Changes in mRNA expression profile underlie phenotypic adaptations in creatine kinase-deficient muscles

A.J.C. de Groof[a], B. Smeets[a], M.J.A. Groot Koerkamp[b], A.N. Mul[b], E.E.W. Janssen[a], H.F. Tabak[b], B. Wieringa[a,*]

[a] *Department of Cell Biology, NCMLS University Medical Center, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands*
[b] *Department of Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

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Abstract We have studied the mechanisms that regulate the remodeling of the glycolytic, mitochondrial and structural network of muscles of creatine kinase M (M-CK)/sarcomeric mitochondrial creatine kinase (ScCKmit) knockout mice by comparison of wild-type and mutant mRNA profiles on cDNA arrays. The magnitudes of changes in mRNA levels were most prominent in M-CK/ScCKmit ($CK^{-/-}$) double mutants but did never exceed those of previously observed changes in protein level for any protein examined. In gastrocnemius of $CK^{-/-}$ mice we measured a 2.5-fold increase in mRNA level for mitochondrial encoded cytochrome *c* oxidase (COX)-III which corresponds to the increase in protein content. The level of the nuclear encoded mRNAs for COX-IV, H^+-ATP synthase-C, adenine nucleotide translocator-1 and insulin-regulatable glucose transporter-4 showed a 1.5-fold increase, also in agreement with protein data. In contrast, no concomitant up-regulation in mRNA and protein content was detected for the mitochondrial inorganic phosphate-carrier, voltage-dependent anion channel and certain glycolytic enzymes. Our results reveal that regulation of transcript level plays an important role, but it is not the only principle involved in the remodeling of mitochondrial and cytosolic design of $CK^{-/-}$ muscles.

Key words: Creatine kinase; Expression profiling; Metabolic signaling; ATP production; Mitochondrial enzyme; Cytochrome *c* oxidase

1. Introduction

Muscle tissue has a high plasticity and is capable of reorganizing its metabolic and structural features along with internal and environmental needs [1,2]. Thus, dynamic regulation of mRNA and protein expression is of fundamental importance. Gene expression in muscle is known to respond to chronic low-frequency stimulation, caloric restriction, hyperthyroidism, or feeding of metabolic inhibitors [3–6]. Also genetic stress in cellular energy metabolism can evoke significant changes in muscle biogenesis [7]. Many of these responses are thought to serve as adaptations to counteract the adverse effect of physiological challenge or gene mutation [1,7–9].

Previously, we have studied changes in the biogenesis of the glycolytic, mitochondrial and structural network in muscles of knockout mice that lack the creatine kinase (CK) isoenzyme subunits sarcomeric mitochondrial creatine kinase (ScCKmit) and muscle-specific cytosolic creatine kinase (M-CK) [10–12]. By understanding the mechanisms that regulate this metabolic and cytoarchitectural remodeling, one can gain insight into the signaling systems that sense the physiological consequences of mutation and help to coordinate the response. Whether these regulatory principles involve alterations in de novo synthesis or altered turnover of gene products, or both, is not known.

CKs (EC 2.7.3.2) are cytosolic and mitochondrial enzymes that catalyze the reaction $MgADP^- + PCr^{2-} + H^+ \Leftrightarrow Cr + MgATP^{2-}$. The CK/phosphocreatine (PCr) system balances adenylates at cellular sites of energy production and consumption by providing PCr as 'temporal energy buffer' and serving as 'high-energy phosphoryl shuttle' [13,14]. Thus, the system protects against effects of ischemia and anoxia, and couples mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis to metabolic demand [15]. The composition and function of the CK circuit differ between different muscle types and functional redundancy with other pathways for high-energy phosphoryl transfer, such as the adenylate kinase, the nucleoside diphosphate kinase (NDPK), and the glycolytic system has been demonstrated [13,16,17].

Using mRNA expression profiling on cDNA arrays, the transcriptional response of clusters of genes to a perturbation in the cellular state can be measured [18]. In this study, we use this technique to examine mutation and cell-type-dependent changes in the levels of individual mRNAs and correlate our findings to previous data on the content of key-metabolic enzymes in normal and CK-deficient muscles. The analysis enabled us to assess the relative importance of transcript level regulation in the response to metabolic stress induced by CK deficiency.

