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Methylation status and chromatin structure of the myostatin gene promoter region in the sea perch *Lateolabrax japonicus* (Perciformes)

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ABSTRACT. Myostatin is a negative regulator of the growth and development of skeletal muscle mass. In fish, myostatin is expressed in several organs in addition to skeletal muscle. To understand the mechanisms regulating myostatin gene expression in the sea perch, *Lateolabrax japonicus*, we examined the methylation status of the myostatin gene promoter region in several tissues (liver, eye, kidney, brain, and heart) isolated from adult specimens. The frequency of methylated cytosines was very low in all tissues, regardless of the level of myostatin expression, suggesting that DNA methylation is not involved in the tissue-specific regulation of myostatin expression. Southern blot analysis of genomic DNA obtained from micrococcal nuclease-treated nuclei showed that chromatin digestion occurs in tissues where the myostatin gene is actively transcribed and that the myostatin gene is protected from micrococcal nuclease in tissues where myostatin is not expressed. The chromatin structure in the myostatin gene region

appears to regulate its expression without DNA methylation.

Key words: Chromatin; DNA methylation; Fish; Micrococcal nuclease; Myostatin

INTRODUCTION

Myostatin, also known as growth differentiation factor 8, is a member of the transforming growth factor (TGF-β) superfamily, which negatively regulates the growth and development of skeletal muscle mass (Lee, 2004). In mammals, myostatin is expressed almost exclusively in skeletal muscle. On the other hand, myostatin appears to be more ubiquitously expressed in fish, suggesting that its contributions to the growth and development of fish are more diverse (Radaelli et al., 2003; Helterline et al., 2007). Consistent with this idea, teleost fishes possess at least two myostatin genes, which are differentially expressed in both muscle and non-muscle tissues, whereas myostatin is encoded by a single gene in mammals (Biga et al., 2005; Kerr et al., 2005; Østbye et al., 2007). Myostatin cDNA has been characterized in a number of commercially important fishes, including rainbow trout (Rescan et al., 2001), Atlantic salmon (Østbye et al., 2001), Mozambique tilapia (Rodgers and Weber, 2001), white bass and striped bass (Rodgers et al., 2001), gilthead sea bream (Maccatrozzo et al., 2001a), catfish (Gregory et al., 2004) and grouper (Ko et al., 2007), among others; and expression of myostatin mRNA has been detected in a variety of tissues and at different stages of development in some fish species (Rescan et al., 2001; Østbye et al., 2001; Rodgers and Weber, 2001; Maccatrozzo et al., 2001a,b; Kocabas et al., 2002; Roberts and Goetz, 2003; Vianello et al., 2003; Xu et al., 2003; Gregory et al., 2004; Ko et al., 2007). Interestingly, Acosta et al. (2005) reported that silencing the myostatin gene produces a giant phenotype in zebrafish, while Lee et al. (2009) showed that the suppression of myostatin with vector-based RNA interference causes a double-muscle effect resulting from hypertrophy in transgenic zebrafish, and Sawatari et al. (2010) recently showed that the introduction of a dominant-negative form of myostatin into the medaka fish *Oryzias latipes* leads to a doubling of the number of its muscle fibers.

Several potential *cis*-acting elements needed for transcriptional activity have been identified in the promoter region of myostatin gene in the gilthead sea bream *Sparus aurata* (Funkenstein et al., 2009). In addition, Ye et al. (2007) identified *cis*-acting elements in the 5' flanking region of myostatin, which may contribute to the tissue specificity of the transcriptional activity of myostatin gene in the sea perch *Lateolabrax japonicus*. However, gene expression also reflects the status of the chromatin (euchromatin or heterochromatin) in the region where the gene is situated. The status of chromatin in particular gene regions is dynamically modulated to control gene expression and other fundamental cellular processes, such as cell proliferation and differentiation (Li, 2002; Felsenfeld and Groudine, 2003; Jaenisch and Bird, 2003).

DNA methylation at cytosine residues is involved in epigenetic regulation that is closely related to heterochromatinization, which suppresses gene expression (Goll and Bestor, 2005; Klose and Bird, 2006; Reik, 2007; Kuroda et al., 2009; Zhang et al., 2010). Furthermore, the myostatin gene promoter regions in a group of closely related fish (belonging to the suborder Percoidei) are highly conserved and contain several CpG dinucleotides within the conserved regions, which could serve as targets for DNA methylation (Figure 1). To better understand the mechanisms regulating myostatin gene expression in fish, we analyzed

the chromatin structure using micrococcal nuclease (MNase) as an enzymatic probe. We also assessed the methylation status of the promoter region of myostatin genes in various tissues from *L. japonicus*.

