

Selective accumulation of MyoD and Myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones

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SUMMARY

Each of the myogenic helix-loop-helix transcription factors (MyoD, Myogenin, Myf-5, and MRF4) is capable of activating muscle-specific gene expression, yet distinct functions have not been ascribed to the individual proteins. We report here that MyoD and Myogenin mRNAs selectively accumulate in hindlimb muscles of the adult rat that differ in contractile properties: MyoD is prevalent in fast twitch and Myogenin in slow twitch muscles. The distribution of MyoD and Myogenin transcripts also differ within a single muscle and correlate with the proportions of fast glycolytic and slow oxidative muscle fibres, respectively. Furthermore, the expression of a transgene consisting of a muscle-specific *cis*-regulatory region from the *myoD* gene controlling *lacZ* was

primarily associated with the fast glycolytic fibres. Alteration of the fast/slow fibre type distribution by thyroid hormone treatment or by cross-reinnervation resulted in a corresponding alteration in the MyoD/Myogenin mRNA expression pattern. These findings show that the expression of specific myogenic helix-loop-helix regulators is under the control of innervation and humoral factors and may mediate differential control of contractile protein gene expression in adult muscle.

Key words: MyoD, Myogenin, skeletal muscle, innervation, thyroid hormone, fibre type

INTRODUCTION

Each muscle of the mature limb is a heterogeneous tissue composed of many parallel muscle fibres that contract at characteristic rates dependent principally on the type of myosin heavy chain (MyHC) they express: fast and slow fibres contain fast and slow MyHC isoforms that display high or low actin-dependent ATPase activity, respectively (Bárány, 1967). Expression of specific MyHC isoforms is also correlated with oxidative metabolic capacity providing a basis for further classification. Adult fast fibres are generally divided into three subtypes, the sluggish oxidative fibres expressing IIa MyHC and two glycolytic subtypes, intermediate speed IIX fibres and the fastest IIB fibres. In addition to MyHC, the different fibre types express distinct isoforms of many other proteins, such as myosin light chain, troponin and sarcoplasmic Ca²⁺-dependent ATPase, all of which contribute to the differences between slow and fast fibres.

Fibre type diversity begins to appear shortly after muscles form in the foetus through interaction between myoblasts

and the limb environment (Chevallier and Kieny, 1982), but independent of innervation (Butler et al., 1982; Condon et al., 1990). At these early stages muscles are still immature: embryonic MyHC isoforms are expressed, nerves are present but do not fire with characteristic fast or slow patterns and muscle cell proliferation and growth proceeds rapidly. Transitions in MyHC gene expression occur in postnatal rodents during the time that innervation is maturing (Rubinstein, 1978; Whalen et al., 1981; Hughes et al., 1993). After weaning, a change in fibre type control occurs so that extrinsic signals, such as neuronal stimuli and hormone levels, become the major determinants of fibre type-specific gene expression (Buller et al., 1960; Pette and Vrbová, 1985; Izumo et al., 1986; Ausoni et al., 1990). To date, the intracellular regulatory molecules that generate and maintain distinct patterns of gene expression in different fibre types have not been defined.

The four myogenic helix-loop-helix (HLH) proteins are candidate regulators of muscle fibre type-specific gene expression (for review, see Weintraub et al., 1991; Buckingham, 1992; Olson, 1992). Consistent with such a role,

they have been shown to activate muscle-specific promoters differentially in transfection studies (Braun et al., 1989; Yutzey et al., 1990; Brennan et al., 1990; Rosenthal et al., 1990; Fujisawa-Sehara et al., 1992), and mRNAs encoding the myogenic HLH proteins display distinct temporal patterns of expression during development when isoform expression is changing rapidly (Sassoon et al., 1989; Ott et al., 1991; Montarras et al., 1991; Hinterberger et al., 1991; Bober et al., 1991; Cusella-De Angelis et al., 1992). However, the lack of correlation between myogenic HLH mRNA expression and specific MyHC isoform expression in myoblast cell lines (Miller, 1990) and during development in vivo (Lyons et al., 1990); the ability of the myogenic HLH proteins to auto- and trans-activate their own expression and to bind to the same DNA sequence (Weintraub et al., 1991); and the fact that knockout of either MyoD or Myf-5 causes no apparent change in fibre type (Braun et al., 1992; Rudnicki et al., 1992) suggest redundant functions for the myogenic HLH proteins.

We show here that MyoD and Myogenin mRNAs are preferentially expressed in adult fast glycolytic and slow muscle fibres, respectively. Moreover, *lacZ* expression controlled by a *cis*-regulatory region of the *myoD* gene was mainly in fast glycolytic muscle fibres. Manipulation of hormone levels or innervation resulted in alterations of MyoD and Myogenin expression that paralleled changes in fibre type. Taken together these results suggest that MyoD and Myogenin may have distinct roles in adult muscle, perhaps in mediating the effects of extrinsic factors on fibre type-specific gene expression.

MATERIALS AND METHODS

RNA isolation and northern analysis

Poly(A)⁺ RNA from the muscles of adult rats (3-6 months old) were analysed by northern blot as described (Peterson et al., 1990). Filters were hybridised simultaneously with random-primed probes synthesised from full-length cDNAs specific to MyoD (1.8 kb; Davis et al., 1987) and Myogenin (1.6 kb; Edmondson and Olson, 1989) mRNAs. Hybridisation with each probe individually demonstrated the size difference and specificity (Peterson et al., 1990). A full-length MRF4 probe (Rhodes and Konieczny, 1989), an actin-coding region probe that recognises all actin transcripts (Peterson et al., 1990), and a 3' untranslated region probe from human slow MHCZ (Saez and Leinwand, 1986) were also used. Antisense oligonucleotide probes, approximately 55 bases in length, complementary to the 3' untranslated regions of rat IIb and IIa (Nadal-Ginard et al., 1982) and IIx MyHC mRNAs (S. Schiaffino, personal communication) were labelled with terminal deoxynucleotidyl transferase. Signals on autoradiograms were quantitated with a laser densitometer.

