

Coexpression of Myosin Heavy Chain 2b with Myosin Heavy Chain 1- Fact or Artefact?

Ida Eržen, Janez Sketelj⁽¹⁾, Onnik Agbulut⁽²⁾, Denise Angaut-Petit⁽³⁾, and Gillian S. Butler-Browne⁽²⁾

Institute of Anatomy and (1) Institute of Pathophysiology, Medical Faculty, Ljubljana, Slovenia, (2) CNRS URA 2115, Faculté de Médecine, Paris, and (3) Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, Gif-sur-Yvette Cedex, France

Abstract

In skeletal muscle, pure fibres expressing one myosin heavy chain (MyHC) isoform, and intermediate fibres, expressing two and exceptionally three MyHCs have been described. When skeletal muscle adapts its fibre type profile to changed functional demands MyHC isoform transformation follows the pathway: MyHC-1 $\leftrightarrow$ MyHC-2a $\leftrightarrow$ MyHC-2x/d $\leftrightarrow$ MyHC-2b. Therefore, in hybrid fibres only successive isoforms from the proposed pathway should coexist. However, jump fibres in which MyHC-1 is co-expressed with MyHC-2x/d have been described recently. The present study describes possible coexpression of MyHC-1 with MyHC-2b in transforming as well as in normal control mouse and rat muscle fibres. The study is only descriptive and provides not sufficient proof to exclude the possible artefact resulting from unknown technical reasons.

Key words: coexpression, mouse, myosin heavy chains, rat.

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An important characteristic of skeletal muscle is its plasticity. Because of this property the muscle is able to adapt its fibre type profile to changing physiological and functional demands. The earliest response can be observed in the changed muscle fibre metabolic profile as well as in the capillary density. Sarcoplasmic, structural and contractile proteins adapt by successive transformation of their protein isoforms (Salmons & Henriksson, 1981; Pette & Vrbova 1985; Pette & Staron, 1990; Pette & Vrbova, 1992; Talmadge *et al.*, 1995; Pette & Staron, 1997; Schiaffino & Reggiani, 1996).

Myosin, the main protein responsible for muscle contraction, consists of heavy and light chains. Myosin heavy chain (MyHC) isoforms are encoded by a multi-gene family and are expressed in a tissue specific and developmentally regulated manner (Wydro *et al.*, 1983; Mahdavi *et al.*, 1987). Skeletal muscle plasticity has been studied extensively by myosin isoform transitions in different experimental models.

In the skeletal muscles of small adult mammals like mouse and rat, most fibres are pure fibres, expressing only one MyHC isoform. However, intermediate fibres, expressing two or more MyHC isoforms do exist in animal and human muscles (Billeter *et al.*, 1980; Staron

& Pette, 1986; Termin *et al.*, 1989; Talmadge *et al.*, 1995; Talmadge *et al.*, 1996; Grossman *et al.*, 1998).

Various factors such as development, innervation, increased and decreased neuromuscular activity, overloading and unloading, hormones and ageing, have been shown to influence the phenotypic expression of skeletal muscle fibres (see Schiaffino and Reggiani, 1996; Pette and Staron, 1997; Talmadge, 2000 for reviews).

Skeletal muscle fibre type transformations are supposed to occur in a graded and orderly sequential manner. MyHC isoform transitions follow the sequence going from the fastest (MyHC-2b) to the slowest (MyHC-1) and vice versa (Mira *et al.*, 1992; Staron & Pette, 1993; d'Albis & Butler-Browne, 1993; Pette & Staron, 1997). Muscle fibres pass through successive steps of MyHC isoform expression. i.e. from MyHC-1 (β slow) $\leftrightarrow$ MyHC-2a $\leftrightarrow$ MyHC-2x/d $\leftrightarrow$ MyHC-2b. During this phase of phenotypic fibre type conversion intermediate fibres can be observed which contain more than one MyHC isoform (Pierobon-Bormioli *et al.*, 1981; Pette & Staron, 1990; Aigner *et al.*, 1993; Talmadge *et al.*, 1995; Caiozzo *et al.*, 1997; Grossman *et al.*, 1998).

