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Id-1 as a possible transcriptional mediator of muscle disuse atrophy

(denervation/tetrodotoxin block/hypertrophy/helix–loop–helix proteins/myogenic factors)

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ABSTRACT **Disuse of muscle leads to atrophy of the fibers. This atrophy is correlated with reduced transcription. We found that when muscle was denervated or paralyzed with a nerve impulse block, the mRNA for Id-1, a negative regulator of transcription, was increased 2- to 7-fold. To test the effect of high Id-1 levels in active muscles, we made transgenic mice in which Id-1 was overexpressed under control of regulatory elements which confer tissue- and fiber-type-specific expression in differentiated muscle cells. Fiber types with high transgene expression were atrophic compared to those in wild-type litter mates. In contrast, fiber types with low transgene expression displayed hypertrophy, presumably caused by an overload due to reduced strength in atrophic synergistic fibers. Apart from the selective effects on fiber caliber, the muscle tissue showed no signs of pathology, and apart from a characteristic slightly lower body weight, the transgenic animals looked and behaved normally. We suggest that in the mature muscle, Id-1 may be involved in regulating muscle fiber size at the transcriptional level during disuse.**

Muscle size and strength are influenced by the way muscles are used. Thus, high levels of activity against heavy loads lead to larger, stronger muscles, whereas low levels of activity lead to smaller, weaker muscles. These changes are mainly due to increases (hypertrophy) and decreases (atrophy) in the diameter of already existing muscle fibers. Muscle atrophy is a serious consequence of many pathological conditions, and more recently it has also received considerable interest because muscle waste is a problem for humans exposed to microgravity during space flight (1). Interest in atrophy as a manifestation of nerve–target interactions is much older. In 1784 Prochaska stated: " . . . numerous physiologists, old and new, have maintained that nutrition is mediated by nerves" (cited in ref. 2). Thus, it was originally thought that the nerve supplied muscle with some kind of "trophic" substance, and this hypothesis has persisted (3). However, in spite of numerous attempts, such substances have not been identified, and muscle atrophy after nerve injury is currently believed to be caused primarily by reduced electrical and mechanical activity (4–6). Besides nerve injury, several other "disuse" models have been employed to reduce electrical and mechanical activity. Thus, nerve impulse block, tenotomy, limb immobilization, and hindlimb suspension all lead to characteristic changes associated with disuse (1, 7).

Transcription of muscle-specific genes is regulated by several different types of DNA-binding proteins. Particularly well studied are the members of the helix–loop–helix (HLH) family, including the so-called myogenic regulators MyoD, myogenin, MRF4, and Myf-5. In tissue culture the myogenic regulators directly or indirectly turn on a variety of muscle specific genes (8, 9). Gene-knockout experiments have proven that absence of myogenin almost completely prevents development of muscle tissue (10, 11), whereas MyoD and Myf-5 seem to be less crucial (12, 13). HLH proteins are thought to bind to DNA as dimers often consisting of one of the ubiquitously expressed E proteins (e.g., E47 or E12) bound to a tissue-specific monomer such as MyoD or myogenin. These E-protein heterodimers form readily and are efficient gene transactivators. Thus, ubiquitous E proteins seem to facilitate transcription in a tissue-specific fashion (9). An opposite role has been proposed for Id proteins (14–17). Id proteins contain the HLH dimerization motif but lack the stretch of basic amino acids that mediates DNA binding. As a consequence, Id heterodimers have low affinity for DNA, and the presence of Id proteins will inhibit HLH-dependent transcription.

Here, we show that Id-1 mRNA (14) is increased under disuse conditions that lead to atrophy. Furthermore, to study the effect of Id-1 overexpression *per se*, we made transgenic mice with Id-1 cDNA fused to the promoter for the myosin light chain (MLC) 1 gene at the 5′ end, and the enhancer for the MLC1/3 gene at the 3′ end. Previous work showed that these regulatory elements conferred strong expression when linked to a reporter gene for chloramphenicol acetyltransferase and that this expression was specific to differentiated muscle (18). In addition, the MLC1/3 elements were shown to confer expression that differed among muscle fiber types in the following way: IIb (highest) > IIx > IIa > I (lowest). In fact, expression was always clearly detectable in type IIb and IIx fibers but was below the detection limit in some type IIa and all type I fibers (19). Assuming that this expression patterns is retained in the Id transgene, the MLC1/3 regulatory elements offer several advantages for studying the effect of Id-1 in muscle fibers of transgenic mice. (*i*) Expression would be directed exclusively to skeletal muscle and not affect other tissues. (*ii*) Expression would be dependent on myogenic activation and be less likely to interfere with muscle differentiation *per se*. This is important because Id-1 inhibits muscle differentiation when overexpressed in myoblasts in culture (16). (*iii*) The level of expression would vary between different fiber types and allow us to study the effects of different levels of transgene expression in groups of fibers that could be identified with fiber-type-specific antibodies. We observed that atrophy was induced selectively in fiber types expected to have high levels of transgene expression, suggesting that Id-1 could be involved in muscle atrophy at the gene regulatory level.

