect the negative regulation of myoblast proliferation inferred from the increased number of fibers in knockout mice.

The MSTN gene made an attractive candidate for the *mh* locus based on the biology observed in the mouse study. The first question to answer was whether a bovine homolog of the gene existed. In fact, cattle have a MSTN gene with extremely high conservation of sequence compared to the mouse gene, with 89% of nucleotide sequence identical in the coding region of the gene, coding for a 375 amino acid protein that is 89% identical to the mouse protein (Kambadur et al., 1997; Grobet et al., 1997; McPherron and Lee, 1997). This conservation allowed the rapid cloning of the bovine version by using PCR primers designed from the mouse sequence. Using these clones, markers were developed to place the gene on the bovine genetic map. It was shown that MSTN maps between COL3A1 and PROC on the cattle map (Figure 1), consistent with the hypothesis that myostatin is the *mh* gene (Smith et al., 1997; Grobet et al., 1997).

It then remained to prove this hypothesis. Because the increased muscle was observed in mice with impaired gene function, it followed that if MSTN is the *mh* gene, then any mutation that disrupts the timing, distribution, level of expression, or function of the protein could be the cause of the phenotype in cattle. Of course, mutations in the protein coding region are not significant if the message is not transcribed at all, so the first analysis determined the expression of myostatin mRNA in BB and normal cattle. As in mice, myostatin expression could be demonstrated early in embryogenesis. No difference between the timing or levels of expression was observed in normal or BB embryos, or in the muscle of adult BB or Pied cattle, suggesting that mutations in the protein itself are likely responsible for the phenotype in these breeds (Kambadur et al., 1997).

Mutations In MSTN

The next step was to determine the sequence encoded by the MSTN gene in normal and double muscled animals. In September 1997, two laboratories simultaneously reported mutation analyses of myostatin in double muscled cattle (Kambadur et al., 1997; Grobet et al., 1997) and a third study was reported the following month (McPherron and Lee, 1997). All three studies showed that double muscled Belgian Blue animals are homozygous for an 11 base pair deletion in the third exon of the gene. This deletion results in a translational frame shift that is predicted to abolish the part of the protein involved in the signaling function of TGFb family members (Figure 3b). Indeed, the portion of the protein that is truncated is nearly identical to that removed in the targeted disruption of the knockout mice. Furthermore, one of the studies found the same deletion in Asturiana breed animals (Grobet et al., 1997). Thus, the Belgian Blue and Asturiana breeds are in essence the same as the knockout mice. In contrast, two of the studies demonstrated that double muscled Piedmontese animals are homozygous for a G to A transition that changes a cysteine to a tyrosine (Kambadur et al., 1997; McPherron and Lee, 1997). In other members of the TGFb family, the cysteine at this position in the signaling portion of the molecule has been shown to participate in an essential disulfide bond, so this single point mutation would be predicted to interfere with function of the protein. Further analysis of other double muscled breeds has now identified 5 total independent mutations, depending on breed type, all of which are predicted to disrupt the function of the protein (Figure 3b; Grobet et al., 1998; T. Smith, unpublished). The only breeds for which mutations have not been found are Limousin and Blond d'Aquitaine. The phenotype for Limousin is not precisely the same as in the others and is normally thought of as a "heavy muscled" breed rather than "double muscled", so there may be genetic factors other than MSTN which can increase muscling. Alternatively, it is possible that these breeds have lower expression levels of myostatin due to promoter mutations, rather than protein coding region defects. Analysis of the promoters for these breeds is underway to address this question. In any case, the discovery of MSTN mutations in double muscled breeds is compelling evidence that abrogation of myostatin function is the cause of double muscling in these breeds, although it is now apparent that the breeds do not all derive from a common ancestral mutation.

