Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the rev-erbAα orphan receptor gene

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Pircher, P. P., Chomez, F., Yu, B., Vennström, B., and Larsson, L.
Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the rev-erbAα orphan receptor gene. Am J Physiol Regul Integr Comp Physiol 288: R482–R490, 2005. First published September 16, 2004; doi:10.1152/ajpregu.00690.2003.—The rev-erbAα orphan receptor plays an important role in the regulation of thyroid hormone receptors, which are encoded by two distinct genes, c-erbA1 and c-erbA2 (TR1 and TR2). The c-erbAα gene is alternatively spliced into α1 (hormone-binding) and α2 (nonhormone-binding) isoforms. Intriguingly, the c-erbAα locus has also been shown to contain an overlapping transcription unit utilizing coding information for the rev-erbAα gene on the opposite strand. Specifically, the rev-erbAα gene overlaps with the thyroid hormone receptor α2 (TRα2)-specific exon, ending 3′ to the thyroid hormone receptor α1 (TRα1) polyadenylation site. rev-erbAα and TRα2 mRNAs are complementary for a stretch of 269 nucleotides because of bidirectional transcription of a partially common exon. The peculiar arrangement of the rev-erbAα gene has led to the suggestion that its transcription could interfere with the expression of the thyroid hormone

The variable expression of myofibrillar protein isoforms is a major determinant of the contractile properties and ATPase activities of skeletal and myocardial fibers (3, 50). The myosin heavy chain (MyHC) molecule is the most abundant myofibrillar protein and is encoded by a multigene family consisting of several members (35). Several isoforms have now been described in rodent skeletal muscles, including one slow isoform (type I or β/slow), three fast (Iia, IIX/d, and IIb), and two developmental (embryonic and neonatal/perinatal/fetal) isoforms, which are all coded by distinct genes (50). Four other MyHC isoforms, slow tonic, superfascial IIm, cardiac-like, and extraocular myosin, are expressed in a few specialized muscles (7, 50). Despite their similarity in primary structure, the expression of different MyHC isoforms is precisely regulated in a tissue-specific and developmental stage-specific manner (50, 52). In addition, innervation patterns (33, 45, 46), altered physiological stimuli (34, 46), and various hormones (19) are known to have a significant impact on MyHC isoform expression.

Skeletal muscle is a major target for hormone action. Thyroid hormone is one of the most potent regulators of skeletal muscle MyHC isoform expression in mammals, and it has been suggested that normal thyroid hormone homeostasis is a prerequisite for normal expression of adult MyHC isoforms during early development. Thyroid hormones act by repressing or activating the genes coding for the different myosin isoforms, and all members of the MyHC multigene family respond to changes in 3,5,3′-triiodo-L-thyronine (T3) levels, although the mode of response is determined in a highly muscle and fiber type-specific manner (20, 40). Both fast- and slow-twitch muscles respond rapidly to both hyper- and hypothyroidism at the MyHC mRNA level (1, 2, 4, 10, 14, 20). The response at the protein level is slower and partially different from that at the mRNA level. In the slow-twitch soleus, similar changes are observed at the protein and mRNA levels, with, for example, fast-to-slow MyHC isoform transition in response to hypothyroidism and the reverse in hyperthyroidism, resulting in altered morphological and contractile properties. In the fast-twitch extensor digitorum longus (EDL) or tibialis anterior muscle, on the other hand, only small or insignificant changes are observed at the protein level in response to hyper- and hypothyroidism despite the significant alterations at the MyHC mRNA level (10, 20, 25, 39, 53).

T3 induces terminal muscle differentiation and regulates the fiber-type composition via direct activation of the muscle-specific myod1 gene family (5, 37). The diverse effects of thyroid hormones are mediated by the intracellular thyroid hormone receptors, which are encoded by two distinct genes, c-erbAα and c-erbAβ. The c-erbAα gene is alternatively spliced into α1 (hormone-binding) and α2 (nonhormone-binding) isoforms. Intriguingly, the c-erbAα locus has also been shown to contain an overlapping transcription unit utilizing coding information for the rev-erbAα gene on the opposite strand. Specifically, the rev-erbAα gene overlaps with the thyroid hormone receptor α2 (TRα2)-specific exon, ending 3′ to the thyroid hormone receptor α1 (TRα1) polyadenylation site. rev-erbAα and TRα2 mRNAs are complementary for a stretch of 269 nucleotides because of bidirectional transcription of a partially common exon. The peculiar arrangement of the rev-erbAα gene has led to the suggestion that its transcription could interfere with the expression of the thyroid hormone
receptor (21, 27) by regulating alternative processing of α1 and α2 mRNAs in vitro by an antisense mechanism (36). Thus relatively modest changes in splice site selection could cause major changes in cellular T3 responsiveness (27). Other orphan receptors, e.g., COUPTF II, RVR, RORα, and RORγ, are abundantly expressed in skeletal muscle (11, 22). These have been reported to be downregulated during myotube formation, and it has been suggested that overexpression of the rev-erbα receptor in myoblasts may block myotube differentiation. Furthermore, repression of myogenesis and of the myoD gene family by rev-erbα has been partly correlated with the ability of this orphan receptor to silence T3/thyroid hormone receptor-dependent gene expression from the myogenin promoter and the thyroid hormone response element (8).