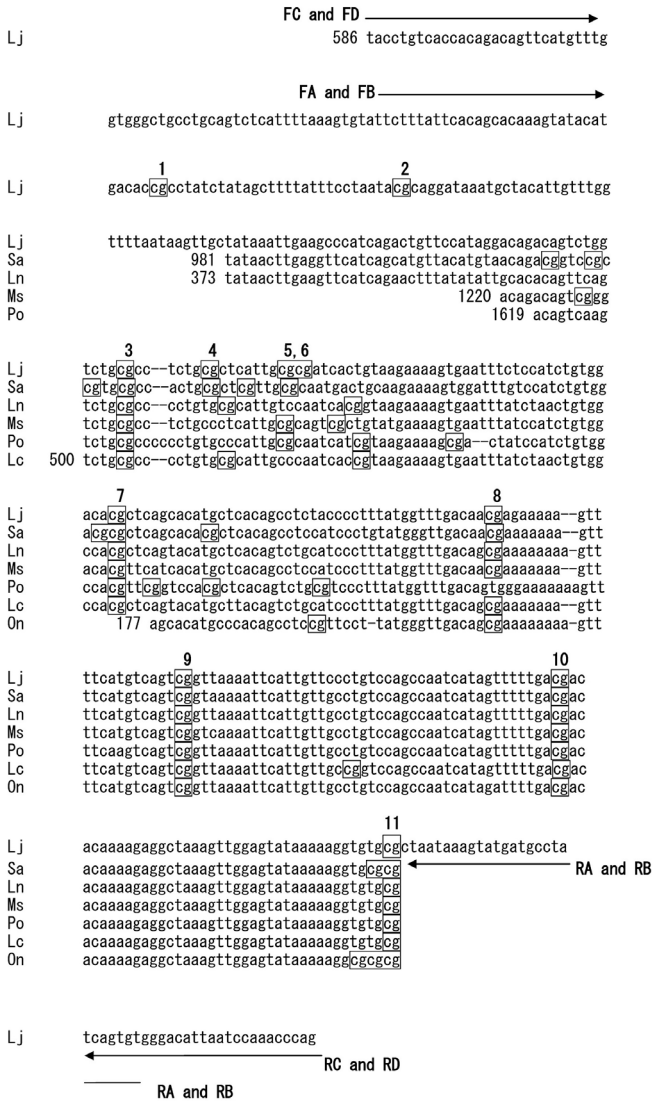

Figure 1. Conserved sequences in the myostatin gene 5' flanking region. Homologous sequences were retrieved from GenBank databases and aligned to identify conserved regions. Sequences are numbered according to the original sequence data. Lj = *Lateolabrax japonicus* (Genbank accession No. AY965685); Sa = *Sparus aurata* (EU881511); Ln = *Lates niloticus* (EF681885); Ms = *Micropterus salmoides* (EF071854); Po = *Paralichthys olivaceus* (DQ997779); Lc = *Lates calcarifer* (EF672685); On = *Oreochromis niloticus* (FJ972683). CpG sites are boxed, and those analyzed by bisulfite sequencing are indicated above the lines (from numbers 1 to 11). Primer sites for methylation analysis are shown by arrows with bold face characters. Primer names (FA, FB, FC, FD, RA, RB, RC, and RD) refer to Table 1.

MATERIAL AND METHODS

DNA primers

The oligonucleotides used as PCR primers are listed in Table 1. Primers were designed to amplify bisulfite-treated genomic DNA, or were used to amplify the probe for Southern blot hybridization.

Table 1. Oligonucleotides used as PCR primers.