*Corresponding author. Fax: (31)-24-3615317; http://www.ncmls.kun.nl/celbio.
E-mail address: b.wieringa@celbi.kun.nl (B. Wieringa).

Abbreviations: ANT, adenine nucleotide translocator; CK, creatine kinase; COX, cytochrome *c* oxidase; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, insulin-regulatable glucose transporter; GPS, gastrocnemius/plantaris/soleus complex of hindlimb muscle; NDPK, nucleoside diphosphate kinase; M-CK, muscle-specific cytosolic creatine kinase; MLC2v/f, myosin light chain-2 (ventricular/fast); OXPHOS, oxidative phosphorylation; PCr, phosphocreatine; P_i, inorganic phosphate; S.E.M., standard error of the mean; SERCA, SR/ER calcium ATPase; ScCKmit, sarcomeric mitochondrial creatine kinase; UCP, uncoupling protein; VDAC, voltage-dependent anion channel

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2. Materials and methods

2.1. Animals and RNA isolation

Animal breeding and housing conformed to the Guidelines for the Care and Use of Laboratory Animals of the Dutch Council, with approval of the Institutional Animal Care and Use Committee at the University of Nijmegen. Individual gastrocnemius muscles from each of four 100-day-old C57Bl/6 or 129/Ola female control mice, or four female M-CK$^{-/-}$ single and CK$^{-/-}$ double knockout mice were dissected and total RNA was prepared using the LiCl–urea method [19]. Soleus muscles from these animals were combined in one sample for RNA preparation. One additional set of RNA samples was prepared from either pooled gastrocnemius or soleus muscles from four 100-day-old female mice (for each different genotype).

RNA preparations of the total gastrocnemius/plantaris/soleus (GPS) complex and liver of female C57Bl/6 and CK$^{-/-}$ animals of 17 and 100 days of age (two animals of each strain per age class) were used to study developmental effects on mitochondrial and nuclear gene expression.

2.2. cDNA probe synthesis

For synthesis of mixed cDNA probe, 2 μg of total RNA was reverse transcribed for 90 min at 42°C in a mixture containing 100 ng/μl oligo(dT) (Eurogentech, Seraing, Belgium), 0.5 mM d(A,T,G)TP, 4 μM dCTP, 5 mM DTT, 30 U RNAse Out, 250 U Superscript II (Life Technologies, Breda, The Netherlands) and 75 μCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Pharmacia, Roosendaal, The Netherlands) in a final volume of 20 μl (in duplicate). The quantity of ^{32}P-labeled total cDNA was assessed after purification over a Sephadex G-50 column (Amersham Pharmacia). cDNA length distribution was routinely monitored by electrophoresis on agarose gels. Before hybridization, the cDNA was treated with 20 μg/ml RNAse A in TES buffer [20] and diluted to 0.8–2.4×10^6 cpm/ml Church hybridization buffer [21]. Marker probe was generated by random primed synthesis of a 1-kb mouse insulin-regulatable aminopeptidase vp165 ds cDNA template. Specific activities of different cDNA probe preparations ranged between 1 and 10×10^6 cpm/μg.

2.3. cDNAs and EST collections

Our collection of cDNA and expressed sequence tag (EST) clones comprises a selection of 134 different mouse DNA sequences for glycolytic enzymes, mitochondrial enzymes and transporters, fatty acid metabolism enzymes, muscle regulatory factors, proteins active in Ca^{2+} signaling, glucose transport, tissue oxygenation as well as cytoskeletal components and transcription factors. Detailed information on the ESTs and construction of the cDNAs is available at www.ncmls.kun.nl/celbio. Plasmid DNAs were grown in *Escherichia coli* HB101, DH10B, DH12S, AG1 and SOLR host cells, isolated, and purified on silica-gel membranes using the Qiagen miniprep procedure (Qiagen, Hilden, Germany).

2.4. Preparation of gridded cDNA arrays on membranes

Plasmid DNAs, diluted at 0.25 μg/μl in 10 mM Tris–HCl/5 mM EDTA supplemented with sucrose (1.5%) and cresol red (0.025%), were gridded on Hybond N$^+$ membranes (sized 11×3 cm; Amersham Pharmacia). Empty plasmids served as negative controls. Macroarrays were generated using a home-built arrayer (see http://cmgm.stanford.edu/pbrown/mguide/index.html), with some modifications from the Brown group. The printhead contained 12 adjustable quilted pens (custom made) with the width of a 384-wells plate (11 cm). The machine was programmed to yield arrays on nylon filters with duplicate spot patterns of each cDNA. Speed of movements of the printhead combined with the adjustment of the pens set the delivered amount of DNA on the filter to 60–80 nl per spot (15–20 ng). With spot sizes of 700–800 μm and a large heart-to-heart distance (3 mm) ^{32}P can be used without the risk of overlapping signals.

