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Abundant expression of myosin heavy-chain IIB RNA in a subset of human masseter muscle fibres

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Abstract

Type IIB fast fibres are typically demonstrated in human skeletal muscle by histochemical staining for the ATPase activity of myosin heavy-chain (MyHC) isoforms. However, the monoclonal antibody specific for the mammalian IIB isoform does not detect MyHC IIB protein in man and MyHC IIX RNA is found in histochemically identified IIB fibres, suggesting that the IIB protein isoform may not be present in man; if this is not so, jaw-closing muscles, which express a diversity of isoforms, are likely candidates for their presence. ATPase histochemistry, immunohistochemistry polyacrylamide gel electrophoresis and in situ hybridization, which included a MyHC IIB-specific mRNA riboprobe, were used to compare the composition and RNA expression of MyHC isoforms in a human jaw-closing muscle, the masseter, an upper limb muscle, the triceps, an abdominal muscle, the external oblique, and a lower limb muscle, the gastrocnemius. The external oblique contained a mixture of histochemically defined type I, IIA and IIB fibres distributed in a mosaic pattern, while the triceps and gastrocnemius contained only type I and IIA fibres. Typical of limb muscle fibres, the MyHC I-specific mRNA probes hybridized with histochemically defined type I fibres, the IIA-specific probes with type IIA fibres and the IIX-specific probes with type IIB fibres. The MyHC IIB mRNA probe hybridized only with a few histochemically defined type I fibres in the sample from the external oblique; in addition to this IIB message, these fibres also expressed RNAs for MyHC I, IIA and IIX. MyHC IIB RNA was abundantly expressed in histochemical and immunohistochemical type IIA fibres of the masseter, together with transcripts for IIA and in some cases IIX. No MyHC IIB protein was detected in fibres and extracts of either the external oblique or masseter by immunohistochemistry, immunoblotting and electrophoresis. Thus, IIB RNA, but not protein, was found in the fibres of two different human skeletal muscles. It is believed this is the first report of the substantial expression of IIB mRNA in man as demonstrated in a subset of masseter fibres, but rarely in limb muscle, and in only a few fibres of the external oblique. These findings provide further evidence for the complexity of myosin gene expression, especially in jaw-closing muscles.

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Keywords

In situ hybridization; Myosin gene expression; ATPase histochemistry; Immunohistochemistry; External oblique muscle; Fibre types

1. Introduction

Variations in skeletal muscle contraction are associated with qualitatively different mATPase activities of myosin heavy-chain protein isoforms. A well-known histochemical system (Brooke and Kaiser, 1970) uses this differential mATPase activity to classify fibres into different types. At present, fibres are usually typed by their mATPase histochemical reactivity and, in addition more recently, by immunological and electrophoretic characterization of myosin heavy-chain protein isoforms. In the Brooke and Kaiser mATPase method, preincubation in mild acidic buffers differentiates fast human muscle fibres into a IIB subclass, which is moderately reactive, and a IIA subclass, which is not reactive. Although pH sensitivity is not identical in skeletal muscle from other mammals, in many species the fibres have been classified into type I, IIA and IIB by histochemical staining. These fibre types have been found biochemically to contain different isoforms (Dalla Libera et al., 1980) and antibodies have been developed that react specifically with either type I, IIA or IIB fibres, which confirm the earlier histochemical classifications (Pierobon-Bormioli et al., 1981). A third subclass of fast fibres, type IIX, has been identified in rat, mouse and guinea-pig limb muscle (Gorza, 1990), based in part upon differential reactivity for a series of myosin heavy chain-specific monoclonal antibodies.

cDNA probes specific to the genes for human myosin heavy-chain I, IIA, and IIX have been prepared (Saez and Leinwand, 1986; Smerdu et al., 1994) to analyse their expression in fibre-type subclasses. The riboprobe for myosin heavy-chain I hybridized with histochemically defined type I fibres and that for IIA with type IIA fibres, but the riboprobe for myosin heavy-chain IIX was found to hybridize with type IIB fibres (Smerdu et al., 1994; Ennion et al., 1995). In addition, the presence of the IIB protein isoform in histochemically defined IIB fibres has not yet been confirmed because, unlike in the rat and mouse, an antibody reactive to human IIB only has not been available. Nevertheless, the gene for human myosin heavy-chain IIB has been identified by RT-PCR (Weiss et al., 1999), indicating that the IIB gene is transcribed. Given that human skeletal muscle has only three main classes of histochemical fibre types, yet genes for type I, IIA, IIX and now IIB have been identified, the fibres that might express message for myosin heavy-chain IIB should be sought.

Human jaw-closing muscles are unusual in comparison to other skeletal muscles in that their fibres cannot always be classified simply by mATPase histochemistry into subtypes I, IIA and IIB, because characteristically they display heterogeneity of myosin heavy-chain proteins (Butler-Browne et al., 1988; Bredman et al., 1991; Sciote et al., 1994). The morphology of these fibres is also unusual in that the type II subclasses are of smaller diameter than the type I subclasses. Human masseter, for example, expresses the myosin heavychain I, IIA, and IIX isoforms, as in limb muscles, but also the developmental (Butler-Browne et al., 1988) and cardiac (Bredman et al., 1991) isoforms. These proteins are found together in a variety of patterns in single fibres when biopsies are obtained from normally functioning, healthy young adult masseters (Sciote et al., 1994). The human jaw-closing muscles must therefore regulate protein expression differently from typical limb muscles, and this includes the expression of an unusually wide variety of myosin heavy-chain genes. Thus, in order to increase the probability of positively detecting IIB gene transcripts in human skeletal muscle, masseter should be included as a possible candidate tissue.

We have now used protein-based methods and in situ hybridization, which included a myosin heavy-chain IIB-specific probe, to compare the myosin heavy-chain isoform composition and RNA expression in the human masseter and in other fibres from the external oblique, gastrocnemius and triceps muscles.

2. Materials and methods

All muscle samples were obtained according to guidelines established by the University Internal Review Board for use of Human Subjects. Fifteen biopsies were obtained by one oral-maxillofacial surgeon, Dr Braun, from the anterior deep portion of masseter of healthy males and females, 18–35 years of age, who were undergoing orthognathic surgery to reposition one or both jaws in conjunction with orthodontic treatment. Samples of two limb and one abdominal muscle were taken to serve as controls for comparison with masseter: one sample of the external oblique from a 57-year-old woman, one of triceps (arm muscle) from a 33-year-old man and one of gastrocnemius (leg muscle) from a 40-year-old woman. These muscles were obtained by one orthopaedic oncology surgeon, Dr Yaw, and represent a limited but diverse sampling of human somatic muscle. Several sections of mouse gastrocnemius were kindly provided by Dr Harry Charleton (Oxford University, UK) for use as a control. Muscle samples were quickly frozen at −70 °C in isopentane with dry ice and cryostat sectioned (10 μm) at −26 °C. Sections for ATPase histochemistry and immunohistochemistry were mounted on coverslips and stored at −70 °C. Sections for in situ hybridization were mounted on 0.2% gelatin-coated slides, fixed with 4% paraformaldehyde in 1× PBS, pH 7.2, washed sequentially in 3× and 1× PBS, and dehydrated with increasing concentrations of ethanol. The sections were air-dried and stored at −70 °C with desiccant. All reagents used for these and subsequent procedures for in situ hybridization were prepared with autoclaved, sterile water treated with 0.2% diethylpyrocarbonate.