Therefore, the fraction of hybrid fibres could drastically increase during processes, which promote muscle fibre

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type transitions, and it was originally thought that the appearance of hybrid fibres represented a transitional state of fibre transformation from one type to the other. However, it was subsequently demonstrated that under appropriate conditions hybrid fibres become increasingly more stable and persist in large numbers for a long period of time. One year after spinal cord transection approximately 50% of all fibres were hybrid fibres (Talmadge *et al.*, 1999). This suggests that under appropriate conditions hybrid fibres may represent a "stable" phenotype.

Co-expression of MyHC-1 with MyHC-2x/d has been described recently in so called 'jump fibres' (Talmadge *et al.*, 1995; Andersen *et al.*, 1999b). It was confirmed in human and rat muscles (Jacobs-El *et al.*, 1993; Talmadge *et al.*, 1995; Grossman *et al.*, 1998; Talmadge *et al.*, 1999; Andersen *et al.*, 1999a; Andersen *et al.*, 1999b) also by demonstrating the presence of mRNA for both MyHC isoforms in the same muscle fibres (Jacobs-El *et al.*, 1993; Andersen *et al.*, 1999a; Andersen *et al.*, 1999b). Co-existence of MyHC-1 with MyHC-2b has been mentioned as a very rare event (Ståhl *et al.*, 1994; Snoj-Cvetko *et al.*, 1996; Caiozzo *et al.*, 1997), however, its presence within numerous fibres of the same muscle has not been described yet.

The aim of the present paper was just to show that a complete co-expression of MyHC-2b with MyHC-1 can be obtained in some muscles, since the phenomenon was described in gastrocnemius and tibialis anterior muscles of one mature mouse, in extensor digitorum longus muscle of two mature rats and in soleus muscle of three rats, in which the peroneus nerve had been transposed to innervate the soleus muscle. Whether both antigens really coexist in the same muscle fibre or the immunohistochemically demonstrated coexpression is artificial remains an open question.

Material and Methods

Material

The animals involved in this study were primarily destined for other purposes. Isolated skeletal muscles were subsequently analysed for the unusual co-expression of MyHC isoforms observed in control and experimental muscles of mice and rats.

Tibialis anterior and gastrocnemius muscles of six mature untreated SWISS NMRI mice (i), extensor digitorum longus and soleus muscles from 45 mature untreated Wistar rats (ii), and 'cross-innervated' regenerating soleus muscles of three Wistar rats (iii) were analysed. In the latter group, the nerve normally innervating extensor digitorum longus muscle was transected and sutured to the distal stump of the transected nerve of the soleus muscle which was injected with bupivacaine (Marcaine, 0.5%; Astra, Södertälje, Sweden), a myotoxic local anaesthetic. Muscles were excised four months later. Soleus muscles of nontreated animals served as a control.

All the excised muscles were frozen in liquid nitrogen and cut into 10 µm transversal sections.

Immunohistochemistry

Muscle sections were first pretreated in a 0.1 M citrate buffer (pH 7.2) for 5 minutes in a microwave oven and thereafter with preimmune rabbit and rat serum diluted 1/5 in phosphate buffered saline (PBS) to reduce background. Monoclonal antibodies against MyHC isoforms MyHC-1 (BAD5), MyHC-2a (SC71), MyHC-2b (BFF3) and MyHC-1 + MyHC-2a + MyHC-2b (negative proof for MyHC-2x/d) (Schiaffino *et al.*, 1986) were applied at +4°C overnight. Peroxidase conjugated rabbit anti mouse IgG (Dako, Denmark) served as the secondary antibody and the peroxidase reaction was visualised with diaminobenzidine tetrahydrochloride.

Electrophoresis of MyHC isoforms

Muscles from normal NMRI mice were extracted on ice for 60 minutes in 4 volumes of extracting buffer (pH 6.5) as described previously (Butler-Browne *et al.*, 1984). Following centrifugation, the supernatants were diluted 1:1 (v/v) with glycerol and stored at -20°C. MyHC isoforms were separated on 8% polyacrylamide gels which were made in the Bio-Rad mini-Protean II dual slab cell system as described previously (Agbulut *et al.*, 1996). Proteins migrated for 31 hours at 72 V (constant voltage) at 4°C. Following migration, the gels were silver-stained according to Blum *et al.* (1987).

