# Sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ATPases, myogenic regulatory factors and myostatin in the regeneration of rat skeletal muscles

**Ph.D. thesis**

Luca Mendler, MD

Institute of Biochemistry
Faculty of Medicine
University of Szeged

2000

# Table of Contents

# 1. List of papers related to the subject of the Thesis

I. **Mendler L**, Zádor E, Dux L and Wuytack F (1998) mRNA levels of myogenic regulatory factors in rat slow and fast muscles regenerating from notexin induced necrosis. *Neuromusc Disord* **8**:533-541

II. #**Mendler L**, #Szakonyi G, #Zádor E, Görbe A, Dux L and Wuytack F (1998) Expression of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPases in the rat extensor digitorum longus (EDL) muscle regenerating from notexin-induced necrosis. *J Muscl Res Cell Mot* **19**(7):777-785
# The authors contributed equally to this work

III. Zádor E, Szakonyi G, Rácz G, **Mendler L**, Ver Heyen M, Lebacq J, Dux L and Wuytack F (1998) Expression of the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ transport ATPase protein isoforms during regeneration from notexin-induced necrosis of rat soleus muscle. *Acta Histochem* **100**(4):355-369

IV. Zádor E, **Mendler L**, Ver Heyen M, Dux L and Wuytack F (1996) Changes in mRNA levels of the sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$ ATPase isoforms in the rat soleus muscle regenerating from notexin-induced necrosis. *Biochem J* **320**:107-113

V. #**Mendler L**, #Zádor E, Dux L and Wuytack F (2000) Myostatin levels in regenerating rat musles and in myogenic cell cultures. *J Muscl Res Cell Mot* (submitted)
# The authors contributed equally to this work

## 2. Abbreviations

| | |
|---|---|
| [α-$^{32}$P]dCTP | dezoxycytidine-5'-triphosphate labelled by radioactive phosphor isotope |
| AGPC | acid-guanidinium-phenol-chlorophorm (method) |
| BFG | basic fibroblast growth factor |
| BrdU | 5'-bromodeoxyuridine |
| BSA | bovine serum albumin |
| bHLH | basic helix-loop-helix (domain) |
| C | deoxycytidine-5'-triphosphate |
| $Ca^{2+}$ | calcium ion |
| cdki | cyclin-dependent kinase inhibitor |
| cDNA | complementary deoxyribonucleic acid |
| DAB | diaminobenzidine |
| $ddH_2O$ | double distilled water |
| DEP | diethyl-pyrocarbonate |
| dNTP | deoxynucleoside triphosphate mix (dATP:dCTP:dGTP:dTTP=1:1:1:1) |
| EDL | extensor digitorum longus (muscle) |
| G | deoxyguanosine-5'-triphosphate |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| HCl | hydrochloric acid |
| HEPES | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (buffer) |
| $H_2O_2$ | hydrogen peroxide |
| Id | inhibitor of DNA-binding (factor) |
| IGF | insulin-like growth factor |
| MADS-box | MCM1, agamous, deficiens, serum response factor |
| MEF2 | myocyte-specific enhancer-binding factor 2 |
| MHC | myosin heavy chain |
| M-MLV RT | Moloney Murine Leukaemia Virus Reverse Transcriptase |
| mpc | myogenic precursor cell |
| MRF | myogenic regulatory factor |
| mRNA | messenger ribonucleic acid |
| myf-3,4,6 | myogenic factor 3,4,6 |
| myoD | myogenic differentiation (factor) |
| NaCl | sodium chloride |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| pax | paired box (protein) |
| Rb | retinoblastoma (protein) |
| RT-PCR | reverse transcription based polymerase chain reaction |
| SDS | sodium lauryl sulphate |
| SERCA | sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase |
| TA | tibialis anterior (muscle) |
| TAD | transactivation domain |
| TBST | Tris buffered saline+Tween-20 (buffer) |

## 3. Summary

Skeletal muscle regeneration involves characteristic time-dependent events of myogenic cell proliferation and differentiation which recapitulates many of the important steps of skeletal muscle development. Our aim was to apply an *in vivo* experimental model of regeneration to study the levels of factors known to play an important role in myogenesis. Necrosis was induced in the slow-twitch soleus and the fast-twitch EDL muscles of rats by intramuscular injection of notexin (a snake venom toxin). Subsequently mononucleated myogenic cells proliferated and fused with each other forming multinucleated myotubes. After differentiation (influenced by re-innervation) mature myofibers were formed. The morphological changes were remarkably similar in both types of muscles, except that in EDL the degeneration/regeneration was delayed probably due to the higher resistance to the toxin. We monitored established and less-known markers of muscle differentiation at different times after notexin administration (up to 28 days). We followed the transcript levels of members of the myogenic regulatory factor (MRF) family (myoD, myf-5, myogenin, MRF4) which show a unique order of activation in myogenesis. Moreover, the patterns of transcript and protein levels of different isoforms of sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) have been analyzed. These pumps are necessary for the muscle relaxation and known to show developmental stage-dependent splicing. Finally, the transcript levels of myostatin, a newly described factor inhibiting muscle growth were also detected. We found that muscle injury induced an increase of the MRF transcript levels in both types of muscles. The order of activation (myoD, myf-5, myogenin and MRF4) was somewhat similar to that reported in development of hindlimb muscles. This increase of the transcription factors was followed by the SERCA transcripts and later on, by the corresponding SERCA proteins. First the neonatal SERCA1b appeared in both muscles, but at the time of re-innervation a switch occured from SERCA1b to the adult fast SERCA1a. In EDL, SERCA1a became dominant, while in the slow-twitch soleus it was replaced by the slow SERCA2a isoform recapitulating the events of muscle development. The time of re-innervation coincided with the switches of the SERCA isoforms, therefore it is likely to remarkably influence the proper ratio of the different isoforms. Myostatin also showed characteristic changes *i.e.* a low level when myoblast proliferation, and an increase to maximum when re-innervation occured. This suggests that the *in situ* synthetized myostatin may take part in the control of growth and differentiaton of regenerating fibers, similar to its role in ontogenesis. However, regenerated muscles did not completely reached the original fiber-type composition, possibly due to the slightly different nature of re-innervation. At the overall level of the whole muscle, the regeneration produced the original fiber size, transcript and protein levels by 28 days after notexin-injection.

# 4. Theoretical overview and introduction

## 4.1. Importance of skeletal muscle regeneration

In recent years further steps have been made for the understanding of the time- and stage-dependent pattern of gene expression in tissue development. Of the many systems that have been experimentally exploited to model developmental control, skeletal muscle may represent one of the best ones (Ludolph and Konieczny 1995). Although embryologic origin of muscle cells is well established, the molecular control of myogenesis and muscle differentiation is not completely understood. Since skeletal muscle regeneration recapitulates many of the principal events of embryonic development, it could be a good model for studying the regulatory mechanisms of muscle differentiation. Moreover, the comparative studies of development and regeneration could help to elucidate the differences between the two events.

On the other hand, the phenomenon of regeneration is important *clinically* since it is part of the pathology of several muscle diseases *e.g.* Duchenne-type hereditary dystrophies and polymyositis (Watkins and Cullen 1987). In Duchenne muscular dystrophy, muscles gradually loose their ability to regenerate probably due to the repeated cycles of degeneration-regeneration. The proper therapy of these diseases has not been found yet. As a potential therapeutical approach, activated myoblasts have been transferred into dystrophic muscles in order to improve their regeneration capacity (Partridge 1991, Dux *et al.* 1993, Karpati *et al.* 1993, Qu *et al.* 1998). Thus, it becomes of interest to learn ways of modulating the outcome of regeneration.

## 4.2. Satellite cells and regeneration

The so called *satellite cells* are considered to be the myogenic precursor cells (mpc) in adult muscle (Mauro 1961). These mononucleated, spindle-shaped cells were first described in uninjured adult muscles as quiescent ones characterized by a condensed chromatin-structure (Mauro 1961, Moss and Leblond 1971). Satellite cells are located

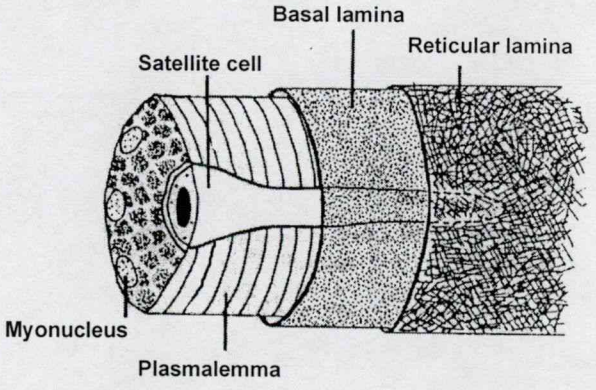


**Figure 1** Location of satellite cell in relation to the myofiber and its layers of extracellular matrix (Bischoff 1993).

in an intimate relationship with the myofibers between the sarcolemma and basal lamina (Fig. 1). They represent 3-8% of the total nuclei of the muscle (Bischoff 1993), whereas the majority of myonuclei belongs to the multinucleated myofibers. Slow muscles contain more satellite cells than fast muscles (Gibson and Schultz 1982), however, there is still some disparity in the literature whether satellite cells of different muscles are committed to produce different muscle-types or predominantly the external *e.g.* neural signals influence the muscle-type formation (Feldman and Stockdale 1991, Schultz 1996, Yablonka-Reuveni and Rivera 1994, Düsterhöft *et al.* 1990, Düsterhöft and Pette 1993, Lagord *et al.* 1998). Satellite cells can be activated by muscle damage, when they re-enter the cell cycle, propagate through the $G_1$, S (DNA synthesis) and $G_2$ phases, and finish the mitosis resulting in active, proliferating myoblasts. There is some evidence that not only satellite cells but also, bone-marrow derived cells (Ferrari *et al.* 1998) and fibroblasts (Gibson *et al.* 1995) might be transformed to myoblasts and involved in the regeneration process. However, the satellite cells are likely to produce the majority of active myoblasts, most of which fuse to primitive, multinucleated myotubes marking the first step of the differentiation process. Myotubes characterized by central nuclei become larger by incorporating new myoblasts and differentiate further by expressing muscle-specific proteins like desmin, myosin, actin, creatine-kinase *etc.* (Schiaffino *et al.* 1989). At the end mature myofibers are formed characterized by peripheral nuclei and the muscle regains its normal size and morphology.

### 4.3. Induction of regeneration. *In vivo* regeneration models

There are several *in vivo* models of skeletal muscle regeneration depending on how muscle injury is generated (Lefaucheur and Sebille 1995b). Mechanical or thermal lesions, ischaemic injuries and myotoxic injections are each suitable to cause muscle damage. *Mechanical* lesions include crush injuries, minced or whole muscle free graft and denervation-devascularization, all of which are associated with *ischaemic* injuries to different extent. The *myotoxins* commonly injected are local anaesthetics like bupivacaine (Saito and Nonaka 1994) and snake venoms like notexin or taipoxin. The different methods to induce muscle injury are not equally effective influencing the time-course and intensity of the subsequent regeneration. The differences may reside *e.g.* in the resistance of satellite cells to injury, the persistence of microvascularization and of sarcolemmal and extracellular matrix structures, the

inflammatory cell response/phagocytosis of the myofiber debris and the proper re-innervation of regenerating muscle cells. *In vitro* studies showed that *satellite cells* resist to myotoxic drugs such as bupivacaine or snake venoms, while they do not survive in ischaemic zones of large free grafted muscles (Philliphs *et al.* 1987). Intact *microvascularization* ensures the survival of satellite cells, thus, disruption of blood supply may impair the regeneration process. The *extracellular matrix* is more resistant to myotoxins than to ischaemic or mechanical injuries and can promote muscle regeneration by assisting the fusion of myoblast to myotubes (Vracko and Benditt 1972) and serving as a reservoir of various growth factors (FGF,TGF-β) (Levine *et al.* 1993) which could regulate *in vivo* muscle regeneration. Moreover, the major component of the basal lamina, laminin has been suggested to stimulate myoblast proliferation in culture (Foster *et al.* 1987). *Inflammatory cells* play also an important role as they are responsible for the phagocytosis and removal of the necrotized tissue indispensable for the proper regeneration (Grounds *et al.* 1992). Furthermore, they are able to release various growth factors (Florini *et al.* 1991) that can control the replication, the differentiation and the fusion of myoblasts. If the inflammatory infiltrate occurs early and intensely, as in the case of myotoxin-treatment (Lefaucheaur and Sebille 1995b), the regeneration seems to be faster and more effective.

Muscle injury of any type can damage *muscle-nerve connection* by destroying the muscle, and consequently, the motor endplates. Thus, the first steps of the regeneration process, *i. e.* the proliferation and the fusion of myoblasts take place in the absence of nerve-endings. No difference was observed in this phase of regeneration of innervated and denervated muscle suggesting that the innervation is dispensible (Sesodia and Cullen 1991). Later, however, after myotubes have been formed, innervation is substantially needed for the maturation and differentiation of myotubes to myofibers (Sesodia and Cullen 1991). If the injury, like mechanical or ischaemic lesions, not only destroys the muscle but also, the nerve endings, the re-innervation of the damaged muscle would be delayed, which in turn results in impaired regeneration.

Briefly, in contrast to mechanical-ischaemic lesions, myotoxins provide an optimal tool for the induction of muscle regeneration leaving the satellite cells, microcirculation, sarcolemma-extracellular matrix and the nerve endings relatively intact.

### 4.4. Notexin-induced regeneration of different muscle-types

The *notexin*-induced regeneration model is one of the myotoxin–induced model systems with all the advantages discussed in the previous chapter. Notexin is the venom of the Australian tiger snake (*Notechis scutatus scutatus)* known to have phospholipase A2 activity (Harris *et al.* 1975). The mechanism of its action is not completely understood since the toxicity of the different components of the toxin does not correlate with their phospholipase activity (Harris and Johnson 1978). However, it has been observed to destroy only the sarcolemma of the myofibers keeping the satellite cells intact. The relative fast *necrosis* accompanied by inflammatory cell response also depends on the type of the injected muscle and, on the quantity and mode of administration. The most sensitive to the toxin are the mitochondria-rich slow-oxidative fibers and muscles containing them, like m. soleus; it shows a uniform necrosis less than 24 hours after its administration (Harris and Johnson 1978). The fast-twitch fibers, and consequently, the predominantly fast muscles like m. extensor digitorum longus (EDL) or tibialis anterior (TA) seem to be more resistant to the toxin resulting in a slower and less uniform necrosis (Harris *et al.* 1975). Notexin can be administered either *systemically or locally*. Systemic injection of the toxin causes rapid neuromuscular and respiratory paralysis before muscle damage could occur preventing the study of degeneration-regeneration (Harris *et al.* 1973). Local (subcutaneous or intramuscular) injection is suitable for inducing necrosis (Harris *et al.* 1975, Harris and Johnson 1978, Sesodia and Cullen 1991, Saito and Nonaka 1994, Zador *et al.* 1996). In case of direct muscle injection, whole-muscle necrosis can be achieved and the effect of surviving fibers can be eliminated.