Primer name	Sequence (5'- 3')	Position in AY965685
FA	TTCTTTATTCACAACACAAAATATACAT	647-674
FB	TTTTTTATTTATAGTATAAAGTATATAT	647-674
RA	ATATTGATAGGTATTATATTTTATTAG	1023-1049
RB	ACACTAATAAACATCATACTTTATTAA	1023-1049
FC	TACCTATCACCACAAACAATTCATATTT	586-613
FD	TATTTGTTATTATAGATAGTTTATGTTT	586-613
RC	TTGGGTTTGGATTAATGTTTTATATTGA	1043-1060
RD	CTAAATTTAAATTAATATCCCACACTAA	1043-1060
LjaMSTNP2F	CCTATCTATAGCTTTTATTTCCTAATA	682-708
LjaMSTNR1	CACACCTTTTTATACTCCAACTTTA	986-1010

Names, sequences and targeted genomic regions are shown.

Nucleus preparation and MNase treatment

Fish samples (40 to 60 cm standard length) were purchased from a fish market at Izumisano Port, Osaka, Japan, and stored at -80°C before use. Individual fish were dissected after thawing under running tap water, and selected tissues (liver, eye, kidney, brain, and heart) were isolated. Each tissue was homogenized using a Teflon homogenizer in TES buffer (10 mM Tris-HCl, 140 mM NaCl, 25 mM EDTA, pH 7.8), after which cells were harvested by centrifugation at 3000 *g* for 10 min. The cell pellets were then resuspended in the same buffer and centrifuged again. The resultant washed cells were suspended in 10 volumes (relative to the packed-cell volume) of hypotonic buffer (10 mM Tris-HCl, 15 mM KCl, 0.5 mM $MgCl_2$, pH 7.8) and left on ice for 10 min. Once swollen, the cells were centrifuged at 1000 *g*, and the cell pellet was resuspended in 5 volumes of hypotonic buffer. The cells were then homogenized using a Dounce glass homogenizer (B-pestle), and the nuclei were harvested by centrifugation and resuspended in the hypotonic buffer. MNase (Sigma-Aldrich) was added to the nucleus suspensions (40 µg/mL), after which the mixtures were incubated at 37°C for 15, 30 or 60 min, and one-third of the original reaction volumes was collected at each time. The nuclease reactions were terminated by adding aliquots (1/20 of the reaction volume) of 0.5 M EDTA, after which the DNA was isolated from each reaction mixture.

Isolation of genomic DNA from MNase-treated cell nuclei

An equal volume of TES buffer containing 1% SDS and 0.5 mg/mL Proteinase K (Invitrogen Life Technologies) was added to the MNase-treated nuclei mentioned above, after which the reaction mixtures were incubated for 30 min at 50°C. The DNA was then extracted using phenol, precipitated with ethanol and dissolved in TE buffer (10 mM Tris HCl, 1 mM

EDTA, pH 7.8). Total DNA isolated from the MNase-treated nuclei was digested using the restriction endonuclease *Taq*I (Takara-Bio), separated on 1.2% agarose gel electrophoresis and subjected to Southern blot hybridization.

Southern blot hybridization

The separated DNA on the agarose gels was transferred to nylon membranes (Hybond™-N+, Amersham Biosciences) using the capillary blot procedure (Southern, 1975), and was probed for the promoter region of the myostatin gene from *L. japonicus*. The probe was then labeled using a PCR DIG Probe Synthesis kit (Roche) with a pair of DNA primers, LjaMSTNP2F and LjaMSTNR1.

Bisulfite treatment of genomic DNA and sequencing

Fish were dissected, and tissue samples from brain, kidney, spleen, liver, heart, eye, muscle, intestine, and gills were homogenized in TES buffer, after which the total genomic DNA was isolated as previously described (Sambrook and Russell, 2001). Samples of genomic DNA were then digested with *Eco*RI (New England Biolabs), and the resultant DNA fragments (500 ng) were subjected to bisulfite modification using an EZ DNA Methylation-Gold kit (Zymo Research). Bisulfite treatment converts cytosines to uracils, but 5-methylcytocines remain intact. Thus, the combination of bisulfite treatment and following PCR enabled us to detect methylated cytosines occurring in genomic DNA (Clark et al., 1994). A set of primers (FC and RC) was used for PCR to amplify the antisense strand of the bisulfite-treated DNA, while another set of primers (FD and RD) was used for PCR to amplify the sense strand of bisulfite-treated DNA. Each PCR product was then subjected to a second PCR with a set of nested primers: FA and RA for antisense strand amplification and FB and RB for sense strand amplification. The PCR was performed using the Ampli Taq Gold 2X PCR Master Mix (Applied Biosystems) with a GeneAmp PCR System 9700 (Applied Biosystems). The amplification protocol entailed initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 2 min, and a final elongation at 72°C for 7 min. The resultant PCR products were cloned into the *Hin*cII site of pUC118 vector. DNA sequencing was performed using the Big Dye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems) and ABI3730 Sequencer (Applied Biosystems).

