Origins of interspecies variation in mammalian muscle metabolic enzymes

K. M. Kocha,* C. E. Genge,* and C. D. Moyes

Department of Biology, Queen’s University, Kingston, Ontario, Canada

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Kocha KM, Genge CE, Moyes CD. Origins of interspecies variation in mammalian muscle metabolic enzymes. Physiol Genomics 43: 873–883, 2011. First published May 17, 2011; doi:10.1152/physiolgenomics.00025.2011.—Do the transcriptional mechanisms that control an individual’s mitochondrial content, PGC1α (peroxisome proliferator-activated receptor γ coactivator-1α) and NRF1 (nuclear respiratory factor-1), also cause differences between species? We explored the determinants of cytochrome c oxidase (COX) activities in muscles from 12 rodents differing 1,000-fold in mass. Hindlimb muscles differed in scaling patterns from isometric (soleus, gastrocnemius) to allometric (tibialis anterior, scaling coefficient = −0.16). Consideration of myonuclear domain reduced the differences within species, but interspecies differences remained. For tibialis anterior, there was no significant scaling relationship in mRNA/g for COX4-1, PGC1α, or NRF1, yet COX4-1 mRNA/g was a good predictor of COX activity (r² = 0.55), PGC1α and NRF1 mRNA correlated with each other (r² = 0.42), and both could predict COX4-1 mRNA (r² = 0.48 and 0.52) and COX activity (r² = 0.55 and 0.49). This paradox was resolved by multivariate analysis, which explained 90% of interspecies variation, about equally partitioned between mass effects and PGC1α (or NRF1) mRNA levels, independent of mass. To explore the determinants of PGC1α mRNA, we analyzed 52 mammalian PGC1α proximal promoters and found no size dependence in regulatory element distribution. Likewise, the activity of PGC1α promoter reporter genes from 30 mammals showed no significant relationship with body mass. Collectively, these studies suggest that not all muscle scale equivalently, but for those that show allometric scaling, transcriptional regulation of the master regulators, PGC1α and NRF1, does not account for scaling patterns, though it does contribute to interspecies differences in COX activities independent of mass.

All animals have the need to alter muscle mitochondrial content during development and in response to physiological and environmental challenges. Central to this remodeling process are transcriptional master regulators, namely peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) and nuclear respiratory factor-1 (NRF1) (20, 33, 34). Mitochondrial biogenesis requires the coordination of hundreds of nuclear genes encoding mitochondrial structural and enzymatic proteins, including the proteins that control replication, transcription, and translation of mitochondrial DNA. This onerous task is mediated by the coactivator PGC1α, which regulates suites of nuclear-encoded metabolic genes through direct and indirect association with DNA-binding transcription factors. Central to control of genes in oxidative phosphorylation are NRF1, NRF2, and nuclear hormone receptors [e.g., peroxisome proliferator-activated receptor (PPAR), retinoic acid receptors, thyroid hormone receptors]. These transcription factors confer upon an individual the capacity to change muscle mitochondrial content during development (5, 13, 24, 25), and in response to exercise (1, 3, 44), dietary manipulations (33, 37), hormonal and energetic changes (2, 18, 22, 33, 45, 46). In this study, we ask if the transcriptional mechanisms that control mitochondrial content in individuals are also responsible for maintaining differences seen between species.

Interspecies differences in the muscle metabolic phenotype can arise for many reasons, such as activity levels and environment challenges. One paradigm that is perhaps most amenable to addressing interspecies differences is the variation arising in relation to body mass, or metabolic scaling. It has been known for more than 100 years that animals differing in body mass show allometric scaling of metabolic rate (29). There is no consensus on either the underlying origins or even the exact mathematical relationships (9, 16, 19, 28, 42, 43), but it is generally accepted that a plot of log mass vs. log mass-specific metabolic rate demonstrates a negative slope, or scaling coefficient (b) ranging from −0.25 to −0.33. Muscle metabolic enzymes also show allometric scale effects in fish (36) and mammals (15), although typically less than is seen in metabolic rate (29, 31). Interestingly, mitochondrial enzymes scale negatively and glycolytic enzymes scale positively. With scaling coefficients of 0.25−0.33, an animal 10 times larger than another would be expected to have about half the oxidative enzyme activity and twice the glycolytic enzyme activity. Simple stochastic models, such as size-dependent differences in turnover of mRNA or protein, cannot easily explain how muscles of larger animals maintain fewer mitochondria yet greater activities of glycolytic enzymes; the scaling patterns in oxidative and glycolytic enzymes appear to demand active regulation.