Western-blot of MyHC isoforms

For western blot analysis, the gels were prepared under the same conditions as above. The proteins were transferred onto nitro-cellulose membranes by active transfer overnight at 30 V (constant voltage). Following transfer, the nitro-cellulose membranes were blocked to eliminate non-specific binding in 3% foetal calf serum diluted in PBS. The blots were then incubated with the primary antibodies BF-F3 (Schiaffino *et al.*, 1986), (1/20) and BA-D5 (1/10) (Schiaffino *et al.*, 1986). The biotin labelled secondary antibody was applied for 45 minutes at room temperature (Dako, Copenhagen, Denmark), followed by the streptavidin biotin HRP complex (Dako, Copenhagen, Denmark). Specific antibody binding was visualised by incubating the membranes in the diaminobenzidine substrate mixture until the bands were sufficiently intense. The reaction was stopped by washing the membranes in tap water.

Results

Immunohistochemistry

In the mouse tibialis anterior (TA) and gastrocnemius (GCM) and the rat extensor digitorum longus muscles immunoperoxidase staining of MyHC isoforms in muscle fibre cross-sections is presented in Figures 1, 2 and 3. One out of the six mice (Figures 1 and 2) and two out

MyHC-1 is co-expressed with MyHC-2b in the same fibre

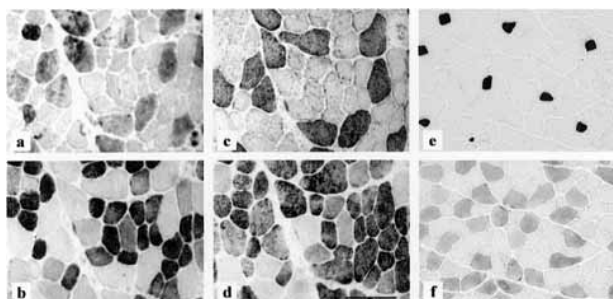


Figure 1. Immunoperoxidase staining in the mouse tibialis anterior muscle (the mouse with unusual immunohistochemical characteristics (a-d) and a control mouse (e, f)) after binding of monoclonal antibodies BAD5 (a, e), BFF3 (c, f), SC71 (b) and BF35 (d) to demonstrate MyHC isoforms MyHC-1 (a, e), MyHC-2b (c, f), MyHC-2a (b) and MyHC-2x/d (d). Scale bar is 100µm.

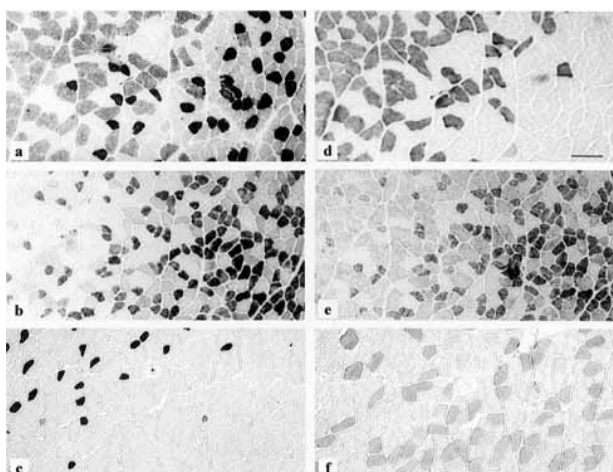


Figure 2. Immunoperoxidase staining in the mouse gastrocnemius muscle (the mouse with unusual immunohistochemical characteristics (a, b, d, e) and a control mouse (c, f)) after binding of monoclonal antibodies BAD5 (a, c), BFF3 (d, f), SC71 (b) and BF35 (e) to demonstrate MyHC isoforms MyHC-1 (a, c), MyHC-2b (d, f), MyHC-2a (b) and MyHC-2x/d (e). Scale bar is 100µm.

of 45 mature rats (Figure 3) showed unusual expression of MyHC isoforms. In the control mouse all fibres that showed positive staining after binding specific antibody against MyHC-2b (BFF3) (Figures 1c, 2d) exhibited an intense staining after binding BAD5, the antibody specific for MyHC-1 isoform (Figures 1a, 2a). In the control rat, all fibres that expressed MyHC-2b (Figure 3d) exhibited an intermediate staining intensity after binding BAD5 (Figure 3a). Pure type 1 fibres exhibited very dark staining. In the mouse, estimated area percentage of hybrid MyHC-1/MyHC-2b expressing fibres was about 15% in tibialis anterior and about 50 to 60% in gastrocnemius muscle. The rest of the fibres in the TA contained MyHC-1, MyHC-2a, MyHC-2a/MyHC-2x/d or

