

ARTICLE

Evidence for Three Fast Myosin Heavy Chain Isoforms in Type II Skeletal Muscle Fibers in the Adult Llama (*Lama glama*)

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SUMMARY Skeletal muscle fiber types classified on the basis of their content of different myosin heavy chain (MHC) isoforms were analyzed in samples from hindlimb muscles of adult sedentary llamas (*Lama glama*) by correlating immunohistochemistry with specific anti-MHC monoclonal antibodies, myofibrillar ATPase (mATPase) histochemistry, and quantitative histochemistry of fiber metabolic and size properties. The immunohistochemical technique allowed the separation of four pure (i.e., expressing a unique MHC isoform) muscle fiber types: one slow-twitch (Type I) and three fast-twitch (Type II) phenotypes. The same four major fiber types could be objectively discriminated with two serial sections stained for mATPase after acid (pH 4.5) and alkaline (pH 10.5) preincubations. The three fast-twitch fiber types were tentatively designated as IIA, IIX, and IIB on the basis of the homologies of their immunoreactivities, acid denaturation of their mATPase activity, size, and metabolic properties expressed at the cellular level with the corresponding isoforms of rat and horse muscles. Acid stability of their mATPase activity increased in the rank order IIA>IIX>IIB. The same was true for size and glycolytic capacity, whereas oxidative capacity decreased in the same rank order IIA>IIX>IIB. In addition to these four pure fibers (I, IIA, IIX, and IIB), four other fiber types with hybrid phenotypes containing two (I+IIA, IIX, and IIXB) or three (IIAXB) MHCs were immunohistochemically delineated. These frequent phenotypes (40% of the semitendinosus muscle fiber composition) had overlapped mATPase staining intensities with their corresponding pure fiber types, so they could not be delineated by mATPase histochemistry. Expression of the three fast adult MHC isoforms was spatially regulated around islets of Type I fibers, with concentric circles of fibers expressing MHC-IIA, then MHC-IIX, and peripherally MHC-IIB. This study demonstrates that three adult fast Type II MHC isoproteins are expressed in skeletal muscle fibers of the llama. The general assumption that the very fast MHC-IIB isoform is expressed only in small mammals can be rejected. (*J Histochem Cytochem* 49:1033–1044, 2001)

KEY WORDS

skeletal muscle
muscle fiber
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SKELETAL MUSCLE CELLS fall into several specialized classes, termed fiber types, which show differences in morphological, contractile, and metabolic properties. Myosin is the most abundant protein in muscle and makes up the primary component of the thick fila-

ments. Its molecule is composed of two heavy chains (MHCs) and four light chains. Both kinds of chains occur as several distinct isoforms coded by different genes (DeNardi et al. 1993). These isoforms represent the best markers of muscle fiber diversity (Schiaffino and Reggiani 1996; Pette and Staron 1997; Bottinelli and Reggiani 2000). Different combinations of MHC isoforms may occur in the same fiber, but the predominant isoform is the main determinant of their functional properties, such as the speed of contraction and fatigue resistance.

From the literature available, it has been suggested

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that the molecular diversity of adult skeletal muscle fibers is species-specific, and important physiological differences between species related to body size have already been reported (Pette and Staron 1990; Schiaffino and Reggiani 1996). In adult skeletal muscle of certain small species of mammals (i.e., rat, mouse, and rabbit), three fast MHC isoforms, termed IIA, IIX, or IID (henceforth IIX), and IIB, have been identified (Bär and Pette 1988; Schiaffino et al. 1989). The differential distribution of these MHCs defines three pure fast fiber types containing a single MHC isoform (IIA, IIX, and IIB) and some hybrid fiber population containing two or three fast MHCs (Gorza 1990; Hämmäläinen and Pette 1993). The relative abundance of the fast MHC IIB isoform decreases among mammals with increasing body size and is not expressed in humans (Smerdu et al. 1994), carnivores (Talmadge et al. 1996), ruminants (Tanabe et al. 1998), and horses (Rivero et al. 1999). In all these species, the differential distribution of their fast MHCs defines only two fast fiber types containing a single MHC isoform (Types IIA and IIX) and an intermediate hybrid fiber population containing the two fast MHCs (Type IIX). The differences among species in the expression of fast MHCs are believed to be based on the structural and functional divergences in homologous MHC isoforms (Schiaffino and Reggiani 1996; Canepari et al. 2000). Therefore, the maximal velocity of shortening of muscle fibers expressing homologous MHC isoforms greatly decreases with increasing body size (Rome et al. 1990). The lack of expression of the MHC-IIB isoform in large mammals has been related to body size and muscle fiber length (Rome et al. 1990). However, some recent studies in adult pigs have showed evidence of the existence of all three fast MHC isoforms, including the MHC-IIB isoform (Lefaucheur et al. 1998; Tanabe et al. 1999), suggesting that the lack of this MHC isoform is not merely a matter of body size (Bottinelli and Reggiani 2000).

The llama (*Lama glama*) is a South American cud-chewing mammal related to the camel, but smaller and without a hump. It was domesticated in the Andes and is now used as a beast of burden and a source of food. The adult body weight of this mammal is 101 ± 18 kg (mean \pm SD) for females and 116 ± 22 kg for males (Nuevo-Freire 1994). The average productive life is about 8–10 years, whereas natural longevity is 14–15 years (Nuevo-Freire 1994). To the authors' knowledge, no previous studies have characterized skeletal muscle fiber types in llamas according to the MHC isoform they express. Therefore, this investigation could increase our current knowledge about the peculiarities and locomotor system in this species. Furthermore, the muscle fiber type classification based on protein expression patterns of MHC could clarify the relationship between meat quality and skeletal muscle

fiber types (Henckel 1989). The working hypothesis is that this species expresses one slow and two fast MHC isoforms because of its relative high body weight. The main purpose of this study was to characterize skeletal muscle fiber types in llamas according to the MHC isoform they express, revealed by immunohistochemistry with a panel of specific anti-MHCs monoclonal antibodies and myofibrillar ATPase histochemistry after acid and alkaline denaturation. Our results show evidence for the existence of four distinct MHC isoforms in adult skeletal muscle of the llama, one slow and three fasts, including the MHC IIB. The differential distribution of these isoforms categorizes four pure fiber types containing a single MHC isoform and a very large number of hybrid fibers with two or three different isoforms.

Materials and Methods

Six elderly (8–10-year-old) and clinically healthy llamas (all males) were used. Semitendinosus muscle, biceps femoris muscle, and vastus lateralis muscle were dissected and cleaned of connective tissues. Muscle samples ($1 \times 1 \times 0.5$ cm) were removed from the midbelly region of these muscles, frozen by immersion in isopentane kept in liquid nitrogen, and stored at -80°C until analyzed.

Samples were transferred to a cryostat at -20°C , serially sectioned at $10\text{-}\mu\text{m}$, and mounted on poly-L-lysine-coated glass slides for immunohistochemistry and histochemistry. Samples from the rat extensor digitorum longus muscle and biopsies from the equine gluteus medius muscle were also removed and used as standard references of skeletal muscle of two well-known species expressing three (rat) and two (horse) fast MHC isoforms. These samples were frozen and processed in the same way as the llama samples.

Serial sections were reacted with a panel of monoclonal antibodies (MAbs) specific for MHC isoforms (Table 1). The specificity of the majority of these MAbs for rat (Schiaffino et al. 1986, 1989) and equine (Rivero et al. 1999) MHC isoforms has been demonstrated. The MAb BF-G6 reacts with rat and human embryonic MHC, but also, when

Table 1 Specificity of monoclonal antibodies (MAbs) against adult rat skeletal myosin heavy chain (MHC) isoforms used in the study^a

MAb	Myosin heavy chain isoforms ^b			
	I	IIA	IIX	IIB
BA-F8	+	–	–	–
SC-75	–	+	+	+
SC-71	–	+	–	–
BF-35	+	+	–	+
S5-8H2	+	–	+	+
BF-G6 ^c	–	–	–	+

^aAccording to Schiaffino et al. (1986, 1989), except for the MAb S5-8H2 (Rivero et al. 1999).

