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Monospecific Antibodies against the Three Mammalian Fast Limb Myosin Heavy Chains

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Skeletal muscle fibres in mammalian limb muscles are of four types: slow, 2A, 2X, and 2B, each characterized by a distinct myosin heavy chain (MyHC) isoform. Existing monoclonal antibodies (mabs) against fast MyHCs lack fibre-type specificity across species and could not positively identify 2X fibres. In this work, mabs were raised against each of the fast MyHCs. These mabs were shown to be monospecific by Western blots and immunohistochemistry in the rat. The advantages of using these mabs for identifying the three fast fibre types and hybrid fibres expressing multiple isoforms were illustrated using rat tibialis anterior muscle. Immunohistochemical analyses confirmed the monospecificity of these mabs in the following additional species: mouse, guinea pig, rabbit, cat, and baboon. 2B fibres were absent in limb muscles of the cat and baboon. These mabs constitute a set of powerful tools for studying muscle fibre types and their transformations.

Key Words: **muscle fibre types; myosin heavy chain; monoclonal antibodies; immunoblotting; immunocytochemistry.**

Skeletal muscle fibres in mammalian limb muscles may be classified phenotypically into four fibre types: slow (type 1) and three fast (types 2A, 2X or 2D, and 2B). These types of fibres differ from each other in structure, function, biochemistry, and histochemistry (1, 2). Functionally, they differ in speed, power, and endurance. Each fibre type contains a different isoform of myosin heavy chain (MyHC) which forms the basis for fibre-type classification. There are thus four isoforms of MyHC, slow, 2A, 2X, and 2B. By transducing energy from ATP at different rates, these MyHCs control the speed and power of muscle contraction (3). The expression of MyHC is subject to physiological parameters such as functional load, pattern of use, and hormonal levels (4). Thus tracking changes in MyHC expression is extremely important in muscle physiology and pathophysiology. Myosin ATPase histochemistry (5) had been used classically to identify fibre types and track changes, but it has largely been replaced by immunohistochemistry using monospecific antibodies to MyHC due to the high resolving power of the latter technique.

Antibodies against structurally distinct myosin isoforms have been used over the past two decades as probes to identify limb muscle fibre types. The initial immunohistochemical identification of the two fast fibre types, then known as 2A and 2B, was established by Pierobon-Bormioli *et al.* (6), using two polyclonal antibodies. Several years later, a third fibre type and MyHC isoform, 2X (or 2D), was discovered using a battery of more specific monoclonal antibodies (7). Type 2A and 2B muscle fibres were labelled with two monospecfic mabs against 2A and 2B MyHCs, respectively. The type 2X muscle fibres were identified by using a combination of mabs, as no monospecific mab against 2X MyHC was available. The 2X muscle fibres were reactive with a mab against all fast MyHCs, unreactive with mabs specific for slow, 2A, and 2B MyHCs, and unlabelled with a mab which reacts against all the other MyHC isoforms. The use of this combination of mabs to identify 2X muscle fibres was awkward and inconvenient. Further, hybrid fibres co-expressing 2X and 2A or 2B MyHC isoforms cannot be clearly identified (8, 9). Another disadvantage with the mabs raised by Schiaffino and co-workers (7) is that their specificities are not conserved across the species, but are restricted to just a few species e.g., the monospecfic mabs against 2A and 2B MyHCs work in rats and mice, but fail to react with guinea pig tissues (8); the monospecific mab against 2A MyHC in the rat reacts with 2A and 2X MyHCs in the rabbit (10); and the mab against 2X and 2B MyHCs in the rat was found to be non-specific in the cat (11). Further, it was not possible to positively identifying the isoforms of

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fast MyHCs expressed in cat (11), horse (12), and cattle (13) with the available mabs.

In this study we raised three monospecific mabs against the three fast MyHCs and showed that their monospecificities are conserved across most mammalian species tested. To illustrate their usefulness, these mabs were used to unambiguously identify the three fast fibre types and to quantify the relative abundance of pure and hybrid fast muscle fibres in the superficial and deep regions of the rat tibialis anterior muscle.

