

## TEMPORAL-SPATIAL MYOSTATIN GENE EXPRESSION IN CHICKEN EMBRYO

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### INTRODUCTION

Myostatin is a key negative regulator of muscle growth that belongs to the transforming growth factor- $\beta$  super family (McPherron *et al.*, 1997). In mouse, deletion of this gene by homologous recombination results in a 2-3 fold increase in muscle mass (McPherron *et al.*, 1997), while in cattle (Grobet *et al.*, 1997; McPherron and Lee, 1997 ; Grobet *et al.*, 1998) and mouse (Szabó *et al.*, 1998) selected for muscle deposition, natural mutations in this gene are associated with muscle hypertrophy and hyperplasia. Moreover, reduced muscle use (Wehling *et al.*, 2000) or muscle-wasting conditions in patients with HIV infection (Gonzales-Cadavid *et al.*, 1998) increase myostatin gene expression. Despite its importance for muscle growth and maintenance, little is known about the ontogeny and spatial distribution of myostatin.

### MATERIAL AND METHODS

**Embryos and tissue samples.** Chicken eggs from the AgRoss line (Agroceres) were incubated at 38-39°C until the embryos reached the desired developmental stage according to Hamburger and Hamilton (1951). Hepatic and skeletal muscle tissues were collected from a 14-day-old chicken and immediately frozen in liquid nitrogen.

**RT-PCR analyses.** Embryos and tissues were dissolved in Trizol LS<sup>®</sup> (Gibco/BRL) according to the methodology described by Chomczynski and Sacchi (1987) for total RNA extraction. The Superscript II RT (Gibco/BRL) kit with oligo dT was used for cDNA synthesis. Myostatin was amplified with specific primers forward (<sup>5</sup>AGTAGCGATGGCTCTTTGGA<sup>3</sup>) and reverse (<sup>5</sup>CTGGGAATGTGACAGCAAGA<sup>3</sup>) located at nucleotide positions 301 (exon 1) and 727 (exon 2) of the chicken myostatin gene (gi|2623569). A deletion mutant of the 427 bp amplicon was constructed by PCR. Using this approach a competitor fragment of 380 bp that maintains the same primer binding sites as the native myostatin gene was created, cloned and used in the semi-quantitative RT-PCR assay.

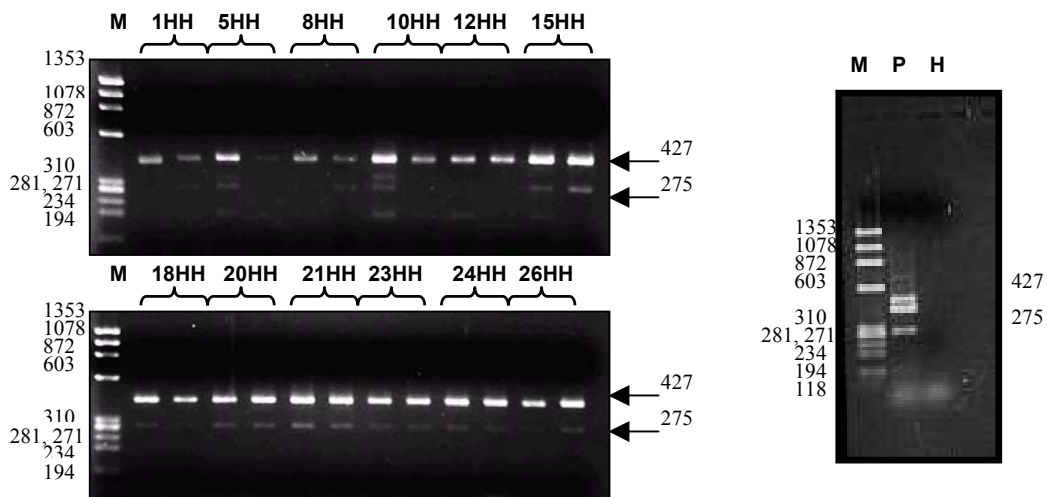
**Sequencing.** The 275 bp and 427 bp myostatin amplicons were gel purified (Sephaglas Band Prep Kit, Amersham Pharmacia Biotech) and sequenced (DYEnamic<sup>™</sup> terminator cycle sequencing kit, Amersham Pharmacia Biotech).

**Whole-mount in situ detection of myostatin.** The procedure developed by Coutinho *et al.* (1992) was used to perform the whole embryo *in situ* hybridizations. Briefly, the 427 bp amplicon was cloned in pGEM-4Z (Promega), digested with *Kpn*I to linearize the plasmid and a digoxigenin-labelled anti-sense RNA probe was synthesized with T7RNA polymerase using the DIG RNA Labelling Kit (SP6/T7) (Boehringer Mannheim). Myostatin transcript signals

were detected by a colorimetric reaction as described by the manufacturer.

## RESULTS AND DISCUSSION

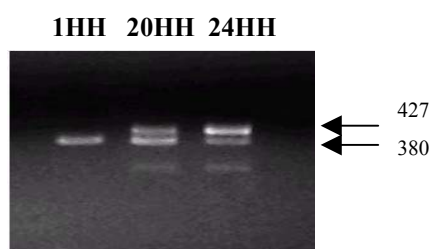
**Temporal myostatin gene expression.** Myostatin transcripts were detected by reverse transcriptase polymerase chain reaction (RT-PCR) in embryos from stages 1HH (immediately after oviposition), 5HH, 8HH, 10HH, 12HH, 15HH, 18HH, 20HH, 21HH, 23HH, 24HH and 26HH (6 days of incubation) (Figure 1A). Analysis of pectoralis muscle of a 14-day-old chicken also revealed the presence of myostatin transcripts, while hepatic tissue of the same animal showed no signal (Figure 1B). In addition, it was interesting to observe the expected PCR product of 427 bp as well as another transcript 275 bp long. Sequencing analysis of both fragments showed that the larger one is a regular transcript while the smaller one is an alternative transcript of myostatin.