Membrane-bound DNAs were denatured in 0.5 N NaOH/1.5 M NaCl, renatured in 0.25 N HCl, and washed in 50 mM phosphate buffer pH 7.2, before the DNA was UV cross-linked at 2 mJ/cm^2 (Stratalinker, Stratagene, La Jolla, CA, USA). Membranes were pre-hybridized overnight at 65°C in Church buffer [21] supplemented with 5×Denhart's reagent [20] and 100 μg/ml single-stranded herring sperm DNA (Boehringer Mannheim, Germany).

2.5. Hybridization, washing and autoradiography

Arrays were hybridized for 4 days at 65°C to the cDNA probe mixture in 5 ml Church hybridization buffer, supplemented with 90 000 cpm of aminopeptidase pv165 cDNA marker probe. Subsequently, the arrays were washed to a final stringency of 0.1×SSC/0.1% SDS at 68°C. Signals were captured and analyzed on a Bio-Rad GS 363 Imaging system (Bio-Rad, Hercules, CA, USA) after an exposure period of 30 min to 96 h. Signal intensity was expressed as the mean cpm of the duplicate spots (Fig. 1A) minus the intensity of the empty vector. Values at least 2-fold above background were collected at the maximum exposure time. Problems with signal saturation were avoided by determining signal intensities at shorter exposure, after which all values were normalized for exposure time.

2.6. Analysis of hybridization results

All values for signal intensities were divided by the value for the mean marker signal intensity. Based on the results of these initial analyses, a designated subset of genes was identified for which no up- or down-regulation was evident. Signal intensities for these cDNAs were subsequently used for normalization [22].

2.7. Quantitative Western blot

Total protein extracts were prepared from colateral GPS complexes of C57Bl/6 and CK$^{-/-}$ mice used for RNA analysis. Preparation of these extracts and semi-quantitative Western blots were performed as described by de Groof et al. [12].

2.8. Statistical analysis

We used a Student's *t*-test for comparison between wild-type, M-CK$^{-/-}$ and CK$^{-/-}$ gastrocnemius muscles (four individual samples, plus pooled sample: $n=5$). Results were considered significant when $P<0.05$ compared to either wild-type strain (C57Bl/6 and 129/Ola), unless stated otherwise. No statistical tests were applied to soleus data, since we used $n=2$ pooled samples.

3. Results

In order to correlate known changes in protein levels in muscles of CK-deficient animals to changes in corresponding mRNA levels, a specially designed array with 134 different cDNAs/ESTs was used. Prior knowledge on gene function, cell-type-specific distribution, isoenzyme expression, and transcript abundance was used in the selection of cDNAs and ESTs for the array. Based on the observation that there was only a 2- to 4-fold change in the level of mitochondrial and glycolytic proteins in gastrocnemius of mice with M-CK or combined M-CK/ScCKmit deficiency [12], we expected that also the corresponding mRNA levels would vary within rather narrow margins. Therefore, we decided to refrain from use of cDNA amplification procedures commonly used for high-density microarray hybridization [23].

3.1. mRNA profile of fast-twitch gastrocnemius is changed by CK absence

A representative array comparison for mRNAs from gastrocnemius muscles of C57Bl/6 and CK$^{-/-}$ mice is shown in Fig. 1A. As anticipated, we still detected residual ScCKmit and M-CK mRNA in CK$^{-/-}$ mice in our assay. For both genes, the gene targeting strategy used led to the formation of aberrant mRNAs and complete loss of M-CK and ScCKmit enzyme coding capacity as demonstrated earlier [10,11].

