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Absence of the Functional Myosin Heavy Chain 2b Isoform in Equine Skeletal Muscles

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ABSTRACT—Nucleotide sequences which included the full coding region for three types of myosin heavy chain (MyHC) isoforms were determined from equine skeletal muscles. The deduced amino acid sequences were 1937, 1938, and 1935 residues for the MyHC-2a, -2x, and -slow, respectively. No MyHC-2b isoform was amplified from the equine muscle cDNA except for one pseudogene fragment. One nucleotide was inserted in the coding region of the equine pseudogene product, a minute amount of which was expressed in the skeletal muscle. The 596 bp sequence of the equine MyHC pseudogene was categorized into the MyHC-2b genes on the phylogenetic tree of the mammalian MyHC genes. These results suggest that an ancestral MyHC-2b gene had lost its function and changed to a pseudogene during the course of horse history. The MyHC genes in some ungulates were analyzed through the PCR amplifications using the MyHC isoform-specific primers to confirm the presence of the MyHC-2b and -2x genes. The exon coding the 3' untranslated region of the MyHC-2x was successfully amplified from the all ungulates examined; however, that of the MyHC-2b gene was amplified only from horses, pigs and lesser mouse deer. The PCR analyses from rhinoceros, sika deer, moose, giraffes, water buffalo, bovine, Japanese serow and sheep genes implied the absence of the MyHC-2b-specific sequence in their genomes. These results suggest that the MyHC-2b gene independently lost its function in some ungulate species.

Key words: myosin heavy chain, muscle, fiber type, ungulate, body size

INTRODUCTION

The molecular basis of muscle contraction is the interaction between myosin and actin molecules associated with ATP hydrolysis as an energy source. The myosin molecule is a heterohexmer consisting of two myosin heavy chains (MyHC) and two pairs of two types of myosin light chains, and the MyHC mainly determines the contractile properties of the myosin molecule. Eight MyHC isoforms are known in mammalian cardiac and skeletal muscle, and four of those eight are expressed in adult skeletal muscles (Schiaffino and Reggiani, 1996). MyHC-slow is the most resistant isoform to fatigue, and is expressed in all mammalian species. The MyHC-2a, -2x and -2b are fast-type isoforms for higher power output in the order 2a<2x<2b. These three fast types were found in small mammalians such as mouse (Weydert *et al*., 1983) and rat (DeNardi *et al*., 1993), but the 2b isoform was not found in large animals until 1997. Moreover, the maximal velocity of the shortening of the muscle fibers expressing homologous MyHC isoforms decreased with increasing body size (Rome *et al*., 1990). These results led a hypothesis that large animals do not express the MyHC-2b isoform in their skeletal muscles. To date, this hypothesis has not been proven. In recent studies, presence of the MyHC-2b mRNA in porcine (Chang and Fernandes, 1997) and human (Weiss *et al*., 1999b) muscles was shown by DNA analyses. Llama also expressed three fast MyHC isoforms, judging from protein analysis (Graziotti *et al*., 2001). However, the bovine muscle showed no MyHC-2b expression through the RT-PCR analysis (Chikuni *et al*., 2004), although the presence of three bands for the fast MyHC isoforms was reported on the SDS-PAGE experiment (Picard *et al*., 1999). Equine skeletal muscle did not express the MyHC-2b isoform at the protein level (Rivero *et al*., 1999), and there is no evidence at the DNA level.

In the present study, the MyHC isoforms expressed in the adult equine skeletal muscle were determined to confirm the lack of the equine MyHC-2b isoform at the DNA level. In addition, the amplification of the MyHC-2b isoform-specific sequence was attempted at some ungulate genomes. These data could indicate the history of the MyHC-2b gene in the history of ungulate species.

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MATERIALS AND METHODS

Animals and genomic DNA preparation

Frozen pieces of equine diaphragm, *semitendinosus* and *longissimus thracis* muscles were obtained from the JRA Equine Research Institute. Those were excised from a male thoroughbred (*Equus caballus*) aged 28 months, and immediately frozen in liquid nitrogen. The frozen tissue samples of giraffes (*Giraffa camelopardalis*), moose (*Alces alces*) and rhinoceros (unidentified) were submitted from the Kanazawa zoo. Genomic DNAs were extracted from the tissues as described by Sambrook *et al*. (1989). The genomic DNAs of pigs (*Sus scrofa*), lesser mouse deer (*Tragulus javanicus*), sika deer (*Cervus nippon*), Japanese serow (*Capricornis crispus*), water buffalo (*Bubalus bubalis*), cattle (*Bos taurus*), sheep (*Ovis aries*) and mouse (*Mus musculus*) were obtained from the DNA stocks in our laboratory, which were prepared using the same procedures.

