

Myosin Heavy Chain 2B isoform is expressed in specialized eye muscles but not in trunk and limb muscles of cattle

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Myosin heavy chain isoforms (MHC) of adult skeletal muscles are codified by four genes named: slow, or type 1, and fast types 2A, 2X and 2B. The slow, 2A and 2X isoforms have been found expressed in all mammalian species studied so far whereas there is a large inter-species variability in the expression of MHC-2B. In this study histochemistry (m-ATPase), immunohistochemistry with the use of specific monoclonal antibodies and RT-PCR were combined together to assess whether the MHC-2B gene is expressed in bovine muscles. ATPase staining and RT-PCR experiments showed that three MHC isoforms (1, 2A, 2X) were expressed in trunk and limb muscles. Slow or type 1 expression was confirmed using a specific antibody (BA-F8) whereas the detection of fast MHC isoforms were validate by means of BF-35 antibody although not by the SC-71 antibody. MHC-2B was absent in limb and trunk muscles, but was present in specialized eye muscles (*rectus lateralis* and *retractor bulbi*) as consistently showed by RT-PCR and reactivity with a specific antibody (BF-F3). Interestingly, a cardiac isoform, MHC- α -cardiac was found to be expressed not only in extraocular muscles but also in masticatory muscles as *masseter*.

Key words: myosin isoforms, MHC-2B, MHC- α cardiac, RT-PCR, m-ATPase, cattle.

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Mammalian skeletal muscle fibres are traditionally classified in distinct groups on the basis of their different histochemical staining. The reactivity for myofibrillar ATPase (m-ATPase) after alkali or acid preincubation identifies acid-resistant or slow fibres and alkali-resistant or fast fibres. These latter can be further distinguished on the basis of different acid and alkaline resistance as 2A and 2B. Combining metabolic enzyme determination and m-ATPase reaction leads to the identification of three types such as β -R, α -R and α -W (Ashmore and Doerr, 1971), also classified as SO (slow oxidative), FOG (fast oxidative-glycolytic) and FG (fast glycolytic) (Peter *et al.*, 1971), or four types, grouped as 1, 2A, 2B oxidative and 2B non-oxidative (Lopez *et al.*, 1992).

The development of polyclonal and monoclonal antibodies against various isoforms of sarcomeric myosin has shown that the differential resistance to acid or alkali pre-incubation depends on the presence of specific isoforms of myosin heavy chains (MHC) (Schiaffino and Reggiani, 1996). Myosin is a hexameric protein composed by two MHC and four MLC (myosin light chain) subunits. Myosin heavy chain isoforms can be considered the molecular markers of fibre type and therefore fibres are indicated using the name of myosin isoform which express. Up to now, nine isoforms of sarcomeric MHC have been found in mammalian skeletal muscles (Schiaffino and Reggiani, 1996): two developmental isoforms (embryonic and neonatal), four adult isoforms (β /slow or type 1, 2A, 2B, 2X or D), the atrial isoform α (or α -cardiac) and two isoforms typical of specialized muscles indicated as extraocular (EO) and masticatory (M).

Only some antibodies are very specific and able to recognize MHC isoforms in different mammalian species (orthologous isoforms). This fact can be explained since orthologous isoforms present a higher degree of aminoacid and/or nucleotide similarity than paralogous isoforms (isoforms present in

the same animal species).

Nucleotide sequences of many MHC and MLC isoforms are available in the database. The exon-intron structure is very similar among different species and the nucleotide sequence is highly conserved. The amino acid identity is higher when MHC orthologs are compared (up to 98%) than among MHC paralogs (up to 78%) (Weiss *et al.*, 1999). Some regions of the MHC molecule are completely identical such as parts of the catalytic site, the actin binding surface and the converter domain, whereas other regions are more variable and more suitable to identify MHC isoforms. Among these variable regions there are: the sequences codifying for surface loops on the myosin head and the two untranslated ends (5' and 3' UTR). Complete sequences of all sarcomeric MHC genes are available in humans and mice and, among livestock animals, three MHC isoforms have been sequenced in the adult skeletal muscle of the cow (Tanabe *et al.*, 1998) and all four in the pig (Chikuni *et al.*, 2001).

There is a large inter-species variability in the expression of adult skeletal muscle MHC isoforms, particularly in MHC-2B expression. In small rodents (mouse, rat) and in rabbit all four MHC isoforms are expressed contributing to the origin of four distinct fibre types, whereas in man MHC-2B gene, although identified in the genome, is not expressed and only MHC 1, 2A and 2X are present in skeletal muscles. Therefore, in man the fibre type previously classified as 2B (conventional 2B) contains MHC-2X (Smerdu *et al.*, 1994). The lack of expression of MHC-2B and the consequent lack of 2B fibres does not reduce the functional variability (maximum shortening velocity or ATPase activity) since the difference between various fibre types is larger in man than in rat or in mouse (Pellegrino *et al.*, 2003). Recently, in some specialized human muscles such as the *masseter*, MHC-2B gene has been found to be expressed by *in situ* hybridisation, but the corresponding protein has not been found (Horton *et al.*, 2001). Moreover, masticatory muscles of several animal species also express MHC- α -cardiac (d'Albis *et al.*, 1991) (Hoh *et al.*, 2000) or MHC-2M (Rowlerson *et al.*, 1981) (Hoh *et al.*, 1999) and muscles controlling eye movement or laryngeal muscles show a composition in MHC isoforms that is even more complex.

