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Analysis of Altered Gene Expression in Rat Soleus Muscle Atrophied by Disuse

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Abstract The present study involved a global analysis of genes whose expression was modified in rat soleus muscle atrophied after hindlimb suspension (HS). HS muscle unloading is a common model for muscle disuse that especially affects antigravity slow-twitch muscles such as the soleus muscle. A cDNA cloning strategy, based on suppression subtractive hybridization technology, led to the construction of two normalized soleus muscle cDNA libraries that were subtracted in opposite directions, i.e., atrophied soleus muscle cDNAs subtracted by control cDNAs and vice versa. Differential screening of the two libraries revealed 34 genes with altered expression in HS soleus muscle, including 11 novel cDNAs, in addition to the 2X and 2B myosin heavy chain genes expressed only in soleus muscles after HS. Gene up- and down-regulations were quantified by reverse Northern blot and classical Northern blot analysis. The 25 genes with known functions fell into seven important functional categories. The homogeneity of gene alterations within each category gave several clues for unraveling the interplay of cellular events implied in the muscle atrophy phenotype. In particular, our results indicate that modulations in slow- and fast-twitch-muscle component balance, the protein synthesis/secretion pathway, and the extracellular matrix/cytoskeleton axis are likely to be key molecular mechanisms of muscle atrophy. In addition, the cloning of novel cDNAs underlined the efficiency of the chosen technical approach and gave novel possibilities to further decipher the molecular mechanisms of muscle atrophy. J. Cell. Biochem. 83: 508–519, 2001.

Key words: suppression subtractive hybridization; hindlimb suspension; muscle genes

Muscle atrophy occurs every time a muscle remains inactive or its activity is reduced for an extended period of time, e.g., during limb immobilization or space flights. It appears to be a highly ordered and regulated process characterized by loss of muscle components with reduction in myofiber size, no reduction in the myofiber population, and maintenance of muscle function, with a capacity for full reversal if muscle utilization returns to normal [Edgerton and Roy, 1996; Tidball et al., 1998]. Different animal models for muscle disuse have been developed, including rat hindlimb suspension (HS). In this model, the consequences are dramatic for antigravity muscles such as the slow-twitch soleus muscle that can exhibit more than 50% atrophy within 2 or 3 weeks [Ohira et al., 1992], in association with a partial transition to a fast-twitch muscle phenotype [Diffee et al., 1991].

Adaptations to the reduction of muscle activity in the HS model were shown to be mediated by modifications in the expression of several components. One of the most spectacular is the partial replacement of the myosin heavy chain (MHC) slow-twitch type 1 isoform by neosynthesized fast-twitch type 2B and 2X isoforms [Takahashi et al., 1991; Talmadge et al., 1996]. As could be expected from the extent of the phenotype modifications, expression of several

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genes was shown to be regulated by muscle activity. Cis-acting elements mediating muscle overloading and/or unloading have already been noted in the regulatory regions of some of them [McCarthy et al., 1999]. Detection of genes regulated by muscle unloading is a potential means of studying the cascade of cellular events triggered by the level of muscle activity. We previously performed differential screening of a muscle cDNA array to isolate four cDNAs differentially expressed in HS and control soleus muscles [Cros et al., 1999]. In the present work, we adapted the recently described suppression subtractive hybridization (SSH) technology [Diatchenko et al., 1996] to enable a systematic search for genes up- or down-regulated in HS atrophied rat soleus muscle. The main advantages of SSH are that the resulting subtracted and normalized cDNA libraries are highly enriched in differentially expressed cDNAs, regardless of the expression level of the corresponding mRNAs, and experiments can be undertaken in an average equipped research laboratory.

We found a total of 36 up- or down-regulated genes in HS soleus muscles after 2 weeks of unloading. Almost all of these expression alterations are reported for the first time. The results highlight the importance and extent of the slow-to-fast twitch muscle type transition. They provide evidence of modulation of the protein synthesis/secretion pathway and the extracellular matrix/cytoskeleton axis as further important mechanisms of muscle atrophy. Interestingly, one third of the cDNAs found are novel cDNAs that have no matches with known gene sequences available in current public databases.

METHODS

Animals and Tissues

After 4 days of acclimatization, female Sprague Dawley rats weighting 200–220 g were randomly divided into two groups of 18 each for HS and control weight-bearing groups. The HS group was submitted to 14 days of suspension according to Morey [1979]. All animals were maintained on a standard diet with water ad libitum and housed in a room with regulated temperature of 22°C and light-dark cycle (12:12). HS and age-matched control animals were euthanized with a lethal dose of pentobarbital sodium. Soleus muscles were excised rapidly, immediately frozen in liquid nitrogen, and stored at −80°C. All further analyses were performed on pools of 36 right and left soleus muscles from each rat group in order to eliminate any individual variations.

Isolation of Total RNA and Poly(A)$^+$ RNA Purification

Isolation of total cellular RNA from 2–3 g pools of 36 soleus muscles was performed using the acid guanidinium thiocyanate phenol–chloroform method [Chomczynski and Sacchi, 1987]. Poly(A)+ RNA purification was carried out on 1.5 mg total RNA samples using the mRNA Separator Kit (Clontech Laboratories, Inc).

Construction and Screening of Subtracted and Normalized cDNA Libraries Using SSH

Subtracted and normalized libraries were generated using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories Inc). Libraries were first screened with subtracted probes then by reverse Northern blot. Modifications of these procedures were performed as previously described [Tkatchenko et al., 2000]. Briefly, cDNA synthesis was performed using 4 μg of poly(A)$^+$ RNA; the first hybridization was carried out for 10 h followed by the second hybridization for 16 h; the final PCR products were separated by 2% agarose gel electrophoresis and short (0.2–1) and long (1–5 kb) fragments were eluted from the gel using dialyzing bags and the QIAquick PCR purification Kit (Qiagen). They were cloned separately into pCRII-TOPO plasmid vector using the TOPO-TA Cloning Kit (Invitrogen). White bacterial colonies were picked into 96-well microtiter plates, grown for 24 h at 37°C, printed with a 96-pin tool onto BIOTRANS nylon filters (ICN Biomedicals, Inc.), and stored at −80°C. After *E. Coli* growth and DNA denaturation, filters were treated with proteinase K for 3 h at 37°C.

