Mammalian Genome 8, 742–744 (1997).

Mammalian Genome

Myostatin maps to the interval containing the bovine *mh* locus

Timothy P.L. Smith, Nestor L. Lopez-Corrales, Steven M. Kappes, Tad S. Sonstegard

U.S. Department of Agriculture, Northern Plains Area, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, Nebraska 68933-0166, USA

Received: 19 June 1997 / Accepted: 2 July 1997

Abstract. Myostatin (GDF-8) is a member of the transforming growth factor-β superfamily and plays a role in muscle growth and development. Mice having targeted disruption of this gene display marked increases in muscle mass, a phenotype similar to the muscular hypertrophy (*mh*) in several cattle breeds. Physical mapping data developed from YAC clones indicate the bovine myostatin gene lies close to the centromere of bovine Chromosome (Chr) 2 (BTA2) at 2q11, indistinguishable from the cytogenetic location of the *mh* locus. In addition, a polymorphism in the second intron of the gene was used to show that myostatin maps within the interval previously shown to contain *mh.* These data suggest myostatin may be the gene causing muscular hypertrophy in cattle.

Introduction

Muscular hypertrophy (*mh*), also known as ''double-muscling'' in cattle, has been recognized as a heritable physiological phenotype for decades (reviewed in Arthur 1995) and is present in Belgian Blue, Piedmontese, and Asturiana de los Valles breeds. Affected animals of the Belgian Blue breed, which have been intensively selected for the trait, display enlarged muscles with a distoproximal and anterioposterior gradient such that the degree of hypotrophy is greatest in the proximal hindquarters. The hypertrophy results primarily from cellular hyperplasia. Other significant effects of the syndrome include a diminished size of internal organs as well as a decrease in the size of external genitalia. Previous genetic studies in a Belgian Blue × Holstein backcross population and Asturiana pedigrees demonstrated that double muscling segregates as a monogenic trait and maps to the centromeric end of the BTA2 linkage group (Charlier et al. 1995; Dunner et al. 1997). Another recent mapping study utilized more markers in this region and genotyped two large half-sib families by use of F_1 Belgian Blue and Piedmontese sires (Casas et al. submitted). This study highlighted the partial recessive character of the trait, as the effect of *mh* on muscle mass of heterozygous *mh*/+ animals was observed to be 1.6 standard deviations higher than +/+ animals. In conjunction with a comparative mapping approach (Fisher et al. 1997; Sonstegard et al. 1997), this analysis narrowed the location of *mh* to an approximately 5-cM interval distal to the Protein C (*PROC*) gene in the vicinity of the alpha collagen type III (*COL3A1*) gene.

A recent study involving the production of mice with homozygous deletions of genes of the TGF-β family has suggested the possibility that myostatin may be a candidate gene for the *mh* locus (McPherron et al. 1997). This family of genes encodes secreted growth factors that are important for the maintenance of tissue homeostasis in the adult animal, as well as playing crucial roles in embryonic development (reviewed in McPherron and Lee 1996). Myostatin is of particular interest because of the phenotype of the knockout mice, which exhibit a severe cellular hyperplasia that results in muscles two to three times the size of wild-type animals. In order to evaluate myostatin as a candidate gene for *mh,* we have mapped bovine myostatin with physical and genetic markers for comparison with the known position of *mh.*

Correspondence to: T.P.L. Smith

Materials and methods

Primers were developed from the reported sequence (GENBANK accession #U84005) of exon 3 of the mouse cDNA for myostatin (primers mg898 and mg1234; numbers refer to the corresponding nucleotide position in the mouse sequence; see below). Optimal conditions for use of these primers were determined with bovine genomic DNA, and the PCR product was cloned and sequenced to verify that the correct locus had been targeted before these primers were used to screen the YAC library. A bovine-specific reverse primer (bg1001) was then developed from the sequence of the PCR product and used in conjunction with a primer designed from the mouse exon 2 sequence (primer mg743) to amplify across intron 2 of the bovine gene from genomic or YAC DNA.

Primer sequences were:

mg898-CCGGAGAGACTTTGGGCTTGACTG
mg1234-TCATGAGCACCCACAGCGGTC
mg743-CAAAATTGGCTCAAACAGCCTGAATC
bg1001-TAATCCAATCCCATCCAAAAGC

Polymerase chain reaction (PCR) was performed in 10 μl total volumes with 100 μM each of all four deoxynucleotides, 0.1% Triton X-100, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 or 2.5 mM $MgCl_2$, and 5–15 ng/μl of DNA depending upon whether the template was bovine genomic or YAC pool DNA, respectively. Thermocyling parameters were 94°C for 4 min, followed by 30–35 cycles of 94°C 1 min, 58°C 1 min, 72°C 1 min (YAC screening) or 2 min (exon 2 amplification), followed by a 4-min elongation step at 72°C. The PCR products were electrophoresed on 1.5% (intron 2 and digestion products) or 3% (exon 3 YAC screening) agarose gels and visualized by staining with ethidium bromide. A *Xcm*I PCR vector (T. Sonstegard, unpublished) was used for cloning of PCR products to be sequenced.