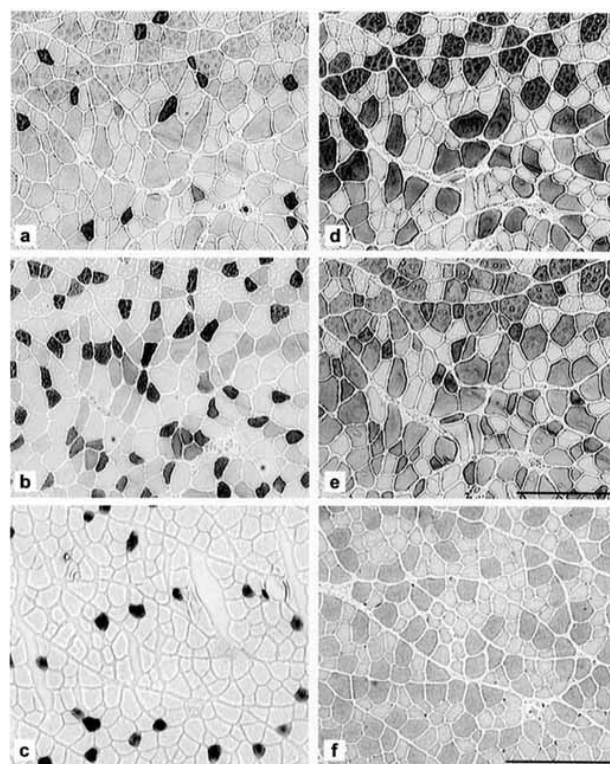


Figure 3. Immunoperoxidase staining in the rat extensor digitorum longus muscle (the rat with unusual immunohistochemical characteristics (a, b, d, e) and a control rat (c, f)) after binding of monoclonal antibodies BAD5 (a, c), BFF3 (d, f), SC71 (b) and BF35 (e) to demonstrate MyHC isoforms MyHC-1 (a, c), MyHC-2b (d, f), MyHC-2a (b) and MyHC-2x/d (e). Scale bar is 100µm.

MyHC-2x/d. In GCM MyHC-1, MyHC-2a/ MyHC-1 and MyHC-2a/2x/d expressing fibres were identified. In the remaining five mice, no co-expression of MyHC-1 with MyHC-2b was found, neither in the TA nor in the GCM muscle (Figures 1e, 1f, 2c, 2f).

In the both rat EDL muscles with unusual MyHC isoform expression the area percentage of hybrid fibres was 45%. No co-expression of MyHC-1 with MyHC-2b was found in about 43 rat muscles, which served as a control in other experiments (Figures 3c, 3f).

Cross-innervated regenerated rat soleus muscles exhibited different levels of staining intensity after binding of BFF3 antibody (MyHC-2b): very strong in one rat (Figure 4g) and less intense in the other two rats (Figures 4b, 4l). However, in all three rats, there was an evident co-expression of MyHC-2b with MyHC-1: many fibers were stained by both BFF3 and BAD5 antibodies on serial sections, respectively. Estimated area percentage of hybrid fibres was about 30%. Control sections, where primary antibody was omitted, showed no background staining (Figure 4o). In nontreated control animals, MyHC-2b (Figure 4j) and MyHC-2x/d could not be detected in their soleus muscle fibers (figure not shown). The majority of