The rev-erbα protein is highly expressed in skeletal muscle (11), but its role in modulation of thyroid hormone action on skeletal muscle MyHC isoform has not, to the best of our knowledge, been investigated. To gain insight into this important aspect of rev-erbα function, we have studied alterations in MyHC isoform expression in mice deficient of the rev-erbα gene. The results have been presented in short form elsewhere (42–44).

MATERIALS AND METHODS

rev-erbα mutant mice. This study was carried out on genetically altered 3- to 5-mo-old mice lacking the rev-erbα gene or the TRα2 gene. The mutant mice were obtained by targeting the coding sequence of the rev-erbα gene or TRα2 and replacing it by a β-galactosidase gene in embryonic stem cells, and transgenic mice devoid of rev-erbα or TRα2 were subsequently generated. The mice were divided into control (+/+; n = 4 in both rev-erbα and TRα2), heterozygous (+/−; n = 5 for rev-erbα and n = 4 for TRα2), and homozygous (−/−; n = 6 for rev-erbα and n = 4 for TRα2) groups. Animals were killed by cervical dislocation, and the soleus and EDL muscles were gently dissected free from surrounding tissue and clamped at approximately the in situ length. The muscle was subsequently weighed, frozen in freon chilled with liquid nitrogen, and stored at −80°C until processed further. The study was approved by the ethical committee at the Karolinska Institute, Stockholm, Sweden (6, 47).

Histochemical analysis of β-galactosidase expression. Fresh soleus and EDL cryostat sections were fixed for 10 min in 0.2% glutaraldehyde in PBS. After three washes in PBS, the samples were first permeabilized in PBS with 2 mM MgCl2, 0.02% NP-40, and 0.01% SDS in PBS. Horseradish peroxidase reactivity was developed for 1 h. All steps were performed at room temperature unless otherwise stated. Sections were preincubated with 5% horse serum or goat serum in PBS to prevent nonspecific binding. Slides were then incubated with anti-MHC antibody overnight at 4°C. Two different monoclonal antibodies were used, i.e., G-6 (IgG) and B-6 (IgG), which recognize embryonic and neonatal MyHC, respectively. Sections were washed three times with PBS-0.2% Tween 20 (PBSTw) and then incubated in 5% horse serum or goat serum in PBS for 1 h. To detect IgG, slides were then incubated in 1:100 biotin-conjugated goat anti-mouse IgG (heavy- and light-chain specific; Vector), diluted in 5% goat serum-PBS. Slides were washed three times in PBSTw for a total of 1 h. Endogenous peroxidase was then blocked by 5% H2O2 in methanol for 20 min at −20°C. After two 10-min washes in PBS, an avidin-biotin complex (Vectastain ABC PK-4002 kit, Vector) was applied to the sections for 1 h. Slides were washed for 5 min in PBS, 10 min in PBSTw twice, 1 min in PBS three times, and then 10 min in PBS. Horseradish peroxidase reactivity was developed for ~1.5 min with 0.6 mg/ml 3,3′-diaminobenzidine, 50 mM Tris·HCl (pH 7.2), and 0.03% CaCl2, to which 0.05% H2O2 was added immediately before staining. Sections were washed in PBS to stop the development and then mounted in glycerin-gelatin. Photographs were taken immediately after mounting (17). The monoclonal antibodies G-6 and B-6 were supplied by S. Schiaffino (48).