cDNA synthesis and sequencing for equine MyHC isoforms

The frozen muscle samples were crushed into fine powder in a liquid nitrogen cold crusher (NRK R-8, Nihon-Rikagakukikai, Japan), and the total RNA was extracted from the frozen powder using an ISOGEN total RNA extraction kit (NipponGene, Japan). Single-stranded cDNA was synthesized by M-MLV reverse transcriptase RNase H minus (TOYOBO, Japan) with the primer 3ADP1T, and then used as a template of the LA-PCR (Takara, Japan) for the full-length amplification of the MyHC-2a, -2x, and -slow with the primer pairs H101/H194, H201/H294, and H403/H494, respectively (Table 1). The LA-PCR products were used as a template for a second round of PCR for direct sequencing on both strands with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, CA) and the 373S DNA sequencer (Perkin-Elmer, CA). The sequences of the primer regions were preliminarily determined by the PCRs on the 5' or 3' un-translated regions (UTR) of the MyHC isoforms. The PCR for the equine MyHC-2b was conducted only on the 3 region with the primer pair MHC191/H392.

PCR analyses of the MyHC-2b and 2x genes

The PCR was conducted on an exon coding the 3' terminus of the coding region and 3'UTR of the MyHC-2b or -2x. The genomic DNA of mouse, horse, rhinoceros, pigs, lesser mouse deer, giraffes, moose, sika deer, Japanese serow, water buffalo, cattle and sheep were used as a template. The mix primer of the 2aC1 and 2xC1 was used as a forward primer, which was designed on the 3' terminus of the coding region to be able to anneal all of the MyHC fast types. The MyHC-2b-specific reverse primer H392 was a porcine MyHC-2b sequence on the 3'UTR, which was determined from the comparison of porcine, human, mouse and rat MyHC-2b sequences. The MyHC-2x specific reverse primer H292 was a bovine MyHC-2x sequence on the 3UTR, which was determined from the comparison of porcine, bovine, human, rabbit, mouse and rat MyHC-2x sequences (Table 1). The PCR procedure was carried out first for 9 min at 95°C, followed by 35 cycles of 0.5 min at 90°C, 0.5 min at 50°C, 0.5 min at 72°C, and finally 7 min at 72°C. The PCR products were applied to 4% NuSieve GTG agarose gel (FMC Bioproducts, ME) and run for 30 min at 100V. The fragments amplified were extracted from the agarose gel and then sequenced through the procedures described above.

Phylogenetic analysis

The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the GENETYX-WIN soft-

Table 1. Nucleotide sequences of the primers used for PCR amplifications

3ADP1T:	5' CTGCAGGAATTCGATATCGAAGCTTGC$(T)_{15}$VN 3'
H101:	5' GCTTGCTAACAAGGACCTCTGAGTTCAGCA 3'
H194:	5' TCAGTCATTCCATAGCGTGAAGGCATGATC 3'
H201:	5' AAGGCCGCATCTCTAAGGCAGGGTCTTTGA 3'
H292:	5' CTGCAAAAATCTTAAGTACAAAATAGAGTG 3'
H294:	5' GCATTTCTTTGGTCACTTTTAGCATTTGGA 3'
H392:	5' GATGATTTATTTCCTTGACATCACATGACA 3'
H403:	5' TCACCTGCAGGCTTGAGCTTCTTTTCTTGTAGACA 3'
H494:	5' CACTTCAAGGAAAATTGCTTTATTCTGCTTCCTCCA 3'
MHC191	5' ACGCAGAAGAAAAGGCCAAGAAGGCCATCA 3'
2aC1:	5' AGCCGGGAGGTTCACACAAAAG 3'
2xC1:	5' AGTCGGGAGGTTCACACAAAAA 3'

Table 2. Identity of amino acid sequences* between the equine, porcine, bovine and human MyHC isoforms

	Pig	Cattle	Human	Horse-2a	Horse-2x
Horse-2a	97.4%	96.9	96.5	–	–
Horse-2x	97.5	97.2	96.4	95.5	–
Horse-slow	97.9	97.9	97.6	81.0	81.4

* The amino acid sequences are deduced from the nucleotide sequences of AB088365 (equine MyHC-2a), AB088366 (equine MyHC-2x), AB088367 (equine MyHC-slow), AB025260(porcine MyHC-,2a), AB02525262(porcine MyHC-2x), AB053226(porcine MyHC-slow), AB059398 (bovine MyHC-2a), AB059399 (bovine MyHC-2x), AB059400 (bovine MyHC-slow), AF111784 (human MyHC-2a), AF111785 (human MyHC-2x) and NM_000257 (human MyHC-slow).

ware version 5.2 (Software Development Co. Ltd., Japan). The trees were based on the nucleotide sequences of the MyHC fast type isoforms using the MyHC-slow as the outgroup. The genetic distance was calculated by Kimura's two-parameter method (Kimura, 1980).

