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Related Expression of MyoD and Myf5 with Myosin Heavy Chain Isoform Types in Bovine Adult Skeletal Muscles

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ABSTRACT—Skeletal muscles are characterized as fast and slow muscles, according to the expression pattern of myosin heavy chain (MyHC) isoforms in the muscle fibers. To investigate the relationships between MyHC isoforms and myogenic regulatory factors (MRFs) including MyoD, Myf5, myogenin, and MRF4 in adult skeletal muscles, expressions of these MRFs in the ten muscles of three cows were analyzed by a semi-quantitative RT-PCR. The results showed that MyoD expression was significantly lower in the lingual muscles (TN), masseter (MS) and diaphragm (DP), which lack MyHC-2x (fast glycolytic) expression and abound with MyHC-slow (slow oxidative) and/or MyHC-2a (fast oxidative), than it was in the pectoralis (PP), psoas major (PM), longissimus thoracis (LT), spinnalis (SP), semitendinosus (ST), semimembranosus (SM), and biceps femoris (BF). In contrast, the Myf5 expression in TN, MS, and DP was significantly higher than in PM, LT, ST, SM, and BF. No significant difference was observed in myogenin and MRF4 expression among the muscles tested. The results suggest that MyoD and Myf5 influence the MyHC isoform expression, although the effects are not decisive in specifying the phenotypes of adult muscles.

Key words: adult muscle, fiber type, Myf5, MyoD, myosin heavy chain isoform

INTRODUCTION

Skeletal muscles can be characterized as functionally distinct fast and slow muscles. Their properties are attributed to the composition of the slow and fast muscle type fibers in individual muscles. The slow and fast muscle fibers contract at characteristic rates dependent principally on the type of myosin heavy chain (MyHC) they express (Schiaffino and Reggiani, 1996). Type I, or slow oxidative, fibers are responsible for the sustained contraction of muscle and employ MyHC slow/β isoform (MyHC-slow), which has a low actin-dependent ATPase activity.

In contrast, fast muscle fibers are recruited for progressively more forceful movements. Adult fast fibers are further classified into four types, according to the type of fast MyHC isoforms they express (Schiaffino and Reggiani, 1996). To date, three fast MyHC genes have been found to have distinct contract rates. The fast oxidative IIA, faster IIX (or IID), and the fastest glycolytic IIB fibers employ MyHC-2a, -2x (or -2d) and -2b genes, respectively. Type IIC fibers express MyHC-slow and -2a in various proportions (Pette and Staron, 1990). In addition to MyHC isoforms, the different types of fibers employ distinct isoforms of other contractile proteins, such as myosin light chains (MyLC) and troponins that are generated by separate genes and/or by mRNA splicing (Schiaffino and Reggiani, 1996).

Muscle fiber specification becomes established from the perinatal stage by hormones (Buckingham, 1992; Muscat *et al*., 1995) and partially by cell lineage (Stockdale, 1997; Wigmore and Dunglison, 1998). In the muscles of adult mammals, muscle fibers can be additionally converted by neuronal stimuli (Stockdale, 1997; Wigmore and Dunglison, 1998). Thus, diversity in the composition of contractile proteins among adult skeletal muscles is established as animals mature (Pette and Staron, 1990).

In the regulation of muscle fiber type, myogenic regulatory factors (MRFs) can mediate extrinsic signals as a direct regulator. Originally these basic helix-loop-helix transcription factors were reported to play essential roles in myogenic cell determination and gene activation in muscle development (Arnold and Braun, 2000). Even in a steady state without extrinsic stimuli, the selective accumulation of MyoD and myogenin transcripts is maintained in the fast and slow muscle fibers, respectively, of adult animals (Hughes *et al*., 1993; Voytik *et al*., 1993; Kraus and Pette, 1997). MyoD knockout mice showed a shift of MyHC isoform expression toward a slower phenotype (Hughes *et al*., 1997) and a