METHODS

Animal Operations. All surgery on mice (B6CBA F_1 hybrids) and rats (Harlan–Sprague–Dawley) was performed under deep Nembutal anesthesia. Denervation was performed by transsecting and reflecting the sciatic nerve in the

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Abbreviations: HLH, helix–loop–helix; MLC, myosin light chain; SV40, simian virus 40; TTX, tetrodotoxin.

thigh. Impulse block in rats was achieved by chronic application of tetrodotoxin (TTX, Behring Diagnostics) locally on the sciatic nerve in the thigh, essentially as described (20). In the present study the TTX solution (40 μg/ml) was delivered by a miniosmotic pump (Alzet 2ML1) implanted subcutaneously on the back of the animals.

RNA Assays. RNA was extracted (21) from muscle tissue in the lower hindlimb. The level of specific mRNA was determined by Northern blotting (22) or RNase protection assays (23).

Transgenic Mice. Id-1 cDNA was released from pMH18ΔR (14) with *Bam*HI and *Eco*RI restriction enzymes, filled in with the Klenow fragment of DNA polymerase I, and cut with *Dra* I in order to remove the endogenous polyadenylylation signal. The resulting fragment was gel purified and ligated into the *Sma* I site of pMDAF(2). This plasmid has a cassette containing the MLC1 promoter, a short polylinker region including a *Sma* I site, the simian virus 40 (SV40) small tumor antigen (t) intron with a polyadenylylation signal, and the MLC1/3 enhancer. The fusion gene was inserted into the genome of mice by conventional transgenic techniques, with modifications as described (24). Five mouse lines were demonstrated to carry the transgene as judged by a PCR assay on DNA extracts from tail tissue (25).

Histology. Standard histological techniques were used to make paraffin and frozen sections. Frozen sections were stained with the following monoclonal antibodies raised against myosin heavy chain: SC-71, BF-35, and BF-F3 (26, 27) and MY-32 (28). Id-1 detection was attempted with a polyclonal antibody (14). The primary antibodies were detected with fluorescein-conjugated anti-IgG and -IgM secondary antibodies (Boehringer Mannheim). Type IIa and IIb fibers were identified with SC-71 and BF-F3 staining, respectively. Type IIx fibers were identified as fibers not stained with BF-35; and type I, as fibers not stained with MY-32. Each section was photographed through a microscope, and montages were made to generate a photograph of the whole muscle cross section. The circumference of single fibers randomly sampled from different parts of the sections was digitized manually on a digitizing tablet coupled to a desktop computer which calculated the fiber cross-sectional areas.

RESULTS

Inactivity Leads to Increased Id-1 mRNA Expression. Transection of the nerve (denervation) is widely used to abolish all nerve-evoked electrical and contractile activity in muscle. We denervated one of the hindlegs of wild-type rats for 4 days, and this led to an ≈3-fold increase in Id-1 mRNA in the muscles (Fig. 1*A*). Since inflammatory processes connected to Wallerian nerve degeneration could have contributed to this response (29), the effect of 4 days of nerve impulse block with TTX, was also investigated. This treatment which does not involve nerve degeneration led to an even stronger (≈7 fold) increase in the Id-1 mRNA (Fig. 1*A*), suggesting that the transient inflammation that accompanies denervation may dampen the induction of Id expression. The effect of denervation was also investigated in wild-type mice. Two days of denervation had no significant effect, but denervation for 10 days induced a higher level of Id-1 mRNA in the denervated leg of all eight mice investigated (Fig. 1*B*). Thus, both denervation and TTX block lead to a significant increase in Id-1 mRNA in rodent muscle.

Id-1 mRNA Is Overexpressed in Transgenic Mice. To examine the effect of Id-1 overexpression in differentiated muscle, we prepared transgenic mice using the Id-1 coding sequence (14) under the regulatory control of the MLC1/3 promoter/enhancer elements. These elements confer tissue-specific expression in differentiated muscle cells in a fiber-type-dependent manner (refs. 18 and 19; see Introduction).