METHODS

Production of mabs. Mabs against specific fast limb MyHC isoforms were isolated while raising mabs against myosins from the cat masseter and rabbit retractor bulbi muscles. Myosin was extracted from tissues as described previously (14). Mab 2F7 was raised against cat masseter myosin. Balb/c mice were injected with SDS denatured masseter myosin (1 mg/mouse, emulsified in Freund's complete adjuvant) as primary antigen. Mabs 6H1 and 10F5 were raised against rabbit retractor bulbi myosin. In order to enhance the immune response, the retractor bulbi myosin was co-adsorbed with an adjuvant peptide to gold particles (15). Myosin (3 mg) was diluted into 15 ml of 10 mM sodium pyrophosphate (NaPPi) pH 6.3. Colloidal gold (150 ml), with particles 15 nm in diameter, was prepared according to Frens (16) and resuspended in 150 ml of 5 mM NaPPi pH 6.3. Colloidal gold was rapidly added to the myosin together with 150 mg of adjuvent peptide (Sigma, St. Louis, MO). The concentration of myosin used was sufficient to stabilise colloidal gold, as assessed by the absence of salt-induced flocculation (17). The gold-myosin conjugate was washed twice in 10 mM NaPPi, pH 6.3, and finally resuspended in 250 μl of 0.3 M NaCl, which was enough for one immunisation. An equal volume of the antigen, either SDS-denatured masseter myosin or retractor bulbi myosin conjugate was emulsified with an equal volume of Freund's complete adjuvant and injected intraperitoneally.

A booster injection with the respective antigen with or without colloidal gold emulsified with Freund's incomplete adjuvant was given 1–2 months after the primary immunisation, and 3–4 days later the spleen cells were isolated and fused with NS1 cells using PEG 4000 (polyethylene glycol, Merck, Hohenbrunn, Germany) (18). Hybridoma cells were plated at limiting dilution and 10–14 days later, hybridoma supernatants were screened using fluorescence immunohistochemistry on cryostat sections of a cat or rabbit muscle tissue block comprising the following muscles: retractor bulbi, masseter, vastus lateralis, and soleus. A fluorescein-labelled rabbit anti-mouse immunoglobulin (Dako Corp., Carpinteria, CA) was used as secondary antibody and the results viewed with a Zeiss (Oberkochen, West Germany) Axioplan microscope equipped with epifluoresence optics. Three hybridoma clones, 2F7, 6H1, and 10F5 were selected as the antibodies they secreted specifically stained type 2A, 2X, and 2B fibres, respectively. These three clones were subcloned to ensure monoclonality and supernatants were collected. Mab 2F7 was isolated from a mouse immunized against cat superfast MyHC, and is reactive against this isoform in cat and dog. However, this MyHC is not expressed in limb muscles (1). An ImmunoType mouse mab isotyping kit (Sigma, St. Louis, MO) was used to determine the immunoglobulin (Ig) classes of the mabs raised in this study. The mabs used in this study were either IgG_1 (2F7) or IgM (6H1, 10F5).

Tissue preparation and immunohistochemical techniques. Muscle blocks were made of the following tissues: adult rat (Sprague-Dawley), guinea pig, cat, and mouse (Balb/c) tibialis anterior (TA); baboon and rabbit vastus lateralis. These blocks were mounted on cork with Tissue-Tek (Miles Scientific, Elkhart, IN), frozen in isopentane cooled in liquid nitrogen, and 10 μm thick sections were cut at −20°C. Indirect immunohistochemical analysis was carried out as previously described (19). In addition to the use of the primary mabs 2F7, 6H1, and 10F5 raised presently, NOQ7-5-4D (hereafter referred to as 5-4D) which reacts against all mammalian skeletal limb slow MyHCs (19, 20) was also used. The secondary antibodies used were a horse-radish peroxidase (HRP)-labelled rabbit anti-mouse immunoglobulin antibody (Dako Corp., Carpinteria, CA) for mabs 2F7 and 5-4D and HRP-labelled goat anti-mouse IgM antibody (Sigma, St. Louis, MO) for mabs 6H1 and 10F5.

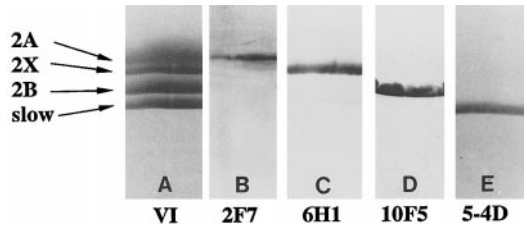

FIG. 1. SDS–PAGE of MyHCs and Westerns from adult rat vastus intermedialis (VI) muscle. Protein-stained SDS gel (A) and Western blots of VI MyHCs stained with mab 2F7 (B) mab 6H1 (C), mab 10F5 (D), and mab 5-4D (E).