To prepare cDNA subtracted probes, final PCR products of subtraction steps were purified using the QIAquick PCR Purification Kit (Qiagen) and adaptors were removed. For reverse Northern blot analysis, a 10 μL volume (200 ng/μL) of each plasmid DNA was mixed with 2 mL of 1.5 M NaCl, 0.5% bromophenol blue, and 200 μL of each sample were transferred onto BIOTRANS nylon membranes using the 96-well Bio-Dot Microfiltration Apparatus (Biorad).

For Northern blot analysis, about 20 μg of total RNA was prepared from pooled soleus muscle (n = 36), differing from the pools used in other steps. mRNA expression levels were standardized according to 18S rRNA hybridization signals.

For all DNA or RNA hybridizations, 50 ng of cDNA were 32P-labeled using the random primers DNA labeling system (Gibco BRL) and probes were purified on Sephadex G50 gel filtration spin columns. HS and control cDNA probes were carefully equilibrated in terms of specific and total activities. Hybridizations were performed overnight at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, 100 mg/mL salmon sperm DNA. The membranes were then washed twice for 20 min in 1 × SSPE, 0.2% SDS at room temperature followed by three 20 min washes in 0.1 × SSPE, 0.2% SDS at 60°C and exposed to Amersham Hyperfilm with intensifying screens or to a phosphorus screen, scanned and quantified with the PCBas program (Fujix).

Rat Muscle cDNA Library Screening

A total of 10^6 phage plaques from a λgt11 rat muscle cDNA library (Clontech Laboratories, Inc.) were screened with randomly primed ^{32}P-labeled cDNA probe. After plaque purification, cDNA phage inserts were purified by polymerase chain reaction (PCR) using specific λgt11 vector amplimers. The procedures were conducted according to the manufacturer's instructions. Hybridizations were carried out as mentioned above.

Plasmid DNA Isolation, Sequencing, and Sequence Analysis

Plasmid DNA was isolated using the Nucleobond AX20 Kit (Macherey-Nagel). Automatic DNA sequencing was carried out by Genome Express SA (Grenoble, France). Sequence homology searches were performed using BLAST and ProfileScan software programs through servers at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the European Molecular Biology network (http://www.ch.embnet.org/index.html). PROSITE documentation was retrieved from the Expasy server (http://www.expasy.ch).

Immunological Detection of Collagen III

For Western blot analysis, whole soleus muscles were minced very fine and total extracts were immediately performed in 2.3% SDS, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8, at 95°C for 5–10 min. After a 13,000*g* centrifugation, the protein concentration of the supernatant was assayed with Biorad protein assay reagent. Fifty micrograms of total protein extracts were electrophoresed in the presence of 1% 2-mercaptoethanol on SDS-8% polyacrylamide slab gels. Proteins were electrophoretically transferred to nitrocellulose membrane. An affinity-purified rabbit anti-collagen III polyclonal antibody (Rockland, Inc.) was used to probe the protein blots [Matus et al., 1980]. An initial blocking step was performed in 5% nonfat milk for 5 h at room temperature. The antibody binding was detected by enhanced chemiluminescence. First and second antibodies were diluted 5,000 times before use.

For immunofluorescence analysis, 8 μm cryostat sections were labeled with the anti-collagen III antibody mentioned above and diluted 200 times. Immunocomplexes were revealed by incubation with a fluoresceine-coupled goat anti-rabbit second antibody. Both incubations were performed for 30 min at 37°C. Sections were mounted in moviol.

RESULTS

Atrophy Progress and RNA Content During 14-Day HS

After 14 days of suspension, HS soleus muscle mass was 71±4 mg (n = 36) and age-matched control soleus mass was 117±8 mg (n = 36), showing an average atrophy of about 40%. RNA contents (μg) per soleus muscle wet weight (mg) were 0.82±0.12 for HS rats and 0.86±0.06 for control rats, indicating no effect of disuse on overall RNA contents relative to soleus muscle wet weight.

SH-Based Cloning of Up- and Down-Regulated cDNAs in HS Soleus Muscle

Our cloning procedure started with the construction of normalized and subtracted cDNA libraries using the SSH technique followed by three screening steps. All steps were similarly and simultaneously performed for up- and down-regulated cDNAs. Two libraries were constructed. The first library was enriched in cDNAs upregulated in HS soleus muscle and was called the "Up" library. To make this library, a total pool of 36 HS soleus muscle cDNAs (called the tester) was normalized and

depleted of cDNAs expressed at lower or comparable levels in control soleus muscle. This was performed by hybridizing the tester with a large excess of a total pool of 36 control soleus muscle cDNAs (called the driver). In addition, we artificially depleted HS soleus muscle tester cDNAs in 2X and 2B MHC cDNAs by adding extra amounts of these cDNAs to control soleus muscle driver cDNAs in an estimated 30-fold excess. This was done to prevent a highly redundant representation of these MHC transcripts already known to be abundantly neosynthesized in HS soleus muscles [Talmadge et al., 1996]. The second library, named the "Down" library, was enriched in cDNAs downregulated in HS soleus muscle by switching the tester and driver cDNAs in the hybridizations.