**Figure 1. RT-PCR analyses of myostatin gene expression in embryonic stages 1HH-26HH (A) and 14-day-old chicken tissue (B). 1HH-26HH, embryonic stages ; P, pectoralis muscle ; H, hepatic tissue ; M,  $\phi$  174/*Hae*III molecular weight marker. Arrows indicate normal (427 bp) and alternative (275 bp) myostatin transcripts**

Since RT-PCR offers no quantification of transcript abundance, a competitive RT-PCR approach was used to investigate if there was an induction of myostatin during early embryonic development. In this assay,  $10^{-5}$  fmoles of a deletion mutant of myostatin (380 bp) were included in the PCR reaction as a competitor molecule. As can be seen in Figure 2, at stage 1HH only the competitive DNA fragment was amplified, indicating that, at this stage, expression level is less than  $10^{-5}$  fmoles. At stage 20HH, the quantity of PCR products of the native and competitive fragments were amplified almost to the same level, showing that at this developmental phase the embryo has roughly  $10^{-5}$  fmoles myostatin transcripts. At stage 24HH, there were more PCR products of the native size, indicating that at this embryonic stage the embryo has more than  $10^{-5}$  fmoles of myostatin transcript. Our results differ from those

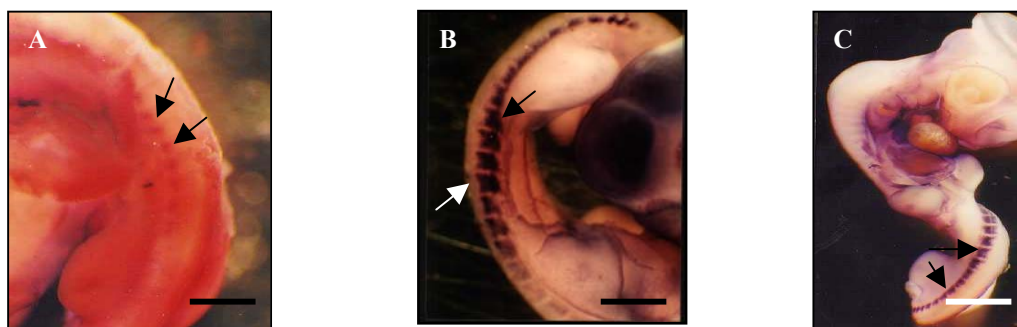
published by Kocamis *et al.* (1999). In their work, myostatin expression was higher in 1HH stage embryos than in stages 20HH and 24HH. However, they did not use an internal control in the PCR reaction, thus making quantification of RT-PCR less reliable.



**Figure 2. Competitive RT-PCR analysis of myostatin expression in embryonic stages 1HH, 20HH and 24HH. Arrows indicate native (427 bp) and competitive (380 bp) amplicons**

**Spatial myostatin gene expression.** Myostatin transcripts were localized in the embryos by whole mount *in situ* hybridization. In embryonic stage 20HH, a weak signal was observed in the ventral region of caudal somites (Figure 3A). Stage 24HH embryos presented higher myostatin expression levels. A more intense myostatin signal was detected in the somites located between the limb buds, with reduced expression in somites located more caudally or cranially (Figures 3B, C). It was interesting to observe that myostatin was not expressed in the whole somite, but only in the hypaxial domain of the myotome. In mouse embryos, McPherron *et al.* (1997) also observed myostatin expression in the somites. However, in their study there was no reduction in myostatin expression in somites located more cranially, as we observed. This difference could be explained because they determined the location of myostatin expression based on the presence of reporter enzyme beta-galactosidase, which has a long half-life and thus does not represent the transcript abundance of myostatin. McPherron *et al.* (1997) also detected expression of myostatin in a transverse section of a somite; however, their result was not reported in enough detail to verify that myostatin is present in the hypaxial region of the somite. The spatial localization of myostatin in the somite is in agreement with its function as a negative regulator of myogenesis, as proposed by McPherron *et al.* (1997). During somite development, cells from the dorsomedial dermomyotome proliferate, migrate beneath the dermomyotome, exit the cell cycle and terminally differentiate (Denetclaw *et al.*, 1997 ; Kahane *et al.*, 1998). Myostatin expression in the hypaxial myotomal region could be responsible for the inhibition of myoblast proliferation in this region.

**Alternative transcript of myostatin.** An interesting finding in our study was the presence of an extra band of 257 bp in the RT-PCR assays. Based on sequence data published at GenBank (gi|2623569) the expected size for myostatin amplification with the primers we designed was 427 bp. However, even when different primers were tested within the same region an extra band was observed (Figures 1 and 2). Sequence analysis of this fragment revealed it to be an alternative transcript of myostatin that has a deletion between nucleotides 373 and 517 of the chicken myostatin mRNA (gi|2623569).



**Figure 3. Whole-mount *in situ* hybridization analyses of myostatin gene expression in embryonic stages 20HH at 35x (A), 24HH at 35x (B) and 24HH at 10x magnification (C). Arrows indicate myostatin mRNA expression**

### CONCLUSION

Our results suggest that myostatin starts to play a role early in development, and its expression pattern in somites is in agreement with its proposed role as a negative regulator of muscle development.

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