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Identification and Characterization of Differentially Expressed Genes in Denervated Muscle

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Denervation results in a series of changes in skeletal muscle. To elucidate the molecular basis underlying these changes, it is important to identify the profile of altered gene expression in skeletal muscle following nerve injury. In the present study, we have examined the differentially expressed genes in denervated gastrocnemius muscle using RNA fingerprinting by arbitrarily primed PCR. Eight differentially expressed mRNA transcripts have been identified. A bilateral regulatory profile can be observed for the up-regulated genes in both denervated and contralateral control muscle following unilateral sciatic nerve injury. The temporal expression profiles of the denervation-regulated genes in muscle during development, together with their dependency on nerve activity, suggest potential functional roles following nerve injury *in vivo.* In particular, the identification of two apoptosis-related genes in denervated muscle provides molecular evidence that the apoptotic process is likely to be involved in the intricate changes that lead to muscle atrophy. Our findings not only allow the identification of novel genes, but also suggest possible functions for some known genes in muscle following nerve injury. Taken together, these findings provide important insights into our understanding of the molecular events in denervated muscle and suggest that the differentially expressed genes may play potential roles during muscle denervation and regeneration.

INTRODUCTION

Nerve injury results in a wide spectrum of molecular and cellular modulation in skeletal muscle, including changes in gene expression. Identification of genes that are differentially expressed in denervated muscle might provide clues to their potential roles in nerve-muscle development and regeneration. For example, the up-regulation of brain-derived neurotrophic factor (BDNF) in denervated gastrocnemius muscle (Koliatsos *et al.*, 1993) is consistent with its ability to rescue degenerating neonatal motor neurons (Sendtner *et al.*, 1992; Yan *et al.*, 1992; Oppenheim *et al.*, 1993). Similarly, induction of insulin-like growth factor may act to protect denervated muscle from damage and facilitate regeneration (Coleman *et al.*, 1995; Barton-Davis, 1998). The dramatic increase in the expression of the alpha component of ciliary neurotrophic factor receptor complex has been proposed to serve an important function in enhancing neuronal regeneration after injury (Ip and Yancopoulos, 1996). Furthermore, the expression of many metabolic molecules such as ferritin heavy chain (Kitahara *et al.*, 1995), adhesion molecules such as neural cell adhesion molecule (Tews *et al.*, 1997a), and extracellular proteases, including urokinase (Tian *et al.*, 1995), also change in response to nerve injury. These adaptive changes act in a concerted manner to maintain muscle cell survival and participate in the remodeling of the neuromuscular synapse.

To further understand the molecular changes that occur in denervated muscle, it is important to identify the profile of differential gene expression following nerve injury. The use of RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) facilitates such studies. The underlying principle for RAP-PCR analysis and other related methods has previously been described (Welsh *et al.*, 1992; Liang *et al.*, 1992; Liversey *et al.*, 1996; Kociok *et al.*, 1998). Comparison of the difference in mRNA composition by RAP-PCR provides a useful approach to identify the event-induced differentially expressed genes (reviewed by McClelland *et al.*, 1995).

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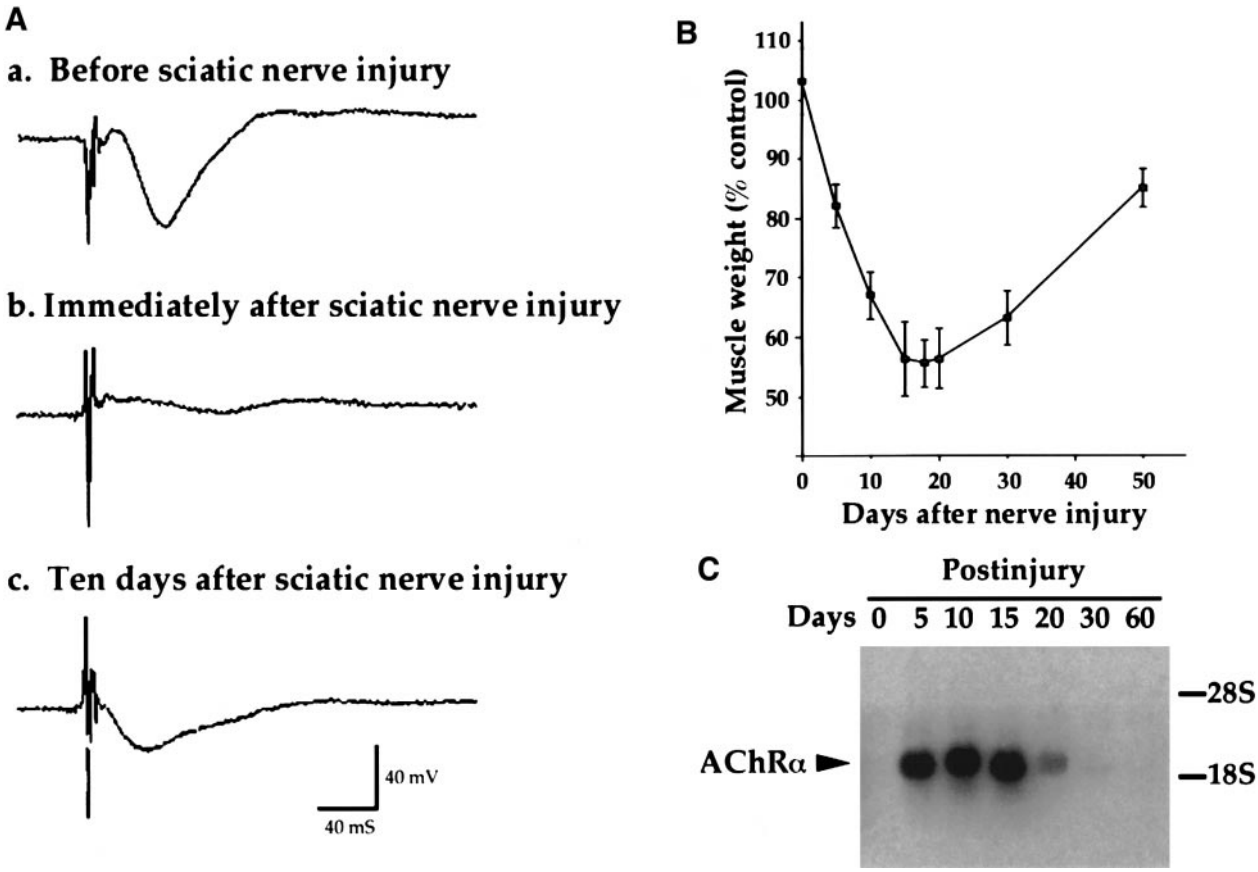

FIG. 1. (A) Electrophysiological evaluation of the model of sciatic nerve injury. Before nerve injury (a), a normal electromyogram could be recorded by stimulating the sciatic nerve trunk which disappeared immediately after nerve injury (b) and reappeared with a smaller amplitude ten days later (c). (B) Changes in the weight of gastrocnemius muscle following sciatic nerve crush. After nerve crush, the weight in gastrocnemius muscle (expressed as a percentage of denervated to contralateral control) decreased rapidly with the minimum level reached at ~15 days after injury, and then gradually recovered at 50 days after injury. Each datapoint represents mean ± SD, n = 5. (C) Northern blot analysis of AChRα transcript in gastrocnemius muscle at 0–60 days following nerve injury. Arrowhead indicated the AChRα transcript detected, while 18S and 28S were depicted on the right.

We have recently employed this strategy to successfully isolate genes induced by retinoic acid in embryonal carcinoma cells (Cheung *et al.,* 1997) and neuregulin-induced genes in muscle (Fu *et al.,* 1999).

In the present study, comparison of mRNA transcripts between denervated and intact gastrocnemius muscle was analyzed by RAP-PCR. A total of eight genes, including two novel genes, were isolated based on their differential expression during the early stages of denervation following sciatic nerve injury. Some of the differentially expressed genes identified may play potential roles in the apoptotic process in denervated muscle. Studies on the developmental profile of gene expression as well as the dependency on neural activity of these identified genes provide important insights into our understanding of the molecular events in denervated muscle.