Determination of MyHC isoform composition. The MyHC composition was determined by SDS-PAGE (23). The total acrylamide and bis concentrations were 4% (wt/vol) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol (26). The ammonium persulfate concentrations were 0.4% and 0.029% in the stacking and separation gels, respectively, and the gel solutions were degassed (<100 mTor) for 15 min at 18°C. Polymerization was subsequently activated by adding TEMED to the stacking (0.1%) and separation gels (0.07%). The muscle was cut at the motor point (soleus) or at its greatest girth (EDL) perpendicular to its longitudinal axis into 10-μm-thick cross sections with a cryotome (2800 Frigocut E, Reichert-Jung, Heidelberg, Germany) at −20°C. Single 10-μm soleus and EDL muscle cross sections were placed in sample buffer in a plastic microcentrifuge tube and stored at −80°C until analyzed. Sample loads were kept small to improve the resolution of the MyHC bands. The gels were placed in the electrophoresis apparatus (SE 600 vertical slab gel unit, Hoefer Scientific Instruments) connected to a power supply and a cooling unit. Electrophoresis was performed at 120 V for 22–24 h with a Tris-glycine electrode buffer (pH 8.3) at 15°C (23). Separating gels were silver stained (12) and subsequently scanned in a soft laser densitometer (Molecular Dynamics, Sunnyvale, CA), with a high spatial resolution (50-μm pixel spacing) and 4,096 optical density level, to determine the relative contents of the MyHC isoforms. The volume integration function was used to quantify the amount of protein, and background activity was subtracted from all pixel values (ImageQuant software v3.3, Molecular Dynamics). Immunoblotting experiments had been conducted previously in this laboratory to determine the migration order of the four MyHC isoforms separated by the type of gels used in this study. The migration order from slowest to fastest migrating MyHC isoform is as follows: Ila-IIX-IB-I (26).

Real-time quantitative PCR. Total RNA was extracted from individual soleus muscles from wild-type controls and heterozygous and homozygous mice with Trizol reagent (Life Technologies, Rockville, MD). Quantification of mRNAs of TRα1 and TRα2 was performed by real-time quantitative RT-PCR with a Perkin-Elmer ABI Prism 7700 sequence detection system. RT-PCR reaction was performed with

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AmpliTaq Gold polymerase (Perkin-Elmer ABI), with 20 ng of total RNA for each reaction.

For quantification of a particular mRNA with real-time RT-PCR, a fluorogenic probe and two primers for PCR (forward and reverse) were synthesized (Perkin-Elmer ABI). The internal oligonucleotide probe was labeled with the fluorescent dye carboxyfluorescein (FAM) at the 5’ end and N,N,N’,N’-tetramethyl-6-carboxyfluorohydamine at the 3’ end. The probe hybridized with the cDNA regions was amplified by PCR. When both dyes are present in an intact probe, N,N,N’,N’-tetramethyl-6-carboxyfluorohydamine acts as a quencher for FAM by absorbing at the FAM emission spectra. When the internally hybridized probe was degraded by the 5’ exonuclease activity of Taq polymerase during PCR, these two dyes were separated in solution, resulting in a subsequent increase in the level of fluorescence in the reaction mixture. Thus the amount of fluorescence released during each amplification cycle was proportional to the amount of specific PCR products generated in that cycle. The 18S RNA was amplified at the same time and used as an internal control. The cycle threshold \( C_t \) values for 18S RNA and those of samples were measured and calculated by computer software (Perkin-Elmer ABI). Relative transcript levels were calculated as \( 2^{-\Delta \Delta C_t} \), in which \( \Delta C_t = |C_{t_{expt}} - C_{t_{ref}\text{plas}}| - C_{t_{ref\text{plas}} - C_{t_{ref\text{plas}}}} \), where the threshold cycle for 18S rRNA, \( C_{t_{ref\text{plas}}} \), is subtracted from the \( C_t \) of the gene of interest, \( C_{t_{expt}} \). This is normalized to the control sample by subtracting the difference between the \( C_t \) for 18S rRNA for the control, \( C_{t_{ref\text{plas}}} \), is subtracted from the \( C_t \) of the gene of interest.

For mice TRA1 and TRA2, the forward primer and probe were 5’-CCG CAA ACA CAA CAT TCC G-3’ and 5’-ACT TCT GGC CCA AGC TGA AGA-3’, respectively. The reverse primer for TRA1 was 5’-GGC GTG GGA GGT CAG TCA-3’. This was designed according to GenBank accession number GI-50385. The reverse primer for TRA2 was 5’-TGC CGC TGC CCC CC-3’. This was designed according to GenBank accession number GI-50389. The same amount of RNA was used for +/-, +/-, and −/− soleus muscles.