Our objective was not to characterize all of the fibre types in any of the muscles sampled or to characterize all the fibre types in any given biopsy of masseter or limb muscle. Especially for masseter, we (Sciote et al., 1994) and others have done this before. It was also beyond our scope to describe all the various possible types and gene expression of myosin heavy chains in human masseter by combining ATPase histochemistry, immunohistochemistry, in situ hybridization and discontinuous SDS–PAGE. In fact, it would very difficult to acquire enough suitable serial sections for all three staining techniques for all isoforms and message in human masseter. So, we started with a more modest goal: to take the myosin heavy-chain genes typically expressed in healthy adult mammalian limb muscle (types I, IIA, IIB and IIX) and determine their pattern of expression in an area of masseter muscle that did not contain α-cardiac or developmental myosin heavy-chain proteins. At first, the limb and abdominal muscles were used only as a control to show the adequacy of the technique, but quite by chance we did find one abdominal skeletal muscle in which the gene for the myosin heavy-chain IIB was expressed.

We did conventional fibre typing by sectioning and staining, and confirmed the content of myosin heavy-chain proteins on glycerol gels before we determined their suitability for in situ hybridization. Five masseter samples shown not to have α-cardiac or developmental protein, and in which morphological features and the number of slides were adequate, were used for in situ hybridization. For limb and abdominal muscle, all four techniques were used routinely. In the samples of gastrocnemius, triceps and external oblique, only the external oblique expressed any myosin heavy-chain IIB gene

Muscle fibre phenotypes were identified by histochemical staining for mATPase activity according to the methods of Brooke and Kaiser (1970), with modifications by Snow et al.

(1982). Sections were preincubated in acidic acetate buffers (pH 4.3–4.5) for the general detection of type I slow fibres and in alkaline barbital–acetate buffers (pH 10.2–10.3) for type II fast fibres. Additional incubations at pH 4.6–4.9 in increments of 0.1 pH units, and at pH 10.4, were made to detect type IIX and type IIB fibres, respectively (Del-Gaudio et al., 1995). This histochemical approach works as well on human jaw-closing and abdominal muscles (Sciote et al., 1994). Fibre types were confirmed in serial sections by immunohistochemistry using the anti-myosin heavy-chain monoclonal antibodies BA-F8 (anti-I), SC-71 (anti-IIA), BF-F3 (anti-IIB), MY-32 (anti-II) (Sciote et al., 1994; Sciote and Rowlerson, 1998), F88112FF8 (anti-α-cardiac; Sera Lab clone) (Sciote et al., 1994), anti–neonatal polyclonal antibody (Scapolo et al., 1991; Sciote et al., 1994) and BF-G6 (anti-embryonic) (Schiaffino et al., 1986), as described previously, but because the BF-F3 hybridoma does not stain histochemically defined IIB fibres in man and no monoclonal antibody against myosin heavy-chain IIX was used, proof of the existence of type IIX fibres was indirect. Mouse hybridomas for monoclonal antibody against myosin heavy-chains I, IIA and IIB were produced by Dr S. Schiaffino (Padova, Italy) and deposited with the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MY-32 is available from the Sigma-Aldrich Chemical Co. (St. Louis). Antibody reactivity in the fibres was detected by an indirect immunoperoxidase method (Sciote et al., 1994), using peroxidase-conjugated antimouse secondary antibodies and development with 3,3′-diaminobenzidine (Vector Laboratories, Inc.) and hydrogen peroxide.

Frozen sections for in situ hybridization were returned to room temperature, rehydrated and rinsed with 1× PBS. Digestion with 30 μg/ml of proteinase *K* and acetylation with 0.25% acetic anhydride were done according to described methods (Zeller and Rogers, 1989). After washes in 0.2× SSC and 1× PBS, sections were completely dehydrated before hybridization. Procedures for hybridization with digoxigenin-labelled riboprobes were adapted from Jostarndt et al. (1994). Clones of 3′-untranslated regions of myosin heavy-chain genes (Saez and Leinwand, 1986; Smerdu et al., 1994; Weiss et al., 1999) were provided by Dr Leslie Leinwand (University of Colorado). These cDNAs included myosin heavy-chain I (170 bp) in pCR Script, IIA (132 bp) in pSK, IIX (121 bp) in pSK and IIB (103 bp) in pCR Script. Restriction enzyme and agarose gel analyses were used to verify the authenticity of each cDNA insert. Riboprobes were synthesized with digoxigenin-labelled UTP in both sense and antisense directions by in vitro transcription from the cDNA templates, according to the manufacturer's guidelines (Roche Molecular Biochemicals/Boehringer-Mannnheim), and purified on Quick Spin™ columns. Riboprobes (200–400 ng/ml) were suspended in 40% deionized formamide, 1× SSC, 10% dextran sulphate, 1× Denhardt solution, 0.67 M NaCl, 0.1 μg/μl yeast tRNA and 0.1 μg/μl salmon sperm DNA, heated at 80 °C for 5 min. Approx. 20 μl of probe was pipetted on to tissue sections, overlaid with silane-treated coverslips and hybridized overnight at 45 °C in a humidified Boekel Slide Moat™ incubator. Coverslips were removed by gentle stirring in 6× SSC, followed by two high-stringency washes at 60 °C for 20 min in 0.5× SSC, 20% formamide, and then brief rinses with 2× SSC at room temperature. Unhybridized probe was digested with 1 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, at 37 °C for 30 min, followed by five washes in 2× SSC at room temperature and another high-stringency wash for 10 min. The sections were rinsed twice with 2× SSC and 0.1 M maleic acid 0.15 M NaCl buffer, pH 7.5. Methods for the detection of hybridized probes were adapted from the manufacturer's protocols (Roche Molecular Biochemicals/Boehringer-Mannnheim), with all incubations done in humidified plastic boxes. Tissue sections were blocked twice for 15 min and 1 h with 5% heat-inactivated BSA in 0.1 M maleic acid 0.15 M NaCl buffer, pH 7.5, at room temperature. Blocking reagent was replaced with sheep anti-digoxigenin Fab–alkaline phosphatase conjugate (1:5000 in 1% BSA, 0.1 M maleic acid 0.15 M NaCl buffer, pH 7.5), incubated overnight at 4 °C, washed with the same buffer and incubated with a donkey anti-sheep Fab–alkaline phosphatase-conjugated secondary antibody (1:1000 in 1% BSA, 0.1 M maleic acid 0.15 M

NaCl buffer, pH 7.5) as before. After several rinses with the same buffer, sections were washed twice with 0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20. Alkaline phosphatase activity was visualized by incubation with 20 μl/ml 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium substrate in 0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20 at room temperature in the dark. After sufficient colour development, the reactions were stopped and the sections washed and mounted with coverslips. No hybridization was found with sense strands to any of the riboprobes used here.