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change of the Ca^{2+} sensitivity of muscle fibers (Metzger *et al*., 1995). Moreover, MyoD gene expression increases when the specific expression of fast MyHC genes increases in slow soleus muscle as a result of thyroid hormone (T3) treatment (Hughes *et al*., 1993). In addition, the T3-responsive region has been found in the upstream region of MyoD gene (Downes *et al*., 1993), but not in that of MyHC-2b gene, suggesting the control of this fiber type by T3 via MyoD regulation. On the other hand, cross-reinnervation of soleus with a fast nerve results in decreased myogenin transcripts in regions induced to express fast MyHC genes (Hughes *et al*., 1993). These studies have suggested that MyoD and myogenin are involved in the specification and maintenance of fast and slow fiber types. In contrast, no relationship between Myf5 or MRF4 and a muscle fiber type has been described to date. In the experiments showing the relationship between MRFs and MyHC isoforms, however, the fiber types have been categorized into only two rough classes, slow and fast, by immunohistochemical detection.

The expression of fiber type-specific genes is maintained to keep the fiber properties, which partially depend on the extrinsic stimuli. The four MRFs can activate the contractile factor genes through MRF's ability to bind to the regulatory regions including E-boxes in the muscle genes, by heterodimerization with the other factors (Arnold and Braun, 2000). Such gene regulations of the contractile proteins can differ among the isoforms. Each of these isoform genes including MyHC can be regulated by a different MRF (Fujisawa-Sehara *et al*., 1992).

Previously we established a method to detect the expression of three bovine MyHC isoforms utilizing the divergent sequences of 5' non-coding region (Tanabe *et al*., 1998). In the present study, to elucidate the maintenance of the diversity of the MyHC isoform expression pattern in adult muscle fiber, we investigated the relationships between the expression of MyHC isoforms and that of MRFs in a variety of bovine skeletal muscles using multiplex RT-PCR. The data show that a selective accumulation of Myf5 mRNA occurs in slower muscles that express MyHC-slow, in contrast to an accumulation of MyoD in faster muscles that express MyHC-2x. Our results raise the possibility of a novel role for Myf5 in affecting the MyHC isoform expression. The data suggest the specific roles of MyoD and Myf5 to regulate the MyHC isoform expression for routine maintenance of fast and slow muscles.

MATERIALS AND METHODS

Treatment of animals and sample preparation

Animals were cared for as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee of National Institute of Livestock and Grassland Science). Three Holstein cows aged 69, 72, and 92 months, were killed by cutting artery after a brain concussion induced by captive-bolt gun stunning. Ten skeletal muscles, namely the lingual muscles (TN), masseter (MS), pectoralis (PP), diaphragm (DP), psoas major (PM), longissimus thoracis (LT), spinnalis (SP), semitendinosus (ST), semimembranosus (SM), and biceps femoris (BF), were excised from each cow within 1 hr after the slaughter. Immediately, small pieces of muscle were prepared from the deep and central part of the muscles and were frozen in liquid nitrogen. The frozen samples were then crushed into fine powder in a liquid nitrogen-cold crusher.

Table 1. Sequences of primers used in this study.

Myf5	fw	CTCAGGAATGCCATCCGCTACATTGAGA
	rv	ATCCAAGCTGGATAAGGAGCTTTTATCCG
MyoD	fr	ATCCTGCGCAACGCCATCCGCTATATCGA
	rv	CTCGCTGTAGTAAGTGCGGTCGTAGCAGT
myogenin	fw	GAGAAGCGCAGACTCAAGAAGGTGAATGA
	rv	TCTGTAGGGTCCGCTGGGAGCAGATGATC
MRF4	fw	GCGAAAGGAGGAGGCTAAAGAAAATCAACG
	rv	TGGAATGATCGGAAACACTTGGCCACTG

fw: forward primer, rv: reverse primer.

