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Overexpression of Myogenin in Muscles of Transgenic Mice: Interaction with Id-1, Negative Crossregulation of Myogenic Factors, and Induction of Extrasynaptic Acetylcholine Receptor Expression

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To investigate the role of myogenin in regulating acetylcholine receptor expression in adult muscle, this muscle-specific basic helix-loop-helix transcription factor was overexpressed in transgenic mice by using regulatory elements conferring strong expression confined to differentiated postmitotic muscle fibers. Many of the transgenic mice died during the first postnatal week, but those that survived into adulthood displayed normal muscle histology, gross morphology, and motor behavior. The mRNA levels of all five acetylcholine receptor subunits (α, β, γ, δ, and ε) were, however, elevated. Also, the level of receptor protein was increased and high levels of receptors were present throughout the extrasynaptic surface membrane of the muscle fibers. Thus, elevated levels of myogenin are apparently sufficient to induce acetylcholine supersensitivity in normally innervated muscle of adult mice. The high neonatal mortality rate of the mice overexpressing myogenin hindered the propagation of a stable line. In an attempt to increase survival, myogenin overexpressers were mated with a line of transgenic mice overexpressing Id-1, a negative regulator that interacts with the basic helix-loop-helix family of transcription factors. The Id-1 transgene apparently worked as a second site suppressor and abolished the high rate of neonatal mortality. This effect indicates that Id-1 and myogenin interact directly or indirectly in these animals. Further study indicated that myogenin overexpression had no effect on the level of endogenous myogenin mRNA, while the levels of myoD and MRF4 mRNAs were reduced. Overexpression of the negative regulator Id-1 increased the mRNA levels of all the myogenic factors. These findings are consistent with a hypothesis suggesting that myogenic factors are influenced by mechanisms that maintain cellular homeostasis.

A family of four basic helix-loop-helix (bHLH) transcription factors (myogenin, myoD, myf-5, and MRF4/herculin) known as myogenic factors have received considerable attention as key regulatory molecules involved in differentiation of skeletal muscle cells. Thus, when these factors are overexpressed in cells in tissue culture (for reviews, see references 42 and 57) or in transgenic animals (39, 51), nonmuscle cells acquire a muscle phenotype. In the present study, we have overexpressed myogenin in fully differentiated muscle cells in order to study the role of this factor in regulating muscle plasticity. In particular, we have been interested in myogenin as a putative messenger in the pathway that links nerve-evoked action potential activity to muscle gene expression. Changes in the pattern or frequency of action potentials in muscle fibers have profound effects on the expression of proteins responsible for contractile properties as well as on the distribution and composition of membrane proteins (for reviews, see references 23, 33, and 43). Thus, for example, elimination of muscle action potentials by denervation leads to atrophy (for a discussion, see reference 20) and development of supersensitivity to acetylcholine (ACh). The latter phenomenon was observed over a century ago by Heidenhain, who studied the effects of nicotine on denervated muscle (26). Supersensitivity has received considerable attention since then, both because of its effects on the pharmacological response of muscle to agonists and because it seems to be prerequisite for successful formation of nerve-muscle synapses (28). It is now clear that ACh supersensitivity is caused by an elevated level of nicotinic ACh receptors (AChR) in the extrasynaptic surface membrane, and it has been shown that denervation causes elevated transcription of AChR subunit genes (subunits α, β, γ, and δ but not subunit ε) in nuclei along the entire length of the muscle fiber (15, 17, 22). These changes are correlated with elevated levels of myogenic factors (5, 10, 14, 38, 41, 62), and tissue culture studies have suggested that myogenic factors can transactivate AChR genes (11, 16, 30, 44, 45, 53). We report here that overexpression of myogenin is sufficient to induce ACh supersensitivity in intact muscle of transgenic mice.

Myogenin, myoD, MRF4, and myf-5 are all muscle-specific proteins that have a high affinity for ubiquitously expressed bHLH proteins from the E2A gene. The resulting heterodimers bind efficiently to DNA and are probably the major form in which myogenic factors transactivate muscle genes (for reviews, see references 42 and 57). Myogenic factors and E2A proteins can also form heterodimers with Id proteins, another group of ubiquitous HLH molecules (1, 8, 29, 54). Id proteins lack the basic region found in positive regulators, and heterodimers containing Id-1 bind poorly to DNA. Thus, Id proteins counteract transactivation by myogenic factors and other bHLH molecules and work as negative regulators. We have previously reported the characteristics of a transgenic mouse line overexpressing Id-1 in skeletal muscle (20). Mating of myogenin overexpressers to these mice allowed us to study interaction between the two transgene products in intact mus-