^b+, Positive reaction for that MAb with that specific MHC isoform; –, no reaction between MAb and MHC isoform.

^cAntibody BF-G6 reacts primarily with the embryonic MHC isoform, but it also binds MHC IIB at a lower affinity (Schiaffino et al. 1986).

used in a more concentrated dilution, with the adult rat MHC IIB isoform (Schiaffino et al. 1986). The MA S5-8H2 reacts with all adult rat, human (unpublished observation), and horse (Rivero et al. 1999) MHCs, except for MHC IIA. An additional serial section was incubated without the primary antibody and used as standard to control nonspecific immunoreactivity. The avidin–biotin–peroxidase complex immunohistochemical procedure was used as previously described (Rivero et al. 1999).

Additional sections were stained for qualitative myofibrillar ATPase activity and acid (pH 4.2–4.6, 2 min) and alkaline (range of pH 10.3–10.6, 10 min) preincubations by using a modification (Nwoye et al. 1982) of the Brooke and Kaiser (1970) method. The optimal pH of the preincubation solution was searched separately for each muscle and for each species in order to distinguish at least three levels of staining intensities after both acid and alkaline preincubations. Two additional serial sections were also stained for succinic dehydrogenase (SDH; Blanco et al. 1988) and α -glycerophosphate dehydrogenase (GPD; Martin et al. 1985), and used to assess oxidative and glycolytic capabilities of single muscle fibers, respectively.

To characterize fiber types according to their MHC content expressed at the protein level and to determine the relative proportion and mean fiber size, a region of the cross-sections containing 150–175 fibers was selected for further analyses. The sections stained for immunohistochemistry, myofibrillar ATPase histochemistry, and metabolic properties (SDH and GPD) were surveyed to find regions free of artifact. Serial sections were visualized and analyzed using a Leica DMLS microscope (Leica Microsistemas; Barcelona, Spain), a Leica high-resolution color charge-coupled device camera (Leica Microsistemas), an eight-bit Matrox meteor frame-grabber (Matrox Electronic Systems; Barcelona, Spain), combined with image-analyzing software (Visiolog 5, Noemi; Microptic, Barcelona, Spain). With the use of the mATPase staining after acid preincubation, a fiber mask was drawn along the cell borders of the desired number of fibers. Images of the remaining immunohistochemical and histochemical sections were then fitted into the fiber mask. Single fibers were subsequently identified and qualitatively classified as positive or negative for a given immunohistochemical reaction. The fiber type distribution of each muscle biopsy was established by immunohistochemistry.

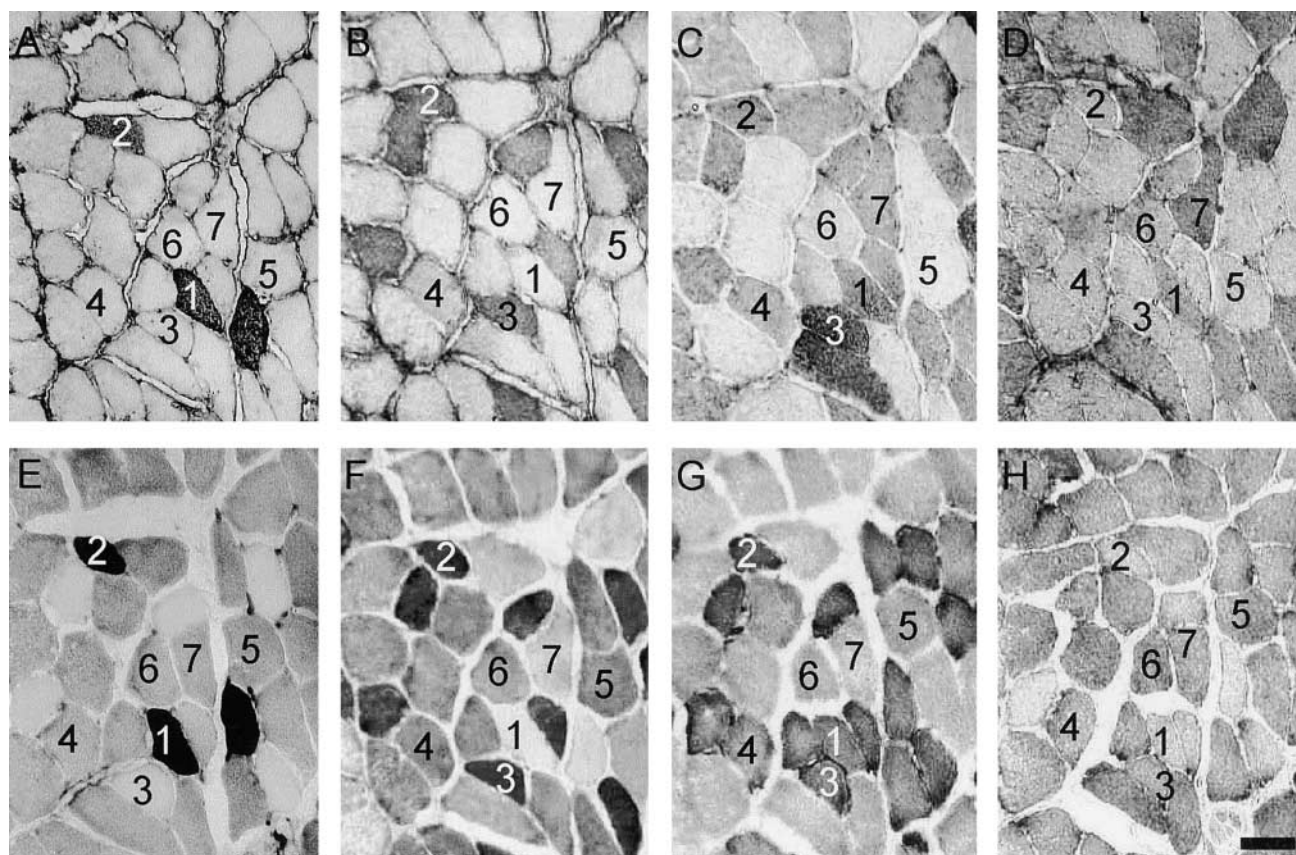


Figure 1 Serial cross-sections of rat control extensor digitorum longus muscle stained by immunohistochemistry with a number of MABs against specific myosin heavy chain (MHC) isoforms (A–D; see Table 1 for specificities) and by enzyme histochemistry of myofibrillar ATPase, succinic dehydrogenase, and α -glycerophosphate dehydrogenase (E–H). (A) BA-F8 MAb (anti-MHC-I). (B) SC-71 (anti-MHC IIA) MAb. (C) BF-35 (anti-MHCs I+IIA+IIB) MAb. (D) BF-G6 (anti-MHC IIB) MAb. (E,F) Myofibrillar ATPase activity after preincubations at pH 4.35 (E) and pH 10.5 (F). (G,H) Succinate dehydrogenase (G) and α -glycerophosphate dehydrogenase (H) activities. The fibers labeled 1, 3, 5, and 7 are “pure” fibers containing MHC I, MHC IIA, MHC IIX, and MHC IIB, respectively. Fibers 2, 4, and 6 are “hybrid” fibers containing MHC I plus MHC IIA (2), MHC IIA plus MHC IIX (4), and MHC IIX plus MHC IIB (6). Bar = 50 μ m.

For each fiber analyzed, a mean optical density (OD) was determined for qualitative mATPase after acid and alkaline preincubation, as well as for the SDH and GPD reactions. In addition, the cross-sectional areas of the same individual muscle fibers were determined in the SDH histochemical reaction. Measurements for each fiber were made in duplicate in two consecutive serial sections, and the mean of both was used to quantify the intensity of the reaction and size of an individual fiber.