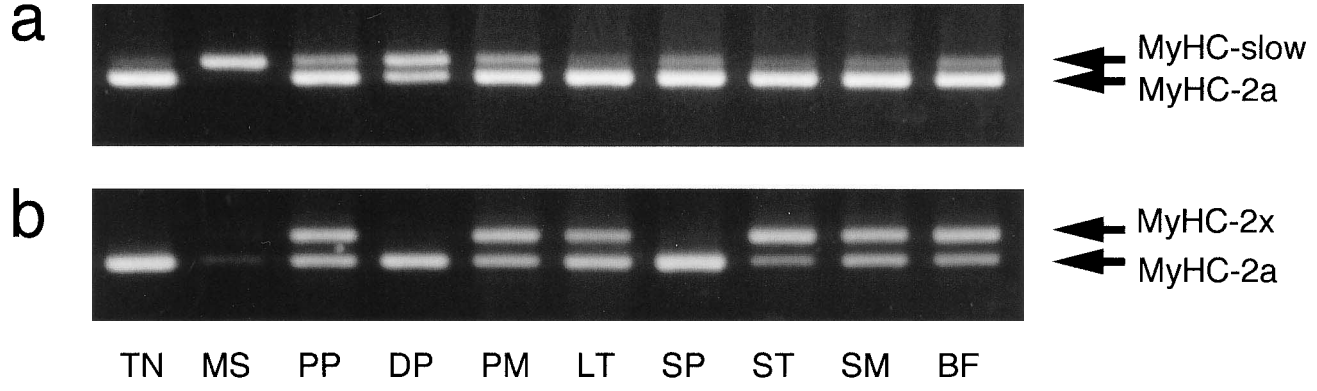


Fig. 1. Electrophoresis of RT-PCR results of myosin heavy chain (MyHC) isoforms in bovine skeletal muscles. (a) Expression pattern of MyHC type slow (MyHC-slow) and MyHC type 2a (MyHC-2a) isoforms. (b) Expression pattern of MyHC-2a and MyHC type IIx (MyHC-2x) isoforms. Specific regions of MyHC-slow (411 bp), MyHC-2a (375 bp), and MyHC-2x (433 bp) cDNAs were amplified. TN: lingual muscles, MS: masseter, PP: pectoralis, DP: diaphragm, PM: psoas major, LT: longissimus thoracis, SP: spinnalis, ST: semitendinosus, SM: semimembranosus, BF: biceps femoris.

mRNA preparation and RT-PCR analysis

The muscle samples were solved in ISOGEN (NipponGene, Tokyo, Japan), and total RNA was extracted from each muscle. The first-strand cDNA was synthesized from 0.5 μg of total RNA using M-MLV Reverse Transcriptase RNase-H minus (Toyobo, Tokyo, Japan) and 3ADP1 as a primer (Muroya *et al*., 2001a).

The sequences of primers for multiplex PCR of the four MRFs are shown in Table 1. MRF primers were designed basically according to bovine Myf5, MyoD, and myogenin cDNA sequences (accession numbers M95684, AF093675, and AF091714 in the DDBJ/EMBL/GenBank nucleotide sequences databases, respectively), but also according to human MyoD and MRF4 (accession numbers X56677 and X52011, respectively) whose bovine sequence are not completely determined. The PCR procedures using AmpliTaq Gold (Perkin-Elmer, Foster City, CA) were carried out first for 9 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C, and finally 7 min at 72°C in the presence of 5% dimethylsulfoxide. In the analysis, the ribosomal protein L7

Table 2. Ratio of MyHC isoforms expressed in bovine skeletal muscles.

MyHC isoform	Percentage of total MyHC isoforms				
	TN	MS	PP	DP	PM
slow	7.4±1.4^{ab}	100^{d}	8.5±2.3^{ab}	42.3±2.2^{c}	8.7±1.5^{ab}
2a	92.6±1.4^{e}	0^{a}	65±12.6^{cd}	57.7±2.2^{c}	39.2±1.5^{b}
2x	0^{a}	0^{a}	26.5±10.2^{b}	0^{a}	52.1±1.5^{d}
MyHC isoform	Percentage of total MyHC isoforms				
	LT	SP	ST	SM	BF
slow	1.7±1.7^{a}	12.5±6.6^{b}	2.0±2.0^{a}	3.5±1.9^{a}	11.9±2.6^{b}
2a	76.4±10.6^{de}	87.5±6.6^{e}	61.1±3.3^{cd}	61.6±6.0^{cd}	53.5±1.5^{bc}
2x	21.9±9.0^{b}	0^{a}	36.9± 5.2^{c}	34.9±4.1^{bc}	34.6±3.5^{b}

