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Differential regulation of MyoD and myogenin mRNA levels by nerve induced muscle activity

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The levels of mRNAs coding for the myogenic factors MyoD and myogenin were measured during synapse formation in developing muscle and in adult muscle, after denervation and reinnervation and after muscle paralysis induced by blocking of neuromuscular transmission by neurotoxins known to alter the density and localization of synaptic proteins such as the acetylcholine receptor (AChR). The mRNA levels of both factors depend on usage of the neuromuscular synapses, but they change to different extents. Myogenin mRNA levels decrease drastically with innervation and increase strongly following blocking of transmission whereas the level of MyoD mRNA showed only a small decrease in response to innervation, denervation or muscle paralysis by neurotoxins. Neither mRNA showed a synapse-related cellular distribution. The results suggest that nerve-induced electrical muscle activity determines the cellular ratio of MyoD and myogenin mRNAs in adult muscle.

MyoD; Myogenin; mRNA level; Muscle activity; Rat

1. INTRODUCTION

With the characterization of MyoD (myoblast determination gene number 1, MyoD1) [1] considerable interest has been focussed on the analysis of factors which induce myogenesis and the expression of muscle-specific structural genes [2]. Other proteins structurally related to MyoD also convert fibroblast (C3H10T1/2) cells into muscle cells and were named myogenin [3,4] myf-5 [5] and Mrf-4 [6] also known as herculin [7] or myf-6 [8]. It is not yet clear how the expression of MyoD and other myogenic factors correlate with the subsequent activation of genes that are expressed specifically in skeletal muscle. There is evidence that MyoD binds directly to regulatory regions of muscle specific genes and activates transcription [1]. Putative MyoD binding sites containing a CANNTG consensus motif have recently been identified on the genes coding for different subunits of the nicotinic acetylcholine receptor (AChR) [9–11]. Two adjacent CANNTG motifs of the chicken AChR α-subunit gene [10] and of the rat muscle AChR γ-subunit gene (Numberger et al., submitted) are involved in transcriptional activation of these genes. On the other hand a single MyoD consensus sequence of the rat muscle AChR ϵ-subunit gene may be necessary to prevent expression in non-muscle cells (Numberger et al., submitted).

Thus far it is not known whether myogenic factors are also directly involved in the regulation of proteins which are localized at the neuromuscular synapse such as the AChR. We have measured the changes in the levels of MyoD- and myogenic-specific mRNA levels during establishment, maintenance and disruption of neuromuscular transmission, during which there are dramatic changes in the level and spatial distribution of synapse-specific mRNAs and proteins.

2. MATERIALS AND METHODS

2.1. *Isolation and surgery of rat muscle*

Triceps muscles from adult Wistar rats (150–300 g) or rats of various postnatal ages, killed with ether, CO_2 or decapitated were removed and immediately frozen in liquid nitrogen. Denervation and reinnervation experiments were performed as described previously [12]. Pharmacological muscle inactivation was induced either by injection of botulinum toxin (BoTX) type A into the triceps muscle or by chronic blockade of nerve signal conduction in the sciatic nerve using tetrodotoxin (TTX) following procedures described by Brenner et al. [13] and Witzemann et al. [27].

2.2. *RNA isolation and Northern blot hybridization analysis*

Total RNA was extracted by the guanidinium isothiocyanate method [12,14]. Samples of RNA (10 μg each) were denatured in 1 M glyoxal and 50% dimethyl sulfoxide [15] electrophoresed on 1.5% agarose gels, transferred [16] to Biodyne membranes (Pall) and crosslinked with the UV Stratalinker from Stratagene. Hybridization was carried out following the procedure recommended by Pall. The membranes were exposed to X-ray film (Kodak X-OMAT) for 1–3 days at −70°C using intensifying screens.

