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Expression of Myosin Heavy Chain Isoforms in Stimulated Fast and Slow Rat Muscles

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The expression of 4 myosin heavy chain (MHC) isoforms was analyzed in the rat soleus (SOL) and extensor digitorum longus (EDL) muscles after denervation and chronic electric stimulation. The stimulation frequencies used were 20 and 150 Hz and the amount of stimulation was either large (20 Hz), intermediate (150 Hz), or small (150 Hz). These patterns resemble some features of normal motor unit activity in SOL and EDL of freely moving rats (Hennig and Lømo, 1985).

The relative expression of each MHC isoform depended strongly on the stimulation pattern. Furthermore, for any particular stimulation pattern, fibers in SOL and EDL expressed different MHCs. Coexistence of different MHC types in the same fiber was frequently observed in stimulated muscles. 20-Hz stimulation preserved normal expression of type 1-MHC in SOL but failed to induce type 1-MHC in type 2 fibers of the EDL, where type 2A- and 2X-MHC expression dominated and type 2B-MHC expression was completely suppressed. 150-Hz low-amount stimulation preserved nearly normal 2B-MHC expression in many type 2 fibers of the EDL but failed to induce type 2B-MHC expression in the SOL, where 2X-MHC became predominant. 150-Hz high-amount stimulation differed from 150-Hz small amount stimulation by suppressing almost all type 2B-MHC expression in EDL and by inducing considerable type 2A-MHC expression in the SOL. Scattered fibers in EDL that were probably the original type 1 fibers responded differently from both type 2 fibers in the EDL and from type 1 fibers in the SOL to stimulation.

The different MHCs expressed by these stimulation patterns corresponded well to the maximum intrinsic shortening velocities determined earlier in the same muscles (Eken and Gundersen, 1988). The incomplete slow-to-fast transformation of V_{max} in SOL can now be attributed to an inability of rat SOL muscles to express type 2B-MHC, whereas the incompletely fast-to-slow transformation of the rat EDL can be attributed to an inability of EDL type 2 fibers to express type 1-MHCs.

These results demonstrate the existence of intrinsic differences between SOL and EDL muscles in the rat and support the concept of different, but partially overlapping, adaptive ranges in these muscles (Westgaard and Lømo, 1988).

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Previous work has demonstrated that the contractile speed and metabolic properties of rat soleus (SOL) and extensor digitorum longus (EDL) muscles can be markedly affected by direct muscle stimulation (Lømo et al., 1974; Eken and Gundersen, 1988; Gorza et al., 1988; Gundersen et al., 1988). In these experiments the stimulation was done on denervated muscles in order to exclude neurotrophic influences and to determine precisely the pattern of activity in the muscle. The experiments showed that stimulation patterns resembling normal EDL motor unit activity maintained essentially normal properties in the EDL and induced incomplete slow-to-fast transformation in the SOL, whereas stimulation patterns resembling normal SOL motor unit activity maintained essentially normal properties in the SOL and induced incomplete fast-to-slow transformation in the EDL. These results are similar to those obtained by cross-reinnervation of rat SOL and EDL muscles (Close, 1969; Gutmann and Carlson, 1975). The fast-to-slow transformation of the rat EDL differs from that observed in innervated fast muscles of the rabbit and the cat after tonic low-frequency stimulation by being less pronounced (Pette et al., 1973; Salmons and Sreter, 1976; Eerbeek et al., 1984).

In the present experiments, rat SOL and EDL muscles were denervated and stimulated for about 2 months with the patterns used in earlier experiments (Eken and Gundersen, 1988; Gundersen et al., 1988). These patterns have features that resemble normal SOL and EDL motor unit activities (Hennig and Lømo, 1985). We have previously shown that intermittent high-frequency stimulation of denervated rat SOL muscles induces the expression of type 2 myosin heavy chains (MHCs) as well as a doubling of intrinsic maximum shortening velocity (Gorza et al., 1988). In the present experiments we have made a more detailed analysis of MHC expression not only in the SOL, but also in the EDL.

The MHCs analyzed were types 1, 2A, and 2B, for which distinct genes have been identified (Izumo et al., 1986) and specific antibodies have been developed (Pierobon Bormioli et al., 1981). In addition, a recently discovered MHC, termed 2X (Schiaffino et al., 1986, 1989), was analyzed. This MHC, which probably corresponds to 1 of the 3 type 2 MHCs recently described by Bär and Pette (1988), is present in a large number of fibers in the normal rat diaphragm and in a smaller number of fibers in normal fast hind limb muscles and appears to be associated with an intermediate speed of shortening (Schiaffino et al., 1988).

Materials and Methods

Denervation and chronic stimulation. Young adult male Wistar rats, weighing 250–350 gm at the beginning of the experiment, were anes-

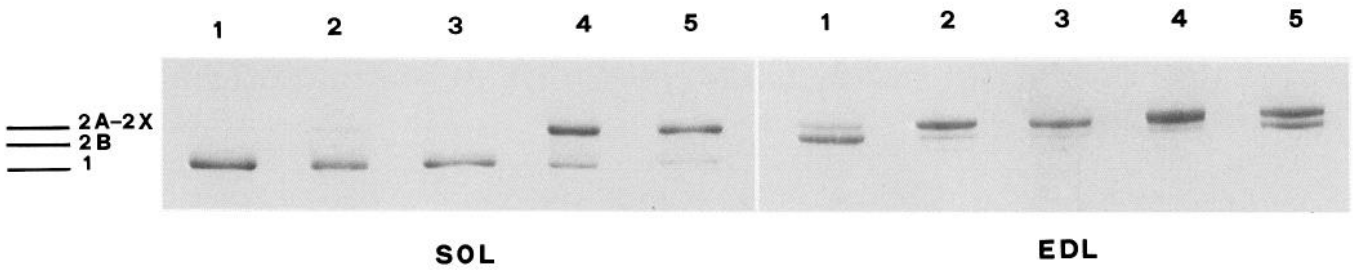

Figure 1. Electrophoretic analysis of MHC isoform expression in normal, denervated, and denervated-plus-stimulated EDL and SOL muscles. Three MHC bands were resolved in SDS-6% polyacrylamide gels and stained with Coomassie blue. Only the region of the gel blots corresponding to the position of the MHCs is shown. *Lane 1,* control muscles; *lane 2,* denervated muscles; *lane 3,* muscles stimulated with the 20-Hz pattern; *lane 4,* muscles stimulated with the 150-Hz high-amount pattern; *lane 5,* muscles stimulated with the 150-Hz low-amount pattern.