Table I summarizes the quantitative data of the cloning procedure. A total of 864 *E. coli* colonies were randomly picked in each of the Up and Down normalized/subtracted libraries. The first screening involved colony differential screening with the Up and Down subtracted cDNA probes. Only *E. coli* colonies giving no hybridization signal with one subtracted probe, and a positive hybridization signal with the probe subtracted in the opposite direction, were selected. The second screening step involved one-end single run sequencing of routinely more than 450 base pairs of the selected *E. coli* plasmid cDNAs. It allowed the elimination of redundant cDNAs and the few empty plasmid vectors. The 2X and 2B MHC cDNAs represented only 10% of the cDNA population; they were excluded from further analysis and are not included in the final number of selected cDNAs (Table I). The last screening step was a quantitative reverse Northern blot comparison of the mRNA expression level in HS and control soleus muscles of the 89 selected non-redundant cDNAs, which were arrayed on duplicate identical low-density membranes (Fig. 1). The hybridization signals detected on phosphorus screens and given by each of the two total pool cDNA probes were standardized by dividing each spot signal intensity by the total of all spot signals for the same membrane. The difference between total signals from each membrane was $< 20\%$. Based on our experience on the reproducibility of quantitative hybridizations, > 1.5-fold differences were considered highly significant since differences as low as 1.3-fold could be reproducibly and significantly detected in three similar independent experiments (unpublished observations). Therefore, cDNAs with a ≤ 1.5 difference of expression between HS and control soleus muscles were not retained.

Characteristics of the cDNAs Found With Significant Altered Expression in HS Soleus Muscle

The final 34 selected cDNAs are listed in Table II, with their expression modifications in HS soleus muscle measured by ratios of reverse Northern blot hybridization signals given by HS versus control soleus muscle probes. This list does not mention two additional differential cDNAs, i.e., the 2X and 2B MHC cDNAs, because of their elimination after the sequencing step, although they were strongly upregulated cDNAs. They were eliminated after the sequencing screening because of their higher redundancy and already known upregulation.

A first set of 14 cDNAs had $\geq 96\%$ homology to already known rat cDNAs and was considered identical to these cDNAs, because of the sequencing errors introduced by one-run sequencing. A second set of 10 cDNAs was highly homologous ($\geq 85\%$ homology) to already known mouse, hamster, taurus or human cDNAs—we considered them as rat cDNA equivalents that were newly cloned in rat. A third set of 10 cDNAs had sequences without any significant matches to known gene sequences in available public databases after complete sequencing of the cDNA inserts, and these cDNAs were considered as novel cDNAs. The cDNAs were classified in eight categories on the basis of their known or unknown cellular functions. Interestingly, very good homogeneity was observed in the up-or

TABLE I. Amounts of Selected Clones After Each Step of the Cloning Procedure

	Up library	Down library
Randomly picked colonies	864	864
Colonies selected after differential screening with subtracted probes	55	71
cDNAs selected after sequencing	26	63
cDNAs selected after reverse Northern blot	8	26

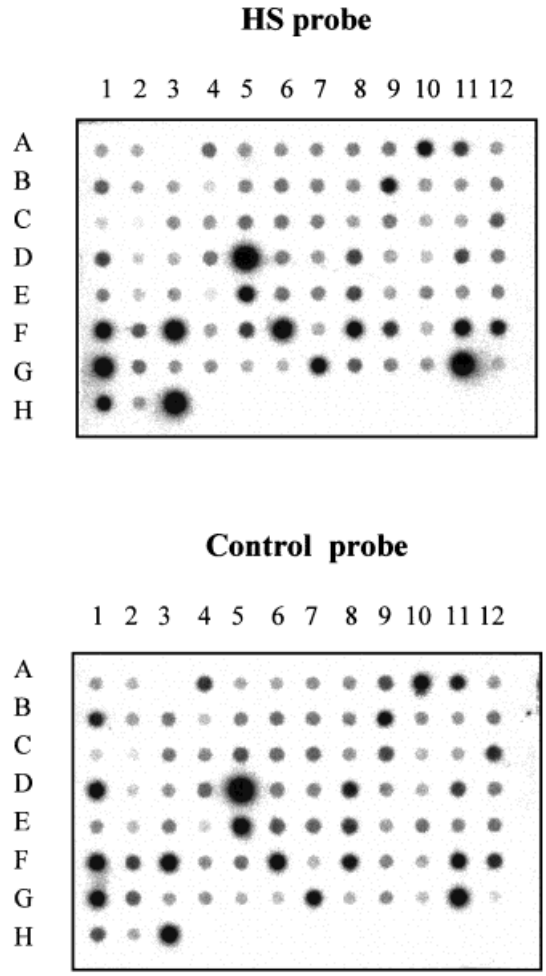

Fig. 1. Reverse Northern blot quantitative hybridization. Two hundred nanograms of the 89 selected plasmid cDNA clones were spotted onto membranes and identical membranes were hybridized in parallel with ^{32}P-labeled total cDNA pool probes prepared from 36 HS (HS probe) and control soleus muscles (Control probe). Hybridization signals were visualized and quantified after exposure to a phosphorus screen. Further standardization was done by dividing each spot signal intensity by the total of all spot signals from each membrane. The resulting ratios between two equivalent spots of each membrane are reported in Table II. Only cDNAs at the A3 and H4 positions did not give a detectable signal. No cDNAs were spotted at the other no-signal positions. Note that the printed picture does not accurately reflect the signal intensity linearity obtained with the phosphorus screen.

down-regulations of each category. So-called cDNAs with unknown/uncertain function included those that were only recently described in one article and without any certain function.

Comparative Expression of a 12-cDNA-Collection Sample Measured by Northern Blotting

To assess the reliability of data obtained using reverse Northern blot, quantitative Northern blot analyses were performed using total RNAs prepared from two other 36 soleus muscle pools rather than the pools used in the previous steps. Twelve up- or down-regulated cDNAs were chosen, with various expression levels and various differential expressions between HS and control soleus muscles (Fig. 2). The Northern blot results were very similar to the reverse Northern blot results (quantitative data not shown). As expected, the few discrepancies concerned the cDNAs that hybridized to several messengers since they were not separated on the reverse Northern dot blots. This was especially the case for the novel 03A12 cDNA. It was found to be 1.7-fold upregulated by reverse Northern blot (Table II) and hybridized on Northern blots to three mRNAs, which were 4.1-, 3.9-, and 3.7-fold upregulated from the shortest to the longest sizes. The upregulation was therefore higher when measured on the separated transcripts.