Quantitative data were averaged according to fiber type and differences between mean values analyzed by a one-way ANOVA. In the presence of a significant F ratio, post hoc comparisons of means were provided by a Fisher's least significant difference test. Statistical significance was accepted at $p < 0.05$.

Results

Rat

Seven muscle fiber types were delineated using IHC MAb against specific MHC isoforms in the control sample of rat muscle (Figures 1A–D; Table 2). Four pure muscle fiber types were identified according to their MHC content: I, IIA, IIX, and IIB (e.g., fibers 1, 3, 5, and 7, respectively, in Figure 1). A few fibers (e.g., fiber 2 in Figure 1) demonstrated coexpression of MHC I and MHC IIA and corresponded to classical IIC fibers (Staron and Hikida 1992). It was possible to distinguish hybrid fibers coexpressing two fast isoforms (IIAX and IIXB; e.g., fibers 4 and 6 in Figure 1).

The four pure fiber types (I, IIA, IIX, and IIB) were also delineated by myofibrillar ATPase (mATPase) histochemistry using combined staining after acid (pH 4.35) and alkaline (pH 10.5) preincubations (Figures 1E and 1F). Type I fibers stained dark after acid preincubation and very light after alkaline preincubation (e.g., fiber 1 in Figures 1E and 1F). The reverse was true for IIA fibers (e.g., fiber 3 in Figures 1E and 1F). Type IIX and IIB fibers stained medium after preincubation at pH 4.35 (e.g., fibers 5 and 7, respectively, in Figure 1E), but IIX fibers stained darker than IIB fibers after alkaline pretreatment (Figure 1F). Quantitative differences in the intensity of staining for mATPase after both acid and alkaline preincubations of these four main fiber types are presented in Table 3. Consequently, these four fiber types could be objectively discriminated with only two sections stained for mATPase histochemistry (Figure 2A). In addition, intermediate hybrid fibers containing MHC I plus MHC IIA were also delineated with these two mATPase histochemical reactions (Figure 2A), because they stained medium to dark after both acid and alkaline preincubations (e.g., fiber 2 in Figures 1E and 1F). Conversely, fast hybrid fibers (IIAX and IIXB) had mean ATPase activities intermediate between those of their respective pure MHC fiber types (Table 3). As a consequence, all these fibers had overlapping ATPase activities and could not be objectively divided into discrete categories (Figure 2A).

Table 2 Immunohistochemical characterization of skeletal muscle fiber types in rat, horse, and llama according to the myosin heavy chain isoform they express^a

MAb	Muscle fiber types ^b							
	1	2	3	4	5	6	7	8
Rat								
BA-F8	+	+	–	–	–	–	–	–
SC-75	–	+	+	+	+	+	+	+
SC-71	–	+	+	+	–	–	–	–
BF-35	+	+	+	+	–	+	+	+
S5-8H2	+	+	–	+	+	+	+	+
BF-G6	–	–	–	–	–	±	±	±
Horse								
BA-F8	+	+	–	–	–	–	–	–
SC-75	–	+	+	+	+	+	+	+
SC-71	–	+	+	+	–	–	–	–
BF-35	+	+	+	+	–	–	–	–
S5-8H2	+	+	–	+	+	+	+	+
BF-G6	+	+	+	–	–	–	–	–
Llama								
BA-F8	+	+	–	–	–	–	–	–
SC-75	–	+	+	+	+	+	+	+
SC-71	–	+	+	+	+	+	–	+
BF-35	+	+	+	+	–	–	–	+
S5-8H2	+	+	–	+	+	+	+	+
BF-G6	–	–	–	–	–	+	+	+

^a+, positive reaction for that specific fiber type with that monoclonal antibody; –, negative reaction for that specific fiber type with that monoclonal antibody; ±, intermediate reaction for that specific fiber type with that monoclonal antibody.

^bThe number of each fiber type (1 to 8) corresponds to those shown in figures.

Muscle fiber types expressing different MHC isoforms also showed important differences in both their intensity of staining for SDH and GPD and their mean cross-sectional areas (Figures 1G and 1H and Table 3).

Horse

Five different fiber populations were demonstrated immunohistochemically according to the MHC isoform they express in the horse gluteus medius muscle (Figures 3A–3E; Table 2). Three were pure fibers containing a unique MHC isoform and were identified as Types I, IIA, and IIX (e.g., fibers labeled 1, 3, and 5 in Figure 3). A few fibers were demonstrated to be hybrid fibers coexpressing MHC I and MHC IIA (e.g., fiber 2 in Figure 3). Many other fibers were hybrid fibers containing the two fast MHC isoforms, i.e., IIA and IIX (e.g., fiber 4 in Figure 3).

On the basis of mATPase reactions after acid (pH 4.45) and alkaline (pH 10.35) preincubations, equine muscle fibers could be objectively divided into three categories (Figures 3F and 3G). Type I fibers were acid-stable and alkaline-labile (e.g., fiber 1 in Figures 3F and 3G). Type IIA fibers were acid-labile and partially alkaline-stable (e.g., fiber 3 in Figures 3F and 3G). Type IIX fibers were partially acid-stable and alkaline-stable (e.g., fiber 5 in Figures 3F and 3G).

Table 3 Mean (\pm SD) optical densities (OD) of myofibrillar ATPase (mATPase) activity after acid and alkaline preincubations, succinic dehydrogenase activity (SDH), α -glycerophosphate dehydrogenase (GPD), and cross-sectional area (CSA, μm^2) of skeletal muscle fiber types in rat, horse, and llama (semitendinosus muscle) according to the myosin heavy chain isoform they express^a

Species/Variable	Muscle fiber types ^b							
	1 I	2 I+IIA	3 IIA	4 IIAX	5 IIX	6 IIXB	7 IIB	8 IIAXB
Rat (n=500)	(n=35)	(n=0)	(n=125)	(n=35)	(n=105)	(n=45)	(n=145)	
mATPase, pH 4.35	0.97 \pm 0.03	0.71 \pm 0.03	0.31 \pm 0.05 ^A	0.32 \pm 0.19 ^A	0.46 \pm 0.09 ^B	0.42 \pm 0.06 ^B	0.43 \pm 0.08 ^B	
mATPase, pH 10.5	0.26 \pm 0.06 ^A	0.53 \pm 0.09	0.75 \pm 0.12	0.56 \pm 0.14 ^B	0.50 \pm 0.10 ^B	0.35 \pm 0.06	0.25 \pm 0.06 ^A	
SDH	0.52 \pm 0.05 ^A	0.55 \pm 0.06 ^{AB}	0.60 \pm 0.09 ^B	0.46 \pm 0.15 ^A	0.32 \pm 0.08	0.24 \pm 0.07 ^C	0.21 \pm 0.07 ^C	
GPD	0.17 \pm 0.02 ^A	0.19 \pm 0.00 ^A	0.25 \pm 0.04 ^B	0.26 \pm 0.01 ^B	0.34 \pm 0.06	0.40 \pm 0.05	0.43 \pm 0.08	
CSA	1655 \pm 272 ^A	1853 \pm 196 ^A	1810 \pm 486 ^{AB}	2271 \pm 555 ^B	2561 \pm 729 ^C	2529 \pm 345 ^C	2900 \pm 693	
Horse (n=410)	(n=85)	(n=5)	(n=200)	(n=80)	(n=40)			
mATPase, pH 4.45	0.77 \pm 0.05 ^A	0.68 \pm 0.01 ^A	0.28 \pm 0.06	0.40 \pm 0.09	0.50 \pm 0.06			
mATPase, pH 10.35	0.27 \pm 0.03	0.54 \pm 0.04 ^A	0.38 \pm 0.07	0.49 \pm 0.08 ^A	0.51 \pm 0.04 ^A			
SDH	0.49 \pm 0.03 ^A	0.50 \pm 0.02 ^A	0.46 \pm 0.05 ^A	0.36 \pm 0.03	0.24 \pm 0.08			
GPD	0.15 \pm 0.04	0.22 \pm 0.01 ^A	0.26 \pm 0.03 ^A	0.35 \pm 0.07	0.64 \pm 0.09			
CSA	1645 \pm 272	2134 \pm 645	2788 \pm 809	3120 \pm 915	4324 \pm 995			
Llama (n=1000)	(n=55)	(n=10)	(n=130)	(n=50)	(n=320)	(n=250)	(n=145)	(n=40)
mATPase, pH 4.5	0.89 \pm 0.10 ^A	0.91 \pm 0.05 ^A	0.03 \pm 0.02	0.23 \pm 0.24 ^B	0.21 \pm 0.09 ^B	0.65 \pm 0.08 ^C	0.65 \pm 0.04 ^C	0.62 \pm 0.08 ^C
mATPase, pH 10.5	0.02 \pm 0.01	0.78 \pm 0.06 ^{AB}	0.30 \pm 0.10	0.54 \pm 0.12 ^{BC}	0.70 \pm 0.11 ^A	0.50 \pm 0.11 ^C	0.49 \pm 0.10 ^C	0.48 \pm 0.13 ^C
SDH	0.85 \pm 0.04 ^A	0.80 \pm 0.02 ^{AB}	0.66 \pm 0.12 ^B	0.55 \pm 0.16 ^C	0.44 \pm 0.09 ^D	0.43 \pm 0.10 ^{CD}	0.34 \pm 0.03	0.59 \pm 0.06 ^C
GPD	0.13 \pm 0.05	0.20 \pm 0.03	0.29 \pm 0.05 ^A	0.28 \pm 0.06 ^A	0.39 \pm 0.06 ^B	0.52 \pm 0.11	0.67 \pm 0.10	0.38 \pm 0.03 ^B
CSA	1435 \pm 507 ^A	1630 \pm 608 ^A	1794 \pm 873 ^A	2204 \pm 789	3172 \pm 1572 ^B	3999 \pm 857 ^C	4040 \pm 1533 ^C	3135 \pm 612 ^B