thetized with Equithesin (0.4 ml/100 gm body weight i.p.). The denervation and stimulation procedures were essentially as described by Gorza et al. (1988). The sciatic nerve was cut and reflected in the thigh, and steel electrodes (AS 632, Cooner Sales Wire Co., Chatsworth, CA) were implanted on EDL or SOL muscles. Stimulation started 1–3 d after the operation and continued for 33–88 d (mean 63 d). Contralateral innervated and denervated muscles were used as control. Three stimulation patterns were used: (1) 200 pulses at 20 Hz every 15 sec, (2) 25 pulses at 150 Hz every 15 sec ("150-Hz high-amount"), and (3) 25 pulses at 150 Hz every 15 min ("150-Hz low-amount"; see Eken and Gundersen, 1988).

Electrophoretic and immunoblotting analysis. Procedures for myosin extraction, SDS-PAGE, and immunoblotting are described in detail elsewhere (Schiaffino et al., 1989). In brief, myosin was isolated from 20–30 mg of tissue by a rapid method that involves first extraction of soluble proteins by a low-salt buffer and then extraction of myosin in a high-salt buffer. MHC isoforms were resolved in SDS–6% polyacrylamide gels and stained with Coomassie blue. The gels were scanned with a Shimadzu CS-930 dual-wavelength TLC densitometer to estimate the relative amount of the MHC bands. For immunoblotting analysis proteins were electrophoretically transferred to nitrocellulose as described by Towbin et al. (1979). The blots were incubated with purified monoclonal antibodies and then with peroxidase-conjugated rabbit antimouse IgG antibodies, and peroxidase activity was revealed using diaminobenzidine as substrate.

Monoclonal antibodies. Monoclonal antibodies were raised in mice using myosin preparations from bovine or rat skeletal muscle as immunogen (Schiaffino et al., 1989). Selected hybridomas were cloned by limiting dilution and grown as ascites in pristane primed mice. Immunoglobulins were purified from ascites and their specificity for the different MHC isoforms was determined by immunoblotting. The following antibodies were used in this study: BF-32, which reacts with type 1- and 2A-MHC;RT-D9, which reacts with type 2X- and 2B-MHC; BF-D5, which reacts with type 1-MHC; SC-71, which reacts with type 2A-MHC;BF-F3, which reacts with type 2B-MHC; and SC-75, which reacts with type 2A-, 2X-, and 2B-MHCs (Schiaffino et al., 1989).

Immunohistochemistry. Serial transverse cryosections of control and stimulated muscles were incubated with appropriate dilutions of monoclonal antibodies and then with peroxidase-conjugated anti-mouse IgG antibodies, using standard procedures (Schiaffino et al., 1989).

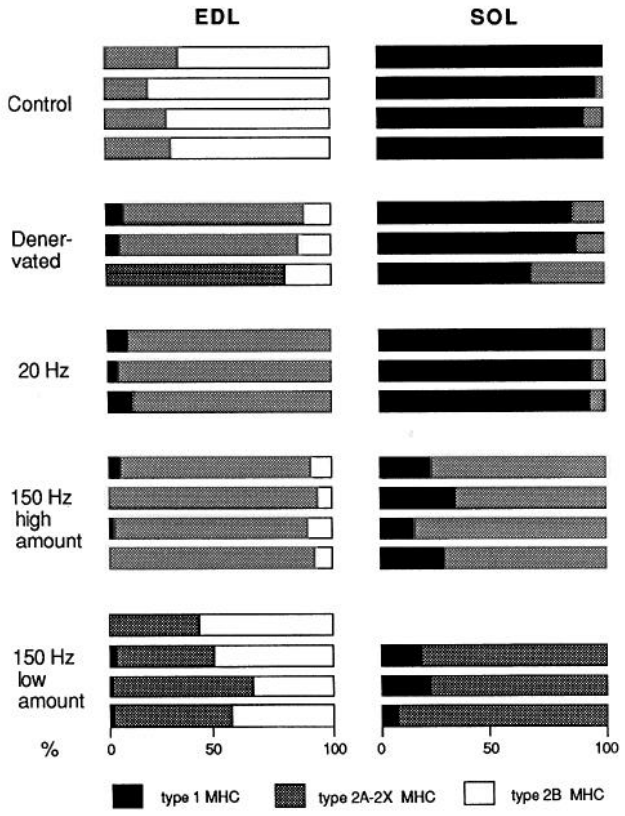

Figure 2. Relative proportions of type 1-, type 2A-2X-, and type 2B-MHC bands based on quantitative densitometric measurements in Coomassie blue–stained SDS–6% polyacrylamide gels of control, denervated, and stimulated muscles. Each bar corresponds to 1 muscle.

Results

MHC isoforms expressed in normal, denervated, and stimulated muscle were identified by SDS-PAGE and by immunoblotting analysis with specific monoclonal antibodies. As previously reported (Schiaffino et al., 1988, 1989), 3 MHC bands can be resolved in Coomassie blue–stained gels: a high-mobility band corresponding to type 1-MHC, an intermediate-mobility band corresponding to type 2B-MHC, and a low-mobility band corresponding to type 2A- and type 2X-MHCs that comigrate in this electrophoretic system.