The *regeneration* after notexin-induced necrosis is relatively fast, similar to the preceeding necrosis (Harris *et al.* 1975, Harris and Johnson 1978, Lefaucheur and Sebille 1995b, Whalen *et al.* 1990). Within 28 days the regenerated muscles regain their original size even in the case of whole-muscle necrosis (Zador *et al.* 1996). However, as mentioned earlier, in the slow-twitch soleus muscles both the necrosis and the onset of regeneration occur faster compared to the fast-twitch EDL muscles, although EDL also completes its regeneration within a month (Harris *et al.* 1975). "Completion" should include both morphological and physiological aspects. In *morphological* sense, regenerated myofibers regain their normal size and shape characterized by peripheral nuclei (Harris *et al.* 1975, Harris and Johnson 1978, Whalen *et al.* 1990, Sesodia and Cullen 1991), though some nuclei are still positioned

centrally. Both the predominant slow myosin isoform (Whalen *et al.* 1990) and the structure of the sarcoplasmic reticulum (Sesodia and Cullen 1991) recover in m. soleus, although the myosin isoform composition becomes more uniform than it was in normal muscles (Whalen *et al.* 1990, Davis *et al.* 1991). Moreover, in the presence of nerve there is a complete recovery of the muscle metabolic capacity (Sesodia *et al.* 1994) ensuring the background for the functional activity. However, in *functional sense* the recovery seems not to be complete in every aspect, although electrophysiological studies showed (Grubb *et al.* 1991, Whalen *et al.* 1990) that regenerated muscles were able to produce normal action potentials and contraction as early as new motor endplates regained their mature form. Other authors, however, reported that in notexin-injected soleus muscle the amplitudes of the contractions remained strongly dependent on external $Ca^{2+}$ concentration, which was a feature of neonatal muscles and not of normal adult muscles (Louboutin *et al.* 1995). This observation refers to others (Vater *et al.* 1992, Whalen *et al.* 1990, Davis *et al.* 1991, Zador *et al.* 1996) supporting that muscle regeneration largely recapitulates muscle development.

### 4.5. Regulation of skeletal muscle differentiation

Skeletal muscle development is a multistep pathway in which mesodermal precursor cells are selected, in response to inductive cues, to form myoblasts that later withdraw from the cell cycle and differentiate (Fig. 3). The breakthrough in understanding of the regulation of myogenesis and muscle differentiation came from the discovery of the MRF (myogenic regulatory factor)- or MyoD-family which include four members: myoD (myf-3 in human), myf-5, myogenin (myf-4 in human) and MRF4 (herculin or myf-6) (Vaidya *et al.* 1992, Ludolph and Konieczny 1995, Olson and Klein 1994, Miller *et al.* 1993, Maione and Amati 1997). They were identified on the basis of their capacity to convert fibroblasts and other nonmuscle cells into skeletal muscle. MRFs are DNA-binding proteins belonging to the 'basic helix-loop-helix' (bHLH) transcription factor-family. They contain a basic domain involved in sequence-specific DNA-binding, and a HLH-domain involved in homo-dimerization or hetero-dimerization with other, non-myogenic HLH proteins known as E-proteins (E12, E47) (Fig. 2). This heterodimerization is necessary for the muscle regulatory factors to be functional *in vivo*. The binding site of bHLH proteins includes a consensus DNA sequence (CANNTAG) termed E-box present in the regulatory regions of several muscle-specific genes

(nicotinic acethylcholine receptor α-subunit, myosin light chain, α-actin, desmin, troponin I, creatine kinase *etc.*) and necessary for their expression (Lowe *et al.* 1998, Fig. 2). The activity of the MRFs can be inhibited by another class of HLH proteins, named Id, which can compete with the MRFs for E-proteins forming Id-E-protein heterodimers. As Id does not contain a basic domain, its heterodimers are not able to bind to the DNA (Miller *et al.* 1993, Ludolph and Konieczny 1995).

Although every MRF binds to the same DNA sequence and also, in fibroblast cultures ectopic expression of each MRF induces muscle differentiation, diverse roles for the various MRFs are suggested by their *in vivo* spatial and temporal expression pattern (Olson and Klein 1994) and also, by the different phenotypes produced in gene knock-out experiments (Olson *et al.* 1996, Rudnicki *et al.* 1993). The specificity of the MRFs may reside in their different transactivation domains (TAD) through which they can cooperate with other factors. *MyoD* and *myf-5* play an important role in muscle determination: somitic precursor cells first express either myoD or myf-5 at a very early embryonic stage (Fig. 3). Both MRFs were reported to remodel the chromatin structure so that previously silent, muscle-specific gene-containing loci can be activated determining the myogenic fate (Gerber *et al.* 1997). They can compensate for each other to certain extent since neither the myoD- nor the myf-5-knockout mice show muscle-defective phenotype but the myoD and myf-5 double-mutant animals do not form muscles (Rudnicki *et al.* 1993). At this point we should mention another transcription factor, *pax-3*, which is a member of the paired box (pax) transcription factor family. It is also supposed to take part in muscle determination (Fig. 3), as in the absence of MRFs pax-3 appears first in cells migrating from the somites to give rise the limb musculature. In contrast, *myogenin* and *MRF4* are expressed later, after proliferating myoblasts have withdrawn from the cell cycle and fused to myotubes (Fig. 3). Myogenin seems to be involved mainly in the direct activation of muscle specific genes, however, it was also reported to be able to substitute for myf-5 under certain conditions (Wang and Jaenisch 1997). MRF-4 may maintain the specific gene expression pattern but it can compensate for the function of myogenin, as well (Zhu and Miller 1997). The story is even more complicated since auto-and cross-activation loops exist between the MRFs that appears to be necessary for the amplification and maintenance of their levels during muscle differentiation (Yun and Wold 1996).

*MEF2-family* of MADS-box transcription factors also plays an essential role in regulating muscle differentiation in tight association with the MRFs (Maione and Amati 1997, Naya and Olson 1999, Yun and Wold 1996; Fig. 2, Fig. 3). MRF-heterodimers and MEF2 can cooperate directly mediated by their DNA-binding domains. MEF2 also recognizes a conserved DNA sequence present in a number of muscle-specific genes able to mediate an E-box independent activation by bHLH myogenic factors (Fig. 2). However, MEF2 is also expressed in other tissues and can interact with several other transcription factors.

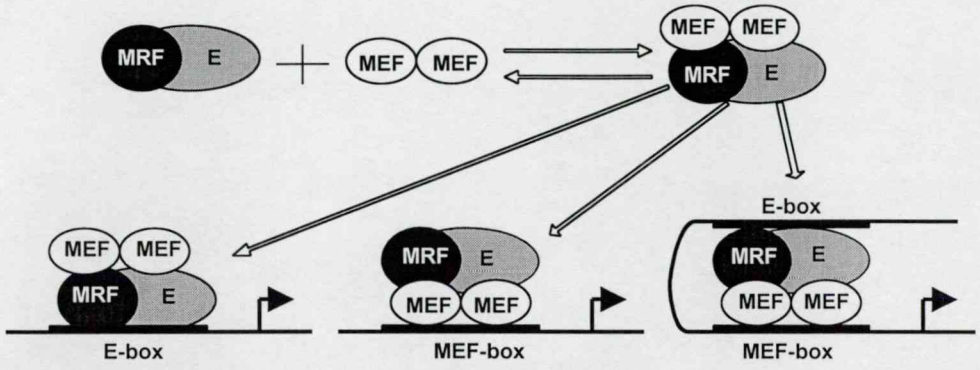


**Figure 2** Models of interaction between the MRF and MEF2 transcription factor family in activating muscle specific genes. MRF-E-protein heterodimers (MRF-E) bind E-box target sites, while MEF2 homodimers (MEF-MEF) bind MEF2 sites. MRF-E-MEF2 complexes are proposed to occupy either E-box or MEF2 sites or both of them. This model suggests a way in which MEF2 factors may engage a strongly muscle-specific regulator (MRF-E) at an MEF2 site. Single-line arrows represent active gene promoters (Yun and Wold 1996)

In summary, the MyoD-and MEF2-families of transcription-factors modulate directly the muscle-specific gene expression. Thus, they are supposed to be the culmination points of any regulatory pathways involved in myogenic differentiation. Moreover, as myoblast proliferation and myogenic differentiation are mutually exclusive events, myoD family also should be coupled to the *regulation of cell cycle*. Indeed, myoD is known to actively control the cell-cycle arrest acting together with the tumor suppressor retinoblastoma (Rb) protein (Maione and Amati 1997). On the other hand, Rb protein is able to induce myoD expression promoting both the cessation of proliferation and the induction of myogenic differentiation. Cyclins, in contrast, can inhibit this functional cooperation by activating cyclin-dependent kinases which hyperphosphorylate the Rb proteins ($Rb\text{-}PO_4$, Fig. 3) and induce the propagation of cells from G1 to S phase (Maione and Amati 1997). However, myoD is also known to transactivate the promoter of cyclin-dependent kinase inhibitors (cdki), like that of

p21 protein, which in turn inhibits the function of these kinases preventing the phosphorylation and inactivation of the Rb protein (Fig. 3).

The role of different *growth factors* playing in muscle differentiation should also be emphasized (Fig. 3). For many years it has been known that the differentiation of cultured myoblasts are negatively regulated by high level of serum or by specific growth factors like bFGF or TGF-β, while insulin and IGF act positively (Florini *et al.* 1991). These factors can independently regulate myoblast proliferation and differentiation. The mechanism of action of FGF or TGF-β seems to involve different proto-oncogenes (fos, myc, jun) which can directly or indirectly inhibit the transcription and/or the activity of MRFs, while IGF is supposed to induce myogenin expression (Florini *et al.* 1991, Maione and Amati 1997).

The regulation of skeletal muscle development should involve the control of the mass of individual muscles and their relation to the whole body mass. Indeed, a new, muscle-specific growth factor called *myostatin* have been reported to be an important negative regulator of muscle growth and development (McPherron *et al.* 1997). Myostatin gene proved to be highly conserved among several species (McPherron and Lee 1997). During embryogenesis, its expression is first restricted to the myotome but later it can be found in many different muscles (McPherron *et al.* 1997). Myostatin knock-out mice are significantly larger than wild-type animals mainly due to an increase in muscle mass, resulting from a combination of cell hyperplasia and hypertrophy (McPherron *et al.* 1997). Moreover, some breeds of cattle characterized by increased muscle mass (double-muscling) *e.g.* Belgian Blue and Piedmontese, have also mutation in the myostatin locus suggesting a general and profound role for myostatin in different species (Grobet *et al.* 1997, Kambadur *et al.* 1997, Smith *et al.* 1997). Similar to other TGF-β family members, myostatin is synthesized as a preproprotein in the muscle and the subsequently secreted (McPherron *et al.* 1997). The active factor might form a dimer but the mechanism of its action remains largely unknown.

The *innervation* is also indispensible for the differentiation process (McLennan 1994); it probably takes part in the determination of different muscle-types (Fig. 3). Whether or not its action is exerted through the different MRFs is not known, however, myoD is predominantly accumulated in fast muscles while myogenin in slow ones, and denervation upregulates the

mRNAs of both factors (Witzemann and Sakmann 1991, Eftimie *et al.* 1991, Adams *et al.* 1995).

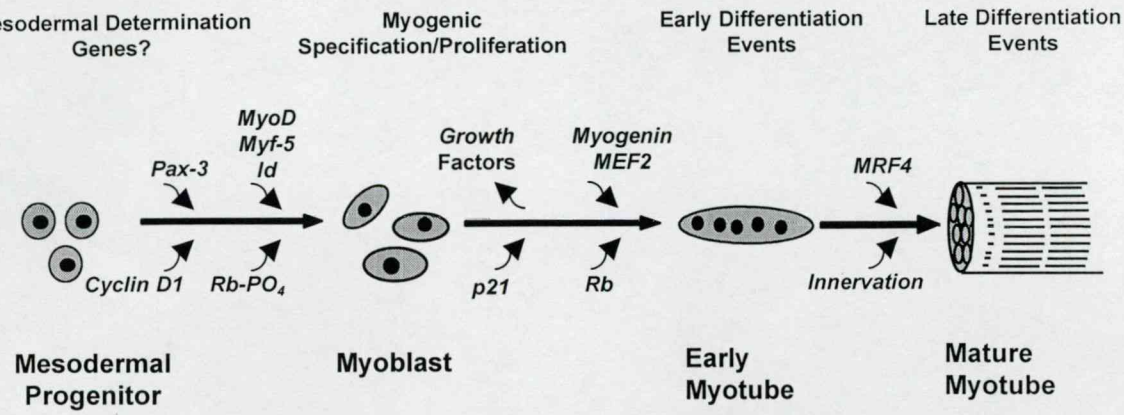


**Figure 3** Summary of the myogenic regulatory cascade model showing the relative linear positions of known factors during skeletal muscle development ( Ludolph and Konieczny 1995; for explanation see text)

### 4.6. Aims and background

Our aim was to apply an experimental model which could be suitable for studying skeletal muscle differentiation *in vivo*. Notexin-induced regeneration of rat skeletal muscle was chosen since it is a well-characterized model from many aspects discussed earlier. We studied the regeneration in its complexity and compared the characteristics of different muscle-types: the slow-twitch soleus and fast-twitch EDL muscles. The amount of the injected toxin was optimized to achieve complete necrosis so that the possible effect of the surviving fibers could be kept at the minimum. We found that, in spite of the comparable weight of the two muscles, three times more snake venom was necessary for eliciting a comparable extent of necrosis in EDL than in soleus muscles. Even after administration of this higher dose of the toxin, remnants of some muscle fibers persisted for up to 3 days in EDL (Fig. 4B,C), whereas virtually all fibers were necrotized already after the first day in soleus (Harris and Johnson 1978, Sesodia and Cullen 1991, Whalen *et al.* 1990). Besides the signs of fiber necrosis, mononucleated cells were also observable very early in both muscles, *i. e.* on day 1 following injection (Fig. 4B). The number of mononucleated cells increased and peaked on day 3 in soleus muscle, while it seemed to occur two days later in EDL. Already on day 5 in soleus and on day 7 in EDL multinucleated myotubes were resulted from myoblast fusions and more matured, but still primitive fibers dominated the whole muscle cross-sectional area (Fig. 4D).

Maturation of fibers was going on until they regained the normal diameter. After 4 weeks the regeneration seemed to be completed in both muscles, although a number of nuclei still occupied a central location within the fibers (Fig. 4E).