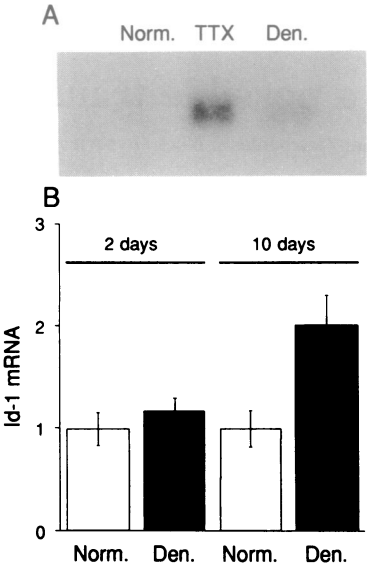

FIG. 1. Levels of Id-1 mRNA in wild-type rats and mice after muscle disuse. (*A*) Levels of Id-1 mRNA measured by Northern blotting of RNA from rat normal muscle (Norm.) or muscle, after 4 days of nerve impulse block with TTX or denervation (Den.). Densitometry of the autoradiographic bands revealed an ≈7- and ≈3-fold increase, respectively, compared with normal muscle. (*B*) Mean and standard error of Id-1 mRNA levels in mouse muscle denervated for 2 days (n = 7) or 10 days (n = 8). Numerical values were obtained by densitometric measurements of autoradiographic bands from RNase protection assays. Intact muscles in the opposite leg served as normal controls. mRNA level is given in relative units, standardized to the mean level for the normal muscles.

Five founder lines of mice which transmitted the transgene to progeny were obtained. Transgene message was detected in four of these lines (04, 29, 95, and 99). While lines 04, 29, and 95 had similar levels of transgene message, the level in line 99 was only about 1/10th the level in the other three lines. Northern blots of transgenic muscle RNA probed with a sequence specific for the SV40-derived 3′ untranslated region revealed two species of 1.4 and 1.7 kb (Fig. 2*A*). We predicted a single species of about 1.7 kb, based on the known properties of the transgene elements and assuming an average poly(A) length of 0.1 kb. Although further experiments will be required to clarify the nature of the shorter species, we believe that it is most likely to arise from aberrant splicing of the SV40 early-region splice acceptor to a cryptic splice donor in the Id coding sequence. Such aberrant splicing with fusion genes using the SV40 intron in a 3′-position has been described (30). To verify that full-length Id-1 transgene RNA was present, we analyzed muscle RNA by RNase protection

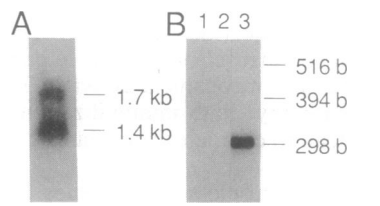

FIG. 2. Measurement of transgene (line 29) mRNA by Northern blotting (*A*) and RNase protection assays (*B*). The Northern blot was probed with a sequence from the SV40 early region which is specific for the transgene. In the RNase protection assay, equal amounts of RNA extracted from muscles of wild-type (lane 2), and transgenic (lane 3) animals were hybridized to radioactively labeled Id-1 antisense sequence extending from nucleotide 484 to the 3′ end of the cDNA (nucleotide 927) (14). The protected fragment of a full-length transgenic message has an expected length of 302 nucleotides. In lane 1 the probe was hybridized with yeast tRNA as a negative control.

assay probed with antisense sequence for the 3′ end of the Id-1 mRNA (Fig. 2*B*). The level of full-length transgene derived Id-1 RNA exceeded the level of endogenous Id-1 mRNA in denervated muscle by ≈50-fold in lines 04, 29, and 95. As discussed below, the significance of the quantitative correlations between transgene expression and the severity of the transgene phenotype cannot be evaluated until more information concerning Id-1 protein levels can be obtained.

Mice Overexpressing Id-1 Appear Normal but Have Lower Body Weight. The transgenic mice looked and moved normally and showed no signs of illness, stress, or neurological disorders. Apart from the differences in muscle fiber size which are described below, a histological evaluation performed on cross sections of paraffin-embedded muscles stained with hematoxylin and eosin revealed no cytological signs of myopathology. Moreover, staining of frozen sections from extensor digitorum longus, tibialis anterior, and soleus muscles with a panel of antibodies against different types of myosin heavy chain (see *Methods*) revealed no major differences in fiber-type composition between transgenic mice and their wild-type litter mates. Thus, there were no signs of the transgene interfering with muscle differentiation.