RESULTS

Bisulfite modification of genomic DNA to evaluate the methylation status

We initially determined the nucleotide sequences of the PCR products obtained with bisulfite-treated DNA (5-18 clones each from the respective samples) (Tables 2 and 3). The efficiency of the bisulfite modification (conversion of C to T) was estimated by counting the numbers of cytosines remaining at CpNs ($N \neq G$), and the conversion frequency was found to be 99% or higher. The number of cytosine residues remaining at CpGs appeared to be above the background level in the sense strands from eye and heart (methylation frequency: 6.36 and 5.68%, respectively), but were at the background level in the complementary strands from these tissues. This may indicate that the methylation frequency was within the error range of

the random sampling. In any case, the frequency of methylated cytosine was very low in the tissues examined, regardless of the level of myostatin gene expression. This runs counter to the suggestion drawn from the results of Ye at al. (2007), and may mean that CpG methylation is not involved in regulating myostatin gene expression in *L. japonicus*.

Table 2. Summary of bisulfite sequencing: relative percentage of unconverted bases after bisulfite treatment.

Tissues	Sense strand		Antisense strand	
	C at CpG	C at CpN (N ≠ G)	G at CpG	G at NpG (N ≠ C)
Intestine	0.00	0.32	4.55	0.68
Brain	1.52	0.66	1.52	0.61
Spleen	2.10	0.49	1.82	0.36
Liver	1.52	0.53	1.52	0.91
Eye	6.36	0.48	0.00	1.12
Kidney	0.76	0.66	1.01	1.01
Gill	3.50	0.37	1.52	0.30
Muscle	1.52	0.32	1.07	0.75
Heart	5.68	0.40	0.91	0.55

Table 3. Summary of bisulfite sequencing: numbers of unconverted cytosines at each CpG site.

CpG position		Intestine	Brain	Spleen	Liver	Eyes	Kidney	Gill	Muscle	Heart
1	Sense strand	0/5	1/12	0/13	1/18	4/10	0/12	2/13	1/12	1/8
	Antisense strand	0/8	0/6	0/10	1/18	0/13	0/9	0/6	0/17	0/10
2	Sense strand	0/5	0/12	0/13	0/18	0/10	1/12	0/13	0/12	0/8
	Antisense strand	0/8	0/6	0/10	0/18	0/13	0/9	0/6	0/17	0/10
3	Sense strand	0/5	0/12	0/13	0/18	2/10	0/12	1/13	0/12	2/8
	Antisense strand	1/8	0/6	0/10	0/18	0/13	1/9	0/6	1/17	0/10
4	Sense strand	0/5	0/12	2/13	0/18	1/10	0/12	2/13	0/12	1/8
	Antisense strand	0/8	0/6	1/10	0/18	0/13	0/9	0/6	0/17	0/10
5	Sense strand	0/5	0/12	0/13	1/18	0/10	0/12	0/13	0/12	0/8
	Antisense strand	1/8	1/6	0/10	0/18	0/13	0/9	0/6	1/17	0/10
6	Sense strand	0/5	0/12	0/13	1/18	0/10	0/12	0/13	0/12	0/8
	Antisense strand	0/8	0/6	0/10	0/18	0/13	0/9	1/6	0/17	0/10
7	Sense strand	0/5	0/12	1/13	0/18	0/10	0/12	0/13	0/12	0/8
	Antisense strand	1/8	0/6	1/10	1/18	0/13	0/9	0/6	0/17	1/10
8	Sense strand	0/5	1/12	0/13	0/18	0/10	0/12	0/13	0/12	0/8
	Antisense strand	0/8	0/6	0/10	0/18	0/13	0/9	0/6	0/17	0/10
9	Sense strand	0/5	0/12	0/13	0/18	0/10	0/12	0/13	0/12	1/8
	Antisense strand	0/8	0/6	0/10	0/18	0/13	0/9	0/6	0/17	0/10
10	Sense strand	0/5	0/12	0/13	0/18	0/10	0/12	0/13	0/12	0/8
	Antisense strand	0/8	0/6	0/10	1/18	0/13	0/9	0/6	0/17	0/10
11	Sense strand	0/5	0/12	0/13	0/18	0/10	0/12	0/13	1/12	0/8
	Antisense strand	0/8	0/6	0/10	0/18	0/13	0/9	0/6	0/17	0/10
Myostatin expression*		+	++	-	-	++	--	+/-	+++	-

The ratios (number of methylated cytosines at CpG) / (number of sequences examined) are shown. The CpG positions are defined in Figure 1. *Expression of myostatin mRNA evaluated by RT-PCR (Ye et al., 2007).