Downregulation of Collagen III Protein in HS Soleus Muscle

One collagen subunit mRNA was found to be highly downregulated in our experiments, based on the downregulation of pro alpha 1 collagen type III (Table II). Because of the crucial role of collagen in extracellular matrix, we investigated the level of expression of collagen III protein. It was comparatively examined in total protein extracts and on cryostat sections from three HS and three control soleus muscles. Western blots and immunofluorescent analysis were carried out with the same purified anti-collagen III polyclonal antibody (Fig. 3). The 138 kDa collagen III band was found to be very faint on HS soleus muscle Western blots and approximately 3.5-fold weaker than on control blots (left panel). This ratio was calculated after lane loading standardization on the basis of either the total protein concentration or the actin band intensity. Although immunofluoresent analysis is not a very efficient quantitative method, a high dilution of the anti-collagen III antibody gave a clearly differential staining between HS soleus muscle and control sections (right panel). Using a 1/200 antibody dilution, an extracellular matrix specific staining was still seen on control soleus muscle sections but almost no staining could be detected on HS soleus muscle sections. The same results were obtained with all the soleus muscles tested. Altered collagen mRNA

TABLE II. Gene Expression Alterations Found in HS Soleus Muscles in Addition to 2X and 2B MHC Upregulations

Dot position (Fig. 2)	Clone name	Identity	Sequence homology (%)	HS upregulation (fold increase)	HS downregulation (fold decrease)
Sarcomere organization					
F3	02D11	Troponin I (fast skeletal muscle isoform)	99 (r)*	2.5	
F6	03B06	Alpha-tropomyosin	99 (r)	2.5	
G8	13D12	Myosin binding protein C (fast skeletal muscle isoform)	89 (h)	2	
H1	14C04	Alpha-actinin-3	96 (m)	2.5	
Calcium homeostasis					
F9	04A03	Parvalbumin	100 (r)	2.5	
G11	13E08	(SERCA1a) sarco/endoplasmic reticulum Ca^{2+} transport ATPase 1a	100 (r)	3.3	
Energy metabolism					
C12	15G01	Succinyl CoA:3-oxoacid CoA transferase precursor	85 (h)		2.2
C5	16E07	NADH dehydrogenase subunit 4	89 (h)		2.2
D8	16G01	Dihydrolipoamide succinyltransferase	96 (r)		2
Synthesis, maturation, and intracellular transport of proteins					
A10	07B03	Heat shock protein 70	99 (r)		2
C3	10D05	Coat protein gamma-cop	87 (t)		1.6
C6	10H06	Amino acid transporter system A2	100 (r)		1.7
D7	16F09	Heat shock protein 105	92 (m)		1.9
E2	17B09	DnaJ-like protein	100 (r)		1.6
F2	18H06	Ran-binding protein 5	92 (h)		1.7
G9	13E02	SEC14L	87 (h)	2.5	
Extracellular matrix and cytoskeleton					
D9	16G05	Laminin B2 chain	94 (m)		1.7
B2	17B01	Pro alpha 1 collagen type III	100 (r)		4.3
E6	17G10	ABP-280 (actin-binding protein 280)	92 (h)		2.3
Transcription and translation regulation					
A11	07F08	Nrf1	96 (m)		1.8
E5	17E08	CNBP (cellular nucleic acid binding protein)	99 (r)		1.8
cDNAs with unknown/uncertain function					
A4	06D12	Adapt78	86 (hm)		2.8
C5	10G08	REX-3	89 (m)		2.1
D3	16E01	Dri 27/ZnT4 protein	99 (r)		1.7
Novel cDNAs					
F5	03A12			1.7	
A9	06H12				2.2
B3	08E04				2.3
B4	08F01				2.7
B8	09C01				1.6
C7	15B05				1.9
C9	15D10				2.6
E3	17C01				2.1
E7	17H01				1.8
E8	18A03				1.7

*r, rat; m, mouse; h, human; t, taurus; hm, hamster.

expression was therefore followed by parallel changes in collagen protein expression.

Tissue Profile Expression of the Differential Novel mRNAs

Two thirds of the novel cDNAs found with altered expression closely matched EST sequences available in public databases. Interestingly, several cDNAs matched only ESTs that were derived from skeletal muscle libraries, which suggested muscle-restricted expression. As a first step to determine the tissue expression patterns of the novel genes found in this work, we investigated their expression in several tissues by Northern blot analysis (Fig. 4). In addition to demonstrating

