ISSN 0370-2731/2000 Proceedings of the New Zealand Society of Animal Production **60:** 90-93

Cloning and characterization of the bovine myostatin promoter

M. SHARMA, J.K. MARTYN*, F. JEANPLONG*, M. THOMAS, MICHAEL P. SPILLER, J.J. BASS AND R. KAMBADUR

Animal Genomics, AgResearch, Hamilton, New Zealand.

ABSTRACT

Myostatin a TGF beta superfamily member has been shown to be present in high levels in the myogenic precursor cells of dermomyotome and in the adult muscle fiber. To identify cis-acting DNA elements involved in myostatin gene expression, we have isolated and sequenced several kilobases of the bovine myostatin promoter region. Sequence analysis of the 1.6 kb immediate upstream region of bovine myostatin revealed several muscle specific transcription factor binding sites such as E-boxes and an MEF2 binding site in this region.

Keywords: Myostatin; promoter; Muscle specific transcription factor binding sites.

INTRODUCTION

Myostatin is a newly found member of the TGF-β superfamily and was first reported to be specifically expressed in skeletal muscle (McPherron *et al.*, 1997). Myostatin null mice show a two to threefold increase in skeletal muscle mass due to an increase in the number of muscle fibers (hyperplasia) and the size of the fibers (hypertrophy) (McPherron *et al.*, 1997). Subsequently, we (Kambadur *et al.*, 1997) and others (Grobet *et al.*, 1997; McPherron and Lee 1997) reported that the Belgian Blue and Piedmontese breeds of cattle, which are characterized by heavy muscling, have mutations in the myostatin coding sequence. Hence myostatin is considered a negative regulator of muscle growth.

Earlier studies have indicated that myostatin gene expression appears to be transcriptionally regulated during development (Kambadur *et al.*, 1997; McPherron *et al.*, 1997). Initially myostatin gene expression is detected in myogenic precursor cells of the myotome compartment of developing somites and the expression is continued in adult axial and paraxial muscles (McPherron *et al.*, 1997). Different axial and paraxial muscles have been shown to express different levels of myostatin (Kambadur *et al.*, 1997). Although the initial reports described the expression of myostatin gene exclusive to skeletal muscle, more recent publications have shown that myostatin mRNA or protein is detected in other tissues. A report using myostatin specific antibodies indicates that myostatin protein is present in cardiomyocytes and purkinje fibers of heart (Sharma *et al.*, 1999) while Ji *et al.*, (1998) detected myostatin mRNA expression in the mammary gland. Also myostatin is present in human skeletal muscle, and its expression is increased in the muscles of HIV-infected men with muscle wasting compared to that in normal men (Gonzalez-Cadavid *et al.*, 1998). Recently (Wehling *et al.*, 2000) have reported higher levels of myostatin mRNA and protein during muscle unloading and a decrease during reloading in fully differentiated muscle. Furthermore higher amounts of myostatin mRNA and protein are observed in fast-twitch muscle than in slow twitch muscle and hence relating myostatin to fiber type specific functions (Carlson *et al.*, 1999). Hence myostatin gene expression appears to be transcriptionally regulated both in pathophysiological conditions and during myogenesis. Hence to gain a better understanding of the regulation of myostatin gene expression, we cloned the promoter for bovine myostatin and analysed the regions within the promoter that activate myostatin gene expression.

EXPERIMENTAL PROCEDURES

Cloning of Myostatin Gene Promoter

Two kilobases of the genomic sequences immediately upstream of the translation start site of myostatin were obtained by inverse PCR (modified protocol of (Pang and Knecht, 1997)).

Sequence analysis

Sequence analysis for transcription factor binding sites was done using MatInspector program (Quandt *et al.*, 1995).