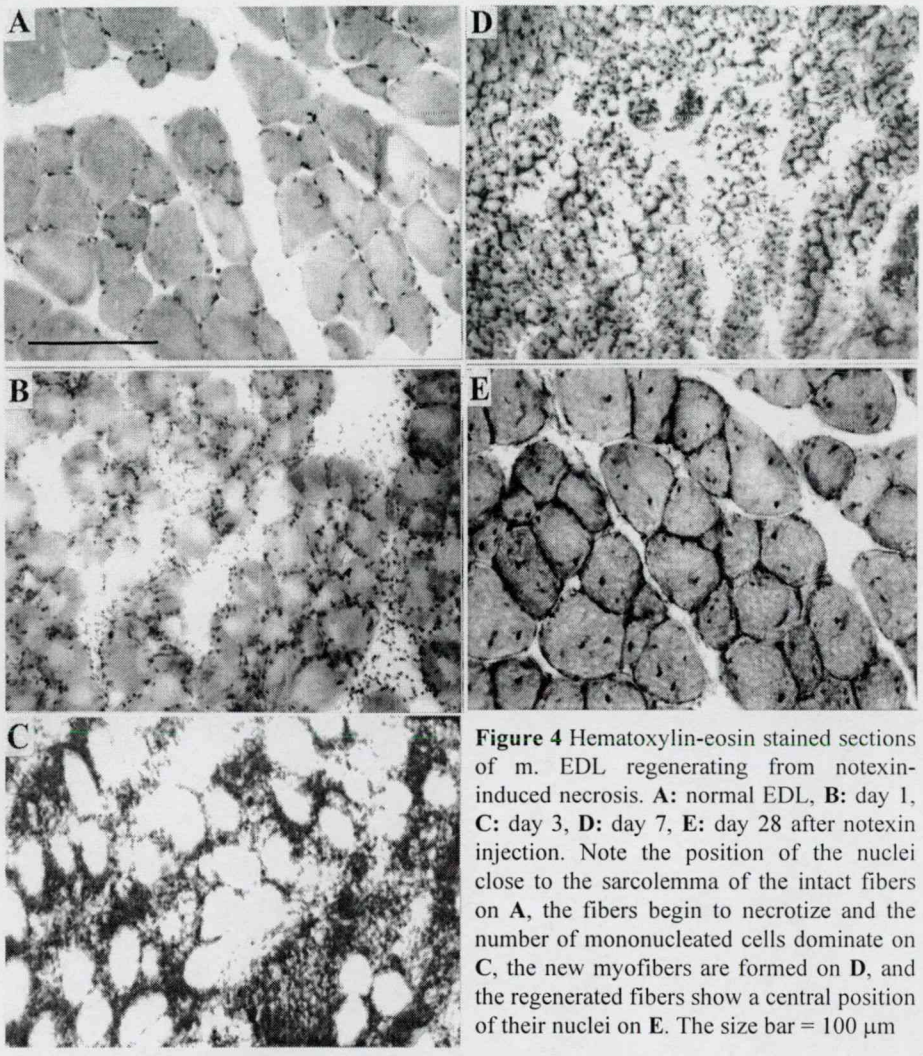


**Figure 4** Hematoxylin-eosin stained sections of m. EDL regenerating from notexin-induced necrosis. **A:** normal EDL, **B:** day 1, **C:** day 3, **D:** day 7, **E:** day 28 after notexin injection. Note the position of the nuclei close to the sarcolemma of the intact fibers on **A**, the fibers begin to necrotize and the number of mononucleated cells dominate on **C**, the new myofibers are formed on **D**, and the regenerated fibers show a central position of their nuclei on **E**. The size bar = 100 μm

Thus, the morphological changes were in good accordance with the results of Harris *et al.* (1975, 1978) and Whalen *et al.* (1990). We also checked the characteristic time-course of cell-division by means of the artificial nucleotide 5'-bromodeoxyuridine (BrdU) which incorporates during the S phase of the cell cycle marking only the dividing cells. The number of the BrdU-positive cells changed in line with the morphological picture, *i.e.* it started to increase on day 1 and peaked on day 3 after notexin-treatment of soleus muscles (unpublished data, Fig. 5). As desmin is a muscle-specific intermedier filament proved to be an amenable marker of activated myogenic cells (Vater *et al.* 1992), we used desmin-immunostaining to

check the myogenic fate of these mononucleated, dividing cells. Desmin-positive myoblasts and myotubes were dominant already on day 3 in soleus (Fig. 5), *i.e.* at the time when the number of dividing cells was the highest, while relatively less cells expressed desmin, especially around the remnants of the necrotic fibers in EDL at this time. The sections stained homogenously for desmin on day 4 in soleus and on day 6 in EDL marking the dominance of myogenic cells (Fig. 5). We also examined the endplate-formation in our regeneration model since its role played in differentiation or regeneration is well-known. As expected, at the time of muscle necrosis almost every motor end-plate had disappeared. New endplates were observed to form between 5-7 days in soleus compared to 7-10 days in EDL, marking the initial phase of re-innervation which coincided with the myotube-formation of both muscles (Fig. 5).

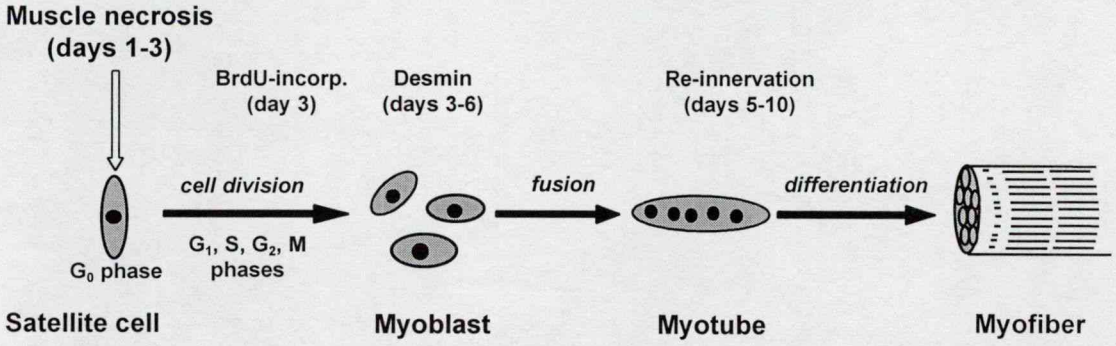


**Figure 5** Scheme of notexin-induced regeneration. Days: days after notexin administration. $G_0$, $G_1$, S, $G_2$, and M: the phases of cell cycle. Muscle necrosis induces the activation of satellite cells which start proliferating (showing the maximum rate on day 3 based on BrdU incorporation) and form desmin-positive myoblasts. Myoblasts fuse to multinucleated myotubes and the differentiation process goes on under the influence of re-innervation until mature myofibers are formed

All of these studies indicated that the time-course of necrosis and the subsequent regeneration is slower (with about 2 days) in EDL than in soleus, which argues in favour of the higher resistance of the fast fibers to the toxin. These markers provided references to the stages of regeneration in which the expression of muscle-specific genes could be investigated.

First we concentrated on the expression pattern of the *sarcoplasmic/endoplasmic reticulum* $Ca^{2+}$*-ATPase (SERCA)*, which pumps $Ca^{2+}$ from the sarcoplasm into the sarcoplasmic reticulum (SR) essential to the relaxation of skeletal muscle. Three types of SERCA genes are known (Burk *et al.* 1989, McLennan *et al.* 1992; Table 1).

| GENE | ISOFORM | EXPRESSION TISSUE |
|---|---|---|
| **SERCA1** | *SERCA1a* | Adult fast-twitch skeletal muscle |
| | *SERCA1b* | Neonatal skeletal muscle |
| **SERCA2** | *SERCA2a* | Slow-twitch skeletal muscle, cardiac muscle and smooth muscle |
| | *SERCA2b* | Smooth muscle and virtually all non-muscle tissues |
| **SERCA3** | *SERCA3a* | Platelets, lymphoid cells, mast cells, endothelial cells, Purkinje neurons, pancreatic islets of Langerhans, salivary glands *etc.* |
| | *SERCA3b* | Mouse pancreatic islets of Langerhans and human kidney |
| | *SERCA3c* | Mouse pancreatic islets of Langerhans and human kidney |

**Table 1** The SERCA gene family (Dode 1998)

The SERCA1 gene is transcribed mainly in fast-twitch skeletal muscles and its pre-mRNA is spliced in a developmental stage-dependent manner into neonatal (SERCA1b) and adult (SERCA1a) mRNAs (Brandl *et al.* 1987, Korczak *et al.* 1988). The SERCA1a protein is abundant in skeletal muscles, however, the expression of the SERCA1b protein has not been investigated yet. The SERCA2 gene is expressed virtually in every cell and its pre-mRNA can be spliced in a tissue-specific manner into 4 classes of mRNAs (Gunteski-Hamblin *et al.* 1988, Eggermont *et al.* 1990, de la Bastie *et al.* 1988). Class 1 is characteristic to slow-twitch skeletal muscle, cardiac muscle and smooth muscle and subsequently translates to SERCA2a protein. Class 2-4 mRNAs are found besides smooth muscles in almost every cell-type translating the "housekeeping" SERCA2b protein. The transcript of the SERCA3 gene is spliced in a tissue-dependent manner into 3 mRNAs present in platelets, lymphoid cells, mast cells, endothelial cells, Purkinje neurons, pancreatic islets of Langerhans *etc.*, and encode for the corresponding proteins (Dode *et al.* 1998, Dode 1998, Bobe *et al.* 1994, Wuytack *et al.* 1994, Wu and Lytton 1993).

As the level of SERCA transcripts and proteins changes during skeletal muscle differentiation, one might expect similar alterations during muscle regeneration. Indeed, we found marked changes at both the mRNA and protein levels of the different SERCAs, part of

which are included in this work from the aspect of the characteristic switches between the SERCA1 and SERCA2, and also, between the SERCA 1a/SERCA1b isoforms.

We also aimed to shed light on the expression of regulatory factors which could participate in the control of the regeneration process, *i.e.* the *myogenic regulatory factors (myoD, myf-5, myogenin, MRF4)* and the growth inhibitory factor *myostatin.* With regard to MRFs, it has been reported (Grounds *et al.* 1992, Füchtbauer and Westphal 1992), that levels of myoD and myogenin are increased during muscle regeneration, but a similar typical sequence of expression of MRFs as seen during normal development has not been described yet for the regenerating muscle. Moreover, embryonic, foetal and new-born myoblasts show a unique expression pattern of MRFs compared to somitic myogenic cells (Smith *et al.* 1993). Denervation and neural stimulation also affect the mRNA levels of MRFs. Since muscle regeneration involves myoblast activation and re-innervation, one might expect changes in expression levels of MRFs (Witzemann and Sakmann 1991, Voytik *et al.* 1993). In addition, since myoD and myogenin were reported to be predominantly expressed in fast-twitch and slow-twitch skeletal muscles, respectively (Hughes *et al.* 1993, Voytik *et al.* 1993), both types of muscle might show different patterns of MRFs during regeneration.

At the end of regeneration newly formed muscles regain their original size. Hence myostatin, which is the negative regulator of muscle growth during development, seem to be a good candidate to have a similar function in regeneration. This question can also be addressed by relating myostatin expression to the known morphological events of regeneration.

## 5. Materials and methods

### 5.1. Induction of regeneration, animal treatment

Wistar rats (300-360g) were used for the experiments. The rats were narcotized by the injection of 1 ml 0.5% sodium pentobarbital per 100 g body weight. A small incision was made on the skin and fascia of the hindlimb so that soleus or EDL muscles were visible and they were directly injected with 20 μg or 60 μg of notexin, respectively, in 200 μl of 0.9% NaCl. After injection the wound was suture-closed. At 1, 3, 5, 7, 10, 21 and 28 days after injection the entire soleus or EDL muscles were dissected and the animals were humanely killed by an overdose of sodium pentobarbital. For each time point at least 3 animals were treated. Control samples were taken from the contralateral muscles injected with 0.9% NaCl.

The dissected muscles were frozen in isopentane cooled by liquid nitrogen and kept at -70 °C. A block of 2 mm-thickness was taken from the central part of each frozen soleus or EDL muscle, it was freeze sectioned and stained by hematoxylin-eosin to monitor the necrosis and the subsequent regeneration. The dose of snake venom to elicit a complete necrosis and the time-course of regeneration were established earlier and found to be remarkably reproducible.

### 5.2. mRNA analysis: reverse transcription-based polimerase chain reaction (RT-PCR)

**5.2.1. Total RNA isolation.** Total RNA was isolated from control and regenerating soleus and EDL muscles by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi 1987). Shortly, muscles were homogenized in 2 ml of Solution D (4M guanidinium thiocyanate, 25mM sodium-citrate pH 7.0, 0.5% N-lauroylsarcosine, 0.1M 2-mercaptoethanol) in a glass/Teflon Potter homogenizer and dispensed in Eppendorf-tubes to 0.5 ml aliquots. After adding 50 µl of sodium acetate (2 M, pH 4.0) followed by a phenol-chloroform-isoamylalcohol extraction, DNA and protein were in the phenol phase and the interphase, whereas RNA remained in the aquous phase. The aquous phase has been precipitated twice with isopropanol thereafter the RNA pellet was washed with ethanol and solved in RNAse-free (diethyl-pyrocarbonate(DEP)-treated) distilled water. RNA concentration and its purity was measured spectrophotometrically (Table 2) and RNA samples were kept at -70 °C until use.

| DAYS AFTER NOTEXIN INJECTION | YIELD (µg/muscle) | |
|---|---|---|
| | M. SOLEUS | M. EDL |
| normal | 95±26 | 55±4.2 |
| 1 | 93±3.4 | 53±8.5 |
| 3 | 98±55 | 66±21 |
| 5 | 185±63 | 57±20 |
| 7 | 194±46 | 66±21 |
| 10 | 174±46 | 85±9.3 |
| 21 | 106±31 | 104±37 |
| 28 | 115±23 | 56±15 |

**Table 2** Total RNA yield in rat soleus and EDL muscles regenerating from notexin-induced muscle necrosis. Total RNA was isolated as described in the Materials and Methods section. The means±S.E.M. for 3 different animals are shown

**5.2.2. Reverse transcription.** Total RNA containing 4-5% mRNA was subjected to an oligo(dT)-primed first strand cDNA-synthesis in a volume of 20 µl at 37 °C for 1 h (2 µg denatured RNA, 1 µl MMLV-RT enzyme [200 U/µl], 4 µl 5x MMLV-RT buffer, 1µl oligo(dT)$_{15}$ [40 µM], 1 µl DTT [0.1 M], 1 µl dNTP mix [10 mM], 0.5 µl RNase inhibitor

[38 U/μl]). The synthesis of the first strand cDNA was followed by the incorporation of [α-$^{32}$P]dCTP and found to be linear in the range of 30-3000 ng input RNA (data not shown).