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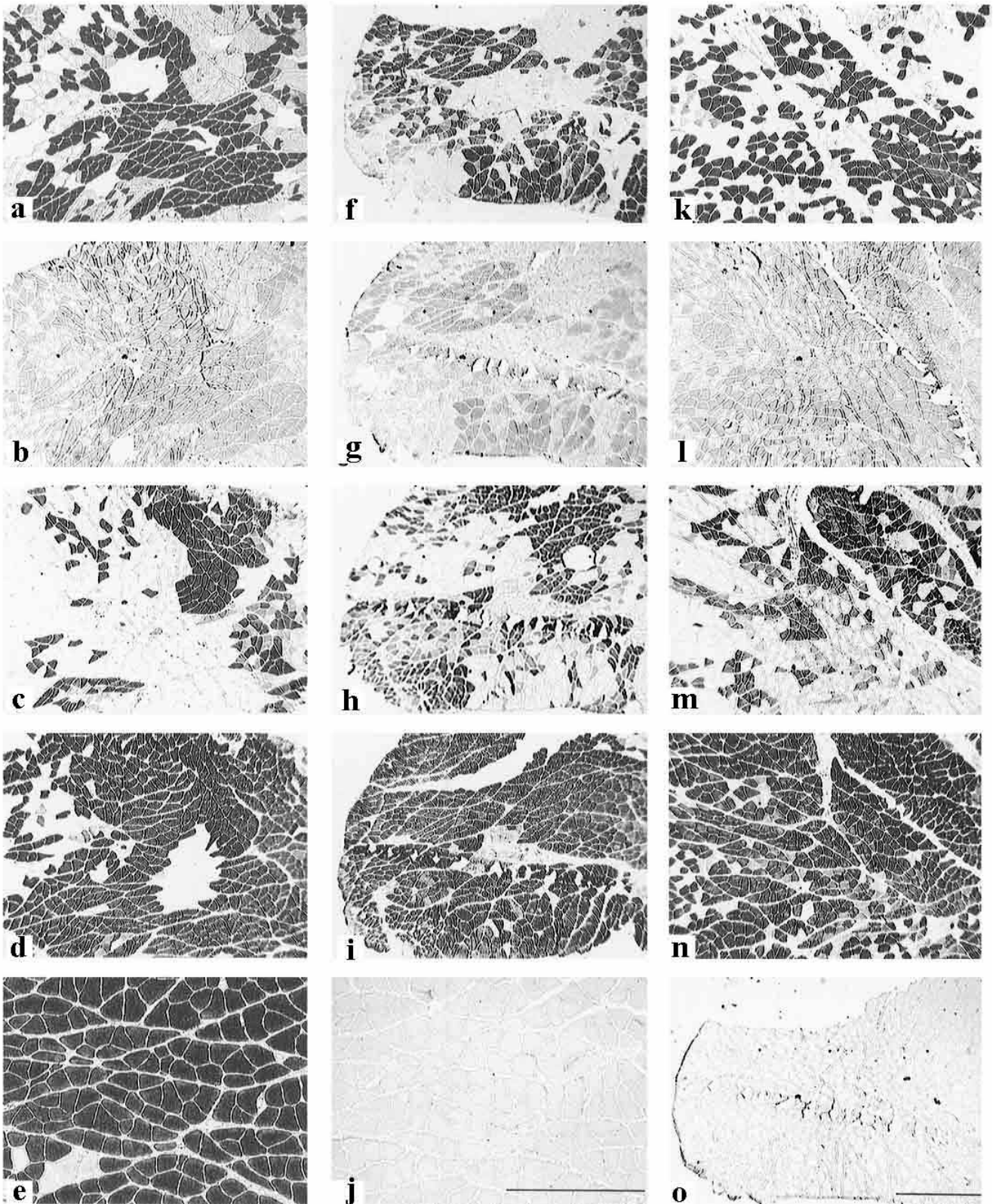


Figure 4. Immunoperoxidase staining after binding of monoclonal antibodies BAD5 (a, f, k), BFF3 (b, g, l), SC71 (c, h, m) and BF35 (d, i, n) to demonstrate MyHC isoforms MyHC-1 (a, f, k), MyHC-2b (b, g, l), MyHC-2a (c, h, m) and MyHC-2x/d (d, i, n) in regenerated cross-innervated soleus muscles of three experimental rats (after bupivacaine injection and transposition of the 'EDL' nerve). The soleus muscle of the control rat is stained for MyHC-1 (e) and MyHC-2b (j). Control section, incubated without the primary antibody is shown in panel (o). Scale bar is 500 μ m.

MyHC-1 is co-expressed with MyHC-2b in the same fibre

the fibres contained MyHC-1 (Figure 4e) and only a few fibres MyHC-2a (not shown).

Electrophoresis and immunoblotting

Specificity of antibodies directed against MyHC-2b (Figure 5a) and MyHC-1 (Figure 5c) was demonstrated by immunoblotting of corresponding gels, where MyHC isoforms had been separated by SDS glycerol gel electrophoresis (Figures 5b, 5d).