RESULTS

Entire sequence of the equine MyHC isoforms

The equine MyHC sequences determined were 5980, 5982, and 5984 bp for the MyHC-2a, -2x, and -slow isoforms, respectively. These nucleotide sequences included full coding regions which encoded 1937 (-2a), 1938 (-2x), and 1935 (-slow) amino acid sequences. Since the entire sequence of the MyHC isoforms is conserved within mammalian species, the amino acid sequences of the equine MyHC isoforms showed high identities to the porcine, bovine, and human MyHC isoforms (Table 2).

The nucleotide sequence data reported in this paper appear in the DDBJ/ EMBL/ GenBank nucleotide sequence databases with the accession numbers AB088365 (equine MyHC-2a), AB088366 (equine MyHC-2x), and AB088367 (equine MyHC-slow).

Sequence comparisons of the equine MyHC functional regions

The typical functional regions of MyHC molecules are shown in Fig. 1. The sequences of the loop 1 and loop 2 regions, which regulated the ATPase activity of MyHC molecules and determined the contracting properties of the MyHC isoforms, were clearly different between the three isoforms. The size and sequence of the loop 1 were similar among the 2a and 2x isoforms of the three species, except for the equine MyHC-2a. The equine MyHC-2a had two amino acids deletions from the 2x isoform on the loop 1 region. The equine slow isoform was different from the fast-type isoforms. On the loop 2 region, the N-terminal portion of the loop 2 region was different not only among the 2a, 2x, and slow isoforms, but also among the equine, porcine, and bovine sequences of the same isoform type.

The equine MyHC-2b pseudogene

The 596 bp fragment of the MyHC 3' region was obtained by the MyHC-2b amplification procedures using the primer pair MHC191/H392 (Fig. 2). The reverse primer H392 was designed on the MyHC-2b-specific sequence of 3'UTR, and the nucleotide sequence of the fragment was similar to the sequences of the MyHC isoforms. However, it contained an additional adenine nucleotide in its sequence. The additional nucleotide changed the reading frames, resulting in a short translation. The attempt at sequencing beyond the fragment failed due to the fact that there was less expression of mRNA or because of the disagreement of primers with the equine gene. A phylogenetic tree of this fragment region was constructed by the neighbor-joining method using the equine, porcine, bovine, and human MyHC sequences, and then compared to the tree of the

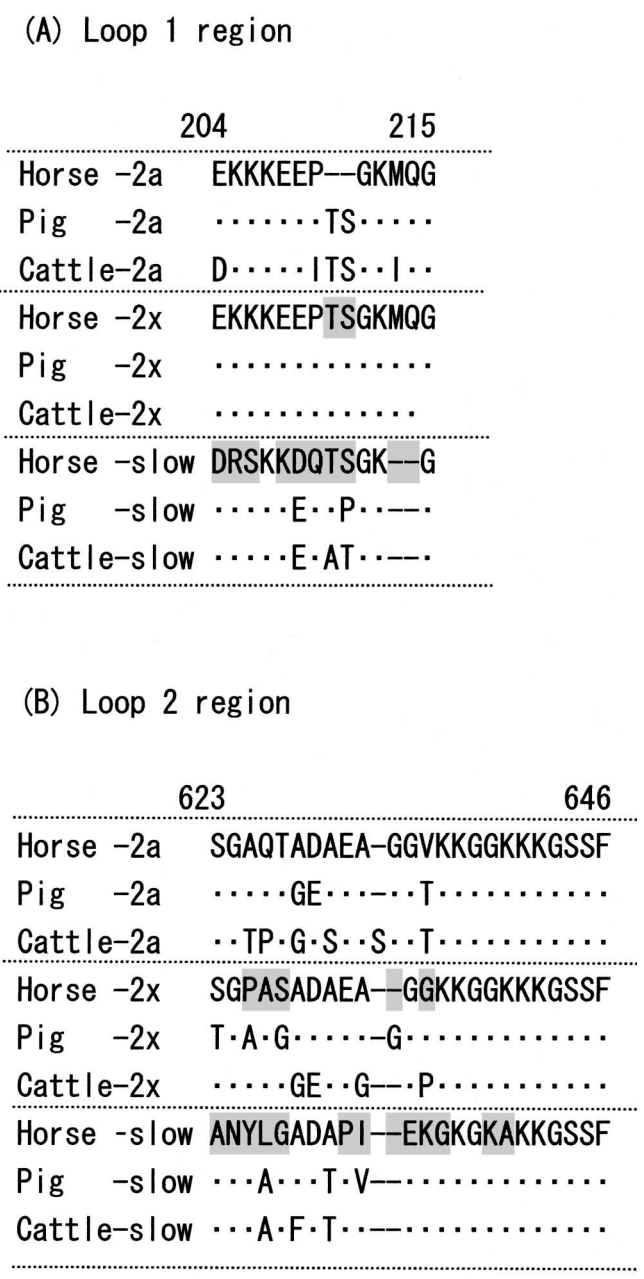

Fig. 1. Alignments of horse, porcine, and bovine MyHC (A) loop1 and (B) loop 2 regions. Dots indicate identical amino acid residues of the porcine and bovine MyHC isoform to that of the equine one. Dashes indicate spaces for alignment. Different amino acid residues of the equine MyHC-2x and -slow from the equine MyHC-2a are shadowed.The amino acid sequences are deduced from the nucleotide sequences; porcine MyHC-2a (AB025260), -2x (AB025262) and -slow (AB053226), bovine MyHC-2a (AB059398), -2x (AB059399) and -slow (AB059400). Horse sequences were determined in this study; equine MyHC-2a (AB088365), -2x (AB088366), and slow (AB088367).