Values are means±SE, n=3. Values with different superscripts in each MyHC isoform differ among muscles at P<0.05. TN: lingual muscles, MS: masseter, PP: pectoralis, DP: diaphragm, PM: psoas major, LT: logissimus thoracis, SP: spinnalis, ST: semitendinosus, SM: semimembranosus, BF: biceps femoris.

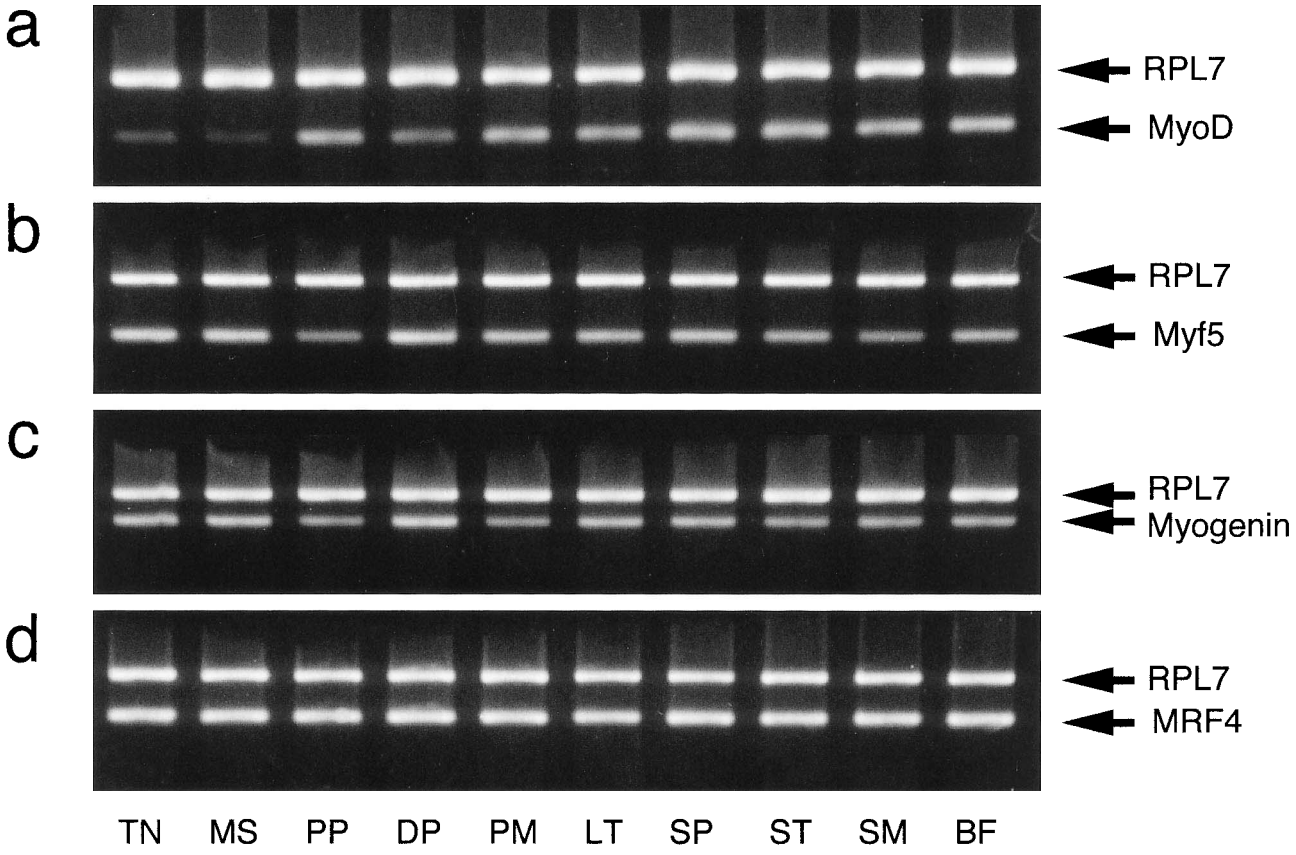
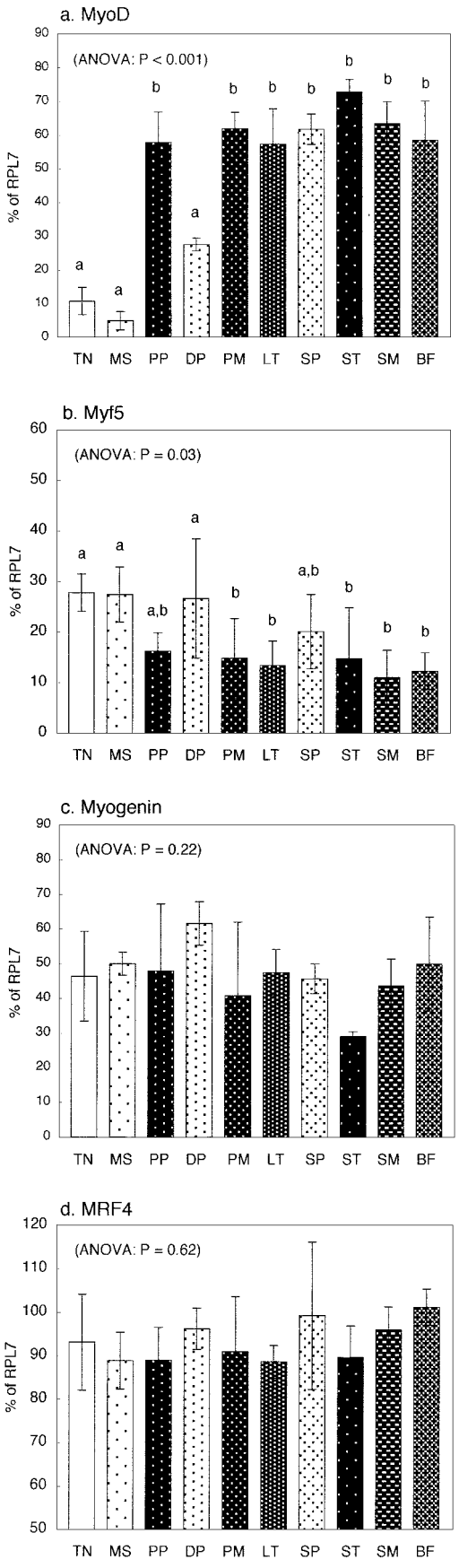


