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Communication

[*Comunicação*]

Temporal expression pattern of myostatin transcripts during chicken embryogenesis

[*Padrão de expressão temporal de transcritos de miostatina durante a embriogênese da galinha*]

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The myogenesis requires the precise temporal and spatial expression of multiple growth factors, which regulate proliferation and differentiation of myoblasts until the formation of the functional tissue. The identification of a new growth and differentiation factor named myostatin has provided new insights for the better understanding of the muscle mass deposition in vertebrates (McPherron and Lee, 1997). The main source of the synthesis and secretion of this growth factor is the skeletal muscle tissue and myostatin transcripts are specifically detected in the myotome during early embryogenesis (Amthor et al., 2002). Mice carrying a targeted disruption of this gene showed a drastic increase in skeletal muscle tissue, resulted by combination of muscle fiber hyperplasia and hypertrophy (McPherron et al., 1997). Additionally, mutation in the sequence of this gene has been associated with the double muscling phenotype in cattle, suggesting that myostatin gene participate actively of muscle mass deposition in these animals (Kambadur et al., 1997). Considering that the spatial expression of myostatin gene has been well characterized by whole-mount *in situ* hybridization assays (McPherron et al., 1997; Amthor et al., 2002), the objective of this report was to estimate the abundance of steady-state mRNA of myostatin gene in chicken embryos at different developmental stages by competitive RT-PCR analysis.

Contrary to *in situ* hybridization assays, the experimental procedure adopted in this study is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amount (Carding et al., 1992). In most cases, a qualitative study is not sufficient to deliver a satisfactory answer concerning the ontogeny of gene of interest. For this reason, quantitative proceedings have preferentially been done for the detection of variations in levels of specific RNA transcripts under different experimental conditions. So, these results provide important insights for the better understanding of the pattern of temporal expression of the myostatin gene during avian embryogenesis.

Fertilized Ross line chicken eggs were incubated in a humidified atmosphere at 37.5±0.3°C. Fifteen to twenty embryos per embryonic stage were collected and staged according to the method described by Hamburger and Hamilton (1951). The developmental stages were HH1, 5, 8, 10, 12, 15, 18, 20, 21, 23, 24 and 26 (sixth day of incubation). Embryos were immediately dissolved in TRIZOL reagent[1] for the total RNA extraction. The single strand cDNA synthesis was carried out from 1µg of total RNA by the reverse transcription reaction at 42°C for 50min in presence of 500ng oligo(dT) primer and 200

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units SuperScript II enzyme[1]. Amplification reactions were performed with 10% of total reverse transcription reaction volume in presence of 1 unit *Taq* DNA polymerase and 0.5µM specific primers as reported by Gabriel et al. (2003). The identity of amplified products was confirmed by sequencing reactions with the Big Dye Terminator kit[2] using an ABI PRISM 377 automated DNA sequencer.

The mRNA levels of myostatin gene were estimated using a modified experimental procedure of quantitative-competitive RT-PCR, as described in detail by Alvares et al. (2003) and Gabriel et al. (2003). The abundance of these transcripts was quantified by direct comparison with standard curves constructed from increasing and known amounts of endogenous cDNA in the presence of the constant amount of an exogenous competitor cDNA. To synthesize the endogenous cDNA fragments of myostatin gene, reverse transcription reaction was performed exactly as described above, since these fragments were identical in size and nucleotide sequence to the cDNA obtained by RT-PCR of embryos. The competitor cDNA fragment was generated by mutational PCR with the antisense deletion primers. Although competitor cDNA fragment present a smaller size, the same primer pairs can be employed to amplify both endogenous and competitor cDNAs. Since the region to which primers bind are common and competitor and endogenous DNA fragments equally compete for the limiting reaction components (nucleotides, primers, and enzyme). A known amount of competitor was directly added to the 1X PCR master mixture to assure that equal amount was present in all samples. Sense primers were 5'-labeled with fluorescein and aliquots of amplified products were submitted to electrophoresis in an automated Laser Fluorescent DNA Sequencer[3]. Following electrophoresis, peaks derived from endogenous and competitor templates were integrated. The logarithmic proportion of endogenous and competitor cDNA peak areas and the logarithm initial concentration of endogenous cDNA were employed to establish a standard curve.
The mathematical equation obtained by linear regression of standard curves was used to determine variations in the mRNA abundance of myostatin gene in embryonic cDNA samples based on the ratio of endogenous to competitor products in individual PCRs. The β-actin expression levels were determined in order to normalize the mRNA abundance of gene of interest. Data corresponding to levels of myostatin transcripts were statistically analyzed by regression analysis using the GLM procedure of SAS® (User's…, 1999).