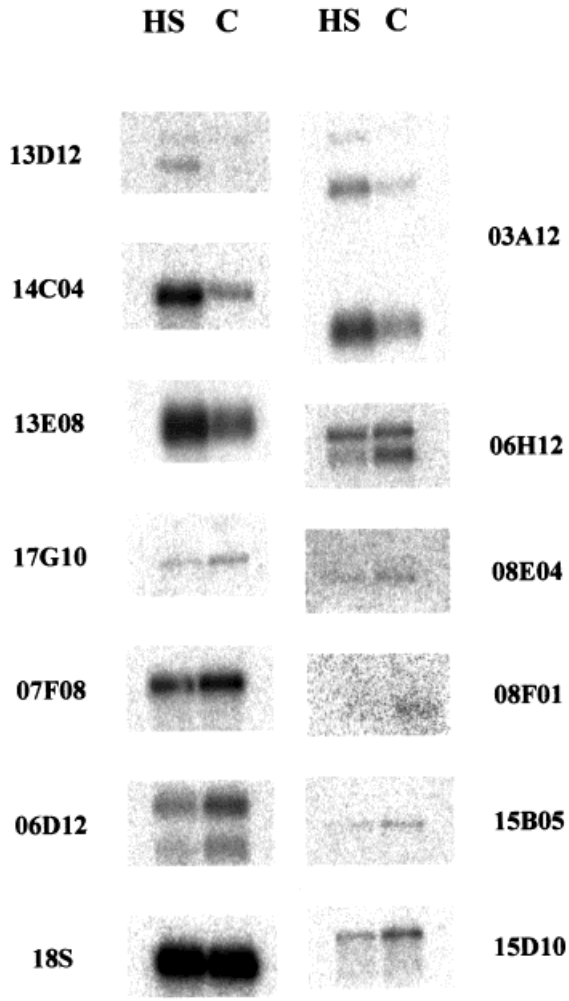

Fig. 2. Northern blot analysis of mRNAs differentially expressed in HS soleus muscles. Twenty micrograms of total RNAs isolated from 14 days HS soleus (HS) and age-matched control (C) were electrophoresed, blotted, hybridized with the listed ^{32}P-labeled cDNA probes, and exposed to a phosphorus screen. cDNA probes are presented in the same order as in Table II. A mammal-specific 18S ribosomal RNA oligonucleotide was used for lane hybridization signal standardization. Note that the printed picture does not accurately reflect the signal intensity linearity obtained with the phosphorus screen.

the presence of specific RNA messengers, the data confirmed the expected muscle preferential expression of most of these genes. In Figure 4, Northern blot hybridization signals are organized from the most skeletal muscle specific (top) to the most ubiquitous expressions (bottom). The strongest expression was found in skeletal and cardiac muscles for most of the genes. The expression was restricted to skeletal muscle or striated muscles for half of them, and only the last two RNA messengers were found in all tissues analyzed. 03A12 hybridized to a major transcript in heart, skeletal muscle, and testis; a second transcript was present only in heart and skeletal muscle and a third transcript was detected only in skeletal muscles, which suggests possible tissue-specific alternative splicing in these three tissues.

09C01 Gene Encodes a Novel Predicted Calcium-Binding Protein

The 09C01 ubiquitous mRNA was 2.4 kb long and the cDNA clone contained 750 bp. We tried to clone a longer cDNA fragment in order to predict a potential function of the encoded protein. A rat skeletal muscle cDNA library was screened with the 09C01 cDNA fragment. A 1670 bp cDNA fragment was isolated. Interestingly, its sequence perfectly matched many mouse ESTs clustered in the Mn.86555 Unigene contig. This contig extended the sequence with 220 bp at the 5′ end. The total 1890 bp sequence is presented in Figure 5. This sequence does not represent the full-length sequence expected from the 2.4-kb length of mRNA, but contains a clear open reading frame encoding a 432 amino acid residue protein. This novel protein presents a central portion, from residue 79 to 399, with 44% identical amino acid residue positions with respect to the CG4662 *Drosophila melanogaster* gene product (Accession No. AAF55702). The *Drosophila* protein sequence was released very recently but without any functional annotation [Adams et al., 2000]. It contains 438 amino acid residues, which is very close to the 09C01 murine protein. The significant similarities between the two proteins validate the 09C01 protein. Two calcium-binding EF-hand motifs were found in the O9C01 protein and are underlined in Figure 5 (PROSITE database Accession No. PS00018). The 09C01 protein is thus very likely a novel member of the large calcium-binding protein family.

DISCUSSION

To identify genes with altered expression in rat HS soleus muscles, we screened two equalized soleus muscle cDNA libraries subtracted in opposite directions using SSH technology [Diatchenko et al., 1996]. Considering the importance of gene expression regulation at the transcriptional level, and the high homogeneity of gene expression alterations found in each functional category, the gene expression

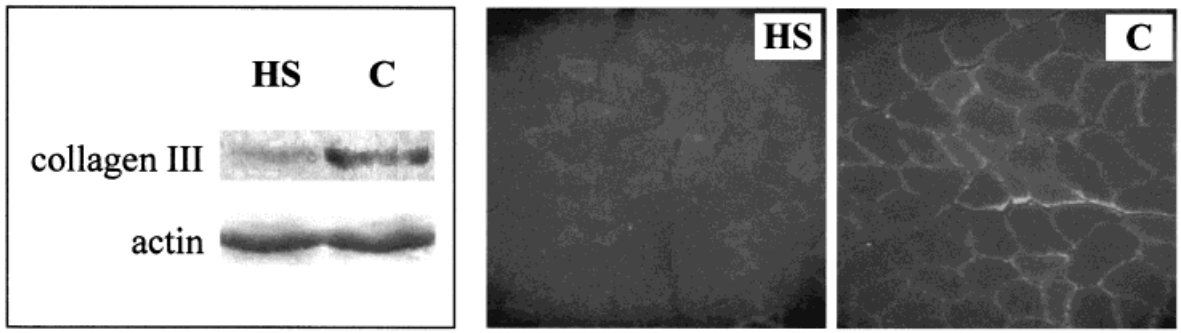

Fig. 3. Comparative protein expression of collagen III. Left panel: representative Western blots of total protein extract from HS and control (C) soleus muscles probed with an anti-collagen III antibody and revealed by enhanced chemiluminescence. Equal lane loading was verified on the blots with the actin band visualized by amido black. Right panel: representative cryostat sections of HS and control soleus muscles successively incubated with an anti-collagen III antibody and a fluorescein-coupled goat anti-rabbit immunoglobulin. The anti-collagen III antibody was diluted 200 times to optimize the labeling difference between HS and control sections. Note the much fainter labeling in the HS soleus muscle section that contains smaller sized fibers than the control section. The pictures were taken under identical conditions at a magnification of 400×. The same Western blot and immunofluorescent results were obtained with the three HS and three control soleus muscle tested.

modifications listed in this work provide important clues about molecular alterations induced by disuse and leading to muscle atrophy. In addition, we showed that one of the strongest gene expression alterations noted in this work, i.e., collagen III downregulation, was followed by strong downregulation of the translated protein. Identification of already known up- or down-regulated genes in muscle atrophy could be considered as a positive control of our results. This is the case for upregulation of 2X and 2B MHC [Cros et al., 1999] and the sarco/endoplasmic reticulum Ca^{2+}-transport ATPase 1a [Schulte et al., 1993]. All other alterations of gene expression found in this study are reported for the first time with this experimental model. They substantially increase the known population of genes whose expression is affected by muscle restriction of activity, thus stressing how much muscle activity contributes to gene regulation.