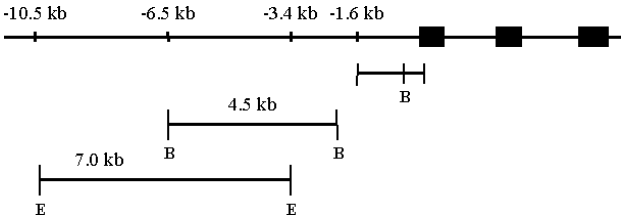
RESULTS

Isolation of bovine myostatin upstream clones

A genomic region of ~1.6 kb which is immediately 5' to the translation start codon ATG of the bovine myostatin gene was amplified from bovine genomic DNA using Partial Inverse Polymerase Chain Reaction. The PCR amplified fragment was cloned into TA cloning vector and sequenced. Using 0.5 kb of the genomic DNA that is 5' most of this 1.6 kb genomic DNA as a probe, a bovine lambda genomic library was screened to isolate a further ~8.4 kb of myostatin upstream region. Various overlapping restriction fragments of the genomic DNA from the lambda clones were sub-cloned into pBluescript vector and analyzed by restriction analysis and sequencing. The transcription start site of bovine myostatin gene has been indicated in the Fig.1B. +1 is assigned to the first transcribed nucleotide.

*Contributed equally to the manuscript.

Figure 1. **Nucleotide sequence of the 5' region of the bovine myostatin gene.** *A*, Restriction fragment map of 10 kb myostatin upstream sequences. Fragments of bovine myostatin genomic clones were subcloned into plasmids at convenient restriction sites (E- EcoRI; B- BglII) and sequenced. *B*, Of the 10-kb sequence of the bovine myostatin, 1600 bp is shown here. The transcription start site has been indicated by a bent arrow. Sequence analysis using MatInspector identified several sequences consistent with previously identified muscle-specific transcription factor-binding sites and other nuclear factor- binding sites. Known TATA boxes are shown in bold; CAAT box is italicised; AP-1 sites are underlined and marked; E-boxes are bold and underlined with bold line; MEF2 binding sequence is underlined with a broken line; GATA boxes are underlined.



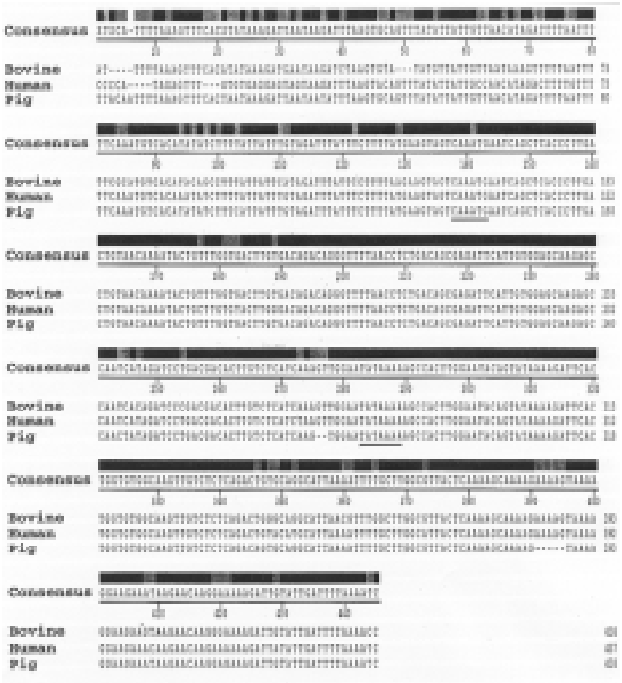
Transcription factor binding sites in myostatin upstream region