MyHC full coding region (Fig. 3). The MyHC-2b group was separated from the other isoform groups on both trees, and the entire shape of the fragment region's tree was similar to the full coding region's tree. The fragment amplified as an equine MyHC-2b was classified into the 2b group on the

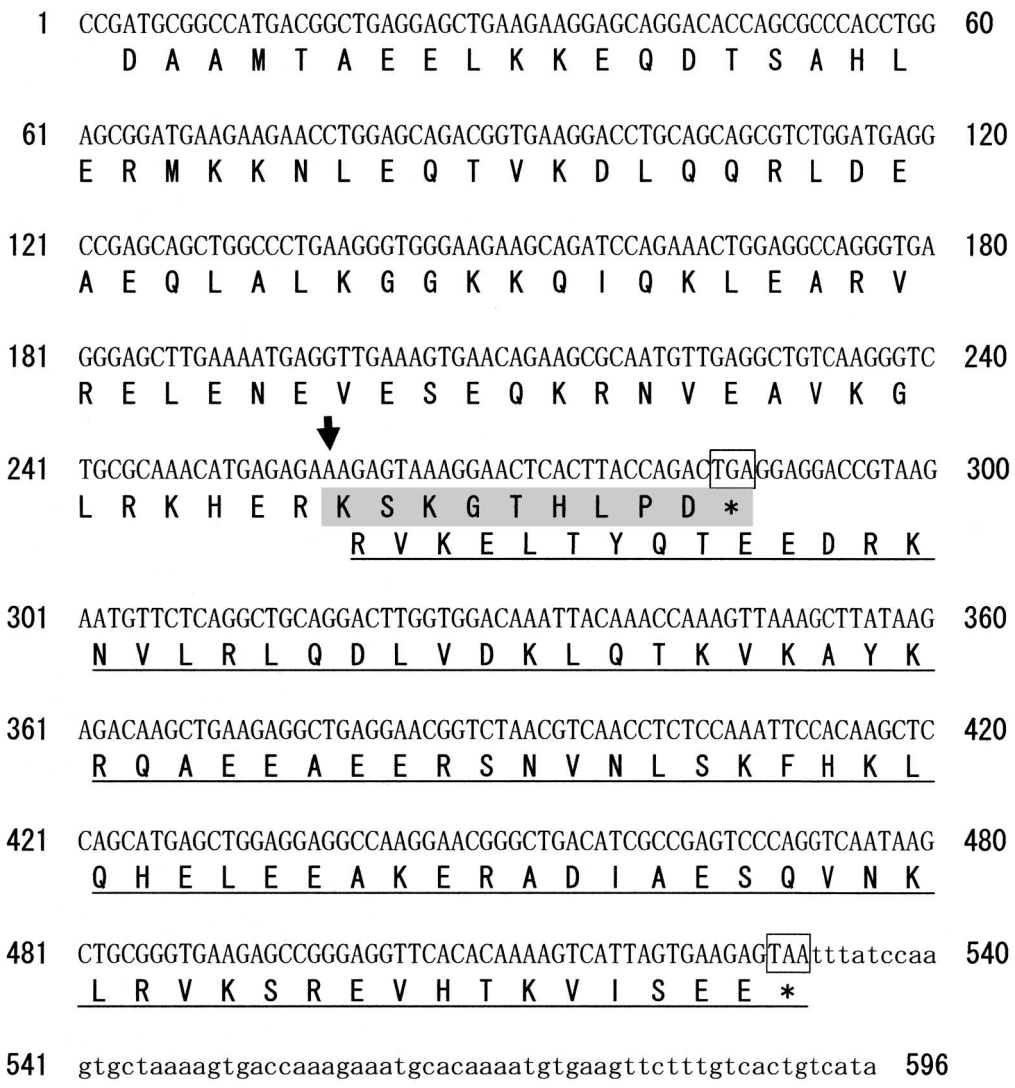

Fig. 2. Nucleotide sequence of the equine MyHC-2b pseudogene fragment. An arrow indicates the insertion of one adenine. The amino acid sequence changed by the insertion is shadowed. The amino acid sequence without the insertion is underlined. Open boxes indicate the stop codon.The nucleotide sequence of the equine MyHC-2b pseudogene fragment appears in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB126008.

phylogenetic tree and had the insertion of one nucleotide causing the frame shift. We thus concluded that this fragment was a product amplified from the MyHC-2b pseudogene.