Expression Alteration of Known Genes

Three mechanisms of muscle atrophy are mainly highlighted at the molecular level according to the number of genes found with altered expression in each category.

i) A slow-twitch towards a fast-twitch partial transition has been widely demonstrated in the slow-twitch soleus muscle after HS [Diffee et al., 1991; McDonald et al., 1994]. However, the exact extent of this shift is still unknown in terms of identity of the involved components. The present results widen the spectrum of genes implied in this transition. Genes from the "sarcomere organization" and the "calcium homeostasis" categories were homogeneously found upregulated in HS soleus muscle. The 2X and 2B MHC genes should also be listed in the "sarcomere organization" category since they had been artificially depleted from the "Up" library and excluded from the analysis. These two categories are essentially made of fast-twitch isoform transcripts; only parvalbumin has no known fast-twitch isoform, but its transcript is more expressed in fast-twitch than in slow-twitch skeletal muscle fibers [Huber and Pette, 1996]. The upregulation of fast-twitch isoform transcripts also very likely includes alpha-actinin-3 and alpha-tropomyosin. The skeletal muscle isoform alpha-actinin-3 was detected only in a subset of type 2 fast muscle fibers, suggesting a fast-twitch isoform [North and Beggs, 1996]. The 03B06-cDNA alpha-tropomyosin clone encodes exons 1, 3, 4, and 5; this set of exons is found in both fast-type-muscle-fibers and nonmuscle transcripts, which only differ by exon 11 [Wieczorek et al., 1988]. Therefore, the 03B06 clone may be part of the fast-type-fiber transcript. The dihydropyridine receptor gene should be added to our "calcium homeostasis" category, since it was found to be 2-fold upregulated in the same animal muscle unloading model [Kandarian et al., 1992]. In contrast, the "energy metabolism" category of genes identified here with an altered expression was found to be homogeneously downregulated, this included genes involved in oxidative energy

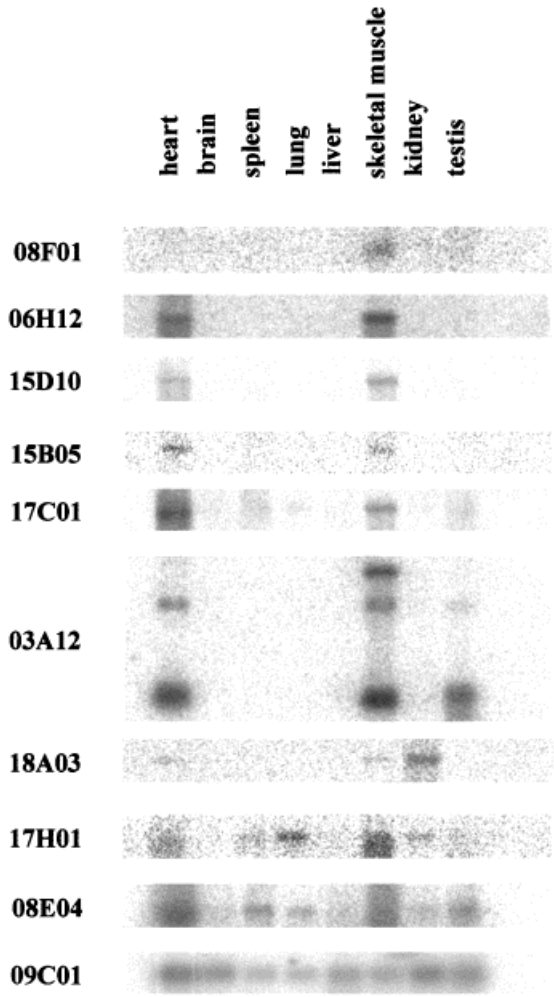

Fig. 4. Novel mRNA tissue expression analyzed on rat adult multiple tissue Northern blots (Clontech Laboratories Inc). Each lane contained 2 μg of Poly(A)$^+$ RNA and equivalent loading was checked by β actin probe hybridization (not shown). Signals were detected on an imaging plate and are ranked in decreasing order of skeletal muscle expression specificity. The sizes of the RNA messengers are (kb): 1.35, 4.2, 11, 11.5, 5.8, 3.8–2.8–1.2, 7, 6.6, 3.5, and 2.4, respectively.

metabolism like the mitochondrial subunit 4 of NADH dehydrogenase and the dihydrolipoamide succinyltransferase citric cycle enzyme. This is in full agreement with the less aerobic energy supply of fast-twitch muscle fibers [Thomason and Booth, 1990]. The phenotype transition to a more fast-twitch type muscle is thus sustained by a coordinated expression regulation of several functional category genes. In addition to the reduction of energy production by oxidative phosphorylation, downregulation of the succinyl CoA:3-oxoacid CoA transferase precursor gene suggests a decrease in oxidative energy production by a more secondary pathway, i.e., the oxidation of ketone bodies, which is first mediated by this enzyme.