RESULTS

Evaluation of the Sciatic Nerve Injury Model

Electrophysiological evaluation of the sciatic nerve injury model used in the present study was performed to confirm the inhibition of conduction of electric impulse after nerve crush. When the trunk of the intact sciatic nerve was stimulated, a normal electromyogram could be recorded in the gastrocnemius muscle, disappeared immediately after injury, and reappeared again

with a relatively smaller amplitude ten days later (Fig. 1A). A dramatic change in muscle weight was also associated with nerve injury (Fig. 1B). The weight of gastrocnemius muscle, expressed as a percentage of denervated to contralateral control, decreased from ~83% on day 5 to ~55% at 15 days after injury. As the nerve reinnervated, the muscle weight recovered gradually to ~85% at 50 days after nerve crush. A dramatic increase in the AChRα transcript was also observed 5 days following nerve crush (Fig. 1C). Taken together, these findings confirmed the validity of the nerve injury model used in the present study. The earlier recovery of the electrical activity across the NMJ might play an important role in facilitating the recovery of muscle from atrophy.

Identification of Differentially Expressed Genes in Denervated Muscle Using RAP-PCR

To identify the differentially expressed genes which might be involved in the process of muscle atrophy and regeneration following sciatic nerve crush, RNA fingerprinting was performed to isolate the differentially expressed genes in denervated gastrocnemius muscle. Total RNA was collected from denervated and intact gastrocnemius muscle at 0, 2, and 5 days after nerve injury, and subjected to RAP-PCR analysis as described under Experimental Methods. RAP-PCR analysis was performed on RNA samples with two dilutions (20 and 100 ng). Only differentially expressed bands that were reproducibly obtained with both dilutions were used for subsequent analysis. After excision of the differentially expressed cDNA fragments from the denaturing gel, the gene fragments were further reamplified, cloned and sequenced. The results were compared with the databases in National Center of Biotechnology Information (NCBI) Genbank using the BLAST server as described under Experimental Methods (Table 1).

Northern Blot Analysis of the Down-Regulated Genes after Denervation

Based on the results of RAP-PCR analysis, a total of eight cDNA fragments were identified. The identity of these cDNA fragments was inferred by comparison with known genes in the database (summarized in Table 1). The mRNA expression of four of these clones, 3.5, 7.11, 8.5, and 10.07, was down-regulated after denervation. The transcript of clone 3.5 (~3 kb), which is 100% identical to rat calpain p94 (calpain-3), was significantly down-regulated to ~10% of control level 5 days after sciatic nerve injury. As the nerve regenerated, it was up-regulated again and recovered to almost the normal level at day 30 (Fig. 2A). The transcripts of clone 7.11 (~3 and ~4 kb), which shares 90% identity with human pre-B cell enhancing factor (PBEF), were down-regulated by ~60% at day 5 and recovered to the normal level at day 20 (Fig. 2B). Clone 8.5 (~1.8 kb) is 100% identical to rat muscle-specific enolase (MSE, $\beta\beta$ enolase). Following nerve injury, a dramatic down-regulation occurred in the denervated muscle (Fig. 2C). Two days after sciatic nerve crush, the expression of MSE decreased and was subsequently reduced to ~10% of control at 5 days after denervation. The mRNA expression of MSE then gradually increased and recovered to ~50% of the basal level at day 20 (Fig. 2C). Clone 10.07 shares 91% identity with human myosin heavy chain (MHC) IIb mRNA. Two transcripts (~6 and ~4 kb) were found to be down-regulated in denervated muscle (Fig. 2D), albeit with a much more delayed time course.

TABLE 1
Denervation Regulated Genes Identified by RAP-PCR Analysis

Clone	Expression profile	Gene identity
3.3	Up-regulated	Novel gene
3.5	Down-regulated	Calpain P94 (calpain-3)
3.7	Up-regulated	Novel gene
5.1	Up-regulated	TNF type 1 receptor associate protein (TRAP-2)
7.11	Down-regulated	Pre-B cell enhancing factor (PBEF)
8.5	Down-regulated	Muscle specific enolase (MSE)
10.07	Down-regulated	Myosin heavy chain IIb (MHC IIb)
12.05	Up-regulated	Glutamine synthetase (GS)

Note. The mRNA expression profile of identified differentially expressed genes in rat denervated skeletal muscle was obtained using Northern blot analysis. Gene identity of RAP-PCR clones was inferred by comparison of the deduced amino acid sequence of cloned cDNA fragments with known genes as described under Experimental Methods.

Northern Blot Analysis of the Up-Regulated Genes after Denervation

The expression of four of the identified genes (clone 5.1, 12.05, 3.3, and 3.7) was up-regulated in denervated muscle. Clone 5.1 is 98% identical to human tumor necrosis factor receptor-associated protein (TRAP-2). Following nerve injury, the transcript (~3.5 kb) was dramatically increased at day 2 and maintained at a high level at day 5. Concomitant with the process of nerve reinnervation, TRAP-2 expression decreased to

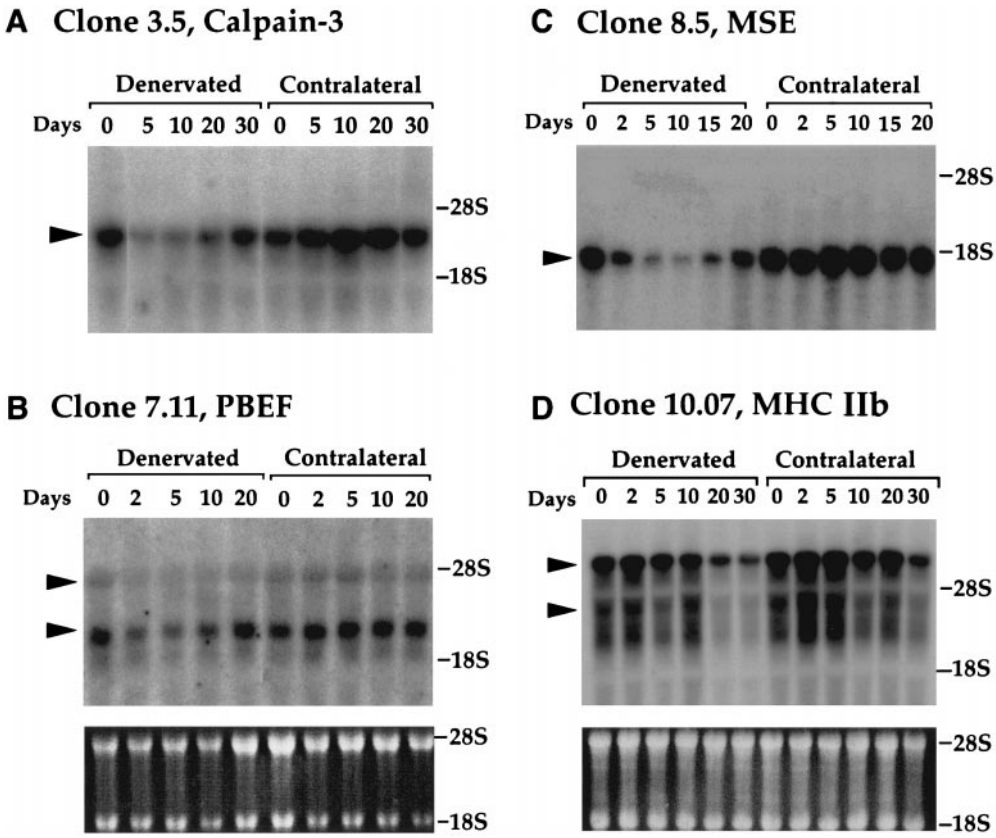

FIG. 2. Northern blot analysis for RAP-PCR clones 3.5, 7.11, 8.5, and 10.07 in denervated muscle. The mRNA expression for these four genes was down-regulated after nerve crush. As the nerve reinnervated, the expression increased and returned to the normal level (except for clone 10.07, which exhibited a more delayed time course for its downregulation). No significant change could be observed in the contralateral muscle. The ethidium-bromide stained gels at the bottom panels indicated the equal loading of RNA. Arrowheads depicted the transcripts detected for these genes, while 18S and 28S were depicted on the right.

the basal level (Fig. 3A). Clone 12.05 shares 100% identity with rat glutamine synthetase (GS), and the two transcripts (~1.5 and ~3.5 kb) were found to be up-regulated following sciatic nerve crush (Fig. 3B).