**5.2.3. PCR-conditions and quantification.** PCR reactions amplifying MRFs (myoD, myogenin, myf-5 and MRF4) (Vaidya *et al.* 1992, Smith *et al.* 1994, De Jaegere *et al.* 1993), the slow-type SERCA2a (Van Den Bosch *et al.* 1994; Fig. 6), myostatin and the internal control of the amplification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Zador *et al.* 1996) were carried out under the conditions given in Table 3.

| PRIMER | NAME | SEQUENCE | PCR CONDITIONS |
|---|---|---|---|
| **GAPDH** | G+ | 5'-tcctgcaccaccaactgcttagcc-3' (nt 528-551) | 94-60-72 °C /1-1-1 min |
| | G- | 5'-tagcccaggatgccctttagtggg-3' (nt 880-904) | 21 cycles |
| **myoD** | + | 5'-tggcgccgctgccttctacg-3' (nt 726-745) | 94-60-72 °C /1-1-1 min |
| | - | 5'-acacggccgcactcttccctg-3' (nt 926-946) | 28 cycles |
| **myf-5** | + | 5'-gagccaagagtagcagccttcg-3' (nt 97-118) | 94-60-72 °C /1-1-1.5 min |
| | - | 5'-gttctttcgggaccagacaggg-3' (nt 516-537) | 28 cycles |
| **myogenin** | + | 5'-gacctgatggagctgtat-3' (nt 115-132) | 94-55-72 °C /1-1-1.5 min |
| | - | 5'-agacaatctcagttgggc-3' (nt 785-802) | 28 cycles |
| **MRF4** | + | 5'-agagactgcccaaggtggagattc-3' (nt 421-444) | 94-60-72 °C /1-1-1 min |
| | - | 5'-aagactgctggaggctgaggcatc-3' (nt 669-692) | 28 cycles |
| **SERCA2a** | $U_f$ | 5'-ctccatctgcctgtccat-3' (nt 1878-1895) | 94-55-72 °C /1-1-1 min |
| | $C_l$ | 5'-gcggttactccagtattg-3' (nt 5527-5544) | 22 cycles |
| **SERCA1a/SERCA1b** | 20 | 5'-ttccatctgcctgtccatgtc-3' (nt 2805-2825) | 94-60-72 °C /1-1-1 min |
| | 23 | 5'-ctggttacttccttctttcgtctt-3' (nt 3029-3052) | 21 cycles |
| **myostatin** | + | 5'-atctgagagccgtcaagactcc-3' (nt 479-500) | 94-58-72 °C /1-1-1 min |
| | - | 5'-cagtcaagcccaaagtctctcc-3' (nt 797-818) | 40 (soleus)/32 (EDL) cycles |

**Table 3** Oligonucleotide primers and PCR conditions used for amplification of reverse transcribed RNA

To measure the relative *SERCA1a/SERCA1b ratios* we followed a different approach. SERCA1 transcripts undergo a developmental-stage dependent splicing so that a 42-bp optional exon is removed in the fast neonatal SERCA1b isoform, but it is retained in the fast adult SERCA1a form. Thus, the ratio of the two splice-variants can be measured by using primers which encompass the optional exon. Primers 20/23 were used in our experiments amplifying a 248-bp adult fragment and a 206-bp neonatal fragment (Fig. 6).

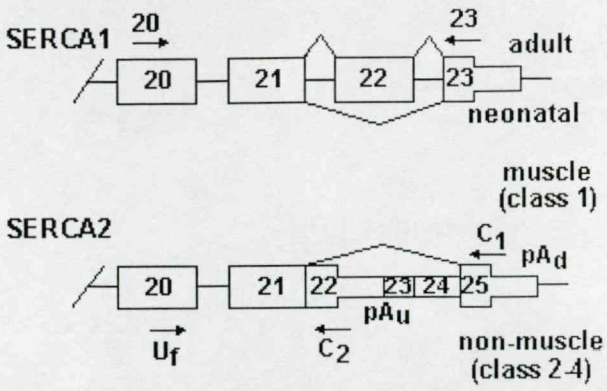


**Figure 6** Positions of primers used to amplify SERCA1a, SERCA1b and SERCA2a (class 1) transcripts. Partial gene structures of the SERCA1 and SERCA2 genes are depicted. Constitutive or optional exons are shown as boxes. The thin lines represent constitutive introns, large boxes represent translated exons. For SERCA1 primers 20 and 23 are used. Exon 22 is retained only in the adult transcript because exon 21 is spliced to exon 23 in the neonatal form. In the muscle-specific transcript of SERCA2a (class 1, amplified by $U_f$ and $C_1$ primers) exon 21 is spliced to exon 25, whereas the non-muscle transcripts (classes 2-4, amplified by $U_f$ and $C_2$ primers) might contain exons 22-24. The drawing is not to scale. $pA_u$ and $pA_d$ are upstream and downstream polyadenilation sites, respectively (Zador *et al.* 1996)

The number of PCR cycles was carefully adjusted to avoid saturation of the amplification system. Amplification products were identified either by their size and fragmentation pattern obtained with the selected restriction enzymes given in Table 4 (MRFs (Fig. 7), SERCAs and GAPDH) or by sequencing (myostatin).

| NAME | PRODUCT SIZE (BP) | DIAGNOSTIC RESTRICTION ENZYME | RESTRICTION PRODUCTS (BP) |
|---|---|---|---|
| **GAPDH** | 377 | BstX I | 196, 181 |
| **myoD** | 221 | Bsa HI | 159, 39, 23 |
| **myf-5** | 441 | Hind III | 235, 122, 84 |
| **myogenin** | 688 | Ava II | 297, 174, 145, 72 |
| **MRF4** | 272 | Pst I | 118, 96, 58 |
| **SERCA2a** | 231 | HinfI | 149, 82 |
| **SERCA1a** | 248 | AvaII | 103, 145 |
| **SERCA1b** | 206 | AvaII | 103, 103 |

**Table 4** Amplified fragments and their identification

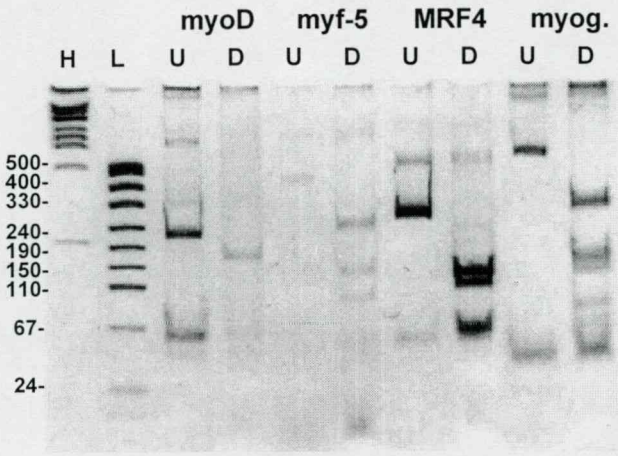


**Figure 7** Identification of PCR fragments of different MRFs. The fragments are separated on a 6% acrylamide gel and visualized by ethidium bromide staining. **H** and **L** are high and low molecular weight markers ($M_r$), respectively. An $M_r$ scale in bp is shown at the left. **U** and **D** refer to undigested and digested fragments with the restriction enzymes given in Table 4. The lengths of the fragments are also given in Table 4. The fragments at or below 60 bp are amplified primer-dimers

In order to radiolabel the PCR fragments for quantification, 5 µl (*i.e.* 1/10 of the volume) of the primary PCR mixture was made up to 50 µl by the same amplification mix in a new tube except that [$\alpha$-$^{32}$P]dCTP was added. Two additional PCR cycles were executed with the same cycle parameters used in the primary PCR. The amplification products were analysed by electrophoresis on 6% acrylamide gels (Sambrook *et al.* 1989). The gels were air-dried and $^{32}$P spots were quantified by means of PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA). As [$\alpha$-$^{32}$P]dCTP was used for labelling, the band intensities were corrected for the CG content of the amplified sequence. For statistical analysis, the Student t-test and the Newman-Keuls test were used.

### 5.3. SERCA protein analysis

#### 5.3.1. Preparation of fragmented muscle membranes and immunoblots

Preliminary experiments on immunoblots showed that in normal soleus approximately 40% of the $Ca^{2+}$-pumps were recovered in the mitochondrial fraction (pellet of a 15 min x 10,000 g spin of the postnuclear supernatant) and approximately 60% in the microsomal fraction (pellet of a 30 min x 200,000 g spin of the postmitochondrial supernatant). Morphological changes which occur during muscle regeneration could cause a redistribution of pumps between mitochondrial and microsomal fractions. Therefore we preferred to use combined mitochondrial/microsomal fractions to avoid preparation artefact.

The soleus and EDL muscles, for which the regeneration state was documented by hematoxylin-eosin staining, were finely minced with scissors and homogenized in 2.5 ml ice-cold 0.25M sucrose, 5mM HEPES pH 7.5 using a glass/Teflon Potter homogenizer. The

homogenates were centrifuged at 1,000 g for 10 min at 4 $^{o}$C and the pellets discarded. The supernatants were centrifuged at 200.000 g for 30 min at 4 $^{o}$C. The pellets, which represented the combined mitochondrial and microsomal fractions, were washed in homogenization buffer and resuspended in 300 µl of 0.25 M sucrose. 5 µl of these suspensions were subjected to electrophoresis on a Laemmli-type of 7.5% SDS PAGE and then electroblotted onto immobilon-P nylon membrane (Millipore). The SERCA1 proteins were detected by a 1:10 dilution of culture supernatant of the A3 mouse anti-SERCA1 monoclonal antibody (Zubrzycka-Gaarn *et al.* 1984). The SERCA2a proteins were detected by a 1:500 dilution of SERCA2a-specific rabbit antisera R-15 (Wuytack *et al.* 1989; Eggermont *et al.* 1990). The blots were quenched in 10mM Tris.HCl pH 7.5, 0.9% NaCl, 0.05% Tween-20 (TBST) for 1 hour. The antibodies were also dissolved in TBST solution. Incubations with primary antibodies and secondary antibodies (peroxidase-conjugated rabbit anti-mouse immunoglobulins or swine anti-rabbit immunoglobulins, 1:1000, Dako A/S, Glostrup, Denmark) lasted for 1 hr. For visualisation of the immunocomplexes nickel-enhanced diaminobenzidine (DAB) precipitations were produced by staining for peroxidase activity in the presence of 0.006% $H_2O_2$ (Wuytack *et al.* 1994). Quantitification was performed by densitometer scanning, using ScanPack 10.1 A20 program (Biometra, Gottingen, Germany). Amounts of sample were always applied within the linear range of the assay as determined by a dilution series of the sarcoplasmic reticulum fraction. Three protein homogenates, each from separate animals, were analyzed for every time point studied and the Student t-test (m. soleus) or the Newman-Keuls test (m. EDL) were used.

Controls to ascertain specifity of the SERCA antibodies were performed. The A3 SERCA1 specific antibody did not cross-react on immunoblots with serial dilutions of combined mitochondrial and microsomal fractions of rat heart known to express SERCA2a but not SERCA1. However, SERCA2a was detected in the same microsomes by the R-15 SERCA2a specific antibody (data not shown).

### 5.3.2. Immunohistochemistry

Soleus and EDL muscles were cryoprotected with 20% sucrose and frozen in isopentane cooled with boiling liquid nitrogen. Serial cryosections of 20 µm thickness were cut and

processed for immunostaining. Sections were incubated in 1% BSA and 10% normal rabbit or goat serum in PBS for 20 min to block non-specific binding sites followed by an overnight incubation with the SERCA1-specific antibody (A3, mouse monoclonal, 1:10) or the SERCA2a-specific rabbit serum (R-15, 1:500), then with the secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulins, Dako, 1:200 or goat anti-rabbit, Sigma, 1:200) for 30 min. The immunocomplexes were visualized by DAB staining of peroxidase activity in the presence of 0.006% $H_2O_2$. Control sections were immunostained in the absence of the primary antibody. Images of the serial sections stained with the SERCA antibodies was compared using Global Vision System software (Analis Belgium, Version 1.2, Namur, Belgium).

## 6. Results

### 6.1. GAPDH mRNA levels in regenerating rat soleus and EDL muscles

The mRNA levels of the housekeeping enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) did not change significantly at the time of muscle necrosis and the subsequent regeneration of both soleus and EDL muscles compared to that of uninjected control muscles (Fig. 8,9). Hence the transcript level of GAPDH enzyme proved to be a good internal control of the RNA recovery and the effectivity of the RT reaction.

### 6.2.Transcript levels of the myogenic regulatory factors (myoD, myf-5, myogenin, MRF4) in regenerating rat soleus and EDL muscles

By an RT-PCR method, we found characteristic changes in the transcript levels of the different myogenic regulatory factors during the regeneration process. In *soleus* muscle, myoD was the first MRF to show an increased mRNA level (Fig. 8A), thus marking the onset of muscle regeneration already on the first day after administration of the toxin. Later on its transcript level reached a maximum on the third day and gradually declined between days 5-21 to about threefold of that of the control and stayed there until day 28.The myf-5 mRNA, in contrast to myoD, decreased on the first day, then showed a maximum 15-fold over the normal level on day 3, thereafter it declined to about half of the normal level at the end of regeneration (Fig. 8A). The myogenin mRNA was not detected on the first day, increased to 9-fold over the normal level on the third day and then declined gradually to the normal level until the end of regeneration (Fig. 8A). The levels of MRF4 mRNA fell below the detection

limit on day 1 (Fig. 8A). It reappeared again on day 3, reached its control level on day 5, after which it tended to oscillate around the normal level.