YAC screening was performed as described (Smith et al. 1996) with the mg898 and mg1234 primers. Fluorescence in situ hybridization (FISH) of the BY261D9 YAC and intestinal alkaline phosphatase (ALPI) cosmid was performed as described (Smith et al. 1996; Lopez-Corrales et al. 1997). Restriction fragment length polymorphism (RFLP) of the intron 2 PCR product was performed by addition of a reaction mix containing 0.5 μl (3 units) of *Hae*III enzyme (Promega), 2.5 μl of 10× reaction buffer (supplied with the enzyme), and 12 μl of H_2O directly to the completed PCR reaction. Digestion was allowed to continue for 2 h at 37°C before the gel was loaded. PCR-RFLP genotypes were scored and analyzed as described (Bishop et al. 1994; Kappes et al. 1997).

Results

The goal of these experiments was to determine whether myostatin represents a possible positional candidate gene for *mh.* Initially, a series of primers were developed from the reported sequence of the mouse cDNA in the portion of exon 3 coding for a series of cysteine residues that is a consensus part of the TGF-β gene family (McPherron et al. 1997). One set of primers, mg898 and mg1234, produced a 337-bp product in human, sheep, porcine, and bovine

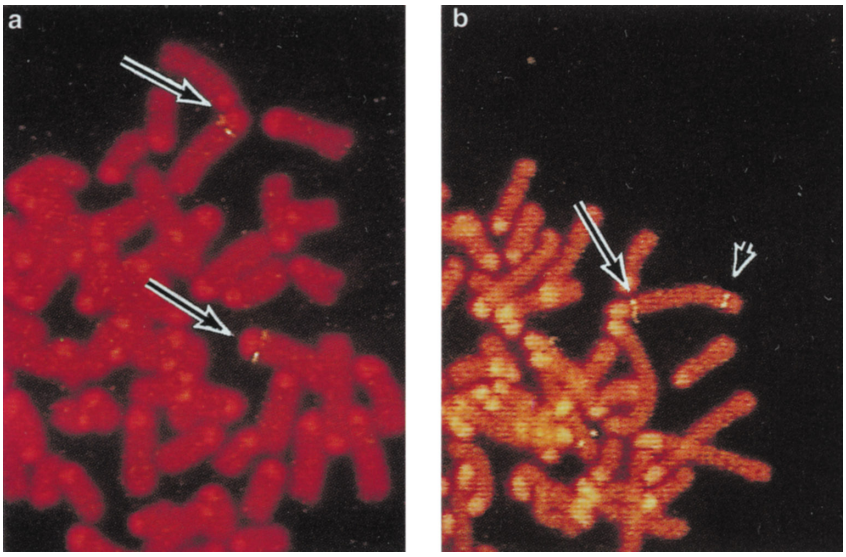

Fig. 1. Physical location of the bovine myostatin gene revealed by FISH of YAC clones. (**a.**) Total DNA from bovine YAC clone BY261D9 was labeled and hybridized to bovine metaphase spreads. Arrows indicate the hybridization signals at 2q11. (**b.**) Verification of the chromosomal identification is provided by cohybridization of the BY261D9 probe with a cosmid probe carrying the *ALPI* gene, which has previously been assigned to 2q44. Arrows indicate the BY261D9 signals and arrowheads indicate the ALPI hybridization signal.

DNA. The product from bovine DNA was cloned, sequenced, and found to have 88.7% identity (excluding the primer sequence) with the mouse sequence and 93.5% identity with the product from human DNA, demonstrating that the primers amplified the desired bovine target sequence.

Previous physical assignments of markers shown to be closely linked to the *mh* locus predict that it lies at BTA2q11 (Sonstegard et al., In press). Our next goal, therefore, was to obtain large insert clones to determine the cytogenetic location of myostatin. The mg898 and mg1234 primers were used to screen the MARC bovine YAC library, and five individual clones were identified. These clones were verified to carry the bovine myostatin gene by amplification with two other sets of primers developed from the mouse sequence (data not shown). Three clones were chosen on the basis of size and used for FISH analysis. Two of the YACs appeared to be chimeric, because they gave more than one hybridization signal (specifically, they gave faint hybridization signals at the telomeres of multiple chromosomes; data not shown). However, all three YACs produced more intense hybridization signals at the centromere of BTA2, and one YAC (BY261D9; approximately 380 kilobases in size) consistently gave only a BTA2 centromeric signal (Fig. 1a). To verify that myostatin is localized close to the BTA2 centromere, the BY261D9 YAC was cohybridized with a cosmid carrying the *ALPI* gene (Fig. 1b) that had previously been mapped to BTA2q44 (Sonstegard et al. in press). These results support the conclusion that bovine myostatin lies at BTA2q11, the predicted position of the *mh* locus (Charlier et al. 1995; Sonstegard et al. in press; Casas et al. submitted).