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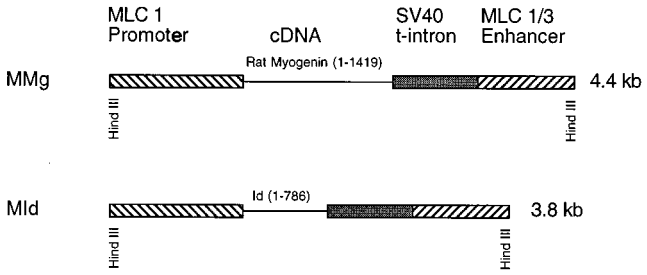

FIG. 1. Maps of the transgenes used in the present study. The endogenous polyadenylation signal was removed from rat myogenin and mouse Id-1 cDNAs. The truncated cDNA sequence was fused to the SV40 early-region t intron and polyadenylation signal. The resulting minigene was placed in a cassette with the MLC1 promoter at the 5′ end and the MLC1/3 enhancer at the 3′ end. The constructs were cut out from the plasmid backbone with *Hin*dIII and gel purified before being injected into mouse embryo pronuclei.

cle. We report that while mice overexpressing myogenin had high rates of neonatal mortality, this was completely eliminated by concomitant overexpression of Id-1.

Myogenic factors have been reported to influence their own expression. Thus, in vitro, high levels of myogenic factors induce even higher levels of expression (positive autoregulation), and products of each of the genes can also transactivate the others (positive crossregulation; for reviews, see references 42 and 57). However, studies of transgenic animals with null mutations in one of the myogenic factor genes suggest that the situation in intact animals might be different (3, 6, 25, 48). We report here that in mice overexpressing myogenin, the mRNA levels of some of the other myogenic factors were reduced, and that in animals overexpressing the negative factor Id-1, the levels of all the myogenic factors were elevated. Both of these findings are consistent with a hypothesis suggesting that myogenic factors are homeostatically regulated by negative feedback in intact mature muscle cells.

MATERIALS AND METHODS

Transgenic mice. The MMg transgene (Fig. 1) was designed to confer overexpression of myogenin only in differentiated muscle fibers. It was made by digesting a full-length rat myogenin *Eco*RI cDNA fragment (63) with *Dra*I in order to remove the endogenous polyadenylation signal. The resulting shorter fragment was ligated directionally into *Eco*RI-*Sma*I-cut pMDAF. pMDAF has a cassette containing the myosin light chain 1 (MLC1) promoter, a short polylinker region including *Eco*RI and *Sma*I sites, the simian virus 40 (SV40) early-region t intron with a polyadenylation signal, and the MLC1/3 enhancer. These elements have been shown previously to confer expression only in differentiated postmitotic muscle fibers (47). The MId transgene, designed to overexpress Id-1 (1), is driven by the same regulatory sequences (Fig. 1) (20).

The MMg and MId transgenes were inserted into the genomes of mice by conventional transgenic techniques, with modifications as described previously (19). Transgenic founders and offspring were identified by PCR assay of DNA extracts from tail tissue (24).

RNA assays. RNA was extracted as described by Chomczynski and Sacchi (7) from hind-limb muscle tissue. The level of specific RNA was determined by Northern (RNA) blotting as described previously (37) or by RNase protection assays as described by Melton et al. (35). For RNase protection assays, the specificity of the protected bands was determined by hybridization against equal amounts of yeast tRNA.

Immunoprecipitation of AChR. Quantitation of AChR in muscle homogenates was performed by immunoprecipitation of receptors labeled with ^{125}I-α-bungarotoxin as described previously (36). In the present study, AChR was precipitated with monoclonal antibody 210 raised against the α subunit or monoclonal antibody 148 raised against the β subunit of the AChR (18, 46). The amount of radioactivity was normalized to the amount of total protein and is reported as a percentage of the level found in normal wild-type muscles in the same experiments.

Determination of AChR in the surface membrane. The density of AChR in the muscle surface membrane was investigated essentially as described by Salpeter (49). Briefly, extensor digitorum longus muscles were excised and incubated with ^{125}I-α-bungarotoxin. After excess toxin had been washed away, the muscles were fixed in glutaraldehyde. Single fibers were subsequently teased from the fixed muscles and arranged on slides. The fibers were covered with a monolayer of photographic film that was premade from a 1:5 dilution of Kodak NTB-2 emulsion. After exposure, the film was developed and the fibers were viewed under dark-field illumination with a 100× objective. AChR density was measured as the number of silver grains in 250-μm^2 fields along the length of the fiber 2 mm on each side of the synapse.