Two novel clones, 3.3 and 3.7, were also found to be up-regulated during denervation. Two transcripts (~1.5 and ~2.4 kb) of clone 3.3 were detected in normal skeletal muscle. Two days following nerve crush, the 1.5 kb transcript of clone 3.3 showed a dramatic increase while the increase observed for the ~2.4 kb transcript was smaller. Interestingly, an extra transcript (~3 kb) was induced at day 2, but rapidly declined (Fig. 3C). Similar to clone 3.3, the transcript of clone 3.7 (~3.5 kb) was significantly up-regulated in denervated muscle 2 days after injury and then decreased rapidly at day 10 (Fig. 3D).

Interestingly, a similar regulatory profile was observed in the contralateral control muscle for these genes upregulated by denervation, albeit with a smaller amplitude and shorter time course (Fig. 3). This bilateral regulatory phenomenon, however, was not obvious for the other four down-regulated genes described above.

Developmental Expression of the Denervation-Regulated Genes in Skeletal Muscle

As a first step to elucidate the functional roles of the denervation-regulated genes in injury and regeneration, developmental expression of these differentially expressed genes was examined in rat skeletal muscle. Low level of expression of calpain-3 mRNA could be detected in skeletal muscle at E21. The transcript was up-regulated throughout the later stages of muscle development (Fig. 4). Similar profile of upregulation was observed for PBEF, MSE, and MHC IIb during muscle

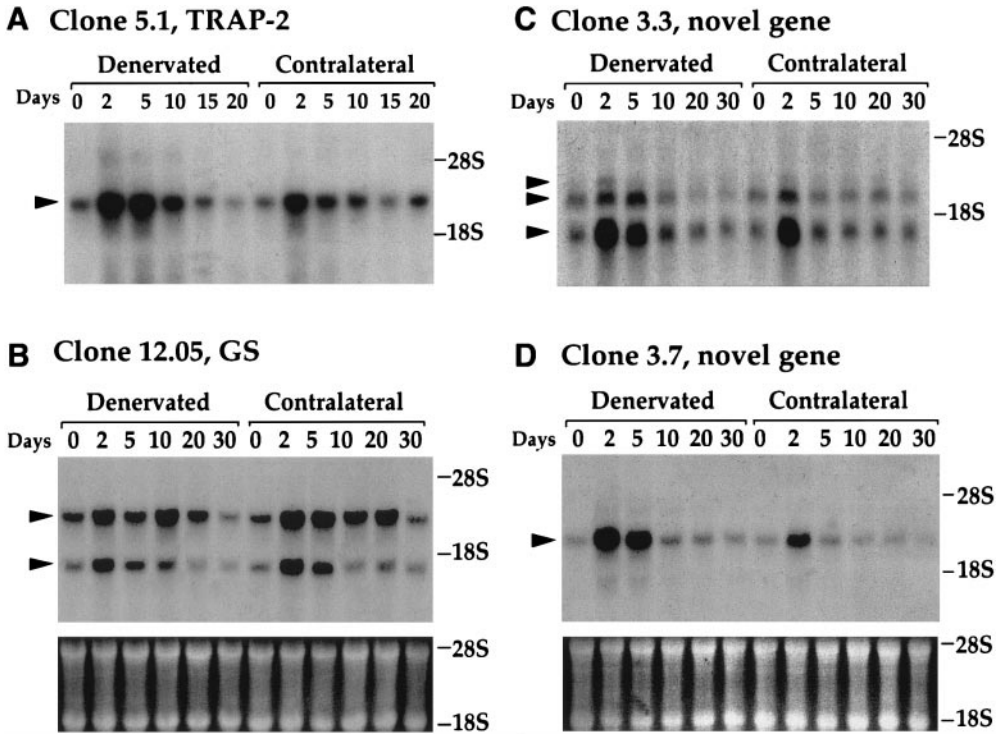

FIG. 3. Northern blot analysis for RAP-PCR clones 5.1, 12.05, 3.3, and 3.7 in denervated muscle. After denervation, the mRNA expression for these four genes was up-regulated. As the nerve reinnervated, the expression decreased and returned to the normal level. Interestingly, a similar up-regulation was also observed in the contralateral control muscle, albeit with a smaller amplitude. The ethidium-bromide-stained gels at the bottom panels indicated the equal loading of RNA. Arrowheads depicted the transcripts detected for these genes, while ribosomal RNA bands (18S and 28S) were depicted on the right.

development (Fig. 4). Thus, for the genes that were down-regulated in denervated muscle, they exhibited a low level of expression early in development. In contrast, the genes that were up-regulated after nerve injury, they were expressed at a more abundant level during early development. For example, TRAP-2 transcript was prominently expressed in early stages during development (e.g., E14) and down-regulated during postnatal stages of P21 and P30 (Fig. 5). Two transcripts of GS were detected in developing rat skeletal muscle; the expression was initially down-regulated and then up-regulated during the later stages of development (Fig. 5).

The developmental gene expression of the two novel clones in skeletal muscle, brain and liver was examined. Three transcripts of clone 3.3 were detected in developing rat muscle. Interestingly, the appearance of the ~2.4kb transcript, which was unique for skeletal muscle, was coincidental with the decrease in expression of the ~3kb transcript. Although the ~1.5kb transcript was predominantly expressed at the early stages of developing muscle, this transcript was down-regulated during development (Fig. 6). No significant changes in the expression of clone 3.3 was observed in the brain during development while in liver, the ~1.5kb transcript was predominantly expressed in the early embryonic stages. On the other hand, the transcript of clone 3.7 could be detected in skeletal muscle and brain, but not liver (Fig. 7). In skeletal muscle, it was detected at E21 and decreased during postnatal stages. The two transcripts of clone 3.7 detected in the brain were expressed at an abundant level in late postnatal stages (Fig. 7).

Regulation of the Differentially Expressed Genes by Nerve Activity

To examine the dependence of the differentially expressed genes on nerve activity, their expression in muscle was examined after TTX-induced paralysis of the sciatic nerve. RT-PCR analysis was performed with the cDNA templates transcribed from TTX-paralyzed gastrocnemius muscle. Pilot experiments were performed for individual gene to determine the linear