Our recent work (Toniolo *et al.*, 2004) has confirmed that among large mammals, pig muscles express all four adult MHC isoforms, slow type 1,

fast 2A, fast 2X and fast 2B. The expression first found with RT-PCR and *in situ* hybridization of all four transcripts (Chang and Fernandes, 1997) (Lefaucheur *et al.*, 2002) has been confirmed by histochemistry, immunohistochemistry and gel-electrophoresis. In trunk and limb skeletal muscles 2B isoform is co-expressed with 2X isoform (2X/B hybrid fibre). Pure 2B fibres are only present in peculiar muscles as *retractor bulbi* and extraocular muscles. The presence of hybrid fibre (2X/B) clarifies the mismatch previously reported between *in situ* hybridisation and protein expression in pig skeletal muscles (Lefaucheur *et al.*, 2002). A possible explanation of this specific expression of MHC-2B in porcine muscles might be found in the selection aimed to increase the meat production. The expression of the 2B gene seems to be associated with high muscular development and also with the expression of the halothane gene responsible for the porcine stress syndrome (Depreux *et al.*, 2002).

The inter-species diversity of the expression of the MHC-2B isoforms stimulates new studies to verify its expression in various species of veterinarian or experimental interest. In this study we aimed to investigate the expression of MHC-2B in bovine muscles. Cattle is bred both for milk and meat production and in some breeds the muscularity has been enhanced by genetic selection to obtain the so called *double muscling* phenotype. In bovine muscles there are still uncertainties regarding the fibre type classification (Picard *et al.*, 1998) (Picard *et al.*, 1999) and the issue is particularly relevant for the *double muscling breeds*. The results obtained by histochemistry (based on mATPase) and immunohistochemistry have identified three fibre types classified as type 1, 2A and *conventional 2B* (it is not clear whether this type contains MHC-2B or 2X) (Picard *et al.*, 1998) (Duris *et al.*, 2000). Gel electrophoresis has separated four bands (Picard *et al.*, 1999) and these results are controversial with the expression studies based on RT-PCR which demonstrate expression of 1, 2A and 2X genes (Tanabe *et al.*, 1998) (Chikuni *et al.*, 2004). Available antibodies can recognize bovine slow myosin, but are not suitable for reliable identification of 2A and 2X fast isoforms. Even the mAb SC71 widely used to recognize myosin 2A in many animal species, does not distinguish type 2A fibres from other fast fibres in bovine muscles (Duris *et al.*, 2000). Genes of bovine MHC isoforms (1, 2A, 2X) have been sequenced (Tanabe *et al.*, 1998) but

the issue about the expression of MHC-2B is still open.

In this study we applied the classical m-ATPase staining, tested several antibodies and validated the conclusions of histochemical and immunohistochemical staining analysing the MHC gene expression by means of RT-PCR. The range of muscles investigated was extended from limb and trunk muscles to specialized extraocular muscles which were suggested as site of possible expression of MHC-2B by previous studies in different animal species (Sartore *et al.*, 1987) (Mascarello and Rowleron, 1992).

Materials and Methods

Sampling of bovine muscles

Muscle samples were collected from adult cows of commercial breeds killed in a slaughter house. Samples of *masseter* (M), *diaphragm pars costalis* (D), *longissimus dorsi* (Ld), *rectus lateralis* an extraocular muscle (EO) and *retractor bulbi* (Rb) muscles were dissected and used for histochemistry and immunohistochemistry. Other samples were taken from the same muscles and from additional muscles such as *extensor carpi radialis* (Ecr) and *pectoralis* (P) and used for RT-PCR analysis. The choice of these different muscles aimed to identify myosin isoforms because, from published data and our preliminary study, these muscles have a different fibre types composition.

Histochemistry and immunohistochemistry

Muscle samples combined into composite blocks were frozen in isopentane cooled with fluid nitrogen and serial sections (10 μ m) were cut in a cryostat. Serial sections were stained for m-ATPase as previously described in detail (Latorre *et al.*, 1993). ATPase staining followed either alkaline pre-incubation (method 1, pH 10.4, 10.5, 10.6 and 10.7 with incubation times for 15 minutes) or acid pre-incubation (method 2, sodium acetate 0.2 M, at pH 4.6, 4.55, 4.5, 4.45, 4.4 for 5 minutes). All methods well distinguished type 1 from type 2 fibres, whereas the separation of fast type 2 fibres was critical. M-ATPase activity after alkaline pre-incubation with increasing pH value allowed us to distinguish fast (positive) from slow (negative) fibres at pH 10.4, and with increasing pH to separate the 2A (positive or moderately positive) from other fast fibres (weakly positive to negative). After 15 min-

utes of pre-incubation at pH 10.7, the m-ATPase activity disappeared in all fibres. M-ATPase activity after acid pre-incubation showed that at pH 4.6 only type 2A was negative while at pH 4.55 and 4.5 the activity progressively disappears in other type 2 fibres. In any case at pH 4.45 all type 2 fibres were negative.

Additional serial sections were stained with the following monoclonal antibodies: BA-F8 (specific for MHC-1 or slow in several species from rat (Bottinelli *et al.*, 1991) to pig (Toniolo *et al.*, 2004), SC-71 (specific for MHC-2A in rat (Bottinelli *et al.*, 1991), BF-35 (specific for MHC-2X in pig (Toniolo *et al.*, 2004), BF-F3 (specific for MHC-2B in rat and in pig (Lefaucheur *et al.*, 2002) and BA-G5 (specific for MHC- α -cardiac (Stal *et al.*, 1994). The immunohistochemical reactions were performed using the Envision method (goat anti-rabbit immunoglobulins conjugated to peroxidase labelled complex, Dako). The primary antiserum was applied overnight at room temperature using different dilutions ranging: from 1:100 to 1:400 for the SC-71, BA-F8 and BF-35 antibodies; from 1:20 to 1:40 for the BF-F3 antibody and from 1:100 to 1:200 for the BA-G5 antibody. After rinsing in PBS buffer (pH 7.2-7.4), the sections were incubated for 30 min at room temperature in Envision system, rinsed again in PBS and the immunoreactive sites visualized using a freshly prepared solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 15 mL of a 0.05 M Tris buffer at pH 7.6, containing 1.5 mL of 0.03% H₂O₂. Antibodies were purchased at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The specificity of the immunostaining was verified: 1) by incubating sections with PBS instead of the specific primary antiserum; 2) by incubating sections with preimmune serum instead of primary antiserum; 3) by incubating sections with PBS instead of secondary antibody.