In situ hybridisation

In situ hybridisation was performed on muscles from adult Wistar rats essentially according to the method of Wilkinson et al. (1987). Specifically, tissue blocks were stored at -70°C then cut at 5-10 µm on a cryostat at -18°C, collected on TESPA-coated RNase-free glass slides, air dried and used the same day for in situ hybridisation. Sections were fixed in 4% paraformaldehyde, treated with 20 µg/ml proteinase K in 50 mM Tris-HCl pH 8.0, 5 mM EDTA, for 8 minutes at room temperature, refixed and then acetylated in 0.5% acetic anhydride in 0.1 M triethanolamine before dehydration in

graded alcohol. Probes were derived from the cDNAs described in Fig. 1, except that antisense and sense riboprobes were synthesised from T7 and T3 promoters present within vector sequences. Hybridisation was for 16-20 hours at 62°C with 10⁵ cts/minute per µl ³⁵S-labelled riboprobes that had been hydrolysed to 500 bp. Following hybridisation, slides were washed in 50% formamide, 1× SSC, 1.4 ml/l -mercaptoethanol at 65°C for 20 minutes, and then 37°C for 1 hour. Unbound probe was hydrolysed with 20 µg/ml RNase A and slides rewashed at 37°C for 30 minutes as above, followed by 2× SSC and 0.1× SSC for 15 minutes each at room temperature. After dehydration, slides were dipped in Kodak NTB-2 emulsion, dried and exposed for 4-15 days at 4°C. Due to the low abundance of MyoD and Myogenin transcripts, 15-day exposures were required, which resulted in relatively high but uniform background signals, comparable to control hybridisations with sense probes in all cases. Actin slides were exposed for 4-6 days, resulting in lower overall background signals. After developing, slides were mounted in Krystallon and photographed on a Nikon FXA microscope.

Immunohistochemistry

MyHC antibodies are described elsewhere (Hughes and Blau, 1992; Hughes et al., 1993). Briefly, unfixed 10-15 µm cryostat sections were incubated with undiluted hybridoma tissue culture supernatant to permit antibody reaction with native MyHC. Biotin-conjugated class-specific anti-mouse Ig (Vector) diluted in PBS with 5% horse serum was applied, followed by avidin-biotin complex horseradish peroxidase (Vectorstain ABC kit) which was visualised using 0.6 mg/ml di-aminobenzidine and CoCl₂. Sections were photographed as described above.

Thyroid hormone treatment

Three month old male Sprague Dawley rats were divided into groups based on body weight. The control group was fed powdered chow and the experimental group was fed chow containing 750 µg 3,3',5-triiodo-L-thyronine (T₃, Sigma) and 5 mg clenbuterol hydrochloride (Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO) per kg ad lib for 3 weeks. The clinical effect of feeding this dose of T₃ in combination with clenbuterol was marked hypermetabolism, hyperphagia and prevention of the anabolic effect of clenbuterol on skeletal muscle. After death, the soleus muscles were rapidly dissected, frozen in liquid nitrogen, and maintained at -70°C until RNA isolation as described above.

Cross-reinnervation

The superficial peroneal nerve of adult Wistar rats was transplanted to the surface of the right soleus muscle. Two weeks later, synapse formation by the implanted nerve was induced by denervating the soleus muscle, as described by Frank et al. (1974). Muscles were analysed 60 days following denervation.

Transgenic mice

A *myoD* genomic fragment from an *ApaI* site at -5870 to a *BspMII* site at +95 (Tapscott et al., 1992) was cloned into the *SmaI* site of the p16.43 betaGal vector (Fire et al., 1990). This vector lacks a conventional poly(A) site but has demonstrated high level protein expression in both stable and transient assays in tissue culture (SJT, data not shown). A fragment defined by the 5' *ApaI* site and the 3' *EagI* site was used for oocyte injections (DNX, Princeton, NJ, USA). Transgenic progeny were detected by DNA analysis and found to express -galactosidase in a wide variety of muscles, but not in any other tissue tested. Female founder #292 was bred to yield progeny #152-3 (5 week old female) and #204-2 (10 week old female) which gave similar -galactosidase staining patterns. A second founder line gave similar results (data not shown). Visualisation of -galactosidase with X-gal was as described in Hughes and Blau (1992).

RESULTS

Ratio of MyoD to Myogenin mRNA differs among muscles

An examination of the mRNAs encoding the myogenic HLH proteins by northern analysis of poly(A)⁺ RNA from five adult rat hindlimb muscles with different contractile properties revealed that the ratios of MyoD to Myogenin mRNA differed among muscles (Fig. 1). Most striking was the observation that muscles composed predominantly of slow fibres (>90%), such as the soleus (SOL) and adductor longus (AL), accumulated high levels of Myogenin and relatively little MyoD mRNA, in agreement with data that Myogenin mRNA preferentially accumulates in the soleus (Rhodes and Konieczny, 1989). By contrast, the extensor digitorum longus (EDL), a fast muscle containing fewer than 10% slow fibres, showed the reverse profile: high levels of MyoD and low levels of Myogenin mRNA. The plantaris (PLAN), a mixed but predominantly fast muscle, showed a MyoD to Myogenin mRNA ratio of 3:2. Muscles that contain substantial numbers of both fast and slow fibres, such as the lateral gastrocnemius (LG), contained high levels of both MyoD and Myogenin mRNA. MRF4 mRNA, the most abundant myogenic HLH transcript expressed in adult muscle, did not differ significantly among muscles. Moreover, Myf-5 mRNA was virtually undetectable in all

muscles tested (data not shown). Thus, muscles with differing fibre type compositions demonstrated differential MyoD and Myogenin mRNA accumulation.

MyoD and Myogenin mRNA localisation correlates with fast and slow muscle regions, respectively

As all muscles are composed of a mixture of slow and fast fibre types, we asked whether Myogenin and/or MyoD transcript accumulation was restricted to particular muscle fibres or regions within individual muscles by *in situ* hybridisation of transverse sections of adult rat hindlimbs (Fig. 2A,B). Adjacent sections were reacted with antibody A4.840 to slow MyHC (Fig. 2C). Qualitative differences in Myogenin and MyoD mRNA accumulation were consistent with the results of the Northern analysis. The Myogenin probe, but not the MyoD probe hybridised to the soleus, a slow muscle. In the superficial lateral gastrocnemius, which is composed of fast glycolytic Iib and Iix fibres, MyoD mRNA was readily apparent (Fig. 2B,G), and Myogenin mRNA accumulation was relatively low (Fig. 2A,E). By contrast, the deep red strip of the lateral gastrocnemius which is composed primarily of slow and fast oxidative Iia fibres, showed strong hybridisation to the Myogenin probe (Fig. 2A,D), but the MyoD signal was not detectable above background (Fig. 2B,F). Thus, individual regions with different functional properties within single muscles, as well as entire muscles, differ in their MyoD/Myogenin mRNA ratio. It is also clear from this analysis that the fast glycolytic fibres in the superficial lateral gastrocnemius contained high MyoD and little detectable Myogenin mRNA, whereas MyoD mRNA was not detectable in the fast oxidative fibres present in the deep lateral gastrocnemius and in low numbers in the soleus. This result suggests that not only does the MyoD/Myogenin mRNA ratio distinguish between fast and slow fibre types, but also between two subclasses of fast fibre types: oxidative and glycolytic.