Isoforms of myosin heavy-chain proteins were identified by electrophoresis in 4% polyacrylamide gels containing 6% glycerol and 0.1% SDS (a modified version of Reiser's glycerol/SDS–PAGE method (Blough et al., 1996; Sciote et al., 2001). A portion of muscle was finely minced on ice, mixed with electrophoresis sample buffer (0.15 M Tris-HCl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.002% bromphenol blue), heated at 100 °C for 5 min and centrifuged to form a pellet of particulate debris. Portions of the solubilized supernatant were applied to gels and electrophoresed at 25 mA for 1.5 h for stacking, followed by 40 mA for 24 h to separate the isoforms. Myosin heavy-chain protein standards produced in previous studies, which contained extracts from human atrial and foetal muscles, were also used in electrophoresis experiments to determine where α-cardiac and neonatal isoforms migrated relative to the I, IIA and IIX adult isoforms. Proteins were visualized by silver staining according to Morrissey (1981). Myosin heavy-chain isoforms were identified by comparing their protein mobilities with those of extracts from single muscle fibres (Sciote and Kentish, 1996) of known phenotype.

The isoforms were also identified in immunological slot blots. Contractile proteins were extracted from whole muscle and single fibres by sonication for 15 min in 40 mM sodium pyrophosphate, 1 mM MgCl2, 1 mM EGTA, adjusted to pH 9.5, which is known to preserve myosin antigenic determinants. Extracts were applied to nitrocellulose membranes under gentle vacuum using a PR600 slot-blot apparatus (Hoeffer Scientific). Non-specific binding was blocked with 0.5% non-fat dry milk in Tris-buffered saline, pH 7.4. Primary and secondary antibodies were the same as those used for immunohistochemistry. Colour reaction was detected using the TMB peroxidase substrate system (Kirkegaard and Perry Laboratories).

3. Results

3.1. Electrophoretic and immunological identification of myosin heavy-chain content in human muscle biopsies

Bands corresponding to the myosin heavy-chains I, IIA and IIX were separated by glycerol SDS–PAGE and their identities confirmed by comparison with single fibres of known myosin heavy-chain content in gels and by immunological analysis of protein slot blots (Fig. 1). In Fig. 1(A) the myosin proteins of both the masseter and external oblique muscles are shown in comparison with samples from human atrial and foetal muscle, and with the single-fibre standards. Bands corresponding to myosin heavy-chain-α and neonatal were not detected in the specimens of external oblique and masseter. In Fig. 2(B), protein blots that show the antigenic activities of the standard single fibres with antibodies to the myosin heavy chains confirm the identity of myosin heavy-chain I (BA-F8), IIA (SC-71) and fast myosin heavy chains (MY-32). The antibody to IIB (BF-F3) was uniformly unreactive with the single- fibre standards (Fig. 1) and with protein extracts of the masseter, external oblique and other human skeletal muscles tested (not shown). The banding pattern of masseter muscle varied according to the sample applied, with greatest variability in the slowest migrating IIX band (sometimes at almost undetectable amounts by silver stain). Extracts

from the external oblique contained type I, IIA and IIX bands, but the triceps and gastrocnemius contained only type I and IIA bands (not shown).

3.2. The presence of myosin heavy-chain IIB transcripts in fibres of human limb muscle

Experiments on triceps and gastrocnemius showed that the results from immunohistochemistry and in situ hybridization were concordant with those from histochemistry. Histochemically defined type I fibres stained positively with the monoclonal antibody and mRNA probe for myosin heavy-chain I; type IIA fibres stained positively with the anti-myosin heavy-chain IIA and fast monoclonals, and with the IIA mRNA probe (results not shown). The external oblique contained histochemically defined type I, IIA and IIB fibres distributed in a mosaic pattern (Fig. 2), and hence represented an example of mixed fibre-type populations, like those seen in another abdominal muscle, the rectus abdominis (Sciote et al., 1994). The external oblique stands as a good control muscle to compare with masseter because the three fibre types are easily discernible, with type I fibres staining at pH 4.3 (Fig. 2a), type II fibres at pH 10.2 (Fig. 2d), and with type I fibres staining darkly and histochemically defined IIB fibres staining intermediately at pH 4.6. (Fig. 2e). The histochemically defined IIB fibres at pH 4.6 (Fig. 2f) hybridized only with the IIX mRNA riboprobe on serial sections (Fig. 2c).

Expression of RNA for I, IIA and IIX, but not IIB, was characteristic of most areas of the external oblique sample, as detected by riboprobes (Fig. 3). The riboprobes used here displayed a generally high degree of specificity, with an additional modest amount of message coexpression involving only the IIA and IIX genes detected (Fig. 3, black and white*).

In the series of stained sections of the external oblique (Fig. 4), type IIA fibres had ATPase reactivity at pH 10.2 (Fig. 4A.d.), but not at pH 4.6 (Fig. 4A.f.) or pH 4.3 (Fig. 4A.c.). Histochemically defined type IIB fibres had ATPase reactivity at pH 10.2, moderate reactivity at pH 4.6 and no reactivity at pH 4.3, yet the majority of these ‘type IIB’ fibres showed IIX mRNA gene expression as well as an immunohistochemical staining pattern consistent with the IIX isoform. Accordingly, fibres that in earlier work had been classified histochemically as ‘type IIB’ (see Fig. 2) are here now termed type IIX. This modification is based upon cumulative evidence from myofibrillar ATPase staining, immunohistochemical staining with anti-myosin heavy-chain monoclonals and riboprobe reactivity to myosin heavy-chain gene transcripts. For the histochemically defined IIB fibres, antibody staining and in situ hybridization with IIX gene transcripts provided enough evidence to define them, subsequently, as type IIX rather than ‘type IIB’ fibres. Both IIA and IIX fibres stained positively for the general anti-fast antibody MY-32 (Fig. 4A.h.). Three representative fibres with close correlation between their myosin heavy-chain protein content and their respective myosin heavy-chain I (Fig. 4A.b.), IIA (Fig. 4A.g.) and IIX (Fig. 4A.e.) RNAs are shown in each panel of Fig. 4 to illustrate the specificity of fibre typing between the several techniques used here and to serve as landmarks. One fibre that coexpressed transcripts of IIA and IIX is designated with an asterisk.

Hybridization of the myosin heavy-chain IIB mRNA probe was detected in only a few areas of the external oblique biopsy (Fig. 4); no such areas were identified in triceps or gastrocnemius samples. The area of tissue sections from in situ hybridization experiments in Fig. 4(A) shows a single, prominent fibre that contains transcripts of myosin heavy-chain IIB (marked →; Fig. 4A.a.), together with I, IIA and IIX. Similar, multiply expressing fibres were found in two other areas of external oblique. These fibres were relatively small, but not exceptionally so as compared with others of the muscle, and showed no overt signs of denervation or abnormality in haematoxylin and eosin-stained sections (not shown). The representative fibre in Fig. 4(A) was examined in at least 60 sections and maintained

suitable morphological features throughout the several analyses used to demonstrate its phenotype and myosin heavy-chain gene expression. Dark staining at pH 4.3 and 4.6, but not at pH 10.2 or with the anti-fast antibody, indicated that it was a type I fibre. In Fig. 4(B), the results of a positive control experiment show that an anti-myosin heavy-chain IIB monoclonal (BF-F3) selectively recognized IIB fibres (Fig. 4B.a; all positively stained fibres) out of the entire fast-fibre population (Fig. 4B.b) and did not cross-react with type I fibres (Fig. 4B.c; fibres identified by black arrows) in mouse gastrocnemius. This same antibody was unreactive when tested with sections of human external oblique, including the area shown in Fig. 2(A) and the two other areas that contained multiply expressing fibres, and in protein-blotting experiments (not shown).