Gene isolation

Genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega) from 300 μ L of bovine blood. Two hundred nanograms of extracted DNA were amplified by Polymerase Chain Reaction (PCR) using degenerate primers: forward 5'GAACAATCCAA(CT)GTCAACC 3' and reverse 5'GAACCTCTC(CT)(AG)CTCTTiAC3', designed on the bases of MHC-2B amino acid

Table 1. Primers used for RT-PCR.

Isoforms	Forward primer (5'-3')	Reverse primer (5'-3')	T _a (°C)	PCR cycles
MHC-1	ACCTTGAGCCCTTCTCT	ACTGTCTTGCCATGTTCTGTCTC	54	28
MHC-2A	GAACAATCCAATACAAATC	TGTGCCTCTCTTCAGTCA	50-45	10td+18
MHC-2X	AGGGTACGTGAACCTGGAAGG	TGCAAAAATCATAAGTACAAAACA	52	28
MHC-2B	CCTGGGACACAGAGTTCTGAAGAGG	GCTTGAGTGCCGCTGATGAGTTGA	58	33

sequences (exon 40) in human and pig (accession number respectively, AF111783 and AB025261). The PCR conditions were: 3 min at 94°C, 40 total cycles with 50 sec at 94°C, 50 sec at annealing temperature progressively reduced from 58 to 52°C in the first twelve cycles, 54°C in the last cycles and an extension step of 50 sec at 72 °C. Five µl of PCR product was electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualised under UV lamp. The PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen) and sequenced (GenBank accession n. AY135646).

RNA expression analysis

Total RNA was extracted from 100 mg of different muscles tissues (as above) using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) and reverse-transcribed with Superscript protocols (Invitrogen, Life technologies, UK) using as primer a mixture of random hexamers. The obtained cDNAs were used as template for RT-PCR expression analysis. Different PCR reactions were conducted under the conditions reported in Table 1, using specific primers designed on the 3'-untranslated regions kindly provided by Prof. Chikuni K. (1, 2A and 2X isoforms). To assess the good quality of RNA and the efficiency of RT reaction a fragment of the bovine β-actin cDNA was amplified.

Two degenerate PCR primers (5' CTCTT(CT)(GT)CTG(AG)G(AG)(GC)ACA(AG) A(GC)TTCT-GAAGA-3' and 5'-ATGGCTTGAGTGCG(CT)-GTGATGAGTTG-3') were designed to amplify a fragment (515 bp) of bovine MHC-2B isoform from *retractor bulbi* cDNA template. The PCR product was cloned into the pCR-II plasmid vector using a TOPO-TA cloning kit (Invitrogen) and sequenced. The nucleotide and putative aminoacid sequences were used to perform, respectively, the BLASTN and the BLASTP searches (available at <http://www.ncbi.nlm.nih.gov/blast>) with default

setting on the complete, non-redundant GenBank database sequences. The new bovine sequence (GenBank accession n. AY227972) was aligned to the related mammalian MHC isoforms using the free Dialign software (available at http://www.genomatix.gsf.de/products/index_dialign.html). The program Mega2 was used to reconstruct phylogenetic tree with the neighbour-joining method.

Subsequently, MHC-2B specific oligonucleotides were designed based on the known 515 bp fragment to perform an RT-PCR on the several muscles examined in the current study (Table 1).

Results

Histochemistry

Samples of *masseter*, *diaphragm*, *longissimus dorsi*, *retractor bulbi* and *rectus lateralis* were stained for m-ATPase after acid and alkaline pre-incubation, using appropriate incubation time and pH which permitted the unambiguous identification of three different fibre types. Figure 1 shows an example of staining on serial sections of *longissimus dorsi* where distinct fibre types can be identified.

Type 1 or slow fibres were easily identified as strong acid stable and the most alkali labile fibre types; these represented the whole fibres population (except for the presence of very few 2C histochemically classified fibres, see later in the text) in the *masseter* muscle, were numerous (about 50%) in *diaphragm*, less abundant in *longissimus* (10%) and only occasionally observed in *retractor bulbi*. In *rectus lateralis* only a few type 1 fibres of small and medium diameter were present. Type 2A fibres were easily distinguished after acid preincubation at pH 4.6 and were more alkaline stable at pH 10.5-10.7 respect than other fibres conventionally classified as type 2B (2* in Figure 1). Type 2A fibres were present in *diaphragm* (where they represent 50% of the fibres, the remaining being type 1, see above) and in *longissimus dorsi* muscles where they are less abundant. In *masseter* and in *diaphragm* some acid resistant fibres, identified as type 1 for their strong acid resistance, showed also a moderate alkali stable activity and could be identified as type 2C (see Figure 3). Fibres with a profile of alkali resistance typical of 2A fibres were also found in *retractor bulbi* and *rectus lateralis*. Conventional type 2B fibres (indicated as 2* in figure 1) appeared to be moderately acid resistant