Statistics. Means and SD were calculated from individual values by standard procedures. A one-way ANOVA was applied to test for the effect of rev-erb{A} or TRA deficiency in the wild-type, heterozygous, and homozygous mouse groups. Differences were considered significant at \( P < 0.05 \).

Table 1. Body weight and soleus, EDL, and TA muscle weights and muscle-to-body weight ratios in wild-type, heterozygous, and homozygous rev-erb{A} mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Soleus Weight, mg (mg/g body wt)</th>
<th>EDL Weight, mg (mg/g body wt)</th>
<th>TA Weight, mg (mg/g body wt)</th>
<th>Heart Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>rev-erb{Aa} (+/+)</td>
<td>5</td>
<td>29 ± 3</td>
<td>8 ± 3 (0.30 ± 0.16)</td>
<td>10 ± 2 (0.36 ± 0.12)</td>
<td>46 ± 5 (1.62 ± 0.35)</td>
<td>156 ± 13 (n = 4)</td>
</tr>
<tr>
<td>rev-erb{Aa} (+/−)</td>
<td>4</td>
<td>28 ± 2</td>
<td>7 ± 1 (0.25 ± 0.06)</td>
<td>9 ± 1 (0.31 ± 0.02)</td>
<td>45 ± 10 (1.61 ± 0.26)</td>
<td>135 ± 24 (n = 5)</td>
</tr>
<tr>
<td>rev-erb{Aa} (−/−)</td>
<td>6</td>
<td>23 ± 4</td>
<td>6 ± 2 (0.29 ± 0.08)</td>
<td>9 ± 3 (0.40 ± 0.14)</td>
<td>36 ± 6 (1.60 ± 0.29)</td>
<td>120 ± 20 (n = 5)</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of mice. EDT, extensor digitorum longus; TA, tibialis anterior; +/+, wild-type; +/-, heterozygous; −/−, homozygous. One-way ANOVA was used to test the effects of receptor knockout on the different variables. *Significantly (\( P < 0.05 \)) different from wild-type mice.

RESULTS

Body weight, muscle weight, and fiber size. In accordance with a previous report, deletion of the rev-erb{Aa} gene was compatible with life and did not cause any obvious anatomic defect, despite the known regulation of rev-erb{Aa} expression during adipocyte and myotube differentiation in vitro (6). However, a lower body weight (21%; \( P < 0.05 \)) was observed in the −/− mice vs. that shown in the wild-type controls. In parallel with the lower body weight, in the −/− mice, the average soleus, EDL, tibialis anterior, and heart muscle weights tended to decrease, with changes of 14–23% in the −/− mice (Table 1). Muscle-to-body weight ratios did not differ between wild-type, +/+, and −/− mice (Table 1).

The average cross-sectional area of the dominating muscle fiber type in the slow-twitch soleus, type I, was 21% smaller in the −/− compared with the other groups of mice, but this difference did not reach the level of statistical significance (Table 2). The size of the soleus type IIA fibers did not differ between the −/−, +/-, and wild-type control groups. In the EDL, the size of the dominating muscle fiber types, i.e., the intermediate and IIB fibers, did not differ between −/−, +/+, and wild-type mice. On the other hand, the type I muscle fibers were twice as large (\( P < 0.05 \)) in the homozygous mice as in the wild-type controls (Table 3). However, type I fiber area measurements in the EDL have to be treated cautiously due to the small number of this fiber type; i.e., fewer type I fibers were measured compared with intermediate and IIB fibers, making these measurements less reliable. In addition, a difference in type I fiber size will not have a significant impact on EDL size and weight due to the small number and size of type I fibers in this muscle.

Expression of the rev-erb{Aa} gene. Enzyme histochemical analyses of EDL and soleus muscle cryosections revealed a

Table 2. Total number of soleus muscle fibers and cross-sectional areas in soleus muscle fiber types classified according to enzyme histochemical stainings for myofibrillar ATPase in +/-, +/-, and −/− rev-erb{Aa} mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total No. of Fibers</th>
<th>Cross-Sectional Area, ( \mu m^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>rev-erb{Aa} (+/+)</td>
<td>5</td>
<td>589 ± 97</td>
<td>1,030 ± 200</td>
</tr>
<tr>
<td>rev-erb{Aa} (+/−)</td>
<td>4</td>
<td>568 ± 98</td>
<td>1,030 ± 310</td>
</tr>
<tr>
<td>rev-erb{Aa} (−/−)</td>
<td>6</td>
<td>529 ± 73</td>
<td>810 ± 220</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of mice. Cross-sectional areas were measured in a total of 50 fibers of types I and IIA.