ii) Protein synthesis seems to be downregulated at both upstream and downstream levels of the translation process. Amino acid transporter A2 is involved in the short-chain neutral amino acid transport [Sugawara et al., 2000]. Ran-binding protein 5 contributes to the nucleocytoplasmic transport pathway and mediates nuclear import of ribosomal proteins [Jakel and Gorlich, 1998]. Its mRNA downregulation suggests a reduction in the assembly of ribosomal subunits in the nucleolus and therefore a reduction in the activity of the translational machinery. At the other end of the protein synthesis process, downregulation of the important heat shock protein 70 and 105 genes and the cochaperone DnaJ-like protein gene could also be interpreted in this context. This list could also contain the alpha B-crystallin gene, encoding a small heat shock protein with molecular chaperone activity preferentially expressed in type 1 skeletal muscle fibers [Groenen et al., 1994; Neufer and Benjamin, 1996], and it was also found to be downregulated in HS rat soleus muscle [Atomi et al., 1991]. If fewer chaperones are present, the folding, assembly, and translocation of nascent proteins across intracellular membranes cannot be performed with the same yield. The observations of Ku et al. [1995] suggested a complementary alternative to the hypothesized HSP downregulation role in protein synthesis. HSP 70 stabilizes the translation complex and facilitates translation; its downregulation would thus slow protein elongation. Interestingly, Naito et al. [2000] recently demonstrated a positive correlation between an experimental upregulation of heat shock proteins and a decrease in HS soleus muscle atrophy, stressing the role of heat shock proteins in the control of muscle atrophy induced by reduced contractile activity. Finally, another aspect of protein synthesis could be altered with downregulation of the coat protein gamma-cop gene. The encoded protein is implicated in vesicular transport of the protein early synthesis/secretory pathway [Harter et al., 1996], and a decrease in any aspect of this transport would mean a decrease in protein post-translational maturation and/or delivery of proteins to their final destinations. Downregulation of essential genes involved in protein synthesis, maturation, and transportation thus appears to be a

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                M   A   A   A   A   G   R   S   A   W   L   A   A   W   G     15
cttccgcaaag ATG GCG GCG GCT GCG GGA AGA AGC GCT TGG CTG GCG GCC TGG GGC    56
 G   R   L   R   R   G   L   A   A   G   R   R   A   V   P   T   R   G     33
GGA AGG TTG CGG CGC GGG CTC GCG GCC GGC CGA CGA GCT GTG CCG ACT CGC GGC   110
 P   L   A   A   A   V   A   G   V   A   L   A   G   A   G   A   A   W     51
CCT CTC GCA GCG GCT GTG GCC GGG GTA GCC CTG GCA GGC GCA GGA GCG GCA TGG   164
 H   H   G   R   V   K   A   A   A   R   E   G   S   R   T   V   S   A     69
CAT CAC GGC CGC GTG AAA GCA GCC GCG CGC GAG GGC TCC CGA ACG GTG TCG GCG   218
 Q   K   N   Y   L   G   P   I   E   K   L   S   L   R   K   Q   R   F     87
CAG AAA AAT TAT TTG GGA CCA ATA GAG AAA CTT TCT CTC CGC AAA CAG CGG TTC   272
 M   Q   F   S   S   L   E   H   D   G   E   Y   Y   M   T   P   R   D   105
ATG CAG TTT TCA TCC CTG GAG CAC GAC GGA GAG TAT TAC ATG ACA CCC CGA GAC   326
 F   L   F   S   V   M   F   E   Q   V   E   R   K   T   L   V   K   K   123
TTC CTC TTC TCA GTC ATG TTT GAG CAA GTG GAG CGT AAA ACA CTG GTC AAG AAG   380
 L   A   K   K   D   I   E   D   V   L   S   G   I   Q   T   A   R   C   141
CTG GCA AAA AAG GAT ATC GAG GAT GTA CTG TCA GGA ATC CAA ACA GCA CGC TGT   434
 G   S   T   F   F   R   D   L   G   D   K   G   V   I   S   Y   T   E   159
GGA TCA ACA TTT TTT AGA GAC CTG GGT GAT AAA GGG GTA ATT TCA TAT ACC GAG   488
 Y   L   F   L   L   T   I   L   T   K   P   H   S   G   F   H   V   A   177
TAT CTT TTC TTG CTT ACA ATC CTC ACA AAA CCT CAC TCT GGG TTC CAT GTT GCT   542
 F   K   M   L   D   V   D   G   N   E   M   I   E   R   K   E   F   V   195
TTT AAA ATG CTG GAT GTG GAT GGG AAT GAG ATG ATC GAG AGG AAG GAG TTT GTT   596
 K   L   Q   K   I   I   S   K   Q   D   G   F   K   T   V   K   T   N   213
AAG CTG CAG AAG ATC ATA AGT AAA CAA GAT GGC TTC AAG ACA GTG AAG ACT AAT   650
 E   T   E   Y   Q   D   P   T   V   K   E   P   G   V   N   T   T   L   231
GAG ACA GAA TAC CAG GAC CCA ACA GTG AAA GAG CCT GGC GTT AAC ACA ACC CTT   704
 Q   V   R   F   F   G   K   R   G   E   K   K   L   H   Y   K   E   F   249
CAA GTG CGT TTC TTT GGG AAA AGA GGA GAA AAA AAA CTC CAT TAT AAA GAA TTT   758
 R   R   F   V   E   N   L   Q   T   E   V   Q   E   M   E   F   L   Q   267
CGA AGA TTT GTG GAA AAT TTA CAA ACA GAA GTT CAA GAA ATG GAA TTC CTT CAG   812
 F   S   K   G   L   N   F   M   R   K   E   D   F   A   E   W   L   L   285
TTC TCT AAA GGC CTG AAT TTC ATG AGA AAA GAA GAC TTT GCA GAA TGG CTA CTT   866
 F   F   T   N   T   E   N   K   D   I   Y   W   R   N   V   R   E   K   303
TTC TTC ACT AAC ACT GAA AAT AAA GAT ATT TAC TGG AGA AAC GTG AGA GAA AAG   920
 L   S   V   G   E   S   I   S   L   D   E   F   K   S   F   C   H   F   321
TTG TCA GTA GGA GAG AGC ATT AGT TTG GAT GAG TTC AAG TCC TTT TGC CAT TTT   974
 T   T   H   L   E   D   F   A   I   A   M   Q   T   F   S   L   A   H   339
ACA ACG CAT TTG GAA GAC TTT GCT ATC GCC ATG CAA ACG TTT AGT TTA GCT CAT  1028
 R   P   V   R   L   A   E   F   K   R   A   V   K   V   A   T   G   Q   357
CGC CCT GTG CGG CTG GCA GAG TTC AAG AGA GCT GTG AAG GTA GCG ACT GGA CAG  1082
 E   L   S   D   N   L   L   D   T   V   F   K   I   F   D   L   D   G   375
GAG CTC TCG GAC AAT CTT CTG GAC ACC GTC TTC AAG ATC TTT GAC CTG GAC GGG  1136
 D   E   C   L   S   H   G   E   F   L   G   V   L   K   N   R   M   H   393
GAC GAG TGC CTC AGC CAC GGC GAG TTC CTT GGG GTA CTG AAA AAC AGG ATG CAC  1190
 R   G   L   W   V   S   Q   Q   Q   S   V   Q   E   Y   W   K   C   V   411
CGA GGC TTA TGG GTG TCA CAG CAG CAG AGT GTG CAA GAA TAC TGG AAG TGT GTG  1244
 K   K   E   S   I   K   G   V   K   E   A   W   R   Q   Q   A   G   K   429
AAG AAG GAA AGC ATC AAG GGA GTA AAG GAA GCC TGG AGA CAA CAA GCC GGC AAG  1298
 G   P   F                                                               432
GGC CCC TTC  taaggggaagctgatggagctccaggagcccagaaagtggtttttccaagagctgactg 1366
tttctatgtcttcattgagtttcttttgagtgtgaagatgtgaagactcaccttctgatttgggggatttgg 1438
gagggatgacgggaggtgaataagtgtgtgccacagtgggaagtcagaggccagcctgtaggagctggttct 1510
ctctgtctaccatgggagtcccagagattgaactcaggtctcgagctttgacatcaagtgcctttacctgat 1582
gagccatctcaccagctccgactccatcatttcttggtgaattttcattacacaaagtattccttttatgaaa 1654
atacacactgctctattaaagacttgtgtacaaagtgtgctttctggagggctaaacaagtcagggaaggaa 1726
cttcattcccaagacaggtactgatcccagaagccgtgatagcttagggaatacttgcagttaaaggcttgc 1798
tccagtgttctttgctccctgccttgaaatagcacataatggcagtgtccggtctggcagcaggatgaagta 1870
tttacattgatacaaaaaaa                                                    1890
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Fig. 5. 09C01 cDNA and predicted encoded protein sequences. Codons are in upper case, the first ATG codon is in bold, the stop codon and the two putative calcium-binding EF-hand motifs are underlined.