Fig. 2. Electrophoresis of RT-PCR results of myogenic regulatory factors in bovine skeletal muscles. (a) MyoD (255 bp), (b) Myf5 (245 bp), (c) myogenin (314 bp), (d) MRF4 (285 bp). Ribosomal protein L7 (RPL7; 393 bp) was adopted as an internal control of the RT-PCR. TN: lingual muscles, MS: masseter, PP: pectoralis, DP: diaphragm, PM: psoas major, LT: longissimus thoracis, SP: spinnalis, ST: semitendinosus, SM: semimembranosus, BF: biceps femoris.



(RPL7) was adopted as a standard (Venuti *et al.*, 1995). The nucleotide sequences of forward and reverse PCR primers for RPL7 were designed based on mouse RPL7 sequences that are completely conserved in human sequences (Muroya *et al.*, 2001a; Muroya *et al.*, 2001b). The primers for RPL7 amplified the cDNA fragments of 393 bp, deduced from the mouse sequence.

The expressions of MyHC isoforms were also analyzed by RT-PCR, using bovine specific primers (Tanabe *et al.*, 1998). The primer sequences were designed based on the data of bovine MyHC-slow, -2a, and -2x cDNAs. The PCR procedures with AmpliTaq Gold were carried out first for 9 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 1 min at 55°C, 30 sec at 72°C, and finally 7 min at 72°C.