The nucleotide sequence of the equine MyHC-2b pseudogene fragment appears in the DDBJ/ EMBL/ GenBank nucleotide sequence databases with the accession number AB126008.

PCR amplification of the equine MyHC-2b fragment from the equine genome

The mix primer of 2aC1 and 2xC1 was used as a forward primer, and the 2b-specific primer H392 was used as a reverse primer to amplify a short fragment from the equine MyHC-2b gene. The fragment region is coded on one exon at the human fast-type MyHC genes, and thus the primer pair designed was assumed to be capable of amplifying the target region of the equine gene. The equine MyhC-2b pseudogene fragment was actually amplified from the equine genome (Fig. 4), and that sequence was in agreement with the equine MyHC-2b pseudogene sequence (data not shown). The same procedure of RT-PCR did not amplify any fragment from the cDNA of equine muscles. The PCR using the 2x-specific primer amplified the MyHC-2x fragment from both the equine genome and cDNAs. These results indicated that the MyHC-2b pseudogene was present in the equine genome and scarcely expressed in equine muscles.

Presence of the MyHC-2b gene in some ungulate species

The MyHC-2b and -2x fragments were amplified from some ungulate genomes through the same procedures as that of Fig. 4 (Fig. 5). The target fragment of the MyHC-2x gene was amplified from all of the ungulate examined.

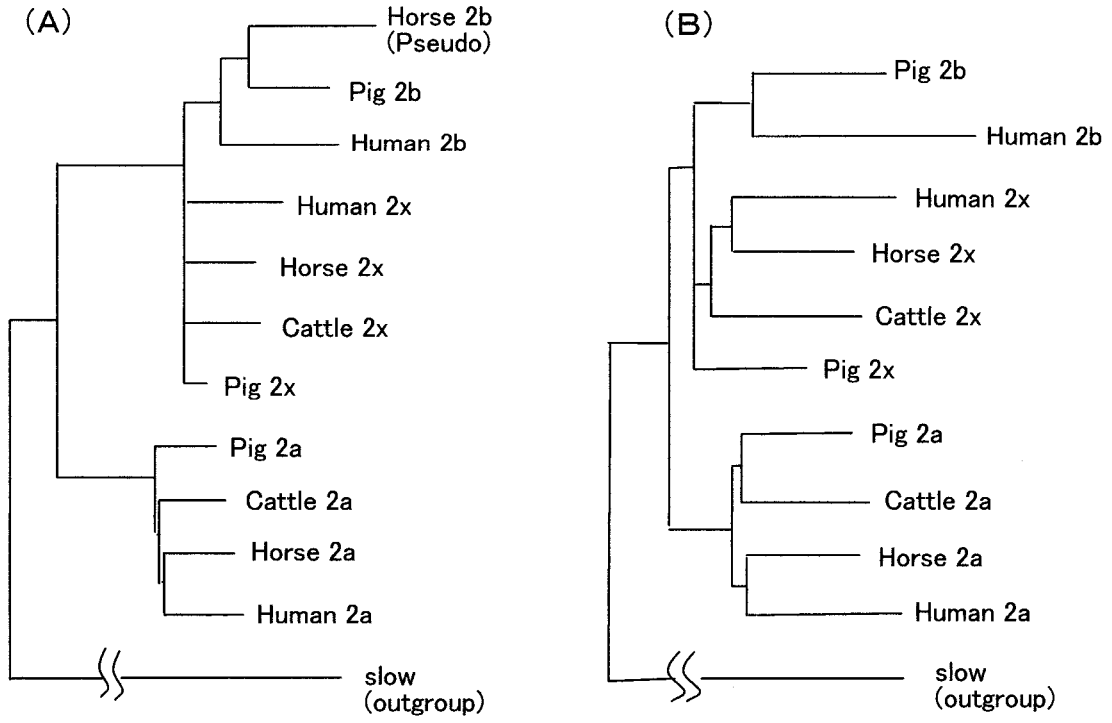

Fig. 3. Phylogenetic trees of myosin heavy chain (MyHC) fast-type isoforms based on (A) the 595 bp nucleotide sequences (except for one additional nucleotide on the pseudogene products) of the 3' fragments and (B) 5808-5823 bp nucleotide sequences for full amino acid coding region. The nucleotide sequences used for the phylogenetic trees were obtained from the DDBJ/EMBL/GenBank databases; porcine MyHC-2a (AB025260), -2b (AB025261), and -2x (AB025262), human MyHC-2a (AF111784), -2b (AF111783), and -2x (AF111785), bovine MyHC-2a (AB059398), and -2x (AB059399). Equine sequences were determined in this study; equine MyHC-2a (AB088365), -2x (AB088366), slow (AB088367) and -pseudo-2b (AB126008). The trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) using the MyHC-slow as the outgroup. The genetic distance was calculated by Kimura's two-parameter method (Kimura, 1980).