Sensitivity of the myostatin gene promoter region to MNase

Southern blot hybridization analysis was carried out to evaluate the structure of the chromatin in the myostatin gene promoter region. DNA in the heterochromatin would be more resistant to MNase, which primarily degrades naked DNA. Myostatin gene regions in brain and eye, which express myostatin (Ye et al., 2007; indicated at the bottom of Table 3), were highly susceptible to MNase (Figure 2A, B). By contrast, the myostatin promoter region was

less susceptible in heart, and that in kidney and liver was even more resistant to MNase (Figure 2B-D), which is consistent with the lower level of myostatin gene expression in these organs. We examined several fish individuals for chromatin analyses, and the profiles of MNase digestion were reproducibly observed.

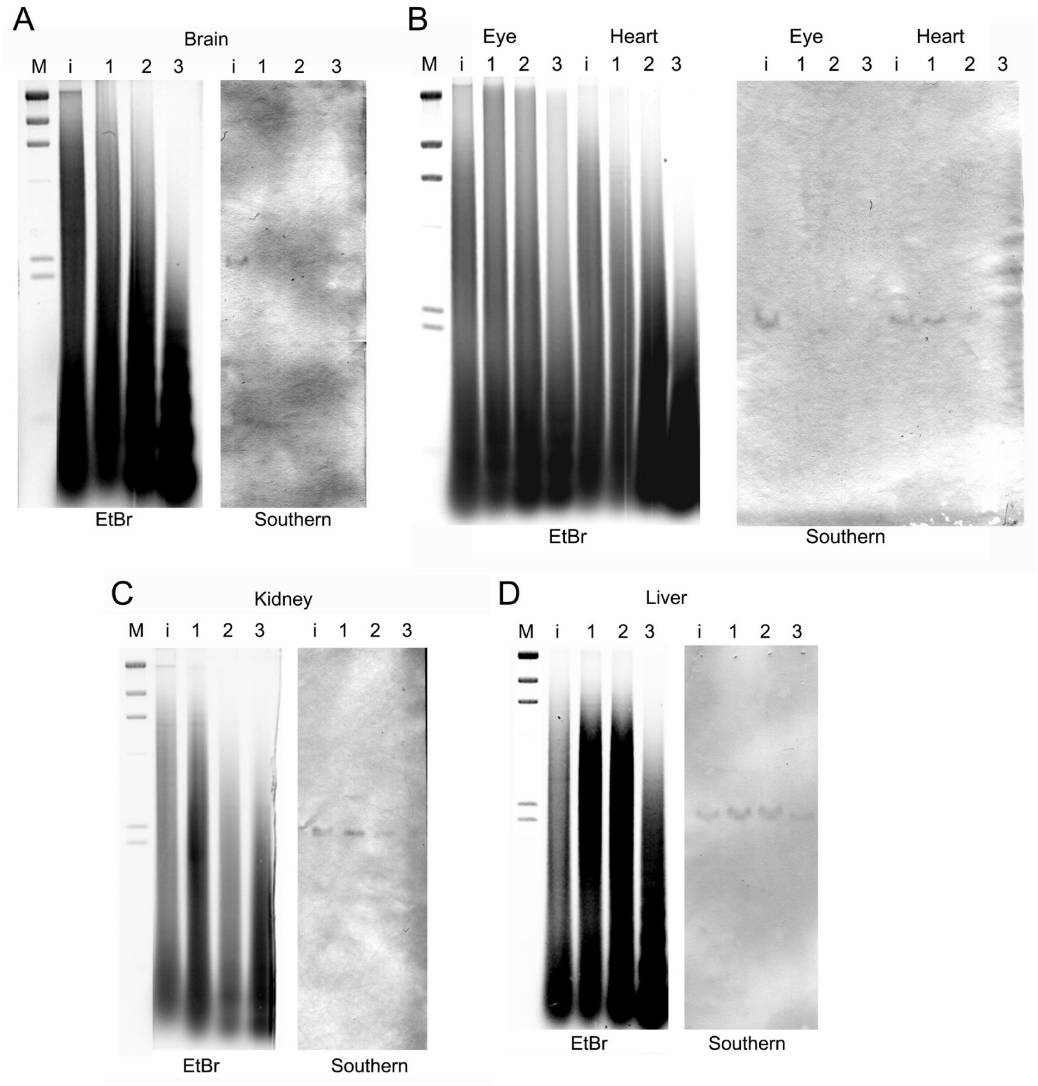

Figure 2. Southern blot analysis of *Taq*I-digested genomic DNA isolated from MNase-treated cell nuclei. Southern blots were probed with digoxigenin-labeled DNA fragments covering position 682 to 1010 of AY965685. *Lane M* = Lambda DNA-*Hin*dIII digest size marker; *lane i* = genomic DNA isolated from intact cell nuclei; *lane 1* = DNA fragments isolated from cell nuclei after 15 min of MNase treatment; *lane 2* = DNA fragments isolated from cell nuclei after 30 min of MNase treatment; *lane 3* = DNA fragments isolated from cell nuclei after 60 min of MNase treatment. **A.** Cell nuclei isolated from brain; **B.** eye and heart; **C.** kidney; **D.** liver.

As widely known, the level of myostatin gene expression is highest in muscle. Currently, we were not able to examine, however, the chromatin structure of the myostatin gene in muscle, because the muscle tissues consist of fibrous proteins (actin and myosin fibers), and the nuclei are destroyed by mechanical shearing and/or the other factors during the homogenization of the muscle. As for gills and intestine, which also express the myostatin gene, the preparation of nuclei contained a high activity of endogenous nuclease, so that the "nucleosome ladders" were not observed after MNase treatment. It was not possible to avoid the effects of nonspecific digestion (shearing, endogenous nucleases, etc.), and these tissues were not submitted to Southern blot analysis.

DISCUSSION