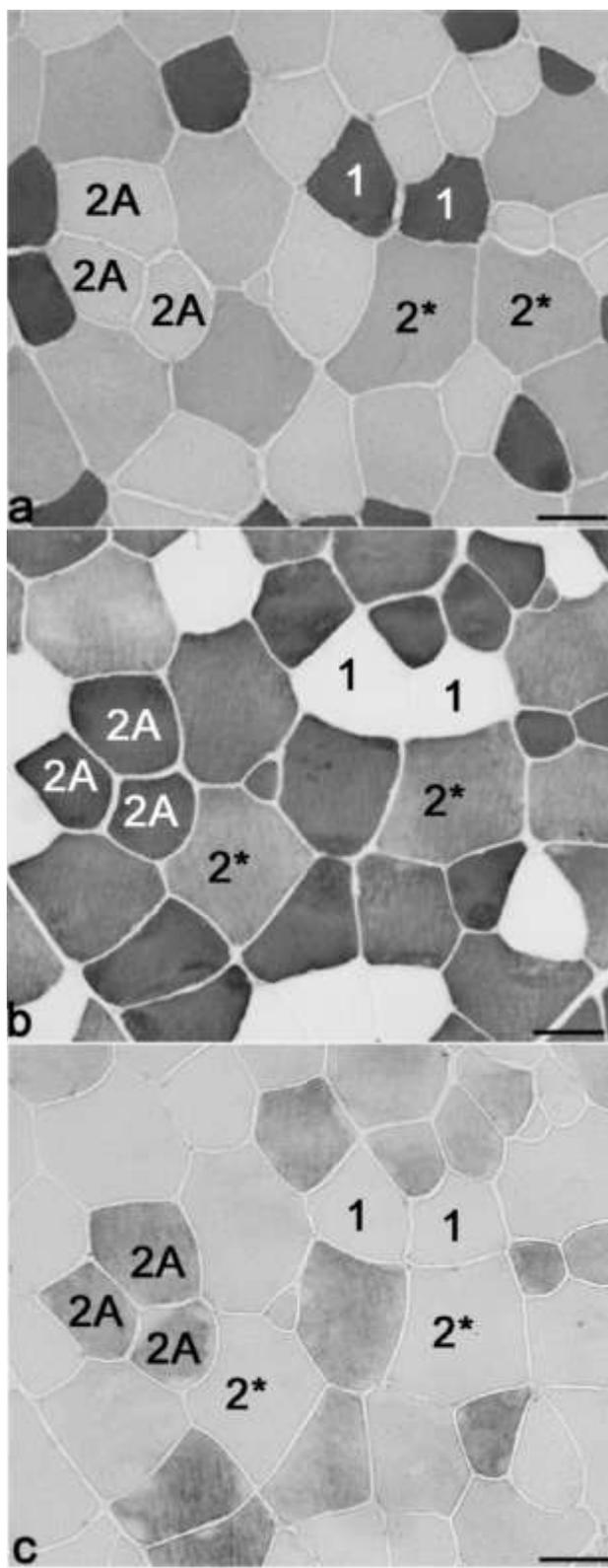


Figure 1. Histochemistry on *longissimus dorsi* muscle. Serial sections were stained for mATPase with a preincubation at (a) acid pH 4.55, at alkali (b) pH 10.5 and (c) pH 10.6. Three fibre types are present: type 1 fibres are evident since they show a strong acid stability and the most alkaline lability; type 2A fibres (the most acid labile after acid preincubation at pH 4.55, the more alkali stable at pH 10.5 and the only fibres positive at 10.6) are easily distinguished from *conventional* type 2B (2*) fibres. Scale bars 40 µm.

(unstained at pH lower than 4.5-4.55) and less alkali resistant than 2A fibres (weakly positive at pH 10.5, negative at pH 10.6). ATPase staining cannot determine whether these *conventional type 2B* fibres express MHC-2B or MHC-2X. In *retractor bulbi* several fibres with variable degrees of alkali and acid resistance were detectable, but it was not possible to classify these fibres on the basis of the simple m-ATPase reaction.

Immunohistochemistry

Serial sections of *masseter*, *diaphragm*, *longissimus dorsi*, *retractor bulbi* and *rectus lateralis* muscles were stained with the antibodies BA-F8, SC-71, BF-35, BF-F3 and BA-G5. Examples are shown in Figure 2 and Figure 3. The immunohistochemical staining with the monoclonal antibodies BA-F8 confirmed the histochemical identification of slow type 1 fibres (Figure 3b). The antibody BF-35 did not react with type 1, was negative or weakly positive with histochemically identified type 2A fibres but was positive in the other type 2 fibres classified histochemically as *conventional 2B* (see Figure 2c). Previous studies in opossum (Sciote and Rowleson, 1998) and in pig (Toniolo *et al.*, 2004) showed that this antibody was specifically reactive with 2X fibres, although it was originally designed to identify 2X fibres since it was positive to all fibre types except for 2X type (Bottinelli *et al.*, 1991) (Schiaffino *et al.*, 1989). This observation suggests that *conventional 2B* fibres are 2X fibres. The antibody SC-71, which was expected to be specific for MHC-2A on the basis of previous studies (Schiaffino *et al.*, 1989) (Bottinelli *et al.*, 1991), did not react specifically with the fibres identified as 2A with m-ATPase, but stained all fast fibres. This surprising result confirmed the observations of Duris and coworkers (Duris *et al.*, 2000) that SC-71 is negative with slow fibres but positive with all fast fibres. Tested at very high dilution SC-71 antibody was negative with 2A fibre but positive with 2X fibres (Figure 2d). The BF-F3, a monoclonal antibody specific for MHC-2B in rat muscles (Bottinelli *et al.*, 1991) (Schiaffino *et al.*, 1989) and moderately positive in pig muscles (Lefaucheur *et al.*, 2002) (Toniolo *et al.*, 2004), was negative in all examined fibres of trunk and limb skeletal muscles but was surprisingly positive in numerous fibres of *retractor bulbi* and *rectus lateralis* (Figure 2e); this fact would suggest the possible presence of MHC-2B in those specialised muscles. Finally, the