To explore the origins of interspecies differences in metabolic enzyme activities, we used rodent skeletal muscles to identify the patterns in J metabolic enzyme activities, both oxidative [cytochrome c oxidase (COX)] and glycolytic [lactate dehydrogenase (LDH)], 2) myonuclear content (mg DNA/g tissue), 3) enzyme transcript levels [citrate synthase (CS)], COX4-1, LDHA, and 4) transcript levels of two putative master regulators, PGC1α and NRF1. To explore the evolutionary variation in PGC1α expression, we compared the promoters of >50 mammals, constructing luciferase reporter genes from a subset to assess evolutionary variation in PGC1α proximal promoter strength.

METHODS

Tissue Samples

In addition to published sequences, we used tissues from various sources to generate DNA sequence, promoter constructs, enzyme measurements, and RNA analyses (Table 1). All collections from live animals were made in accordance with an approved Queen’s University Animal Care Committee protocol.
Enzyme and RNA analyses. Animals used for tissue enzyme and RNA analyses were derived from both lab and field collections. Charles River Canada supplied lab mice (Mus musculus CD-1 males), gerbils (Meriones unguiculatus), and hamsters (Mesocricetus auratus). Rats (Rattus norvegicus Sprague-Dawley) were sentinel males from the animal care centre at Queen’s University. These samples were euthanized using pentobarbital 150 mg/kg. Female guinea pigs (Cavia porcellus) were control animals used in the Reynolds and Table 1.

Table 1. Species used for enzyme, promoter, and reporter gene analyses

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E, enzyme; P, promoter; R, reporter gene.
Brien lab at Queen’s University and were anaesthetized and decapitated. Dwarf hamsters (Phodopus campbelli) were retired from the Wynn-Edwards colony at Queen’s University and were killed by cervical dislocation. Groundhogs (Marmota monax) muscles were collected on our behalf by Cornell University staff from their colony.

Wild rodents were trapped using Sherman box traps (North American deer mice, Peromyscus maniculatus; eastern chipmunks, Tamias striatus) or Havahart traps (eastern grey squirrels, Sciurus carolinensis; American red squirrels, Tamiasciurus hudsonicus). Animals were euthanized using 150 mg/kg pentobarbital after being removed from the trap. Beaver (Castor canadensis) samples were contributed by a local trapper as part of a pest removal, killed by rifle.

DNA analysis. Samples from many local species were obtained from buccal cell swabs of living specimens (human, Homo sapiens; domestic dog, Canis lupus familiaris; housecat, Felis catus), meat from a local butcher (domestic sheep, Ovis aries), road kill (porcupine, Erethizon dorsatum), or through hunters and trappers (moose, Alces alces; white-tailed deer, Odocoileus virginianus; black bear, Ursus americanus; least weasel, Mustela nivalis; American mink, Neovison vison; fisher, Martes pennant; raccoon, Procyon lotor; wolf, Canis lupus; coyote, Canis latrans; meadow vole, Microtus pennsylvanicus). Many samples of exotics were obtained from colleagues in Biology (mouflon, Ovis aries orientalis; chamois, Rupicapra rupicapra; muskox, Ovibos moschatus; giraffe, Giraffa camelopardalis; rhinoceros, Diceros bicornis; tapir, Tapirus bairdii; lynx, Lynx canadensis; brown hyena, Hyaena brunnea; polar bear, Ursus maritimus; gorilla, Gorilla gorilla). Others were provided by Kevin Campbell (University of Manitoba; masked shrew, Sorex cinereus; water shrew, Sorex palustris; star-nosed mole, Condylura cristata; coast mole, Scapanus orarius). The Metro Toronto Zoo provided samples of lion (Panthera leo) and capybara (Hydrochoerus hydrochaeris). In each case, DNA sequences were based upon a single individual.

Enzyme Analyses

We made measurements from whole gastrocnemius (GAST), soleus (SOL), and tibialis anterior (TA) from 12 rodents from 5 families: Muridae: lab mouse, deer mouse, gerbil, lab rat; Cricetidae: dwarf hamsters, Syrian hamsters; Sciuridae: eastern chipmunks, eastern grey squirrels, American red squirrels, groundhogs; Caviidae: guinea pigs; Castoridae: beaver. Muscles were excised from euthanized animals and flash-frozen in liquid nitrogen. GAST, SOL, and TA were collected from each species except groundhog, for which no SOL was available, and the TA is called tibialis cranialis.

Muscles for enzyme analyses were powdered in liquid nitrogen and stored at −80°C. Between 50 and 75 mg of powdered tissue were homogenized in 20 vol of extraction buffer (20 mM Tris, 0.6 mM lauryl maltoside, pH 8.0) using a ground glass homogenizer on ice. COX was assayed with 20 mM Tris (pH 8.0), 0.6 mM lauryl maltoside, and 100 µM cytochrome c. Cytochrome c was reduced with an excess of ascorbate, dialyzed exhaustively against 20 mM Tris (pH 8.0), and then frozen in aliquots. Each sample was assayed in a 96-well plate using a Molecular Devices SpectroMax Plus spectrophotometer (Sunnyvale, CA) held at a temperature of 25°C. This assay was done immediately after homogenization in quadruplicate with samples diluted 40-fold in extraction buffer and read for 90 s at a wavelength of 550 nm.