Table 3. Total number of EDL muscle fibers and cross-sectional areas in EDL muscle fiber types classified according to enzyme histochemical stainings for myofibrillar ATPase in +/-, +/-, and −/− rev-erb{Aa} mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total No. of Fibers</th>
<th>Cross-Sectional Area, ( \mu m^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>rev-erb{Aa} (+/+)</td>
<td>5</td>
<td>622 ± 224</td>
<td>220 ± 20</td>
</tr>
<tr>
<td>rev-erb{Aa} (+/−)</td>
<td>4</td>
<td>549 ± 178</td>
<td>390 ± 180</td>
</tr>
<tr>
<td>rev-erb{Aa} (−/−)</td>
<td>6</td>
<td>559 ± 301</td>
<td>470 ± 60*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no of mice. Cross-sectional areas were measured in a total of 50 type IIB fibers and 50 or as many as possible of type I (13 ± 5, 6–23) and intermediate fibers (32 ± 1, 20–50). One-way ANOVA was used to test the effects of receptor knockout on the different variables. *Significantly (\( P < 0.05 \)) different from wild-type mice.
normal organization of oxidative, intermediate, and glycolytic fibers in both heterozygous and homozygous mice. The expression of the targeted rev-erbAα gene promoter was determined by measurement of β-galactosidase activity in EDL and soleus muscle fibers from wild-type, heterozygous, and homozygous mutant animals. The muscle fibers were much more strongly stained in the homozygous mice compared with the wild-type or heterozygous mice. This is in accordance with previous observations in cerebellar Purkinje cells and the concept that the rev-erbAα protein normally represses its own expression in vivo (6).

In the fast-twitch EDL, the rev-erbAα gene showed uniform expression within individual muscle cells from both +/− and −/− mice (Fig. 1). In the heterozygotes, type I and intermediate fibers, i.e., the fibers with the highest mitochondrial enzyme activity, showed an intermediate β-galactosidase staining. The large type IIB fibers with the lowest mitochondrial enzyme activity, on the other hand, displayed a weak β-galactosidase staining (Fig. 1). As mentioned above, the rev-erbAα protein represses its own transcription in wild-type and heterozygous mice, resulting in a very strong staining in the homozygous animals due to the lack of autorepression (Fig. 1).

In the slow-twitch soleus, type I and IIA fibers, i.e., muscle fibers expressing the β/slow and the IIA MyHC isoform, are predominant. A small number of soleus muscle cells coexpress β/slow and IIA MyHC isoforms in variable proportions, i.e., fibers labeled as type IIC according to enzyme histochemistry (Fig. 2). More complex β-galactosidase staining was observed in soleus than in EDL. Although less distinct than in EDL, a link between β-galactosidase staining intensity and enzyme histochemical fiber type was also noted in the soleus. That is, in heterozygous animals, β-galactosidase staining was inter-

![Fig. 1. Extensor digitorum longus (EDL) muscle from heterozygous (A) and homozygous (B) rev-erbAα mice. Serial EDL muscle sections were stained for myofibrillar ATPase after acid preincubations (a: pH 4.5; c: 4.3), for β-galactosidase (b), and for succinic dehydrogenase (d). Type I (circles), intermediate (triangles), and type IIB (squares) fibers are indicated with the different stainings. Bars = 50 μm.](http://ajpregu.physiology.org/Content/Full/288/2/R485.jpg)
mediate in type I fibers and very weak to intermediate in fibers of type IIA and IIC (Fig. 2). In the heterozygous mutants, variations in β-galactosidase gene expression were also observed within individual muscle cells. Consistent with the results in the EDL, the staining was stronger in the homozygous animals and was slightly stronger in type IIA than in type I fibers (Fig. 2). Typically, type IIC fibers showed a staining pattern intermediate between types I and IIA in both heterozygous and homozygous animals. This is consistent with the MyHC isoform expression in this fiber type and the variable staining between different IIC fibers, which could, at least in part, be related to the variable expression of type I and IIA MyHC isoforms in different IIC fibers. In both EDL and soleus, incubation times were 2 h in the homozygous animals due to the strong β-galactosidase staining intensity; muscle sections from heterozygous animals, however, were incubated for 5 h (Figs. 1 and 2).

A few muscle spindles were observed in the soleus cross sections, and the β-galactosidase gene is expressed in both intra- and extrafusal muscle fibers (Fig. 2). Although a detailed analysis of intrafusal spindle fibers was beyond the scope of this study, the strongest staining intensity was observed in intrafusal fibers from homozygous animals (Fig. 3).