Figure 1 shows representative MHC bands from normal (lane 1), denervated (lane 2), or denervated and stimulated (lanes 3–5) EDL and SOL muscles. The relative amounts of MHC, as determined by densitometric analyses of Coomassie blue–stained gels in 3–4 muscles of each experimental group, are presented in Figure 2.

As shown in Figures 1 and 2, the normal EDL muscles contained large amounts of type 2B-MHC (mean 73.1%) and smaller amounts of type 2A-2X MHC (26.8%), whereas the denervated EDL contained predominantly type 2A-2X-MHC (79.2%). Type 2A-2X predominated also in EDL muscles stimulated with the 20 Hz (91.3%) and 150 Hz high-amount (89.0%) pattern, whereas the EDL muscles stimulated with the 150 Hz low-amount pattern contained almost equal amounts of type 2B- (45.2%) and type 2A-2X- (52.6%) MHCs. Interestingly, the slow,

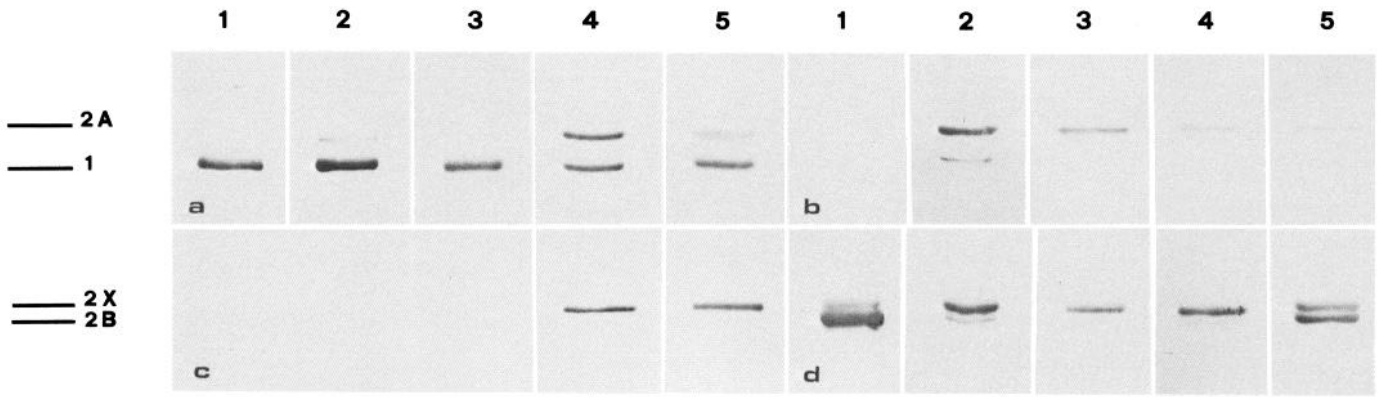

Figure 3. Immunoblotting analysis of MHC isoform expression in normal, denervated, and denervated-plus-stimulated EDL and SOL muscles. The myosin preparations shown in Figure 1 were transferred onto nitrocellulose and reacted with monoclonal antibodies specific for type 1- and 2A-MHCs (*a, b*) or 2X- and 2B-MHCs (*c, d*). Only the region of the blots corresponding to the position of MHCs is shown. *Lane 1,* control muscles; *lane 2,* denervated muscles; *lane 3,* muscles stimulated with the 20-Hz pattern; *lane 4,* muscles stimulated with the 150-Hz high-amount pattern; *lane 5,* muscles stimulated with the 150-Hz low-amount pattern.