Although the Id-1 transgenic mice appeared normal in most respects, they were usually slightly smaller than nontransgenic littermates. Systematic weighing revealed a small, but clear, weight difference throughout the growth period. On average, transgenic animals from lines 29, 04, and 95 weighed 16%, 10%, and 9% less than wild-type littermates. This was a specific effect of transgene expression, since transgenic mice from line 99, which displayed only weak transgene expression (1/10th of the RNA signal in the other lines) had normal body weights. We thus conclude that apart from the effects described below, overexpression of Id-1 in muscles leads to phenotypically lighter though otherwise normal animals.

Muscle Fibers Overexpressing Id-1 Become Atrophic. Because the MLC regulatory elements have been shown to promote very different levels of reporter gene expression in different muscle fiber types, we analyzed histological sections in which specific fiber types were identified with monoclonal antibodies raised against myosin heavy chain (see *Methods*). We found that the cross-sectional area of type IIb fibers, which exhibit the highest level of MLC promoter/enhancer-driven expression, was on average 25% smaller than that of IIb fibers in wild-type littermates (Fig. 3). This difference was statistically significant (Mann–Whitney *U* test, $P < 0.0001$). The effect on type IIx fibers, which are expected to have a weaker transgene expression, was smaller (11% atrophy) and more variable (Fig. 3). The atrophy of IIx fibers as a population was, however, statistically significant ($P = 0.006$). These findings suggest that fibers expressing high levels of the Id-1 transgene become atrophic in a dose-dependent manner.

Fibers with Low Transgene Activity Become Hypertrophic. We also investigated fiber types where MLC1/3 regulatory elements confer lower transgene expression (19). Type IIa fibers of transgenic animals were more variable in size than those of wild-type littermates (Fig. 4), perhaps due to variability in transgene expression (19). However, although some fibers were small, on average the IIa fibers of transgenic animals were 34% *larger* and thus displayed a significant hypertrophy ($P < 0.0001$). When type I fibers were measured, the hypertrophy of the transgenic fibers was even more clear: the Id-1 transgenic type I fibers were on average 50% larger than wild-type fibers ($P < 0.0001$) (Fig. 4). We conclude that in the fiber types where Id-1 is overexpressed at low levels, or not at all, the muscle fibers become increasingly hypertrophic with decreasing transgene expression. We interpret this effect to be a work load-induced hypertrophy. Previous studies have shown that when work overload is induced—e.g., by severing the fibers of part of a muscle, by partial denervation, or by cutting the tendon of one of a synergistic group of muscles—a hypertrophy of the remaining intact fibers results (31). In normal animals, the IIb fibers are generally the strongest fibers, and we propose that when these fibers are weakened through a reduction in cross-sectional area, the type I and type IIa fibers are overloaded. Such compensatory mechanisms might also have had a moderating effect on the atrophy observed in IIb and IIx

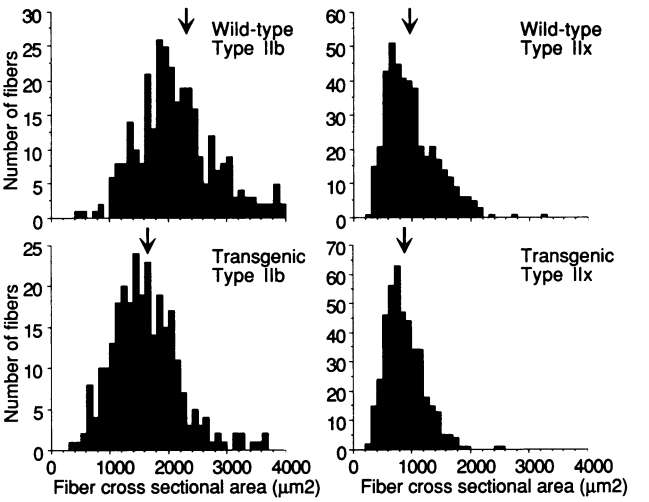

FIG. 3. Histograms of cross-sectional areas of muscle fibers from tibialis anterior in Id-1-overexpressing mice (line 29) and wild-type littermates. For IIb fibers, data were pooled from four sex-matched sibling-pairs that all showed similar differences. The material consisted of 317 and 284 fibers from wild-type and Id-1-overexpressing mice, respectively. For IIx fibers two sibling pairs showed a significant atrophy, while for two others the transgene had no significant effect. Shown are the pooled data of all four pairs; 430 and 429 fibers were measured in wild-type and Id-1-overexpressing mice, respectively. Arrows above the histograms indicate the mean cross-sectional areas.