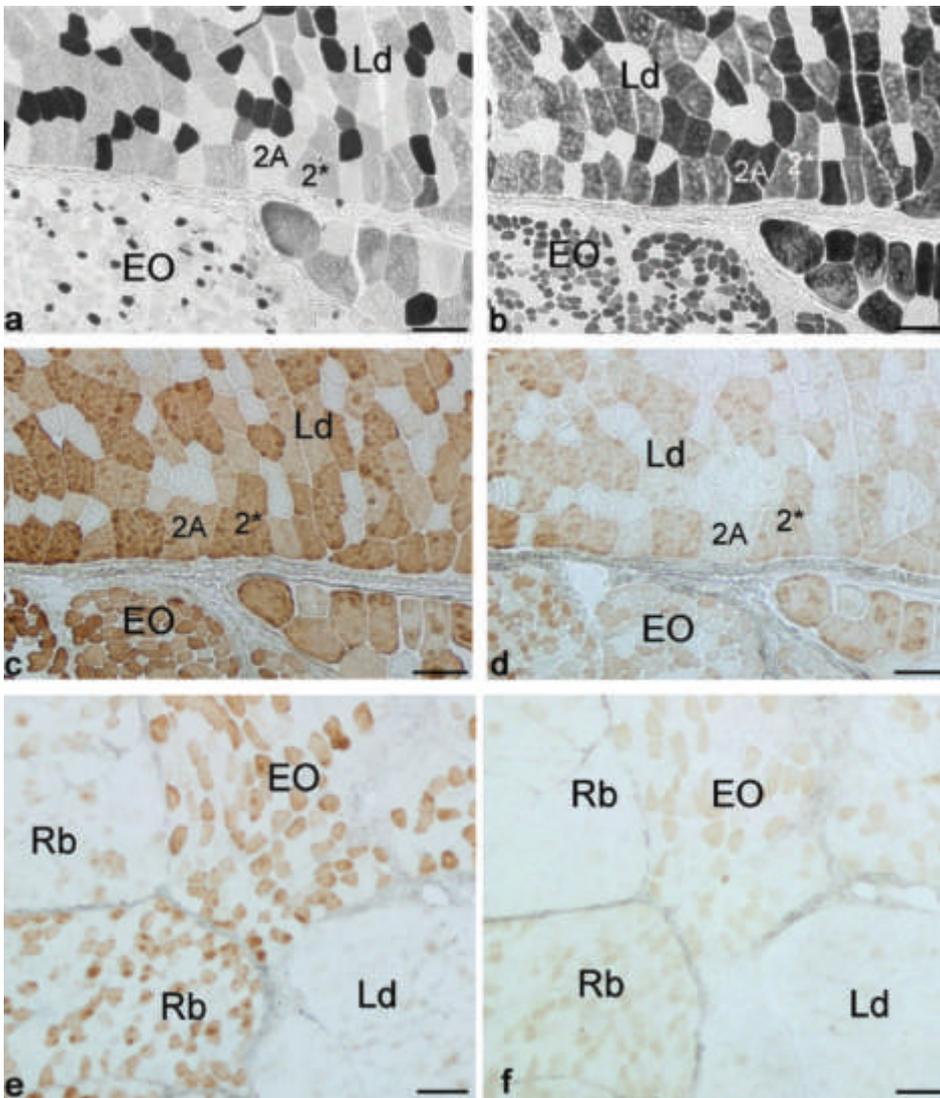


Figure 2. Top four panels (a, b, c, d). Histochemistry and immunohistochemistry on a composite block of *longissimus dorsalis* (Ld) and extraocular (*Rectus lateralis*) (EO) muscles. Serial sections were stained for m-ATPase activity after (a) acid at pH 4.5 and (b) alkaline at pH 10.6 preincubation and with monoclonal antibodies (mAbs) for (c) BF-35 and (d) SC-71. The conventional 2B fibres (2*) are strongly positive to BF-35 mAb suggesting that these fibre are 2x type (c). The histochemical type 2A fibres are moderately positive or negative, depending on the dilution used with BF-35 mAb (c) but are surprisingly negative to SC-71 mAb (d); the latter mAb is therefore not useful to identify 2A fibres. Scale bars 100 μm.

Bottom two panels (e-f). Immunohistochemistry of composite block of extraocular (*rectus lateralis*) (EO), *retractor bulbi* (Rb) and *longissimus dorsalis* (Ld) muscles. Two serial sections stained for (e) BF-F3 and (f) BAG-5 mAbs. Using BF-F3, a mAb specific for 2B-MHC (e), positive fibres are present in EO and Rb muscles but not in skeletal muscles as shown in *longissimus* muscle (Ld). In eye muscles some fibres are also positive to BAG-5 mAb (f), suggesting the presence of MHC- α -cardiac. Scale bars 100 μm.

antibody BA-G5, specific for MHC- α -cardiac was tested and found to be reactive with the alkaline stable fibres of *masseter* and in numerous fibres of *retractor bulbi* and *rectus lateralis* (Figure 2f and Figure 3d).