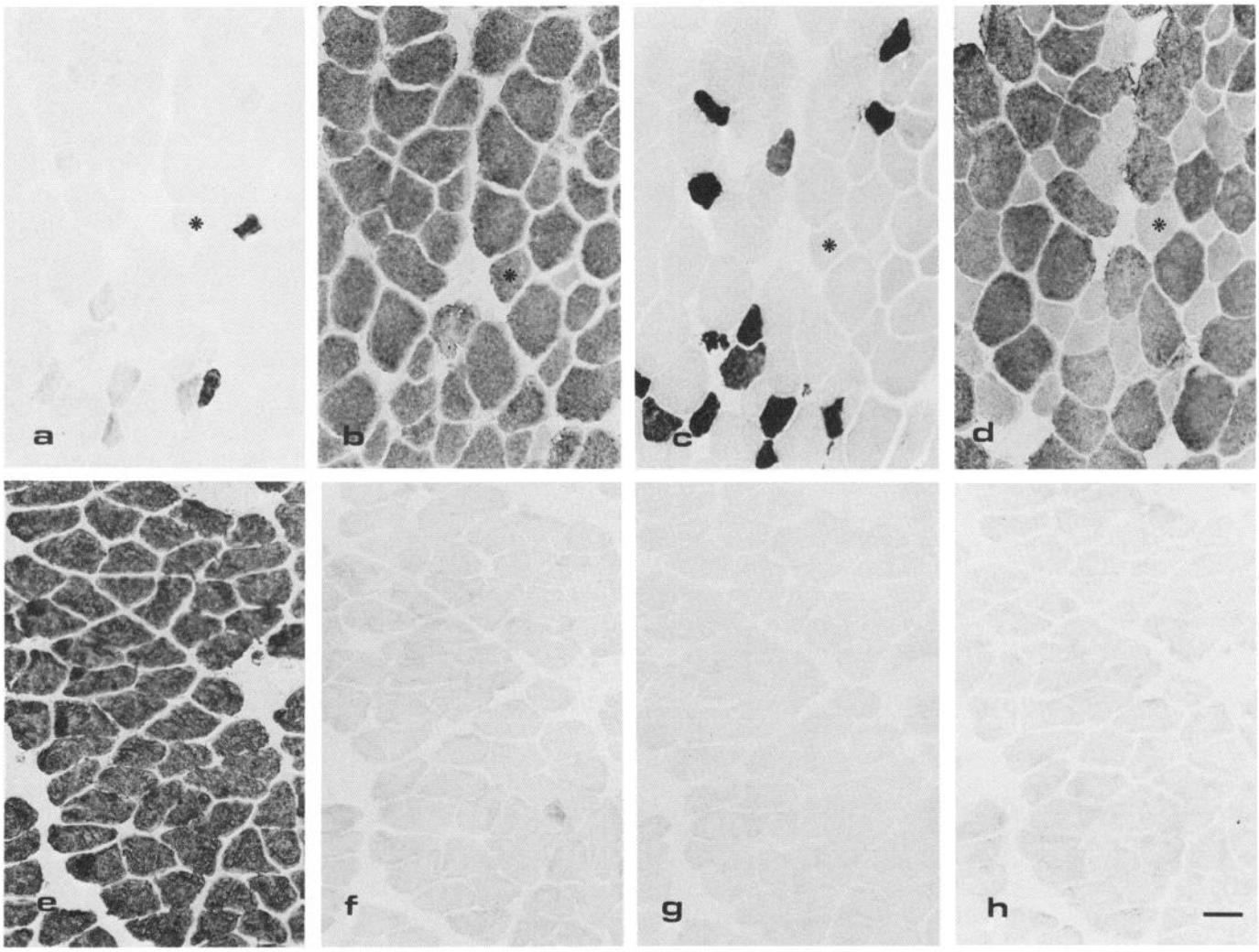

Figure 4. Immunoperoxidase staining of serial cryosections of control EDL (*a–d*) and SOL (*e–h*) with monoclonal antibodies specific for type 1-MHC (*a, e*), all type 2 MHCs (*b, f*), type 2A-MHC (*c, g*), and 2B-MHC (*d, h*). *Asterisk* marks a type 2X fiber, which stains for type 2-MHC but not for type 2A- and 2B-MHCs.

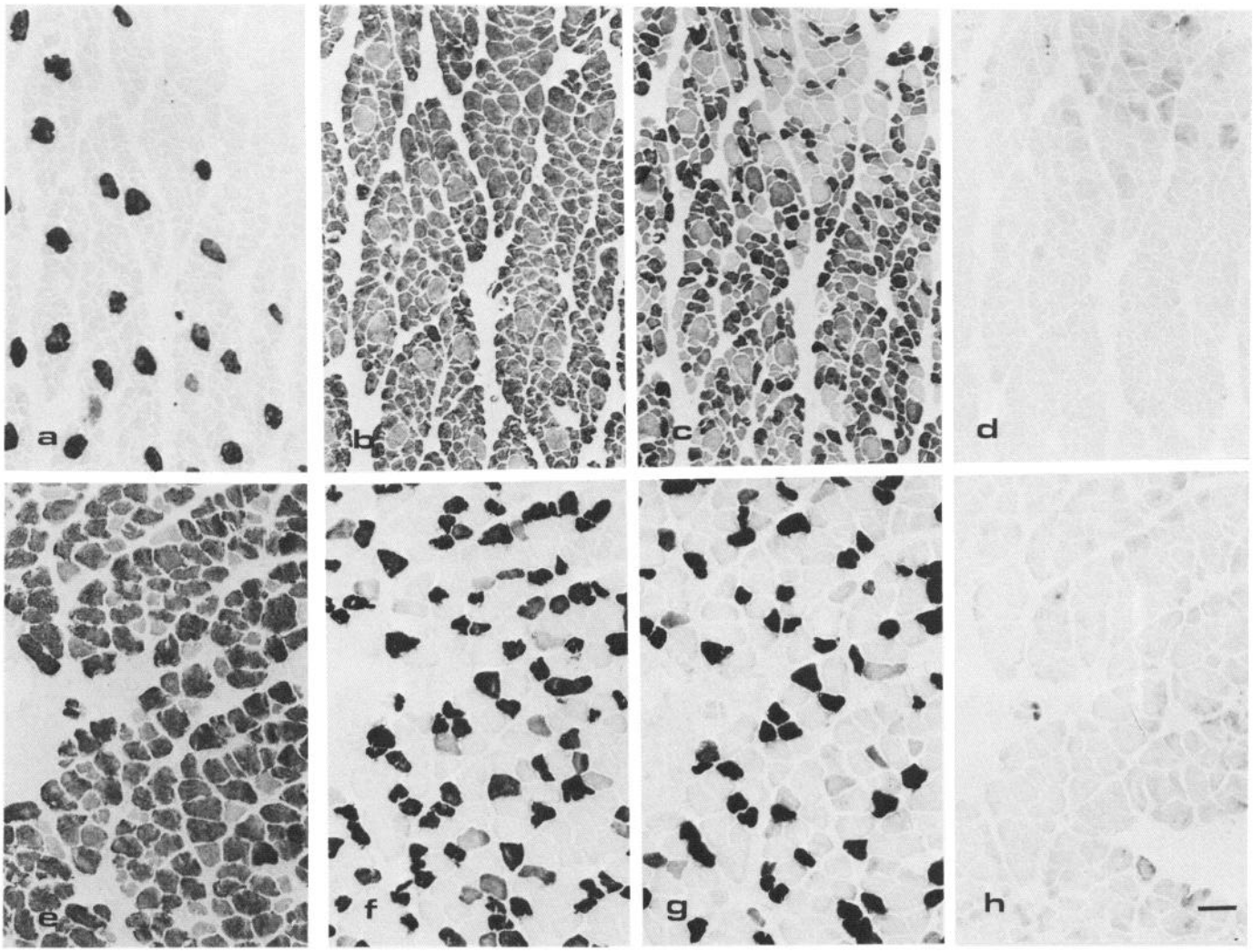

Figure 5. Immunoperoxidase staining of serial cryosections of denervated EDL (*a–d*) and SOL (*e–h*) with monoclonal antibodies specific for type 1-MHC (*a, e*), all type 2 MHCs (*b, f*), type 2A-MHC (*c, g*), and 2B-MHC (*d, h*).