We were unable to achieve single fibre resolution with these *in situ* hybridisations because of the low abundance of the MyoD and Myogenin mRNAs in adult muscle. Moreover, the specific signals generated by both the Myogenin and MyoD probes were localised to the periphery of the muscle fibres, preventing an assignment of signal to individual fibres. This peripheral localisation of mRNA is consistent with the location of the fibre nuclei that lie subjacent to the plasma membrane (Russell and Dix, 1992). Myogenin mRNA demonstrated an annular distribution in slow regions (Fig. 2A,D), whereas MyoD appeared more punctate in fast regions (Fig. 2B,G). Control experiments showed that even actin mRNA, which is highly abundant in muscle fibre cytoplasm, displayed a similar annular distribution in slow and a punctate pattern in fast muscle (Fig. 2H,I). This apparent difference in location may simply reflect the greater density of muscle cell nuclei in slow muscle tissue (Schmalbruch, 1985).

While the Myogenin signal showed an annular pattern in slow regions (Fig. 2A,D), rare intense Myogenin⁺ 'dots' were detected in all muscles (Fig. 2E, arrows). That the punctate Myogenin dots represent actively dividing or differentiating myoblasts is suggested by the size, location and rarity of dots, and the frequent occurrence of dots in pairs (23%, of 113 dots 26 appeared in pairs). An alternative

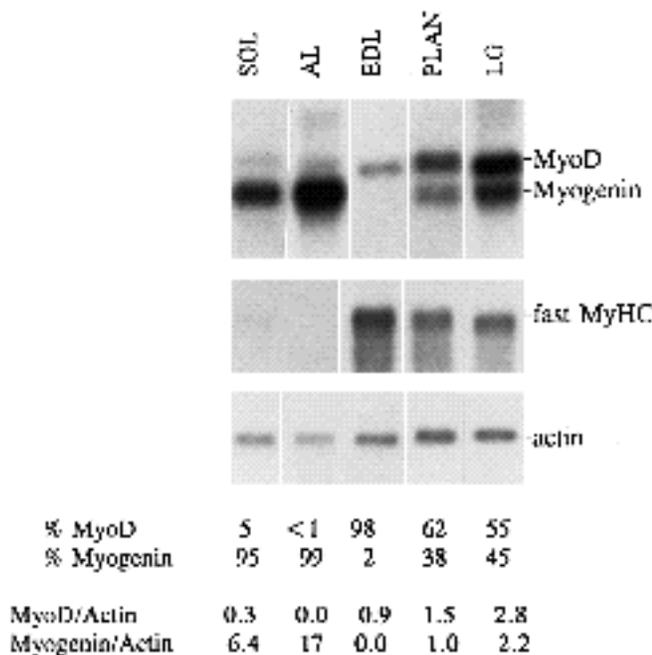


Fig. 1. Ratio of MyoD to Myogenin mRNA differs among muscles. Poly(A)⁺ RNA (3 µg/lane) from adult rat muscles were analysed by northern blot. Filters were hybridised simultaneously with probes specific to MyoD and Myogenin mRNAs. The filter was stripped and rehybridised sequentially with the probes indicated. The MyoD/Myogenin blot was exposed for 4 days, fast MyHC Iia for 12 hours, and actin for 10 minutes. Similar results were obtained with Wistar, Sprague Dawley and Fisher 344 rats. SOL, soleus; AL, adductor longus; EDL, extensor digitorum longus; PLAN, plantaris; LG, lateral gastrocnemius.