Electrophoretic and immunoblotting analysis. Myosin used for electrophretic analysis was extracted from the rat vastus intermedialis as described previously (14). High-resolution SDS–PAGE was performed according to (21). Large format gels were run in a Hoefer Scientific SE 600 unit (Hoefer Scientific Instruments, San Francisco, CA). One slight modification was the addition of 10 mM 2-mercaptoethanol in the upper electrode buffer, which improves band resolution (22, 23). The gels were stained with Coomassie Brilliant Blue. MyHC bands were Western blotted and stained immunochemically as previously described (10).

Quantification of muscle fibre type distribution and measurement of fibre cross-sectional area. The percentages of slow, 2A, 2X, and 2B fibres in both the superficial and deep portions of the tibialis anterior muscle were calculated based on photomicrographs of tissue sections stained by MyHC immunohistochemistry. The total number of fibres counted was 600 from 3 fields for each of the deep and the superficial regions. Fibres were classified as hybrid fibres if they were significantly stained with two of the anti-MyHC mabs. The cross-sectional areas of each muscle fibre type were calculated using an image processing system consisting of a Zeiss Axioplan microscope with an attached video camera, image processor, MacIntosh computer, and NIH-IMAGE software. All statistical comparisons were performed using the unpaired two-tailed Student's *t*-test.

RESULTS AND DISCUSSION

The immunoreactivities of mabs 2F7, 6H1, and 10F5 raised in this study with electrophoretically separated MyHCs from rat vastus intermedius muscle are shown in Fig. 1. Vastus intermedius was used because all three fast MyHCs were well represented. Rat limb MyHCs are known to migrate in high resolution SDS gels in the following order: 2A < 2X < 2B < β/slow (24). Figure 1A shows protein stained MyHC bands from the rat vastus intermedius while Figs. 1B–1E show Western blots of rat vastus intermedius stained with mabs 2F7, 6H1, 10F5, and anti-slow MyHC mab 5-4D, respectively. Mab 2F7 reacts with the 2A MyHC band, mab 6H1 reacts with the 2X MyHC band, and mab 10F5 reacts with the 2B MyHC band. Each mab reacted with only a single MyHC band.