2.3. Hybridization probes

Hybridization probes were labelled with [α-^{32}P]dCTP using the random primed labelling kit from Boehringer (Mannheim) following procedures based on the method of Feinberg and Vogelstein [17]. Ex-

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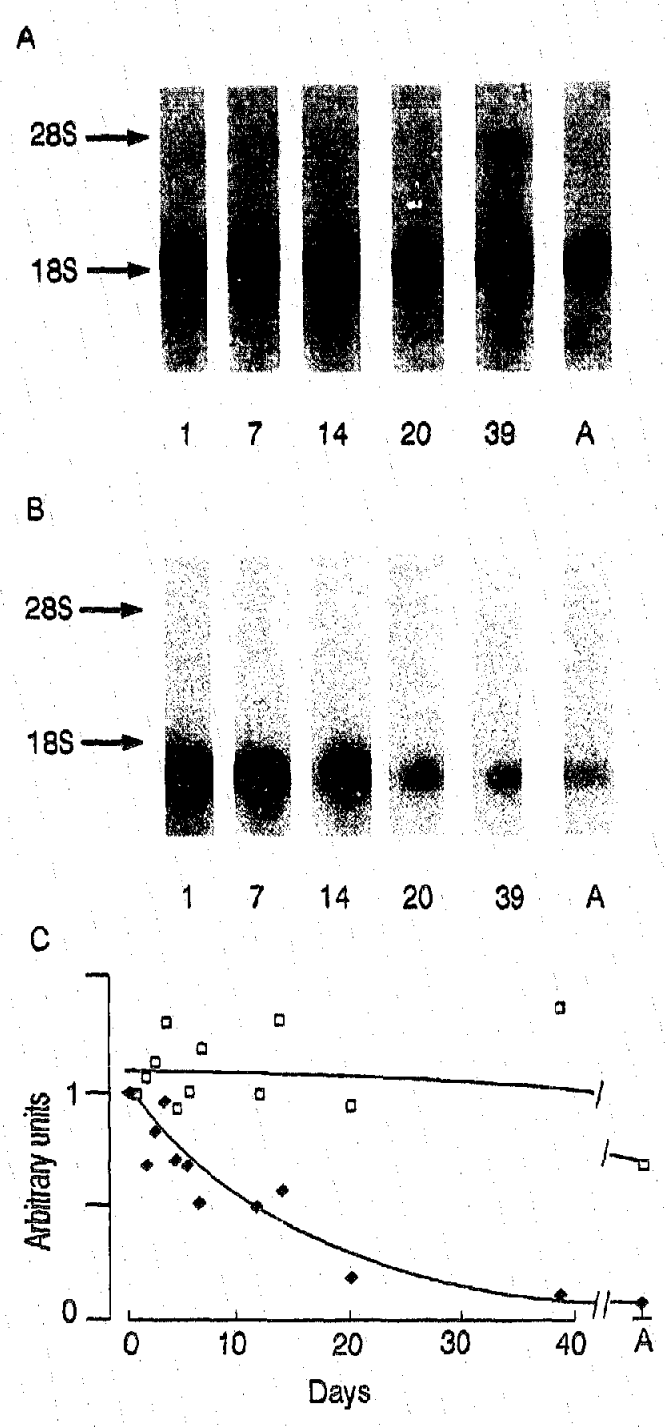

Fig. 1. Northern blot hybridization analysis of MyoD and myogenin transcripts during postnatal development of rat muscle. (A) MyoD mRNA, and (B) myogenin mRNA, show autoradiograms of Northern blots which were analyzed by hybridizing with ^{32}P-labelled probes (section 2) derived from the corresponding full length cDNAs [1,4]. The positions of rat ribosomal RNAs are given. (C) Graphic representation of the relative changes of the MyoD and myogenin mRNA levels during postnatal development. The relative contents of the respective mRNAs (given in arbitrary units) were plotted as a function of days after birth; the value for adult muscle is indicated by 'A'. The values are normalized with the values measured at day 1 after birth for MyoD and myogenin mRNA. Lines are drawn by eye.

cess of incorporated radioactive nucleotides was removed using spin-columns from Biorad. The specific activities of the probes ranged from $1\text{-}2\times10^{9}$ cpm/μg DNA. MyoD [1] and myogenin [4] specific probes were derived from full length cDNA clones kindly provided by Dr Lassar and Dr Olson.

2.4. *Evaluation of autoradiograms*

Equal loading of total RNA was ascertained by ethidium bromide staining of the agarose gels. The ribosomal 18S and 28S RNA bands served as internal standards. Loading increasing amounts (2–15 μg) of total RNA showed a linear increase in specific hybridization signals. In some cases the same blots were rehybridized using AChR subunit or actin mRNA specific probes (Witzemann et al., in preparation) to confirm the specificity of the observed changes in mRNA content (not shown). Autoradiograms were scanned with the dual wavelength scanner from Shimadzu (CS-910). For densitometric evaluation care was taken to scan autoradiograms whose densities were in the linear response range of the film.