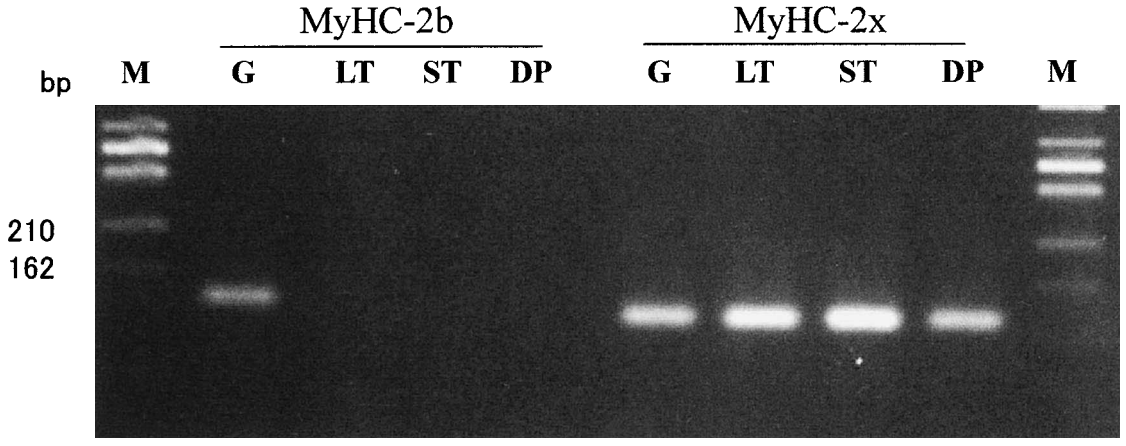

Fig. 4. PCR amplifications of the MyHC-2b and -2x fragments from equine gene and cDNA. M, makerX174/HincII digest. G, equine gene; LT, cDNA from *longissimus thracis*; ST, cDNA from *semitendinosus*; DP, cDNA from diaphragm.

Sequencing work showed that these fragments corresponded to the MyHC-2x isoform (Fig. 6). The PCR product of the MyHC-2b gene was amplified only from horses, pigs and lesser mouse deer. The sequences of the porcine and equine fragments were in agreement with the porcine MyHC-2b and the equine MyHC-2b pseudogene sequences, respectively. That of the lesser mouse deer corresponds to the MyHC-2b isoform (Fig. 6). The PCRs for the MyHC-2b amplified no products from rhinoceros, sika deer, moose, giraffes, water buffalo, bovine, Japanese serow and sheep genes.

These nucleotide sequence data appear in the DDBJ/EMBL/ GenBank nucleotide sequence databases with the accession numbers AB126009 AB126016.