Discussion

Based on immunohistochemical reactions with monoclonal antibodies whose specificity for binding to particular myosin heavy chain isoforms was proved electrophoretically, the present report points to the possibility that in addition to the recently described co-expression of MyHC-1 with MyHC-2x/d in jump fibres (Talmadge *et al.*, 1995; Andersen, *et al.* 1999b), MyHC-1 can also be co-expressed with MyHC-2b in hybrid fibres. To unequivocally prove this statement additionally the presence of transcripts of both isoforms should have been demonstrated and co-existence of both isoforms should be proved within the same fibre using electrophoresis. Since the material described in this study was used for other purposes and the phenomenon of co-expression was noticed only later additional analysis of the material is not possible.

Although indications of the possible co-expression of MyHC-1 with MyHC-2b have been mentioned in the literature (Stahl *et al.*, 1994; Snoj-Cvetko *et al.*, 1996; Caiozzo *et al.*, 1997) in contrast to this study, coexistence of both MyHC isoforms was described only in individual muscle fibres and the phenomenon has not been generally accepted.

We were well aware that the observed MyHC-2b and MyHC-1 co-expression in a high percentage of muscle fibres is very unusual. Therefore, we carefully checked possibilities that might lead to an artificial co-localisation of both MyHC isoforms in the same muscle

fibres of the samples analysed in this study. (i) The results obtained in this study proved to be reproducible since equal results were obtained on the same material in three independent immunoperoxidase stainings. (ii) Immunoperoxidase reaction was clear with no background staining and control sections were completely unstained (Figure 3).

(iii) The observed MyHC isoform co-expression is most probably not due to the lack of antibody specificity. By using SDS glycerol gel electrophoresis and immunoblotting in parallel experiments, we have proved that BFF3 antibody specifically binds to MyHC-2b and BAD5 antibody to MyHC-1, from both rat and mouse muscles (Figure 3). However, this does not exclude a possible cross-reaction with another unknown antigen that might be present in some muscles in fibres expressing MyHC-1.

(iv) The BFF3 antibody, as described by Schiaffino *et al.* (1989) is an IgM, in contrast to the other antibodies, which are all IgGs. This is probably the reason why the staining intensity is weaker after immunoperoxidase detection with this antibody compared to the reaction obtained after binding of antibodies against other MyHC isoforms.

In the present study possible co-expression of MyHC-1 with MyHC-2b has been found even in mature muscles of mouse and rat, what indicates that the described phenotype can be stable. However, in general it is a rare event.

In regenerating cross-innervated rat soleus muscles, all muscle fibres that showed positive immunohistochemical reaction after binding of the monoclonal antibody against MyHC-2b, also co-expressed MyHC-1. However, the immunohistochemically estimated level of MyHC-2b was different in different animals suggesting that the observed MyHC co-expression in experimental rats was probably only transient. In agreement with this, in our further experiments (results not shown) where we analysed Marcaine injected soleus muscles five months after peroneal nerve transposition, there was not a trace of the co-expression of MHC-1 with MHC-2b. This is also reinforced by the recent data of Agbulut *et al.* (personal communication, manuscript in preparation) who have showed by SDS glycerol gel electrophoresis that MHC-2b is transiently expressed in the soleus muscle both during development and regeneration of the soleus muscle.

Intermediate fibres, expressing two or more MyHC isoforms have been described in animal and human muscles (Billeter *et al.*, 1980; Pierobon-Bormioli *et al.*, 1981; Pette & Staron, 1990; Aigner *et al.*, 1993; Talmadge *et al.*, 1995; Sant'Ana Pereira *et al.*, 1995; Andersen & Schiaffino, 1997; Caiozzo *et al.*, 1997; Grossman *et al.*, 1998). The question as to whether the intermediate fibres represent transitional fibres, resulting from transformation of one fibre into another, or represent a stable fibre type with a mixed myosin composition, has been addressed in

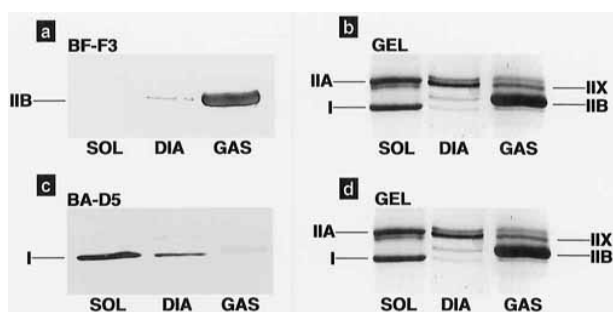


Figure 5. Electrophoretic separation (b, d) and western blot analysis (a, c) of MyHC isoforms of mouse soleus, diaphragm and gastrocnemius muscles after binding of monoclonal antibodies BFF3 (a) BAD5 (c) to confirm the specificity of these antibodies for MyHC-2b (a) and MyHC-1 (c). SOL - soleus, DIA - diaphragm, GAS - gastrocnemius.