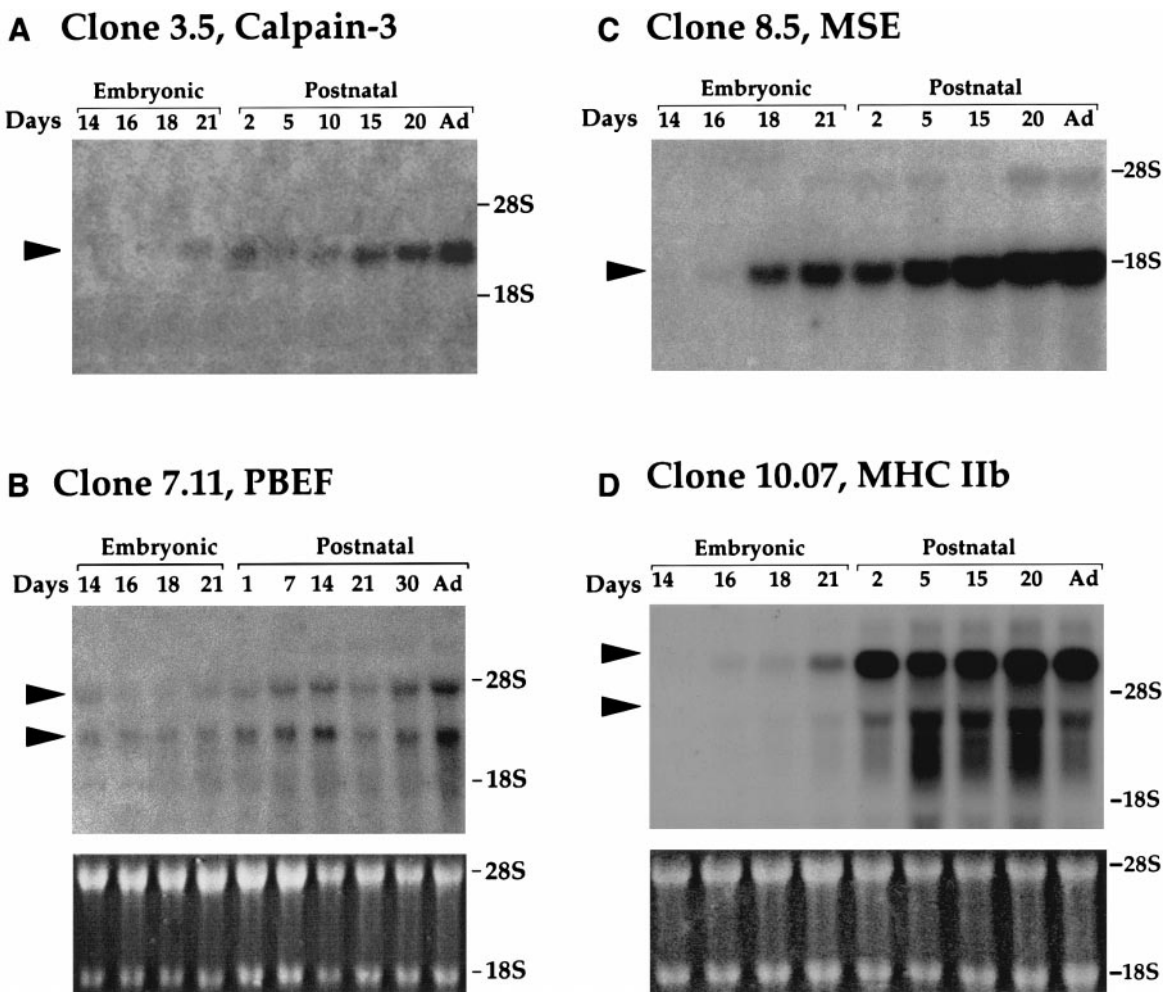

FIG. 4. Northern blot analysis for RAP-PCR clones calpain-3, PBEF, MSE, and MHC IIb in skeletal muscle during development. Total RNA was extracted from rat skeletal muscle [from embryonic day 14 to adult (Ad)]. The mRNA expression of these four genes was up-regulated throughout the developmental stages. Ribosomal RNA bands (18S and 28S) were indicated on the right while arrowheads depicted the mRNA transcripts detected for these four genes. The ethidium-bromide stained gels at the bottom panels showed the equal loading of RNA.

range of the PCR using different number of cycles (data not shown). Subsequent RT-PCR analysis was performed with the same initial cDNA concentration and the cycle number, which was confirmed to be on the linear range (e.g., 26 cycles). Both GAPDH and cyclophilin were simultaneously amplified to serve as the internal control of the amount of RNA. Following TTX-paralysis, Clones 3.3, 3.7, and TRAP-2 mRNA expression was significantly up-regulated, in a manner similar to that observed in denervated muscle (Fig. 8). On the other hand, reduced expression of calpain-3, PBEF, and MSE was observed in the TTX-treated muscle, similar to the decrease observed after denervation. The expression of MHC IIb and GS mRNA, however, did not exhibit any significant change following 2 days of TTX treatment (Fig. 8). To confirm whether longer period of TTX treatment would result in the same regulatory profile, the treatment period was extended to 4 and 6 days and Northern blot analysis was performed (Fig. 9). The change in message levels in paralyzed muscle was compared with that observed in the denervated muscle in the same experiment. The effectiveness of TTX block was confirmed by the increase in the mRNA expression of AChRα. TTX-induced paralysis was able to induce changes in gene expression in a manner similar to denervation (Fig. 9). Furthermore, an increase in the expression of GS was observed after a longer period of TTX treatment for 6 days (Fig. 9).

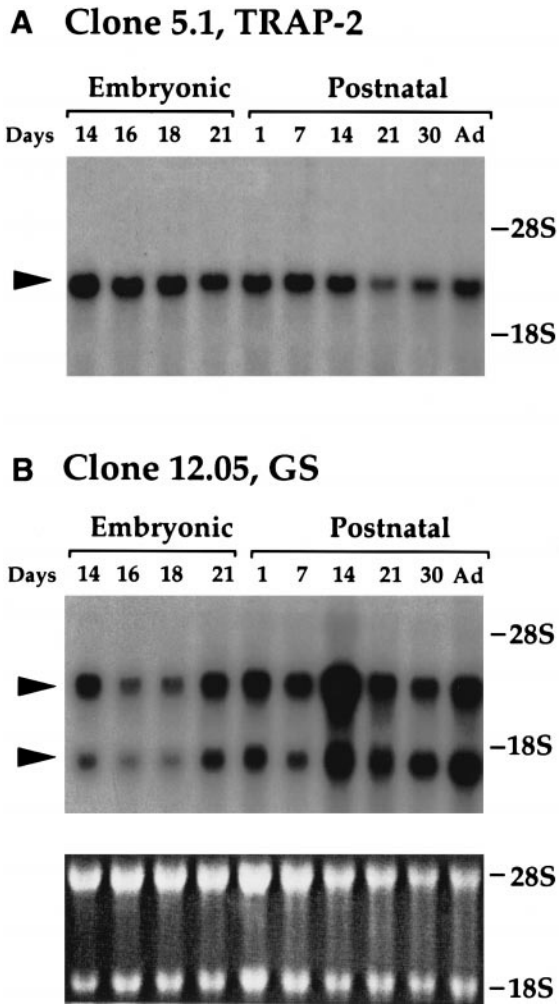

FIG. 5. Northern blot analysis for RAP-PCR clones TRAP-2 and GS in skeletal muscle during development. Total RNA was extracted from rat skeletal muscle [from embryonic day 14 to adult (Ad)]. Ribosomal RNA bands (18S and 28S) were indicated on the right while arrowheads depicted the detected mRNA transcripts. The ethidium-bromide stained gel at the bottom panel showed the equal loading of RNA.

DISCUSSION

In the present study, we have utilized RAP-PCR analysis to identify genes that are differentially expressed in skeletal muscle following nerve injury. We report here that a total of eight cDNA fragments were cloned and analyzed based on the RNA fingerprints of differentially expressed transcripts in denervated muscle. During the early stages of denervation, four genes (clones 3.3, 3.7, TRAP-2, and GS) showed up-regulation while the other four (calpain-3, PBEF, MSE, and MHC IIb) were down-regulated. These differentially expressed genes may potentially play functional roles in the intricate processes involved in denervation and regeneration. The identification of TRAP-2 and calpain-3 as denervation-regulated genes provides molecular evidence that activation of the apoptotic signaling pathway plays an important role in skeletal muscle in response to nerve injury. More importantly, of these eight identified genes, two denervation-regulated novel genes (clones 3.3 and 3.7) have been isolated. Interestingly, some of the mRNA species that are well-known to be up-regulated by denervation (e.g., AChR subunits) are not among the genes identified in denervated muscle in our study. One key feature of the RAP-PCR is its ability to identify differentially expressed genes without prior knowledge of their exact sequences; the pattern of RNA fingerprints can be affected by the primer sets used in each reaction. The preferential annealing of the primers to the transcripts determines which candidate genes can be subsequently identified following RAP-PCR. Since denervation results in altered expression of numerous genes and we did not target at a specific group of genes,

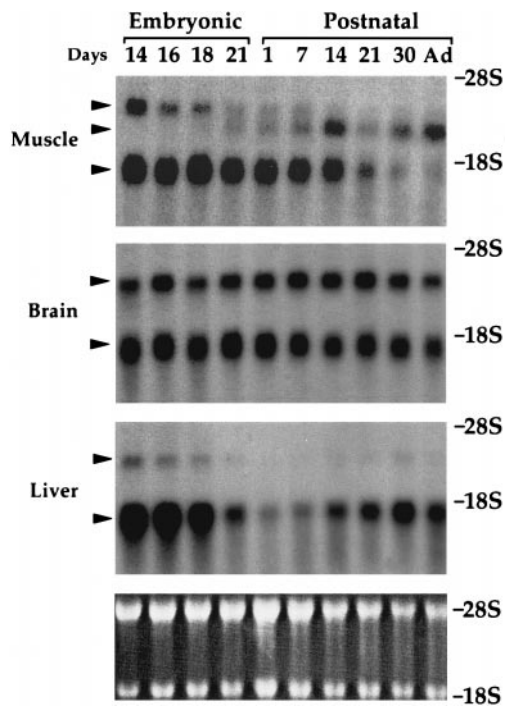

FIG. 6. Developmental mRNA expression of novel clone 3.3 in rat tissues. Total RNA was extracted from rat skeletal muscle, brain, and liver [from embryonic day 14 to adult (Ad)]. Ribosomal RNA bands (18S and 28S) were indicated on the right while arrowheads depicted the detected mRNA transcripts for clone 3.3. The ethidium-bromide stained gel at the bottom panel showed the equal loading of RNA.