^aWithin a row, means with the same letter are not statistically different ($p>0.05$).

^bThe number of each fiber type (1 to 8) corresponds to those shown in Figures.

Quantitative differences observed in the staining intensity of the three major fiber types for both mATPase methods were statistically significant (Table 3), and they could be discriminated into discrete clusters of fibers (Figure 2B). Type C fibers were also histochemically delineated (e.g., fiber 2 in Figures 3F and 3G). A continuum in the staining intensity for mATPase was observed, however, between pure IIA and IIX fibers (Figures 3F and 3G). In fact, hybrid IIAX fibers (e.g., fiber 4 in Figures 3F and 3G) had lower mATPase activity after acid preincubation than pure IIX fibers, but this activity was not different between the two fiber types after alkaline pretreatment (Table 3). As a consequence, hybrid Type IIAX fibers were graphically scattered across their respective pure MHC fiber types (Figure 2B).

The MHC content also had a significant impact on SDH and GPD activities, as well as on areas, of equine skeletal muscle fiber types (Figures 3H and 3I; Table 3).

Llama

Eight different fiber populations were demonstrated immunohistochemically in hindlimb muscles of adult llamas with the panel of MAbs specific for MHC isoforms used in the present study (Figures 4A–4F; Table 2). These phenotypes were classified as four pure fibers containing a single MHC isoform (named I, IIA, IIX, and IIB) and four hybrid fibers with two (i.e., I+IIA, IIAX, and IIXB) or three (i.e., IIAXB) different

MHC isoforms. Despite this, the immunoreactivity patterns of these MAbs to MHC isoforms in llama skeletal muscle fibers were not identical to those recorded in rat and horse skeletal muscle fibers (Table 2), and the results of the present study are not conclusive regarding the identity of the MHC isoforms expressed in llama skeletal muscle. However, we have adopted a conventional terminology to describe the various muscle fiber phenotypes found in this species to maintain a consistent nomenclature across different species.

On the basis of mATPase reactions after acid (pH 4.5) and alkaline (pH 10.5) preincubations, the muscle fibers could be divided into four basic categories (Figures 4G, 4H, 5A, and 5B). Type I fibers stained dark after acid and light after alkaline preincubations (e.g., fiber 1 in Figures 4G and 4H). Type IIA stained light after acid preincubation and medium after alkaline pretreatment (e.g., fiber 3 in Figures 4G and 4H). Type IIX fibers had moderate mATPase activity after acid and high activity after alkaline preincubations (e.g., fiber 5 in Figures 4G and 4H). Type IIB fibers also stained medium by mATPase after acid and alkaline preincubations, but they stained darker than IIX fibers after acid pretreatment (e.g., fiber 7 in Figures 4G and 4H). To sum up, after acid preincubation, Type I fibers had the highest mATPase activity, followed in the rank order by IIB>IIX>IIA (Figure 5A; Table 3). The mATPase activity after alkaline preincubation increased in rank order I<IIA<IIB<IIX (Figure

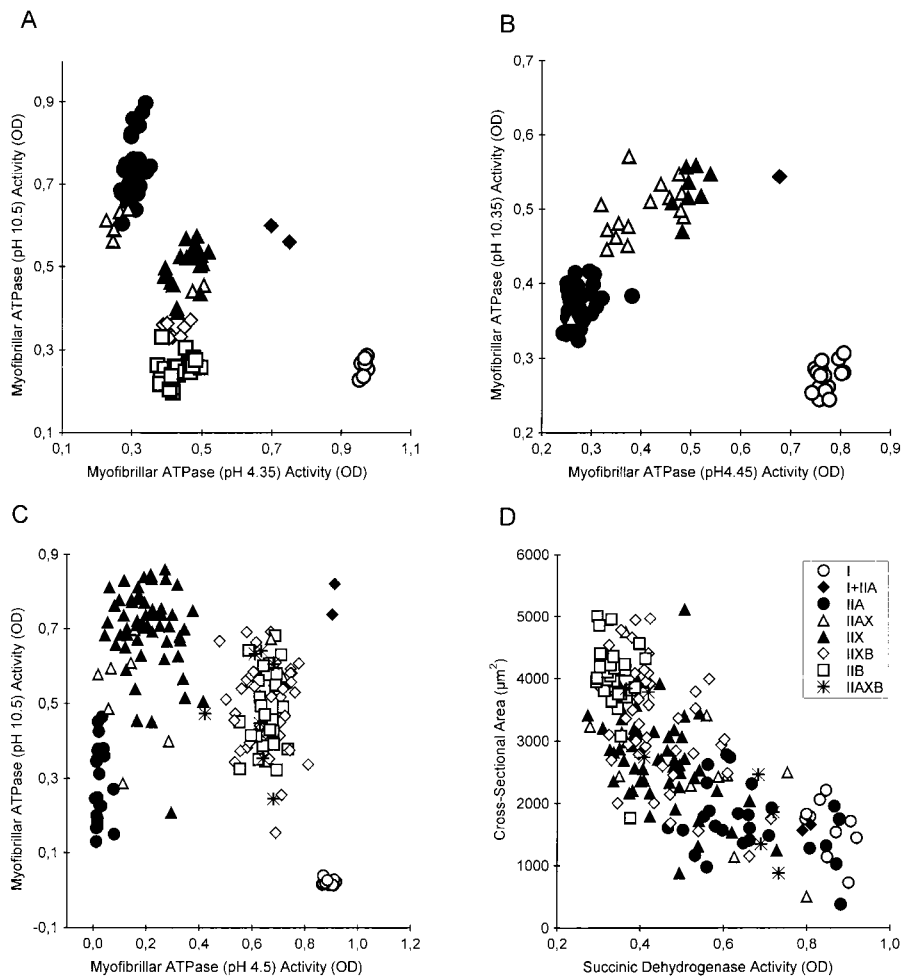


Figure 2 Relationship between the optical density (OD) of individual muscle fibers identified according to the myosin heavy chain they express and stained for qualitative myofibrillar ATPase after acid and alkaline pre-incubations for rat (A), horse (B), and llama (C) muscles. Clusters of four (rat and llama) and three (horse) pure (i.e., expressing a unique myosin heavy chain isoform) fiber could be objectively delineated, but hybrid fast fibers were scattered between their corresponding pure fiber phenotypes. The linear relationship between the OD of individual muscle fibers stained with succinic dehydrogenase and cross-sectional area for the llama muscle is also shown in D.