Normalized profiling data for fast-twitch gastrocnemius of wild-type and CK$^{-/-}$ mice are depicted in the scatter graph of Fig. 1B. Strikingly, five out of eight affected signals in gastrocnemius muscle represent mRNAs encoding mitochondrial proteins. Mitochondrial DNA encoded cytochrome *c* oxidase (COX)-III showed a 2.5-fold up-regulation. Its presence in the cDNA mixture results from annealing of oligo(dT) to A-rich

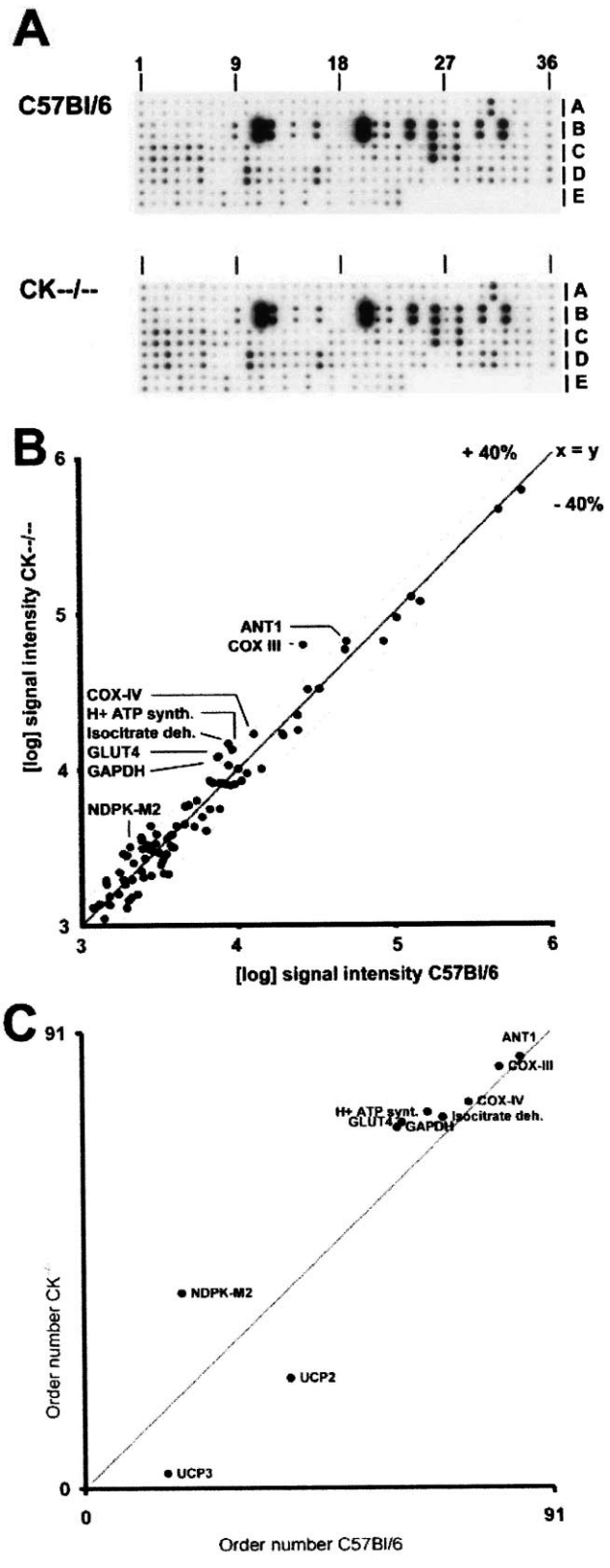


Fig. 1. A: ^{32}P images of arrays from C57Bl/6 wild-type and $CK^{-/-}$ gastrocnemius muscles. B9, ScCKmit; B11, M-CK; B20, actin; B28, COX-III; C2, COX-IV; C3, isocitrate dehydrogenase; C32, GLUT4; D3, H^+-ATP synthase-C. B: Scatter graph representation of array data of C57Bl/6 (*x*-axis) and $CK^{-/-}$ gastrocnemius ($n=4$). Axes show signal intensities after normalization. The dark solid line represents equal expression levels in wild-type and $CK^{-/-}$ samples, the gray solid line marks 40% difference in signal intensity. C: Order of appearance representation of comparative data of C57Bl/6 (*x*-axis) and $CK^{-/-}$ gastrocnemius ($n=4$). UCP3 (16,3); NDPK-M2 (19,39); UCP2 (40,22); GADPH (61,72); GLUT4 (62,73); H^+-ATP synthase-C (67,75); isocitrate dehydrogenase (70,74); COX-IV (75,77); COX-III (81,84); ANT1 (85,86).