The abundance of myostatin mRNA was estimated in chicken embryos at distinct developmental stages by competitive RT-PCR analysis. As observed in Fig. 1, basal levels of myostatin mRNA were detected in embryos between stages HH1 and HH15. These results differ from those reported by Amthor et al. (2002) that determined, by whole-mount *in situ* hybridization assays, the onset of myostatin expression at stage 13. These apparently contradictory effects could be explained since the employed RT-PCR analysis these produce highly accurate quantitative results in comparison to those obtained by *in situ* hybridization assays. The expression of this transcript was strongly upregulated at stage HH24, followed by reducing in these levels from stage HH26, as observed in Fig. 1. Accentuated levels of myostatin mRNA detected at stage HH24 reinforce those findings of Amthor et al. (2002) that observed an enhancement in myostatin expression during a considerable time after the formation of the sclerotome, dermomyotome, and myotome somite development in chicken embryos. It is yet characterized that myostatin is also activated during skeletal muscle formation in bovine embryos from day 16 of gestation until adulthood, when primary myoblasts initiate the fusion and differentiation in myofibers and that its expression in adult muscles is less significant than during fetal development (Oldham et al., 1999). Such preliminary findings describe relevant information concerning the timing and abundance of myostatin gene during *in ovo* development. Since the number of skeletal

[1] Invitrogen Corporation. Carlsbad. USA.
[2] Applied Biosystems Corporation, Foster City, USA.
[3] A.L.F.™, Pharmacia LKB Biotechnology, Carlsbad, USA.

muscle fibers in adult birds depends on the balance between proliferation and differentiation of precursor cells during the prenatal life (Fredette and Landmesser, 1991) and based on the fact that myostatin is a potent negative regulator of myogenesis, further studies should be done in order to generate new approaches for manipulation of the myostatin gene activity during early development. Thus, it will provoke the late activation of this gene during embryogenesis, probably resulting in adult birds with increased skeletal muscle mass.

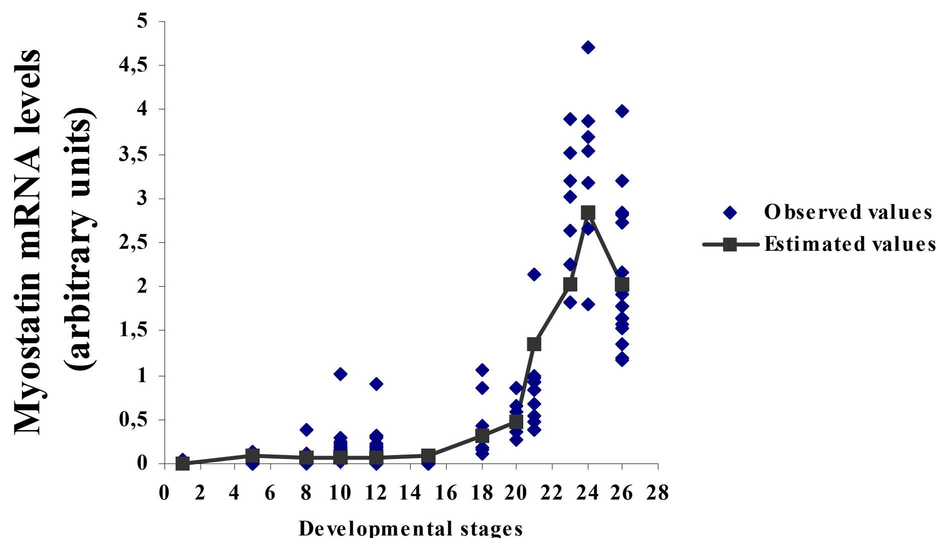

Figure 1. Pattern of temporal expression of myostatin transcripts in chicken embryos during *in ovo* development. Estimated values (presented as arbitrary units) corresponding to levels of myostatin mRNA at analyzed developmental stages were HH1 – 0.009, HH5 – 0.037, HH8 – 0.048, HH10 – 0.133, HH12 – 0.149, HH15 – 0.188, HH18 – 0.351, HH20 – 0.48, HH21 – 1.42, HH23 – 2.015, HH24 – 2.797 and HH26 – 2.097.

Keywords: chicken embryo, myostatin, temporal expression, mRNA abundance, competitive RT-PCR

RESUMO

No presente estudo, estimou-se a abundância dos transcritos da miostatina foi estimada durante a embriogênese de galinha por análises de RT-PCR competitiva. Níveis basais de mRNA desse gene foram detectados até o estádio HH15, enquanto acúmulos significativos nesses níveis foram observados apenas no estádio HH24, seguido por redução na abundância desses transcritos a partir do estádio HH26. Tais descobertas preliminares proporcionam informações relevantes sobre a ativação do fator de crescimento miostatina durante o desenvolvimento in ovo de aves.

Palavras-chave: embrião de galinha, miostatina, expressão temporal, abundância de mRNA, RT-PCR competitiva

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