The combined results of histochemical (m-ATPase reaction) and immunohistochemical staining showed that *masseter* muscle is composed mainly by type 1 fibres expressing MHC-slow (Figure 3b). The composition of the fibres classified histochemically as 2C was clarified by immunohistochemistry. These fibres were negative for SC-71 and BF-35 antibodies but were positive with BA-G5, specific for MHC- α -cardiac (Figure 3d). Thus, it seems likely that histochemically 2C fibres of the *masseter* muscle contain MHC- α -cardiac in addition to MHC-slow. The diaphragm muscle is composed by virtually equal proportions of slow fibres and fast fibres. These latter fibres are negative with

SC-71 and with BF-35 used at low concentration and can be therefore classified as 2A fibres mainly on the basis of the histochemical m-ATPase reaction. The *longissimus dorsalis* muscle is composed by a few type 1 and a few type 2A fibres while an high number of *conventional* 2B fibres which are positive with BF-35 (Figure 3c) and negative with BF-F3 (Figure 2e) were present. These latter fibres can be therefore identified as 2X fibres. *Retractor bulbi* and *rectus lateralis* were studied to find a possible expression of MHC-2B in specialised muscles. The positive reaction of several fibres with BF-F3 is in favour of the view that in these muscles, MHC-2B protein is actually expressed (Figure 2e). Both *retractor bulbi* and *rectus lateralis* express also MHC- α -cardiac (Figure 2f). In *retractor bulbi* fibres are predominantly fast (2A, 2X and likely also 2B) with a scarce presence of slow fibres. In *rectus lateralis* the fibre type distribution is likely

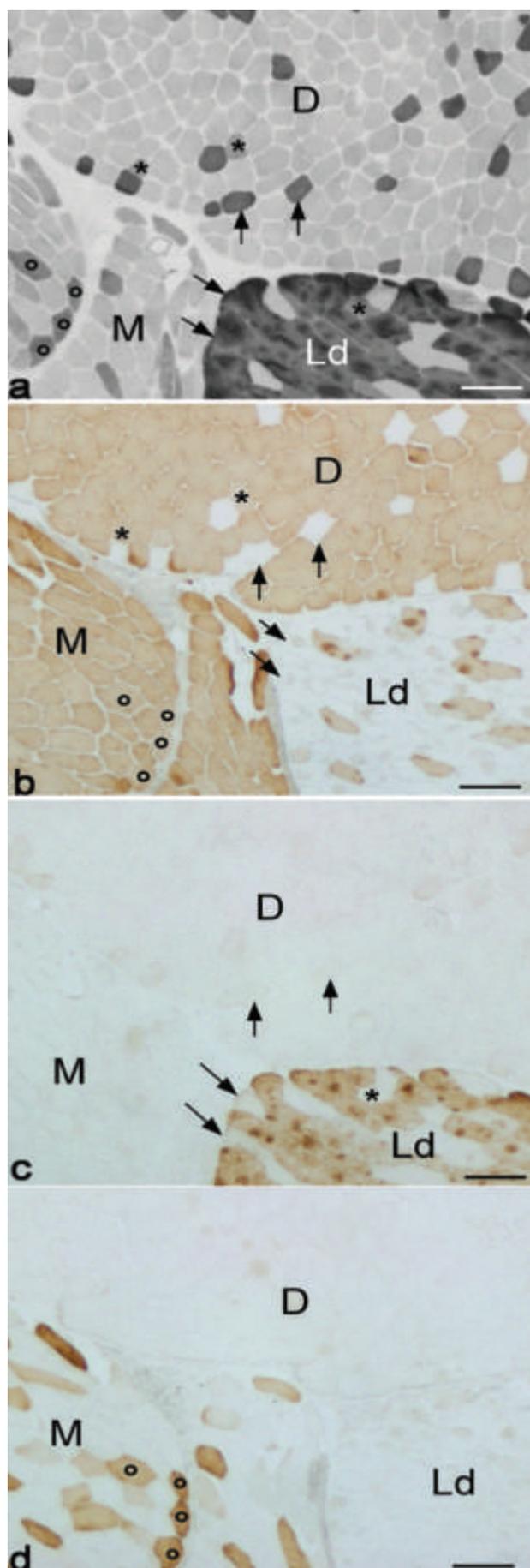


Figure 3. Histochemistry and immunohistochemistry on a composite block of *masseter* (M), *diaphragm* (D) and *longissimus dorsi* (Ld) muscles. Serial sections were stained for mATPase after (a) alkaline preincubation at pH 10.2 and with monoclonal antibodies: (b) BA-F8 specific for MHC-1; (c) BF-35 mainly specific for MHC-2X and (d) BAG-5 for MHC- α -cardiac. Slow or type 1 fibres, negative with mATPase staining after alkaline preincubation (a) and positive to BA-F8 mAb (b), represent the total population in *masseter*, in *diaphragm* are very numerous and in *longissimus* are only a few. Type 2A fibres (arrows) are alkaline stable (a), negative to BA-F8 mAb (b) and also negative to some dilution (1:400 i.e.) of BF-35 (c); these fibres are very few in these samples of *diaphragm* and *longissimus* muscles. In *longissimus* muscle type 2X fibres, alkaline stable at pH 10.2 (a) and positive to BF-35 mAb (c), are the predominant type.

In all three muscles, some fibres (*, °) show an intermediate positivity after a moderate alkaline preincubation (a). In *masseter* these moderately positive fibres (°) are positive to the mAb specific for MHC- α -cardiac (d), suggesting that they co-express MHC- α -cardiac and MHC-1 because all *masseter* fibres are positive to BA-F8 mAb (b). In *diaphragm* and *longissimus* muscles this type (*) likely corresponds to 2C type, as they are negative to BAG-5. Scale bars 100 μ m.

more complex due to the presence of developmental MHC isoforms and MHC-E0.

MHC gene expression

The conclusions reached with the combination of histochemistry and immunohistochemistry were validated with the determination of MHC isoforms using RT-PCR with specific primers.