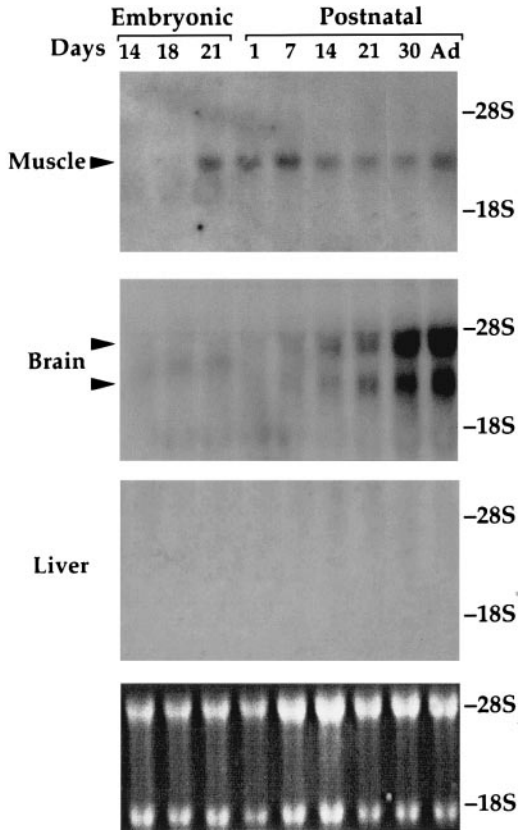

FIG. 7. Developmental mRNA expression of novel clone 3.7 in rat tissues. Total RNA was extracted from rat skeletal muscle, brain, and liver [from embryonic day 14 to adult (Ad)]. Ribosomal RNA bands (18S and 28S) were indicated on the right, while arrowheads depicted the detected mRNA transcripts. The ethidium-bromide-stained gel at the bottom panel showed the equal loading of RNA.

such as receptors, it is reasonable that the arbitrary primers used in our study did not pick up the transcript encoding AChR subunits. The eight differentially expressed genes reported in this study constitute a subset of this enormous pool of genes that are amplified by the arbitary primers.

Regulation of Apoptosis-Related Genes in Denervated Muscle

Two of the genes identified to be regulated by denervation in this study, TRAP-2 and calpain-3, have been suggested to be involved in apoptosis. TRAP-2 binds specifically to the intracellular domain of the type 1 receptor for tumor necrosis factor (TNF), at a site upstream to the region involved in the induction of cell death (Boldin *et al.,* 1995; Tsurumi *et al.,* 1996). Since this interaction may play a functional role in apoptosis mediated by TNF receptor (Vilcek and Lee 1991), our finding on the upregulation of TRAP-2 after nerve injury is consistent with the possibility that apoptotic process is involved in denervation. It has been proposed that long-term muscle survival needs innervation-induced superathreshold activity, while apoptosis can be initiated by the loss of functional innervation. Increase in the magnitude of fiber apoptosis has been reported following denervation and muscle paralysis (Trachtenberg, 1998). Furthermore, denervated muscle reveals stronger immunoreactive activity of apoptosis-related protein (such as bcl-2 and bcl XL) and high rates of DNA fragmentation (Tews *et al.,* 1997b). It is possible that denervation may prompt muscle fiber to activate an intrinsic "suicide" program to undergo apoptosis, which may contribute to muscle atrophy. Thus, the rapid and dramatic regulation of TRAP-2 observed after denervation provides important molecular evidence to support the involvement of apoptotic event in denervated muscle. Interestingly, we have recently obtained evidence that clone 3.3, which is also upregulated by denervation, also plays an important role in regulating apoptosis (unpublished data).

Calpain-3 is a member of the calcium-dependent neutral protease (CANP) large subunit family (reviewed by Sorimachi *et al.,* 1997; Kinbara *et al.,* 1998). The involvement of the calpain family in apoptosis has been reported for different cell types including hippocampal neurons (reviewed by Sorimachi *et al.,* 1997). The expression of calpain-3 has previously been reported to decrease during interleukin-6 induced muscle atrophy (Tsujinaka *et al.,* 1996). Mutations in this gene are also known to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A) (Richard *et al.,* 1995). It has been suggested that calpain-3 may play an active role in signal transduction and its function is essential to the muscle system. It is possible that the loss of function of calpain-3 might lead to the activation of proteases, including other members in the calpain family. Decrease in the expression of calpain-3, such as that observed in LGMD2A or following denervation in the present study, would therefore result in muscle atrophy.

Other Known Genes That Are Differentially Expressed in Denervated Skeletal Muscle

MSE and GS, two of the known genes identified in the present study, are metabolic molecules, which participate in the physiological processes in muscle. MSE is a glycolytic enzyme (2-phospho-d-glycerate hydrolase) that is predominantly expressed in mature muscle. Our

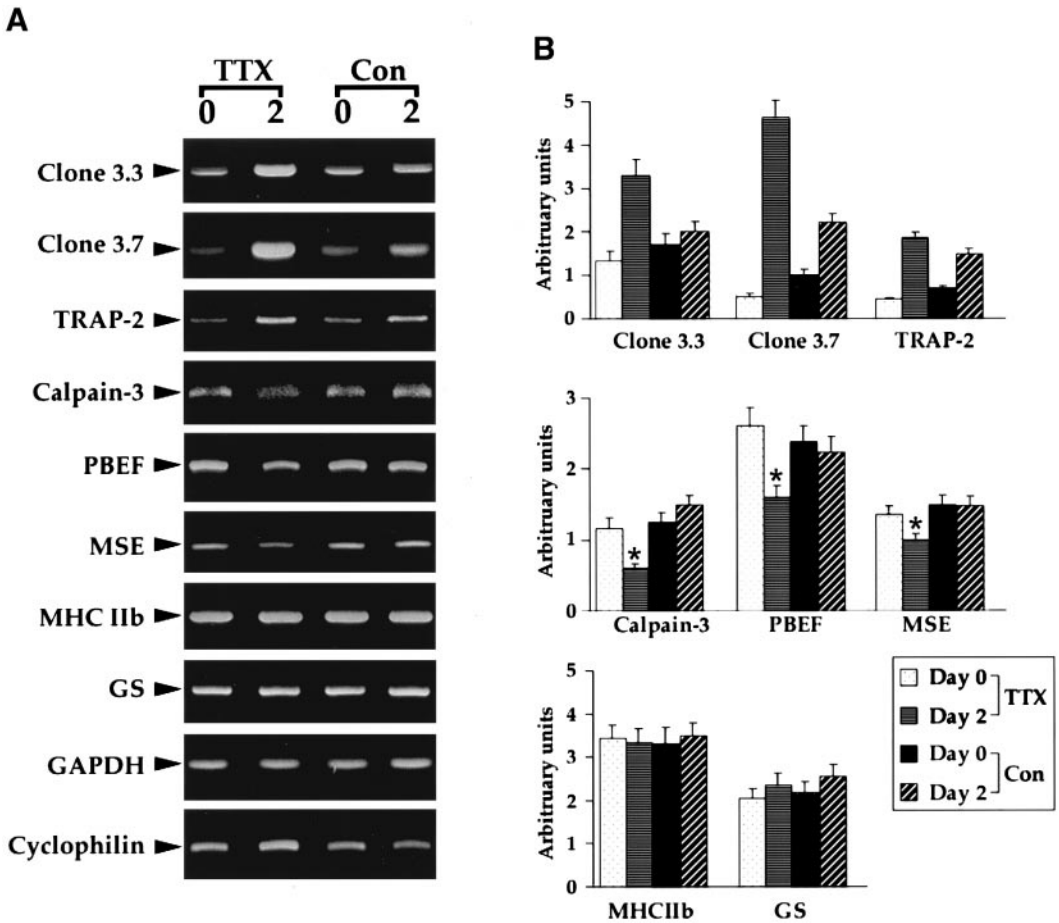

FIG. 8. Regulation of the mRNA expression of the RAP-PCR clones by neural activity. (A) RT-PCR analysis was employed to examine the mRNA expression of the RAP-PCR clones after *in vivo* TTX treatment. Total RNA was extracted from gastrocnemius muscle paralyzed with TTX or from contralateral control muscle (Con) at 0 or 2 days. GAPDH and cyclophilin were amplified simultaneously as the internal control. (B) Quantitation of the RT-PCR results was performed as described under Experimental Methods and expressed as arbitrary units (mean ± SD, $n = 3$). $^{**}P < 0.005$ and $^{*}P < 0.05$ compared with day 0 control, unpaired t test. The regulatory profile of the genes upregulated by TTX is depicted in the upper panel, downregulated genes in the middle panel and genes unaffected by 2-day treatment of TTX in the bottom panel.

finding on the dramatic down-regulation of MSE mRNA after nerve injury is consistent with the previous report that the MSE protein was drastically decreased in denervated muscle (Kato *et al.*, 1985). Since MSE expression and regulation is thought to be closely correlated with the functional and differentiation status of muscle, decreased expression of MSE might indicate the loss of the differentiated phenotype of muscle after denervation. Unlike MSE, the expression of GS is up-regulated following denervation. GS catalyzes the final step in the glutamine synthetic pathway and can be increased under stress conditions (Rannels and Jefferson, 1980). The increased expression of GS in denervated muscle can catalyze the conversion of degraded amino acids to glutamine, which can be released and serves as the energy source for other tissues.