RESULTS

Transgene expression. The present results were derived from transgenic MMg line 96, which overexpresses myogenin, and transgenic MId line 29, which overexpresses Id-1 (see Materials and Methods). Northern blot analysis with a transgene-specific probe revealed one MMg transcript at 2.4 kb and two species at approximately 1.4 kb in total RNA extracted from MMg muscle (Fig. 2A). We predicted a single RNA species at about 2.4 kb, on the basis of the known properties of the transgene elements and assuming an average poly(A) length of 0.2 kb. To clarify the nature of the smaller RNA fragments, we mapped the RNA derived from the rat myogenin transgene by an RNase protection assay. In addition to the full-length RNA transcript, we found two shorter fragments consistent with transcripts having 3′ truncations at positions corresponding to codons 157 and 159 (Fig. 2B). When RNA samples from MMg and control muscles were assayed with a mouse myogenin probe, a single large fragment corresponding to endogenous mouse myogenin mRNA was found in both wild-type and MMg animals (Fig. 2C). In the MMg mice only, additional, smaller and more abundant fragments resulting from imperfect protection of the mouse probe by rat transgene-derived RNA were also detected (Fig. 2C). Densitometry of these fragments revealed that the signals from the transgene-derived RNA were more than 100-fold stronger than the RNA signal from the endogenous myogenin gene. Although further experiments will be required to clarify the cause of the truncations in the transgenic RNA coding sequence, we believe that they most likely arise from aberrant splicing of the SV40 early-region splice acceptor to a cryptic splice donor in the myogenin coding sequence. Such aberrant splicing with fusion genes with the SV40 early region in a 3′ position has been described previously (27). Notably, experiments with transfected cells indicate that recombinant myogenin truncated at amino acid 158 retains most of its ability to transactivate muscle promoters (52). Thus, even the shorter proteins predicted from the most abundant truncated transgenic RNA species in the present study are expected to retain biological activity.

The characteristics of MId line 29 overexpressing Id-1 have been described previously, and the expression pattern was similar to the one conferred by the MMg transgene. Briefly, high levels of mRNA from the Id-1-encoding transgene were observed in muscle, exceeding the signal from the endogenous gene by approximately 50-fold (20).

Effects of myogenin and Id-1 on survival. For most transgenes driven by MLC regulatory sequences, we have routinely found that 10 to 20% of the offspring from injected embryos are transgenic and that approximately 70% of these express the transgene (50). However, for the MMg transgene we obtained few transgenic founders, and most of those we obtained did not express detectable levels of transgene-derived RNA. One female founder (no. 96), however, produced pups that frequently died soon after birth, and these pups were found to express MMg RNA. In an attempt to "rescue" this transgene, we mated mouse 96 with a male from MId line 29. The rationale was that in doubly transgenic mice, the excess Id-1 from the MId transgene might prevent some of the excess myogenin from the MMg transgene from binding to DNA and thereby