**MyHC isoform composition.** In the fast-twitch EDL, the IIx and IIb MyHC isoforms were strongly dominant in rev-erbAα wild-type, +/−, and −/− mice, and there was no significant difference in MyHC isoform expression between the groups (Fig. 4).

In the slow-twitch soleus, one-way factor analysis demonstrated significant differences in MyHC isoform expression between wild-type and the rev-erbAα-deficient mouse groups. Compared with the type I MyHC content in wild-type controls (47.1 ± 6.1%), a moderate increase was observed in +/−...
(58.5 ± 5.6%; P < 0.05) and a more pronounced increase (68.5 ± 6.0%; P < 0.001) in \(rev-erbA\) mice (Fig. 4). The altered type I MyHC isoform expression was accompanied by a corresponding decrease in type Ila MyHC content. The type Ix MyHC content was very low, and the IIb MyHC isoform was absent in the soleus muscles in all three groups.

Neither embryonic nor neonatal MyHC isoforms were detected in any of the EDL or soleus muscles from the wild-type or \(rev-erbA\) mutant mice by electrophoretic or immunocytochemical techniques (data not shown).

**TR\(1\) and TR\(2\) mRNA expression.** Because thyroid hormone exerts strong control on MyHC isoform expression via thyroid hormone receptors (54), we determined the TR\(1\) and TR\(2\) receptor mRNA levels in the soleus. Quantitative real-time RT-PCR measurements showed that TR\(1\) mRNA levels were 65% lower (P < 0.01) in homozygous than in wild-type mice. The heterozygous animals had 45% lower TR\(2\) mRNA levels than wild-type controls, but this difference was not statistically different. This resulted in a tendency toward an increased TR\(1\)-to-TR\(2\) ratio in the \(rev-erbA\)-deficient mice (Table 4).

**MyHC isoform composition in TR\(2\)-deficient mice.** TR\(1\) expression and thyroid hormone \(\beta\)-receptor expression have strong impacts on skeletal muscle expression of MyHC isoforms, especially in the slow-twitch soleus (54). The influence of the nonligand-binding TR\(2\) on skeletal muscle MyHC isoform expression, on the other hand, remains unknown. In an additional series of experiments, we therefore measured MyHC isoform expression in EDL and soleus muscles from four TR\(2\) wild-type, four heterozygous, and four homozygous \(rev-erbA\) mutant mice of age 4-5 mo.

Late-onset growth retardation has been reported in TR\(2\)-deficient mice (47), but no signs of lower body weight were observed in the present study. This absence of a growth reduction might be due to the lower age and smaller sample size in this study. The body and soleus weights did not differ between the wild-type (28.9 ± 2.4 g; 6.3 ± 1.2 mg), +/−
MyHC isoform expression in rev-erbAα mutant mice

Table 4. TRα1 and TRα2 expression in +/+ , +/−, and −/− rev-erbAα mutant mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TRα1</th>
<th>TRα2</th>
<th>TRα1 to TRα2</th>
</tr>
</thead>
<tbody>
<tr>
<td>rev-erbAα (+/+)</td>
<td>4</td>
<td>1.05±0.24</td>
<td>0.80±0.24</td>
<td>1.43±0.42</td>
</tr>
<tr>
<td>rev-erbAα (+/−)</td>
<td>4</td>
<td>1.17±0.15</td>
<td>0.44±0.14</td>
<td>2.33±0.27</td>
</tr>
<tr>
<td>rev-erbAα (−/−)</td>
<td>5</td>
<td>0.93±0.35</td>
<td>0.29±0.18*</td>
<td>3.55±1.48</td>
</tr>
</tbody>
</table>

Arbitrary units; n = no. of mice. TRα1 and TRα2, thyroid hormone α1 and α2 receptor, respectively. One-way ANOVA was used to test the effects of receptor knockout on the different variables. *Significantly (P < 0.01) different from wild-type mice.