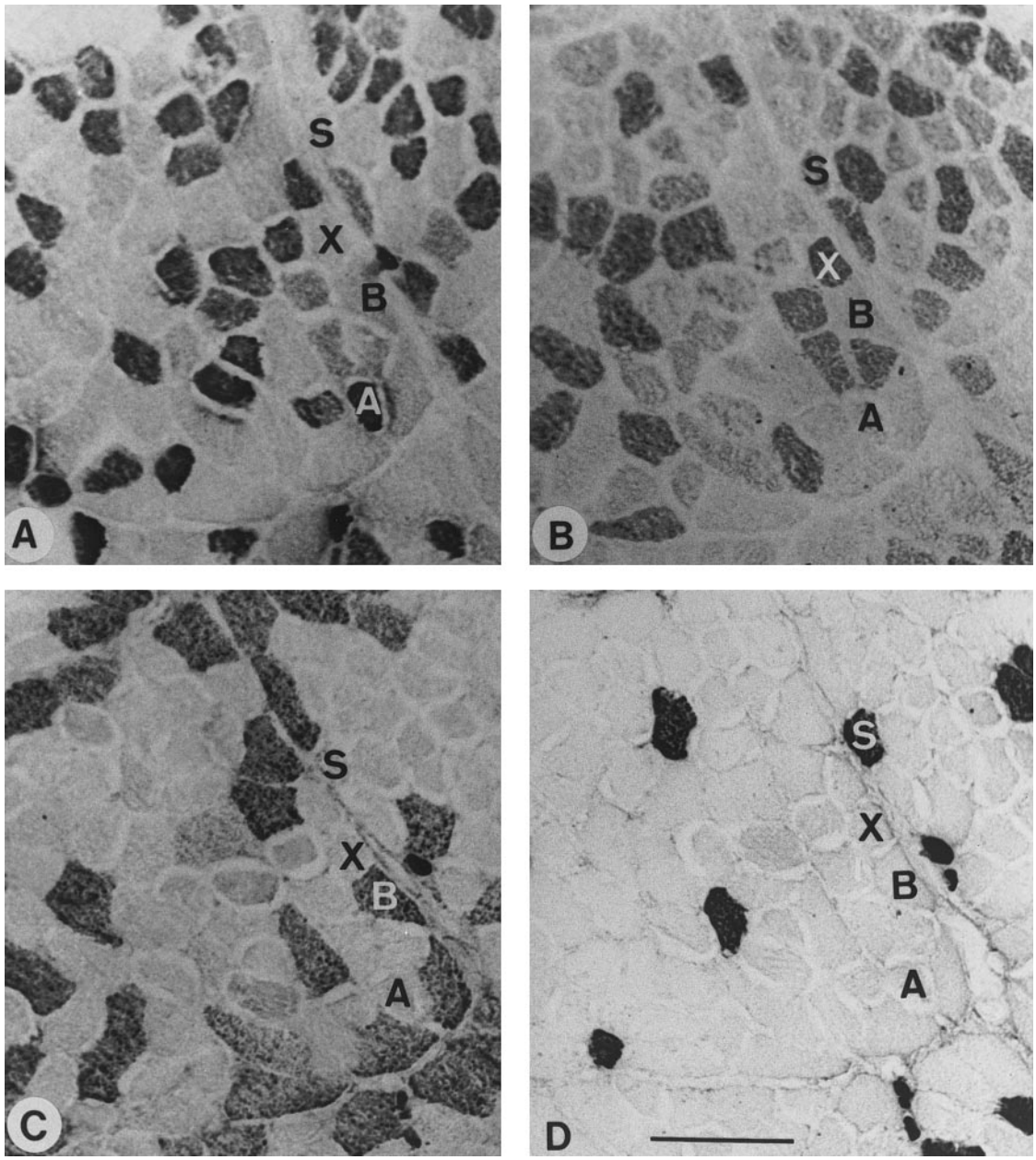

FIG. 2. Immunoperoxidase staining of semi-serial sections of the deep region of the tibialis anterior muscle from an adult rat with anti-2A MyHC mab 2F7 (A), anti-2X MyHC mab 6H1 (B), anti-2B MyHC mab 10F5 (C), and anti-slow MyHC mab 5-4D (D). Fibres labeled A, X, B, and S represent fibre types 2A, 2X, 2B, and slow, respectively. Scale bar = 100 μm.

The mabs 2F7, 6H1, and 10F5 stained three distinct populations of fast fibres in rat TA muscle sections (Fig. 2): mab 2F7 stained 2A muscle fibres (Fig. 2A); mab 6H1 stained 2X muscle fibres (Fig. 2B); and mab 10F5 stained 2B muscle fibres (Fig. 2C). In addition to the pure fibres, there were also a number of hybrid fibre populations containing type 1 plus 2A MyHCs, type 2A plus 2X MyHCs, and type 2X plus 2B MyHCs.

The fibre type compositions in the deep and superficial regions of the TA are shown in Table 1. Previous studies on fibre type composition of the rat TA did not take into consideration the vast differences between the deep and superficial regions (8, 25). The deep region of the TA consisted of approximately equal proportions of the three fast fibre types: type 2A (29%); type 2X (26%); and type 2B (28%), together with a few slow fibres (6%). In contrast, the superficial tibialis anterior had no slow fibres, a few type 2A fibres (2%), a moderate proportion of type 2X fibres (18%) and a large proportion of type 2B fibres (76%). Hybrid fibres, expressing two fast MyHCs, were more abundant in the deep region than the superficial region. In the deep

TABLE 1

Relative Abundance of Various Types of Fibres in the Superficial and Deep Regions of the Tibialis Anterior Muscle of The Rat[a]

Fibre type	Abundance (%) (superficial region)	Abundance (%) (deep region)
Slow	0	6
Slow/2A	0	<1
2A	2	29
2A/2X	1	6
2X	18	26
2X/2B	3	5
2B	76	28

[a] Total number of fibres counted was 600 from three fields in each region.

region there were 6% type 2A/2X fibres and 5% type 2X/2B fibres, whilst in the superficial region there were only 1% type 2A/2X fibres and 3% type 2X/2B fibres. A previous study used a panel of 6 mabs together with differences in succinic dehydrogenase and myosin ATPase staining to obtain the relative abundance of the three fast limb fibre types (8): 4.2% type 2A/2X fibres and 5.8% type 2X/2B fibres in rat TA types (8). In another study, a combination of myosin ATPase histochemistry and single fibre electrophoresis was used to characterise the muscle fibre types in the rat TA (25). It was found to comprise 3% type 2A/2X fibres and 10% type 2X/2B fibres. The fibre type profile and prevalence of hybrid fibres seen in our immunohistochemical study is broadly similar to those of the two previous studies, but were obtained with considerable ease and reliability.