Samples were loaded onto agarose gel in the following order: *pectoralis* (P), *diaphragm* (D), *longissimus dorsi* (Ld), *masseter* (M), *extensor carpi radialis* (Ecr), *extraocular (rectus lateralis)* (EO) and *retractor bulbi* (Rb). Size standard (s) used was 1Kb Plus DNA Ladder (Invitrogen). The slow type 1 (fragment of 229 bp) was present in all muscles. The fast type 2A (fragment of 189 bp) was absent in *masseter* muscle. The 2X myosin (fragment of 440 bp) was expressed in *pectoralis*, *longissimus dorsi*, *extensor carpi radialis*, *rectus lateralis* (EO) and *retractor bulbi*. The 2B isoform was only expressed in peculiar muscles such as *rectus lateralis* and *retractor bulbi*.

Transcripts of slow, 2A and 2X MHC isoforms were detected using specific primers from the 3' and 5' non-coding region as described in Materials and Methods. Figure 4 shows that in *masseter* only the slow type 1 was detected and in *diaphragm* only the slow type 1 and the fast 2A isoforms were observed whereas in all other muscles examined the expression of type 1, 2A and 2X isoforms were detected.

As a first step to extend the analysis to MHC-2B, it has been used a PCR approach with degenerate primers for amplifying a bovine genomic DNA frag-

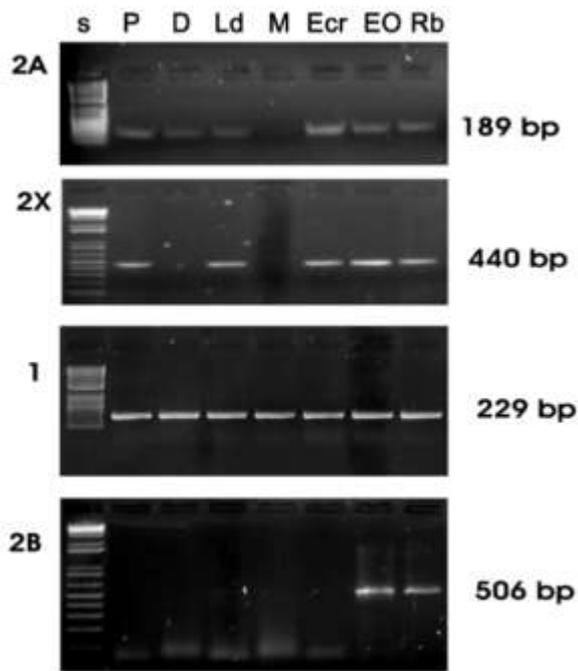


Figure 4. RT-PCR expression analysis of bovine myosin isoform (1, 2A, 2X and 2B) on different skeletal muscle tissue.

ment. The PCR product (GenBank accession n. AY135646) corresponded to a conserved region among MHC isoforms (exon 40) and showed a high aminoacid similarity to both human and pig 2B and 2X isoforms (96%). Thus, to elucidate the presence of 2B transcripts, specific primers have been designed from the exon 16, (forward primer) which presents the highly variable sequence known as loop 2, and from the exon 21 (reverse primer) and used on cDNA obtained from *retractor bulbi*. The PCR product obtained from these experiments (GenBank accession n. AY227972) showed a high sequence similarity to 2B when compared to human and pig sequence (93% nucleotide identity) whereas the nucleotide identity showed a lower score with human 2X (90%), pig 2X (91%) and bovine 2X (91%).

The phylogenetic tree shows an evident evolutionary distance between fast and slow isoforms (dashed line square and continuous line square). The hatched square groups all fast isoforms. However, it is possible to recognise at least four subgroups within the fast family: 2A type is always linked to 2X (dark grey circle), embryonic and extraocular (grey circle) isoforms are well separated from the other fast MHC. The light grey circle represents the 2B MHC subgroup, in which our partial bovine sequence is located together with the

other 2B published sequences. The tree node that divides 2B subgroup from 2A/2X subgroup is supported by a strong bootstrap value. Numbers at tree nodes refer to percent bootstrap values after 500 replicates; the scale bar refers to a phylogenetic distance of 10 nucleotidic substitutions per site.

The neighbour-joining tree (Figure 5) define the position of the novel sequence within the MHC-2B orthologous group together with the other two known 2B sequences (human and porcine). Moreover, strong bootstrap values confirmed that orthologous MHC isoforms are phylogenetically closer than the paralogous isoforms and four major MHC groups (2A, 2X, 2B and 1) are evident in Figure 5. RT-PCR carried out in several bovine muscles with the specific primers for MHC-2B provided unambiguous evidence that MHC-2B is expressed in *retractor bulbi* and extraocular muscles only (see Figure 4, panel 2B). Indeed, the immunohistochemical approach showed that type 2B is present along with the other three MHC isoforms only in these two muscles (see Figure 2e).

Discussion

In this study we showed that the gene coding for MHC-2B is present in the bovine genome although its expression is restricted to specialized muscles. This finding was based on the combined use of immunohistochemical staining and RT-PCR. The monoclonal antibody BF-F3 which had been proved to be specific for MHC-2B (Schiaffino *et al.*, 1989) (Bottinelli *et al.*, 1991) (Lefaucheur *et al.*, 2002) reacted only with muscle fibres of *retractor bulbi* and *rectus lateralis* (muscles where RT-PCR experiments indicated that MHC-2B RNA is present) and did not react with any other trunk or limb muscles (where RT-PCR did not show the presence of the 2B specific RNA). The presence of MHC-2B mRNA in specialized eye muscles has been confirmed by sequencing a cDNA fragment used to design specific primers for RT-PCR experiments.