3.3. The presence of myosin heavy-chain IIB transcripts in fibres of human masseter

Masseter biopsies contained two main fibre types, I and IIA, when classified histochemically and immunohistochemically (Fig. 5). The type I fibres stained with the type I antibody (Fig. 5b) and were reactive for mATPase activity at pH 4.6 (Fig. 5j). The IIA fibres stained with the IIA (Fig. 5f) and general fast (Fig. 5h) antibodies, were unreactive for mATPase at pH 4.6, and were reactive at pH 10.2 (Fig. 5i). The diameters of type I fibres in the masseter were similar to those in the external oblique, but masseter type II fibres were much smaller in diameter than those of the trunk muscle (Fig. 2A). There were a few small-diameter fibres, which could not be classified as type I or IIA, that were found by subsequent antibody staining to contain more than one isoform. The presence of small-diameter IIA fibres and the coexpression of myosin proteins, especially in the small-fibre population, agree with the reported characteristics of the masseter (Sciote et al., 1994), so that the biopsies used here are fair representatives of this muscle. Although the developmental isoforms (neonatal and embryonic) and the cardiac-α isoform have earlier been found in masseter, the present investigation was restricted to biopsies in which only the I, IIA and IIX isoforms were expressed.

When compared with results from in situ hybridization, all fibres classified as type I expressed message for myosin heavy-chain I (Fig. 5a; e.g. fibre marked □; see also Table 1). However, when examined by the same criteria, fibres classified as IIA were divided into two subgroups, those that expressed IIA protein and mRNA (Fig. 5c; e.g. fibre marked*) and those that expressed message for more than one myosin heavy chain. The combinations of message in the coexpressing type IIA fibres included IIA with IIB (Fig. 5e; e.g. fibre marked →), IIA with IIX (Fig. 5d; e.g. fibre marked ●) and IIA with IIX and IIB (e.g fibre marked ➜). Detail of the fibres coexpressing IIA with IIX and IIB that contained transcripts for myosin heavy-chain IIB is shown at high magnification in Fig. 5(k). In contrast to the external oblique, an abundance of IIB transcripts was seen consistently in the small fibres of the human masseter over all areas of the several biopsies examined. The IIB antibody BF-F3, which is reactive for histochemically defined IIB fibres in mouse muscle (Fig. 4B), was unreactive for all masseter fibres (Fig. 5g).

A very small number of fibres contained more than one myosin protein isoform. One such fibre (+ in Fig. 5) expressed type I and IIX protein, having ATPase activity at both pH 10.2 and 4.6, was more reactive with the type I than the general type II antibody, and contained message for myosin heavy-chain I only. Another fibre (♦ in Fig. 5) contained large amounts of IIX protein, small amounts of type I and IIA proteins, and message for myosin heavy-chain IIA only.

4. Discussion

We have used in situ hybridization to identify RNA for myosin heavy-chain IIB in fibres of a trunk skeletal muscle, the external oblique, and in the jaw-closing masseter. Conventional

methods for the identification of myosin heavy-chain isoforms, which include mATPase histochemistry, immunohistochemistry and gel electrophoresis, were used to characterize and describe fibres in which the transcripts for IIB were found. In the external oblique the RNA for IIB was expressed in a few type I fibres that also expressed transcripts for I, IIA and IIX. IIB gene expression was very rare in this abdominal muscle, and in additional samples, one from the upper limb, the triceps, and one from the lower limb, the gastrocnemius, no IIB mRNA could be detected. In the masseter the RNA for myosin heavychain IIB was abundantly expressed in histochemical and immunohistochemical type IIA fibres that also expressed transcripts for IIA and, in some cases, IIX. IIB protein was not detected in any fibres or extracts from either muscle using an antibody that reacts specifically with this protein in mice. These data provide direct evidence that IIB message is transcribed, yet support other information suggesting that IIB protein is not present in human skeletal muscle.

Histochemical classification of fibre types in human jaw-closing muscles has been particularly difficult because coexpression of myosin heavy-chain proteins gives variable ATPase reactivities; nonetheless, one histochemical study on human masseter muscle did identify type IIB fibres, together with types I, IIA and others that coexpressed myosin heavy chains (Eriksson and Thornell, 1983). Human muscle fibres classified histochemically as type IIB were later found to hybridize with a riboprobe for myosin heavy-chain IIX and were characterized immunohistochemically as containing the IIX protein (Smerdu et al., 1994); similar results also came from experiments on single human fibres (Ennion et al., 1995), but IIB riboprobes and antibodies were not used in either of these studies. IIB protein has been detected indirectly in areas of human masseter where histochemical type IIB fibres may be found, using a monoclonal antibody against bovine muscle from the VII1H3 hybridoma that reportedly is reactive with myosin heavy-chain I and IIB isoforms (Monemi et al., 1999). A recent study using this same hybridoma on human lateral pterygoid and digastric muscles, however, reports reactivity for I and IIX proteins and not IIB (Monemi et al., 2000). With a new panel of monoclonal antibodies, including some specific for myosin heavy-chains IIA, IIX and IIB, Lucas et al. (2000) found evidence for IIB fibres and protein in the rabbit, but not in the cat or baboon. Collectively, these data suggest that the presence of true IIB fibres containing myosin heavy-chain IIB is variable among mammalian species and may not occur in human.

Because histochemically defined type IIB fibres of human skeletal muscle are known to contain IIX transcripts, some coexpression of IIB with IIX mRNAs was expected in the external oblique and masseter, but no such exclusive combination was detected in either muscle. IIB and IIX have apparently separate functions, as demonstrated by the severely affected phenotype in mice made genetically null for myosin heavy-chain IID/X compared with those lacking IIB (Acakpo-Satchivi et al., 1997). Lefaucheur et al. (1998), using in situ hybridization with 3′-untranslated region riboprobes to the porcine myosin heavy-chain genes, report that 51% of the total number of histochemical type IIB fibres in the pig longissimus muscle contain only IIB transcripts while 31% coexpress RNA for IIB with IIX. However, it is unclear whether the histochemical type IIB fibres that singly express IIB RNA were a pure IIB phenotype, because immunohistochemical analyses of myosin heavy-chain proteins were done with general anti-fast and anti-slow antibodies. These IIB RNA-containing fibres, as well as those of the human masseter and external oblique described here, may represent an as yet unclassified phenotype in moderate to large mammals whose patterns of expression and function are unknown.