basic component of the muscle atrophy process. Consequently, the quantitative repression of numerous proteins would be due to a decrease in the translation rate and post-translational events, such as protein folding, maturation, and transport controlled by a limited number of downregulated genes. From this global viewpoint, it is more difficult to interpret the upregulation of the only SEC14L gene, another gene involved in the intracellular transport system. This gene encodes a phosphatidylinositol/phosphatidylcholine transfer protein essential for vesicle budding from the Golgi complex [Sha et al., 1998]. It could be speculated that the

above-mentioned downregulations also cause compensatory upregulations.

iii) Genes specific to the extracellular matrix and cytoskeleton were found to be another main downregulated target in muscle atrophy. This was observed for two genes encoding major components of the extracellular matrix, i.e., pro alpha 1 collagen type III and laminin B2 chains. The high collagen mRNA downregulation was shown by Western blot and immunofluorescent analysis to be followed by downregulation of the encoded protein. Multiple cis-acting elements were recently identified in the laminin B2 chain gene promoter, indicating that this gene transcription is highly regulated by several transcription factors from different signaling pathways [O'Neill et al., 1997]. These results show that a decrease of extracellular matrix components contributes to muscle atrophy along with the decrease in myofiber component amounts. Regarding the cytoskeleton, an actin-binding protein encoding gene, i.e., actin-binding protein 280, was also found to be downregulated. The encoded protein is located in the peripheral cytoplasm where it links the actin cytoskeleton to membrane glycoproteins [Gorlin et al., 1990]. Downregulation of this category of genes could be related to the lower extent of mechanical forces applied to the non-contracting HS soleus muscle, which does not need the same mechanical protection. This interpretation is supported by the mechanoprotection function provided by the actin-binding protein 280, as observed by Glogauer et al. [1998].

Novel Gene Expression Alteration

Finally, the cloning of 10 novel cDNAs highlighted the efficiency of the cloning strategy for isolating novel genes. Interestingly, most of the novel genes that were found with altered expression in HS soleus muscles exhibited restricted or preferential expression in striated muscles. This suggests that the molecular mechanisms , which determine the muscle atrophy phenotype, are largely muscle specific. Besides alteration of ubiquitous mechanisms such as protein synthesis and maturation, several muscle-specific components are involved in this process. As expected, this specificity also applied among known genes found in this study with, for instance, the alteration of several genes encoding muscle contractile proteins. The cloning of novel cDNAs considerably boosts the interest of our global approach and paves the way to many further investigations. All of these novel muscle cDNAs were shown to hybridize to specific RNA messengers. Because of the important cellular functions affected by muscle atrophy, it could be assumed that some novel genes might have very significant roles and facilitate further analysis of molecular mechanisms involved in skeletal muscle atrophy. As an example, we further analyzed the novel 09C01 gene. The sequence of the predicted translated product confirmed the novelty of this finding. The existence of two calcium-binding EF hand motifs, which are shared by numerous proteins with an already clearly established function, strongly suggests a calcium regulated role for the 09C01 protein. Considering the importance of cellular functions mediated by calcium in muscle fiber, we are currently investigating the functions of this novel gene. Such studies may ultimately lead to identification of new therapeutic targets for controlling muscle mass in very common muscle activity reduction situations.

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