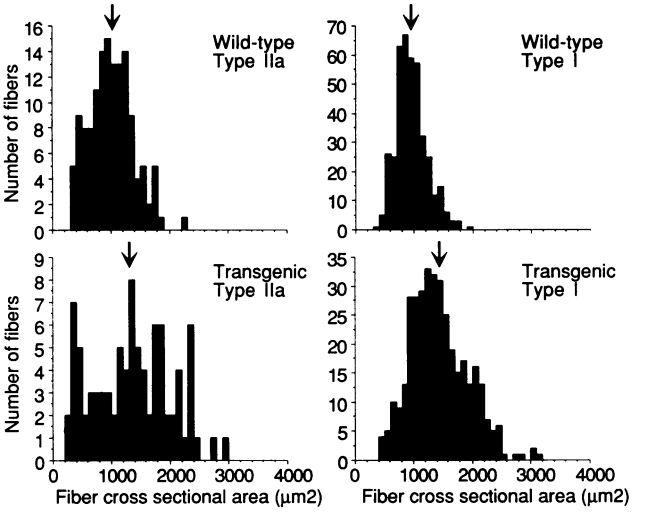

FIG. 4. Histograms of muscle fiber cross-sectional areas from soleus muscles of Id-1-overexpressing mice (line 29) and wild-type littermates. For IIa fibers, data were pooled from two sex-matched sibling pairs that both showed similar differences. The material consisted of 83 and 68 fibers from wild-type and Id-1-overexpressing mice, respectively. For type I fibers data were pooled from three pairs that all showed similar differences. The material consisted of 400 and 364 fibers from wild-type and Id-1-overexpressing mice, respectively. Arrows above the histograms indicate the mean cross-sectional areas.

fibers. These fibers might have been even more atrophic if the work load and amount of activity experienced for each fiber were the same as in wild-type animals. The 25% reduction in cross-sectional area observed in transgenic IIb fibers is quantitatively similar to the effect of 1 week of denervation in the rat, where longer denervations lead to more pronounced effects (32).

In summary, we observed a range of effects on fiber size, apparently depending on the level of transgene expression (Fig. 5). We suggest that this phenomenon is caused by two superimposed and counteracting mechanisms: a primary Id-1 induced atrophy and a secondary work-induced compensatory hypertrophy in fibers less affected by the transgene.

DISCUSSION

Is Id a Regulator of Muscle Fiber Size? We have shown that the level of Id-1 is increased in muscle under conditions which lead to disuse atrophy. Furthermore, atrophy is induced when Id-1 is overexpressed by a transgene in active muscle fibers. These findings suggest a role for Id-1 in the regulation of muscle fiber size. Because Id-1 RNA levels are normally high in undifferentiated myoblasts and decrease upon terminal differentiation, Benezra *et al.* (14) have proposed that Id-1 may negatively regulate myogenesis. Indeed, when Id-1 was overexpressed in myoblasts by using a strong viral promoter, differentiation was inhibited (16). In our experiments, Id-1 expression was controlled by muscle-specific elements that are fully active only in differentiated muscle (18, 33). Thus, we did not expect that MLC promoter/enhancer-controlled Id-1 expression would affect muscle formation, and indeed no gross signs of histo- or cytological abnormality were observed in the transgenic muscle tissue. Our data suggest that Id-1 might play a different role in muscle fibers that are already formed and that the elevated levels of this protein during disuse, or in transgenic mice, is related to the development of atrophy. In the transgenic animals the MLC regulatory elements are active during the entire period of muscle growth that follows muscle differentiation; thus both acute Id-1 expression and the presence of high levels of Id-1 message throughout this period might have contributed to the atrophy. Nonetheless, our data support a role for Id-1 in the regulation of muscle fiber size in addition to its role in muscle differentiation.