LDH was assayed in 20 mM HEPES (pH 7.0), with 1 mM pyruvate and 0.2 mM NADH. Homogenates were diluted 40-fold in assay buffer (20 mM HEPES, pH 7.0) immediately prior to the assay to obtain acceptable rates.

The enzyme homologues were also used to measure DNA content of rodent muscle, an indicator of myonuclear content. Aliquots of homogenates were digested in proteinase K (0.2 mg/ml) in 200 mM NaCl, 20 mM Tris, 50 mM EDTA, 0.10% SDS (pH 8.0). The digest was treated with RNase A (10 µg/ml) and then quantified using PicoGreen against standards treated in the same manner.

RNA Analyses

RNA was extracted from ~30–50 mg of powdered rodent TA muscle using the Qiagen RNeasy kit (Mississauga, ON, Canada) following manufacturer’s instructions. RNA was quantified using the 260 nm absorbance on the Molecular Devices SpectroMax Plus spectrophotometer. From this total RNA, cDNA was made from 2 µg RNA using the Qiagen QuantiTect Rev. Transcription kit (Mississauga, ON, Canada) as per the manufacturer’s instructions. Real-time primers (Table 2) were constructed for COX4-1, NRF-1, PGC1α, CS, and LDHA based upon near perfect homologies in published sequences from rodents and nonrodents. To compare transcript levels between species, it was essential that the primers for a particular gene were identical for all species, with no mismatches or wobble sites. For each species, we verified that the real-time PCR reaction was linear with respect to template concentration and amplification generated a product that demonstrated a single dissociation curve. A species was excluded from the analysis when it failed to generate an appropriate dilution series or when the PCR product produced multiple dissociation peaks. Analysis was performed on each cDNA of interest and normalized to β-actin (BACT) to determine the threshold cycle (CT). The relative cDNA for a gene of interest is expressed as 2ΔΔCT (BACT – GENE of INTEREST).

The values for each gene and species were transformed into relative cDNA levels per g tissue by multiplying mg RNA per g tissue. The resulting values for multiple genes (relative mRNA per g tissue) are then arbitrarily adjusted to fit within the same values to facilitate comparisons between genes.

Real-time analysis was conducted on an ABI 7500 RT-PCR system (Foster City, CA) using 10 min at 95°C (1 cycle) followed by 40 cycles of 15 s at 95°C, 30 s at annealing temperature and 36 s at 72°C. Reactions (25 µl) contained 1 × SYBR green master mix (Roche Applied Sciences, Basel, Switzerland), 100 ng cDNA, and 0.58 µM of each primer.

COX is a multimeric protein and it was not feasible to measure the expression of all subunits. COX4-1 is the muscle isoform for the subunit COX4. We developed consensus primers based upon published sequences that enabled us to amplify single products of the appropriate size in all species except American red squirrel and Syrian hamster, which yielded double dissociation peaks.

LDH is a tetrameric protein composed of subunits from five genes. In skeletal muscle, the enzyme is composed essentially from muscle subunits encoded by LDHA. We developed consensus primers based upon published sequences that enable us to amplify single products of the appropriate size in all species except beaver.

CS is a homodimeric protein with no paralogs. Its consensus primers based upon published sequences that enable us to amplify a single product of the appropriate size in all species except beaver.

Table 2. Primers used for rodent mRNA analysis

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<td>ATGCCATCTGGCTGGAAGG</td>
<td>103</td>
<td>59</td>
</tr>
</tbody>
</table>

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single products of the appropriate size in all species except guinea pig, which yielded a double dissociation peak.

NRF1 is a monomeric protein with no paralogs. The real-time primers were able to amplify appropriate amplicons from all species.

PGC1α is a monomeric protein with no isoforms, although 2 paralogs exist with overlapping functions. The primers were developed specific for PGC1α and amplified a single product in all species.

Promoter Analyses

Promoter sequence analysis began with surveys of published genomic sequences. Using this information, a proximal promoter (~700 bp) was amplified from various taxa to generate long promoter sequences. Beyond this point, there was very little homology between species. From these sequences, we designed internal primers that were able to amplify a shorter proximal promoter (~600 bp) from a subset of species to conduct reporter gene analyses.