3. RESULTS

3.1. *MyoD and myogenic mRNA levels changed differentially during postnatal development*

Total RNA isolated from lower leg muscle from rat was analyzed by Northern blot hybridization. Autoradiograms obtained upon hybridization with ^{32}P-labelled MyoD and myogenin specific cDNA-probes are shown in Fig. 1A, B. The same blot was hybridized first with a MyoD-specific probe and then, after washing, with a myogenin-specific probe. MyoD mRNA remains at about the same level during the first 6 weeks of postnatal development, myogenin mRNA levels on the other hand are higher at birth and decrease drastically within the first 3 weeks of development.

Densitometric evaluation of the autoradiograms in Fig. 1 A, B and of additional experiments are summarized in Fig. 1C, where the relative amounts of MyoD and myogenin transcripts are plotted as a function of postnatal age. The results suggest that MyoD and myogenin mRNAs may respond differently to synapse formation and maturation or may follow different predetermined development programs postnatally.

3.2. *Innervation of muscle down-regulates mRNA levels of MyoD and myogenin*

To discriminate whether the differences in the postnatal changes of MyoD and myogenin mRNA level are solely a consequence of myogenic differentiation or whether it is also affected by neural signals, total RNA was isolated from muscles at 7 days of age which were, however, denervated at day 1 after birth. MyoD mRNA levels, which do not change during normal postnatal development, are clearly increased in 7 day (or 11 day, not shown) denervated muscles and the normally occurring postnatal decrease of myogenin specific mRNA is prevented by early denervation (Fig. 2A), since the myogenin mRNA levels are significantly higher in the denervated muscle when compared to the normally innervated control muscle (Fig. 2B). These results suggest

that both myogenic factors are decreased postnatally in response to innervation. The specificity of these changes is supported by experiments where the same RNA extracts were hybridized with probes specific for the AChR subunit mRNAs and actin mRNA (not shown). Denervation of neonatal muscles produced increased levels of AChR subunit mRNAs when compared to the contents found in normally developing