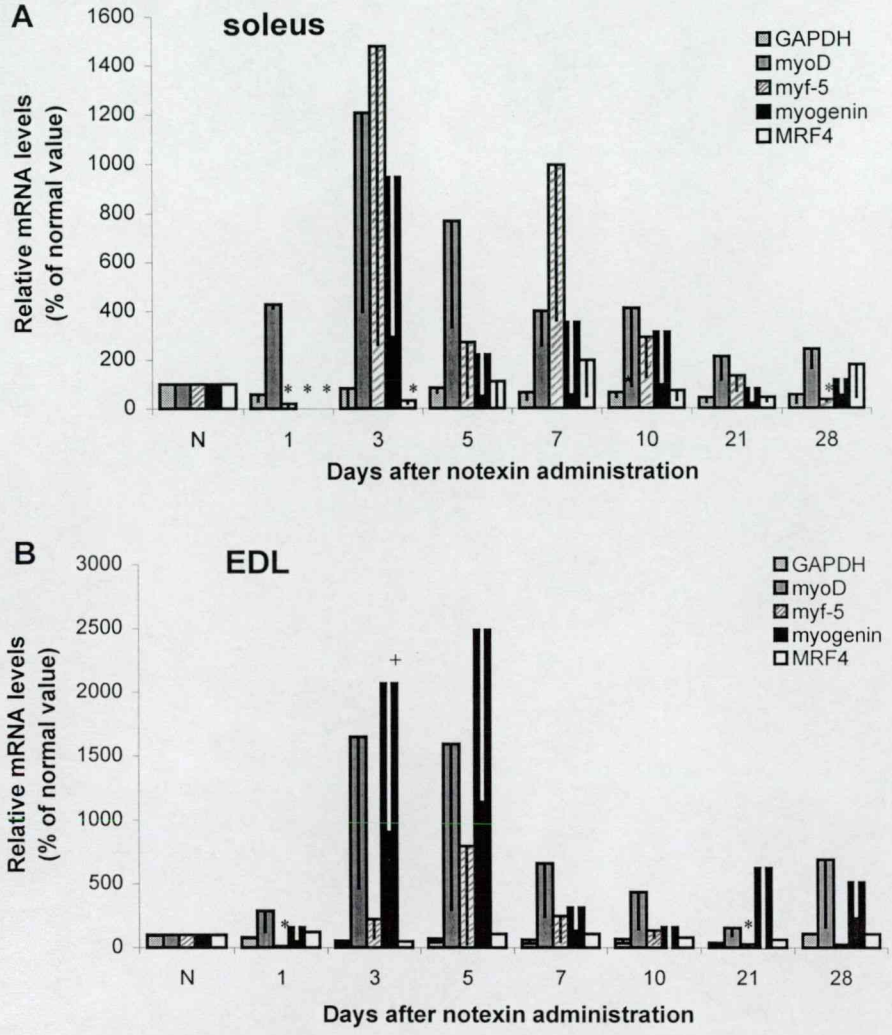


**Figure 8** Reverse transcriptase-PCR-detected changes in MRF and GAPDH mRNA levels during notexin-induced regeneration of the rat soleus (**A**) and EDL (**B**) muscles. The columns show mean values of 3 different animals, the vertical bars in columns are standard errors of mean value (SEM). The labels of the columns show: N, normal muscle; 1-28 days after notexin administration. Significant changes are labelled with either (*)-$p<0.01$ or (+)-$p<0.05$. The values given in the ordinate are relative values that cannot be compared for the different factors. The levels of the internal control GAPDH remained constant. MyoD increased in both muscles on day 1 and reached a maximum on days 3-5. Myf-5 abolished from both the soleus and EDL on day 1 but increased many times above normal on day 3 or 5, respectively. Similarly, myogenin and MRF-4 disappeared from soleus but not from EDL on the first day. Myogenin showed its maximum on days 3-5 in both muscles while MRF-4 increased on day 7 in soleus and remained relatively unchanged in EDL

During regeneration of *EDL* the mRNA level of myoD increased on the first day, stayed at 16-fold above the normal level on day 3 and 5, declined on days 7-21 but then increased to 6-fold over the normal level at the end of regeneration (Fig. 8B). In contrast to myoD, the myf-5 mRNA almost disappeared on the first day, it increased on days 3-5 and gradually

declined below the normal level during the subsequent period of regeneration (Fig. 8B). The myogenin mRNA increased on the first five days, declined on days 7-10 and then tended to increase slightly towards day 28 (Fig. 8B). The level of MRF4 mRNA did not change on the first day of regeneration but it showed a transient slight decline on day 3, then it kept to the level of the control between days 5 and 28 (Fig. 8B).

### 6.3. SERCA1a/SERCA1b and SERCA2a mRNA levels in regenerating rat EDL muscles

In the slow-twitch soleus muscles characteristic switches from the neonatal SERCA1b to the adult SERCA1a and finally, from SERCA1 to the slow-type SERCA2a transcripts were shown during the notexin-induced necrosis/regeneration (Zador *et al.* 1996).

*In EDL,* mRNA levels of the fast-twitch SERCA1 isoform were always more abundant than that of the slow-twitch SERCA2 form. In control muscles, the adult SERCA1a form was the most prominent one and only a trace amount of the neonatal SERCA1b mRNA was found (Fig. 9A).

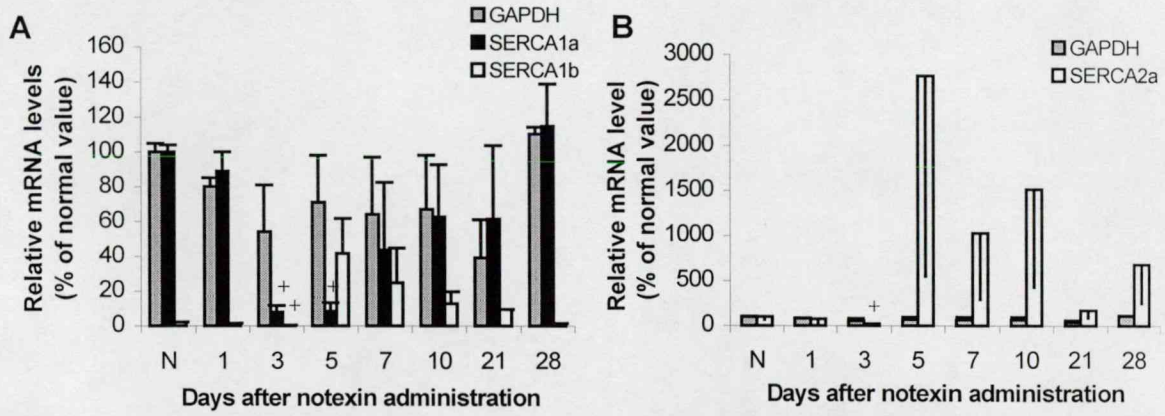


**Figure 9** RT-PCR detected changes of GAPDH, SERCA 1a/1b (**A**) and SERCA 2a (**B**) transcripts during the regeneration of EDL from notexin-induced necrosis. The mean values on the ordinate are relative values normalized to the mean values of the controls (N) which are taken as 100 %. Column labels are the same as in Fig. 8. (+) means significant difference ($p<0.05$). The level of the GAPDH transcript remained relatively constant (see also in Fig. 8). In normal EDL the adult fast SERCA1a was the dominating isoform (**A**). During regeneration the neonatal SERCA1b appeared first but after one week the SERCA1a dominated again (**A**). The slow SERCA2a transcript showed only a transient increase at the time of the SERCA1b/SERCA1a switch (**B**)

On the first day their absolute levels were only slightly lower, but on the third day the level of mRNAs of both SERCA1 isoforms dropped 10-fold lower. Similar to soleus, it was the SERCA1b transcript, which increased first on day 5, and later it was gradually replaced by the adult SERCA1a isoform. After 28 days of regeneration, the levels of SERCA1a and SERCA1b were back to normal. The SERCA2 transcript levels were always much lower than

those of SERCA1, but during recovery from necrosis (days 5 to 21) it showed a transient increase (Fig. 9B) for which mostly the muscle-specific SERCA2a isoform accounted.

### 6.4. Levels of SERCA1 and SERCA2a proteins in regenerating rat soleus and EDL muscles

Levels of SERCA1 and SERCA2a proteins were assessed by Western blotting of the combined mitochondrial/microsomal muscle fractions throughout the necrosis/regeneration process. *In soleus,* both the fast SERCA1 and the slow SERCA2a protein levels were strongly reduced 3 days after administration of the toxin. The SERCA1 protein gradually recovered between day 5 and 21, while SERCA2a protein reached a level at approximately 1/3 of the control level on day 5 and remained there on days 7 and 10, as well. On day 21, however, a further increase in the SERCA2a level was observed to a value similar to the control and this persisted on day 28 (Fig. 10A).

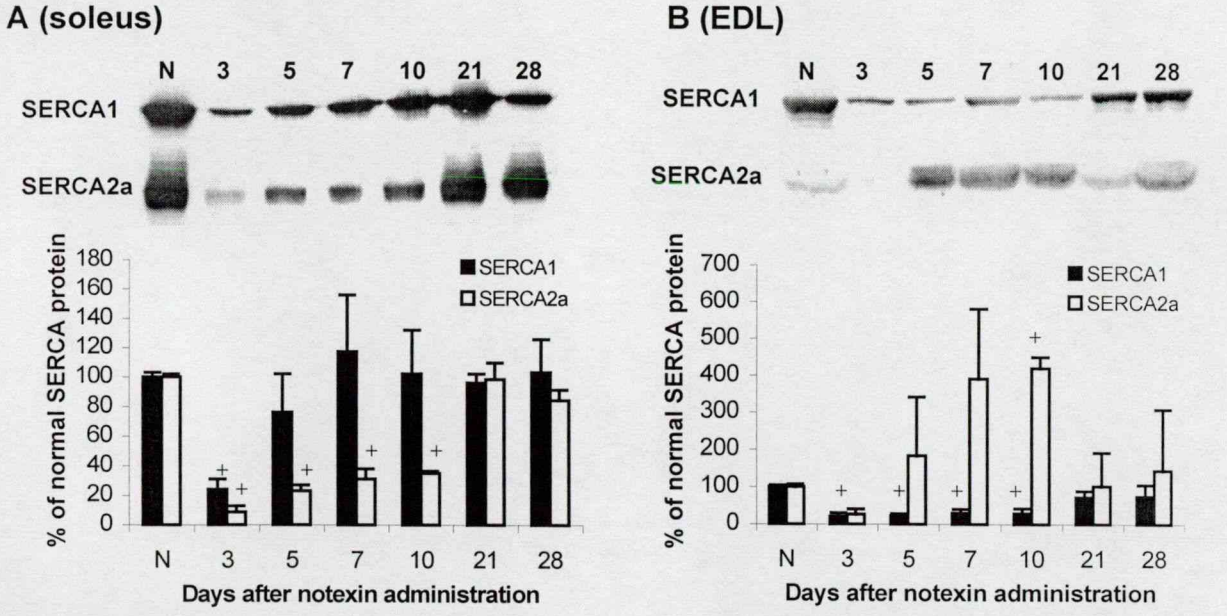


**Figure 10** Immunoblot quantification of SERCA1 and SERCA2a isoforms during the regeneration of soleus (**A**) and EDL (**B**) muscles. Inserts show representative immunoblots of the SERCA proteins (116 kD) stained with either A3 monoclonal antibody (SERCA1-specific) or R-15 antiserum (SERCA2a-specific). Equivalent parts of the membrane fractions were analyzed and the protein contents of the fractions were not significantly different in the stages of regeneration. N, normal muscles; 3-28 days after notexin administration. For each time-point 3 animals were analyzed. The values are normalized to that of the control and given as its percentage. Vertical bars show ±S.E.M., (+) means significant changes ($p<0.05$). In soleus (**A**) the level of SERCA1 significantly decreased on day 3, while the level of SERCA2a was lower on days 3,5,7,10. In EDL (**B**), the levels of SERCA1 significantly decreased on days 3-10, however, the level of SERCA2a decreased on day 3 and increased on day 10

*In EDL*, similar to soleus, both the fast SERCA1 and the slow SERCA2a protein levels were much below the control on day 3. However, SERCA1 level did not recover until day 10, then it increased to the control level on days 21-28 (Fig. 10B). The level of SERCA2a showed a transient increase on day 5 and 10, declined on day 21 and remained low until day 28 as in normal EDL (Fig. 10B).

### 6.5. Immunohistochemistry of SERCA1 and SERCA2a proteins in regenerating rat soleus and EDL muscles

In normal slow-twitch *soleus* muscles the slow-type SERCA2a protein is the predominant isoform, however, a number of fibers (about 25 %) express exclusively the fast-type SERCA1 protein, besides a few fibers which coexpress both isoforms (Fig.11A,B). During regeneration profound changes occured at the levels of the different isoforms as revealed by immunoblots, although at the end of regeneration muscles showed similar levels to those of controls. This change was difficult to follow at the levels of fiber-types because the presence of fast and slow fibers were uniform in each regenerating fibers and no fiber expressing only one of the SERCA isoforms was distinguishable until day 21. After 4 weeks of regeneration the number of SERCA1 expressing fibers dramatically declined, while nearly all fibers uniformly expressed SERCA2a (Fig. 11C,D). Thus, the fiber-type composition was not the same as in normal soleus.

Similar to soleus, the regeneration altered the fiber-type organisation of the fast-twitch *EDL* muscles, which are characterized by the predominant expression of the fast SERCA1 isoform (about 97 % of the fibers; Fig. 11E). Although the number of SERCA1 or SERCA2 expressing fibers did not change, the number of hybrid fibers expressing both isoforms increased after 28 days of regeneration compared to that of normal EDL (Fig. 11G,H). Another characteristic effect of regeneration was that while in normal EDL, the few fibers expressing SERCA2a appeared as single individual fibers interspersed among the SERCA1 positive ones (Fig. 11F), in regenerated EDL most of these fibers occurred in groups (Fig. 11H).

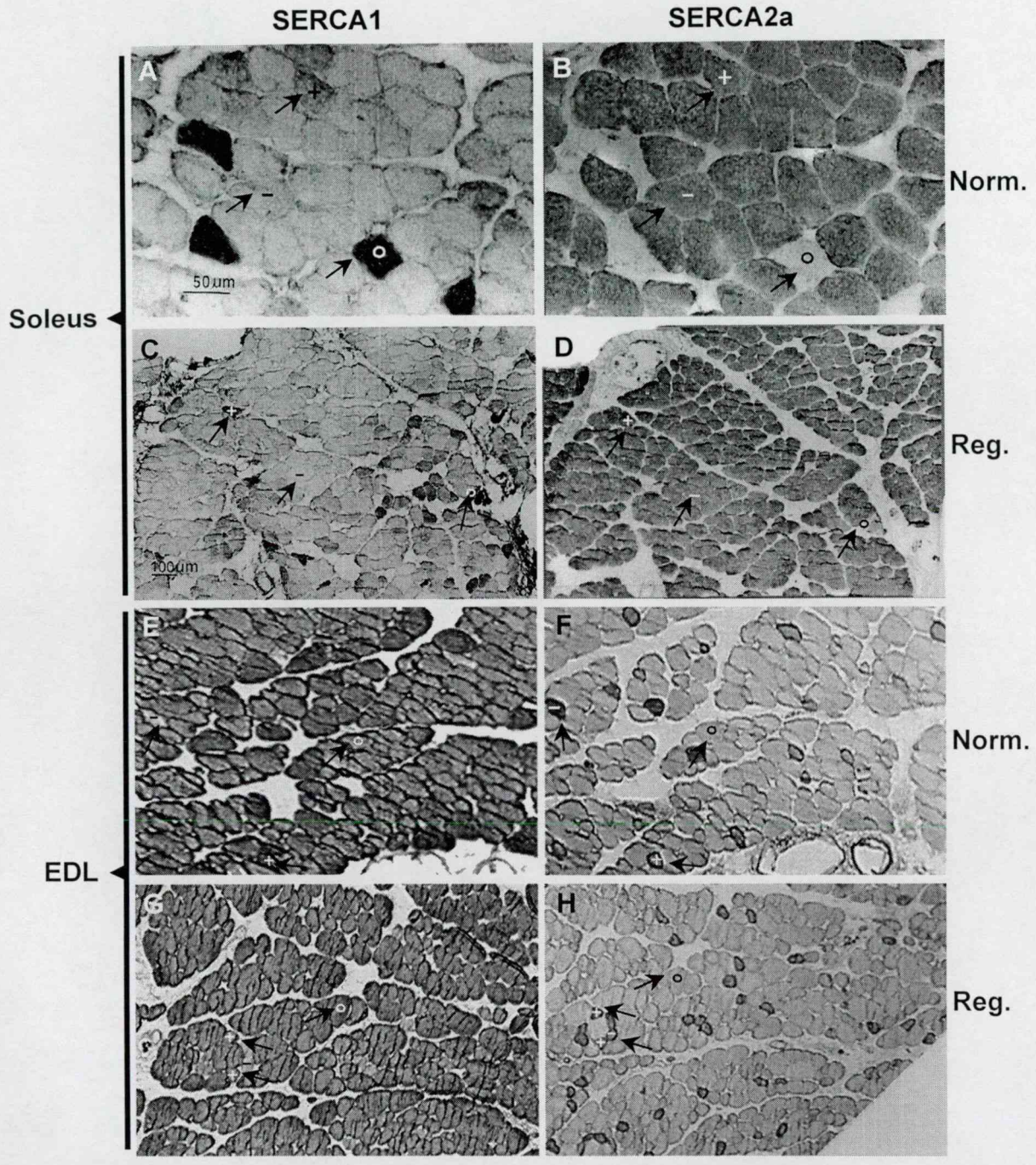


**Figure 11** Immunocytochemical detection of SERCA protein expression in fibers of normal and regenerated (on day 28) soleus and EDL muscles. Serial transversal sections stained either by the A3 monoclonal antibody (SERCA1-specific) or by the R-15 antiserum (SERCA2a-specific) are shown. The scale bars are 50 µm (A, B) or 100 µm (C-H). Arrows show the characteristic fibers labelled with the following symbols: (o) fibers stained for the fast SERCA1 only; (-) fibers stained for the slow SERCA2a only, (+) fibers stained for both SERCA1 and SERCA2a (hybrid fibers). In normal soleus most fibers were stained for the slow SERCA2a (**B**) but fibers expressing SERCA1 (**A**) or both isoforms (**A, B**) were also present. After 28 days of regeneration less fibers expressed SERCA1 (**C**) and almost every fiber was stained uniformly for SERCA2a (**D**). In normal EDL most fibers expressed the SERCA1 isoform (**E**) with some slow fibers (SERCA2a-positive) interspersed among them (**F**). In regenerated EDL, however, the slow SERCA2a-positive fibers formed groups (**H**) and the number of the hybrid fibers increased (**G, H**)