Most fibres in the representative sections of the external oblique were homogeneous for histochemical phenotype and myosin heavy-chain gene transcripts, with only limited coexpression. In contrast, representative sections of the masseter displayed an array of

coexpressions for fast myosin heavy-chain RNAs and proteins (summarized in Table 1). Coexpressions among the myosin heavy-chain genes are thought to indicate dynamic molecular transitions that occur as part of phenotypic changes in muscle fibres (Pette and Staron, 1997) and some physiological correlates of these processes have been investigated in man (Andersen and Schiaffino, 1997; Andersen et al., 1999). The hybrid fibres seen here in the masseter may be the result of phenotypic transitions, although the stable expression of different amounts of isoforms could be occurring. Known examples of such hybrids include the histochemical type IM and IIC fibres (Eriksson and Thornell, 1983) that are often detected in human masseter. IM fibres express more type I than IIA protein and IIC fibres express more type IIA than I. No single factor is known to determine the coexpression of myosin heavy chains in the masseter, but embryological origin, patterns of development and innervation, and stretch-induced hypertrophy are all believed to contribute to its complexity of fibre types (reviewed in Sciote and Morris, 2000).

The coexpression and plasticity of fibre phenotypes suggest that all of the myosin heavy-chain genes are transcriptionally competent and capable of being expressed simultaneously, thus raising questions about their regulation in skeletal muscle. In man the genes for the cardiac isoforms reside on chromosome 14, whereas the genes for skeletal muscle proteins are located in a 350-kb cluster on chromosome 17, arranged in the 5′–3′ sequential order of embryonic-IIA-IIX-IIB-perinatal-extraocular (Weiss et al., 1999). Overall, the spatial arrangement of the myosin heavy-chain genes does not correspond with their temporal pattern of expression, but their genomic organization does appear to be important in other ways. For example, in the IID/X null animal model there occurs a compensatory increase among type IIA fibres while IIB fibres remain relatively unchanged (Sartorius et al., 1998), suggesting that control of expression may be more closely linked between IIX and IIA than to IIB. Promoter sites and regulatory elements for the genes of mouse myosin heavy-chain I (Thompson et al., 1991; McCarthy et al., 1999) and IIB (Lakich et al., 1998; Swoap, 1998) have been investigated, but are not well characterized in man. Hughes et al. (1993) showed that, among the known myogenic regulatory factors, high and low ratios of myogenin to MyoD mRNA are associated, respectively, with the slow and fast muscles of the rat. Recently, Walters et al. (2000) have reported that another factor, myogenic regulatory factor-4, is preferentially expressed in slow fibres of the rat gastrocnemius but not of the soleus, indicating that regulation is not only fibre- but also muscle-specific.

Our evidence of IIB gene message without detectable B protein isoform strongly suggests that one of the main regulatory steps in the expression of human myosin heavy-chain genes is post-translational processing. Although details of the regulation these genes are not known, there are several possibilities for persistent cellular mechanisms. Foremost, because all of the adult myosin heavy-chain mRNA gene transcripts are robustly expressed in some isolated fibres of the external oblique, this expression does not appear to be transitional, but may rather be a stable phenotype in skeletal muscle. This possibility is supported by the other cited reports of multiply expressing fibres in human skeletal muscle and by evidence of the long-term persistence of such fibres in the rat soleus (Talmadge et al., 1999). The stable coexpression of several myosin genes in a single fibre could result from either uniform transcription in nuclei that are functionally coordinated or possibly from non-uniform transcription among heterogeneous nuclei. The possibility of non-uniform transcription is supported by evidence that myosin heavy-chain proteins may remain localized in domains near the individual myonuclei where their synthesis originated (Pavlath et al., 1989) and that expression can be nonuniform in coexpressing single fibres (Ennion et al., 1995; Sant’Ana Pereira et al., 1995). Additionally, evidence from studies in regenerating muscle of normal and transgenic mice indicates that not all myonuclei are simultaneously active for the transcription of specific genes (Newlands et al., 1998). Accordingly,

regulation along the length of a skeletal muscle fibre is thought to be independent, with coordination of expression occurring in domains of adjacent nuclei.

Whatever the functional state of their constituent nuclei, the multiply expressing fibres in our samples of external oblique all displayed a protein phenotype that was unequivocally I/slow, indicating that only the message for myosin heavy-chain I had been translated, and masseter fibres expressing IIB mRNA most often displayed IIA mRNA and IIA protein isoform. Thus, mechanisms must be present for the selective trafficking and/or processing of the other myosin heavy-chain mRNAs that were seen by in situ hybridization. Shoemaker et al. (1999) report that localization patterns of myosin heavy-chain mRNAs are similar in both homogeneous and coexpressing fibres of rat muscle, with message for I and IIA found in peripheral areas while IIX and IIB are evenly distributed throughout. They suggest that small differences in protein-binding motifs in the 3′-untranslated regions of the myosin heavychain transcripts may determine their preferential localization to cytoskeletal elements in muscle fibres. DaCosta et al. (2000) report that differential splicing occurs in two exons of the 5′-untranslated region of myosin heavy-chain-perinatal transcripts of hind-limb muscles from 50-day-old foetal pigs. However, the way in which these mechanisms contribute to differential processing of myosin heavy-chain RNAs in adult skeletal muscle is not yet understood.

In conclusion, transcripts for myosin heavy-chain IIB were identified in fibres of human masseter and external oblique muscles, but their presence was not correlated with any specific fibre type or accompanied by detectable B protein. The basis for the unusual patterns of IIB transcription, as well as the high degree of myosin heavy-chain coexpression in masseter, where IIB transcripts were abundant in a subset of fibres, is unclear. Further studies using single-fibre techniques and IIB-specific antibodies should help in understanding the transcriptional and post-transcriptional regulation of IIB expression and the molecular basis for the diversity of myosin heavy-chain expression in human muscle.

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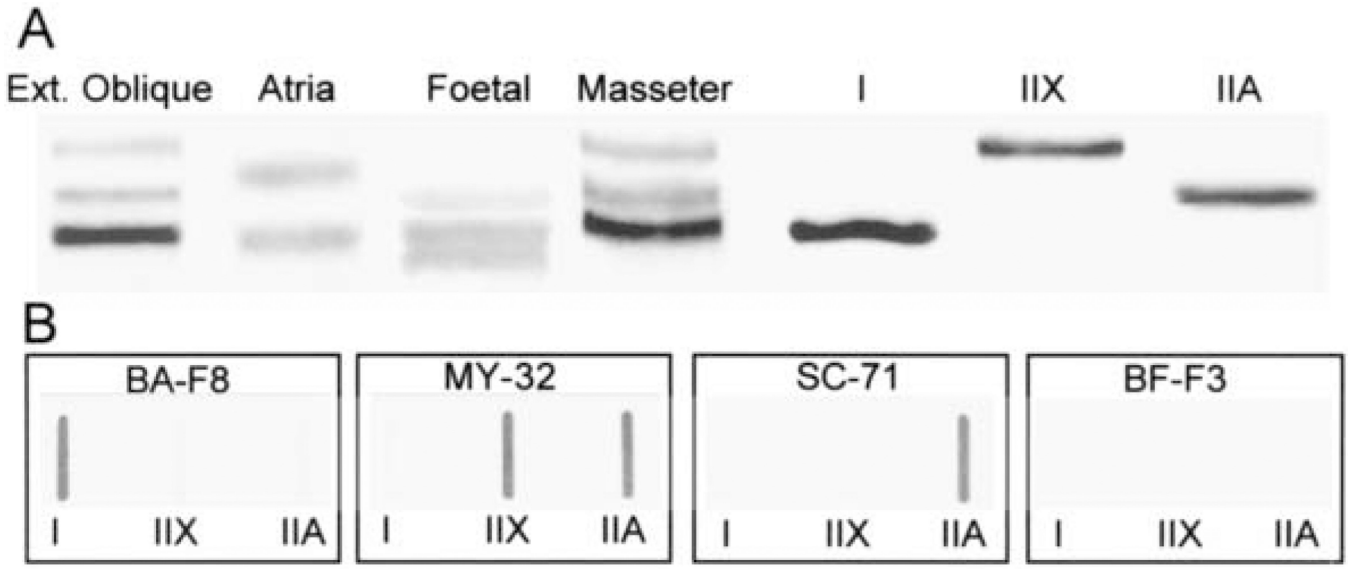