Brief quantification of the expressions of MyHC isoforms and MRFs, and statistical analysis

The results of multiplex PCR of MyHC isoforms or MRFs were semi-quantitatively analyzed using a brief densitometric method. The PCR products were applied to electrophoresis in 4% agarose gel, and then the gel was stained by 0.5 μg/ml ethidium bromide. The images were taken by scanner and were converted to electronic picture files. The ratio of each MyHC isoform or MRF to control band in electrophoresis gel was calculated by NIH image 1.61 software (NIH Division of Computer Research and Technology, National Institute of Health, Bethesda, MD). MyHC isoform expression was estimated as the relative value of MyHC-slow or -2x to MyHC-2a, and expression of each MRF was relatively estimated to RPL7. The results were statistically analyzed by ANOVA, followed by least significant difference method among the muscles.

RESULTS

MyHC isoforms show various expression patterns in bovine muscles

First, to see which type of MyHC gene is expressed in the muscles, we analyzed the expression of MyHC-slow, -2a, and -2x by RT-PCR. The fastest type of MyHC-2b mRNA could not be detected in bovine skeletal muscle, because of the lack of MyHC-2b expression (Tanabe *et al.*, 1998; Chikuni, unpublished data). The primers for MyHC-slow, -2a, and -2x amplified the cDNA fragments of 411 bp, 375 bp, and 433 bp, respectively (Tanabe *et al.*, 1998). The RT-PCR results showed that MS did not express the MyHC-2a and -2x (Figs. 1a, b; Table 2), which indicates the exclusive expression of MyHC-slow in the muscle. In DP, the expression of both the MyHC-slow and the fast type of MyHC-2a but not of MyHC-2x was observed (Fig. 1b). TN and SP mostly expressed MyHC-2a and a small amount of MyHC-slow but did not express the faster type MyHC-2x.

Fig. 3. Expression pattern of myogenic regulatory factors in bovine skeletal muscles. Values are means±SE, n=3. The ratio of expression of MyoD (a), Myf5 (b), myogenin (c), and MRF4 (d) to ribosomal protein L7 was quantified from the multiplex RT-PCR results in a densitometric method. The values of MRFs expression were calculated from the data of three cows and are indicated as percentages of RPL7 expression in graphs. Columns with different characters differ among muscles at P<0.05. TN: lingual muscles, MS: masseter, PP: pectoralis, DP: diaphragm, PM: psoas major, LT: longissimus thoracis, SP: spinnalis, ST: semitendinosus, SM: semimembranosus, BF: biceps femoris.

These findings indicate the faster character of TN and SP in comparison with MS and DP in cattle. The expression pattern of the other muscles tested showed traces of MyHC-slow and an abundance of MyHC-2a and -2x, indicating what may be further faster composition in bovine muscles, especially in PM (Table 2). This expression pattern of MyHC isoforms in the ten muscles was consistent in the three cows tested and was consistent with the previous study (Tanabe *et al*., 1998). Thus, these data indicate that MyHC isoform composition differed among bovine skeletal muscles.

MyoD is expressed in MyHC-2x-expressing muscles but less in non-MyHC-2x-expressing muscles

To investigate the relationships between the expression of MyHC isoforms and that of MRFs, we analyzed the expression of MRFs in the bovine muscles by multiplex RT-PCR (Fig. 2a–d), then performed densitometric semi-quantification. The primers for MyoD, Myf5, myogenin, and MRF4 amplified the predicted cDNA fragments of 255 bp, 245 bp, 314 bp, and 284 bp, respectively, as deduced from the sequences of bovine and the other species. The expression of MyoD considerably differed among the muscles tested (Fig. 3a). According to the quantification results, in TN, MS, and DP, where MyHC-2x was not expressed, MyoD expression was evaluated to be less than 30% of RPL7 expression. In particular, MyoD expressions in TN and MS were slight (less than 11% of RPL7), and were significantly weaker than that in DP (27.5%) ($P<0.05$). In contrast, MyoD expressions in PP, PM, LT, SP, ST, SM, and BF were evaluated to be more than 55% of those of RPL7. The differences in MyoD expression among TN, MS, or DP and in each of the rest muscles were significant ($P<0.05$).