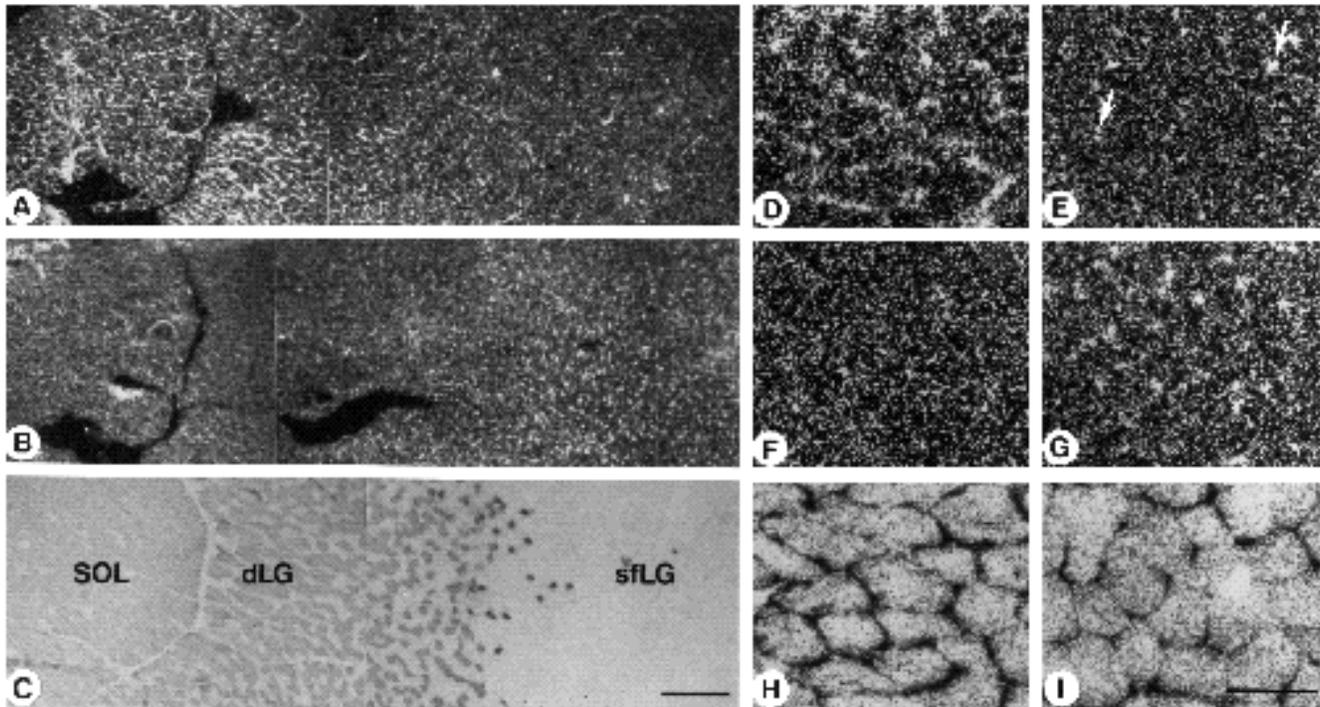


Fig. 2. MyoD and Myogenin mRNA localisation correlates with fast and slow muscle regions, respectively. In situ hybridisation using antisense riboprobes to Myogenin (A,D,E), MyoD (B,F,G), and actin-coding region (H,I) was performed on serial frozen sections of the soleus (SOL), the deep red strip (dLG) and superficial (sfLG) regions of the lateral gastrocnemius of the hindlimb from adult rats. Immediately adjacent serial sections (C) were reacted with antibody A4.840 to slow MyHC and visualised as described previously (Hughes and Blau, 1992; Hughes et al., 1993). High magnification micrographs of the dLG (D,F,H) and sfLG (E,G,I) are shown. Myogenin signal showed an annular pattern in slow regions (A,D,E), and rare intense Myogenin⁺ 'dots' (arrows) in all muscles. MyoD showed a punctate pattern in fast regions but signals were not detected above background in slow muscles (B,F,G). A,B,D-G are dark-field micrographs, C,H,I are bright-field micrographs. A-C: bar, 500 μ m; D-I: bar, 100 μ m.

explanation, that Myogenin dots reflect Myogenin mRNA accumulation at neuromuscular junctions, seems unlikely as we did not detect any differences in the frequency of Myogenin dots along the length of the muscles in which neuromuscular junctions are located in the midbelly region away from myotendinous junctions (Bevan and Steinbach, 1977) (data not shown). Thus, although various structural features of the muscle may contribute to the different hybridisation patterns with MyoD and Myogenin probes, fibre type composition appears to be a major component.

The *myoD* promoter drives *lacZ* expression in a subset of fast fibres

Although supporting a correlation between MyoD and Myogenin expression and specific fibre types, our in situ hybridisation analysis was unable to discriminate at the single fibre level. To examine in greater detail the differential expression of MyoD, a transgenic mouse was constructed using a DNA fragment from the 5' flanking region of the murine *myoD* gene, from -5870 to +95, driving *nslacZ*, which encodes a modified β -galactosidase that is targeted to nuclei because of the addition of an SV40 nuclear localisation signal to the N terminus. This *myoD* regulatory region has been shown to confer muscle specificity in vitro (Tapscott et al., 1992). Tissue sections from the mice contained β -galactosidase activity localised to nuclei in skeletal muscles, but not in nerve, bone or smooth muscle.

Expression of β -galactosidase differed between mouse muscles just as did endogenous MyoD expression in rats: in fast muscles numerous β -galactosidase⁺ blue nuclei were observed at the periphery of muscle fibres, whereas in the soleus (which in the mouse contains approximately equal numbers of slow and fast oxidative IIA fibres), little β -galactosidase was detected (Fig. 3A,B). The frequency of β -galactosidase⁺ nuclei in many fast muscles was too high to be accounted for by the rare satellite myoblasts present (Schmalbruch, 1985; Fig. 3C), suggesting that most β -galactosidase⁺ nuclei were contained within muscle fibres. Moreover, rare β -galactosidase⁺ nuclei were observed in the central position typical of regenerated fibre segments (Bourke and Ontell, 1984), demonstrating conclusively that at least some transgene expression was within muscle fibres (Fig. 3B, arrowheads).

The pattern of β -galactosidase expression allowed the determination of *myoD* promoter activity on a cell-by-cell basis. In the plantaris and deep lateral gastrocnemius muscles which in the mouse contain a mixture of fibre types, the β -galactosidase⁺ nuclei were primarily associated with fast non-IIa fibres as demonstrated by a lack of reactivity with N2.261, an antibody that detects both slow and type IIA fast MyHC isoforms in adult mammalian muscle (Hughes et al., 1993 and Fig. 3D). In contrast, in the soleus, which is composed entirely of either slow or type IIA fast fibres, few weakly β -galactosidase⁺ nuclei were detected (Fig. 3D).

Conversely, in the deep medial gastrocnemius, a muscle that also contains a mixture of different muscle fibre types, the β -galactosidase⁺ nuclei were primarily associated with fast IIb fibres that were identified by the expression of IIb MyHC using antibody BF-F3 (Schiaffino et al., 1989; Fig. 3E). Thus, the *myoD* cis-regulatory region tested here restricts gene expression to non-IIa fast muscle fibres.

Cross-reinnervation of the soleus with a fast nerve results in decreased Myogenin mRNA expression in regions induced to express fast MyHC

If the correlation between muscle phenotype and MyoD/Myogenin mRNA levels reflects a causal relationship, one might expect that manipulations that alter muscle

phenotype would be accompanied by corresponding alterations in the relative levels of MyoD and Myogenin transcripts. To test this possibility, the rat soleus muscle was cross-reinnervated with the superficial peroneal nerve that normally innervates the peroneus longus, a predominantly fast muscle. Cross-reinnervation of the soleus by a nerve from a fast muscle causes fibre type transformation by an increase in the expression of fast MyHC, shown by reactivity with antibody N3.36 that recognises all fast MyHC isoforms (Fig. 4B,D) and loss of slow MyHC (Buller et al., 1960; Hoh et al., 1980 and data not shown). Considerable variation in the proportion of fibres successfully cross-reinnervated following denervation was observed among the three rats analysed. Areas of atrophied fibres (Fig. 4B, arrow) that hybridised strongly with both the Myogenin