20-Hz pattern suppressed all fast type 2B-MHC expression but failed to induce marked slow type 1-MHC expression (8.8%).

The normal, denervated, and denervated plus 20 Hz-stimulated SOL muscles contained predominantly type 1-MHC (96.9, 78.5, and 93.7%, respectively) and small amounts of type 2A-2X-MHCs. In contrast, SOL muscles stimulated at 150 Hz (high- and low-amount) contained predominantly 2A-2X-MHCs (75.1 and 84.3%) and small amounts of type 1-MHC. Under no conditions did SOL muscles express type 2B-MHC.

To distinguish further between the different MHCs, such as those shown in Figure 1, the proteins were transferred to nitrocellulose and stained with monoclonal antibodies against type 1-2A-MHCs (Fig. 3*a*) and type 2X-2B-MHCs (Fig. 3*b*). As shown in Figure 3, 20-Hz stimulation induced only type 1-MHC expression in SOL and predominantly type 2X-MHC expression in EDL. In contrast, 150-Hz high-amount stimulation induced roughly equal amounts of type 2X- and 2A-MHCs in SOL and predominantly 2X-MHC in EDL, whereas 150-Hz-low amount-stimulation induced predominantly type 2X- and some type 1-MHC in SOL and approximately equal amounts of type 2X- and 2B-MHCs in EDL.

The MHC composition of stimulated muscles was also investigated at the single-fiber level by immunoperoxidase staining of serial cryosections. A monoclonal antibody reactive with all 3 type 2-MHCs and monoclonal antibodies specific for type 1-, 2A-, and 2B-MHCs were used for this study. As shown in Figure 4, control SOL muscles were homogeneously composed of type 1 fibers, whereas control EDL muscles contained 3 subpopulations of type 2 fibers (2A, 2X, and 2B) and rare type 1 fibers. Type 2X fibers were reactive for type 2-MHC, but unreactive for 2A- and 2B-MHCs. Fibers reacting for both type 1- and 2A-MHC or both 2A- and 2X-MHC were very rare.

Denervation induced expression of 2A-MHC in many fibers in SOL and a striking decrease in 2B-MHC reactivity in most fibers in EDL (Fig. 5). Denervation atrophy affected all fibers in both muscles, except type 1 fibers in EDL. This finding may explain why type 1-MHC is detected in denervated but not in normal EDL by SDS-PAGE (see Figs. 1 and 3).

The 20-Hz stimulation pattern maintained the normal histochemical fiber type profile in SOL and induced a complete disappearance of 2B-MHC in EDL (Fig. 6). In EDL, many type 2 fibers coexpressed type 2A- and 2X-MHCs, while fibers ap-

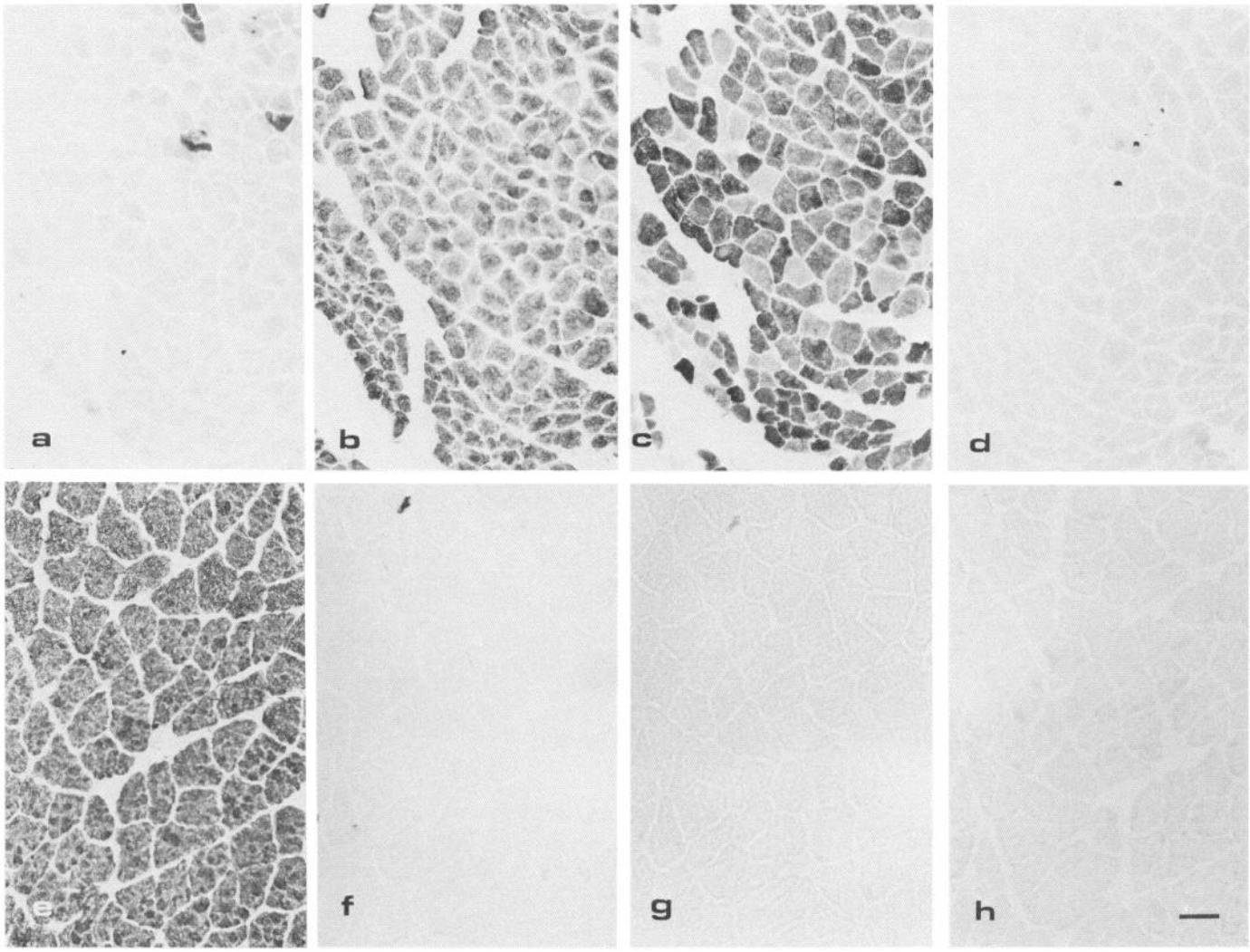

Figure 6. Immunoperoxidase staining of serial cryosections of 20-Hz-stimulated EDL (*a–d*) an SOL (*e–h*) with monoclonal antibodies specific for type 1-MHC (*a, e*), all type 2 MHCs (*b, f*), type 2A-MHC (*c, g*), and 2B-MHC (*d, h*).