Table 5. Relative contents of MyHC isoforms in soleus and EDL muscles from TRα2+/+, +/−, and −/− mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Soleus Type I, %</th>
<th>Soleus Type IIa, %</th>
<th>Soleus Type IIx, %</th>
<th>Soleus Type IIb, %</th>
<th>EDL Type IX, %</th>
<th>EDL Type Iib, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα2 (+/+)</td>
<td>4</td>
<td>42±2</td>
<td>37±7</td>
<td>17±6</td>
<td>3±3</td>
<td>23±2</td>
<td>77±2</td>
</tr>
<tr>
<td>TRα2 (+/−)</td>
<td>4</td>
<td>42±2</td>
<td>40±2</td>
<td>16±3</td>
<td>2±2</td>
<td>26±3</td>
<td>74±3</td>
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<tr>
<td>TRα2 (−/−)</td>
<td>4</td>
<td>40±2</td>
<td>40±2</td>
<td>20±2</td>
<td>0±0</td>
<td>20±2</td>
<td>80±2</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = no. of mice. MyHC, myosin heavy chain.

Discussion

The major findings in this study are as follows. 1) Fiber type-specific, i.e., MyHC isoform-specific, expression of the rev-erbAα protein was observed in both the EDL and soleus. In the EDL, the heterozygous animals showed the strongest β-galactosidase staining in the type I and intermediate fibers, whereas the opposite pattern was seen in the homozygous animals; i.e., type IIB fibers displayed the strongest staining intensity. In the soleus, the link to fiber type/MyHC isoform was less distinct than in the EDL, but there was a general trend toward stronger β-galactosidase staining in the type I fibers in the heterozygous mice, whereas the type IIA fibers showed the strongest staining intensity in the homozygous mice. 2) Heterozygous and homozygous rev-erbAα mice showed a significant fast (type Ia)-to-β/slow (type I) MyHC isoform transformation in the slow-twitch soleus muscle. 3) The TRα2 content was lower in the rev-erbAα-deficient mice. However, soleus MyHC isoform composition did not differ between wild-type and TRα2-deficient mice, indicating that the lower TRα2 content in the rev-erbAα-deficient mice is an unlikely mechanism underlying the altered soleus muscle MyHC isoform composition.

Effects of rev-erbAα deficiency on MyHC isoform composition. The mechanism of action of T3 in skeletal muscle is complex and not fully understood, but there are several lines of evidence indicating that T3 targets the myogenic helix-loop-helix transcription factors (the myoD gene family) and the contractile proteins via skeletal muscle nuclear receptors (37).

It has been postulated that, during muscle differentiation, the rev-erbAα orphan protein modulates the T3 response of skeletal muscle via direct activation of the muscle-specific myoD gene family. In vitro experiments have shown abundant levels of rev-erbAα mRNA in dividing myoblasts, followed by depressed rev-erbAα mRNA levels and the appearance of muscle-specific mRNAs when the myoblasts have differentiated into postmitotic multinucleated myotubes (8). The importance of the rev-erbAα orphan protein during muscle differentiation was further supported by the complete abolishment of muscle differentiation in muscle cells overexpressing rev-erbAα (8). Based on these in vitro experiments, Downes et al. (8) concluded that rev-erbAα acts as a negative regulator of myogenesis by targeting the myoD family, presumably by disrupting thyroid hormone receptor-homodimer and thyroid hormone receptor-retinoid X receptor heterodimer formation on thyroid response elements in the promoter myoD family members.

In adult mice, rev-erbAα mRNA is an ubiquitously expressed transcript, and the most abundant levels have been observed in brown fat, skeletal muscle, and brain (6, 11, 27). In the adult homozygous and heterozygous rev-erbAα mutant mice, fast- and slow-twitch skeletal muscles showed a normal distribution of muscle fiber types and mitochondrial enzyme activities. Only marginal anatomic and morphological differences were observed between the wild-type, heterozygous, and homozygous mice, such as a slightly lower body weight, unaltered muscle-to-body weight ratios, and smaller muscle fiber sizes (type I fibers in the soleus) in the homozygous mice, suggesting a mild general growth retardation.

Intriguingly, the alterations observed in the soleus from the rev-erbAα mutant mice were reminiscent of observations in response to hypothyroidism; i.e., the relative content of the β/slow (type I) MyHC increased from the wild-type controls to the mutant mice, with the highest proportion in the homozygous animals and intermediate values in the heterozygous mice. Circulating T3 and thyroxine levels have been reported to be normal in rev-erbAα mutant mice (6), and hypothyroidism is an unlikely mechanism underlying the different MyHC composition in control and rev-erbAα mutant mice. However, different tissue concentrations or altered hormone deiodination cannot be completely ruled out as an underlying mechanism. It is interesting to note that rev-erbAα suppresses the activity of thyroid hormone receptors and thyroid hormone responsive elements in myotubes (8), indicating that the rev-erbAα may play a different role in adult and developing skeletal muscle.