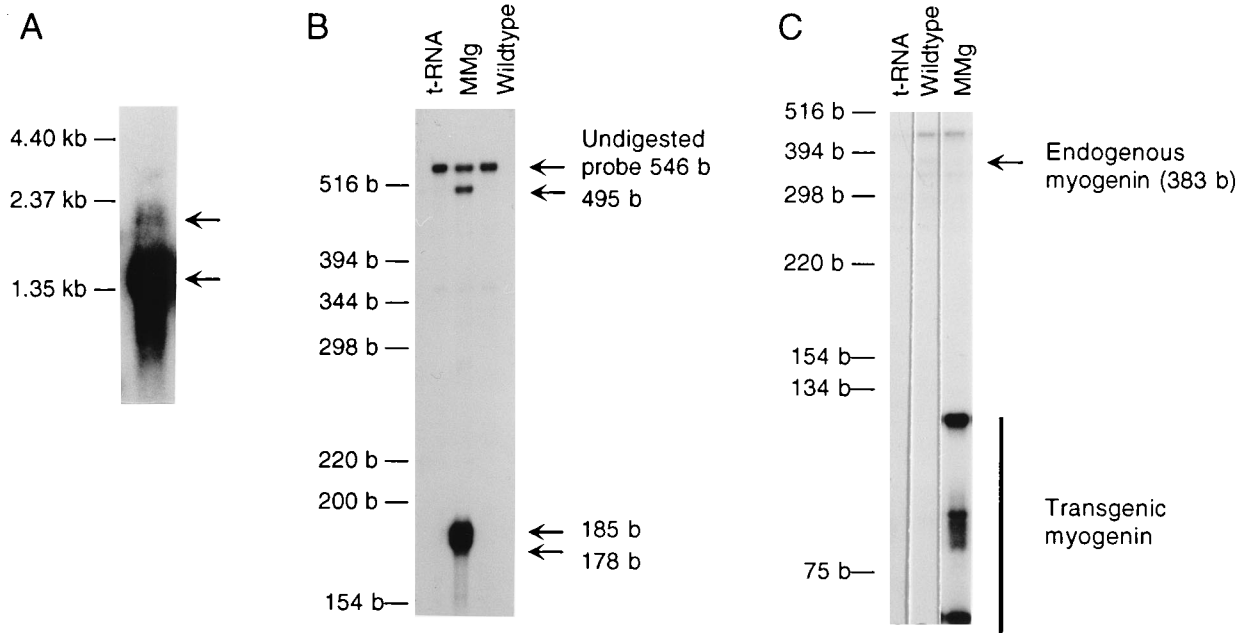

FIG. 2. Expression of RNA from the MMg transgene. (A) Northern blot of RNA extracted from muscle of MMg transgenic mice and probed with SV40 early-region sequence (see Materials and Methods for further details). (B) RNase protection assay of RNA extracted from muscle of MMg transgenic mice in order to test the integrity of the 3′ part of the transgenic coding sequence. The full coding sequence of rat myogenin is 869 bp (63). The probe was made essentially to protect the 3′ part of this sequence and was constructed from a template containing the 495 bases of sequence between the *Stu*I site at base 398 of the coding sequence and the *Sty*I site located 31 bases into the 3′ noncoding sequence. In addition, the probe contained 51 bases of polylinker sequence, totaling 546 bases. The assay displayed three protected fragments calculated to be 495, 185, and 178 bases, respectively. The 495-base fragment corresponds to probe sequence protected by full-length mRNA. The two shorter fragments represent probe protected by myogenin sequence that was truncated at positions corresponding to codons 157 and 159. (See Results for further details.) (C) RNase protection assay of RNA extracted from wild-type and MMg transgenic animals and probed with mouse myogenin cDNA sequence (13). Probe was made for the 383 bases of 3′ noncoding sequence downstream of the *Apa*LI site at position 1098. A band at 383 bases was interpreted to represent mRNA from the endogenous gene, while the smaller bands, which were present only in MMg animals, correspond to transgenic RNA.

counteract the deleterious effects that myogenin had on survival. The mating resulted in viable double-transgenic mice (MMg+MId). The improved survival conferred on the MMg animals by concomitant presence of the MId transgene indicated that the MMg and MId transgene products indeed interacted either directly or indirectly. We documented this interaction quantitatively for 368 offspring resulting from backcrossing double-transgenic mice to wild-type mice. Assuming that there is no bias in uterine mortality and that the two transgenes are located on different somatic chromosomes, Mendelian genetics predicts four possible genotypes occurring with equal frequency at birth (wild type, MMg, MId, and MMg+MId). Test results for 111 newborn pups (day 0) confirmed this prediction (Fig. 3). In contrast, when 257 mice were tested 5 to 11 days postnatally, only 3% were found to display the MMg genotype, confirming that this genotype was lethal for a large fraction of offspring in the first postnatal week. About one-third of the surviving animals were double transgenic (MMg+MId), and this genotype was present at about the same frequency as was the wild-type genotype (Fig. 3). In other words, the MMg+MId animals survived to the same extent as did wild-type animals; thus, the MId transgene completely compensated for the adverse effects of the MMg transgene on survival.

We have not been able to elucidate what caused the high neonatal mortality rate in the MMg mice. Upon visual inspection at birth, MMg, MId, MMg+MId, and wild-type pups were indistinguishable; the transgenic pups displayed no gross behavioral or anatomical abnormalities, and they were able to suckle, since milk could be observed in their stomachs. Histological evaluation of the muscles from MMg animals also revealed no signs of pathology, and in vivo visualization (32, 55) revealed no particular abnormalities in pre- or postsynaptic structures (56). The few MMg animals that survived into adulthood also had no obvious abnormalities in gross morphology, motor behavior, or muscle histology. Systematic weighing revealed, however, that MMg mice were slightly lighter than their wild-type littermates (Fig. 4). A similar lower weight was found also with the MId mice (confirming previous observations in reference 20) and in the MMg+MId mice (Fig. 4). Thus, with respect to animal weight the presence of either one or both transgenes led to a reduction, but unlike the effect on

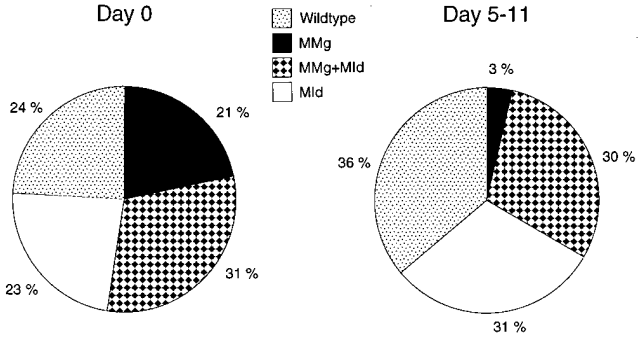

FIG. 3. Distribution of genotypes in offspring resulting from backcrosses of double-transgenic mice hemizygous for both the MMg and the MId transgenes. Offspring were tested at birth (Day 0) or later (Day 5-11). Genotype was determined by a PCR assay of tissue samples taken from each animal (24). A total of 111 animals were tested at day 0, and 257 animals were tested at days 5 to 11.