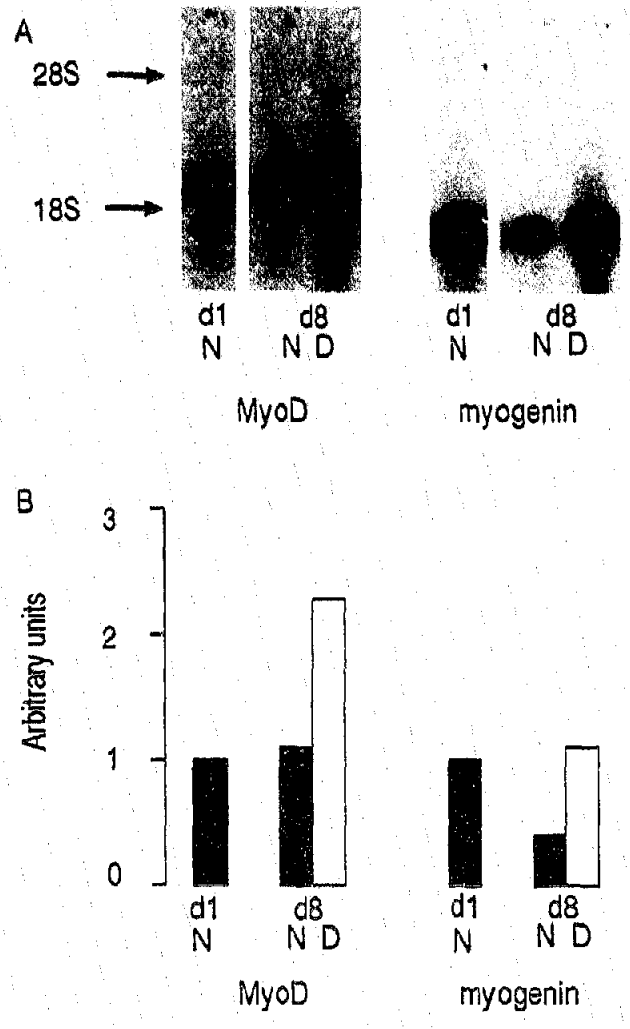

Fig. 2. Postnatal changes in MyoD and myogenin mRNA levels depend on innervation. Total RNA was prepared from postnatal muscle one day after birth. At the same time muscle was denervated and the total RNA was isolated 7 days later from the denervated and the contralateral, innervated muscle as described before [18]. RNA hybridization analysis was performed as detailed in section 2. (A) Autoradiograms show MyoD and myogenin specific mRNAs. Positions of the rat ribosomal RNAs are indicated. Below the corresponding autoradiograms the postnatal stages analyzed are given, d1 (postnatal day 1) and d8 (postnatal day 8). N, RNA isolated from innervated muscle. D, RNA isolated from denervated muscle. (B) Graphic representations of the densitometric evaluation of the autoradiograms. Arbitrary units are values normalized using the values measured at day 1 after birth for MyoD and myogenin mRNA. N (filled bars) and D (open bars) represent RNA from normal and denervated muscle at day 1 and day 8 after birth.

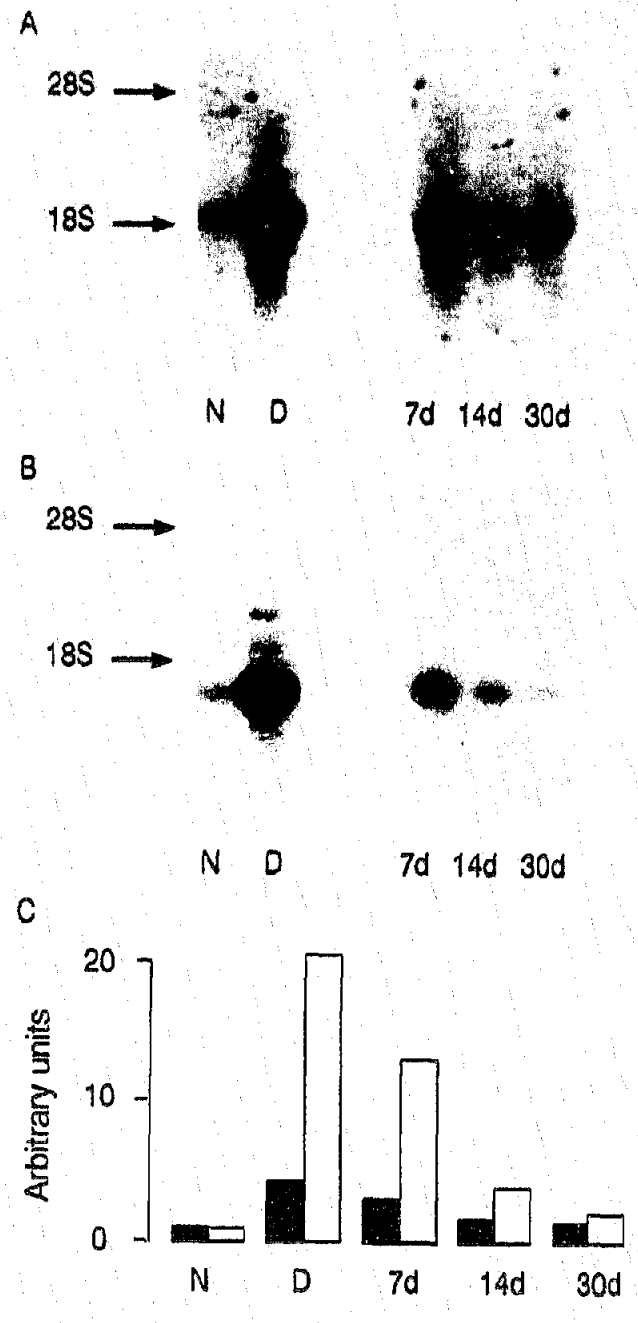

Fig. 3. MyoD and myogenin mRNA content of adult muscle is down-regulated by innervation. Total RNA was isolated from adult, innervated muscle and from muscle denervated for 7 days and subjected to RNA hybridization analysis. Reinnervation experiments were performed on rat diaphragm where functional reinnervation had been followed in parallel on separate muscle preparations [12]. N and D indicate normal and denervated muscle. Days 7, 14, 30 correspond to the time following mechanical nerve crush after which reinnervation occurs beginning at day 7 [12]. (A) Autoradiogram representing muscular content of MyoD mRNA. (B) Autoradiogram of the blot used in (A) but hybridized with a myogenin-specific probe. The positions of rat ribosomal RNAs are indicated in (A) and (B). (C) Graphic representation of the densitometric evaluation of the autograms shown in (A) for MyoD (filled bars) and (B) for myogenin (open bars). Arbitrary units are values obtained by normalizing with the values measured for normal, innervated muscle.

muscles whereas reduced amounts of actin transcripts were observed [18].