Myf5 is preferentially expressed in MyHC-slow-expressing muscles

A significant specific pattern of Myf5 expression among the muscles was also observed. Myf5 expression in TN, MS, and DP was high (27.8, 27.4, and 26.7% of RPL7 expression, respectively; Fig. 3b). In SP, which showed a pattern of MyHC isoform expression similar to that in TN, Myf5 expression was at an intermediate level (20.1% of RPL7), but was not significantly different from the rest of the muscles. Myf5 expressions in all the MyHC-2x-expressing muscles (PM, LT, ST, SM, and BF) were significantly weaker than in TN, MS, and DP ($P<0.05$). The highest expression of Myf5 in MyHC-2x-expressing muscles, observed in PP, was evaluated to be only 16.2% of RPL7 expression.

No significant specificity in myogenin or MRF4 expression is observed among muscles

Myogenin expression seemed to differ among the bovine muscles (Fig. 2c), but the differences were not significant according to the results of ANOVA ($P=0.22$). Among the muscles tested, the myogenin expression in the slower muscles TN, MS, DP, and SP was evaluated to be 46.4, 50.0, 61.6, and 45.6% of RPL7, respectively, whereas it was 47.9, 40.7, 47.4, 28.9, 43.6, and 49.9% in the faster muscles PP, PM, LT, ST, SM, and BF, respectively.

MRF4 showed a relatively higher expression than the other MRFs in each of the bovine muscles (evaluated to be more than 88% of RPL7). No difference in MRF4 expression among the muscles was observed ($P=0.62$, Fig. 3d).

DISCUSSION

In the present study, MyHC isoform composition differed among the ten skeletal muscles, as shown in previous studies of protein level (Young and Davey, 1981; Young, 1982; Manabe *et al*., 1995) and mRNA level determined by multiplex RT-PCR (Tanabe *et al*., 1998). Although three fast-type MyHC isoforms have not been distinguished from each other in terms of protein level, the previous studies also showed good correlation of expression between the mRNA level and the protein level of MyHC slow or fast isoforms. The difference in the expression of MyHC isoforms among muscles varies among mammalian species (Manabe *et al*., 1995; Tanabe *et al*., 1998). The exclusive expression of MyHC-slow observed in bovine MS is a characteristic of ruminant animals (Young and Davey, 1981; Manabe *et al*., 1995), presumably because of their sustained chewing behavior. These observations suggest the flexibility of expression pattern of MyHC isoforms in each muscle in response to a specific physiological condition.

Expression of MyoD also differed among the bovine muscles. MyoD expression was lower in TN, MS, and DP, which lacked MyHC-2x expression and abounded with MyHC-slow and/or MyHC-2a, than it was in PP, PM, LT, SP, ST, SM, and BF. In SP, a high MyoD expression but not any MyHC-2x was observed. As shown in the previous studies investigating MyoD expression in skeletal muscles, MyoD gene products are preferentially accumulated in the fast muscle fibers of adult rats (Hughes *et al*., 1993; Voytik *et al*., 1993; Kraus and Pette, 1997). Moreover, MyoD knockout mice showed a shift of MyHC isoform expression toward a slower phenotype (Hughes *et al*., 1997) and a change of the Ca^{2+} sensitivity of muscle fibers (Metzger *et al*., 1995), suggesting the contribution of MyoD to fast fiber type specification. On the other hand, it has also been shown that the extent of the shift in the fiber type caused by MyoD gene knockout differed among muscles (Hughes *et al*., 1997); this suggests that the contribution of MyoD varies among muscles. The reason for the difference in MyoD expression among the bovine muscles remains unknown and should be further studied to elucidate the role of MyoD in muscle fiber formation.