The expression of the MHC isoforms 1, 2A and 2X has been previously confirmed by RT-PCR in limb and trunk muscles (Chikuni *et al.*, 2004). All three isoforms are expressed in the *longissimus dorsi*, whereas only types 1 and 2A are expressed in *diaphragm* and only type 1 is expressed in *masseter*. This differential distribution has allowed the comparison with the results of m-ATPase histochemical staining. Three types of fibres with specific sensitiv-

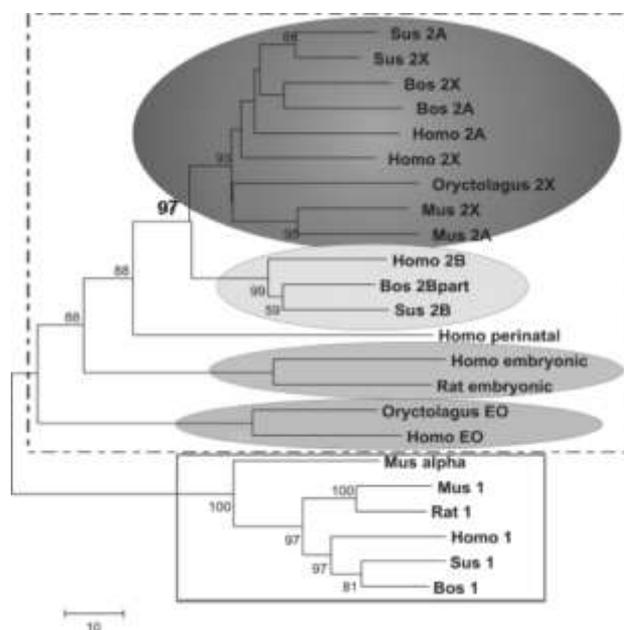


Figure 5. Neighbor-joining tree of mammalian myosin heavy chain isoforms (1, 2A, 2B, 2X, perinatal, embryonic, extraocular and α -cardiac).

ity to alkali or acid pre-incubation can be clearly identified: type 1 and type 2A show the expected reactivity while the third type, conventionally called type 2B, must correspond to 2X fibres on the basis of RT-PCR results. Immunohistochemical approach could only partly give support to this interpretation: as expected type 1 fibres are reactive with BA-F8, well known to be specific for MHC-slow. BF-35 showed a general reactivity with fast fibres, although fibres identified as *conventional 2B* which express MHC-2X, showed a stronger reactivity. Previous studies in opossum (Sciote and Rowleson, 1998) and in pig (Toniolo *et al.*, 2004, for further explanation about the BF35 clone used in this study) have shown that BF35 is specifically reactive with 2X fibres although it was originally described as reactive with all isoforms except for 2X fibres (Schiaffino *et al.*, 1989) (Bottinelli *et al.*, 1991).

SC-71 showed a stronger reaction for 2X fibres respect than 2A fibres and these latter fibres became negative when the antibody was used at very low concentration. Also Duris *et al.* (2000) showed that SC-71 is not able to distinguish type 2 fibres. Probably, the lack of specificity of the antibody SC-71 can be explained on the basis of a sequence similarity between bovine MHC-2X and MHC-2A. The bovine MHC-2A shows very high similarity not only with

orthologous MHC-2A in pig (93%), horse (92%), man (91%), but also with paralogous bovine MHC-2X (92%). The similarity with other MHC-2X is lower; 89% with MHC-2X of pig and horse and 88% with human MHC-2X. These observations could justify the lack of specificity of this antibody. Our results underline that when monoclonal antibodies are used in different species it is necessary to verify their specificity by means of several approaches such as RT-PCR or Western Blotting. In this research it has been chosen the former method since in our previous study conducted in pig (Toniolo *et al.*, 2004) antibodies used in Western blots experiments had not distinguished fast fibres (in particular the 2A from the 2X).

Histochemical m-ATPase staining showed the presence of type 2C fibres in *masseter*. The first interpretation of this type of m-ATP staining is the co-existence of slow and MHC-2A in the same fibre. However RT-PCR did not show expression of MHC-2A in bovine *masseter*, thus excluding this interpretation. Attempts to assess which MHC isoform might be co-expressed with MHC-slow lead to demonstrate the reactivity with an antibody specific for MHC- α -cardiac. This finding is unexpected and interesting. The expression of MHC- α -cardiac had been demonstrated in masticatory muscles of several species but not yet in ruminants: in marsupials MHC- α -cardiac is the only isoform present in *masseter* (Hoh *et al.*, 2000) while in rabbit and man it is present together with slow and 2A isoforms (Bredman *et al.*, 1991) (d'Albis *et al.*, 1991). In this study we observed that in bovine *masseter*, the α -cardiac isoform is co-expressed with the slow type in some hybrid fibres. As suggested by Hoh *et al.*, (2000) in the case of marsupials, the expression of this particular isoform may be related with specific features of mastication in herbivores because the presence of α -cardiac fibres in masticatory muscles ensure rapid comminution of food into fine particles necessary for efficient fermentation in the gastro-enteric tract.

In conclusion, in this study we provided the first evidence in favour of the expression of MHC-2B in some specialized bovine muscles as *extraocular* and *retractor bulbi*. Fibres expressing MHC-2B are characterized by the highest speed of shortening in all animal species until now studied (Toniolo *et al.*, 2004) (Pellegrino *et al.*, 2003): it is likely that the mechanical features of these fibres can be exploited in the best way to produce fast rotations of the eye ball. The expression pattern of bovine MHC iso-

forms is thus very different from that we have recently reported in pig (Toniolo *et al.*, 2004), where MHC-2B is widely expressed in trunk and limb muscles. Since in the Toniolo *et al.* (2004) paper we hypothesised that the abundant expression of MHC-2B in porcine muscles was a consequence of selective pressure leading to increase meat production and neglect posture or locomotion requirements, it will be of interest to study whether in bovine breeds characterized by exceptional muscle development, known as *double muscling phenotype*, the 2B isoform is more widely expressed.

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