PBEF is a 52-kDa secreted protein which can increase the pre-B cell colony formation activity of stem cell factor and interleukin-7 (Samal *et al.*, 1994). Our study provides the first demonstration that the expression of PBEF could be regulated in denervated muscle in a neural activity-dependent manner. The significance in the decreased expression of PBEF after denervation, however, remains to be elucidated. Similar to PBEF, MHC IIb mRNA was downregulated by nerve injury, albeit with a much delayed time course. This down-regulation is consistent with a recent report that MHC IIb protein expression was decreased in tibialis anterior

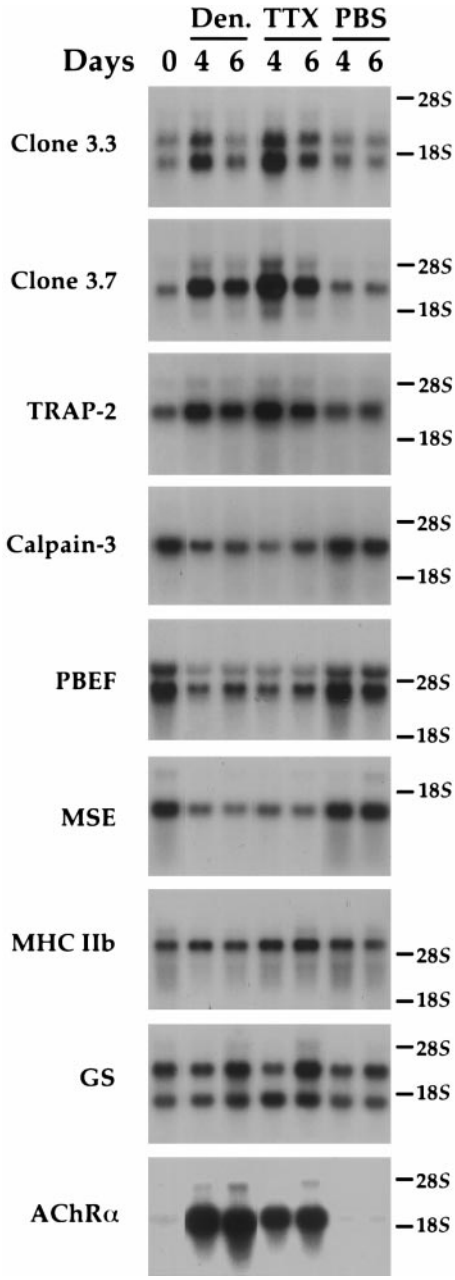

FIG. 9. Northern blot analysis of the mRNA expression of the RAP-PCR clones following *in vivo* TTX treatment. The expression of the RAP-PCR clones in gastrocnemius muscle was examined at 0, 4, or 6 days after denervation (Den.), TTX treatment or buffer (PBS) control treatment. The genes examined were indicated on the left, while 18S and 28S were depicted on the right.

following denervation (Huey and Bodine, 1998). The reduced expression of MHC IIb might be related to the impaired function of muscle contraction after denervation.

Bilateral Regulatory Pattern of the Upregulated Genes after Unilateral Nerve Injury

All of the up-regulated clones (clone 3.3, 3.7, TRAP-2, and GS) exhibit a similar expression pattern in the contralateral intact muscle after unilateral nerve injury, albeit with a smaller amplitude and shorter time course. A similar phenomenon in nervous system has recently been reviewed by Koltzenburg *et al.* (1999). Following peripheral nerve lesion, the expression of several molecules has been reported to change in the contralateral nonlesioned structures, in a manner similar to that observed in the lesioned ipsilateral side (e.g., Zhou *et al.*, 1996). The gene regulation observed on the contralateral side could be mediated by systemic factor(s), which, in reponse to nerve injury, is released into circulation and act on the target muscle. Alternatively, the transmedian nerve communication at the spinal cord and/or brain level can transfer the injury information from lesioned side to the intact side and result in the bilateral pattern of regulation. Our finding on the up-regulation of the genes in the contralateral control muscle following *in vivo* TTX treatment lends support to the hypothesis of transmedian nerve communication, suggesting that nerve paralysis on one side will also result in the regulation of genes in the contralateral control muscle.

Developmental Expression Profile and Nerve Activity Dependence of the Denervation-Regulated Genes in Muscle

Analysis of the molecular events during development facilitates our understanding of the processes that occur in the skeletal muscle following nerve injury. Comparison of the gene expression during development and regeneration reveals that denervated muscle undergoes dedifferentiation and revert to the embryonic state with recovery of immature properties. For example, calpain-3, PBEF, MSE, and MHC IIb, representing genes that are downregulated in denervated muscle, exhibit an up-regulatory profile during development (Fig. 10). Changes in the expression of the other four up-regulated genes (TRAP-2, GS, clone 3.3, and clone 3.7) are more complicated during development, but the overall higher level of expression during embryonic stages is consistent with the increase in expression following denervation (Fig. 10). Interestingly, the transcript profiles of clone 3.3 during development indicate that there are three transcripts in late embryonic and early postnatal stage, while in adulthood and early embryonic stages, only two transcripts can be observed. After denervation, however, three transcripts of clone 3.3 can be