Nerve-evoked activations of skeletal muscle have a strong influence on myofibrillar protein synthesis and muscle contractility (32). A dramatic increase in physical activity levels over a long time induces fast-to-slow MyHC isoform transition in skeletal muscle (16). Although phenotypic measurements of...
the rev-erbAα mutant mice were not investigated in detail by our group, or to the best of our knowledge by any other group, we did not observe any obvious differences in physical activity patterns between wild-type, heterozygous, or homozygous animals. Thus altered physical activity levels appear to be an unlikely mechanism underlying the increased relative content of the β/slow (type I) MyHC isoform in the soleus from heterozygous and homozygous mice.

Fiber-type-specific expression of the rev-erbAα protein. In both the fast-twitch EDL and the slow-twitch soleus muscles, rev-erbAα protein expression was linked to enzyme histochemical fiber type determined by myofibrillar ATPase staining pattern. The strongest β-galactosidase staining intensity was observed in fast-twitch (types IIA, intermediate, and IIB) in homozygous and in slow-twitch muscle fibers in heterozygous mice. However, differences in the rev-erbAα protein expression were also observed between the two muscles, such as a larger variability in the rev-erbAα protein expression between as well as within muscle fibers of the same histochemical type in the soleus than in the EDL muscle.

The rev-erbAα protein may modulate thyroid hormone action via the myoD gene family; i.e., rev-erbAα downregulates thyroid receptor/T3-mediated transcriptional activity from the myogenin promoter and thyroid hormone response element (8). The observation by Hughes et al. (18) that myogenin transcripts are selectively expressed in slow-twitch muscles could in part explain the upregulation of the β/slow MyHC isoform in the soleus of rev-erbAα-deficient mice. Furthermore, rodent soleus muscle contains different populations of type I fibers that are indistinguishable with respect to enzyme histochemical fiber type and MyHC isoform expression but differ from each other in the response to denervation and to thyroid hormone (49). The variable expression of the rev-erbAα gene between and within soleus fibers and the modulatory role of the rev-erbAα gene in thyroid hormone action on MyHC synthesis may accordingly contribute to, or cause, the variable response to circulating thyroid hormone levels and nuclear thyroid hormone receptor deficiency in the slow-twitch soleus (25, 28, 29, 53, 54).

A detailed presentation of the rev-erbAα protein expression in intrafusal muscle fibers is beyond the scope of this manuscript. However, it is interesting to note the similar β-galactosidase staining pattern in intrafusal and extrafusal muscle fibers, i.e., a stronger staining intensity in homoyzgous than in heterozygous animals. Intrafusal muscle fibers express MyHC isoforms that are not expressed in extrafusal limb muscles (30, 31, 41), and this may explain the weaker β-galactosidase staining intensity in intrafusal than in extrafusal muscle fibers.

Effects of the mutant TRα locus. The targeting of the TRα2 gene ablated the expression of the TRα2 protein and resulted in an unavoidable three- to sixfold increase in TRα1 as a result of precluding splicing of the nuclear RNA to a TRα2 mRNA (13, 47). The TRα2-deficient mice exhibited both characteristics of hypothyroidism, such as low levels of circulating thyroid hormone, and hyperthyroidism, such as high heart rate. These features have been ascribed to the increased levels of TRα1 and not the lack of TRα2, since the heterozygous mice showed a phenotype intermediate between the wild-type control and the TRα2-deficient mice (47), indicating that tissue availability to T3 may determine the proportion of ligand-bound vs. ligand-free TRα1 and hence also the effects on target gene expression.

Despite the lower levels of thyroid hormone, soleus MyHC isoform expression did not differ between TRα2-deficient and control mice. It is therefore suggested that the lower TRα2 expression is an unlikely mechanism underlying the higher type I MyHC content in the rev-erbAα-deficient mice; rather, the data indicate that overexpression of TRα1 in the TRα2-deficient mice counteracts the effects of hypothyroidism. It is therefore suggested that the rev-erbAα orphan protein functions as a physiologically relevant regulator of myosin synthesis by modulating TRα1 expression. This is supported by muscle-type-specific differences in MyHC isoform and TRα1 expression in response to hypothyroidism (51). In the slow-twitch soleus muscle, hypothyroidism is characterized by an increased content of the type I MyHC isoform and decreased TRα1 expression. In the fast-twitch EDL muscle, on the other hand, hypothyroidism has no significant impact on either MyHC isoform or TRα1 expression (51).

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