Earlier work has shown that the size of muscle fibers is correlated with the type of MyHC expressed (9, 26, 27). The mean cross-sectional areas of fibers containing the various MyHC isoforms in the present study are reported in Table 2. The three pure fast fibre types were statistically different with regard to their fibre size ($P < 0.05$). Type 2A fibres were smallest (815 ± 221 {SD} μm^2), type 2B fibres were largest (1904 ± 570 μm^2), and type 2X fibres were intermediate (1305 ± 374 μm^2). This relationship between fibre size and MyHC expressed confirms previous observations in numerous rat muscles (9, 26, 27). The hybrid 2A/2X fibres had a mean cross-sectional area which was intermediate between type 2A and 2X fibres, which was statistically different from 2A but not 2X fibre types ($P < 0.05$). The mean cross-sectional area of type 2X/2B fibres was slightly greater than the pure type 2B fibres and was statistically different from both pure 2X and 2B fibre types ($P < 0.05$) (Table 2). These results are similar to those seen in a previous study in the rat medial gastrocnemius muscle (9). The fact that 2X/2B fibres displayed a larger mean diameter than 2B fibres may indicate that they were 2B fibres that had been receiving high rates of stimulation, causing transient hypertrophy, and a fibre type transition to 2X fibres.

In order to determine whether the specificities of the mabs raised in this study are conserved across mammalian species, cryostat sections from fast limb were stained with mabs 2F7, 6H1, 10F5, and 5-4D from the following species: guinea pig; mouse; rabbit; cat; and baboon. In the fast muscles of the two other rodents, guinea pig, and mouse, mabs 2F7, 6H1, and 10F5 stained 2A, 2X, and 2B fibre types, respectively (data not shown). The three mabs 2F7, 6H1, and 10F5 also stained three distinct fibre types in rabbit fast muscle (Figs. 3A, 3D, and 3G). In the cat and baboon, the fast fibres were of two types, those that stained with mab 2F7 (type 2A) (Figs. 3B and 3C) and those that stained with mab 6H1 (type 2X) (Figs. 3E and 3F). The mab 10F5 failed to stain any fibres in these two species (Figs. 3H and 3I), suggesting that these two species don't have 2B muscle fibres. Talmadge *et al.* (11) has suggested that cat fast muscle has no 2B muscle fibres, however the 2X fibres were identified by their failure to react with two mabs: one against rat 2B MyHC and another against rat slow, 2A and 2B MyHCs. The use of the specific 2X MyHC mab in this study has led to a positive identification of the 2X muscle fibres in the cat. Previous studies on human limb muscle based on *in situ* hybrization (28) and molecular analysis of isolated single fibres (29), suggested that human limb fibres do not express 2B MyHC. The absence of 2B fibres in the baboon, first described here, suggests that other primates, like humans, generally do not express 2B MyHC in their limb muscles.

This paper describes three mabs monospecific for the three fast 2A, 2X, and 2B MyHCs expressed in eutherian mammalian limb muscles. The specificities of these mabs were verified in representatives of four orders of eutherians (rodent, lagomorph, carnivore, and primate) as well as two orders of marsupials (Zhong, Lucas, and Hoh, unpublished observations). These

TABLE 2

Cross-Sectional Areas of Different Fibre Types According to the Myosin Heavy Chain Isoform They Express in the Deep Region of the Rat Tibialis Anterior

Fibre type	Mean area ± SD (μm^2)	Range (μm^2)
2A	815 ± 221 (n = 197)†	412–1951
Slow	915 ± 258 (n = 107)†	434–1709
2A/2X	1236 ± 242 (n = 33)**	798–1763
2X	1305 ± 374 (n = 191)*	428–2260
2B	1904 ± 570 (n = 138)†	822–3340
2X/2B	2149 ± 515 (n = 30)†	956–3305

† $P < 0.05$ compared with all other fibre types.
* $P < 0.05$ compared with all other fibre types except 2A/2X.
** $P < 0.05$ compared with all other fibre types except 2X.