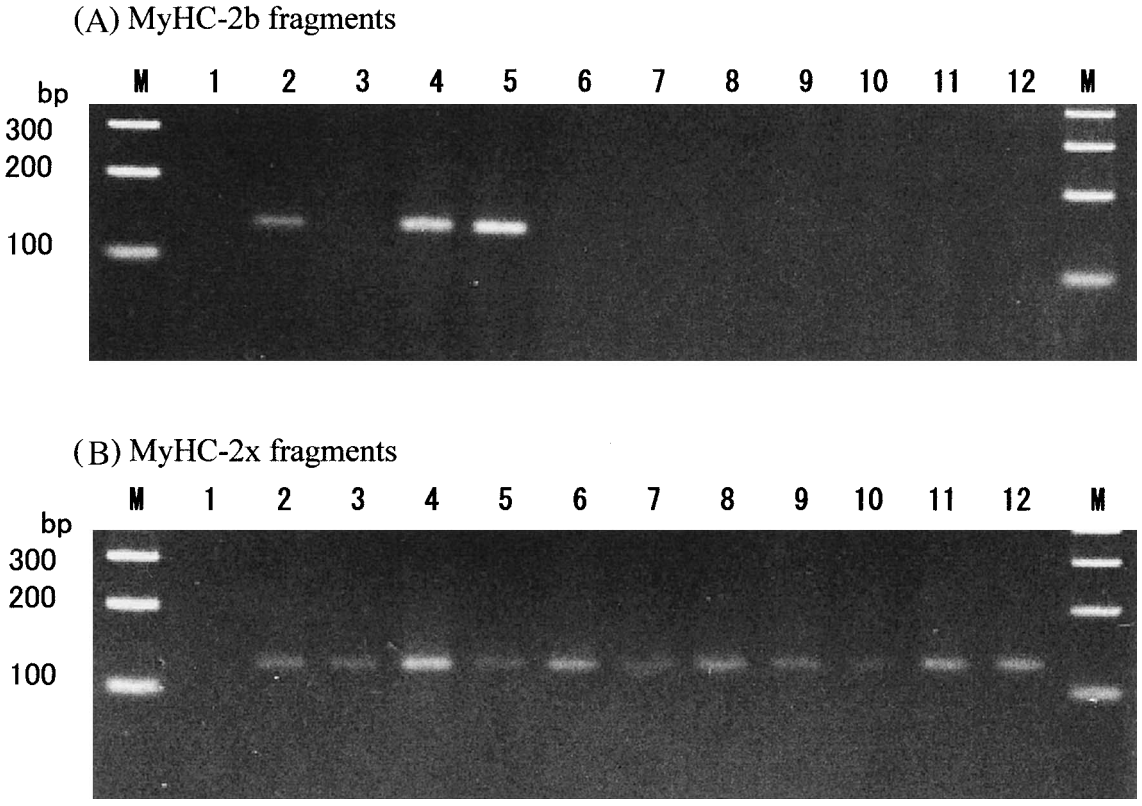

Fig. 5. PCR amplifications of the MyHC-2b and -2x fragments from some ungulate genes. M, maker; 1, mouse; 2, horse; 3, rhinoceros; 4, pig; 5, mouse deer; 6, sika deer; 7, moose; 8, giraffe; 9, cattle; 10, water buffalo; 11, Japanese serow; 12, sheep.

(A) MyHC-2b fragments

```
Pig              TCATAAGTGAAGAGTAATTCATCTAAATGCACAAAA--GTGACCAAAGAAATGCACAAAATGTGAAAATCTTTGTCACTGTTATA  83
Horse(pseudo)    ····T·····T···C··G···TA···--------------------------------GT···········C···  82
Human            ·····TCT····A·AG···AT·················G·····GT···········CC·G  85
Mouse deer       ·T··C··C·····TCT·····A···G--------------------------------T···········C·A·  83
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(B) MyHC-2x fragments