stretches in the COX-III RNA. Nuclear DNA encoded mRNAs for COX-IV, H^+-ATP synthase-C, adenine nucleotide translocator-1 (ANT1), isocitrate dehydrogenase (all mitochondrial), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NDPK-M2 and insulin-regulatable glucose transporter (GLUT4) were 1.3- to 1.6-fold increased in $CK^{-/-}$ muscles. No significant effects were seen for these mRNAs in M-CK single mutant mice, except for the COX-III and GAPDH signals. Quantitative measurements on single RNA preparations and on pooled gastrocnemius muscle samples yielded identical results (not shown), data are therefore presented in a combined fashion in Table 1.

Because all values ranged within 96–248% of those in C57Bl/6 wild-type mice, we decided to analyze mRNA quantities also by inter-comparison in an 'order of signal intensity' plot (Fig. 1C), which renders comparison independent of the normalization procedure used. One caveat with this approach is that the magnitude of the shift in ranking position will be rather small for (the few) transcripts with a relatively high expression level (such as COX-III), and rather large for transcripts with a low expression level (where many transcripts cluster together). Consistent increases in signal intensity ranking position were again observed for all genes mentioned in Table 1. In addition, the uncoupling protein-2 (UCP2) mRNA level showed a drastic shift in ranking order. However, this observation may be non-significant as the shift was based on a decrease of only 35% at low signal intensity ($P=0.12$; $n=4$ compared to C57Bl/6 and 129/Ola). Also the UCP3 transcript level showed a 33% decrease, but with substantial differences between wild-type strains C57Bl/6 and 129/Ola (not shown). Effects of genetic background variation can therefore not be fully excluded for this latter mRNA.

3.2. mRNA profile of slow-twitch soleus is changed by CK absence

We have previously shown that the nature and magnitude of the compensatory adaptations in muscles of CK-deficient mice are strongly cell-type dependent [10–12]. Changes in mRNA transcript profiles of M-$CK^{-/-}$, $CK^{-/-}$, C57Bl/6 and 129/Ola mice from pooled slow-twitch soleus RNA (two sets) were therefore also determined. Although values for a conspicuous high number of signals were closer to background than in the gastrocnemius arrays (not shown), again several ESTs/cDNAs displaying a 1.2- to 2.5-fold change in signal intensity were identified (Table 2), including COX-III, nuclear encoded mitochondrial transcripts and GLUT4. Consistently, changes were only evident in $CK^{-/-}$ and not in M-$CK^{-/-}$ soleus, the only notable exception being ventricular myosin light chain-2 (MLC2v) mRNA which displayed a 30% and 80% increase in these mutant muscles, respectively. Interestingly, converse changes in SR/ER calcium ATPase-1 (SERCA1; −45% decrease) and SERCA2 (+33% increase) mRNA levels resulted in a relative shift in favor of SERCA2 expression in $CK^{-/-}$ soleus.

Table 1
RNA expression changes in gastrocnemius

EST/cDNA	C57Bl/6		M-CK$^{-/-}$		CK$^{-/-}$		129/Ola	
	RNA	Protein[a]	RNA	Protein[a]	RNA	Protein[a]	RNA	Protein[a]
COX-III	100 ± 5.6	nd	142 ± 5.7*,#	nd	248 ± 37*,§	nd	123 ± 6.5†	nd
Isocitrate dehydrogenase	100 ± 8.7	nd	129 ± 16	nd	145 ± 11†,¶	nd	103 ± 9.9	nd
H$^+$-ATP synthase-C	100 ± 7.3	nd	127 ± 12‡	nd	166 ± 20†,¶	nd	101 ± 14	nd
COX-IV	100 ± 10	100	116 ± 13	197	134 ± 8.3†,¶	427	96 ± 11	121
ANT1	100 ± 1.2	100	107 ± 6.4	181	133 ± 7.2*,¶	251	98 ± 11	146
GAPDH	100 ± 8.2	nd	152 ± 11*,#	nd	157 ± 19†,#	nd	104 ± 19	nd
GLUT4	100 ± 8.7	100	101 ± 5.6	160	161 ± 19†,¶	204	116 ± 5.6	161
NDPK-M2	100 ± 6.0	nd	113 ± 10	nd	158 ± 12*	nd	126 ± 14	nd

Values represent percentage ± S.E.M. ($n = 5$). *$P < 0.1$ vs. C57Bl/6; †$P < 0.05$ vs. C57Bl/6; ‡$P < 0.10$ vs. C57Bl/6; §$P < 0.01$ vs. 129/Ola; ¶$P < 0.05$ vs. 129/Ola; #$P < 0.10$ vs. 129/Ola. nd, not done.
[a]Data from de Groof et al. [12].