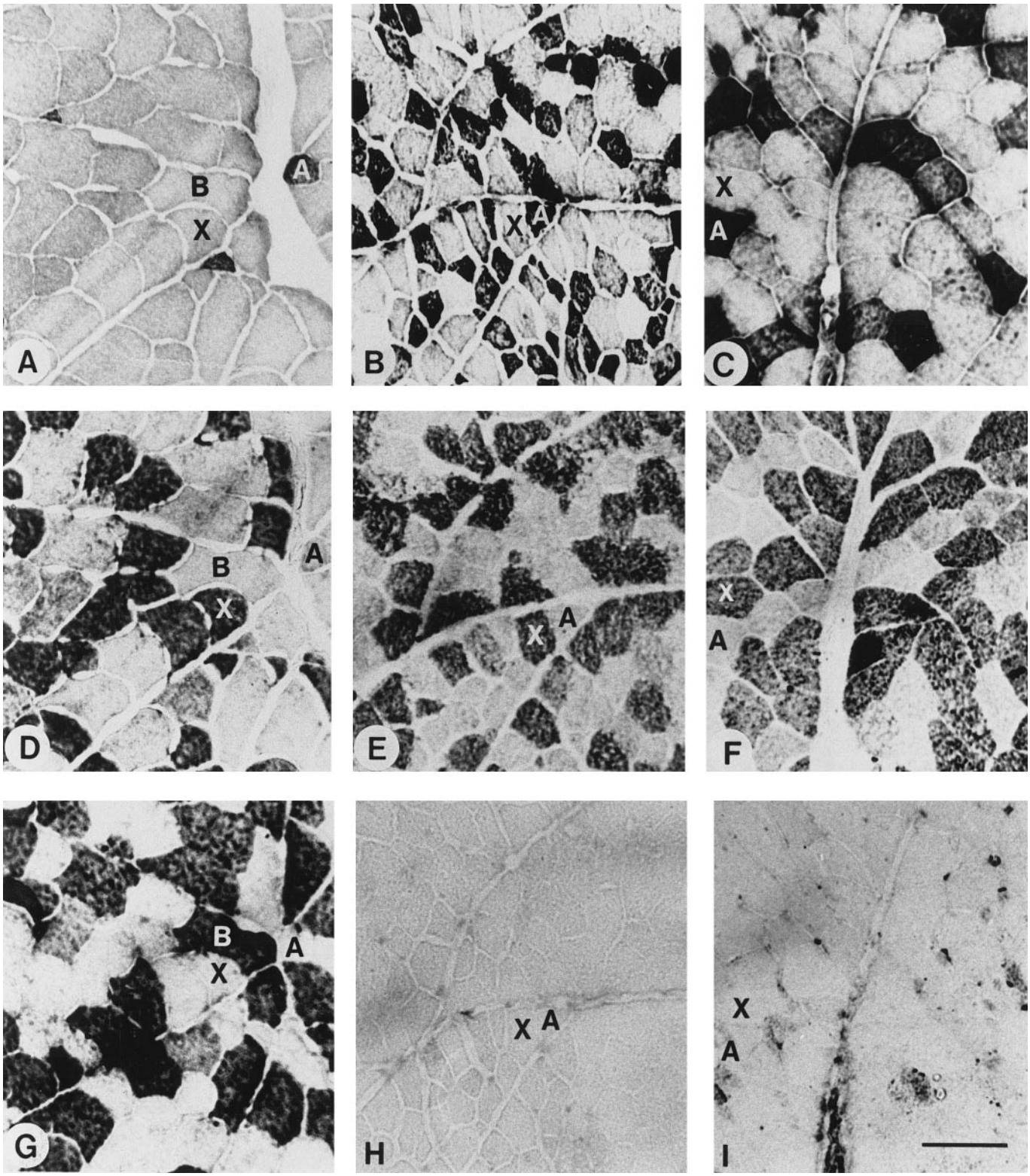

FIG. 3. Immunoperoxidase staining of semi-serial sections of adult rabbit vastus lateralis (A, D, G), cat tibialis anterior (B, E, H), and baboon vastus lateralis (C, F, I) with mab 2F7 (A–C), mab 6H1 (D–F), and mab 10F5 (G–I). Fibres labeled A, X, and B represent fibre types 2A, 2X, and 2B, respectively. Scale bar = 100 μm.

findings suggest that the epitopes for these mabs on the respective MyHCs are highly conserved. The use of monospecific antibodies against 2A, 2X, and 2B MyHCs is advantageous as it simplifies the identification of the three fast muscle fibre types in muscle sections and allows unambiguous identification of the hybrid muscle fibres. These specific mabs will serve as important tools for studying mammalian muscle fibre types and of their changes in pathological conditions or following experimental procedures that involve altering neural, hormonal, and mechanical influences.

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