5B; Table 3). Once again, hybrid fibers had intermediate staining intensities for mATPase histochemistry (Table 3). When the relationship between acid and alkaline ATPase activities of the same individual fibers was plotted, a clear grouping of muscle fiber types with homogeneous MHC content was possible in the llama (Figure 2C). Hybrid C fibers also were delineated, but fast hybrid fibers (i.e., IIAX, IIXB, and IIAXB) were admixed with their respective MHC pure phenotypes (Figure 2C).

The staining intensities of SDH (Figures 4I and 5C) and GPD (not shown) activities also formed a continuum, but differences could be elicited between fiber types of presumed homogeneous MHC content (Table 3). Type I fibers had the highest SDH activity, followed in rank order by IIA>IIX>IIB. The staining intensities of GPD reaction was consistently ranked according to fiber type such that IIB>IIX>IIA>I. Hybrid fibers generally showed intermediate SDH and GPD activities in between their respective pure MHC fiber types. The cross-sectional areas of llama muscle fibers also varied significantly according to the MHC isoform

they express (Table 3). Type I and IIA fibers were the smallest, type IIB the largest, and type IIX of intermediate fiber size. There was an inverse relationship between fiber area and fiber SDH activity ($r=0.81$, $p<0.001$) in the llama muscle, but this relationship did not allow a certain grouping of myofibers (Figure 2D).

After a population of 972 fibers in the semitendinosus muscle of the llama were examined, 58.7% of fibers were pure phenotypes (i.e., expressing a single MHC isoprotein), whereas the remaining 41.3% were hybrid fibers with two or even three MHCs. Within the pure phenotypes, IIX fiber was the most common (37.9%), followed in rank order by IIB (14.4%), IIA (3.6%), and I (2.8%). The most common hybrid phenotype was IIXB (23.5%), followed by IIAXB (11.3%), IIAX (6.1%), and I+IIA (0.4%).

Distribution of the three fast fiber types in skeletal muscle fiber types was spatially regulated around typical islets of Type I fibers (Figure 6A). Fibers expressing MHC IIA were contiguous to those expressing MHC I (Figures 6B and 6C). Pure IIX fibers were in the direct vicinity of Type I and IIA fibers, and hybrid

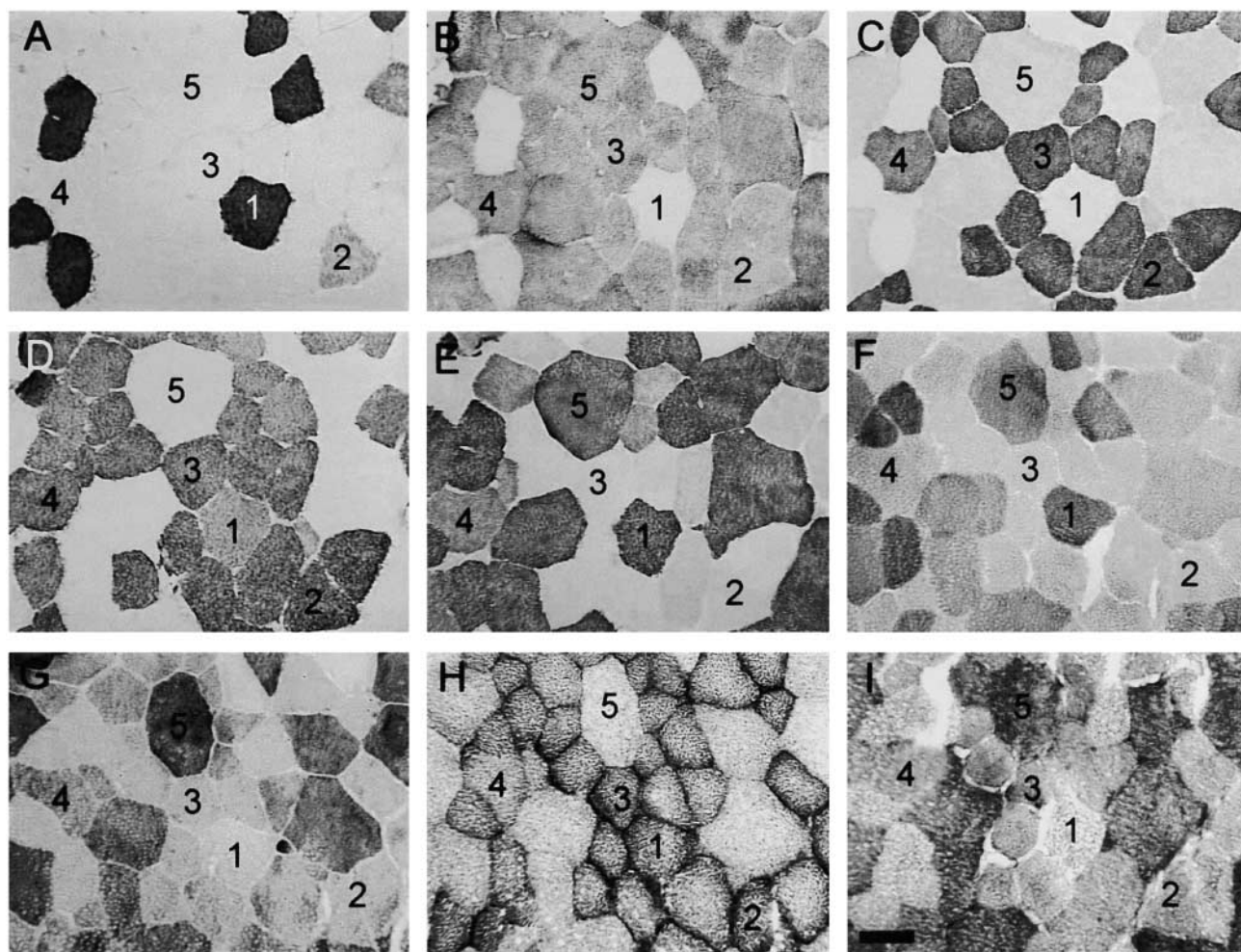


Figure 3 Serial cross-sections of horse control gluteus medius muscle stained by immunohistochemistry with a number of MABs against specific myosin heavy chain (MHC) isoforms (A–E; see Table 1 for specificities) and by enzyme histochemistry of myofibrillar ATPase, succinic dehydrogenase and α -glycerophosphate dehydrogenase (F–I). (A) BA-F8 MAB (anti-MHC I). (B) Fast MAB (anti-MHCs IIA+IIX+IIB). (C) SC-71 (anti-MHC IIA) MAB. (D) BF-35 (anti-MHCs I+IIA+IIB) MAB. (E) S5-8H2 (anti-MHC I+IIX+IIB) MAB. (F,G) Myofibrillar ATPase activity after pre-incubations at pH 4.45 (F) and pH 10.35 (G). (H,I) Succinate dehydrogenase (H) and α -glycerophosphate dehydrogenase (I) activities. The fibers labeled 1, 3, and 5 are “pure” fibers containing MHC I, MHC IIA, and MHC IIX, respectively. Fibers 2 and 4 are “hybrid” fibers containing MHC I plus MHC IIA (2), and MHC IIA plus MHC IIX (4). Bar = 50 μ m.

IIXB and IIAXB were located mostly within primary fascicles between the islets of Type I fibers (Figure 6D). However, pure IIB fibers were located mainly at the periphery of the rosettes near the edges of primary fascicles (Figure 6E).