Fig. 1.

Identification of myosin heavy-chain (MyHC) protein isoforms in human muscle. (A) Samples from external oblique and masseter were compared with samples from human atrial and foetal muscle, as well as with single fibres homogeneous for isoforms I, IIA and IIX. The mobilities of proteins from a whole homogenate of external oblique and masseter, separated in 9% polyacrylamide gels containing 0.1% SDS and 6% glycerol and visualized by silver staining, correspond to those of similarly prepared type I, IIX and IIA fibres used as standards. Human atrial samples contained MyHC type I and α, and human foetal samples contained MyHC type I, IIA and neonatal. (B) The identities of the MyHC proteins in the standard single fibres were confirmed in immunological slot blots using BA-F8 (anti-type I), MY-32 (anti-type II), SC-71 (anti- type IIA) and BF-F3 (anti-type IIB) antibodies. No antigenic activity for type IIB was detected in any of the single fibres.

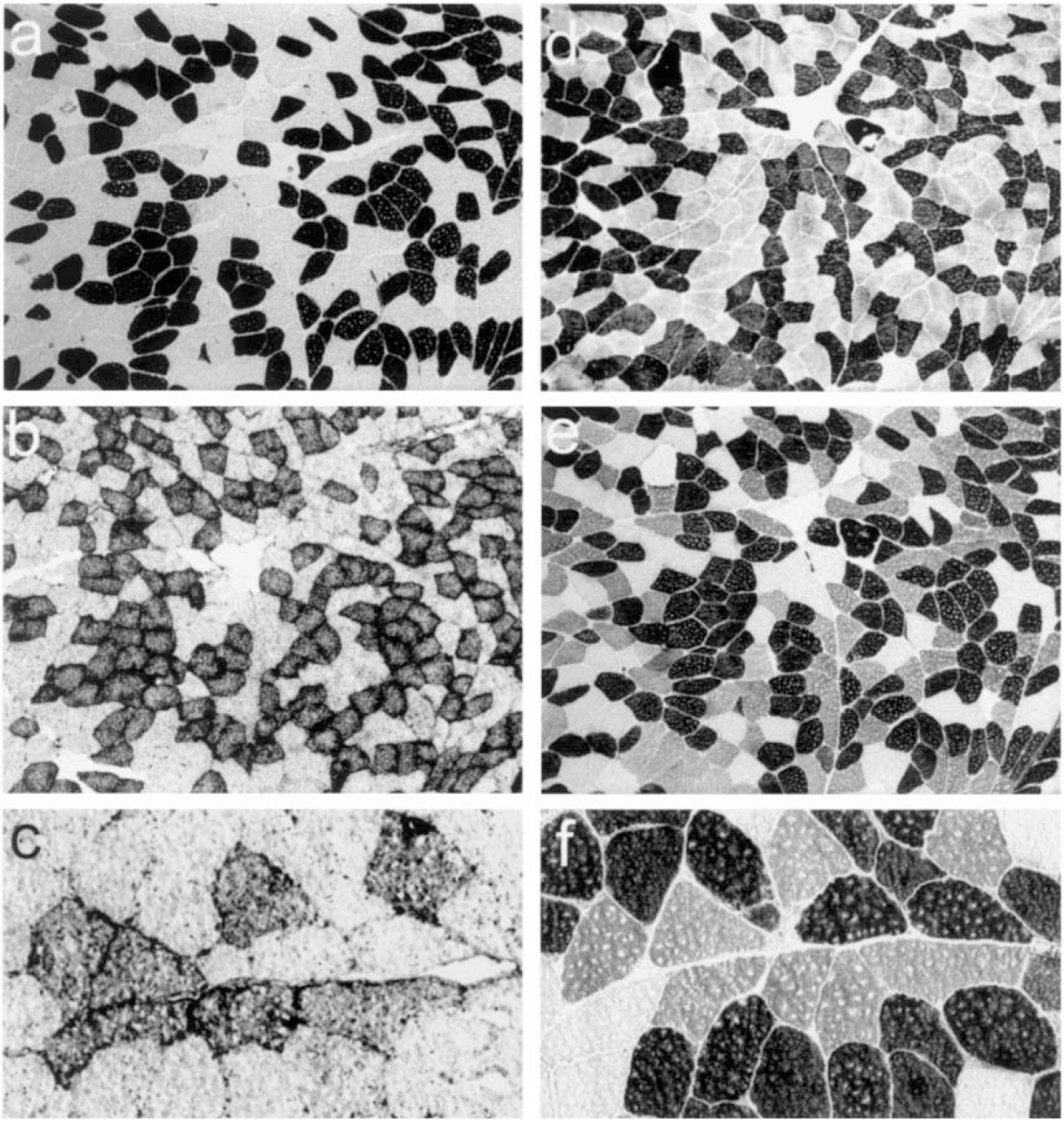

Fig. 2.

Distribution of slow and fast fibre types in serial cryosections of human external oblique muscle. Type I slow fibres are shown (a) stained positively by the histochemical method for myofibrillar ATPase after preincubation at pH 4.3 and (b) by in situ hybridization with a digoxigenin-labelled riboprobe for the expression of myosin heavy chain (MyHC) I/β. (d) Type II/fast fibres are shown stained positively for myofibrillar ATPase after preincubation at pH 10.2. (e) The fibres with intermediate staining after preincubation at pH 4.6 are histochemically defined type IIB. (f) In higher-power views, these intermediately staining histochemical type IIB fibres at pH 4.6 hybridize only with the IIX mRNA riboprobe (c). (a), (b), (d), (e)×200; (c), (f)×600.