In evaluating this hypothesis we have tried to take account of the quantitative aspects of Id-1 expression. In denervated or TTX-blocked muscles, Id-1 mRNA expression increased

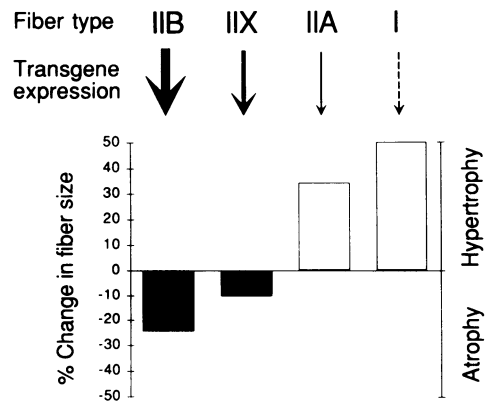

FIG. 5. Summary of the effect of Id-1 overexpression in various fiber types. The thickness of the arrows illustrates the level of transgene expression conferred by the MLC1/3 regulatory sequences when coupled to a chloramphenicol acetyltransferase reporter gene in transgenic mice (19). In type I fibers the expression was below the detection limit (dashed arrow). Change was calculated as change in mean size based on the data shown in Figs. 3 and 4.

only 2- to 7-fold. However, experiments with Id-1 overexpression in undifferentiated myoblasts have shown that even a 5-fold overexpression resulted in a dramatic block of myogenesis *in vitro* (16). Thus, even moderate changes in the level of Id-1 can be of functional importance. In our transgenic experiments, we used regulatory elements that conferred Id-1 RNA levels about 50 times those of wild-type mice. If the levels of Id-1 protein are proportionately high, the atrophy we observe may be caused by unphysiologically high levels of Id-1, although the lack of cytological abnormalities speaks against a nonspecific deleterious effect on the cells. Our attempts to assay Id-1 protein by immunoprecipitation and immunohistochemistry were unsuccessful. This may be due to the characteristics of the antiserum we have used or to the very low levels of Id-1 protein, even in the transgenic mice. In fact, to our knowledge, Id-1 protein has been detected only *in vitro* in cultured myoblasts and overexpressing myotubes transfected with an Id-1 expression vector (16). In those studies, expression of Id-1 RNA correlated poorly with protein levels, leading the authors to conclude that muscle cells negatively regulate Id-1 at a posttranscriptional level. Therefore, if Id-1 protein levels are also regulated *in vivo*, the excess of transgene RNA that we observe may not reflect functional protein levels. Clearly, this question can be resolved only by developing better methods for the detection of Id-1 protein in muscle tissue.

Disuse Induces a Complex Gene-Regulatory Response. Besides atrophy, the most prominent effect of denervation or nerve block in muscle is development of supersensitivity to acetylcholine. Supersensitivity involves an increase in transcription, probably of all five acetylcholine receptor subunit genes (α, β, γ, δ, and ε) (34). On the other hand, disuse decreases transcription of several other identified genes—e.g., α-actin (35, 36), cytochrome *c* (35), αB-crystallin (37), and glycogen phosphorylase (38). In fact, disuse seems to induce a complex response involving up- and down-regulation of a large number of genes (39). It is therefore not surprising that the pattern of changes in the level of transcription factors is also complex. Thus, disuse increases not only the negative regulator Id-1 but also the myogenic factors which work as positive regulators (40–43). It might appear paradoxical that both negative and positive regulators are turned on at the same time. However, since the primary action of Id-1 is to bind to E proteins, a high level of Id-1 will not simply reduce the level of active HLH molecules but may also facilitate formation of homodimers (14). Although the functional importance of homodimers is unknown, they can bind strongly to DNA (44) and could work as more selective regulators of transcription. One could also speculate that the different regulatory proteins work in different nuclei; for example, some data indicate that transcription of acetylcholine receptor genes is increased in only a subset of nuclei after denervation (45).

"Immediate early" genes are genes that can be turned on rapidly in response to stimuli, and without prior protein synthesis. Several immediate early genes are turned on by denervation, including c-*jun*, *junB*, and c-*fos* (46, 47). Interestingly, Fos and possibly also Jun interact negatively with myogenic regulators (48, 49). The immediate early genes might thus play a role similar to Id after denervation. Since Id-1 responded to denervation only after several days (Fig. 1*B*), the products of the immediate early genes could be important for the initial response to disuse, whereas Id could be important for maintenance and long-term effects.

In summary, the pattern of transcription factors induced by disuse mimics an earlier developmental stage with high levels of Id, myogenic regulators, and immediate early gene products. This "dedifferentiation" is manifested also in the appearance of several functionally important muscle proteins found normally only in the immature animal, such as acetyl-

choline receptors with γ subunits (50), TTX-resistant sodium channels (51), and various contractile proteins (52, 53). The present data show that overexpression of one of the transcriptional regulators that is increased during disuse, Id-1, induces atrophy, which is one of the major phenotypic consequences of disuse.

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