Discussion

By using different MABs, it was possible to identify four MHC isoforms in adult llama skeletal muscle: the MHC- β /slow or MHC I and three fast (Table 4). Whereas the identity of one of these three fast MHC isoforms seems to be clearly an MHC IIA isoform, the present results are not conclusive regarding the final characterization and identification of the other two

fast MHCs. We have provisionally referred to these isoforms as IIX and IIB on the basis of their immunolabeling, enzyme histochemistry, and size homologies at the cellular level with the corresponding isoforms of rat and horse muscles (Table 4). This adoption is based on the assumption that the structure of each MHC isotype is generally conserved between species, whereas greater sequence divergence may be found between different isotypes within the same species (Schiaffino and Reggiani 1996). However, some important differences in the primary structure of the llama IIX and IIB MHC isoforms with regard to those of rat and horse muscles were evident from the different staining pattern of some MABs. The unequal reactivity of the fibers containing the MHC IIX with the MAB SC-71,

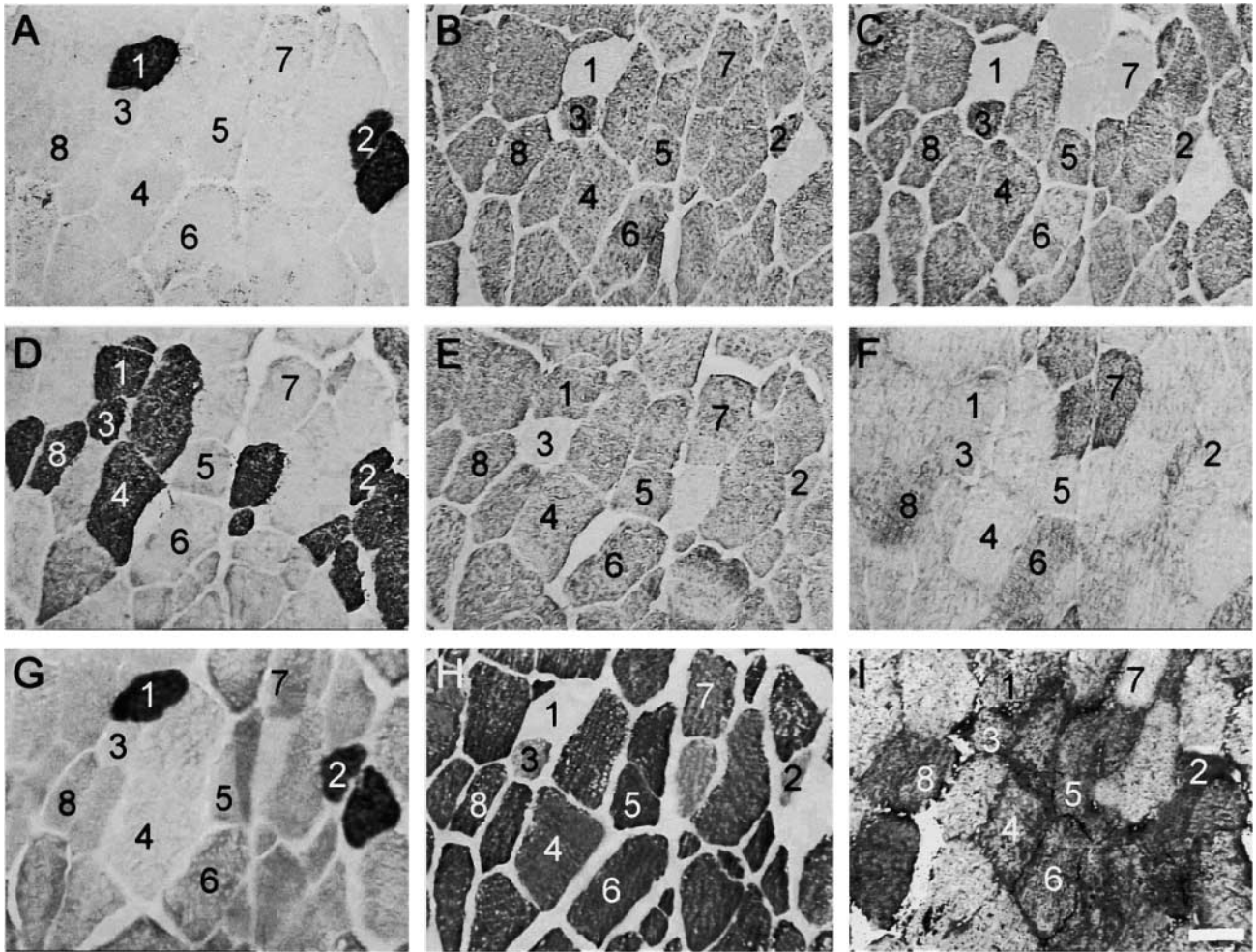


Figure 4 Serial cross-sections of llama (*Lama glama*) semitendinosus muscle stained by immunohistochemistry with a number of MABs against specific myosin heavy chain (MHC) isoforms (A–F; see Table 1 for specificities) and by enzyme histochemistry of myofibrillar ATPase and succinate dehydrogenase (G–I). (A) BA-F8 MAB (anti-MHC I). (B) SC-75 MAB (anti-MHC IIA+IIX+IIB). (C) SC-71 (anti-MHC IIA) MAB. (D) BF-35 (anti-MHC I+IIA+IIB) MAB. (E) S5-8H2 (anti-MHCs I+IIX+IIB) MAB. (F) BF-G6 (anti-MHC IIB) MAB. (G,H) Myofibrillar ATPase activity after preincubations at pH 4.5 (G) and pH 10.5 (H). (I) Succinate dehydrogenase activity. The fibers labeled 1, 3, 5, and 7 are “pure” fibers containing MHC I, MHC IIA, MHC IIX, and MHC IIB, respectively. Fibers 2, 4, 6, and 8 are “hybrid” fibers containing MHC I plus MHC IIA (2), MHC IIA plus MHC IIX (4), MHC IIX plus MHC IIB (6), and MHC IIA plus MHC IIX plus MHC IIB (8). Bar = 50 μ m.

specific for MHC IIA, between llama and rat/horse muscles (Table 4), shows that llama MHC IIX has common epitopes with the MHC IIA of rat and horse. Similarly, immunoreactivity of llama MHC IIB was very close to that of rat MHC IIB, but this isoform was not recognized in llama by the MAB BF-35, directed against all (including IIB) rat MHCs except the MHC IIX (Table 4). Consequently, further studies are required to precisely characterize these MHCs in the llama.

Fiber typing in mammalian skeletal muscle has extensively been identified by myofibrillar ATPase histochemical methods based on acid (Brooke and Kaiser 1970) and alkaline (Guth and Samaha 1970) denaturation. Only three major fiber types [one slow-twitch

(Type I) and two fast-twitch (Types IIA and IIB)] could be distinguished with these traditional methods in mature skeletal muscle of the majority of mammals (Matoba et al. 1985). By applying combined and more refined mATPase histochemical methods, three pure fast Type II fibers can be delineated in skeletal muscle of a number of rodents (Gorza 1990; Rowleson 1991; Hämaläinen and Pette 1993). The identification of three fast-twitch fiber populations can also be obtained in skeletal muscle of a large mammal such as the llama with the simultaneous application of two different mATPase histochemical stainings (Figure 2C). The acid stability of mATPase activity of Type II fibers in llama increased consistently in the rank order IIA>IIX>IIB (Table 4). Identical patterns have al-

