Differences in histone modifications between slow- and fast-twitch muscle of adult rats and following overload, denervation, or valproic acid administration

Fuminori Kawano,1 Keisuke Nimura,2 Saki Ishino,3 Naoya Nakai,4 Ken Nakata,5 and Yoshinobu Ohira6

1Graduate School of Health Sciences, Matsumoto University, Matsumoto, Nagano, Japan; 2Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan; 3Center for Medical Research and Education, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan; 4School of Human Cultures, University of Shiga Prefecture, Hikone, Shiga, Japan; 5Medicine for Sports and Performing Arts, Graduate School of Medicine, Osaka University, Toyonaka, Osaka, Japan; and 6Graduate School of Sports Sciences, Doshisha University, Kyotanabe City, Kyoto, Japan

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Kawano F, Nimura K, Ishino S, Nakai N, Nakata K, Ohira Y. Differences in histone modifications between slow- and fast-twitch muscle of adult rats and following overload, denervation, or valproic acid administration. J Appl Physiol 119: 1042–1052, 2015. First published September 24, 2015; doi:10.1152/japplphysiol.00289.2015.—Numerous studies have reported alterations in skeletal muscle properties and phenotypes in response to various stimuli such as exercise, unloading, and gene mutation. However, a shift in muscle fiber phenotype from fast twitch to slow twitch is not completely induced by stimuli. This limitation is hypothesized to result from the epigenetic differences between muscle types. The main purpose of the present study was to identify the differences in histone modification for the plantaris (fast) and soleus (slow) muscles of adult rats. Genome-wide analysis by chromatin immunoprecipitation followed by DNA sequencing revealed that trimethylation at lysine 4 and acetylation of histone 3, which occurs at transcriptionally active gene loci, was less prevalent in the genes specific to the slow-twitch soleus muscle. Conversely, gene loci specific to the fast-twitch plantaris muscle were associated with the aforementioned histone modifications. We also found that upregulation of slow genes in the plantaris muscle, which are related to enhanced muscular activity, is not associated with activating histone modifications. Furthermore, silencing of muscle activity by denervation caused the displacement of acetylated histone and RNA polymerase II (Pol II) in 5′ ends of genes in plantaris, but minor effects were observed in soleus. Increased recruitment of Pol II induced by forced acetylation of histone was also suppressed in valproic acid-treated soleus. Our present data indicate that the slow-twitch soleus muscle, which has a unique set of histone modifications, which may be induced by the preservation of the genetic backbone against physiological stimuli.

epigenetics; fast and slow skeletal muscle; gene expression; muscle activity

SLOW-TWITCH SKELETAL MUSCLES are characterized by an ability for high energy expenditure, glucose uptake, and resistance to both stress and fatigue (13, 19, 23). Skeletal muscles acquire these characteristics in accordance with their tonic contractile activity, which is necessary to maintain posture against gravity (6, 14, 17, 18, 24). Running exercise augments the characteristics of fast-twitch muscles and produces muscle fibers that are more oxidative and insulin sensitive, but do not show any phenotypic transformations [e.g., a shift in myosin heavy chain (MHC) isoforms] (20, 30). Overexpression of paxilloxome proliferator-activated receptor gamma coactivator 1 alpha, one of the central transcriptional coregulators of mitochondrial biogenesis (4), improves skeletal muscle endurance, but does not affect muscle fiber type (35). Furthermore, myogenin 1, also known as calsarcin 2, inhibits the activation of calcineurin and nuclear factor of activated T cells, which activates genes specific to slow-twitch muscle fibers (7). However, the knockout of myogenin 1 in mice does not result in an increase in the population of slow fibers that express type I MHC (type I fibers) in fast-twitch gastrocnemius muscle, whereas in myogenin 1-knockout mice, the oxidative metabolism is enhanced (7). Conversely, loss of muscle activity triggers a phenotypic shift in muscle characteristics toward those of fast-twitch fibers, with continued inactivity resulting in muscle fiber atrophy. Previous studies have demonstrated that slow-twitch muscles such as the soleus replace type I fibers with hybrid fibers that coexpress type I and II MHC immediately after the unloading/inactivation experienced during space flight (2, 32), tail suspension (25, 26), or denervation (28) in rodents. These results suggest the existence of some underlying differences between fast- and slow-twitch muscle fibers that prevent their interconversion.