DNA purification. DNA from tissues was used to provide material for promoter characterization. Tissue samples were digested in proteinase K, as described in the enzyme analyses section. The DNA was extracted in an equal volume of phenol-chloroform-isooamy alcohol (25:24:1). After centrifugation (10 min at 1,700 g), the aqueous phase was recovered and DNA was precipitated by adding 0.1 vol of ammonium acetate (7.5 M) and 2 vol of 100% ethanol, followed by centrifugation (3 min at 1,700 g). The pellet was washed with 70% ethanol, air-dried, and resuspended in 250 µl TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0). DNA purity was assessed using absorbance at 260 nm and 280 nm and then quantified based on the 260 nm reading.

Published PGC1α promoter sequences. Promoter sequences were available for several placental mammals from GenBank or ENSEMBL databases (Table 1). In each species for which genomic data was reliable, we downloaded and aligned about 700 bp of sequence immediately proximal to the start codon of the PGC1α gene.

There was complete conservation of the arrangement of the gene between species in that there was no TATA box evident and a very short 5′-untranslated region upstream of the translation start codon. Additionally, the sequence of the gene corresponding to the NH2 terminus of the protein was very highly conserved, permitting the design of a universal reverse primer that worked for all mammals examined.

Sequencing novel promoters. Clade-specific consensus primers were developed for about 700 bp (Table 3) of the proximal promoter region. Beyond this region, there was considerable divergence between even closely related species. This long promoter was amplified between even closely related species. This long promoter was amplified region. Beyond this region, there was considerable divergence between even closely related species. This long promoter was amplified.

Table 3. Primers to amplify long (~900 bp) promoter and reporter genes

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer for Long Promoter (5′–3′)</th>
<th>Reverse Primer for Long Promoter (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodents</td>
<td>CATGCCTTTGCAAACACTGCTCTAAATTAG</td>
<td>TACGCTCGATGTCAGCTCAG</td>
</tr>
<tr>
<td>Dwarf hamster</td>
<td>GCTTTTCAAGACCTGCCCTAAATTAG</td>
<td></td>
</tr>
<tr>
<td>Carnivores</td>
<td>TTTCACAGACCTGCCCTCCTG</td>
<td></td>
</tr>
<tr>
<td>Cetartiodactyla</td>
<td>CATCCTGTCGTTTCAAAAGCGCT</td>
<td></td>
</tr>
<tr>
<td>Giraffe</td>
<td>CCTGCTTTCAACACTGCCCTC</td>
<td></td>
</tr>
<tr>
<td>Rhinoceros, tapir</td>
<td>CATCGCTTTGTTTCAAAAGCGCT</td>
<td></td>
</tr>
<tr>
<td>Primates and eulipotyphia</td>
<td>GCTTTTCAACACTGCCCTC</td>
<td></td>
</tr>
</tbody>
</table>
The room temperature. The Plasmid mix (10 μl DMEM with 500 ng luciferase reporter plasmid and 50 ng Renilla plasmid) was transferred to the FuGene mix (40 μl DMEM with 1.5 μl FuGene), then incubated at room temperature. After 30 min, the FuGene/plasmid/DMEM mixture was added drop-wise into one well of the 50% confluent 12-well plates, gently agitated, and returned to the incubator. After 24 h the medium was changed to differentiation media (DMEM, 2% horse serum, with penicillin, streptomycin, and neomycin). After 3 additional days, cells were collected for luciferase measurements. Luciferase expression was determined using the Dual Luciferase Assay System (Promega) according to the manufacturer’s instructions on a luminometer (Lmax; Molecular Devices, Sunnydale, CA) with automatic injectors. Cells were harvested in 100 μl of passive lysis buffer and frozen at −80°C. To measure firefly luciferase, 100 μl of luciferase assay reagent (LARII, Promega) were added to 10 μl of room-temperature cell lysate in a white 96-well plate. Relative light units were measured for 30 s, with a 1 s premeasurement delay. To measure Renilla luciferase, 100 μl of Stop & Glo solution (Promega) were then added to the same well, and relative light units were again measured for 30 s, with a 2 s delay.

Statistics

The variation in number of animals used for each measurement complicated the analyses. We had only one beaver sample, but we conducted all analyses on this species with pseudoreplication. Regression analyses used means for each species.

The influence of body mass on enzyme activities was assessed using a simple linear regression model. To assess if transcription factor mRNA could be used to predict downstream events, we compared the 12 means of each parameter using simple linear regression and multiple regression.

RESULTS

The perception that mammalian muscle enzymes show an allometric scaling pattern have been based upon relatively few studies that focus on single select muscles and employ distantly related species (29). Our first goal was to assess if allometric scaling of metabolic enzymes was evident in one family of mammals (rodents).