3.3. Developmental timing of adaptive changes in muscle mRNA profile

CK isoenzyme expression profiles in normal muscles change during maturation and growth to adulthood [13]. Adaptations in mRNA levels in response to CK deficiency may therefore have a flexible character during the first months of life. Comparison of RNA expression levels between whole GPS muscles of CK$^{-/-}$ and wild-type animals at 17 and 100 days of age (Table 3) demonstrated that up-regulation of mRNAs encoding mitochondrial proteins, MLC2v and GLUT4 is already evident early in life. Strikingly, at 17 days we found an increase in mRNA content for voltage-dependent anion channel-3 (VDAC3), and the inorganic phosphate (P_i)-carrier and 2-oxoglutarate/malate carrier. Increase in mRNA content for these proteins was not seen in animals at 100 days of age.

3.4. Are organ-related effects involved in CK-related changes in mRNA profile?

To ascertain that changes in mRNA profile are not an effect of altered endocrine signaling or whole-body metabolism, and are confined to CK-deficient muscles only, we also performed mRNA array analysis of liver from 100-day-old CK$^{-/-}$ and wild-type mice. Liver was chosen because it is central in whole-body energy metabolism [14], but should not be affected by 'internal' metabolic stress as CKs are not expressed in this organ. Comparison of mutant and control livers yielded no significant differences (Fig. 2A; quantitative data not shown). Unlike in muscle, also the balance in expression levels for COX-IV and COX-III, representatives for the nuclear and mitochondrial transcript populations, respectively, was essentially unchanged (Fig. 2B).

3.5. Linear relationship between changes in mRNA and protein levels?

Relative changes in protein (values taken from [12]) and mRNA content in CK-deficient gastrocnemius are compared in Tables 1 and 2. There is simultaneous change in protein and mRNA levels for ANT, COX-IV (see below) and GLUT4, but not for VDAC, and P_i-carrier. To analyze this phenomenon in more detail we assessed the relationship between mitochondrial COX-III and nuclear COX-IV mRNA and protein levels in greater detail. Semi-quantitative Western blot analysis of the protein content in two wild-type and two mutant GPS muscles (Fig. 2C) showed a 2.6-fold up-regulation of COX-III protein content, in line with the 2.5-fold increase in the level of COX-III mRNA in the CK$^{-/-}$ mouse. By contrast, no strict coupling between the increase in COX-IV protein and mRNA content was seen (2.6-fold vs. 1.5-fold up-regulation, respectively).

4. Discussion

We used conventional mRNA profiling on cDNA arrays to obtain insight into mechanisms that play a role in sensing of – and the response to – metabolic stress in the CK-mediated high-energy phosphoryl shuttle. Earlier our group had already uncovered important functional and structural differences between M-CK$^{-/-}$, CK$^{-/-}$ and wild-type muscles [10,11,15], most of which were associated with rather subtle changes in the level of a diverse series of mitochondrial, cytoarchitectural and glycolytic proteins [12]. Based on these findings we have postulated that most – if not all – of these changes may in fact have a compensatory character, serving to ameliorate the adverse physiological effects of gene ablation [10,11]. Similar

Table 2
RNA expression changes in soleus (mean values of two sets of pooled samples)

EST/cDNA	C57Bl/6		M-CK$^{-/-}$		CK$^{-/-}$		129/Ola	
	RNA	Protein[a]	RNA	Protein[a]	RNA	Protein[a]	RNA	Protein[a]
COX-III	100	nd	97	nd	247	nd	123	nd
Isocitrate dehydrogenase	100	nd	73	nd	117	nd	87	nd
H$^+$-ATP synthase-C	100	nd	84	nd	125	nd	94	nd
COX-IV	100	100	92	105	123	182	101	91
ANT1	100	100	91	89	138	137	91	95
GLUT4	100	100	101	145	136	155	106	137
MLC2v	100	nd	132	nd	180	nd	98	nd
SERCA1	100	nd	89	nd	55	nd	105	nd
SERCA2	100	nd	125	nd	133	nd	105	nd

nd, not done.
[a]Data from de Groof et al. [12].

compensatory remodeling, involving an increased mitochondrial density or alteration in O_2 diffusion pathway(s), has meanwhile been observed in ANT1 [7,24] and myoglobin [8,9,25] knockout mice and is a normal response to physiological challenge [1].