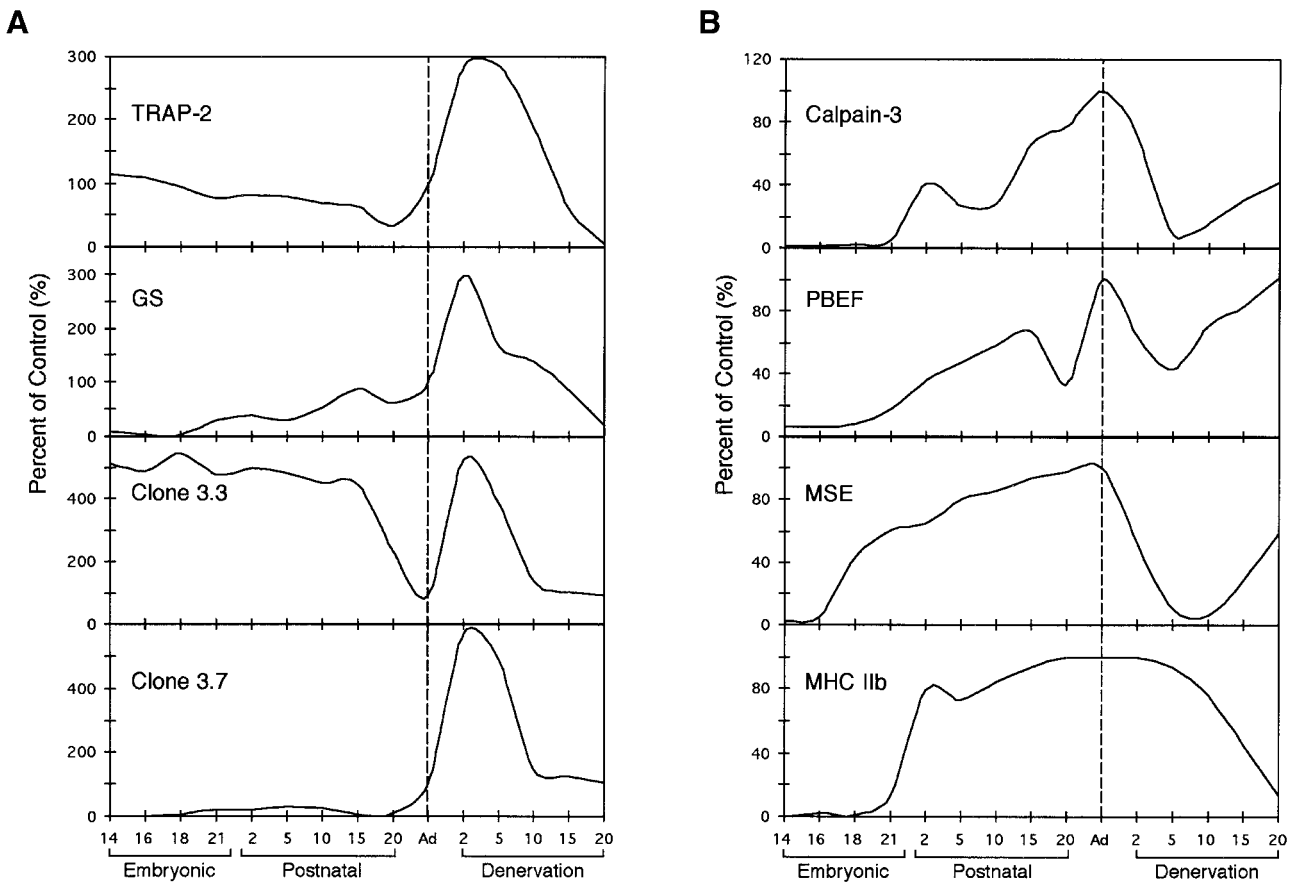

FIG. 10. Comparison of the mRNA expression of the RAP-PCR clones during development and following denervation. The quantitative data was normalized as a percentage of adult gastrocnemius muscle (which was set at 100%). The dotted line refers to the adult stage, with the developmental profile to the left and injury profile to the right. The profiles presented for GS and clone 3.3 represented the quantitation results obtained for the smaller transcripts. Those clones that were upregulated or downregulated following nerve injury were depicted in A or B, respectively.

induced in adult skeletal muscle. Thus, the profile of mRNA transcripts observed is consistent with the reversion of the denervation-induced dedifferentiation process to the late embryonic/early postnatal stages.

Blockade of the normal conduction of electrical signals by TTX treatment results in a denervated status of the intact muscle by inducing the loss of neural activity. We have examined the changes in gene expression patterns in muscle after TTX-induced paralysis. These changes, consistent with the observation from results obtained after nerve injury, show that the regulation of almost all of the differentially expressed genes (clones 3.3 and 3.7, TRAP-2, calpain-3, PBEF, MSE, and GS) is dependent on nerve activity. The expression of MHC IIb mRNA, however, was not signficantly affected following up to 6 days of TTX treatment. It is possible that the manifestation of the TTX-induced change in gene expression of MHC IIb may require longer than a 6-day period. Alternatively, it may be regulated by a growth factor-dependent mechanism. Recent evidence from our laboratory demonstrated that the expression of MHC IIb could be regulated by neuregulin (unpublished data).

Taken together, the panel of denervation-regulated genes identified in the present study further demonstrates that RAP-PCR analysis is a useful approach in isolating differentially expressed genes that are involved in various cellular responses. For example, the regulation of apoptosis-related genes in denervated muscle suggests that activation of the apoptotic signaling pathway may play an important role in skeletal muscle in response to nerve injury. Moreover, the panel of denervation-regulated genes identified indicates the complexity of the signaling events in muscle that ulti-

mately leads to muscle atrophy and regeneration. Mapping out the precise functions of these genes and identifying other denervation-induced genes will provide insights into our understanding of the molecular basis of denervation.

EXPERIMENTAL METHODS

Animal Surgery and Electrophysiological Evaluation

Adult male SD rats (~250 g) were purchased from the animal center of Chinese Academy of Sciences (Shanghai). The procedure of sciatic nerve crush was performed as previously described (Hantai *et al.*, 1990; Ip *et al.*, 1996). Briefly, the sciatic nerve on the left side of the rat was exposed under sterile condition. Forceps protected by silicon tube were used to crush the trunk of the sciatic nerve for 40 s at the mid-thigh level with a standard pressure meter. The extent of damage of the sciatic nerve was evaluated by electrophysiological approach. The stimulating electrodes were placed on the nerve proximal to the crushed site and the recording electrodes were inserted into gastrocnemius muscle to record the action potential after the evoke from the trunk of sciatic nerve. The muscle action potentials were recorded before injury, immediately after injury, and 10 days after injury.

Sciatic Nerve Paralysis by Tetrodotoxin (TTX)

Paralysis of the sciatic nerve was performed by TTX (Sigma, USA) as described (Michel *et al.*, 1994). Rats were anesthetized by isoflurane inhalation, which produces general anesthesia with loss of consciousness, analgesia, suppression of reflex activity, and muscle relaxation. An osmotic minipump (Alzet 2002, Alza Corp., U.S.A.), containing 180 μg/ml TTX, 200 IU penicillin and 200 μg/ml streptomycin in Hank's solution was implanted subcutaneously and positioned by surgical silk. TTX was delivered to sciatic nerve by silastic tubing (i.d. 0.078 cm, 10 cm in length) connecting the minipump. TTX was delivered at a rate of 4.5 μg/day with a pumping rate of 1 μl/h. Control experiments were performed using Hank's solution without TTX. Two days later, gastrocnemius muscle was collected for total RNA extraction.

Sample Collection and Total RNA Extraction

The operated animals were sacrificed at various days (0–30 days) after denervation. Gastrocnemius muscles from operated and contralateral control sides were excised and frozen for further analysis. In addition, the skeletal muscle (from hind limb), brain, and liver tissues of normal rat from embryonic and postnatal stages (from E 14 to adult) were also dissected. Total RNA were extracted from the tissue samples as previously described (Ip *et al.*, 1995). RNA was resuspended in DEPC-water and concentration was estimated by measuring the optical density at 260 nm using a spectrophotometer.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis

RT-PCR reaction was performed according to instruction from supplier (Gibco BRL, U.S.A.) as previously described (Fu *et al.*, 1999). Northern blot analysis was performed as previously described (Ip *et al.*, 1995). Briefly, 15 μg of total RNA was electrophoresed, transferred onto nitrocellulose membrane, and cross-linked by UV irradiation. Cloned DNA fragments were labeled with [^{32}P]dCTP by Megaprime DNA labeling kit (Amersham, U.S.A.). Hybridization was performed in 0.5 M sodium phosphorate buffer at 65°C, and the membrane was washed with 2× SSC and 0.1% SDS at 65°C. The semi-dried membrane was then wrapped and exposed to Kodak X-ray film at −80°C. Data in the present study have been confirmed in at least three individual experiments; the results shown are representative of the data obtained. Quantitation was performed using the integrated density and concentration analysis provided by the EagleSight software (Strategene, U.S.A.).

RNA Fingerprinting by RAP-PCR

Reverse transcription and arbitrarily primed PCR reaction were performed as previously described (Welsh *et al.*, 1992; Cheung *et al.*, 1997). Briefly, total RNA was treated with DNase I and reverse transcription reaction was performed using a first arbitrary primer (RT-primer) at 37°C for 1 h. The reaction mixture contains 1× PCR reaction buffer, 50 μM each dNTP, 3 mM $MgCl_2$, 0.05 μCi/μl [^{32}P]dCTP, 1 μM each of two primers (RT-primer and the other arbitrary primer). PCR cycles were composed of one low-stringency amplification (annealed at 37°C) followed by 30 high-stringency cycles (annealed at 55°C). PCR products were separated on 6% denatured polyacrylamide gel. Differentially expressed PCR bands were excised from the gel and reamplified with the same primers at high stringency. Reamplified cDNA fragments were gel-purified with Qiaex II kit (Qiagen, Germany), according to the sup-

plier's instruction, and subcloned into pBluescript II KS(+) (Stratagene) by blunt end ligation. Size of the cloned fragments ranged from 400 to 600 bp. Double-stranded DNA sequencing was performed with T7 DNA polymerase sequencing kit (Amersham). At least five individual clones from each amplified product were sequenced. Arbitrary primers used in the RAP-PCR reaction were as follows. (1) GTAAAACGACGGCCAGT; (2) ATCAACATCCACGACTGG; (3) CGAGGTCGACGGTATC; (4) GCCCAGCATGTAAGTAAT; (5) GTAAAACGACGGCCAGT; (6) TCAGTACTGCGGACGCCA.