The effect of denervation and subsequent reinnervation of adult muscle on the levels of MyoD and myogenin transcripts is shown in Fig. 3. Following denervation MyoD mRNA increases only about 3-5-fold (Fig. 3A) whereas myogenin shows a more drastic response and increased by at least one order of magnitude (Fig. 3B, C). The strong increase in myogenin mRNA is quantitatively comparable to the increase of AChR α-, γ- and δ-subunit mRNAs following denervation [19]. Actin mRNA is, however, decreased in denervated muscle [27] again supporting the specificity of the observed changes in MyoD and myogenin mRNA levels. MyoD as well as myogenin mRNA levels decrease as a consequence of reinnervation and reach within about 3 weeks the levels found in normally innervated muscle (Fig. 3). Preliminary experiments demonstrate in addition that both mRNAs are reduced to normal levels when the denervated muscle is electrically stimulated.

3.3. *Muscle paralysis changes MyoD and myogenin mRNA levels*

Surgical denervation of the muscle leads not only to muscle inactivity, except for fibrillatory electrical activity, but also to the degeneration of the nerve terminals which is what may be responsible for denervation-like changes (for review see [20]). To determine the effects of only muscle paralysis, with the neuromuscular synapse left structurally intact, nerve-induced muscle activity was blocked pharmacologically by botulinum toxin (BoTX) and tetrodotoxin (TTX).

The changes induced by blocking of neuromuscular transmission with BoTX (for review see [21]) are summarized in Fig. 4. Both MyoD (Fig. 4A) and myogenin mRNAs (Fig. 4B) are significantly elevated already 2 days after BoTX administration. Within 7 days of BoTX administration the mRNA levels are increased by about 4-fold for MyoD and about 21-fold for myogenin which is similar to the increase observed upon surgical denervation of the muscle. TTX applied by a nerve-cuff also induces muscle paralysis. However, in contrast to BoTX-blocked muscles, vesicular acetylcholine is still released spontaneously with TTX (for review see [22,23]). In TTX-blocked muscle MyoD mRNA levels increase only slightly and the increase appears to correspond to the small increase observed in denervated muscle (Fig. 4A). Myogenin mRNA levels increase more drastically but the increase is only 50-60% of the amount observed after surgical denervation (Fig. 4B).

3.4. *Cellular distribution of MyoD and myogenin mRNA*

As a consequence of innervation the AChR subunit mRNAs become mainly restricted to the synaptic regions during postnatal development [24]. It was

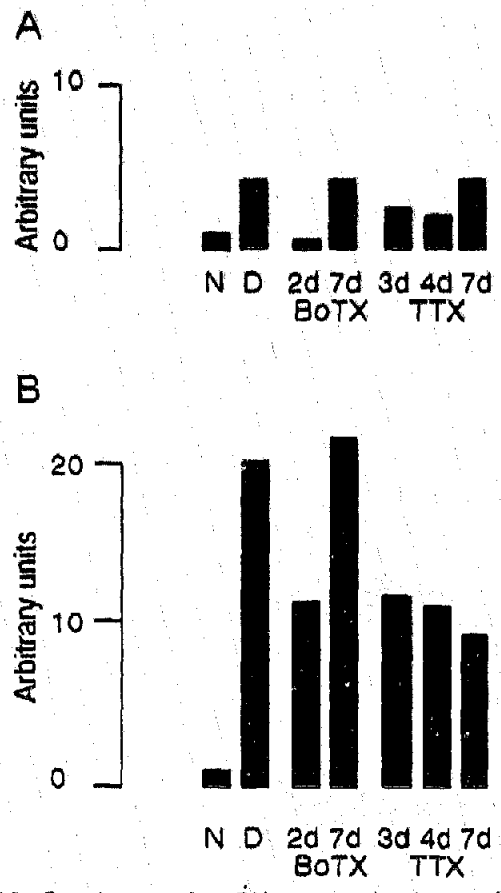

Fig. 4. MyoD and myogenin mRNA content in pharmacologically paralysed muscles. Total RNA was isolated from triceps muscle paralysed by injection of botulinum toxin (BoTX) or by chronic blockade of impulse conduction in the sciatic nerve with tetrodotoxin (TTX) as described in [13]. RNA hybridization analysis was performed using MyoD and myogenin specific probes. N and D indicate RNA isolated from normal and denervated muscle. BoTX indicates mRNA content in muscle paralysed by BoTX for 2 and 7 days (2d, 7d) and TTX mRNA content in muscle paralysed by TTX for 3, 4 and 7 days (3d, 4d, 7d). Autoradiograms were densitometrically scanned and the values are shown by the bar histograms. Arbitrary units correspond to values normalized by the values measured for mRNA content of normal innervated muscle. (A) MyoD mRNA. (B) Myogenin mRNA.