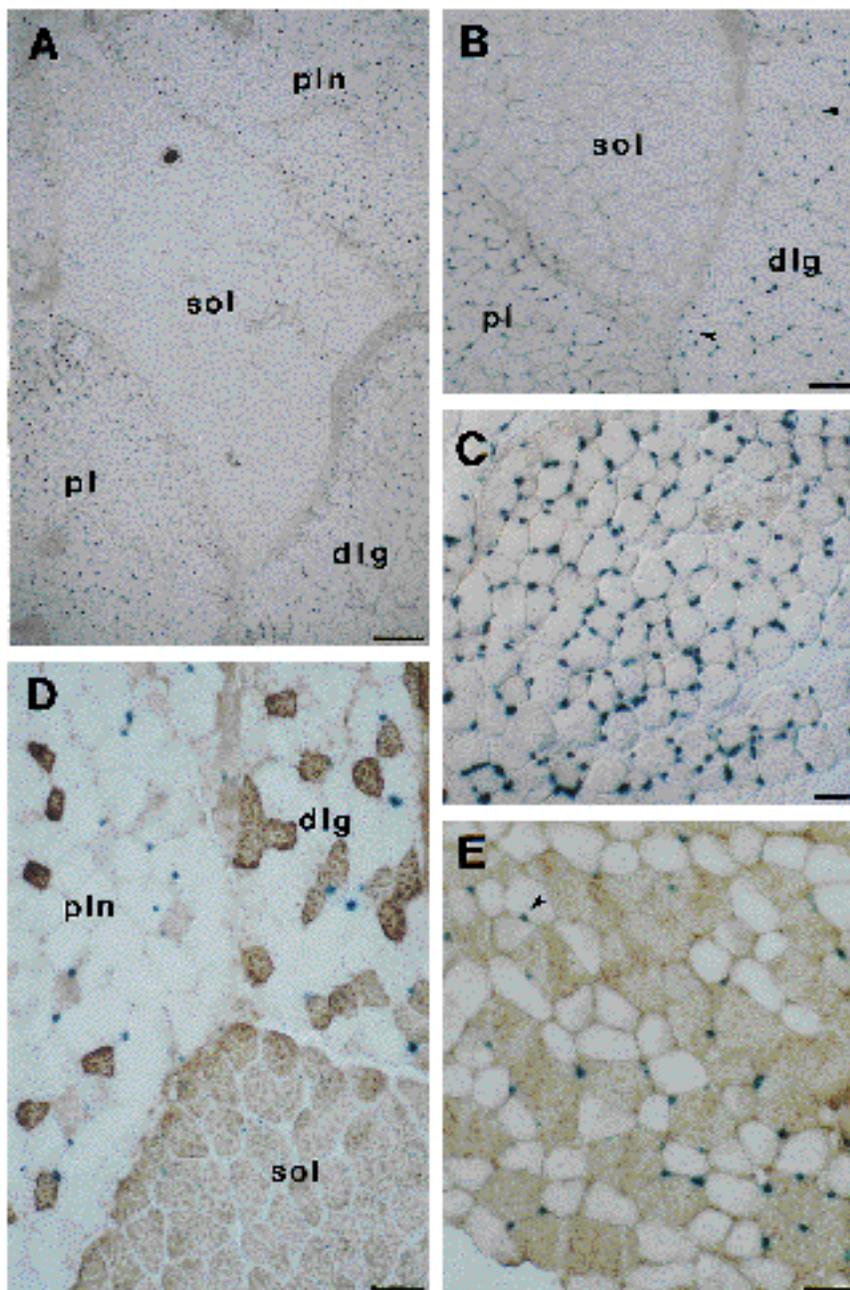


Fig. 3. *lacZ* driven by *myoD* regulatory sequences mimics MyoD mRNA expression in transgenic mice. Serial adjacent cryostat sections from *myoD/nlslacZ* transgenic mice were reacted either after paraformaldehyde fixation for optimal visualisation of β -galactosidase alone (A-C), or without fixation to permit detection of β -galactosidase and MyHC isoforms (D,E) as described (Hughes and Blau, 1992). Low power micrographs show the lack of β -galactosidase⁺ nuclei in soleus (sol), while such nuclei are frequent in adjacent muscles that contained fast fibres such as lateral gastrocnemius (dlg), plantaris (pln), and peroneus longus (pl) (A,B). Rare β -galactosidase⁺ nuclei in a central location within muscle fibres (B, arrowheads) are indicative of a regenerated muscle fibre. The fast flexor halucis longus muscle contained many intensely β -galactosidase⁺ muscle fibre nuclei (C). Note that the intensity of β -galactosidase activity varied substantially between fast muscles. Litter mates without the transgene contain no detectable β -galactosidase activity in any muscle. Fibres in the lateral gastrocnemius and plantaris, contain β -galactosidase⁺ nuclei in fibres that fail to stain with N2.261, an antibody that reacts with epitopes on both slow and fast IIa MyHC in adult mammalian muscle (Hughes et al., 1993)(D). Fibres of the slow soleus muscle, which are entirely slow or fast IIa, contain low levels of β -galactosidase reactivity (D). The deep medial gastrocnemius muscle contained numerous β -galactosidase⁺ nuclei localised primarily at the periphery of fibres that react with BF-F3, an antibody specific to IIb MyHC (E). Arrowhead indicates a rare β -galactosidase⁺ nucleus clearly not associated with BF-F3⁺ fibres. A: bar, 200 μ m; B,C: bars, 100 μ m; D,E: bars, 50 μ m.