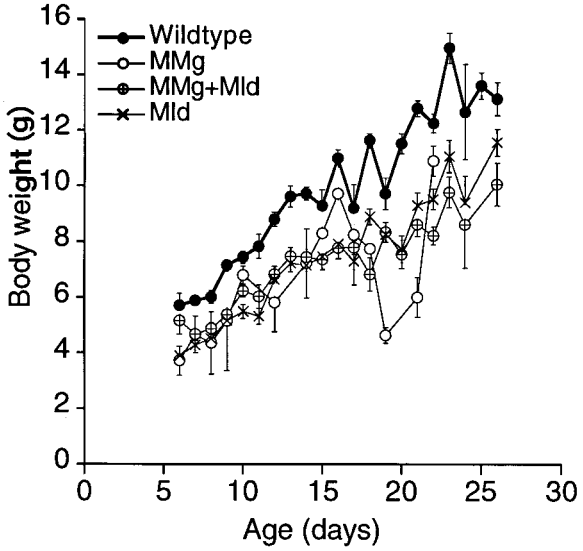

FIG. 4. Growth curves for animals of different transgenic genotypes. Each datum point is the mean ± standard error (error bars) for 2 to 32 observations (on average, 12 observations). Some error bars are omitted for the sake of clarity.

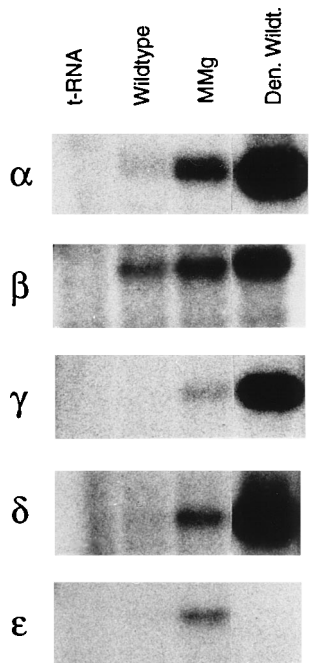

FIG. 5. RNase protection assays of muscle RNA from MMg transgenic and wild-type mice probed with sequences for the different AChR subunits. The probes used have been described previously (34). Den. Wildt., denervated wild type.

survival, there were no signs of the two transgenes interacting to affect weight.

Myogenin overexpression induces increase in AChR mRNA. Once we achieved a stable supply of MMg animals, our first aim was to investigate the possible role of myogenin in developing ACh supersensitivity. Normal newborn animals are known to have supersensitive muscles and high levels of mRNA for receptor subunits (for a review, see reference 23). Even so, the newborn MMg mice tended to have levels of mRNA for the α subunit and particularly the γ subunit even higher than those found in wild-type littermates. When adult MMg mice were compared with wild-type mice of similar age, the MMg animals were found to have clearly higher levels of mRNA for all the five AChR subunits (α, β, γ, δ, and ε), although the induction was less than that obtained by denervation in the wild type (Fig. 5). An exception was mRNA for the ε subunit: in this case the effect of overexpressing myogenin was surprisingly strong, since denervation had no detectable positive effect (Fig. 5).

Myogenin overexpression induces increase in AChR protein. In order to test if the upregulation of AChR genes leads to increased levels of AChR protein, binding of ^{125}I-α-bungarotoxin to Triton X-100 extracts of wild-type and MMg muscles was measured. Toxin binding was increased twofold in the MMg mice in comparison with binding observed for extracts from wild-type mice (Table 1). In double-transgenic animals (MMg+MId) the increase was reduced towards normal values, and in MId animals there was less toxin binding than in wild-type animals. These results were obtained with antibodies against the α subunit, which can bind toxin as a free monomeric subunit, but similar results were obtained with antibodies against the β subunit, which results in immunoprecipitation of ^{125}I-α-bungarotoxin only with fully assembled receptor (2). We conclude that overexpression of myogenin increased the level of fully assembled AChR. Furthermore, concomitant expression of Id-1 to a large degree reversed this effect.

Myogenin overexpression induces AChR in the extrasynaptic surface membrane. The hallmark of supersensitivity is that the AChR are distributed in high concentrations outside the synaptic area along the entire length of the muscle fibers. To investigate whether the increase in AChR mRNA and protein was accompanied by the presence of extrasynaptic AChR in the intact muscle fibers, living extensor digitorum longus muscles from the MMg animals were incubated with ^{125}I-α-bungarotoxin. The binding to the surface AChR of teased single fibers was then investigated by exposing the fibers to photographic film. The amount of AChR was estimated from the number of silver grains obtained after development of the film. Fibers from MMg mice induced higher numbers of silver grains in the extrasynaptic region over several millimeters along the fiber than did those from wild-type mice, which had extrasynaptic silver grain densities close to background level (Fig. 6). We conclude that although the levels of extrajunctional AChR were not as high as in denervated muscle (Fig. 6), overexpression of myogenin induced an increase in AChR inserted into the extrasynaptic surface membrane. In addition, similar measurements for fibers from MMg+MId animals indicated that presence of the MId transgene reduced the extrajunctional level of AChR towards normal (data not shown) and thus confirmed the results obtained by immunoprecipitation with muscles from mice of this genotype (Table 1).