The FISH analysis suggested that myostatin may be a positional candidate gene for *mh.* In order to increase the resolution and provide further support for this hypothesis, we developed a PCR-RFLP marker, using primers that amplify across intron 2 of the gene. A forward primer (mg743) was developed from exon 2 sequence of the mouse gene, and a reverse primer (bg1001) was developed from the bovine exon 3 sequence (see Materials and methods). These primers produce an approximately 2200-bp product from bovine genomic DNA that includes 250 bp of exon sequence. A polymorphism in the MARC reference population (Bishop et al. 1994; Kappes et al. 1997) revealed by the enzyme *Hae*III was used to identify 91 informative meioses and provide the basis for linkage mapping of myostatin.

The TWOPOINT option of the CRIMAP program version 2.4 (Green et al. 1990) resulted in significant two-point linkage of the myostatin PCR-RFLP only with markers in the BTA2 linkage group. The maximum LOD score for any BTA2 marker and myostatin was 24.40 with marker *BM81124,* which lies near the centromeric end of the BTA2 linkage group (Kappes et al. 1997; Sonstegard et al. 1997). LOD scores greater than 5 were also found for myostatin and eight other markers from the centromeric end of BTA2. The CRIMAP program was then used to position the marker within the linkage group. The FLIPS3 and FLIPS4 options indicated four marker orders that result in scores differing by less than 3 LOD, but all of these possible orders place the myostatin gene between the centromere and the *COL3A1* gene. The order with the highest LOD score was *PROC*–(0.8)–*BM81124*–(0.0)–*BY5*–(0.0)–*TGLA44*–(1.9)–**myostatin**–(2.0)–*COL3A1*–(1.0)–*BM3627* (numbers in parentheses indicate cM distance between markers).

Discussion

The FISH data in combination with the PCR-RFLP linkage analysis show that the location of the myostatin gene is indistinguishable from that of the *mh* locus at the resolution of the mapping study (approximately 3–5 cM). Thus, the bovine myostatin gene represents a positional candidate for the gene causing the muscular hypertrophy observed in Belgian Blue cattle. Previous to the discovery of this gene, a comparative mapping study of BTA2 with human Chr 2 had suggested other possible positional candidates known to have effects on muscle development (Sonstegard et al. 1997). These include Gli2, a zinc finger protein of the kruppel family for which other members are known to affect limb development (Matsumoto et al. 1996); Bin1, a myc-interacting protein containing an SH3 domain whose expression is linked to development of myotubes from myoblasts (Negorev et al. 1996); Grb14, a member of a family of genes that are targets for PDGF-regulated serine kinases, of which one (Grb2) is known to bind activated growth factor receptors and couple the receptor to the RAS pathway (Baker et al. 1996); and En1 (engrailed), a homeobox gene involved in segmentation/pattern formation in insects (summarized in Patel et al. 1989). All four of these genes lie in the vicinity of the PROC gene on the human map, although their position on the bovine map has yet to be verified. In addition, there are a significant number of expressed sequence tags (ESTs) that lie in this vicinity on the human map, including some derived from skeletal muscle, that also represent positional candidate genes.

Although all these genes represent formal candidates for the *mh* locus, the similarity of the phenotype of the knockout mice to the homozygous *mh/mh* cattle makes myostatin the most attractive

candidate. The fact that it acts as a negative regulator of muscle growth, as evidenced by the increased muscle in mice lacking the gene, is highly suggestive that simple deletion or point mutation that decreases the activity or expression of the gene would suffice to create the observed phenotype. Other members of this family of genes act by formation of active dimers capable of interacting with signal-transducing ligands, and indeed it appears that myostatin exists as a dimer (McPherron et al. 1997). This mechanism suggests that a mutation either in the promoter region, affecting expression timing or levels, or in the coding region affecting dimerization, receptor binding, or signal transduction, may underlie the *mh* phenotype.

Interestingly, the mouse study found little difference between wild-type (+/+) mice and heterozygous (−/+) animals in muscle weights. This sharply contrasts with the situation in cattle, which show significant effects of a single copy of the *mh* allele (Casas et al. submitted). A possible explanation for this difference could be that the *mh* mutation in cattle results in normal levels of a defective protein, which leads to the formation of defective dimers with the protein from the normal copy. The formation of all three classes of *mh/mh*, *mh*/+, and +/+ dimers could lead to the intermediate phenotype observed in *mh*/+ cattle through competitive inhibition by the nonfunctional dimers. In contrast, the heterozygous knockout mice have only functional dimers made from the single, uninterrupted wild-type allele.