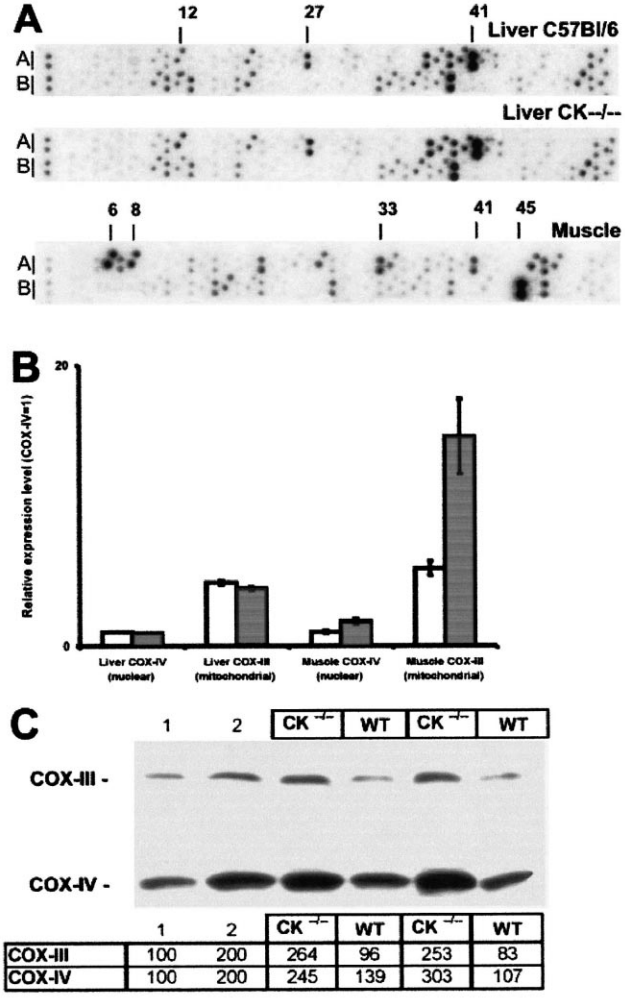
We demonstrate here that part of this plasticity is regulated by adaptation in the steady state level of mRNAs. As might have been anticipated, the magnitudes of changes in mRNA levels did never exceed those of the changes in protein level. Moreover, for most mRNAs the effects are significantly more prominent in $CK^{-/-}$ than in M-$CK^{-/-}$ mice, in accordance with the protein findings. For COX-IV, ANT, and GLUT4, changes in mRNA and protein content were significantly correlated, albeit not in a 1:1 relationship. Strikingly, although P_i-carrier and VDAC protein content were significantly (2- to 4-fold) increased, we did not detect changes in mRNA level for these proteins in 100-day-old $CK^{-/-}$ gastrocnemius. Only in 17-day-old GPS complex we did see a modest (1.5-fold) increase in the level of mRNAs for these proteins. Moreover, with the exception of GAPDH mRNA, we did not find an effect on the expression levels of glycolytic mRNAs like those for lactate dehydrogenase, aldolase or pyruvate kinase-M. Earlier we demonstrated that lactate dehydrogenase (and possibly aldolase) undergoes an isoenzyme shift which is most notable in soleus of $CK^{-/-}$ animals [12]. We may therefore have to use isoform-specific probes in future cDNA array analyses to identify any effects at the transcript level for these proteins.