parently corresponding to original type 1 fibers coexpressed type 1- and 2A-MHCs.

In EDL, the 150-Hz small-amount pattern preserve type 2B-MHC expression in many fibers (Fig. 7). These muscles differed from normal, however, by having almost no type 2A fibers, and by having a few, apparently original type 1, fibers coexpressing type 1- and 2X-MHCs. In contrast, after stimulation with the 150-Hz high-amount pattern, only very few fibers contained type 2B-MHC, the rest expressing only type 2X-MHC since they were reactive for type 2-MHC but unreactive for 2A- and 2B-MHCs. Interestingly, a small number of fibers in both the 20-Hz- and the 150-Hz-stimulated EDL muscles expressed type 1-MHC (Figs. 6 and 7). As these fibers had a distribution similar to that of type 1 fibers in the normal EDL (Fig. 4), they were probably original type 1 fibers. In addition, clusters of fibers staining for type 1-MHC were observed in some areas of some 20-Hz-stimulated EDL muscles.

In SOL, both stimulation patterns at 150 Hz induced type 2-MHC expression (Fig. 8). For the 150-Hz low-amount pattern the expression was exclusively of the type 2X isoform as the fibers did not react with either anti-2A- or anti-2B-MHC antibodies. For the 150-Hz high-amount pattern, however, there was a significant accumulation of 2A-MHC. In addition, as previously described for SOL muscles stimulated at 100 Hz (Gorza et al., 1988; Schiaffino et al., 1988), most fibers retained significant reactivity for type 1-MHC. Three distinct MHCs, type 1-, 2A- and 2X-MHC, were thus coexpressed in most fibers in the 10-Hz high-amount pattern group (Fig. 8, *e–g*).

Discussion

Two main conclusions can be drawn from this work: first, that muscle stimulation strongly affects the expression of MHCs in rat SOL and EDL muscles, and second, that the same stimulus patterns result in different expressions of MHCs in SOL and EDL and apparently also in type 1 and type 2 fibers of the EDL. As MHC expression in striated muscle appears to mainly regulated at the transcriptional level (Izumo et al., 1986), these results indicate that MHC isogenes can be turned on and off by activity pattern in a muscle-type- and fiber-type-specific manner.

A major finding is that depending on the stimulation pattern, the SOL may express types 1-, 2A-, and 2X-, but not 2B-MHCs,