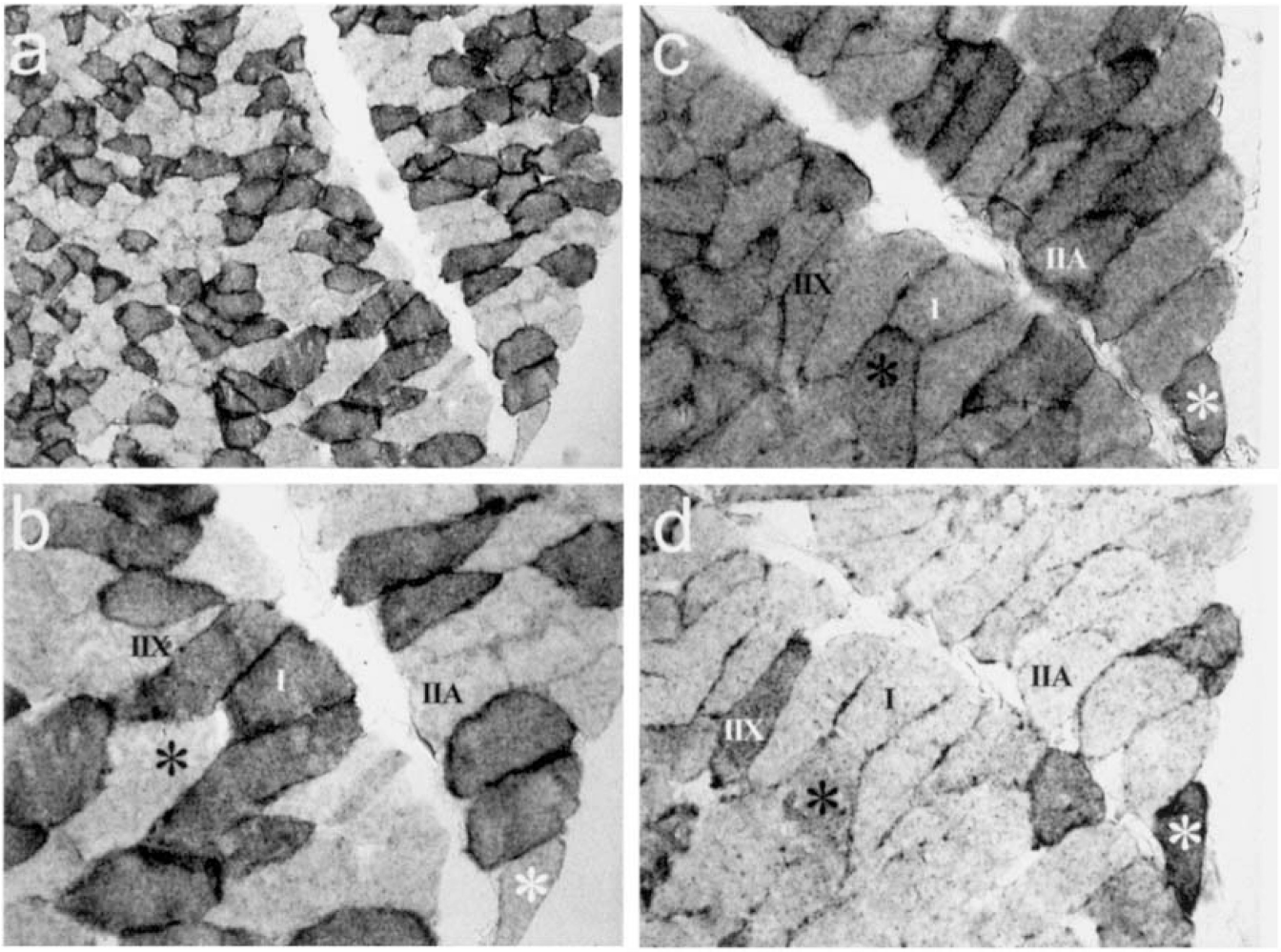

Fig. 3.

Expression of myosin heavy-chain (MyHC) isoform transcripts in fibres of serial sections from the human external oblique muscle determined by in situ hybridization with digoxigenin-labelled 3′-untranslated region riboprobes. MyHC-I/β-expressing fibres are shown at (a) ×200 and (b) ×400 magnifications; (c) MyHC-IIA- and (d) -IIX-expressing fibres are shown at ×400 magnification. The positions of fibres representative of each type and fibres that coexpress MyHC-IIA and -IIX are indicated (b,c,d). Among the coexpressing fibres, one displayed MyHC-IIA > MyHC-IIX (*) and the other MyHC-IIX > MHC-IIA (white *).

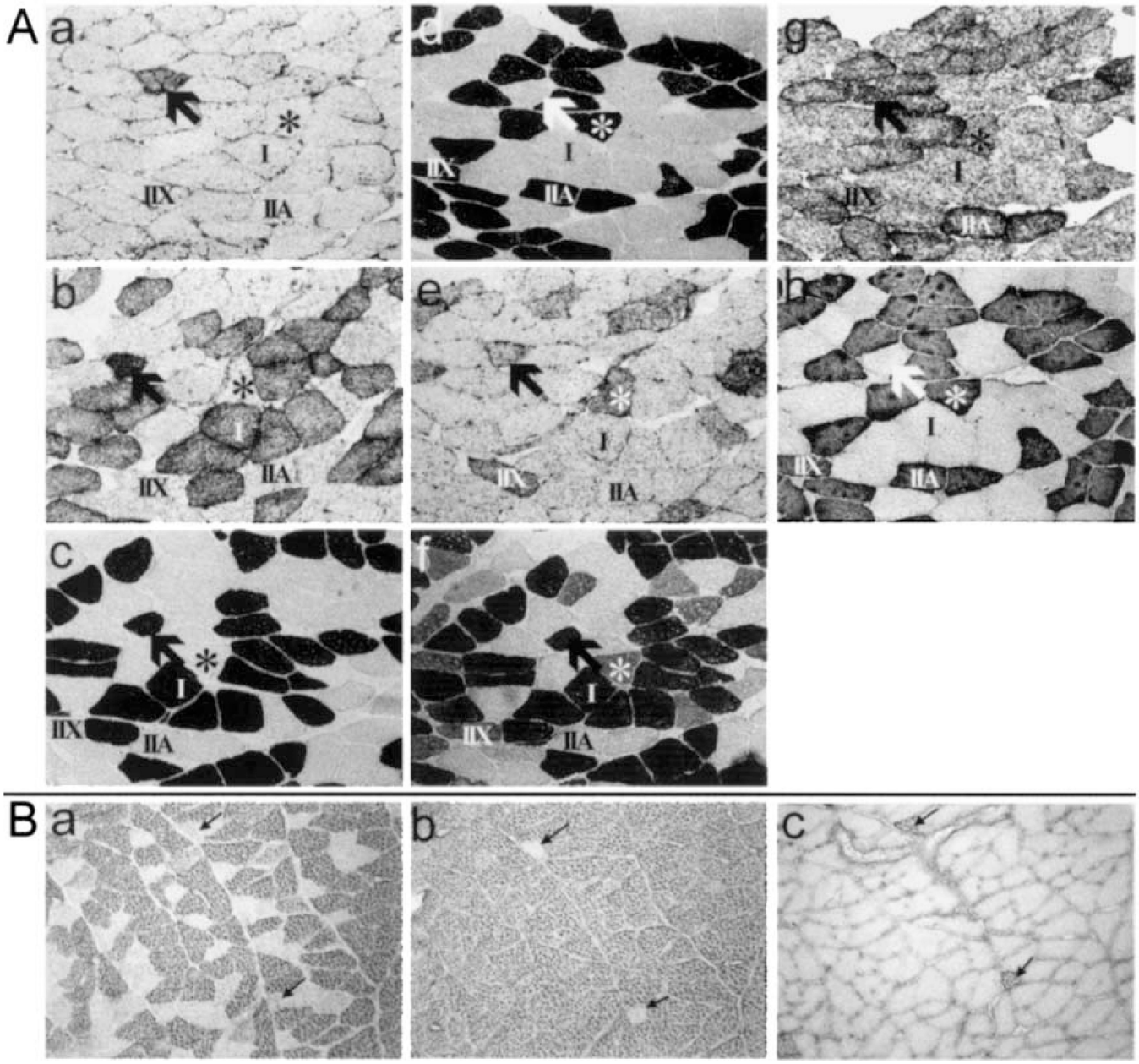

Fig. 4.