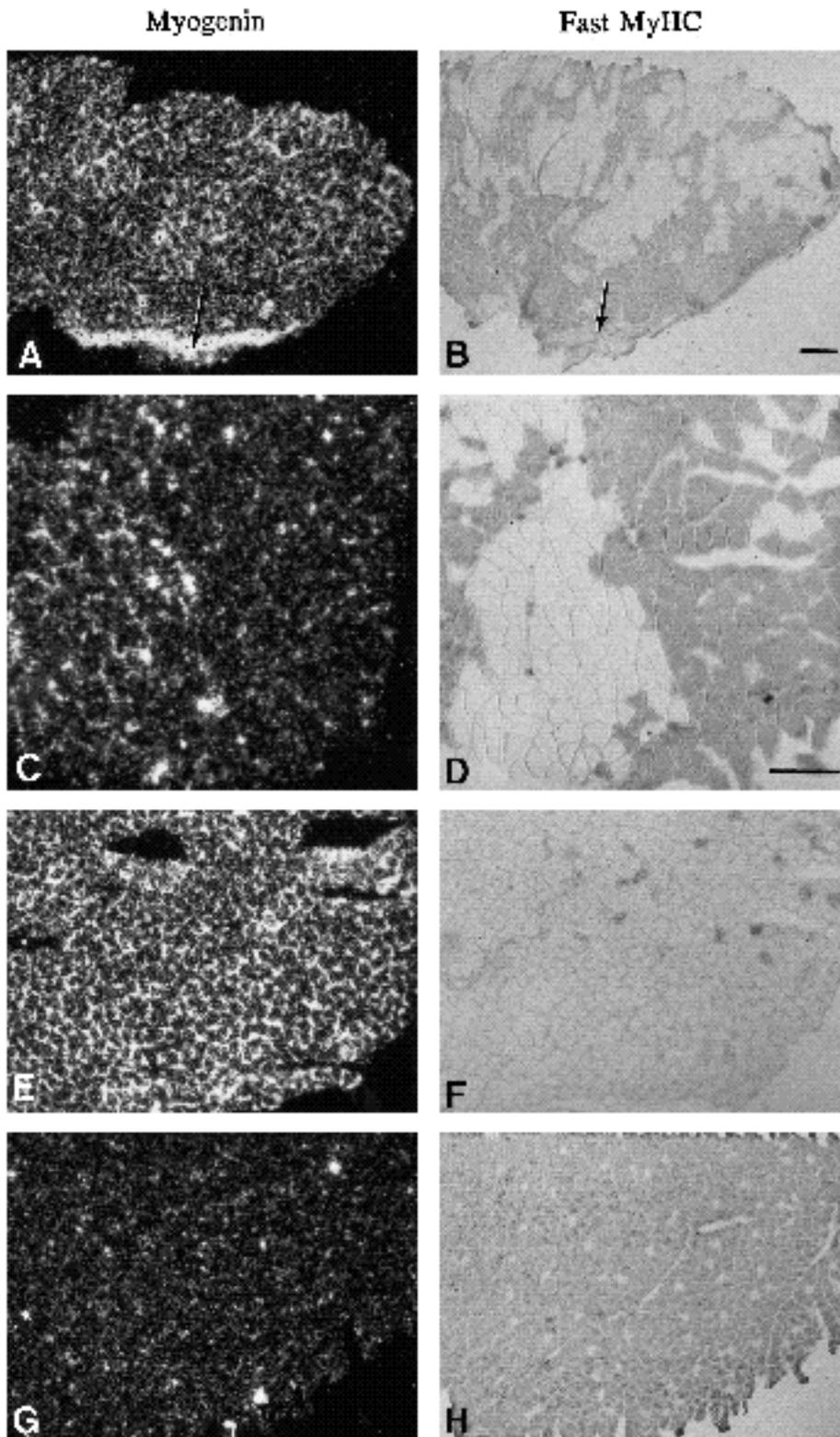


Fig. 4. Cross-reinnervation of the soleus with a fast nerve results in decreased Myogenin mRNA expression in regions induced to express fast MyHC. Serial frozen sections of the cross-reinnervated soleus (A,B), contralateral control soleus (E,F), and contralateral control extensor digitorum longus (G,H) were subjected to in situ hybridisation with the Myogenin antisense probe (A,E,G), or reacted with antibody N3.36 (Hughes and Blau, 1992) to all fast MyHC isoforms (B,F,H). Higher magnification micrographs of a second cross-reinnervated soleus are shown (C,D). The arrows in A and B indicate a region of denervation and strong Myogenin hybridisation. Note the smaller size of the cross-reinnervated soleus (A) compared to the control (E) due to atrophy of denervated fibres. Bars, 100 μ m.

(Fig. 4A, arrow) and MyoD probes (data not shown) were evident, consistent with our observation that northern analysis of mRNA from cross-reinnervated muscles gave variable but elevated Myogenin and MyoD mRNA signals (data not shown). These hybridisation signals apparently resulted from lack of effective reinnervation of some fibres

following denervation, a situation that leads to high level expression of both MyoD and Myogenin mRNAs (Eftimie et al., 1991; Witzemann and Sakmann, 1991; Piette et al., 1992; Neville et al., 1992; Buonanno et al., 1992) and to fibre atrophy (Jolesz and Sreter, 1981). The induction of MyoD mRNA by denervation made its use as a marker of

the conversion of slow fibres to a fast phenotype ambiguous. However, analysis of the loss of Myogenin mRNA accumulation in fibres that had been successfully cross-reinnervated was still feasible. In each of three animals, the annular Myogenin mRNA signal was decreased in those fibres of the soleus where cross-reinnervation had induced fast and decreased slow MyHC expression (compare Fig. 4A and B). In contralateral control muscles, the annular Myogenin signal was clearly visible throughout the slow soleus muscle (Fig. 4E,F), whereas the fast extensor digitorum longus had little Myogenin mRNA (Fig. 4G,H). A higher magnification micrograph of another experimental soleus clearly showed the fibre type grouping characteristic of cross-reinnervation (Kugelberg et al., 1970), and that only slow regions continued to express Myogenin mRNA (compare Fig. 4C and D). This decline in Myogenin mRNA in areas expressing fast and losing slow MyHC is consistent with a shift in the pattern of gene expression from that of the soleus to that of a fast muscle. Thus, maintenance of different fibre phenotypes in adult muscles correlates with low but distinctly different levels of the myogenic HLH mRNAs.

Thyroid hormone treatment results in activation of both *MyoD* and fast MyHC gene expression in the slow soleus

To test further the correlation between MyoD and Myogenin mRNA accumulation and fibre phenotype, and to avoid complications of denervation, the phenotype of the soleus was altered by hormone treatment. Adult rats were treated for three weeks with thyroid hormone (T_3) which has been shown to result in activation of fast MyHC gene expression in the soleus (Izumo et al., 1986; Gustafson et al., 1986). To maximize fast MyHC gene expression, rats were also treated with clenbuterol, a β_2 -receptor agonist, which has also been shown to cause slow to fast conversion (Zeman et al., 1988). Northern analysis of poly(A)⁺ RNA from the soleus of two treated and two control rats demonstrated a dramatic increase in MyoD mRNA in the experimental soleus muscles (Fig. 5). The treated muscles also showed a significant increase in fast oxidative type IIa and intermediate type IIx MyHC mRNA expression. Type IIb MyHC mRNA was detectable in treated muscles only after long exposures of the blots to film. No significant change in either slow MyHC, MRF4 or Myogenin mRNA accumulation was detected. It has yet to be determined if prolonged treatment with T_3 and clenbuterol will result in further fibre type transformation (Izumo et al., 1986; Gustafson et al., 1986). In addition, the data do not allow us to determine whether the change in MyoD expression precedes or simply parallels the changes in MyHC expression. However, these results are consistent with the hypothesis that MyoD is involved in controlling fast glycolytic and Myogenin slow MyHC gene expression.