MyHC-1 is co-expressed with MyHC-2b in the same fibre

different studies (Talmadge *et al.*, 1995; Andersen & Schiaffino, 1997; Talmadge, 2000). In muscles, exposed to experimental procedures that promoted fast to slow or slow to fast fibre type transitions, some fibres have been demonstrated to express two MyHC isoforms for a long time after the original experimental trigger had ceased (Talmadge *et al.*, 1999). Therefore, one can assume that intermediate fibres probably represent a stable fibre type (Talmadge, 2000).

Existence of 'jump fibres' in experimentally modified muscles indicates that MyHC isoform transitions may not always follow the pathway from MyHC-1 (β slow) $\leftrightarrow$ MyHC-2a $\leftrightarrow$ MyHC-2x/d $\leftrightarrow$ MyHC-2b and *vice versa*. When a muscle has to adapt quickly to a new physiological or experimentally induced conditions, protein transcription might skip one or two MyHC encoding genes, resulting in the co-expression of MyHC-1 with MyHC-2x/d (without MyHC-2a) (Talmadge *et al.*, 1995; Andersen & Schiaffino, 1997; Grossman *et al.*, 1998; Andersen, *et al.* 1999b) or turn over times of these intermediate MHC isoforms may vary. However, in animal muscles the induced percentage of jump fibres was much higher than that in human muscles (Talmadge *et al.*, 1995; Grossman *et al.*, 1998; Andersen *et al.*, 1999b).

Myonuclear discoordination in mRNA expression could result from the fact that some myonuclei might be pre-programmed to maintain a slow phenotype, or they might be more resistant to adaptation than other myonuclei. On the other hand, each myonucleus may transcribe multiple MyHC genes more or less simultaneously. In 30-day spinal cord transected rat soleus, 40% of the fibres co-express MyHC-1 with MyHC-2x/d and a similar phenomenon has been described after space flight (Talmadge *et al.*, 1995; Grossman *et al.*, 1998). These findings demonstrate that at least some fibres or myonuclei have the ability to 'jump' from type MyHC-1 to type MyHC-2x/d expression rapidly and perhaps without first expressing type MyHC-2a. Hence, slow to fast transformation in the rat soleus does not necessarily have to occur through a sequential conversion from type 1 $\square$ 2a $\square$ 2x/d $\square$ 2b.

The present study suggests the possibility that muscle fibre transformations can be even faster and two isoforms can be skipped. Persistence of this unusual phenotype even in mature control animals, in which no experimental procedure that promotes fibre type transformation was induced, is difficult to explain. If every myonucleus has an equal chance to express all MyHC isoforms then intrinsic and extrinsic factors might be those that favour expression of one or the other gene. For the maintenance of expression of a single MyHC isoform, such as that normally observed in control fast and slow hindlimb muscles either proper electrical activation or load bearing or both are required (Talmadge, 2000).

In summary, we have found that the co-expression of MyHC-1 with MyHC-2b can occur not only within single

fibres in different muscles but also in the majority of fibres in muscles of a mature mouse and rat and in muscle samples of some experimental rats. However, the methods that we have used could not provide sufficient proof for an unequivocal coexistence of both antigens within the same muscle fibre. Subsequently they did not allow us to give any reliable explanation of the mechanism underlying this unusual co-expression of MyHC isoforms in mice and rats, therefore we leave this question open.

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Address correspondence to:

Ida Eržen, Institute of Anatomy, Medical Faculty, Korytkova 2, 1000 Ljubljana, Slovenia, tel. +01 543 73 12, fax +01 543 73 01, Email ida.erzen@mf.uni-lj.si.

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MyHC-1 is co-expressed with MyHC-2b in the same fibre

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