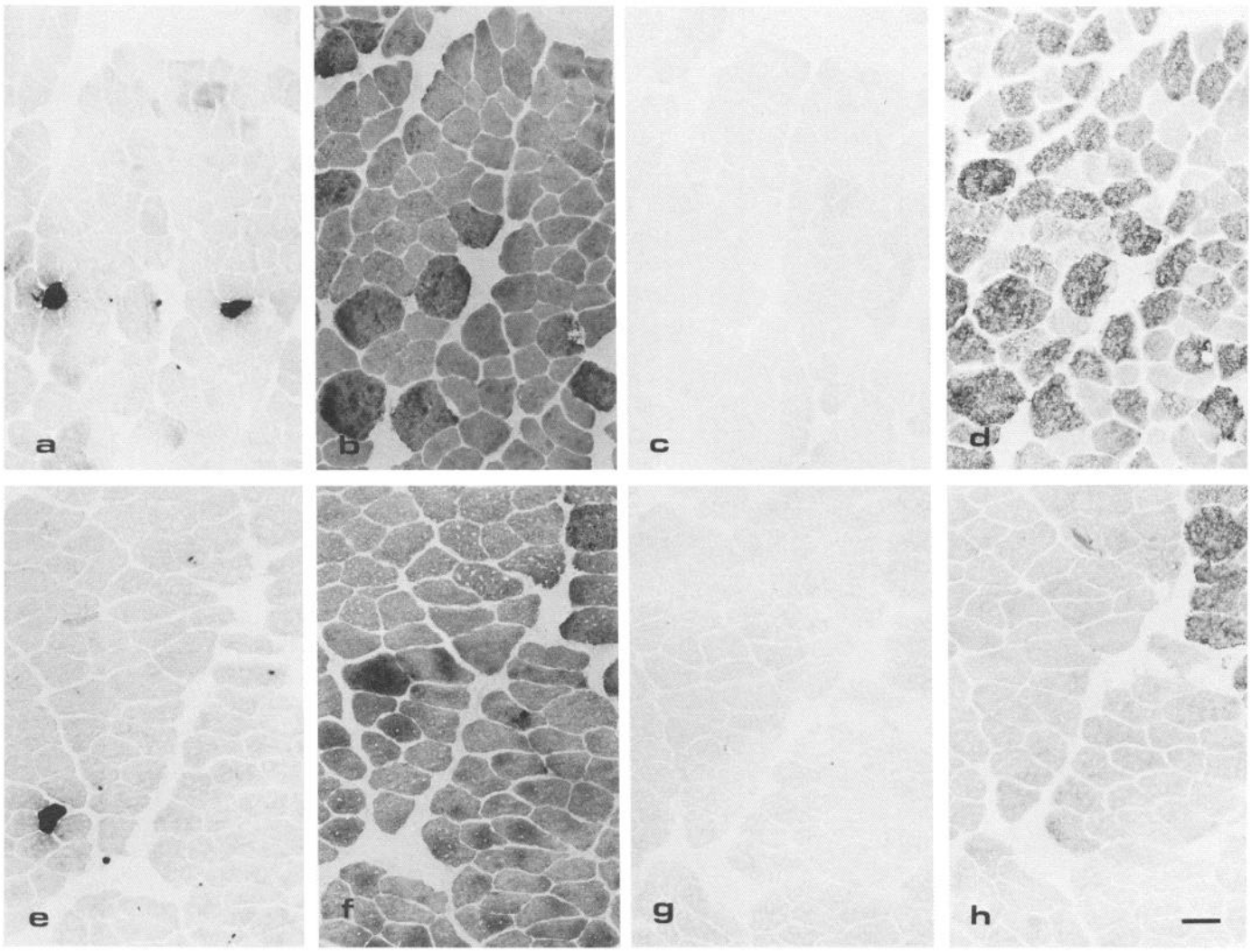

Figure 7. Serial cryosections of EDL stimulated with the 150-Hz low-amount pattern (*a–d*) and with the 150-Hz high-amount pattern (*e–h*). Immunoperoxidase staining with monoclonal antibodies specific for type 1-MHC (*a, e*), all type 2 MHCs (*b, f*), type 2A-MHC (*c, g*), and 2B-MHC (*d, h*).

whereas EDL type 2 fibers may express types 2B-, 2X-, and 2A-, but not 1-MHCs. This result suggests that tissue-specific differences between SOL and EDL render the gene coding for type 2B-MHC unavailable for activity-dependent expression in the SOL and the gene coding for type 1-MHC unavailable for activity-dependent expression in the EDL type 2 fibers.

In SOL, the expression of type 2A- and 2X-MHCs is turned off by 20-Hz stimulation and turned on by 150-Hz stimulation. The expression of type 2A-MHC is more pronounced during large amounts of 150-Hz stimulation than during small amounts of 150-Hz stimulation, whereas the converse is true for the expression of type 2X-MHC. This result indicates that large amounts of high-frequency activity up-regulate the expression of 2A-MHC and down-regulate the expression of 2X-MHC. Also, the expression of type 1-MHC can be graded by stimulation in the SOL. Thus, 94% of the MHCs expressed during 20-Hz stimulation is of type 1, whereas the corresponding mean percentages for the other stimulation patterns are 25% (150-Hz high-amount) and 16% (150-Hz low-amount).

In EDL, expression of type 2B-MHC is turned off by 20-Hz stimulation, very weakly turned on by 150-Hz high-amount, and strongly turned on by 150-Hz low-amount stimulation. In addition, there is marked expression of 2X-MHC and weak expression of 2A-MHC during 150-Hz high-amount stimulation. In contrast, in the SOL the dominant effect of 150-Hz high-amount stimulation is expression of 2A-MHC.

The different responses of SOL and EDL to identical stimulation point to intrinsically different properties in these fast and slow muscles. Similar intrinsic differences between rat SOL and EDL muscles have recently been demonstrated by thyroid hormone stimulation (Izumo et al., 1986). In addition, there appear to be intrinsic differences between type 1 and type 2 fibers in EDL. Thus, a few scattered fibers in the EDL, which probably correspond to the original type 1 fibers, express type 1-MHC during 150-Hz stimulation, whereas the type 2 fibers do not. Assuming that these scattered fibers are the original type 1 fibers, then the type 1 fibers in the EDL also differ from the type 1 fibers in the SOL. For example, during 150-Hz high-amount stimulation the type 1 fibers in EDL do not express type 2A-MHC, whereas in SOL they do. And during 20-Hz stimulation type 1 fibers in EDL express type 2A-MHC, whereas in SOL they do not.