Epigenetic modification plays a crucial role in mediating cell fate. Trimethylation at lysine 4 of histone 3 (H3K4me3) and acetylation of histone 3 (H3) typically mark transcriptionally active promoters and gene bodies in various human cells (3, 37, 38). For instance, the histones present at the MyoD-binding sites in skeletal muscle cells are acetylated during embryonic myogenesis, which allows MyoD to regulate the expression of skeletal muscle-specific genes (21, 36). The critical role of epigenetic histone modifications in this process suggests that histone modifications can mediate cell differentiation. Pandorfo et al. (27) reported alterations in the active histone modifications of H3K4me3 and H3 acetylation, based on the upregulation and downregulation of type I, IIx, and IIb MHC genes in the plantaris and soleus muscles or after hindlimb unloading; in contrast, it was found that the histone modifications in type IIa MHC gene did not respond to unloading despite downregulation of gene expression in the soleus. These results suggest that gene expression is not simply regulated by the levels of histone modification in skeletal muscles. However, histone modifications in skeletal muscles are not well understood across the genome. Therefore, the main purpose of the present work was to identify the major differences in histone modifications and the regulation of gene expression between fast- and slow-twitch muscles in adult rats.

MATERIALS AND METHODS

Animal care. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the Physiological Society of Japan. The study was also submitted for reprint requests and other correspondence: Fuminori Kawano, Graduate School of Health Sciences, Matsumoto University, Niimiura 2095-1, Matsumoto, Nagano 390-1295, Japan (e-mail: fuminori.kawano@matsu.ac.jp).
approved by the Animal Use Committee at the Graduate School of Medicine, Osaka University (approval 22-071). All experiments in the present study used male Wistar Hannover rats (Nihon CLEA, Tokyo, Japan). We used 8-wk-old animals to compare normal muscles with those that underwent denervation or administration of valproic acid (VPA, Wako, Osaka, Japan), and 14-wk-old animals to investigate the effects of overloading. For the study in neonatal rats, littermates were caged with their mother for nursing. Three adult rats or a single littermate were housed in cages (28/11003 45/11003 20 cm) in an animal room under a 12:12-h light:dark cycle with temperature and humidity maintained at 20–24°C and 40–60%, respectively. A commercial solid diet (CE-2; Nihon CLEA) and water were supplied ad libitum. All surgeries and samplings were performed under anesthesia with i.p. injection of sodium pentobarbital (5 mg/100 g body wt). Samplings of the plantaris and soleus muscles of adult rats were performed after cardiac excision to reduce the blood proteins within the muscle. To avoid the effects of acute ischemia, the muscles were sampled immediately after the cardiac excision. In neonatal rats, the plantaris and soleus muscles were sampled immediately after decapitation on postnatal day 2, 7, 14, or 21 (n = 3 for each time point). Muscles were frozen in liquid nitrogen and stored at −80°C until analysis. Three animals in individual groups were used to compare the differences between normal plantaris and soleus muscles, and six animals were used to compare muscle overloading, denervation, or VPA treatment.

Table 1. Sequences of primer pairs for ChIP-qPCR analysis

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Position</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actn3</td>
<td>TSS–200bp</td>
<td>GCCTGAGCAAGATGATGATG</td>
<td>ACTCACTTTCCGCTGCTGTT</td>
</tr>
<tr>
<td>Cd24</td>
<td>TSS–200bp</td>
<td>GGGAAGCAAGGTATGCTCTG</td>
<td>TTTGCTAACACGCACTTTCA</td>
</tr>
<tr>
<td>Gtp2</td>
<td>TSS–200bp</td>
<td>AGGTGAAAGGCTGAGGATGA</td>
<td>CAAAGTACCTGGCCACAGAC</td>
</tr>
<tr>
<td>Mstn</td>
<td>TSS–200bp</td>
<td>GGGCCACTGGATCTAAAGAG</td>
<td>TTGGTCTGCGAGCTGATGGA</td>
</tr>
<tr>
<td>Bdh1</td>
<td>TSS–200bp</td>
<td>CAGTTTTAGGGGTCCGGAGA</td>
<td>AGACCCTCTTAACGCAAGAC</td>
</tr>
<tr>
<td>Lgi1</td>
<td>TSS–200bp</td>
<td>TCGAGAAAGGATCGGAAATGC</td>
<td>CTGGAAGAGATCTGCGATTT</td>
</tr>
<tr>
<td>Paccrg</td>
<td>TSS–200bp</td>
<td>CTAGCCTTGAGATCGGAGAC</td>
<td>AGAGATGGGTGGATGAGAGA</td>
</tr>
<tr>
<td>Scl16a7</td>
<td>TSS–200bp</td>
<td>TAAATCGGAGAGTTGATGAG</td>
<td>ACAAGATGAGAGAAGGAGA</td>
</tr>
<tr>
<td>Myh7</td>
<td>TSS–200bp</td>
<td>CAGTTTTAGGGGTCCGGAGA</td>
<td>AGACCCTCTTAACGCAAGAC</td>
</tr>
<tr>
<td>Hspb1</td>
<td>TSS–200bp</td>
<td>TCGAGGAAGGATCGGAAATGC</td>
<td>CTGGAAGAGATCTGCGATTT</td>
</tr>
</tbody>
</table>

bp, base pairs; ChIP, chromatin immunoprecipitation; qPCR, quantitative polymerase chain reaction; TSS, transcription start site.
Muscle treatment. Responses of muscular properties to overloading, denervation, and administration of VPA were studied. Overloading of plantaris muscle was performed. The distal tendons of left soleus and gastrocnemius muscles were completely transected to avoid reattachment of tendons to the plantaris. Denervation was performed by transaction (~5 mm) of the left sciatic nerve at the gluteal level. No treatments were given to the rats used as controls. Sampling of soleus and/or plantaris was performed in both normal and experimental groups 28 days after surgery. The muscle was cleaned of excess fat and connective tissue and pinned on a cork, thereby keeping the muscle stretched at an optimum length. The muscle was frozen in liquid nitrogen-cooled isopentane and stored at −80°C until analysis.