### 6.6. Transcript levels of myostatin in regenerating soleus and EDL muscles

A relatively low level of myostatin mRNA was detected in control, non-injected *soleus* muscles (Fig. 12A). However, on the first day after notexin administration it disappeared almost completely from the necrotized muscle ($p<0.01$) and reappeared only on the third day, but still at a significantly lower level. Starting from day 3, the expression level of myostatin increased until it reached a maximum two and a half-fold above the control value on day 7 ($p<0.05$; Fig. 12A). From this time on the myostatin transcript declined gradually and returned to normal level at the completion of regeneration.

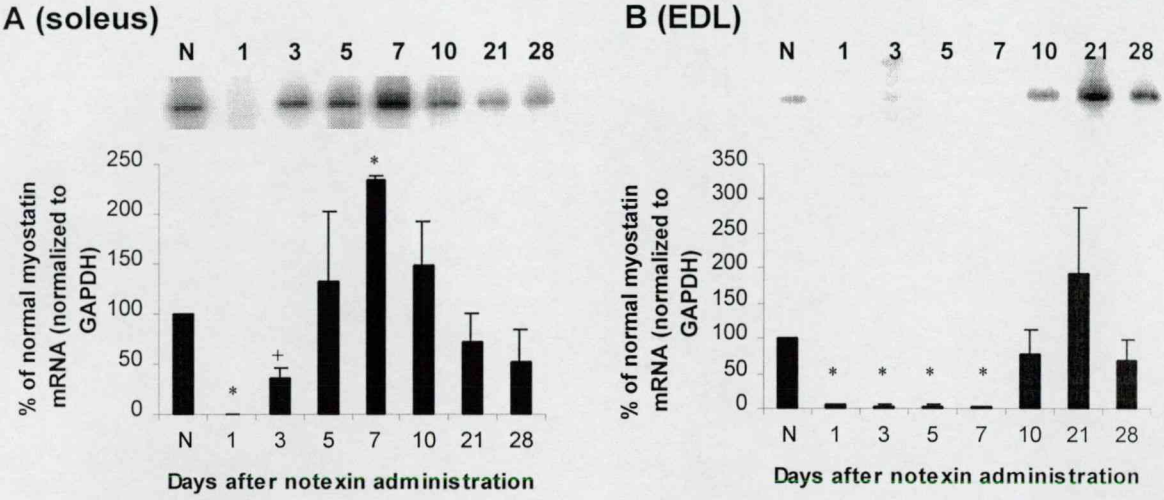


**Figure 12** Changes in myostatin mRNA levels normalized to those of GAPDH in regenerating rat soleus (**A**) and EDL (**B**) muscles. Inserts show phosphorimages of a typical RT-PCR amplification of a 340 bp fragment of the myostatin mRNA. The columns represent mean values obtained from three different animals and expressed as percentage of control muscles. Vertical bars indicate ±S.E.M., significant changes are labelled: (*) $p<0.01$ or (+) $p<0.05$. Labels are as follows: N, control muscles; 1-28 days after notexin injection. The myostatin transcript disappeared from both muscles at the time of necrosis (day 1 for soleus and days 1-3 for EDL). In soleus, its level started to increase on day 3 and reached a maximum on day 7, while in EDL the reappearance of the transcript was delayed

*EDL* muscles showed largely similar levels of myostatin mRNA upon induction of necrosis/regeneration, except that the reappearance of the transcript was delayed compared to soleus (Fig. 12B). On the first day myostatin mRNA level declined dramatically ($p<0.01$) and remained almost unmeasurable until day 10. Then it reappeared again and showed a tendency to increase to a maximum above the control on day 21, although this peak was statistically not significant because of the large scatter of the data. Finally myostatin mRNA reached roughly the same value as in control muscles on day 28.

## 7. Discussion

Morphological studies by another group (Harris *et al.* 1975, Harris and Johnson 1978) and our laboratory indicated that the dynamics of regeneration from notexin-induced necrosis of rat soleus and EDL muscles differ from each other. The accumulation of myoD and myogenin mRNAs was also different in normal soleus and EDL (Hughes *et al.* 1993, Voytik *et al.* 1993). In spite of these differences we found that the levels of mRNA for the early-acting myogenic factors (myoD and myf-5) showed quite similar time-courses in the two regenerating muscles. On the first day after notexin-treatment the myoD mRNA levels increased dramatically in both muscles, probably marking the activation of satellite cells and the beginning of regeneration (Fig. 13; Ludolph and Konieczny 1995, Grounds *et al.* 1992, Füchtbauer and Westphal 1992, Levinovitz *et al.* 1992, Smith *et al.* 1994, Schultz 1994, Cornelison and Wold 1997). The comparable time-course of changes in myoD mRNA was particularly remarkable because the complete necrosis occurred later in EDL than in soleus. The notexin-damaged fibers probably activated the satellite cells both in soleus and EDL with a similar time-course, but unknown factors may have delayed the necrosis and the regeneration in the fast-glycolytic fibers (Harris *et al.* 1975, Harris and Johnson 1978).

The mRNA level of myf-5, which is also known to be activated early in normal myogenesis (Olson and Klein 1994, Ludolph and Konieczny 1995), dropped to levels below the detection limit on the first day (Fig. 13). This suggests that myf-5 is regulated differently from myoD in the regenerating muscle. It is well-documented that the transcript level of myf-5 increased in mice lacking a functional myoD gene (Rudnicki *et al.* 1993). It might be that the two transcription factors are regulated in an opposite way in the early regeneration and at day 28, similar to what is seen in normal development. The change of myoD and myf-5 mRNA levels at the beginning of regeneration suggests that satellite cells respond in a similar time-course in both types of muscles in spite of their different resistance to the toxin.

The transcript levels of the late-acting myogenic regulatory factors (myogenin and MRF4) changed in a different way in the regenerating muscles; they disappeared from soleus on day 1 but remained normal in EDL ($p<0.05$). This again probably reflects the difference in the time-course of necrosis of the two muscles *i.e.* the mRNAs of the late MRFs disappear together with the muscle fibers in soleus, while they persist in the still surviving fibers in

EDL. On day 3, when myoblasts are already activated in soleus, the mRNA levels of both myogenin and MRF4 increased. However, in EDL, only the transcript of myogenin increased, probably reflecting the gradual replacement of the necrotizing fibers by the new myoblasts and myotubes, whereas the mRNA of MRF4 was maintained at the normal level.

As discussed earlier, the re-innervation is an essential step for the normal progression of muscle regeneration (Sesodia and Cullen 1991). In accordance with Grubb *et al.* (1991) we also observed that the formation of neuromuscular endplates takes place between 5-7 days in soleus and 7-10 days in EDL after notexin-treatment. The transcription of myoD and myogenin is induced by denervation and repressed by neural stimulation (Eftimie *et al.* 1991, Adams *et al.* 1995, Hughes *et al.* 1993). Therefore we might expect a repression of myoD and myogenin mRNA levels after the new muscle fibers have become re-innervated. In line with this hypothesis, the levels of myoD and myogenin mRNAs declined in the later stages of regeneration for both types of muscle. A similar change was observed for myf-5, but not for MRF4 which remained relatively constant during the second half of regeneration. After 4 weeks the mRNA levels for each of the myogenic factors returned to nearly normal levels. In conclusion, the only important difference in MRF mRNA expressions of EDL and of soleus muscles was that myogenin and MRF4 mRNAs did not disappear from the EDL on the first day. This may be explained by the persistence of the differentiated muscle fibers at that time in EDL, but not in soleus. Apart from this, the changes in the mRNA levels of MRFs were essentially similar in both muscles during the regeneration process. This suggests that the myoblasts activated during the regeneration of slow and fast muscles express myoD, myf-5 and myogenin in a similar time-dependent pattern.

Generally it is accepted that the order of activation of MRFs in the early muscle development is: myf-5/myoD, myogenin and MRF4 (Olson and Klein 1994, Ludolph and Konieczny 1995, Schiaffino *et al.* 1996). We found that the increase of their mRNA levels followed a similar pattern (for both types of muscle) with an earlier uprise of myoD than that of myf-5 during muscle regeneration (Fig. 13). The MRFs also precede the expression of a number of muscle-specific genes in muscle development (Olson and Klein 1994, Ludolph and Konieczny 1995, Schiaffino *et al.* 1996). It was reported that the mRNA of myogenin increases before those of the sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase isoforms in developing muscles (Arai *et al.* 1992). We also found that the uprise of each MRF mRNA

preceded that of the SERCA forms during regeneration of soleus and EDL muscles (Fig. 13). In particular, on day 1 in soleus and day 3 in EDL, the SERCA transcripts disappeared from the muscles and their levels remained low until day 5 (Zador *et al.* 1996). This also confirms that the necrosis of the fibers in EDL is slower than in soleus. At first, the neonatal SERCA1b mRNA increased on day 5 suggesting that it is an early molecular marker of the myotube formation in both muscles, but apparently it occurs later than that of the MRFs (Fig. 13). A similar increase of the neonatal SERCA1b isoform was also observed in normal development (Brandl *et al.* 1986, Brandl *et al.* 1987). This shows that the initial pattern of activation of the muscle-specific SERCA isoforms in notexin-induced regeneration is similar to that in normal development. After the first peak of the SERCA1b mRNA level, large increases of the transcript levels of SERCA1a and SERCA2a were observed around day 7 in both muscles (Zador *et al.* 1996), exactly when re-innervation of the new fibers occured. Thus, re-innervation might have induced a switch from the neonatal SERCA1b to the adult fast SERCA1a, and a few days later from SERCA1 to the slow-type SERCA2a mRNA (Fig. 13). In soleus muscle (Zador *et al.* 1996), after 3 weeks of regeneration the SERCA1a isoform was replaced by the slow SERCA2a and the SERCA2a/SERCA1a ratio reached a situation (ratio:3.9) found in normal adult soleus (Wu and Lytton 1993). However, this latter increase of the slow SERCA isoform in the regenerating EDL was negligible and transient, similar to the changes found in ontogenesis (Brandl *et al.* 1987). This suggests that during the later stages of re-differentiation the fast SERCA1 gene expression is suppressed whereas the slow SERCA2 expression is stimulated in the slow-twitch soleus muscles. A similar switch between SERCA1 and SERCA2 isoform was observed after chronic low-frequency stimulation of fast-twitch muscle (Briggs at al. 1990, Leberer and Pette 1989). Chronic mechanical overload of rat fast-twitch muscle increased the expression of SERCA2 (mRNA and protein) two-fold, while the SERCA1 level declined (Kandarian *et al.* 1994). In contrast, denervation of the slow-twitch muscle of rats and rabbits was reported to lower the levels of the slow SERCA2 mRNA and protein without affecting the fast SERCA1 expression (Salvatori *et al.* 1988, Schulte *et al.* 1994). Remarkably similar changes in expression patterns as we observed for the SERCA pumps in soleus were also reported for the myosin isoforms. Here too, from the multiple myosin isoforms *i.e.* embryonic, neonatal, fast and slow isoforms, only the slow one becomes exclusively expressed in the final stage of the notexin-induced

regeneration of the innervated soleus (Whalen *et al.* 1990). It is therefore likely that while regeneration process could elicit the expression of the SERCA transcripts, the functional innervation highly influences the proper ratio of their isoforms.

When we studied the SERCA protein expression during the regeneration it was not possible to deduce the molar ratio of SERCA1 and SERCA2 proteins from the immunoreactions as we did it for the mRNA levels, because different antibodies were used. Moreover, the monoclonal antibody A3 does not discriminate between neonatal and adult variants of SERCA1. The only difference between these two isoforms is confined to their extreme C-termini where terminal G994 of the adult form is replaced by an extended tail 994-DPEDERRK-1001 in the neonatal form (Wu and Lytton 1993). This might explain why a specific antibody for SERCA1a is not available. This precluded the demonstration of a putative increase in the level of the neonatal protein, which might accompany the corresponding increase of its mRNA. Assumed that the first increase in the SERCA1 protein level must be ascribed to the neonatal SERCA1b, the changes of SERCA protein levels followed those of SERCA transcripts during the regeneration of both muscles. Therefore, the SERCA1 and SERCA2 expressions are likely to be mainly pretranslationally controlled.