TABLE 1. Immunoprecipitation of AChR

Genotype	Subunit α		Subunit β	
	% of wild-type radioactivity (mean ± SE)	Muscles (*n*)	% of wild-type radioactivity (mean ± SE)	Muscles (*n*)
Wild type	100	4	100	2
MMg	205 ± 14	4	170 ± 13	2
MMg+MId	135 ± 29	4	110 ± 63	2
MId	60 ± 12	4	33 ± 14	3
Wild type denervated	913 ± 161	2	476 ± 175	2

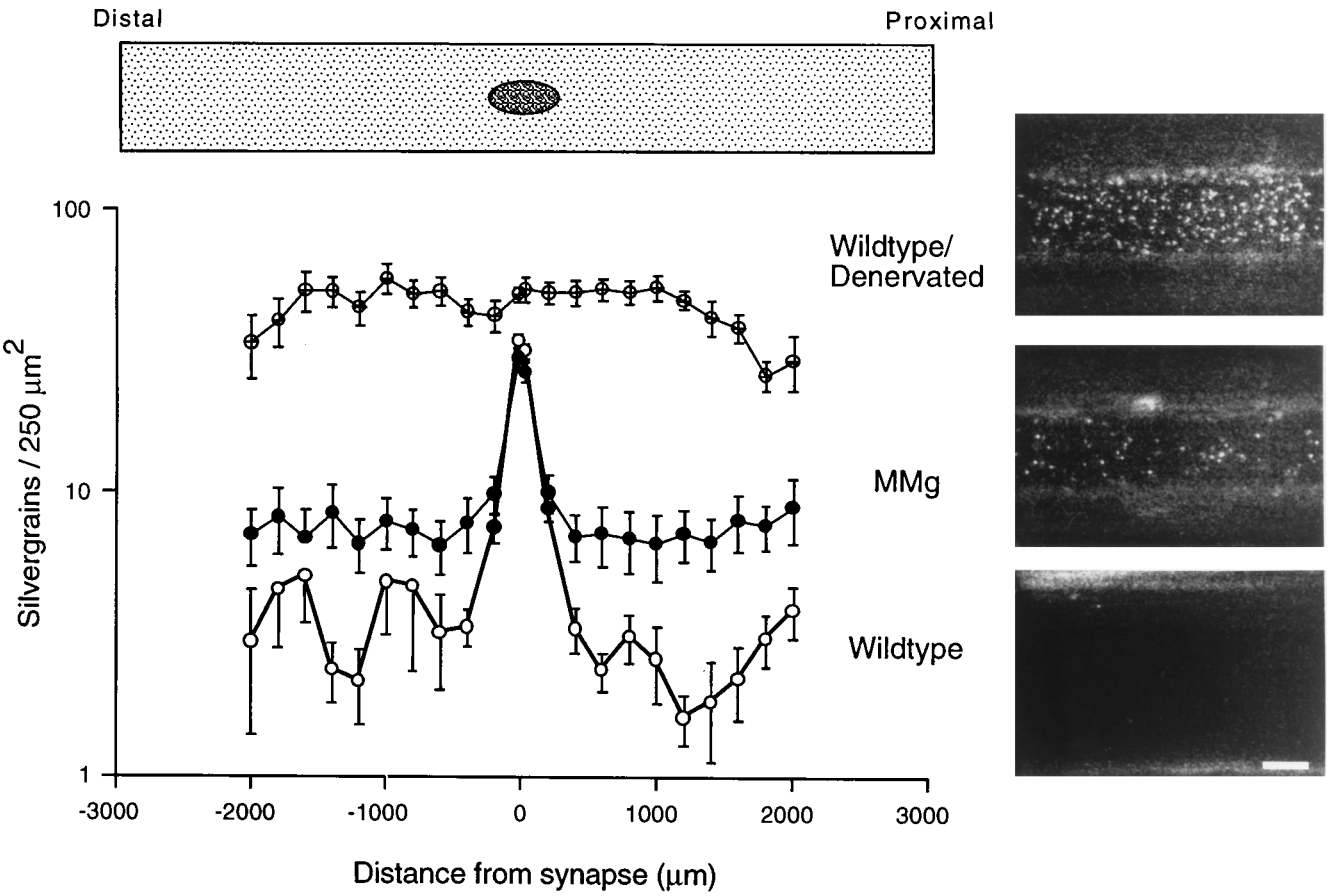

FIG. 6. Measurements of AChR density along single muscle fibers (see Materials and Methods for details). AChR density was visualized by autoradiography of single muscle fibers from muscles that had been incubated with ^{125}I-α-bungarotoxin. The fibers were subsequently covered with a monolayer of photographic emulsion. After exposure and development, silver grains were counted in 250-μm^2 fields along the length of the fiber, 2 mm on each side of the synapse (indicated in the sketch at the top by a stippled oval). The number of silver grains is given on a logarithmic scale. Each datum point represents the mean ± standard error (error bars) for 3 to 27 fibers (on average, 18 fibers). Background density of silver grains on the film outside the fiber was 0 to 4 grains per 250-μm^2 field. Results are from MMg mice (solid symbols) and from normal (white symbols) and denervated (crossed symbols) wild-type mice. One side of some error bars was removed for the sake of clarity. Grain densities at the synapse proper were not measurable. Measurements were done as close to the synapse as 25 μm; because of stray radiation from the synapse, however, these measurements were not accurate. The right-hand pictures are dark-field micrographs from the extrasynaptic area of fibers from MMg mice and from normal and denervated wild-type mice. Scale bar, 10 μm.

Myogenin and Id-1 affect the expression of other bHLH genes. In vitro experiments have suggested that myogenic factors interact with their own promoters, resulting in a positive cascade of myogenic factor expression. Our transgenic mice provided an opportunity to explore this question in vivo. RNA levels for all the myogenic factors (endogenous myogenin, MRF4, myf-5, and myoD) were measured in total RNA from hind-limb muscle extracts of MMg, MId, MMg+MId, and wild-type mice. We found that overexpression of myogenin had no major effects on the level of endogenous myogenin RNA. Moreover, myogenin overexpression had no positive effect on the RNA levels of any of the other myogenic factors (Fig. 7; Table 2). On the contrary, in the MMg mice, RNA levels for two of the factors, MRF4 and MyoD, were reduced compared with those in wild-type mice (Fig. 7; Table 2). Similarly, on the basis of tissue culture cotransfection studies, overexpression of Id-1 would be expected to attenuate expression of myogenic factors (11, 21). In the MId animals, however, the RNA levels for all the myogenic factors were increased (Fig. 7; Table 2). Both the finding that myogenin overexpression led to a decrease in the expression of other myogenic factors and the finding that Id-1 overexpression led to an increase are consis-