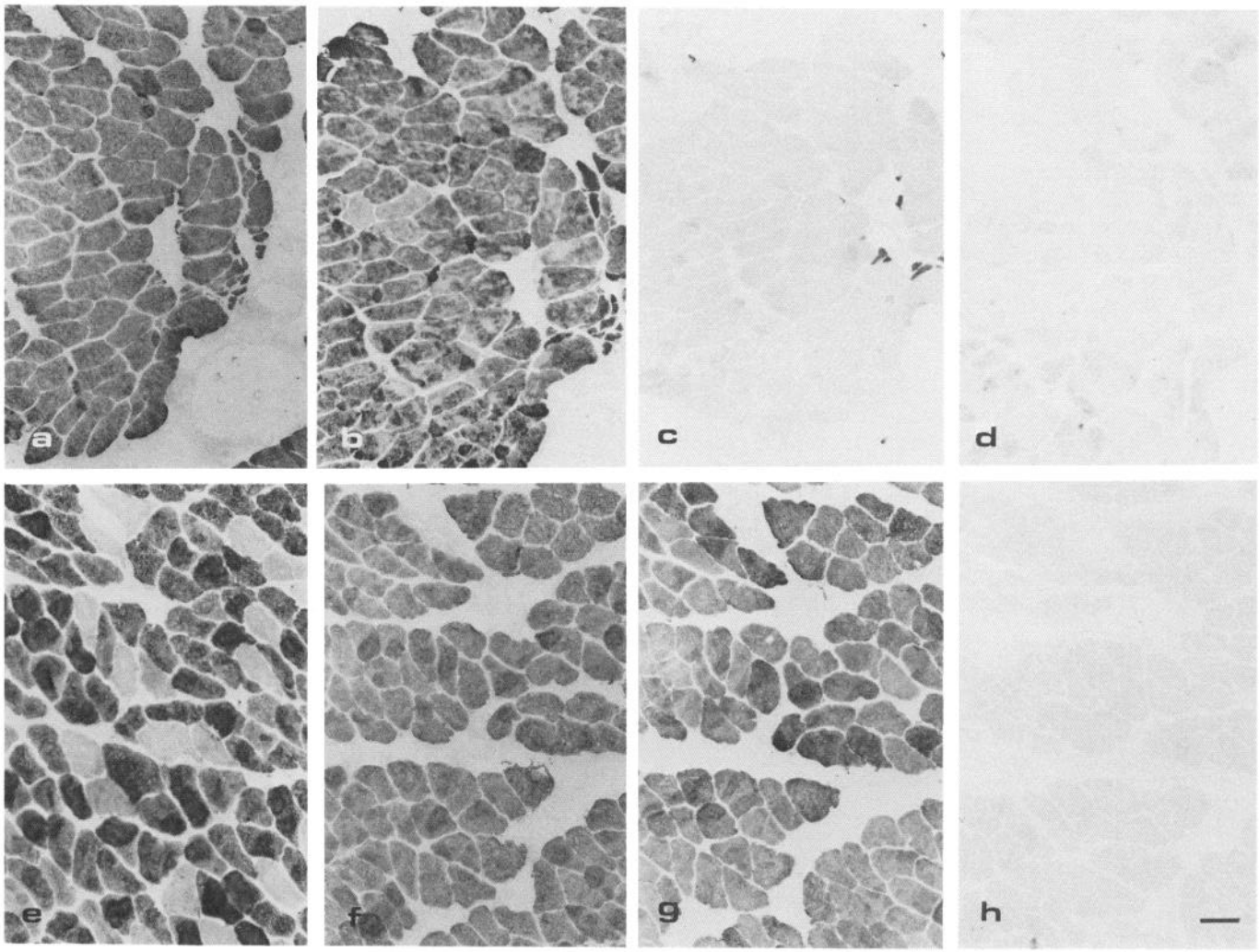

Figure 8. Serial cryosections of SOL stimulated with the 150-Hz low-amount pattern (*a–d*) and with the 150-Hz high-amount pattern (*e–h*). Immunoperoxidase staining with monoclonal antibodies specific for type 1-MHC (*a, e*), all type 2 MHCs (*b, f*), type 2A-MHC (*c, g*), and 2B-MHC (*d, h*).

The coexistence of fast and slow myosin has previously been reported in transforming fibers of stimulated rabbit muscles (Rubinstein et al., 1978; Maier et al., 1988). This study shows that normal rat SOL and EDL muscles rarely contain more than 1 MHC. In contrast, stimulated muscle fibers often contained 2, and in one case even 3, MHCs. These coexpressions were different in different fiber types and provide further evidence of intrinsic fiber differences and resistance to complete fiber type transformation.

Recent observations indicate that maximum shortening velocity at the sarcomere level (intrinsic V_{max}) is determined by the type of MHC expressed in the muscle fiber (Reiser et al., 1985). There is also evidence that V_{max} of fibers expressing type 2A-MHC (Sweeney et al., 1986; Eddinger and Moss, 1987) or predominantly (85%) type 2X-MHC (Schiaffino et al., 1988) is intermediate between that of fibers expressing type 1- and type 2B-MHCs. The present results together with those of Eken and Gundersen (1988) are consistent with this conclusion. In control and 20-Hz-stimulated SOL muscles, which express predominantly type 1-MHC (99% and 94%, respectively), V_{max} is about 5 fiber lengths per sec, whereas in control EDL muscles that express predominantly type 2B-MHCs (68–81%), V_{max} is about 15 fiber lengths per sec. Moreover, in the 20-Hz-stimulated EDL muscles, which express predominantly the intermediate type 2A-2X-MHCs, V_{max} is also intermediate (11 fiber lengths per sec). Thus, the incomplete fast-to-slow transformation of V_{max} in EDL by 20-Hz stimulation can now be attributed to the inability of the EDL to express type 1-MHC and to the predominant expression of the intermediate types 2A- and 2X-MHCs. Similarly, the incomplete slow-to-fast transformation of V_{max} in high-frequency-stimulated SOL muscles can be attributed to the failure of the SOL to express type 2B-MHC as well as to the predominant expression of type 2A-2X-MHCs and a small residual expression of type 1-MHC.

Which aspect of a stimulation pattern is responsible for turning an MHC gene on or off is not very clear. The frequency of stimulation has a clear effect on twitch speed in rat SOL, but not in rat EDL or cat peroneus longus muscles (Kernell et al., 1987; Westgaard and Lømo, 1988; T. Eken and K. Gundersen, personal communication). Intrinsic V_{max} in SOL and EDL muscles of the rat appears to be regulated by amount rather than frequency of impulse activity (Eken and Gundersen, personal com-

munication). In the tibialis anterior of the rabbit moderate amounts of stimulation transform type 2B fibers to type 2A fibers (Mabuchi et al., 1982), while larger amounts of stimulation result in transformation to atypical type 1 fibers (Staron et al., 1987). Also, differences in train duration appear to affect V_{max} (Eken and Gundersen, 1988) and MHC expression (our unpublished observation).

In conclusion, it appears that the properties of SOL and EDL in the rat can be graded by muscle activity within different adaptive ranges (Westgaard and Lømo, 1988). For the twitch contraction time the adaptive range extends from 12 to 45 msec in the SOL and from 10 to 25 msec in the EDL (Westgaard and Lømo, 1988). For intrinsic V_{max} the adaptive range, based on the relatively few stimulation patterns used so far, extends from 11 to 5 fiber lengths per sec in SOL and from 15 to 11 fiber lengths per sec in EDL (Eken and Gundersen, 1988). Finally, with respect to MHC expression, the SOL shows an adaptive range which includes type 1-, 2A-, and 2X-, but not 2B-MHCs, whereas EDL type 2 fibers show an adaptive range which includes type 2B-, 2X-, and 2A-, but not type 1-MHCs. It will be of interest to investigate intermediate stages during the transformation of stimulated muscles to determine whether there is an obligatory sequence of MHC expression within these adaptive ranges.

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