Metabolic Enzymes in Rodents

Twelve species of lab-reared and wild-caught rodents were analyzed for hindlimb muscle nucleic acids (Table 4), enzyme activities, using the mitochondrial enzyme COX and the glycolytic enzyme LDH. COX and LDH were measured in enzyme homogenates and expressed both per g tissue and per mg DNA, to take into account differences in myonuclear domain. For COX-specific activities (Fig. 1) only TA showed a statistically significant scaling pattern whether expressed per g tissue (b = −0.16, P = 0.0089) or per mg DNA (b = −0.14, P = 0.0021).
Neither GAST nor SOL showed a significant mass dependence (per g tissue, GAST $P = 0.36$, SOL $P = 0.91$; per mg DNA, GAST $P = 0.30$, SOL $P = 0.054$). For LDH-specific activities (Fig. 2), only TA showed a significant scaling relationship and only when expressed per g tissue ($b = +0.11, r^2 = 0.034$; per mg DNA, $P = 0.068$), whereas GAST and SOL showed no significant mass dependence (per g tissue, GAST $P = 0.14$, SOL $P = 0.061$; per mg DNA, GAST $P = 0.19$, SOL $P = 0.74$).

Previous studies have reported a reciprocal relationship between oxidative and glycolytic enzymes (15, 29, 36). When the muscles of different-sized rodents were compared in terms of the ratio of LDH/COX (Fig. 2), there was a strong size dependence in GAST ($b = 0.19$, $P = 0.36$; per mg DNA, GAST $P = 0.09$, SOL $P = 0.74$). In each case, transcript levels were assessed by real-time PCR, corrected with $\beta$-actin mRNA, and multiplied by mean RNA/g (Table 4) to obtain a relative measure of transcripts per g tissue.

$NRF1$, $PGC1\alpha$, and COX4-1 mRNA. The first approach we employed was to express $COX4-1$, $NRF1$, and $PGC1\alpha$ transcripts relative to body mass using the traditional scaling axes (Fig. 3). There was no significant effect of body mass on the mRNA of these genes ($COX4-1 P = 0.11$, $NRF1 P = 0.20$, $PGC1\alpha P = 0.58$).

We also performed correlational analyses of the data to determine the relationships between each mRNA and its downstream effect (Fig. 4). $COX4-1$ mRNA could predict 55% of the COX activity (Fig. 4A). The mRNA levels of $NRF1$ and $PGC1\alpha$ correlated with each other ($r^2 = 0.42$). Both $NRF1$ (Fig. 4B, $r^2 = 0.52$) and $PGC1\alpha$ (Fig. 4C, $r^2 = 0.48$) were strong predictors of $COX4-1$ mRNA and COX activity ($r^2 = 0.55$ and 0.49, respectively) (data not shown).

Next, the entire data set was analyzed using a multiple linear regression approach to examine the influences of body mass, $PGC1\alpha$ mRNA, $NRF1$ mRNA, and $COX4-1$ mRNA. Omission of $COX4-1$ mRNA from the analyses had negligible effects. As mRNA for $NRF1$ and $PGC1\alpha$ were strongly correlated, subsequent statements about $PGC1\alpha$ mRNA also apply to $NRF1$ mRNA. A model using $PGC1\alpha$ mRNA and body mass could explain 90% of the variation in COX activity (Fig. 4D). The standardized partial regression coefficients indicate that $PGC1\alpha$ (standard beta $= 0.63$) and mass (standard beta $= -0.60$) contribute about equally to explaining variation in COX (Table 5). Thus, neither $PGC1\alpha$ nor $NRF1$ mRNA predicts the effects of body mass, but either explains how individual species deviate from the value predicted from mass alone.

![Fig. 2. Catalytic activities for lactate dehydrogenase (LDH) in 3 rodent hindlimb muscles.](http://physiolgenomics.physiology.org/)}
**LDHA, LDHB, and CS.** The analysis of TA mRNA also provided the opportunity to explore other targets of interest including transcripts for another mitochondrial enzyme (CS) and a representative glycolytic enzyme (LDH).

CS mRNA (Fig. 3E) was found to have a much stronger relationship with body mass ($r^2 = 0.46$) and display a slope ($b = -0.29, P = 0.022$) that was higher than COX4-1 transcripts or COX itself. Regrettably, we did not measure CS activity in this study.

When LDHA mRNA was expressed relative to body mass (Fig. 3C), no scaling emerged ($b = -0.038, P = 0.84$) in contrast to the positive scaling seen for the enzyme ($b = +0.11$). There was no correlation between LDH activity and LDHA transcript levels. LDHB mRNA (data not shown) showed a negative allometric scaling relationship ($b = -0.30, P = 0.07$).