Sequence Analysis

The sequences of the cDNA fragments were submitted to the National Center of Biotechnology Information (NCBI) for homology alignment using the BLAST program. Nucleotide sequences were compared with the GenBank and EMBL databases. Deduced amino acid sequences were compared with the CDS translations of the GenBank databases and the amino acid sequences from the PDB, SwissProt, Spudate, and PIR databases. TBLASTX was also performed with the dbEST database at the NCBI.

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REFERENCES

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N., and Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. *Proc. Natl. Acad. Sci. USA* **95:** 15603–15607.

Boldin, M. P., Mett, I. L., and Wallach, D. (1995). A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its "death domain." *FEBS Lett.* **367:** 39–44.

Cheung, W. M., Chu, A. H., and Ip, N. Y. (1997). Identification of candidate genes induced by retinoic acid in embryonal carcinoma cells. *J. Neurochem.* **68:** 1882–1888.

Coleman, M. E., DeMayo, F., Yin, K. C., Lee, H. M., Geske, R., Montgomery, C., and Schwartz, R. J. (1995). Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. *J. Biol. Chem.* **270:** 12109–12116.

Fu, A. K. Y., Cheung, W. M. W., Ip, F. C. F., and Ip, N. Y. (1999). Identification of genes induced by neuregulin in cultured myotubes. *Mol. Cell. Neurosci.* **14:** 241–253.

Hantai, D., Rao, J. S., and Festoff, B. W. (1990). Rapid neural regulation of muscle urokinase-like plasminogen activator as defined by nerve crush. *Proc. Natl. Acad. Sci. USA* **87:** 2926–2930.

Huey, K. A., and Bodine, S. C. (1998). Changes in myosin mRNA and protein expression in denervated rat soleus and tibialis anterior. *Eur. J. Biochem.* **256:** 45–50.

Ip, N. Y., and Yancopoulos, G. D. (1996). The neurotrophins and CNTF: Two families of collaborative neurotrophic factors. *Annu. Rev. Neurosci.* **19:** 491–515.

Ip, F. C. F., Fu, A. K. Y., Tsim, K. W. K., and Ip, N. Y. (1995). Cloning of the alpha component of the chick ciliary neurotrophic factor receptor: Developmental expression and down-regulation in denervated skeletal muscle. *J. Neurochem.* **65:** 2393–2400.

Ip, F. C. F., Fu, A. K. Y., Tsim, K. W. K., and Ip, N. Y. (1996). Differential expression of ciliary neurotrophic factor receptor in skeletal muscle of chick and rat after nerve injury. *J. Neurochem.* **67:** 1607–1612.

Kato, K., Shimizu, A., Semba, R., and Satoh, T. (1985). Tissue distribution, developmental profiles and effect of denervation of enolase isozymes in rat muscles. *Biochim. Biophys. Acta.* **841:** 50–58.

Kinbara, K., Ishiura, S., Tomioka, S., Sorimachi, H., Jeong, S. Y., Amano, S., Kawasaki, H., Kolmerer, B., Kimura, S., Labeit, S., and Suzuki, K. (1998). Purification of native p94, a muscle-specific calpain, and characterization of its autolysis. *Biochem. J.* **335:** 589–596.

Kitahara, T., Kiryu, S., Takeda, N., Kubo, T., and Kiyama, H. (1995). Up-regulation of ferritin heavy chain mRNA expression in the rat skeletal muscle after denervation: Detected by means of differential display. *Neurosci. Res.* **23:** 353–360.

Kociok, N., Unfried, K., Esser, P., Krott, R., Schraermeyer, U., and Heimann, K. (1998). The nonradioisotopic representation of differentially expressed mRNA by a combination of RNA fingerprinting and differential display. *Mol. Biotechnol.* **9:** 25–33.

Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H., and Price, D. L. (1993). Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons *in vivo. Neuron* **10:** 359–367.

Koltzenburg, M., Wall, P. D., and McMahon, S. B. (1999). Does the right side know what the left is doing? *Trends Neurosci.* **22:** 122–127.

Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257:** 967–971.

Livesey, F. J., and Hunt, S. P. (1996). Identifying changes in gene expression in the nervous system: mRNA differential display. *Trends Neurosci.* **19:** 84–88.

McClelland, M., Mathieu-Daude, F., and Welsh, J. (1995). RNA fingerprinting and differential display using arbitrarily primed PCR. *Trends Genet.* **11:** 242–246.

Michel, R. N., Cowper, G., Chi, M. M., Manchester, J. K., Falter, H., and Lowry, O. H. (1994). Effects of tetrodotoxin-induced neural inactivation of single muscle fiber metabolic enzymes. *Am. J. Physiol.* **267:** C55–C66.

Oppenheim, R. W., Prevette, D., Haverkamp, L. J., Houenou, L., Yin, Q. W., and McManaman, J. (1993). Biological studies of a putative avian muscle-derived neurotrophic factor that prevents naturally occurring motoneuron death in vivo. *J. Neurobiol.* **24:** 1065–1079.

Rannels, S. R., and Jefferson, L. S. (1980). Effects of glucocorticoids on

muscle protein turnover in perfused rat hemicorpus. *Am. J. Physiol.* **238:** E564–E572.

Richard, I., Broux, O., Allamand, V., Fougerousse, F., Chiannilkulchai, N., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., and Roudaut, C. (1995). Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **81:** 27–40.

Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S., and McNiece, I. (1994). Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol. Cell. Biol.* **14:** 1431–1437.

Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H., and Barde, Y. A. (1992). Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature* **360:** 757–759.

Sorimachi, H., Ishiura, S., and Suzuki, K. (1997). Structure and physiological function of calpains. *Biochem. J.* **328:** 721–732.

Tews, D. S., Goebel, H. H., Schneider, I., Gunkel, A., Stennert, E., and Neiss, W. F. (1997a). Expression profile of stress proteins, intermediate filaments, and adhesion molecules in experimentally denervated and reinnervated rat facial muscle. *Exp. Neurol.* **146:** 125–134.

Tews, D. S., Goebel, H. H., Schneider, I., Gunkel, A., Stennert, E., and Neiss, W. F. (1997b). DNA-fragmentation and expression of apoptosis-related proteins in experimentally denervated and reinnervated rat facial muscle. *Neuropathol. Appl. Neurobiol.* **23:** 141–149.

Tian, W. H., Festoff, B. W., Blot, S., Diaz, J., and Hantai, D. (1995). Synaptic transmission blockade increases plasminogen activator activity in mouse skeletal muscle poisoned with botulinum toxin type A. *Synapse* **20:** 24–32.

Trachtenberg, J. T. (1998). Fiber apoptosis in developing rat muscles is regulated by activity. *Dev. Biol.* **196:** 193–203.

Tsujinaka, T., Fujita, J., Ebisui, C., Yano, M., Kominami, E., Suzuki, K., Tanaka, K., Katsume, A., Ohsugi, Y., Shiozaki, H., and Monden, M. (1996). Interleukin 6 receptor antibody inhibits muscle atrophy and modulates proteolytic systems in interleukin 6 transgenic mice. *J. Clin. Invest.* **97:** 244–249.

Tsurumi, C., Shimizu, Y., Saeki, M., Kato, S., Demartino, G. N., Slaughter, C. A., Fujimuro, M., Yokosawa, H., Yamasaki, M., Hendil, K. B., Toh-e, A., Tanahashi, N., and Tanaka, K. (1996). cDNA cloning and functional analysis of the p97 subunit of the 26S proteasome, a polypeptide identical to the type-1 tumor-necrosis-factor-receptor-associated protein-2 / 55.11. *Eur. J. Biochem.* **239:** 912–921.

Vilcek, J., and Lee, T. H. (1991). Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **266:** 7313–7316.

Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. (1992). Arbitrarily primed PCR fingerprinting of RNA. *Nucleic Acids Res.* **20:** 4965–4970.

Yan, Q., Elliott, J., and Snider, W. D. (1992). Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* **360:** 753–755.

Zhou, X. F., Rush, R. A., and Mclachlan, E. M. (1996). Differential expression of the P75 nerve growth factor receptor in glia and neurons of the rat dorsal root ganglia after peripheral nerve transection. *J. Neurosci.* **16:** 2901–2911.

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