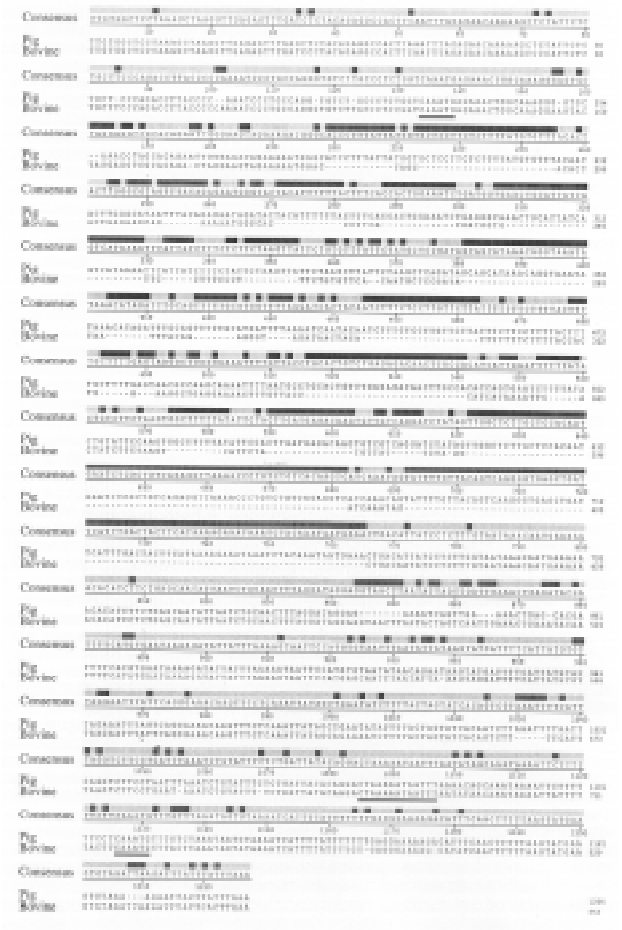
DNA sequence of the 10 kb of upstream region was analyzed by Lasergene and MatInspector software for restriction analysis and nuclear factor binding elements. DNA sequence of ~ 1.6 kb with various nuclear factor binding sites which is relevant to this communication is shown in Fig 1B. Analogous to a typical mammalian basal promoter, we have found one CAAT box (-73 to -69) and two different TATA boxes within 400 bp from the transcriptional start site. MatInspector analysis revealed that there are several different putative binding sites for transcription factors located in myostatin upstream sequences (Fig 1B). These include both muscle specific transcription factor binding sites and other gene specific nuclear factor binding sites. Among the muscle specific transcription factors MEF2 binding site is located within 500 bp of the enhancer sequences while the four E- boxes are located at -175, -410, -1034 and –1176 nucleotides. Furthermore, several sequences consistent with binding sites for AP1, GATA, GATA1, GATA2, GATA3 are also present.

Conservation of myostatin upstream sequences during evolution.

The GenBank data base was searched for any matches to bovine myostatin upstream sequences. An entry containing pig myostatin upstream sequences (AJ133580) was recovered. Recently 440 bp of human myostatin upstream sequences have been reported by (Ferrell *et al.*, 1999). Hence we aligned 440 bp of myostatin upstream sequences from human, cattle and pig using Megalign software. Comparison of these sequences revealed that there is a 86% conservation between human and bovine; 89% conservation between bovine and porcine myostatin upstream sequences. However, the similarity between porcine and human myostatin upstream sequences was 88%. In the 440 bp of upstream sequences the TATA box and the E-box are conserved in all three species. Beyond –440, we could compare only bovine and porcine sequences since human sequences were not available and we found a relatively weak homology (56%) in this region. Nevertheless E-Boxes and MEF-2 binding sequences were found to be conserved in both the species within this region. Since the comparisons were constrained by the "cluster homology", several gaps had to be introduced in the porcine upstream sequences for best homology alignment (Fig 2B in here).

Figure 2. Myostatin upstream sequences are well conserved during evolution. *A*, Alignment of 440 bp of myostatin upstream sequences from human, cattle and pig. Non-conserved nucleotides are indicated by solid bars. The consensus nucleotide sequence is shown at the top. The TATA box, and the E-box (underlined) are conserved in three species. *B*, Comparison of bovine and pig further upstream sequences (beyond 440). The consensus nucleotides sequence is shown at the top. Gaps in the porcine sequences have been introduced for the best homology alignment. The E-boxes and MEF2 binding sites (underlined) are conserved between the two species.





DISCUSSION

Myostatin, a member of TGF beta superfamily, is a Growth and Differentiation Factor (GDF-8) that is predominately expressed in skeletal muscle. Myostatin expression is first detected in the dermomyotome compartment of somites during embryonic myogenesis in day 10.5 mouse embryo (McPherron *et al.,* 1997). The expression then continues into adult muscle fiber. Expression analysis performed in bovine and porcine tissue indicated that myostatin expression is transcriptionally regulated during development (Ji *et al.,* 1998). Furthermore it is also reported that different axial and paraxial muscles express different amounts of myostatin (Kambadur *et al.,* 1997). Hence to understand the molecular basis of tissue and stage specific expression of myostatin we have cloned and characterized 10 kb of the bovine myostatin promoter and enhancer region.