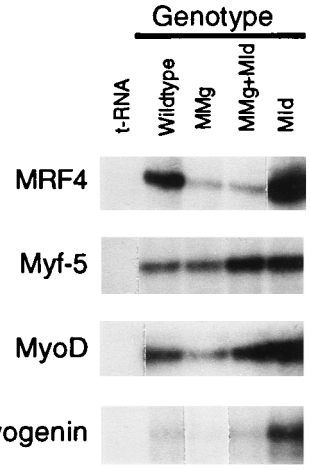

FIG. 7. RNase protection assays of RNA extracted from muscle of mice of different transgenic genotypes. The RNA was probed with sequences of the mouse myogenic factors. For myogenin, the probe used was the same as the one described in the legend to Fig. 2. Here, only the large-molecular-size band (495 bases), specific for the endogenous gene, is shown. The other probes were the same as those used by Miner and Wold (40).

TABLE 2. mRNA levels relative to those for the wild type

Factor	Change in mRNA level for genotype[a]:		
	MMg	MMg+MId	MId
MRF4	−	−	+
myf-5	0	+	+
myoD	−	0	+
Myogenin (endogenous)	0	0	+

[a] 0, no reliable change; +, increase; −, decrease.

tent with negative feedback loops being in operation, resulting in a homeostatic regulation of myogenic factors.

DISCUSSION

Myogenin as a mediator of extrasynaptic ACh supersensitivity. Our data indicate that overexpression of myogenin is sufficient to induce elevated levels of AChR in the extrasynaptic surface membrane of muscle fibers. This induction of ACh supersensitivity suggests that myogenin and possibly also the other myogenic factors are messengers in the signaling pathway connecting action potential activity to gene expression in muscle. The simplest mechanism would be that myogenin binds to E boxes in the AChR genes either as homodimers or bound to proteins from the E2A gene. We cannot exclude, however, the possibility that in the transgenic animals myogenin exerts its effects indirectly or that the observed effects are caused by myogenin working in concert with other transcription factors in a complex network. Qualitatively, overexpression of myogenin mimicked the effects of denervation with respect to AChR mRNA and protein levels, and with respect to the anatomical distribution of AChR in the cell membrane. The increases were, however, less pronounced than those caused by denervation. A trivial reason for this difference might be related to the transgene being expressed only in a subset of fibers, primarily the fast-type 2B and 2X fibers (9). A more interesting explanation for the strong effect of denervation is related to the fact that denervation has other effects in addition to increasing the levels of myogenin. Thus, in denervated muscles the levels of all the myogenic factors are increased (5, 10, 14, 38, 41, 62) while in the myogenin-overexpressing mice these factors were unchanged or reduced (present data). Denervation also induces dephosphorylation of myogenin, and since myogenin is activated by dephosphorylation this would boost the effect of elevated levels (31, 35a).