In the present study, we have found selective accumulation of Myf5 mRNA among the muscles. Possibly due to its relatively low expression compared to the other MRFs, Myf5 mRNA or protein has rarely been detected in previous studies, but Myf5 is certainly expressed in adult muscles (Voytik *et al*., 1993). MS and DP abounded with MyHC-slow mRNA, and TN and SP mostly expressed MyHC-2a. In

these slower muscles lacking MyHC-2x expression, Myf5 expression was higher than that in the other MyHC-2x-expressing muscles. Thus, Myf5 mRNA preferentially accumulated in slow muscles, which suggests its selective expression in slow muscle fibers or satellite cells. Although little is known about the role of Myf5 in specific MyHC isoform expression, Myf5 may contribute to preparing the fiber type specification in satellite cells where Myf5 is expressed preferentially in adult muscles (Beauchamp *et al*., 2000). However, previous studies suggested that Myf5 expressed in satellite cells does not influence selective slow or fast fiber formation, and neither does MyoD (Cornelison and Wold, 1997; Mendler *et al*., 1998; Cooper *et al*., 1999). In these studies, the fiber-independent accumulations of Myf5 and MyoD were observed by cell culture analysis or in the regenerating process caused by drugs. The expression of the MRFs may be altered in such an emergent condition, in comparison with the expression in routine fiber maintenance.

Myogenin is preferentially expressed in steady-state and stimulated slow muscles, which suggests its selective accumulation in slow fibers (Hughes *et al*., 1993; Voytik *et al*., 1993; Kraus and Pette, 1997). Although myogenin mRNA seemed to also accumulate selectively in some muscles in our study, no significant difference in myogenin mRNA accumulation among bovine slow and fast muscles was observed. In previous studies, the selective myogenin mRNA accumulation in slow muscles has not always been observed. Myogenin mRNA was not detected in slow fibers within a fast muscle, and a fast muscle, plantaris, was observed to express a considerable amount of myogenin mRNA (Hughes *et al*., 1993). As demonstrated by severe muscle defects in myogenin knockout mice, which could not be rescued by the other MRFs, myogenin plays crucial roles that are common in all skeletal muscles (Nabeshima *et al*., 1993; Rawls *et al*., 1995; Valdez *et al*., 2000). The absence of a significant difference in myogenin expression among the muscles may reflect myogenin's common roles in both fiber types. Furthermore, myogenin overexpression in transgenic mice altered the expression of the genes involved in oxidative metabolism but did not alter that of MyHC isoforms (Hughes *et al*., 1999). Therefore, although myogenin tends to be expressed selectively in slow fiber specification, it is more likely that myogenin does not regulate MyHC isoform genes.

The Myf5 expression in the bovine muscles showed the reverse pattern of MyoD expression. This finding suggests that MyoD and Myf5 have distinct roles in the activation of MyHC-2x and -slow genes, respectively. This possibility is also raised by the fiber type-specific regulation of the other genes of contractile proteins. MyLC isoform genes are differently trans-activated by the corresponding MRFs (Fujisawa-Sehara *et al*., 1992). In addition, fiber type-specific regulatory elements have been found in MyHC-slow genes (Knotts *et al*., 1996; Wiedenman *et al*., 1996) and in troponin-I or troponin-C isoform genes (Corin *et al*., 1995; Nakayama *et al*., 1996). Furthermore, the cooperative modulation by MRFs with the other transcription factors, such as MEF2 family proteins, is also suggested (Molkentin *et al*., 1995; Wu *et al*., 2000). However, the influence of the threshold of each MRF or of the ratio of one MRF to the others in MyHC isoform gene expression remains unknown.

No significant difference in MRF4 expression among the muscles was observed in this study. Our results support the conclusions of previous studies and show no tendency toward an MRF4 expression pattern in relation to muscle fiber type (Hughes *et al*., 1993; Voytik *et al*., 1993; Kraus and Pette, 1997). High MRF4 expression may be required for the maintenance of muscle fibers independently of the fiber type. The absence of a difference in the MRF4 expression among the muscles suggests that MRF4 does not participate in maintaining fiber type diversity in adult muscles.

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