Basal promoter and muscle specific transcription factor binding sites of myostatin upstream sequences are well conserved during evolution

A Genbank search with the 1.6 kb bovine myostatin enhancer region identified an entry of the porcine myostatin upstream sequence that has a high degree of homology to bovine myostatin genomic DNA. Recently Ferrell *et al.*, (1999) have published ~440 bp of human myostatin upstream sequences. Thus all three mammalian promoter sequences (up to 440 bp) were aligned to evaluate the structural conservation of myostatin promoter sequences (Fig 2A) during evolution. The results indicate that there is an 86% conservation between human and bovine, 89% conservation between bovine and pig and 88% conservation between pig and human. Since the further (1.2 kb) upstream sequences were available only for cattle and pig, we compared the conservation of myostatin enhancer sequences between these two species only. Although there is a significant homology in the basal promoter region of human, bovine and porcine myostatin, there is a relatively weak homology in the further upstream sequences in pig and cattle. However the MRF binding sequence elements E-boxes and MEF2 binding sequence are well conserved in both pig and cattle. Thus the observed similarity in developmental expression pattern of myostatin gene in cattle and pig could be due to conserved muscle specific cis-elements in the myostatin enhancer. In summary, we show that the myostatin enhancer sequences contain several muscle specific and other cis-elements. Structurally and functionally myostatin promoter appears to be well conserved during evolution.

ACKNOWLEDGEMENTS

We are grateful to Brett Langley for his critical help in graphics and reading of the manuscript. Thanks are also due to Dr Allan Crawford for his continued support and critically reading the manuscript. We are indebted to the Foundation of Research and Technology (New Zealand) for financial support.

REFERENCES

Carlson, C.J., F.W. Booth, and S.E. Gordon. 1999. Skeletal muscle myostatin mRNA expression is fiber-type specific and increases

during hindlimb unloading. *American Journal of Physiology* **277**: R601-6.

Ferrell, R.E., V. Conte, E.C. Lawrence, S.M. Roth, J.M. Hagberg, and B.F. Hurley. 1999. Frequent sequence variation in the human myostatin (GDF8) gene as a marker for analysis of muscle-related phenotypes. *Genomics* **62**: 203-7.

Gonzalez-Cadavid, N.F., W.E. Taylor, K. Yarasheski, I. Sinha-Hikim, K. Ma, S. Ezzat, R. Shen, R. Lalani, S. Asa, M. Mamita, G. Nair, S. Arver, and S. Bhasin. 1998. Organization of the human myostatin gene and expression in healthy men and HIV-infected men with muscle wasting. *Proceedings of National Academy of Sciences U S A* **95**: 14938-43.

Grobet, L., L.J. Martin, D. Poncelet, D. Pirottin, B. Brouwers, J. Riquet, A. Schoeberlein, S. Dunner, F. Menissier, J. Massabanda, R. Fries, R. Hanset, and M. Georges. 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle [see comments]. *Nature Genetics* **17**: 71-4.

Ji, S., R.L. Losinski, S.G. Cornelius, G.R. Frank, G.M. Willis, D.E. Gerrard, F.F. Depreux, and M.E. Spurlock. 1998. Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *American Journal of Physiology* **275**: R1265-73.

Kambadur, R., M. Sharma, T.P. Smith, and J.J. Bass. 1997. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. *Genome Research* **7**: 910-6.

McPherron, A.C., A.M. Lawler, and S.J. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**: 83-90.

McPherron, A.C. and S.J. Lee. 1997. Double muscling in cattle due to mutations in the myostatin gene. *Proceedings of National Academy of Sciences U S A* **94**: 12457-61.

Pang, K.M. and D.A. Knecht. 1997. Partial inverse PCR: a technique for cloning flanking sequences. *Biotechniques* **22**: 1046-8.

Quandt, K., K. Frech, H. Karas, E. Wingender, and T. Werner. 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Research* **23:** 4878-84.

Sharma, M., R. Kambadur, K.G. Matthews, W.G. Somers, G.P. Devlin, J.V. Conaglen, P.J. Fowke, and J.J. Bass. 1999. Myostatin, a transforming growth factor-beta superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. *Journal of Cellular Physiology* l**180**: 1-9.

Wehling, M., B. Cai, and J.G. Tidball. 2000. Modulation of myostatin expression during modified muscle use. *FASEB JOURNAL* **14**: 103-10.