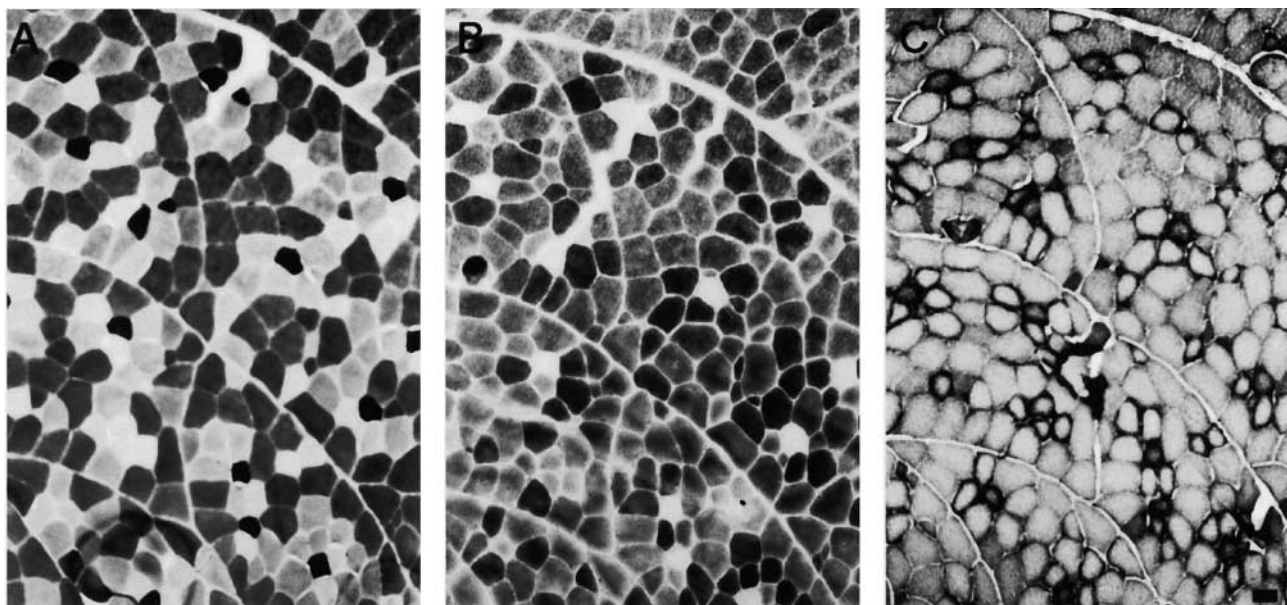


Figure 5 Serial cross-sections of llama vastus lateralis muscle stained by enzyme histochemistry of myofibrillar ATPase (A,B) and succinate dehydrogenase (C). (A,B) Myofibrillar ATPase activity after preincubations at pH 4.5 (A) and pH 10.5 (B). (C) Succinate dehydrogenase activity. Four levels of staining intensities can be observed for both acid and alkaline stabilities of mATPase activity. Nevertheless, a continuum in the staining intensity was clearly observed between subgroups of fast fiber types. Bar = 50 μ m.

ready been reported in the mouse skeletal muscle (Gorza 1990; Rowleson 1991). By contrast, the acid stability of mATPase activity of IIX and IIB fibers in rat muscle was identical because they could be not objectively delineated (Table 4). Another important interspecies variability was observed in the alkaline stability of the mATPase of Type II fibers (Table 4). Whereas in rat muscle the alkaline stability of mATPase activity of type II fiber types decreased in the rank order IIA>IIX>IIB, in llama muscle the rank order was IIX>IIB>IIA (Table 4). In the horse, Type IIX fibers also were more alkaline-stable than IIA fibers (Table 4). Nevertheless, both acid and alkaline stability of mATPase activity of skeletal muscle fiber types can vary greatly according to the mATPase histochemical method used (Latorre et al. 1993). This means that acid and alkaline stabilities of mATPase are not homogeneous in the same fiber types of different species.

The limitation of histochemical ATPase procedures is clearly illustrated in the present study by the fact that a large number (30% of those examined) of histochemically designated IIB fibers in the llama actually are hybrid IIXB (27%) and IIAXB (13%) fibers. Similarly, a large number (6% of the fiber population) of hybrid Type IIAX fibers were admixed with their corresponding pure phenotypes. These data emphasize the advantages of an immunohistochemical analysis to detect fibers with mixed MHC composition.

The phenotypic differences in fiber size, oxidative capacity and glycolytic capacity seen between llama fi-

ber types were, in general, linked to the MHC content and very similar to those observed in rat muscle (Rivero et al. 1998; and present results). In llama, as in rat muscle (Rivero et al. 1998), Type IIX fibers are smaller, more oxidative, and less glycolytic than IIB fibers, whereas they are larger, less oxidative, and more glycolytic than Type IIA fibers. Furthermore, the inverse relationship between oxidative and fiber size in the llama muscle is in agreement with that previously reported in rat (Rivero et al. 1998). Fibers containing MHC-IIB are generally larger and have lower SDH activities than fibers that display higher fatigue resistance (I and IIA) MHC isoforms. Nevertheless, this relationship is not conserved across the full range of mammalian species, because in dog skeletal muscle the larger Type IIB fibers (named by the authors "II-Dog") usually have an uncommon very high oxidative capacity (Latorre et al. 1993).

Results from the present study have demonstrated the presence of MHC IIB isoprotein at a very high percentage (49.2% in semitendinosus muscle) of the fibers in several hindlimb muscles of adult llamas. Interestingly, the percentage of Type II fibers was greater than 90% in all these muscles. In agreement with our data, Lefaucheur et al. (1998) also reported the presence of MHC IIB transcripts in about 67% of the fibers in pig longissimus muscle (predominantly composed of fast-contracting fibers), but they were not expressed in the red portion of the semitendinosus muscle (predominantly composed of slow-contracting