DISCUSSION

Role of myogenic HLH proteins in fibre type-specific gene expression

We have examined the distribution of the mRNAs encoding the myogenic HLH transcription factors to determine if their distribution in adult muscle made them potential regulators

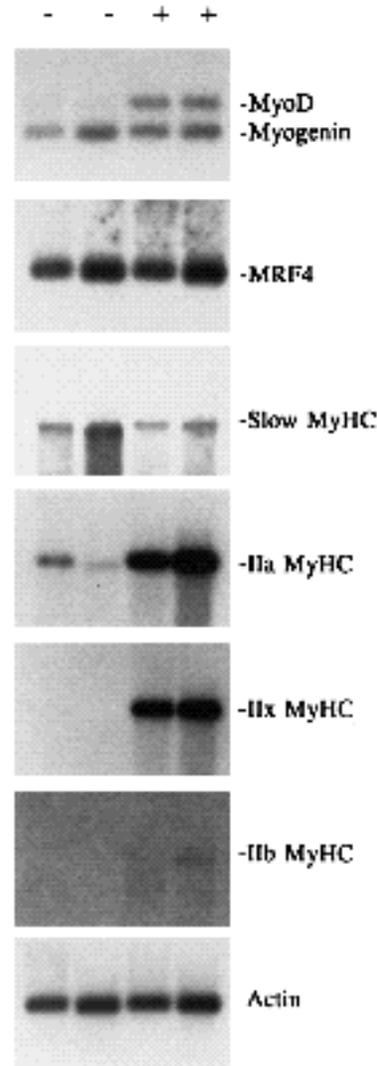


Fig. 5. Thyroid hormone and clenbuterol treatment results in activation of both *MyoD* and fast MyHC gene expression in the soleus. Poly(A)⁺ RNA (3 μ g/lane) from the soleus of control rats (-) and rats treated for 3 weeks with T_3 and clenbuterol (+) were analysed by northern blot. The filter was hybridised simultaneously with probes specific to MyoD and Myogenin mRNAs. The filter was stripped and rehybridised sequentially with probes to slow MyHC and actin, and with oligonucleotides complementary to the central 55 bases of the 3' UTR of rat IIa, IIx, and IIb MyHC mRNAs. Exposure times were as in Fig. 1, except for IIb MyHC which was exposed for 6 days.

of specific muscle fibre types. We found that muscles with differing fibre type compositions, as measured by expression of specific MyHC isoforms, have distinct MyoD and Myogenin mRNA accumulation, with MyoD preferentially present in fast and Myogenin in slow muscle regions. MyoD accumulation appeared highest in the fastest regions, that is those containing type IIx and/or IIb fibres, an observation that was supported by the preferential expression of β -galactosidase in fast IIb fibres of MyoD-nlslacZ transgenic mice. Furthermore, several manipulations that alter muscle fibre type-specific gene expression were associated with consistent changes in myogenic HLH mRNA expression.

However, despite this suggestive data, there does not appear to be an absolute correlation between accumulation of MyoD and Myogenin mRNA and specific fibre types: Myogenin mRNA was not detectable over background in the rare slow fibres present in some predominantly fast regions. Moreover, the predominantly fast plantaris muscle accumulated considerable Myogenin mRNA. These apparent discrepancies could be accounted for by translational or post-translational control mechanisms preventing mRNA levels from accurately predicting protein activity, particularly as

Myogenin mRNA is expressed substantially before Myogenin protein accumulates in the developing embryo (Cusella-De Angelis et al., 1992). Both phosphorylation, which can modulate the function of the myogenic HLH proteins (Martin et al., 1992; Li et al., 1992), and the presence of appropriate partners for heterodimer formation (Murre et al., 1989), are also likely to influence myogenic HLH protein activity.

In addition to these factors, the control of fibre type-specific gene expression *in vivo* is likely to be even more complicated. Both threshold and relative levels of MyoD, Myogenin and MRF4, acting in combination with other muscle-specific transcription factors, such as the MEF2/RSRF family or M-CAT-binding factor (Mar and Ordahl, 1990; Cserjesi and Olson, 1991; Pollock and Treisman, 1991; Yu et al., 1992), could lead to subtle differences in MyHC gene expression within muscle fibres, and ultimately to the continuum of fibre types present in the adult animal.

How might the myogenic HLH transcription factors act to restrict expression of muscle genes to specific fibre types? Rosenthal et al. (1990) and Fujisawa-Sehara et al. (1992) have shown in transient transfection assays that MyoD is more effective than Myogenin, Myf-5 or MRF4 in activating the fast fibre-specific myosin light chain 1/3 enhancer. As this enhancer directs expression of a reporter gene specifically to fast muscle fibres in transgenic mice (Donoghue et al., 1991), these findings suggest that individual genes may respond differently to distinct members of the myogenic HLH family. Such a view could explain how low levels of MyoD or Myogenin protein might act to control fibre type-specific gene expression even though MRF4 mRNA is accumulated to much higher levels than either MyoD or Myogenin mRNAs in adult muscle fibres. Indeed, in co-transfection assays, MRF4 has proved less effective than MyoD or Myogenin in trans-activating most genes tested (Yutzey et al., 1990). As the differential activity of the myogenic HLH proteins appears to map to regions outside the DNA-binding domains that are not well conserved between the different proteins (Chakraborty and Olson, 1991; Mak et al., 1992), it is possible that the myogenic HLH proteins discriminate between muscle-specific enhancers *in vivo*, perhaps through interaction with distinct coregulators.

How does the differential accumulation of MyoD and Myogenin mRNAs arise? Analysis of the expression of a *myoD-nlslacZ* transgene in adult mice suggested that MyoD activity, which appears to be restricted to a subset of fast fibres (non-IIa), is controlled at the level of transcription. The *myoD cis*-regulatory element located between -5870 and +95 may be responsive to novel transcription factors present in these fast muscle fibres of the adult. Alternatively, this enhancer may be auto-activated by endogenous MyoD present within the non-IIa fast fibres. However, we cannot rule out the possibility that the MyoD mRNA is preferentially stable in fast fibres and that 95 bases of the 5' untranslated region (present within the transgene) confers such stability, although *in vitro* studies have not demonstrated a regulatory function for this region (Tapscott et al., 1992). In any case, these results are consistent with our observations that MyoD mRNA preferentially accumulates in fast non-

IIa fibres. Recently, another *myoD cis*-regulatory element located approximately 20 kb 5' to the start site of transcription, outside the region tested here, has been found to promote expression of a transgene in embryonic muscle (Goldhamer et al., 1992). Whether this element also shows fibre type-specific expression is not known, but it is possible that different *cis*-regulatory elements may be responsible for controlling *myoD* gene expression at different stages of development.

The myogenic HLH proteins may also have roles in adult muscle, independent of fibre type. It is likely that some of the signal we detect is in myoblasts that have not yet fused into fibres. Whereas the majority of satellite myoblasts are quiescent, the infrequent, intense punctate dots observed with the Myogenin probe in all muscle regions may correspond to the subpopulation of myoblasts that are proliferating or differentiating, as suggested by studies of regenerating muscle (Grounds et al., 1992; Füchtbauer and Westphal, 1992). This explanation is consistent with the observation that Myogenin mRNA levels increase as dividing myoblasts withdraw from the cell cycle and differentiate *in vitro* (Wright et al., 1989). Another possibility is that all fibre types with the same MyHC expression pattern are not identical. It has been suggested that muscle fibres differ in their intrinsic adaptive range, that is the spectrum of fibre types over which the electrical activity of nerves can modify them (Westgaard and Lømo, 1988, see below). Perhaps myogenic HLH proteins encode information that restricts fibres to a subset of possible final phenotypes, rather than controlling fibre type-specific gene expression *per se*.