**PGC1α Promoter Analyses in Mammals**

The proximal promoters were analyzed for a selection of laurasiatherians and euarchontoglierean mammals that were primarily terrestrial (i.e., excluding cetaceans) and nonfliers (i.e., excluding bats). We compiled sequences from the Ensembl database representing ~800 bp upstream of the transcription start site beyond which even closely related taxa were highly divergent. Taxon-specific primers were used to amplify ~700 bp of proximal promoter from other species. For a subset of mammals, a segment of ~600 bp of proximal promoter was subcloned into a luciferase reporter gene to assess promoter strength.

**Promoter sequence.** We did not expect that analysis of 600 bp of the PGC1α promoter would faithfully reflect taxonomic relationships, but the tree generated from the PGC1α promoter (Fig. 5) displays fairly good agreement with accepted phylogenetic relationships (38). The branch lengths within and between taxa suggest the promoter is diverging much faster in rodents than other taxa.

Proximal promoters were analyzed for the presence/absence of putative transcription factor binding sites (Fig. 5). In the proximal 600 bp of mouse sequence there are two insulin-responsive sequences (IRS) (478 and 315 bp relative to translation start codon), E1 box (169), an inverted MyoD site (144), HNF3 site (96), NF-Y site (74), and another E1 box (21). There were some common themes but many exceptions to the arrangement of regulatory elements in PGC1α promoters from other mammals.

The distribution on IRS elements was also highly variable, but with many taxon-specific patterns. All members of Artiodactyla and Carnivora had a single site, while Perissodactyla and Eulipotyphia had none. Within rodents, sciuromorphs had no IRS, caviomorphs possessed a single distal IRS, and murids possessed a second more proximal IRS.

A distal CRE site was present in every species except deermouse, lab mouse, meadow vole, muskrat, and capybara.

**Table 5. Estimates from multivariate analyses**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate (95% CI)</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-0.077 (-0.14 to -0.013)</td>
<td>2.7</td>
<td>0.02</td>
</tr>
<tr>
<td>logPGC1α</td>
<td>0.41 (0.25-0.57)</td>
<td>5.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>logBodyMass</td>
<td>-0.14 (-0.19 to -0.08)</td>
<td>5.6</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

CI, confidence interval.
A second CREB site (TGACGCCA) was seen in every species (−50), but inverted (not depicted on Fig. 5).

Elements for the myogenic factors (MyoD site and E1 box) were present in all species. Two E1 boxes were seen in every species in the same positions. A MyoD site was present in every species, though always in the (−) orientation.

An HNF3 site occurred in all species except 2 (of 7) artiodactyla and the caviomorph rodents (porcupine, guinea pig, capybara).

Sp1 sites were present in every species, though there was some variants in number and sequence. The first element GGCTGGG was present in every species (−211) except deer-mouse (GGTTGGG). The same Sp1 sequence was seen in the (−) orientation in each species, in each case separated by 10 nucleotides from the first (not depicted on Fig. 5). A third Sp1-like sequence (GGGGCTGGT) was found more proximal (−114) in each species except coyote and the three caviomorphs.

Some species appeared to lack an NF-Y site: bushbaby, Caviidae (guinea pig and capybara), and horse. Most species had a single NF-Y site, but a second more proximal NF-Y site was found in some groups: 6 (of 8) Artiodactylidae, 4 Eulipotyphla (moles and shrews), 2 Lagomorpha (rabbit and pika), and a single primate (gorilla).
Promoter activity. Proximal promoters from a subset of these species were used to construct luciferase reporter genes (Table 1). The expression of firefly luciferase and Renilla luciferase were measured in two mouse myoblast lines: C2C12 and SoI8 cells. Luciferase activities were measured after 3 days of differentiation. When all species were considered, an isometric relationship between body mass and PGC1α promoter strength was seen in both SoI8 cells and C2C12 cells (Fig. 5C).

DISCUSSION

Although much has been learned about the mechanisms by which an animal changes metabolic enzyme levels, much less is known about the origins of differences seen between species. Elegant gene regulatory mechanisms coordinate gene expression to maintain the integrity of complex pathways during metabolic remodeling. An evolutionary change that generates a difference between species cannot compromise the capacity of an individual to undertake compensatory metabolic remodeling. Thus differences in metabolic enzyme activities between species may arise by mechanisms that are distinct from those involved in metabolic remodeling.