(A) Distribution of slow and fast fibre types in cryosections of human external oblique muscle: (a) in situ hybridization with myosin heavy-chain (MyHC) IIB riboprobe; (b) in situ hybridization with MyHC I/β riboprobe; (c) myofibrillar ATPase activity after preincubation in pH 4.3 buffer; (d) myofibrillar ATPase activity after preincubation in pH 10.2 buffer; (e) in situ hybridization with MyHC IIX riboprobe; (f) myofibrillar ATPase activity after preincubation in pH 4.6 buffer; (g) in situ hybridization with MyHC IIA riboprobe; (h) staining with anti-MyHC-type II antibody (MY-32). (B) Serial cryosections of mouse gastrocnemius muscle: (a) staining with anti-MyHC IIB antibody (BF-F3); (b) staining with anti-MyHC-II antibody (MY-32); (c) staining with anti-MyHC-I antibody (BA-F8). Arrows identify type I fibres. All ×200.

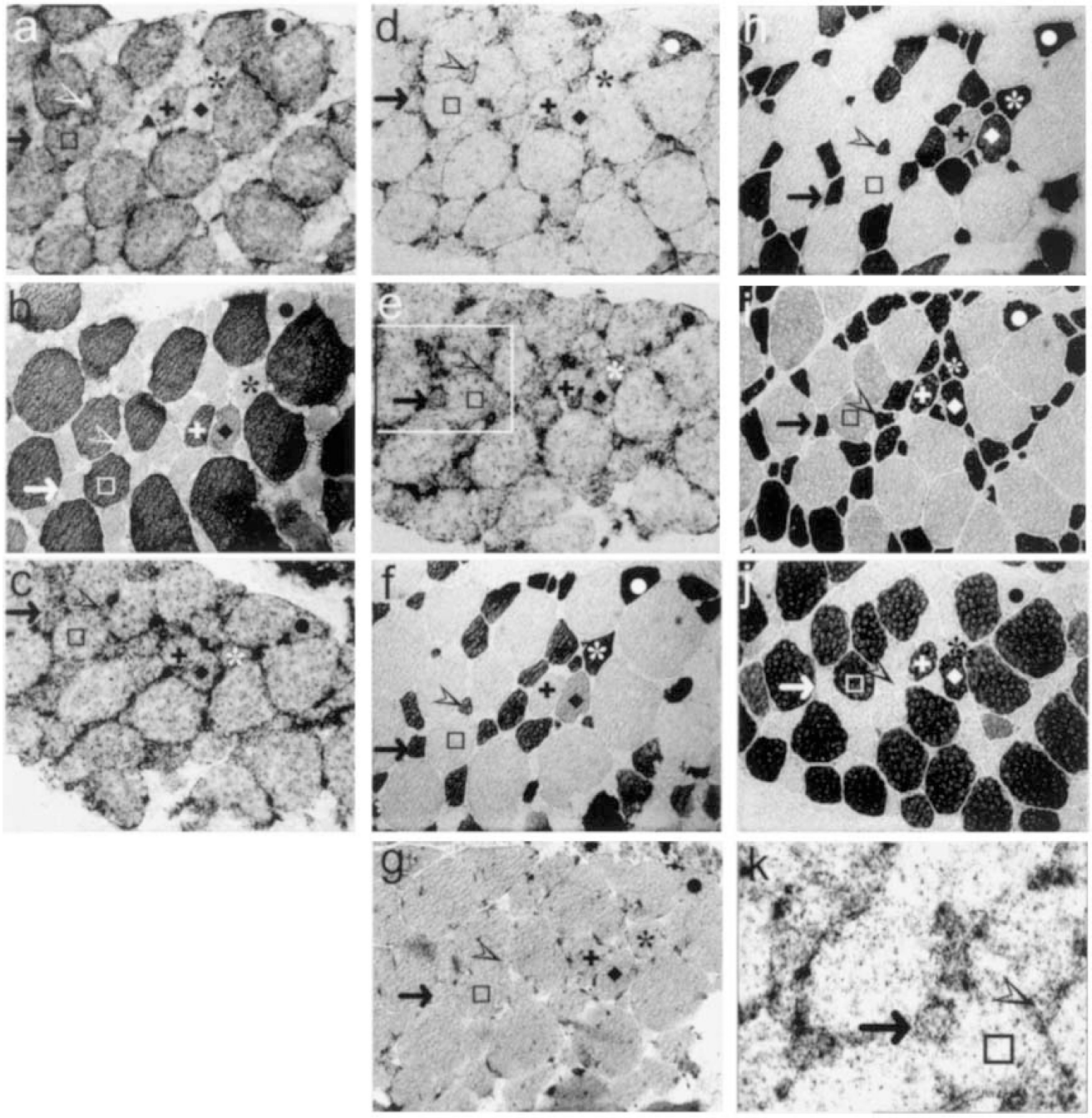

Fig. 5.

Distribution of slow and fast fibre types in serial cryosections of human masseter: (a) in situ hybridization with myosin heavy-chain (MyHC) I riboprobe; (b) staining with anti-MyHC-I antibody (BA-F8); (c) in situ hybridization with MyHC-IIA riboprobe; (d) in situ hybridization with MyHC-IIX riboprobe; (e) in situ hybridization with MyHC-IIB riboprobe; (f) staining with anti-MyHC-IIA antibody (SC-71); (g) staining with anti-MyHC-IIB antibody (BF-F3), haematoxylin and eosin counterstain; (h) staining with anti-MyHC-type II antibody (MY-32); (i) mATPase reactivity after preincubation in pH 10.2 buffer; (j) mATPase reactivity after preincubation in pH 4.6 buffer; (k) oil-immersion high-power view of insert from panel (e) (IIB hybridization). All at ×400, except for panel (k), ×600.

Table 1

MyHC protein and mRNA content in masseter fibres

Symbol	Protein content	Histochemistry		Riboprobe			
		pH l0.2	pH 4.6	I	IIA	IIX	IIB
□	I	−	+	+	−	−	−
✻	IIA	+	−	−	+	−	−
➜	IIA	+	−	−	+	−	+
●	IIA	+	−	−	+	+	−
➢	IIA	+	−	−	+	+	+
✚	I > IIX	+	+	+	−	−	−
♦	IIX > I,IIA	+	+	−	+	−	−