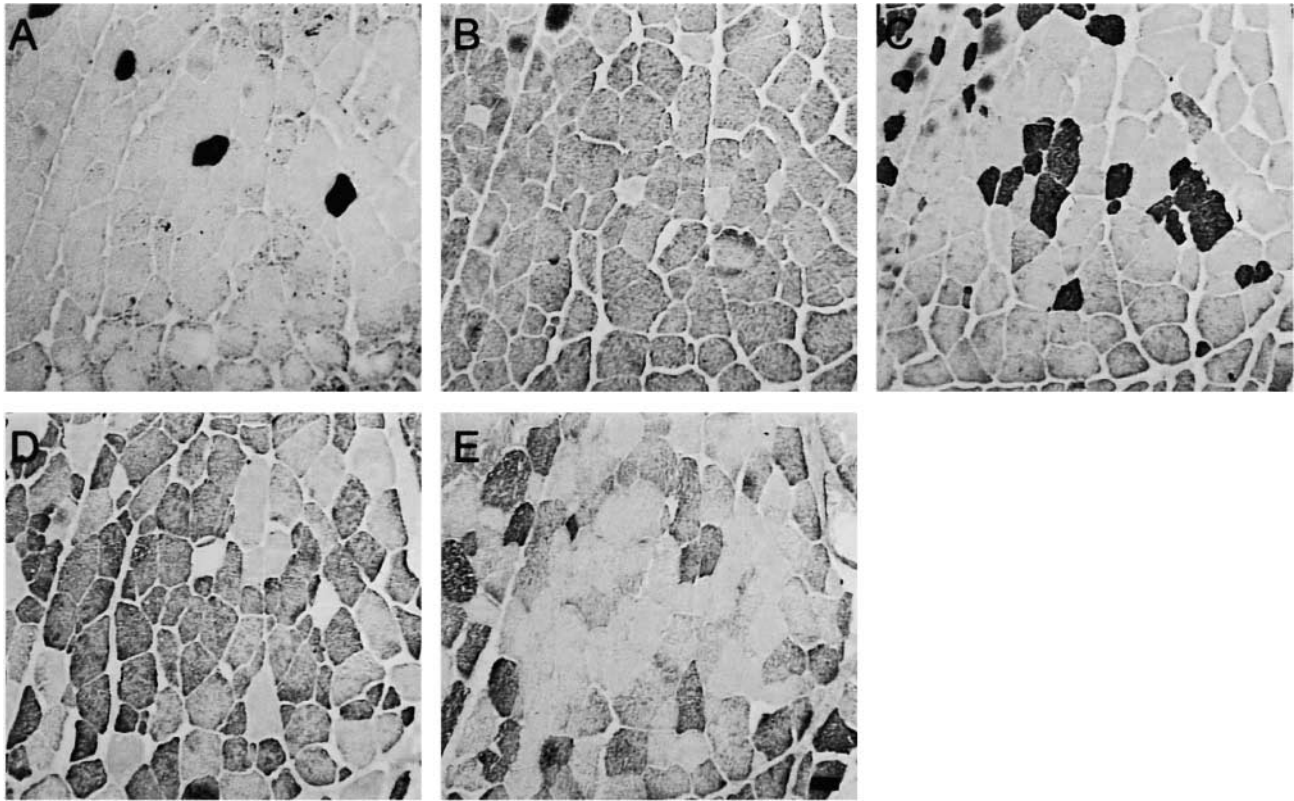


Figure 6 Spatial distribution of skeletal muscle fiber types in the llama. Serial sections of semitendinosus muscle were stained with MABs against specific myosin heavy chain (MHC) isoforms. (A) BA-F8 (specific for MHC I). (B) S5-8H2 (specific for all MHCs except MHC IIA). (C) BF-35 (specific for MHCs I+IIA in the llama). (D) SC-71 (specific for MHCs IIA+IIX in the llama). (E) BF-G6 (specific for MHC IIB in the llama). Note the low percentage of Type I (positive in A) and Type IIA (negative in B) fibers, the high percentage of fibers expressing MHC IIX (positive in B and D), and the moderate number of fibers expressing MHC IIB (positive in E). Furthermore, note that the three Type II fibers are spatially distributed around the typical islets of Type I fibers encountered in llama skeletal muscle. Thus, fibers expressing MHC IIA are contiguous to Type I fibers forming typical rosettes of muscle fibers (both positive in C). Fibers expressing MHC IIX are in the direct vicinity of Type I and IIA fibers (D), and fibers containing MHC IIB are located mainly at the periphery of the rosettes near the edges of primary fascicles (E). Bar = 50 μ m.

muscle fiber). Tanabe et al. (1999), however, reported only 30% of MHC IIB transcripts in pig longissimus thoracis muscle, but 60% in the white portion of the semitendinosus muscle. Expression of MHC IIB transcripts was not observed in pig semiespinalis muscle, tongue, and diaphragm (all composed predominantly of slow Type I fibers). Overall, these results may indicate that the expression of the very fast MHC IIB may also be considered muscle-specific. It is also reasonable to postulate that the MHC IIB gene could also be expressed in other larger mammals, and also very probably in selected fast-contracting muscles or in selected portions of these muscles in a number of large mammals, including those in which the expression of this isoform has not yet been identified. In fact, although no fibers containing MHC IIB in human muscles have been found until now, the gene coding for this isoform is present in the human genome and has been localized to chromosome 17 (Bottinelli and Reggiani 2000).

The differential distribution of the four MHC isoforms identified in llama skeletal muscle defines four major fiber types containing a single MHC isoform (i.e., I, IIA, IIX, and IIB) and a number of intermediate hybrid fiber populations containing (a) both MHC-I and MHC-IIA (i.e., Type I+IIA MHC fibers or Type "C" fibers), (b) two of the three fast MHCs (i.e., Type IIX and IIB fibers), or (c) even the three fast MHC isoforms (i.e., Type IIX fibers). The large number of fibers expressing IIB and/or IIX MHC isoforms (93.2% of those examined in semitendinosus muscle) may well be related to the very low level of fitness of animals included in the study, because inactivity induces a transition of MHC isoform in the order I→IIA→IIX→IIB (Pette and Staron 1997). Hybrid fibers are often assumed to represent a very low population in normal sedentary mammals, and their physiological meaning has been related to transitional fibers resulting from transformation of one fiber type into another (Pette and Staron 1997). Current data, how-

Table 4 Comparative immunohistochemical reactivity with anti-MHC monoclonal antibodies, histochemical intensity with myosin ATPase after acid and alkaline preincubations, succinic dehydrogenase (SDH), and α -glycerophosphate dehydrogenase (GPD), and relative fiber size of pure muscle fiber types identified according to the MHC isoform they express

	Monoclonal antibodies ^a						Histochemistry ^b				
	BA-F8	SC-75	SC-71	BF-35	S5-8H2	BF-G6	Acid ATPase	Alkaline ATPase	SDH	GPD	Size ^c
Rat											
I	+	-	-	+	+	-	++++	+	+++	+	+
IIA	-	+	+	+	-	-	+	+++	++++	++	+
IIX	-	+	-	-	+	-	++	++	++	+++	++
IIB	-	+	-	+	+	+	++	+	+	++++	+++
Horse											
I	+	-	-	+	+	+	+++	+	+++	+	+
IIA	-	+	+	+	-	+	+	++	+++	++	++
IIX	-	+	-	-	+	-	++	+++	+	+++	+++
Llama											
I	+	-	-	+	+	-	++++	+	++++	+	+
IIA	-	+	+	+	-	-	+	++	+++	++	+
IIX	-	+	+	-	+	-	++	++++	++	+++	++
IIB	-	+	-	-	+	+	+++	+++	+	++++	+++

^aFor monoclonal antibodies: + and -, positive and negative, respectively, reaction for that specific fiber type with that monoclonal antibody.

^bFor histochemistry: +, ++, +++, and +++++, light, intermediate, dark, and very dark staining, respectively, for that specific fiber type with that histochemical staining.

^cFor size: +, ++, and +++, small, intermediate, and large, respectively.

ever, indicate that a considerable percentage of these fibers (41.3% in semitendinosus muscle) exist in the skeletal muscle of sedentary llamas, so they should be considered as stable fibers with mixed MHC composition. Similar results have been obtained in a wide variety of mammals (Talmadge et al. 1996; Lefaucheur et al. 1998; Andersen et al. 1999; Linnane et al. 1999).

Some previous studies have revealed fibers that simultaneously coexpress three different MHC isoforms in muscles of elderly subjects (Andersen et al. 1999). A similar phenotype (IIAXB fibers) was also frequently observed in the present study. This observation might be associated with the extensive neuropathological changes that normally occur in elderly individuals and/or might be the consequence of some genetically regulated endogenous program setting with advancing age (reviewed by Andersen et al. 1999).

Finally, another interesting observation of the present study is the spatial distribution of muscle fiber types in llama skeletal muscle (Figure 6). Whereas in most mammalian species muscle fibers belonging to the same motor unit are randomly distributed and are mixed with other muscle units, exhibiting a classical "mosaic" pattern, llama muscle exhibits unique rosette patterns consisting of islets of a single slow fiber, surrounded by concentric circles of fast fibers expressing successively MHC IIA, then IIX, and finally IIB. Accordingly, the vast majority of the fibers expressing MHC IIB are located at the periphery of primary fascicles. Similar spatial distribution patterns have already been described in pig muscles (Lefaucheur et al. 1998), but the reason for such a peculiar distribution is not fully known.

This study demonstrates that at least four adult MHC isoforms, one slow-twitch and three fast-twitch, are expressed at the protein level in llama limb muscles. These isoforms have provisionally been referred to as Types I, IIA, IIX, and IIB, based on the homologies of their immunoreactivities with a panel of MAbs, acid and alkaline stabilities of mATPase histochemistry, and their metabolic and size properties with the corresponding isoforms of rat and horse muscles. Nevertheless, further studies are needed to fully characterize these MHC isoforms. The present study confirms that the very fast MHC-IIB isoform is not exclusively expressed in skeletal muscles of small species of mammals but is also present in predominantly fast-contracting muscles of larger mammals such as the llama. It is noteworthy that conventional IIB fibers, as defined by traditional mATPase histochemistry, constitute a heterogeneous population and should be characterized either as pure (IIB) or as hybrid (IIXB and IIAXB) phenotypes. This improvement in accuracy of muscle fiber typing is of practical importance to better understand the involvement of fiber types in locomotor, growth, and meat quality traits of the llama.

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