A surprising difference between myogenin overexpression and denervation was the effect on the mRNA of ε subunit. While ε-subunit mRNA levels were strongly elevated in the MMg mice, denervation had only small and variable effects on this subunit (Fig. 5) (34, 59–61). The ε-subunit promoter is sensitive to inactivity (22), but its expression is confined to a few synaptic nuclei even after denervation (4, 22). The present findings indicate that the ε gene is no less sensitive to elevated levels of myogenin than the other AChR subunit genes are. The effect of myogenin on the ε subunit is intriguing since it is a synapse-specific subunit. Myogenic factors are, however, apparently not selectively expressed at the synapse (58), and it remains unclear if myogenic factors play any role in regulating the high-level expression of AChR that occurs in synaptic cell membranes. Nonetheless, our data support the notion that myogenic factors are involved in activity-dependent regulation of AChR in extrasynaptic muscle cell membranes.

Id-1 overexpression prevents myogenin-induced death. About 90% of MMg mice died within the first postnatal week, but coexpression of Id-1 resulted in virtually normal survival of these mice. The most straightforward explanation for this rescue is that Id-1 binds to myogenin and/or E2A proteins and thereby prevents the lethal effects of excess myogenin.

We have not been able to establish the reason for the lethal effects of the MMg transgene. Both the MMg and the MId transgenes were designed to be expressed exclusively in postmitotic skeletal muscle (47), and we speculate that deaths are caused by some mild form of muscle impairment. Presence of the MId transgene led to a partial reversal of myogenin's effect on AChR expression, and it is likely that the ability of Id-1 to rescue the animals is related to reduced activation of AChR and/or some other downstream genes in the muscle cells. Mildly impaired muscle function might reduce suckling or breathing and result in deaths due to dehydration and/or suffocation. Nonetheless, in the present study a genetic defect caused by the myogenin transgene was compensated by genetic manipulation of a completely different gene, the Id-1 gene. While such second site suppressor effects are common in lower species, this is, to our knowledge, the first example from mammals.

Homeostatic regulation of bHLH molecules in mature muscle fibers? Experiments using tissue culture have indicated that myogenin can activate its own promoter (positive autoregulation) and promoters of other myogenic factors (positive crossregulation) (see, e.g., reference 12). In the myogenin-overexpressing mice we observed no sign of positive autoregulation or positive crossregulation. Thus, the level of endogenous myogenin mRNA was unaltered, and also none of the other factors showed an increase. In fact, mRNA levels for myoD and MRF4 were decreased in the MMg mice, suggesting negative crossregulation of these genes by myogenin. This notion of negative crossregulation between the myogenic factors was supported by the finding that mRNAs for all of them were increased in the MId animals, where the excess of Id-1 is expected to reduce the functional levels of bHLH molecules in general. Similar to what we found, previous experiments with mice having a null mutation in the myogenin gene revealed failure to display autoregulation; thus, the activation patterns for the myogenin promoter, at least during early development, were unaltered (6). With respect to crossregulation of other myogenic factors, it was reported that myoD was unaltered in myogenin mutant mice (25) while mRNA levels for MRF4 were moderately decreased. The latter finding could be interpreted as a sign of positive crossregulation, but since muscle mass was severely decreased, the effect could be indirect, due to a decrease in the number of cells expressing MRF4 (25). In mice with a knockout mutation in the myf-5 gene, no sign of crossregulation was observed, since all three of the remaining myogenic factors were present at normal levels (3, 48). Similarly, in mice with a knockout mutation in the myoD gene, myogenin and MRF4 were present at normal levels. Interestingly, however, a reciprocal relationship between the levels of myoD and myf-5 was found: myf-5 increased in a dose-dependent manner depending on whether one or both alleles of myoD were mutated. All of these findings support the idea of negative crossregulation.

While phenomena observed to occur in transgenic animals might reflect the developmental history of the animals rather than gene regulatory loops operating in the muscle cells, the postulation of negative crossregulation between myogenic factors is an attractive hypothesis. Differentiated muscle fibers have relatively low levels of myogenic factors, and positive cross- and autoregulation would lead to inherently unstable conditions because small perturbations would be amplified;

negative feedback regulation, on the other hand, would promote steady-state conditions.

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