Clearly further examination of the timing and level of expression, as well as the amino acid sequence of the protein in normal and *mh* animals, will help to resolve these issues and determine whether myostatin is the *mh* gene. Studies are currently under way to sequence the genes of normal and heavy-muscled cattle breeds to find any underlying mutations. In addition, studies of other breeds will be conducted to determine whether there is allelic variation affecting muscling with less severe effects than the *mh* allele. It is an exciting possibility that the discovery of the myostatin gene may eventually lead to treatments that increase muscle growth in meat animals with normal myostatin genes, or delay the expression of hypertrophy in heavy-muscled breeds to avoid the problems of dystocia normally associated with Belgian Blue animals.

Acknowledgments. We thank Kevin Tennill and Renée Godtel for expert technical assistance; Sherry Kluver for manuscript preparation; and Jan Watts for photographic services.

References

Arthur PF (1995) Double muscling in cattle: a review. Aust J Agric Res 46, 1493–1515

Baker E, Sutherland GR, Sutherland RL, Daly RJ (1996) Assignment of the human *GRB14* gene to chromosome 2q22-q24 by fluorescence *in situ* hybridization. Genomics 36, 218–220

Bishop MD, Kappes SM, Keele JW, Stone RT, Hawkins GA, Fries R, Solinas-Toldo S, Grosz MD, Sunden SLF, Yoo J, Cundiff LV, Beattie CW (1994) A genetic linkage map for cattle. Genetics 136, 619–639

Casas E, Keele JW, Shackelford SD, Sonstegard TS, Smith TPL, Koohmaraie M, Kappes SM, Stone RT (1997) Association of the double-muscling locus on bovine chromosome two (BTA2) with carcass traits. J Anim Sci, submitted

Charlier C, Coppieters W, Farnir F, Grobet L, Leroy PL, Michaux C, Mni M, Schwers A, Vanmanshoven P, Hanset R, Georges M (1995) The *mh* causing double-muscling in cattle maps to bovine Chromosome 2. Mamm Genome 6, 788–792

Dunner S, Charlier C, Farnir F, Brouwers B, Canon J, Georges M (1997) Towards interbreed IBD fine mapping of the *mh* locus: double-muscling in the Asturiana de los Valles breed involves the same locus as in the Belgian Blue cattle breed. Mamm. Genome 8, 430–435

Fisher SR, Beever JE, Lewin HA (1997) Genetic mapping of *COL3A1* to bovine chromosome 2. Mamm Genome 8, 76–77

Green P, Falls K, Crooks S (1990) Documentation for CRI-MAP, version 2.4. Washington University School of Medicine, St. Louis, Mo

Kappes SM, Keele JW, Stone RT, McGraw RA, Sonstegard TS, Smith TPL, Lopez-Corrales NL, Beattie CW (1997) A second generation linkage map of the bovine genome. Genome Res 7, 235–249

Lopez-Corrales NL, Sonstegard TS, Smith TPL, Beattie CW (1997) Physical assignment of glucagon and nebulin in cattle, sheep and goat. Mamm Genome 8, 428–429

Matsumoto N, Fujimoto M, Kato R, Niikawa N (1996) Assignment of the human *GL12* gene to 2q14 by fluorescence *in situ* hybridization. Genomics 36, 220–221

McPherron AC, Lee S-J (1996) In *Growth Factors and Cytokines in Health and Disease*, Vol. 1B, D. LeRoith and C. Bondy, eds. (JAI, Greenwich), pp. 357–393

McPherron AC, Lawler AM, Lee S-J (1997) Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387, 83–90

Negorev D, Riethman H, Wechsler-Reya R, Sakamuro D, Prendergast GC, Simon D (1996) The Bin1 gene localizes to human chromosome 2q14 by PCR analysis of somatic cell hybrids and fluorescence *in situ* hybridization. Genomics 33, 329–331

Patel NH, Martin-Blanco E, Coleman KG, Poole SJ, Ellis MC, Kornberg TB, Goodman CS (1989) Expression of engrailed proteins in arthropods, annelids, and chordates. Cell 58, 955–968

Smith TPL, Alexander LJ, Sonstegard T, Beattie CW, Broom MF (1996) Construction and characterization of a highly redundant large insert YAC library of the bovine genome. Mamm Genome 7, 155–156

Sonstegard TS, Lopez-Corrales NL, Kappes SM, Beattie CW, Smith TPL (1997) Comparative mapping of bovine and human chromosome 2 identifies segments of conserved synteny containing the bovine *mh* locus. Mamm. Genome, 8, 750–754