Perspectives

The value of the cattle genome map in the effort to find the "double muscle gene" is apparent, as is the need for close ties between maps of livestock, the highly developed human map, and the maps of easily manipulable species such as mice. In all likelihood, the search for the mh locus has been completed. This is a significant finding that provides an important basis for research into muscle development and fat deposition in all livestock species. The question that remains is, how do we make use of this information? In spite of popular press claims to the contrary, the discovery of myostatin as the agent of double

muscling is unlikely to dramatically change cattle production in the near term. Breeders have been able to use MSTN knockout in cattle since double muscling was first recognized, long before the underlying gene was known, and the limits of the use of these mutations have been tied to the negative aspects of the double muscled syndrome that are independent of the knowledge of the gene. However, recent studies suggest that animals with only one functional copy of MSTN (i.e. heterozygous for one of the knockout mutations, *mh*/+ genotype) have a significant increase in retail product yield, and produce leaner meat, while having less increase in birth weight than *mh/mh* animals and a lower incidence of calving difficulties (Table 1; Casas et al., 1998). One possible system to utilize the carcass advantages of *mh*/+ animals is a terminal sire mating system, using a *mh/mh* bull and normal cows. The drawback to this system is the need to purchase replacement animals or to have a separate herd to provide replacements, both of which represent inefficiencies. Another, more efficient system is maintaining *mh* in single copy through the use of a direct genetic test for *mh* alleles (Fahrenkrug et al., submitted) on potential breeding animals in a herd segregating one or more of the *mh* mutations. Using a direct test to classify the genotype of each bull and cow can prevent matings between two *mh*/+ animals and avoid the production of *mh/mh* offspring, thus minimizing calving difficulty while maintaining the advantage of *mh*/+ carcasses (Keele et al., submitted).

Table 1. Mean and standard errors for birth weight (BWT), retail product yield (RPYD), rib eye area (REA), yield grade (YG), marbling (MAR), fat thickness (FAT), estimated kidney, pelvic, and heart fat (KPH), calving ease (CE) and tenderness measured as shear force at 3 (S3) and 14 (S14) day postmortem, and effect of the *mh* allele [(*mh*/+) - (+/+)] as proportion of the residual standard deviation.

Trait	Mean ± SE	Residual Difference[a]	SD Units
BWT, kg	40.3 ± .2813	4.56	.41
RPDY, %	66.4 ± .199	4.39	1.60
REA, cm^2	85.2 ± .48	1.3	1.35
YG	2.13 ± .35	- .74	- 1.42
MAR	500 ± 3.14	50.92	- 1.01
FAT, cm	.7 ± .018	- .099	- .84
KPH, %	2.8 ± .033	- .43	- .86
CE	1.2 ± .05	NS	NS
SH3	4.6 ± .05	NS	NS
SH14	3.5 ± .033	NS	NS

[a] Differences are presented in the units for the traits in the first column. NS = not significant (P>.05). Casas et al., J. Anim. Sci. (In press).

The increased muscle mass of *mh*/+ animals indicates that a single functional copy of MSTN cannot compensate for the damaged copy, suggesting relatively small changes in the amount of the protein can lead to dramatic differences in muscle fiber number. This raises the tantalizing possibility that muscle size in normal breeds could be influenced by interfering with myostatin activity, since it would only require small changes. However, this requires that specific, nongenetic methods for inhibiting activity be discovered which will probably not be realized immediately. The fact that myostatin is expressed in early development, and hyperplasia is evident prenatally, suggests that the target may have to be embryonic. Further study (probably in mice) is needed to determine if muscle mass can be affected through the myostatin pathway postnatally, a feature that would make it much more amenable to manipulation in normal breeds. Encouragingly, myostatin is expressed in adult muscle, suggesting it may be regulating muscle cell proliferation (perhaps through satellite cells) throughout life. If this is true, it may some day be possible to induce muscle hypertrophy in adult animals at a time most convenient for production, e.g. in the feedlot.

An immediate use of the identification of the mh gene is the ability to accurately genotype animals for the presence of known myostatin mutations. This information will be valuable to breeders, whether they are trying to breed for or against the double muscling trait. It will also aid germplasm evaluation studies that include double muscle breed components by providing direct assessment of the number of mh alleles present in any animal, without the need for parental data. It will spur the search for allelic variation in myostatin in other meat animal species, that may have less effect than the complete abrogation of function in double muscled cattle. Studies are underway to determine if genetic manipulation of myostatin through transgenesis or antisense RNA technology can be useful approaches to increasing muscle mass in swine or poultry. The fact that myostatin is a negative regulator of muscle growth is especially encouraging, since any treatment that interferes with its function should increase muscle mass. Such interference is likely to be easier to accomplish than manipulation of positive growth factors has been, and we already know what the consequences of complete abrogation are to all systems in the animal. In summary, the identification of the mh locus demonstrates how all facets of genome research in multiple species can act in concert to advance our understanding of genetic variation and the fundamental biology of production traits in livestock.

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