Scaling of Metabolic Enzyme Activities

Our goal was to assess the underlying mechanisms that determine why muscle metabolic enzymes differ among species, focusing on patterns related to body size. More than 50 years ago, it was first recognized that muscle mitochondrial enzymes showed negative allometric scaling (29). Likewise, it has been known for more than 30 years that glycolytic enzymes show positive allometric scaling in fish (36) and mammals (15). The magnitude and origin of the scaling of mitochondrial enzymes have received the most attention in recent years. One possible confounding influence is a difference in myonuclear content. Whether considering it as myonuclear domain (fiber volume per nucleus) or nuclear content (DNA per g tissue), the abundance of nuclei can in principle have a dramatic effect on constitutive gene expression. Each nucleus produces mRNA to provide the precursors in support of the surrounding cytoplasm (32). It follows that more nuclei per g tissue would lead to more precursors per g tissue (14, 31, 39). Thus, higher levels of nuclear gene transcripts (per g tissue) can arise even if there are no differences in gene expression per se. There is a strong relationship between myonuclear domain size and body mass in both fast twitch and slow twitch mammalian muscle fibers (26). In this study on rodents, we found that only TA showed significant negative scaling of mitochondrial enzymes (per g) and positive scaling of glycolytic enzymes (per g), with little effect when myonuclear domain was considered. However, when COX was expressed relative to DNA, differences between muscles diminished and the scaling coefficients converged. Thus, consideration of myonuclear content reduced the differences between muscles of a species, but not differences between species. Previous studies with fish reached the same conclusion: expressing mitochondrial enzyme activities relative to DNA content largely negated differences in mitochondrial enzymes in relation to size (10) and between fibers (8) but did not affect differences between species (8).

Though the main focus of this study is the impact of body size, it is also important to consider the potential impact of other factors, including lifestyle and phylogenetic relationships (23). Squirrels possessed COX activities that were higher than would be expected based on the regression of all rodents. The interspecies differences were most obvious when comparing chipmunk to gerbil, red squirrel to hamster, and eastern gray squirrel to guinea pig. The squirrels have ~50% more COX activity than the other size-matched rodents. It is not possible from our data set to distinguish between factors such as phylogenetic signatures (sciuromorphs vs. muroids), activity levels (active vs. more sedentary), or rearing conditions (wild vs. lab bred or colony reared). Allometric patterns in metabolic rate can be influenced by diet and habitat (28) and activity levels (41). Body mass is thought to be the major factor in interspecies differences in metabolic rate, accounting for up to 96% of variation with size (28). Though the differences in metabolic enzymes seen between species may arise from complex evolutionary and physiological factors, the more proximate question is how the muscle cell determines the activities of metabolic enzymes and the extent to which the differences arise from genetics or environment.

Transcriptional Origins of Interspecies Differences in COX activity in TA Muscle

Regardless of whether the differences in COX are linked to size or other factors, the interspecies differences seen in TA muscle COX afford an opportunity to explore the underlying basis of the variation. Most of the work on transcriptional master controllers has been performed on mice, rats and humans, but in these models mitochondrial content has been shown to be under transcriptional control in response to energy challenges such as exercise and dietary stress (20, 34). The question becomes whether interspecies differences are driven by the same factors. Therefore, we asked if the “master controllers” of mitochondrial biogenesis were regulated in a way that would account for scaling patterns in mitochondrial enzymes. Transcriptional control of the genes for PGC1α and NRF1 is central to the capacity of these factors to act as master regulators of mitochondrial biogenesis (1, 6, 7, 18, 20, 22, 34, 46). Consequently, we expected their expression to drive differences seen in COX4-1 mRNA and ultimately COX activity.

COX activity in TA muscle (Fig. 1C) showed a clear scaling pattern ($b = −0.16$) and body mass explained about half of the interspecies differences in COX activities ($r^2 = 0.51$). Surprisingly, there was no significant regression between body mass and mRNA for COX4-1, PGC1α, or NRF1 (Fig. 3), yet each of these mRNAs was a good predictor of COX activity (Fig. 4). When all of the parameters were evaluated in a multivariate analysis, the paradoxical patterns were more clearly resolved. As expected, PGC1α and NRF1 mRNA were good predictors of COX4-1 mRNA, which in turn was a good predictor of COX activity. This simple transcription pathway could predict about half of the differences seen in COX activity between species. However, body mass, independently of PGC1α or NRF1 transcript levels, also explained about half of the variation in COX activities among these animals. Thus, COX activity is determined in equal measure by unknown mechanisms linked to body mass (independent of PGC1α or NRF1 transcript levels) and by PGC1α and NRF1 (independent of body mass). In more general terms, the COX activity in an animal may be determined by its body mass, but PGC1α and/or NRF1 regulate the
actual COX activity to be higher or lower than value predicted by mass alone.

We have little evidence of the mechanisms determining interspecies differences in COX activity acting independently of PGC1α and NRF1 transcript levels. In adaptive remodeling of individuals, posttranscriptional regulation of activity plays a role in the function of both PGC1α [e.g., SIRT1-mediated acetylation (33)] and NRF1 [serine kinase-dependent phosphorylation (17)]. Alternately, there may be a role for other transcription factors known to affect mitochondrial gene expression, such as NRF2, PPAR, thyroid hormone receptors, or retinoic acid receptors (20, 34). As a first attempt to explain regulation of interspecies differences, we further explored the mechanisms by which the differences in mRNA levels of the transcription factors arise between species, focusing on the proximal promoter of PGC1α. The NRF1 gene has a very large and variable 5′-untranslated region, with considerable variation between species, making this promoter difficult to analyze across species. Our analysis of the PGC1α gene was limited to ~700 bp of proximal promoter. More distal regions of the promoter have considerable sequence variation that precludes cloning novel sequences through consensus primers.