therefore of interest to find out whether innervation would also contribute to an uneven cellular distribution of the MyoD and myogenin mRNAs. Rat diaphragm was dissected into synapse-rich and synapse-free muscle strips and total RNA was extracted for RNA hybridization analysis. The results show that MyoD and myogenin mRNAs are present in approximately equal amounts in both muscle regions and thus display an uniform cellular distribution (Fig. 5A). Denervation causes, for both myogenic factors, an increase in synaptic as well as extrasynaptic regions which corresponds to the increases of the mRNA levels present in total muscle (Fig. 5B). MyoD and myogenin mRNA expression appears therefore not to be confined to specific cellular regions such as the innervated region of the muscle.

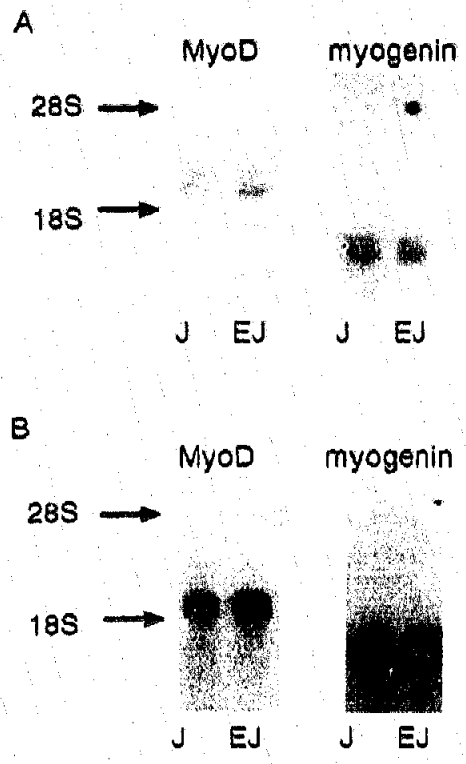

Fig. 5. Lack of synapse specific location of MyoD and myogenin mRNAs in normal and denervated muscle. Rat diaphragm was dissected into synapse-rich (J) and synapse-free (EJ) muscle strips [12] and total RNA was extracted for RNA hybridization analysis. (A) Autoradiograms obtained using MyoD and myogenin specific probes as indicated and total RNA from normal innervated diaphragm. (B) Autoradiograms obtained using MyoD and myogenin specific probes as indicated and total RNA from 7 days denervated diaphragm.

4. DISCUSSION

The results show that the muscular contents of MyoD and myogenin mRNA change in several experimental situations where the interaction between the motor nerve and muscle is altered, namely during postnatal development, denervation, reinnervation, and toxin induced muscle paralysis. Innervation reduces the mRNA levels of both factors, but to different extents. Whereas the level of MyoD shows only relatively small changes in abundance when neuromuscular transmission is established or interrupted (about 2–5-fold) the level of myogenin mRNA seems to be more tightly controlled by the nerve showing much larger changes (about 20-fold). The experiments with neuromuscular block by toxins, which leave the nerve-muscle contact physically intact but block functional transmission, suggest that a signal related to the nerve-induced electrical activity of the muscle is critical for reduction of MyoD and myogenin mRNA in adult muscle. The difference in mRNA levels between denervated and BoTX paralyzed muscle on the one hand and TTX paralyzed muscle on the other hand suggests that, in addition, a neutrophic factor independent of muscle activity may also be involved in the reduction of myogenin levels.

A correlation between the time course of either MyoD and myogenin mRNA levels and AChR subunit specific mRNA levels is, if present at all, rather weak. Possibly, coordinated changes can be found during denervation and reinnervation between myogenin mRNA levels in adult muscle and changes in α-, γ- and δ-subunit mRNAs [27] whereas for the β- and ϵ-subunit mRNAs no correlation is detectable. Also the developmental changes are only weakly, if at all, correlated [18].