Although it is known that many active gene promoters in the human genome show little DNA methylation (Weber et al., 2007), DNA methylation at cytosines has not been well studied as an epigenetic trait in fish. There is reportedly the absence of genome-wide changes in DNA methylation during early embryogenesis in zebrafish (MacLeod et al., 1999), although DNA methylation in the promoter region appears to correlate with vitellogenin I gene expression in this fish (Strömqvist et al., 2010). In the medaka fish *Oryzias latipes*, by contrast, the vast majority of CCGG sites are methylated during early embryonic development, and the extent of the methylation at these sites does not change, or changes very little, during the remaining stages of embryogenesis (Walter et al., 2002).

In the present study, we analyzed the methylation status of the 5' flanking region of the myostatin gene in various tissues from *L. japonicus*. Bisulfite treatment and subsequent PCR revealed nearly complete conversion of cytosines to thymines at CpNs ($N \neq G$), so that very few cytosines remained at CpGs (Table 2). This may mean that the methylation system is not utilized for tissue-specific regulation of myostatin gene. Sequence comparison among closely related species has shown that the 5' flanking regions of myostatin genes are highly conserved (Figure 1). The majority of the variation within these conserved regions is caused by indels at contiguous nucleotides (oligo-C or oligo-A) and transitional substitutions. While several CpG sites are situated within the conserved regions, transitional substitutions (CpG to TpG or CpG to CpA) are observed in the unconserved CpG sites. Methylated cytosines tend to change to thymines through spontaneous deamination (Ehrlich et al., 1986), so that 5-methyl-CpG may be substituted by TpG or CpA on an evolutionary time scale. Thus, DNA methylation may not be involved in myostatin gene regulation, but may instead reflect evolutionary processes.

Our MNase assays suggest that the myostatin gene is compacted into heterochromatin in tissues where it is not expressed, such as liver and kidney, whereas it is susceptible to MNase in the eye and brain, which do express myostatin. These results are consistent with the well-known fact that transcriptionally inactive chromatin is relatively resistant to MNase digestion, while openly configured chromatin containing transcriptionally active genes is selectively solubilized by MNase (Tata and Baker, 1978). Our present results suggest that heterochromatinization in tissues where myostatin is not expressed is regulated by mechanisms that do not require DNA methylation.

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