For COX-IV mRNA we see that a moderate (34%) increase in mRNA level still allows a greater than 2-fold increase in protein product in $CK^{-/-}$ mice. Between changes in COX-III mRNA and protein content, a better correlation was found. Similar discrepancies between responses in nuclear and mitochondrial transcripts after electrical stimulation in muscle were reported by Williams et al. [26]. The cellular concentration of mitochondrial COX mRNAs (COX-I, -II, -III) is largely determined by mtDNA copy number, and is higher than that of nuclear encoded COX mRNAs (COX-IV) [27]. Both transcriptional and posttranscriptional regulatory principles have been proposed to explain how ultimately equimolar ratios of translation products assemble as subunits into the COX holoenzyme [28], and how protein expression levels of OXPHOS genes are adjusted to changes in energy requirements [29,30]. Taken together, our data suggest that both transcriptional and translational control of nuclear encoded mitochondrial transcripts are involved in the remodeling of muscle in our CK mutants, whereas mitochondrial encoded genes are mainly controlled by transcriptional regulation.

Table 3
RNA expression changes in GPS complexes of 17-day-old and 100-day-old mice (wild-type C57Bl/6 = 100%)

EST/cDNA	$CK^{-/-}$ 17 days[a]	$CK^{-/-}$ 100 days[b]
COX-III	142	234
Isocitrate dehydrogenase	148	154
H^+-ATP synthase-C	145	152
COX-IV	120	150
ANT1	130	124
GAPDH	98	128
GLUT4	142	138
VDAC3	148	107
P_i-carrier	145	114
2-oxoglutarate/malate carrier	145	118
MLC2v	199	129

[a]17 days: results of pairwise comparison of two pooled C57Bl/6 and two pooled $CK^{-/-}$ GPS complexes.
[b]100 days: results of comparisons between two C57Bl/6 and two $CK^{-/-}$ GPS complexes.



	1	2	CK -/-	WT	CK -/-	WT
COX-III	100	200	264	96	253	83
COX-IV	100	200	245	139	303	107

Fig. 2. A: Representative arrays from C57Bl/6 and $CK^{-/-}$ liver (100 days) and C57Bl/6 soleus muscle. A6, M-CK; A7, adenylate kinase 1; A8, ScCKmit; A12, fatty acid synthase; A27, ANT2; A33, SERCA1; A39, COX-IV; A41, COX-III; B13, hexokinase 1; B45, actin. B: COX-III and COX-IV mRNA expression levels in C57Bl/6 (white bars) and $CK^{-/-}$ liver and GPS muscle of two 100-day-old mice. Error bars indicate standard error of the mean (S.E.M.) of the signals in two hybridization experiments. The expression level of COX-IV in C57Bl/6 liver and GPS muscle was arbitrarily set to 1 for comparison. C: Quantitative Western blot for COX-III (22 kDa) and COX-IV (17 kDa). Two wild-type samples (WT: 1-fold and 2-fold protein concentration) were loaded to facilitate densitometric analysis of band intensities (WT = 100%).

We found no effects on steady state levels of the mRNAs for muscle transcription factors MyoD and MRF4, whereas myogenin and Myf5 mRNAs were not reliably detectable (data not shown). Also the mitochondrial transcription factor A (Tfam) showed no change in mRNA level, whereas NRF1, NRF2a and NRF2b could not be quantified reliably. For calcineurin, a key regulatory factor linking calcium homeostasis to gene expression, we observed no change in mRNA levels. Of course, our results do not exclude the possibility that these factors themselves do have an active role in regu-

lating the phenotypic adaptations, but effects at the transcript level are not evident.

Whatever the regulatory principle(s) involved, our profiling data give further support to the contention that the mRNA and protein changes have a compensatory role. The increase in mRNA content for several mitochondrial proteins could reflect the increased mitochondrial density and greater need for increased mitochondrial ATP production [11]. A further compensatory mechanism might involve the mitochondrial proton homeostasis, reflected in the 1.6-fold up-regulation of H^+-ATP synthase-C and 0.65-fold down-regulation of UCP2 (and UCP3). Although further study is necessary, we envisage a model where proton leak via UCP is prohibited and more protons enter the mitochondrion via the H^+-ATP synthase complex, thereby providing greater efficiency in ATP production [31]. The increase in MLC2v expression could reflect a changed isoenzyme composition of MLC2 (a possible isoenzyme shift from MLC2f to MLC2v), leading to altered energy consumption in the contractile apparatus.

In conclusion, our array data show that the observed phenotypic adaptations in CK-deficient mice are caused by a concerted remodeling of different muscle characteristics, with an important role for regulation of transcript level.

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