PGC1α Promoter Analyses

We analyzed ~600 bp of the PGC1α promoter from the start codon to an upstream conserved element for IRS. We assessed the location, number, and arrangement of transcription factor binding elements, and used the sequence to construct neighboring trees. We were surprised to find such a short sequence provided a faithful rendering of mammalian phylogeny (38). Each gene has functional constraints that can cause them to evolve in ways that misrepresent phylogeny (1, 11). Noncoding regions, such as promoters and introns, can experience many point mutations, substitutions, insertions, and deletions with little selective penalty. However, promoters, as well as introns, possess sequences that are conserved because they are specific binding sites (11, 12, 27).

The variation seen in the promoters for mammals we studied fell into three categories: sequence variation between elements, presence or absence of specific elements, and arrangement of elements. The overall variation in sequence was sufficient to derive a robust tree (Fig. 5A) that depicted mammalian phylogeny despite the size (38). The most remarkable feature of the tree was the unexpectedly long branch lengths within rodents. The factor driving this divergence is not immediately clear, but in addition to overall sequence variation there was considerable variation in the representation of specific elements, particularly more distal IRS and NFAT sites (Fig. 5B).

Elements may act as binding sites in vitro or in engineered settings but be transcriptionally irrelevant in vivo (40). Most of the elements identified in this study have been shown to bind transcription factors and regulate transcription in some physiological context (20, 34). These include sites involved in constitutive expression and basic cellular function, such as sites for Sp1, NF-Y, and HNF3, sites that impart sensitivity to metabolism, including CRE, and IRS sites, and muscle-specific factors, such as MyoD sites and E-boxes. For most of these elements, there were instances when select species showed gain or loss but in ways that reflected no particular relationship to size pattern. There were also many potential sites for nuclear hormone receptors, and there was considerable diversity in the number and arrangement of these elements though no phylogenetic or mass-dependent pattern was apparent.

We used reporter genes to assess the activity of the PGC1α promoter in rodents and other placental mammals. There was no simple allometric relationship observed, despite sampling from species that differed in body mass by 1,000-fold. The lack of difference in promoter strength between species, but a clear difference in mRNA levels points to the potential role for more distal elements (46). There is also a potential role for hormones, such as insulin-like growth factors and thyroid hormone, which are known to exert effects on mitochondrial gene expression (6, 7, 20–22, 34).

Reciprocal Regulation of OXPHOS and Glycolytic Genes

Somero and Childress (36) were the first to note the reciprocal pattern in scaling of mitochondrial and glycolytic enzymes, the same relationship observed in our study and others (10, 15). It is unclear whether this pattern arises as a result of independent regulatory pathways or a single pathway with reciprocal effects. The current study provides evidence that these two aspects of the metabolic phenotype, mitochondrial and glycolytic enzyme levels, arise through different cellular mechanisms. In the case of LDH, the scaling coefficients for LDHA mRNA and catalytic activity were quite dissimilar. LDH activity showed positive scaling, whereas LDHA mRNA showed no mass dependence. One possible explanation invokes a role for cytoplasmic protein turnover. Larger animals have a lower metabolic rate, which is likely accompanied by decreases in protein damage and turnover. Thus, larger animals may have less need for LDH synthesis to maintain their LDH activities. We have seen in other studies that larger animals have lower than expected LDHA mRNA, given their LDH activities, which we also attributed to cytoplasmic protein turnover (4).

In summary, our data have provided important insights into the mechanisms by which allometric scaling of metabolic enzymes arise in muscle. Not all muscles show allometric scaling of metabolic enzymes, though all muscles show a reciprocal relationship between oxidative and glycolytic enzymes. In muscles that show allometric scaling of COX, the differences can be attributed in roughly equal parts to transcriptional regulation through NRF1 and PGC1α and body mass effects acting through other cellular mechanisms, such as posttranscriptional effects on NRF1 and PGC1α activities (e.g., SIRT1 acetylation, MAPK phosphorylation) or other transcriptional regulators. Remarkably, almost half of the variation in COX seen between species is consistent with a simple model of transcriptional regulation of NRF1 and PGC1α activities. How their respective promoters are controlled to achieve this difference remains unanswered but sequences differences in the proximal promoter do not appear to explain why PGC1α mRNA levels vary among species.

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