That the members of the myogenic HLH family may set limits on the subsequent differentiation of muscle fibres is reminiscent of the finding that the Myc family, also HLH proteins, seems to restrict differentiation in the brain (Bernard et al., 1992). *c-myc*, *N-myc* and *L-myc* are all expressed at relatively high levels in the developing brain. As differentiation proceeds, N-Myc becomes restricted to glial cells and L-Myc to neuronal cells, with c-Myc expression continuing in both cell types, analogous to MyoD, Myogenin, and MRF4 expression in muscle, respectively.

Role of myogenic HLH proteins in developing muscle

In embryonic muscle where the expression of the myogenic HLH molecules has been studied in greatest detail, high levels of both MyoD and Myogenin mRNAs seem to be present in all muscles independent of their fast/slow phenotype (Hinterberger et al., 1991; Bober et al., 1991). Myotubes in tissue culture, which often express all four members of the myogenic HLH family (Peterson et al., 1992), resemble embryonic muscles *in vivo* (Whalen et al., 1981; Schiaffino et al., 1988). Even in those cell lines that differentially express the myogenic HLH proteins, no correlation with specific MyHC isoforms is detected (Miller, 1990), consistent with the situation in embryonic muscle. Similarly, denervated adult muscles re-express high levels of both MyoD and Myogenin, as well as embryonic isoforms of MyHC (Eftimie et al., 1991; Witzemann and Sakmann, 1991; Piette et al., 1992; Neville et al., 1992; Buonanno et al., 1992). In contrast to these situations, we find that the

myogenic HLH mRNAs are differentially expressed in innervated adult muscle, suggesting that these molecules may have distinct roles at different stages of development.

Muscle can form in the absence of either MyoD or Myf-5 (Rudnicki et al., 1992; Braun et al., 1992), yet MyoD^{ml} mice lacking a functional *myoD* gene up-regulate the expression of Myf-5, which is almost undetectable in the wild-type mouse muscle following birth (Rudnicki et al., 1992). Therefore, it is possible that Myf-5 may be able to substitute functionally for MyoD, accounting for the lack of gross fibre type abnormality in the muscles of neonatal MyoD^{ml} mice. Alternatively, defects in fibre type distributions in the MyoD^{ml} mice may only become apparent in the adult following the maturation of innervation and the appearance of fibre type-specific myogenic HLH gene expression. In either case, these results are compatible with our hypothesis that maintenance of fibre type-specific gene expression in adult muscles is dependent upon the presence of low but distinctly different levels of the myogenic HLH proteins in different fibre types.

Hormonal influences on myogenic HLH gene expression and fibre type

Both thyroid hormone (T₃) and clenbuterol shift MyHC expression toward faster isoforms (Ianuzzo et al., 1977; Zeman et al., 1988). In addition, Russell et al. (1988) have shown that the induction of fast MyHC mRNA will occur even in denervated muscles, suggesting that the effect of T₃ is not neurally mediated. Our results show that combined treatment with T₃ and clenbuterol causes significant increases in IIa and IIx MyHC mRNA and to a much lesser extent IIb mRNA in the soleus, consistent with the idea that fibre type transformation proceeds from IIa>IIx>IIb (Kirschbaum et al., 1990a). Thus, the dramatic increase in MyoD mRNA in the soleus under these conditions precedes conversion to the fastest phenotype (Gustafson et al., 1986; Izumo et al., 1986). Although hormone treatment modifies MyoD and fast MyHC expression, it had no significant effect on Myogenin or slow MyHC mRNAs in the soleus, consistent with co-expression of fast and slow MyHC isoforms under these conditions (Gustafson et al., 1986; Izumo et al., 1986) and maintaining the correlation between myogenic HLH and MyHC gene expression. It has yet to be determined whether the thyroid hormone receptor bound to T₃ activates *myoD* gene transcription directly. Once expressed, MyoD could interact with the thyroid hormone receptor to effect the expression of downstream genes in a manner analogous to AP1 and glucocorticoid receptor (Miner et al., 1991).

Myogenic HLH proteins may mediate the effects of electrical activity on muscle fibre gene expression

Innervation controls the phenotype of adult muscle fibres (Buller et al., 1960). Many of the effects of innervation, for example the suppression of acetylcholine receptor expression in extrajunctional regions of muscle fibres or the expression of particular MyHC isoforms, are controlled by the electrical activity of the fibre, not by distinct trophic factors provided by fast and slow nerves (Goldman et al., 1988; Gorza et al., 1988; Kirschbaum et al., 1990b; Ausoni et al., 1990). That electrical activity induces changes in

muscle gene expression implies that the regulators of gene expression within the muscle fibre must sense and integrate, over time, the electrical signals from the nerve. It is clear that the loss of electrical stimulation can lead to up-regulation of myogenic HLH mRNAs (Fig. 4; Eftimie et al., 1991; Witzemann and Sakmann, 1991; Piette et al., 1992; Neville et al., 1992; Buonanno et al., 1992), suggesting that the myogenic HLH transcription factors could mediate the effects of the nerve. Our data confirm these observations and show that the effect of denervation in increasing myogenic HLH mRNA levels persists for at least two months, specifically in those muscle fibres that are denervated. Most significantly, we find that slow fibres converted to a faster phenotype by cross-reinnervation have decreased Myogenin mRNA levels. As the effects of cross-reinnervations are mediated through the different pattern of electrical activity that the new nerve imposes upon the muscle (Ausoni et al., 1990), our data suggest that the myogenic HLH genes can respond to the pattern of electrical activity, not simply to its presence or absence. Thus, the myogenic HLH proteins may be the intracellular mediators through which electrical activity regulates gene expression, and therefore, the contractile activity of innervated muscle fibres. Elucidation of the signal transduction pathway utilised to convert changes in electrical activity into changes in gene expression will be of great interest.

CONCLUSION

The data presented here strongly suggest that MyoD and Myogenin may be intermediaries in the mechanisms whereby a variety of stimuli are integrated to allow adaptation of adult muscle fibres to the demands placed upon them during adult life. To test this hypothesis, it will be necessary to manipulate their levels of expression in the intact organism and to determine how the genes encoding the myogenic HLH proteins are themselves controlled.

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