Although the normal levels of SERCA proteins were restored, in the regenerated soleus more fibers expressed the slow SERCA2a isoform meanwhile less fibers showed the fast SERCA1 positivity than in normal muscles. This finding indicates that the regenerated soleus, even after a prolonged period of time, at least in this respect, does not completely return to its condition prior to necrosis. As mentioned earlier, the contractile activity was also reported to change in regenerated soleus muscles (Louboutin *et al.* 1995). Though in our experiments the affected leg appeared to be normal after 4 weeks of regeneration, it was not tested for contractility. Thus, the a correlation between the transition of regenerated soleus muscle towards a more slow-type of muscle and the possible changes in contractile activity can be studied in later experiments. After a similar period of recovery from notexin-induced necrosis, an increased ratio of slow/fast myosin containing fibers has also been described (Whalen *et al.* 1990). In our experiments, the SERCA1 expressing fibers were found to co-express the slow and fast SERCA isoforms. This is again similar to what was observed for myosin isoforms, where co-expression of fast and slow isoforms was also shown (Whalen *et al.* 1990). The parallel changes in expression of SERCA and myosin isoforms may reflect that contraction

and relaxation are tightly coupled at the molecular level and are possibly influenced by common regulatory mechanisms. In rats, each fiber receives its own innervation from individual motor units (Torella *et al.* 1993) and chronic low-frequency stimulation studies led to the conclusion that different stimulation patterns are required for the induction of SERCA2a or SERCA1 (Leberer *et al.* 1989), similar to that of slow and fast myosin isoforms (Thomas and Ranatunga 1993). Co-expression of slow and fast type of SERCA pumps both in normal and regenerated soleus suggests co-induction of SERCA1 and SERCA2a. Co-expression of low levels of SERCA2a with the dominant SERCA1 pump has also been reported in normal fast-twitch muscles (Wu and Lytton 1993). Moreover, after chronic stimulation of fast-twitch muscles the number of the hybrid fibers co-expressing both the slow and fast SERCA isoforms increased. In accordance with this we found that after 28 days of regeneration fast-twitch EDL muscles contained more hybrid fibers than the normal ones suggesting that the slow nerve-endings are slightly more efficient at re-innervating the regenerated fibres than the fast ones. A competition for re-innervation of regenerating fibers is also supported by the close spatial association of the slow SERCA2a expressing (type I) fibers in regenerated EDL muscles.

In conclusion, during notexin-induced regeneration of soleus and EDL muscles the transcripts and proteins of SERCA show a similar time-dependent expression pattern which recapitulates those observed during normal muscle development (Fig. 13; Brandl *et al.* 1987). However, the exact mechanism of the regulation of the expression of SERCA isoforms in regeneration is not known. As mentioned earlier the increase of the level of myogenic regulatory factors (MRFs) precedes the expression of SERCAs. However, in the later stages of regeneration no differences were observed between the fast-twitch EDL and the slow-twitch soleus in respect to their MRF expression. This suggests that the SERCA1 and SERCA2 levels are not directly controlled by these transcription factors.

As revealed by several authors (see introduction), the muscle regeneration after notexin-induced necrosis, at least in morphological sense, seemes to be almost complete. Both the diameter of the regenerated muscle fibers and the size of the whole muscle were similar to uninjured control muscles. Hence we expected that myostatin, which is supposed to be an important regulator of skelatal muscle growth, would show characteristic time-dependent changes. Indeed, the changes in myostatin mRNA levels were largely similar in regenerating

soleus and EDL muscles, except that the uprise of myostatin occurred some days earlier in soleus than in EDL. This could be partially explained by a difference in the dynamics of regeneration. At the time of necrosis, the myostatin mRNA, similar to those of α-skeletal muscle actin (Mendler *et al.* 1998 [I.]) and SERCAs, completely disappeared from both muscles. In soleus, cell proliferation showed a maximum on day 3 (based on BrdU incorporation), followed by fusion and formation of new myotubes and myofibers from day 5 onwards, while in EDL these phenomena occured about two days later (see introduction). The level of myostatin mRNA began to increase around this time, *i.e.* after the fusion of myoblasts to myotubes (Fig. 13), consequently, it reached the maximum when myotubes and myofibers were actively growing and differentiating then decreased again to normal level when both types of regenerating muscles regained the size and morphology similar to controls. Therefore, the different time-course of the myostatin expression in soleus compared to that in EDL seems to be the consequence of the different dynamics of regeneration, rather than that of the difference in myoblast composition as we discussed it earlier for the mRNA levels of myogenic regulatory factors. The expression pattern observed in the course of regeneration, *i.e.* the initial up-regulation of myostatin after myoblast fusion followed later on by the gradual decrease to normal level, shows similarities to that found in porcine skeletal muscles during the normal development (Ji *et al.* 1998). Based on these data, one could conclude that myocyte fusion might be important for upregulating the myostatin expression *in vivo*, although *in vitro* fusion-defective myogenic cells are also able to upregulate the myostatin transcript level (data not shown). It is worth considering that changes of myostatin mRNA levels in regenerating muscles occur in parallel with those of insulin-like growth factor (IGF-I) receptor and of IGF-II, which were correlating with myotube formation (Levinovitz *et al.* 1992, Marsh *et al.* 1997). In contrast, other growth factors: FGF, TGF-β1 and IGF-I were shown to play a role in myotube formation of primary myoblasts (Lefaucheur and Sebille 1995), although their mRNA levels were not changing at that time. Despite these similarities, there is no evidence for a regulating factor that might control myostatin expression directly during muscle regeneration. Since the essential role of re-innervation is well-known in many aspects of regeneration (Sesodia and Cullen 1991, Sesodia *et al.* 1994, Voytik *et al.* 1993), one might expect that the formation of new endplates could somehow modulate the myostatin mRNA levels in the regeneration process, as well. In our *in vivo* experiments the increase of

myostatin transcript levels coincided with the time of re-innervation both in EDL and soleus muscles and it decreased when the new endplates were established. If we presumed that myostatin protein expression follows a similar pattern to that of its mRNA, we could conclude that myostatin might influence the late phase of the regeneration characterized by differentiation rather than active cell proliferation. However, we have evidence (data not shown) that myostatin protein did not follow the level of its mRNA in the first part of regeneration. This suggests that myostatin may have an origin outside the damaged muscle. It is only after day 5 in soleus and day 7 in EDL, that the transcript levels seem to be high enough to support translation in the regenerating muscle itself (Fig. 13). Based on this data it looks like that myostatin is present during the whole process of muscle necrosis and the subsequent regeneration possibly influencing both cell proliferation and differentiation. Thus, myostatin is not only a regulator of prenatal muscle development (Slack 1997, Lee and McPherron 1999) but seems to be a good candidate for the same function in regeneration.

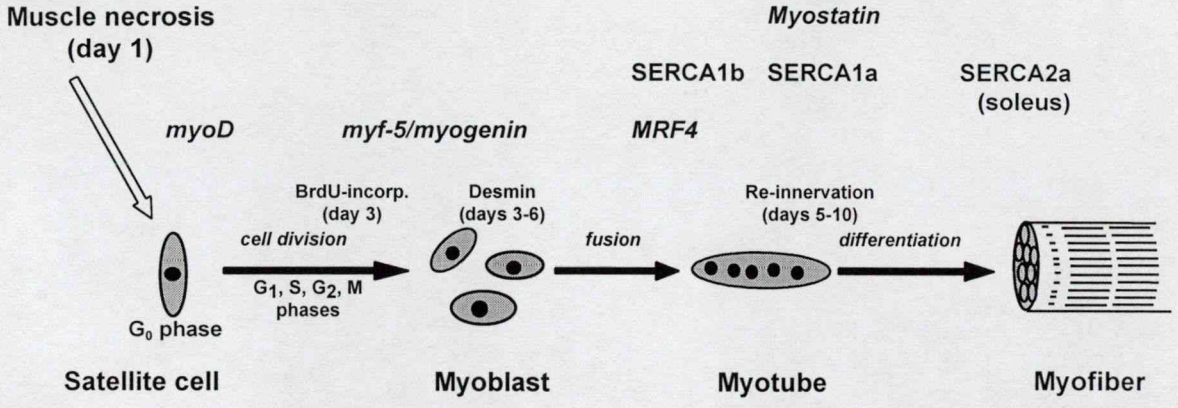


**Figure 13** Schematic representation of the time-dependent expression of the investigated markers in notexin-induced regeneration. Scheme and labels are the same as in Fig. 5. The names of MRFs, SERCAs and myostatin show the first appearance/increase of their mRNAs relative to the characteristic morphological events. Soleus and EDL showed differences in SERCA expression, that is, in the slow soleus the SERCA2a dominated the regenerated muscle, while in the fast EDL SERCA1a remained the dominant isoform (SERCA 2a only increased transiently at the time of re-innervation which is not shown on the scheme). The SERCA proteins followed the changes of their mRNAs with some delay

## 8. Conclusions

1. We have established and characterized the morphological and biochemical aspects of the *in vivo* regeneration of a slow- and a fast-type skeletal muscle (m. soleus and m. EDL, respectively) following notexin-induced necrosis. The fast fibers of EDL showed higher resistance to the toxin resulting in a delayed and less synchronous degeneration/regeneration process than in the soleus.
2. The order of activation of the muscle-specific transcription factors (myoD, myf-5, myogenin, MRF4) and of the different isoforms of SERCAs were similar to that found in muscle development suggesting that the expressions of muscle-specific genes during regeneration largely recapitulate those events of myogenesis. The expression pattern of MRFs showed little, if any, differences in the different muscle-types. Similarly, both the regenerating soleus and the EDL muscles expressed the neonatal SERCA1b isoform, which therefore seems to be a reliable marker of regeneration.
3. Re-innervation profoundly changed the expression of the MRFs and the SERCA isoforms in accordance with the role of innervation in ontogenesis. Re-innervation seemed to be essential to form the original fast or slow character of the regenerated muscle. For some reasons, however, the slow nerve-endings were likely to be more effective in the re-innervation of new fibers and this changed the fiber-type composition and the expression pattern of SERCAs.
4. We found that myostatin mRNA was present in regenerating muscle and reached the maximum at the time of myotube growth and differentiation. This implies that myostatin, which inhibits muscle growth during development, may also have a role in the regenerating muscle.

## 9. Acknowledgements

This work was supported by the 3-year doctoral fellowship of the Hungarian Ministry of Education; the grant MKM 273 from the Hungarian Ministry of Education; the grant OTKA T006277 from the Hungarian Scientific Research Found; the grant B-10/96 from OMFB and the grant BIL 96/15 Ministerie Vlaamse Gemeenschap, Belgium.

I greatly acknowledge to Professor László Dux for inviting me to join to the department as a student research worker and for providing me the possibility to resume the scientific work as a PhD student.

I am especially thankful to my promoter Ernô Zádor, who was supporting me without sparing his time and energy over the past few years. His guidance and encouragement was indispensible all the time.

I am greatful to Professor Frank Wuytack, who gave me the opportunity to work in his laboratory and showed me the way how to live and work abroad.

I am thankful to Gábor Rácz for his suggestions, advice and help in preparing the figures and editing the text of the Thesis.

Many thanks to Balásházy Istvánné for the skillful technical assistance.

Without my husband and my family I have not been able to face all the challenges I had to. I am especially thankful to them.

## 10. References


1. Adams L, Carlson BM, Henderson L and Goldman D (1995) Adaptation of nicotinic acetylcholine receptor, myogenin, and MRF4 gene expression to long-term muscle denervation. *J Cell Biol* **131**:1341-1349
2. Arai M, Otsu K, McLennan DH and Periasamy M (1992) Regulation of sarcoplasmic reticulum gene expression during cardiac and skeletal muscle development. *Am J Physiol* **262**:C614-620
3. Bischoff R (1993) The satellite cell and muscle regeneration. in: *Myology. Basic and Clinical.* vol.1:pp97-118 (Engel AG and Franzini-Armstrong C, eds) 2nd ed. McGraw-Hill, Inc.
4. Bobe R, Bredoux R, Wuytack F, Quarck R, Kovács T, Papp B, Corvazier E, Magnier C and Enouf J (1994) The rat platelet 97-kDa $Ca^{2+}$-ATPase isoform is the sarcoplasmic reticulum $Ca^{2+}$-ATPase 3 protein. *J Biol Chem* **269**:1417-1424
5. Brandl CJ, Green NM, Korczak B and McLennan DH (1986) Two $Ca^{2+}$-ATPase genes: homologies and mechanical implications of deduced amino acid sequences. *Cell* **44**:597-607
6. Brandl JB, Deleon S, Martin DR and McLennan DH (1987) Adult forms of the $Ca^{2+}$-ATPase of sarcoplasmic reticulum: expression in developing skeletal muscle. *J Biol Chem* **262**:3768-3774
7. Briggs FN, Lee KF, Fehér JJ, Wechsler AS, Ohlendick K and Campbell K (1990) Ca-ATPase isozyme expression in sarcoplasmic reticulum is altered by chronic stimulation of skeletal muscle. *FEBS Lett* **259**:269-272
8. Burk SE, Lytton J, McLennan DH and Shull GE (1989) cDNA cloning, functional expression, and mRNA tissue-distribution of a third organellar $Ca^{2+}$ pump. *J Biol Chem* **264**:18561-18568
9. Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid-guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156-159
10. Cornelison DDW and Wold BJ (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol* **191**:270-283

11. Davis CE, Harris JB, Nicholson LV (1991) Myosin isoform transitions and physiological properties of regenerated and re-innervated soleus muscles of the rat. *Neuromusc Disord* **1**:411-21

12. De Jaegere S, Wuytack F, De Smedt H,Van Den Bosch L and Casteels R (1993) Alternative processing of the gene transcripts encoding a plasma-membrane and a sarco/endoplasmic reticulum $Ca^{2+}$ pump during differentiation of $BC_3H1$ muscle cells. *Biochim Biophys Acta* **1173**:188-194

13. De la Bastie D, Wisnewsky C, Schwartz K and Lompré A-M (1988) ($Ca^{2+}$+$Mg^{2+}$) dependent ATPase mRNA from smooth muscle sarcoplasmic reticulum differs from that in cardiac and fast skeletal muscles. *FEBS Lett* **229**:45-48

14. Dode L (1998) Identification and characterization of the human sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 3 (SERCA3) gene. Ph.D. thesis. Acta Biomedica Lovaniensia 178, Leuven University Press.