The lack of correspondence between mRNA levels of myogenic factors and AChR subunit mRNAs in various experimental conditions for whole muscle mRNA levels is also seen for the cellular distribution of mRNAs. The mRNAs of both factors do not show a synapse-related cellular distribution, thus an involvement of these myogenic factors in the establishment and maintenance of synaptic localization of AChR subunit specific mRNA is unlikely.

The differential regulation of the muscular contents of MyoD and myogenin mRNAs by nerve-induced muscle activity indicates that the ratio of these two factors could be selectively affected by muscle activity. The finding that denervation-triggered activation of AChR-genes require de novo synthesis of a transcriptional activator [25] raises the question whether myogenic factors such as myogenin transactivate AChR-subunit genes upon denervation. MyoD binding elements are located in the upstream sequences of AChR subunit genes and there is evidence that they regulate the transcription of α- [10] and γ- (Numberger et al., in preparation) subunit genes in primary muscle cultures. The α-, γ- and δ-subunit mRNAs seem to be regulated, at least partially, by a signal induced by electrical activity of the muscle [26,27]. Possibly myogenin, being also tightly regulated by electrical muscle activity or a related factor, could be involved in the regulation of extrasynaptic α- and γ- and δ-subunit mRNA levels in adult muscle.

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REFERENCES

[1] Davis, R.L., Weintraub, H. and Lasser, A.B. (1987) Cell 51, 987–1000.
[2] Weintraub, H., Tapscott, S.J., Davis, R.L., Thayer, M.J., Adam, M.A., Lassar, A.B. and Miller, A.D. (1989) Proc. Natl. Acad. Sci. USA 86, 5434–5438.
[3] Wright, E.W., Sasson, D.A. and Lin, V.K. (1989) Cell 56, 607–617.
[4] Edmondson, D.G. and Olson, E.N. (1989) Genes Dev. 3, 628–640.
[5] Braun, T., Buschhausen-Denker, G., Bober, E. and Arnold, H.H. (1989) EMBO J. 8, 701–709.

[6] Rhodes, S.J. and Konieczny, S.F. (1989) Genes Dev. 3, 2050–2061.
[7] Miner, J.H. and Wold, B. (1990) Proc. Natl. Acad. Sci. USA 87, 1089–1093.
[8] Braun, T., Bober, E., Winter, B., Rosenthal, N. and Arnold, H.H. (1990) EMBO J. 9, 821–831.
[9] Baldwin, T.J. and Burden, S.J. (1989) Nature 341, 716–720.
[10] Piette, J., Bessereau, J.-L., Huchet, M. and Changeux, J.-P. (1990) Nature 345, 353–355.
[11] Wang, X.-M., Tsay, H.-J. and Schmidt, J. (1990) EMBO J. 9, 783–790.
[12] Witzemann, V., Barg, B., Nishikawa, Y., Sakmann, B. and Numa, S. (1987) FEBS Lett. 223, 104–112.
[13] Brenner, H.R., Lomo, T. and Williamson, R. (1987) J. Physiol. 388, 367–381.
[14] Chirgwin, T.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[15] McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. USA 74, 4835–4838.
[16] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[17] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[18] Witzemann, V., Barg, B., Criado, M., Stein, E. and Sakmann, B. (1989) FEBS Lett. 242, 419–424.
[19] Witzemann, V., Stein, E., Barg, B., Konno, T., Koenen, M., Kues, W., Criado, M., Hofmann, M. and Sakmann, B. (1990) Eur. J. Biochem. 194, 437–448.
[20] Cangiano, A. (1985) Neuroscience 14, 963–971.
[21] Habermann, E. and Dreyer, F. (1986) Curr. Top. Microbiol. Immunol. 129, 93–179.
[22] McArdle, J.J. (1983) Progr. Neurobiol. 21, 135–198.
[23] Thesleff, S. (1989) Quart. J. Exp. Physiol. 74, 1003–1017.
[24] Brenner, H.R., Witzemann, V. and Sakmann, B. (1990) Nature 344, 544–547.
[25] Tsay, H.-J., Neville, C.M. and Schmidt, J. (1990) FEBS Lett. 274, 69–72.
[26] Goldman, D., Brenner, H.R. and Heinemann, S. (1988) Neuron 1, 329–333.
[27] Witzemann, V., Brenner, H.R. and Sakmann, B. (1991) J. Cell Biol. (in press).