```
Cattle           TCATCAGTGAAGAGTAACTCATCCAAATGCTAAAAAGTGACCAAAGAAATGCACAAAATGTGAAAATC  68
Pig              ····A·····T·····C·····C·····  68
Horse            ····T·····T·----------------GT··  67
Human            ····A·····T·T···T··C····G···G·····  68
Rhinoceros       ····A·····-----------------T···  67
Mouse deer       ·····C·····C·····  68
Sika deer        ·····  68
Moose            ·····  68
Giraffe          ·····  68
Water buffalo    ·····C·····G·····  68
Japanese serow   ·····  68
Sheep            ·····  68
```

Fig. 6. Partial sequences of the ungulate myosin heavy chain (MyHC) 2b and 2x isoforms. Dots indicate identical nucleotides to (A) the porcine or (B) the bovine sequences. Dashes indicate spaces for alignment. Open boxes indicate the stop codon. The nucleotide sequences determined in this study appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB126008-AB126016.

DISCUSSION

The absence of the equine MyHC-2b isoform was reported at the protein level (Rivero *et al*., 1999), and the pseudogene of the equine MyHC-2b isoform was identified in the present study. These results indicated that there is no expression of the functional MyHC-2b isoform in equine skeletal muscles. To confirm this, RT-PCR amplifications were conducted with the equine cDNA and gene by using the MyHC-2b-specific reverse primer H392 (Fig. 4). If a functional MyHC-2b gene is present in the equine genome, it could be amplified together with the pseudogene from both the cDNA and genome by the PCR. The electrophoresis in Figure 4 shows no visible fragment from the equine cDNAs prepared from *semitendinosus* and *longissimus thracis* muscles, in which the 2B type fiber is abundant. This result means that there is no expression of the MyHC-2b-like sequence, except for the minute expression of the pseudogene product in the equine muscles. We thus concluded that the MyHC-2b isoform is not functional in equine skeletal muscles.

Body size is one of the major factors affecting the functioning of organisms. Hill (1950) predicted that the velocity of the shortening of muscle fibers during locomotion increases as body size decreases; small animals have to take many more steps to cover the same distance because of their shorter legs. When running at the same speed as larger animals, small animals have higher stride frequencies and consume energy at higher rates. Taylor *et al*.(1982) reported, based on data from 62 avian and mammalian species, that the metabolic energy consumption of muscles during locomotion decreased with the increase of body mass. However, stresses acting on bone and muscle increase as body size increase. The ability of bones to resist force depends on tissue cross-sectional areas, which decrease in proportion to an animals weight. As a way to adapt to this problem, large animals align their limbs more closely with the ground reaction force by size-dependent (Biewener, 1989). Another way adaptation to this problem occurs is by decreasing in the velocity of muscle shortening by changing the composition and types of muscle fibers in large animals. The lack of MyHC-2b, which is the fastest type in the isoforms, would reduce the stresses in bones and the metabolic energy consumption in muscles.

The changes in the amino acid sequence of the MyHC isoforms are also effective in tuning the contractile properties of skeletal muscles. The length of the equine MyHC-2a on loop 1 was two amino acids shorter than the porcine and bovine isoforms (Fig. 1). The rate of ADP release in MyHC molecules was a function of loop size and flexibility, with the larger loops giving faster rates of ADP release (Sweeney *et al*., 1998). The two amino acids shorter sequence in the equine MyHC-2a could indicate slower ATP hydrolysis on the IIA type fibers than in the other animals. The sequential diversity in the loop 2 region also suggests the differences in contractile properties among animal species (Fig. 1). Although the experiments constructing the chimaeric myosins indicated the importance of the loop 2 region for ATPase activities (Ueda *et al*., 1994), the relationships between the amino acid sequence of the loop 2 region and function are unclear. These sequence data would reflect the differences in contractile properties among animal species which have diverse activities.

The MyHC isoforms expressed in skeletal muscles are members of the myosin superfamily, which would derive from nonmuscle myosin by multiple duplications (Goodson and Spudich, 1993; OOta and Saitou, 1999). The ancestral fast-type MyHC gene diverged from the slow type gene early in evolution, and then the next duplications produced some fast-type genes. Presently, the fast-type MyHC genes are clustered on a narrow segment of a single chromosome in both human and mouse genomes. The order, transcriptional orientation, and relative intergenic distance of the fast-type genes are remarkably conserved between the two species. This fact supports the idea that these loci were formed by gene duplications that occurred before the divergence of these species between ~75 and 110 million years ago (Weiss *et al*., 1999a). The phylogenetic trees in Fig. 3 are consistent with this idea; animals have multiple MyHC isoforms, which are clustered within each isoform group.

It has been reported in some papers that pigs, which are ungulates, express the functional MyHC-2b isoform in their skeletal muscles (Chang and Fernandes, 1997; Lefaucheur *et al*., 1998; Chikuni *et al*., 2001). This implies that the common ancestral animal of the order Perissodactyla and the order Artiodactyla kept the four types of MyHC isoforms, but horses and cattle had lost the functional MyHC-2b gene in their history. In general, pseudo genes change their sequences more rapidly than their original genes, because of no restriction of physiological function. The sequence of the equine pseudogene is similar to that of the active MyHC-2b isoforms of pigs and humans; therefore, the small-sized ancestors of the horses might have an active MyHC-2b gene and then have lost the function according to the increase of their body size. The fragment of the rhinoceros MyHC-2b isoform was not amplified from the genome, because there may be more substitutions in the rhinoceros MyHC-2b sequence than in the equine one. The rhinoceros MyHC-2b may have lost the function before the equine one did. The MyHC-2b sequence was amplified from the pig (family Suidae) and lesser mouse deer (family Tragulidae), but not from giraffe (family Giraffidae), sika deer, moose (family Cervidae), cattle, water buffalo, Japanese serow and sheep (family Bovidae) in the order Artiodactyla (Figs. 5 and 6). The ancestral animal of the suborder Ruminantia may have lost its functional MyHC-2b gene before the divergence of the family Giraffidae or the family Cervidae (Fig. 7). These results suggest that the loss of function of MyHC-2b occurred independently in the history of ungulate animals.

The hypothesis that large animals do not express the MyHC-2b isoform in their skeletal muscles was undermined when human and porcine MyHC-2b isoforms were determined (Weiss *et al,* 1999b; Chang and Fernandes, 1997). It

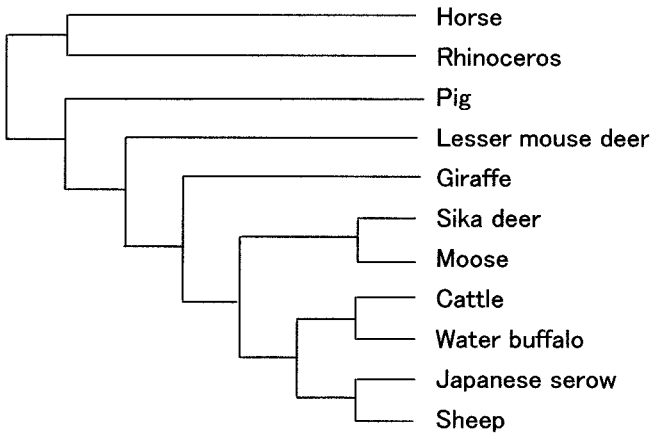

Fig. 7. Phylogenetic relationships among the 11 ungulates examined. The tree of relationships was based on published papers (Matthee *et al*., 2001; Chikuni *et al*., 1995).

was found that the lack of the MyHC-2b isoform was not only a matter of body size, but that the history of the animals could affect the composition of the MyHC isoforms in skeletal muscles. Sheep, Japanese serow, and sika deer, which are similar in size to pigs, did not show the MyHC-2b sequences, because their ancestral animal may have lost the functional MyHC-2b gene. However, body size is considered one of the major factors determining the existence of the MyHC-2b isoform, because the larger animals of the orders Artiodactyla and Perissodactyla examined did not show the presence of the functional MyHC-2b gene. The lack of the MyHC-2b isoform would be advantageous or neutral, at least not deleterious for large animals.

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