15. Dode L, De Greef C, Mountian I, Attard M, Town MM, Casteels R and Wuytack F (1998) Structure of the human sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 3 gene. *J Biol Chem* **273**:13982-13994

16. Dux L (1993) Muscle Relaxation and Sarcoplasmic Function in Different Muscle Types. *Rev Physiol Biochem Pharmacol* **122**:69-147

17. Dux L, Cooper C, Sewry CA and Dubowitz V (1993) *Notechis scutatus* venom increases the yield of proliferating muscle cells from biopsies of normal and dystrophic canine muscle-a possible source for myoblast transfer studies. *Neuromusc Disord* **3**: 23-29

18. Düsterhöft S and Pette D (1993) Satellite cells from slow rat muscle express slow myosin under appropriate culture conditions. *Differentiation* **53**:25-33

19. Düsterhöft S, Yablonka-Reuveni Z and Pette D (1990) Characterization of myosin isoforms in satellite cell cultures from adult rat diaphragm, soleus and tibialis anterior muscles. *Differentiation* **45**:185-191

20. Eftimie R, Brenner HR and Buonanno A (1991) Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. *Proc Natl Acad Sci* **88**:1349-1353

21. Eggermont JA, Wuytack F, Verbist J and Casteels R (1990) Expression of endoplasmic reticulum $Ca^{2+}$-pump isoforms and of phospholamban in pig smooth-muscle tissues. *Biochem J* **318**:689-699

22. Eggermont JA, Wuytack F and Casteels R (1990) Characterization of the mRNAs encoding the gene 2 sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$-pump in pig smooth muscle. *Biochem J* **266**:901-907

23. Feldman JL and Stockdale FE (1991) Skeletal muscle satellite cell diversity: satellite cells from fibers of different type in cell culture. *Dev Biol* **143**:320-334

24. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G and Mavilio F (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279**:1528-1530

25. Florini JR, Ewton DZ and Magri KA (1991) Hormones, growth factors, and myogenic differentiation. *Ann Rev Physiol* **53**:201-216

26. Foster RF, Thompson JM and Kaufmann SJ (1987) A laminin substrate promotes myogenesis in rat skeletal muscle cultures: analysis of replication and development using anti-desmin and anti-BrdUrd monoclonal antibodies. *Dev Biol* **122**:11-20

27. Füchtbauer E-M and Westphal H (1992) MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Dev Dynamics* **193**:34-39

28. Gerber AN, Klesert TR, Bergstrom DA and Tapscott SJ (1997) Two domains of MyoD mediate transcriptional activation of genes in repressive chromatin: a mechanism for lineage determination in myogenesis. *Genes Dev* **11**:436-450

29. Gibson AJ, Karasinski J, Relvas J, Moss J, Sherratt TG, Strong PN and Watt DJ (1995) Dermal fibroblasts convert to a myogenic lineage in mdx mouse muscle. *J Cell Sci* **108**: 207-214

30. Gibson MC and Schultz E (1982) The distribution of satellite cells and their relationship to specific fibre types in the soleus and extensor digitorum longus muscles. *Anat Rec* **202**:329-337

31. Grobet L, Royo Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R and Georges M (1997) A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat Genet* **17**: 71-74

32. Grounds MD, Garrett KL, Lai MC, Wright WE and Beilharz MW (1992) Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res* **267**:99-104

33. Grubb BD, Harris JB and Schofield IS (1991) Neuromuscular transmission at newly formed neuromuscular junctions in the regenerating soleus muscle of the rat. *J Physiol* **441**:405-421

34. Gunteski-Hamblin AM, Greeb J and Shull GE (1988) A novel $Ca^{2+}$ pump expressed in brain, kidney, and stomach is encoded by an alternative transcript of the slow-twitch muscle sarcoplasmic reticulum $Ca^{2+}$-ATPase gene. Identification of cDNAs encoding $Ca^{2+}$ and other cation-transporting ATPases using an oligonucleotide probe derived from the ATP-binding site. *J Biol Chem* **263**:15032-40

35. Harris JB, Johnson MA and Karlsson E (1975) Pathological responses of rat skeletal muscle for a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin Exp Pharmacol Physiol* **2**:383-404

36. Harris JB and Johnson MA (1978) Further observations on the pathological responses of rat skeletal muscle to toxins isolated from the venom of the australian tiger snake. *Clin Exp Pharmacol Physiol* **5**:587-600

37. Harris JB, Karlsson E and Thesleff S (1973) Effects of an isolated toxin from Australian tiger snake (*Notechis scutatus scutatus*) venom at the mammalian neuromuscular junction. *Br J Pharmacol* **47**:141-146

38. Hughes SM, Taylor JM, Tapscott SJ, Gurley CM, Carter WJ and Peterson CA (1993) Selective accumulation of myoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* **118**:1137-1147

39. Ji S, Losinski RL, Cornelius SG, Frank GR, Willis GM, Gerrard DE, Depreux FFS, Spurlock ME (1998) Myostatin expression in porcine tissues: tissue specificity and developmental and postnatal regulation. *Am J Physiol* **275**:R1265-1273

40. Kambadur R, Sharma M, Smith TPL and Bass JJ (1997) Mutations in *myostatin* (*GDF-8*) in Double-Muscled Belgian Blue and Piedmontese Cattle. *Genome Res* **7**:910-916

41. Kandarian SC, Peters DG, Taylor JA and Williams JH (1994) Skeletal muscle overload upregulates the sarcoplasmic reticulum slow calcium pump gene. *Am J Physiol* **266**:C1190-1197

42. Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttmann R, Holland P, Koch PA, Shoubridge E, Spence D, Vanasse M, et al (1993) Myoblast transfer in Duchenne muscular dystrophy. *Ann Neurol* **34**:8-17

43. Korczak B, Zarain-Herzberg A, Brandl CJ, Ingles CJ, Green MN and McLennan DH (1988) Structure of the rabbit fast-twitch skeletal muscle $Ca^{2+}$ ATPase gene. *J Biol Chem* **263**:4813-4819

44. Lagord C, Soulet L, Bonavoud S, Bassaglia Y, Rey C, Barlovatz-Meimon G, Gautron J and Martelly I (1998) Differential myogenicity of satellite cells isolated from extensor digitorum longus (EDL) and soleus rat muscles revealed *in vitro*. *Cell Tissue Res* **291**:455-468

45. Leberer E and Pette D (1986) Immunochemical quantitation of sarcoplasmic reticulum $Ca^{2+}$-ATPase, of calsequestrin and of parvalbumin in rabbit skeletal muscles of defined fiber-composition. *Eur J Biochem* **156**:489-496

46. Lee SJ and McPherron AC (1999) Myostatin and the control of skeletal muscle mass. *Curr Opin Genet Dev* **9**:604-607

47. Lefaucheur JP and Sebille A (1995a) Muscle regeneration following injury can be modified in vivo by immune neutralization of basic fibroblast growth factor, transforming growth factor β1 or insulin-like growth factor I. *J Neuroimmunol* **57**:85-91

48. Lefaucheur JP and Sebille A (1995b) The cellular events of injured muscle regeneration depend on the nature of the injury. *Neuromusc Disorders* **5**:501-509

49. Levine JH, Moses HL, Gold LI and Nanney LB (1993) Spatial and temporal patterns of immunoreactive Transforming Growth Factor β1, β2, and β3 during excisional wound repair. *Am J Pathol* **143**:368-380

50. Levinovitz A, Jennische E, Oldfors A, Edwall D and Norstedt G (1992) Activation of insulin-like growth factor II expression during skeletal muscle regeneration in the rat: correlation with myotube formation. *Mol Endocrin* **6**:1227-1234

51. Louboutin J-P, Fichter-Gagnepain V and Noireaud J (1995) Comparison of contractile properties between developing and regenerating soleus muscle: influence of external calcium concentration upon contractility. *Muscle Nerve* **18**:1292-1299

52. Lowe DA, Lund T and Alway SE (1998) Hypertrophy-stimulated myogenic regulatory factor mRNA increases are attenuated in fast muscle of aged quails. *Am J Physiol* **275**: C155-166

53. Ludolph DC and Konieczny SF (1995) Transcription factor families: muscling in on the myogenic program. *FASEB J* **9**:1595-1604

54. Maione R and Amati P (1997) Interdependence between muscle differentiation and cell-cycle control. *Biochim Biophys Acta* **1322**:M19-M30

55. Marsh DR, Criswell DS, Hamilton MT, Booth FW (1997) Association of insulin-like growth factor mRNA expressions with muscle regeneration in young, adult, and old rats. *Am J Physiol* **273**:R353-358

56. Mauro A (1961) Satellite cells of skeletal muscle fibers. *J Biophys Biochem Cytol* **9**:493-495

57. McLennan DH, Toyofuku T and Lytton J (1992) Structure-function relationship in sarcoplasmic or endoplasmic reticulum type $Ca^{2+}$ pumps. *Ann NY Acad Sci* **671**:1-10

58. McLennan IS (1994) Neurogenic and myogenic regulation of skeletal muscle formation: a critical re-evaluation. *Progress in Neurobiology* **44**:119-140

59. McPherron AC, Lawler AM and Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* **387**:83-90

60. McPherron AC and Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci* **94**:12457-12461

61. Miller JB, Everitt EA, Smith TH, Block NE and Dominov JA (1993) Cellular and Molecular Diversity in Skeletal Muscle Development: News from in vitro and in vivo. *BioEssays* **15**:191-195

62. Moss FP and Leblond CP (1971) Satellite cells as a source of nuclei in muscles of growing rats. *Anat Rec* **170**:421-436

63. Naya F and Olson E (1999) MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. *Curr Op Cell Biol* **11**:683-688

64. Olson EN and Klein WH (1994) bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes and Devel* **8**:1-8

65. Olson EN, Arnold HH, Rigby PW and Wold BJ (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell* **85**:1-4

66. Partridge TA (1991) Myoblast transfer: a possible therapy for inherited myopathies? *Muscle and Nerve* **14**:197-212

67. Philliphs GD, Lu D, Mitashov VI and Carlson BM (1987) Survival of myogenic cells in freely grafted rat rectus femoris and extensor digitorum longus muscles. *Am J Anat* **180**:365-372

68. Qu Z, Balkir L, van Deutekom JC, Robbins PD, Pruchnic R and Huard J (1998) Development of approaches to improve cell survival in myoblast transfer therapy. *J Cell Biol* **142**:1257-67

69. Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold H-H and Jaenisch R (1993) MyoD and myf-5 is required for the formation of skeletal muscle. *Cell* **75**:1351-1359

70. Saito Y and Nonaka I (1994) Initiation of satellite cell replication in bupivacaine-induced myonecrosis. *Acta Neuropathol* **88**:252-257

71. Salvatori S, Damiani E, Zorzato F, Volpe P, Pierobon S, Quaglino Jr D, Salviati G and Margreth A (1988) Denervation-induced proliferative changes of triads in rabbit skeletal muscle. *Muscle Nerve* **11**:1246-1259

72. Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning. A laboratory manual (Cold Spring Harbor Laboratory Press)

73. Schiaffino S, Gorsa L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K and Lomo T (1989) Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Mot* **10:**197-205

74. Schulte L, Peters D, Taylor J, Navarro J and Kandarian S (1994) Sarcoplasmic reticulum $Ca^{2+}$-pump expression in denervated skeletal muscle. *Am J Physiol* **267**:C617-C622

75. Schultz E (1996) Satellite cell proliferative compartments in growing skeletal muscles. *Dev Biol* **175**:84-94

76. Schultz E and McCormick KM (1994) Skeletal muscle satellite cells. *Rev Physiol Biochem Pharmacol* **123**:214-257

77. Sesodia S and Cullen MJ (1991) The effect of denervation on the morphology of regenerating rat soleus muscles. *Acta Neuropathol* **82**:21-32

78. Sesodia S, Choksi RM and Nemeth PM (1994) Nerve-dependent recovery of metabolic pathways in regenerating soleus muscles. *J Muscle Res Cell Motil* **15**:573-81

79. Slack JMW (1997) Growth control: Action mouse. Current Biol 7:R467-469

80. Smith CK, Janney MJ and Allen RE (1994) Temporal expression of myogenic regulatory genes during activation, proliferation and differentiation of rat skeletal muscle satellite cells. *J Cell Physiol* **159**:379-385

81. Smith TH, Block NE, Rhodes SJ, Konieczny SF and Miller JB (1993) A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. *Development* **117**:1125-1133

82. Smith TPL, Lopez-Corrales NL, Kappes SM and Sonstegard TS (1997) Myostatin maps to the interval containing the bovine *mh* locus. *Mamm Genome* **8**:742-744

83. Thomas PE and Ranatunga KW (1993) Factors effecting muscle fiber transformation in cross-reinnervated muscle. *Muscle Nerve* **16**:193-199

84. Torella JR, Fouces V, Palomeque J and Viscor G (1993) A combined myosin ATPase and acetylcholinesterase histochemical method for the demonstration of fiber-types and their innervation pattern in skeletal muscle. *Histochemistry* **99**:369-372

85. Yablonka-Reuveni Z and Rivera AJ (1994) Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibres. *Dev Biol* **164**:588-603

86. Yun K and Wold B (1996) Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr Opin Cell Biol* **8**:877-889

87. Vaidya TB, Rhodes SJ, Moore JL, Sharman DA, Konieczny SF and Taparowsky EJ (1992) Isolation and structural analysis of the rat myoD gene. *Gene* **116**:223-230

88. Van Den Bosch L, Eggermont J, De Smedt H, Mertens L, Wuytack F and Casteels R (1994) Regulation of splicing is responsible for the expression of the muscle-specific 2a isoform of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase. *Biochem J* **302**:559-566

89. Vater R, Cullen MJ and Harris JB (1992) The fate of desmin and titin during the degeneration and regeneration of the soleus muscle of the rat. *Acta Neuropathol* **84**:278-288

90. Voytik SL, Przyborski M, Badylak SF and Konieczny F (1993) Differential expression of muscle regulatory factor genes in normal and denervated adult rat hindlimb muscles. *Dev Dynamics* **198**:214-224

91. Vracko RE and Benditt EP (1972) Basal lamina: the scaffold for orderly cell replacement. Observations on regeneration of injured skeletal muscle fibers and capillaries. *J Cell Biol* **55**:406-419

92. Wang Y and Jaenisch R (1997) Myogenin can substitute for Myf5 in promoting myogenesis but less efficiently. *Development* **124**:2507-2513

93. Watkins SC and Cullen MJ (1987) A qualitative and quantitative study of the ultrastructure of regenerating muscle fibers in Duchenne muscular dystrophy and polymyositis. *J Neurol Sci* **32**:181-192

94. Whalen RG, Harris JB, Butler-Browne GS and Sesodia S (1990) Expression of myosin isoforms during notexin-induced regeneration of rat soleus muscles. *Dev Biol* **141**:24-40

95. Witzemann V and Sakmann B (1991) Differential regulation of MyoD anf myogenin mRNA levels by nerve induced muscle activity. *FEBS Lett* **282**:259-264

96. Wu KD and Lytton J (1993) Molecular cloning and quantification of sarcoplasmic reticulum $Ca^{2+}$-ATPase isoforms in rat muscles. *Am J Physiol* **264**:C333-341

97. Wuytack F, Papp B, Verboomen H, Raeymaekers L, Dode L, Bobe R, Enouf J, Bokkala S, Authi KS and Casteels R (1994) A sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 3-type $Ca^{2+}$ pump is expressed in platelets, in lymphoid cells, and in mast cells. *J Biol Chem* **269**:1410-1416

98. Zador E, Mendler L, Ver Heyen M, Dux L and Wuytack F (1996) Changes in mRNA levels of the sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$-ATPase isoforms in the rat soleus muscle regenerating from notexin-induced necrosis. *Biochem J* **320**:107-113

99. Zhu Z and Miller JB (1997) MRF4 can substitute for myogenin during early stages of myogenesis. *Dev Dynamics* **209**:233-241

100. Zubrzycka-Gaarn E, MacDonald G, Phillips L, Jorgensen AO and McLennan DH (1984) Monoclonal antibodies to the $Ca^{2+}$+$Mg^{2+}$-dependent ATPase of sarcoplasmic reticulum identify polymorphic forms of the enzyme and indicate the presence in the enzyme of a classical high-affinity $Ca^{2+}$ binding site. *J Bioenerg Biomembr* **16**:441-64

# 11. Annex

## Papers related to the subject of the Thesis