Right muscles were not used for any analyses.

Animals were given i.p. injections of 200 mg/ml VPA diluted in saline (300 mg/kg body wt) daily for 7 days. On the final day, injections were given 2 h before muscle sampling. Animals in the control group were injected with an equal volume of saline following the same schedule and methods. Soleus and plantaris muscles were sampled bilaterally and stored as stated above.

RNA extraction. A piece of muscle (10–20 mg) or whole muscle from neonatal rat was homogenized in 1 ml of ISOGEN (Nippon Gene, Tokyo, Japan). RNA extraction was performed following the manufacturer’s instructions. The final pellet of RNA was resuspended in ultrapure water. For quantitative PCR (qPCR), cDNA was synthesized from 400 ng of total RNA using a SuperScript VILO master mix (Invitrogen, Waltham, MA). The mixture of RNA was incubated at 42°C for 60 min followed by inactivation of enzyme at 85°C for 5 min. Complementary DNA was diluted to 1/20 by ultrapure water and stored at −20°C until analysis.

Microarray and selection of specific genes. We used a commercial service for microarray analysis, which included CodeLink Bioarray (Applied Microarrays, Tempe, AZ) and SurePrint G3 Rat GE 8 × 60K Microarray (Agilent Technologies, Santa Clara, CA). Comparisons between plantaris and soleus muscles were double-checked using two different arrays, and the RNA sample obtained from the overload experiment was determined by CodeLink Bioarray only. Full data of gene expression using Agilent Microarray are available on the Gene Expression Omnibus (GEO) database managed by the National Center for Biological Information.

![Fig. 2. Comparison of gene expression and histone modification between normal plantaris (PL) and soleus (Sol) muscles. A: relative expression levels of fast and slow genes in the PL (open bars) and Sol (closed bars) normalized by Rp121 (internal control). B: the model indicates the sites analyzed by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR). The distribution of active histone markers H3K4me3 (C and D) and H3 acetylation (E and F) at the sites from the transcription start site (TSS) to 200 bp downstream (C and E) and from 200 to 400 bp downstream (D and F), normalized by the amount of DNA used for the reaction, are shown. The values of ChIP-qPCR data are expressed as % input. Dotted lines in C–F indicate the levels of negative controls tested with rabbit IgG. Values are means ± SE. *P < 0.05.](http://jap.physiology.org/content/105/5/1044)
for Biological Information (identification number GSE62028). Data containing comparisons between plantaris and soleus, or overloaded and normal plantaris obtained from CodeLink Bioarray are accessible as an additional file from the author because the Bioarray data format does not universally translate for publication on the APS web site. Type-specific fast genes were selected on the basis of the following requirements: 1) the expression level in the normal plantaris was greater than twofold higher compared with the normal soleus, and 2) the genes were not upregulated greater than twofold by overloading in the plantaris, because we speculated that fast genes may not be upregulated when the muscle is transformed toward slower properties in response to overloading. Furthermore, the top four genes with the greatest differences between normal plantaris and soleus were selected as fast-specific genes. Slow-specific genes were searched with the reverse requirements. The expression level of the identified genes was quantified and confirmed by qPCR using the specific Taqman probes (Applied Biosystems, Waltham, MA) shown in the section Quantitative PCR.

Chromatin immunoprecipitation. Muscle segments (20–40 mg) were homogenized in cooled PBS containing protease inhibitors (Millipore, Billerica, MA) and 5 mM butyrate. After centrifugation at 12,000 g, the pellet was fixed in 1% paraformaldehyde on ice for 10 min followed by quenching in 200 mM glycine. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, 1% SDS, and 10 mM EDTA, pH 8.0) and sonicated using a Sonifier 250 (Branson, Swedenboro, NJ). For chromatin immunoprecipitation (ChIP)-qPCR, 12 s of continuous sonication followed by 30 s on ice was repeated eight times. This procedure resulted in 900 bp of peak and 500 bp of average DNA fragment size. For ChIP followed by DNA sequencing (ChIP-seq), 20 s of continuous sonication followed by a 30-s interval on ice was repeated 15 times, resulting in 300 bp of peak and average DNA fragment size. After centrifugation at 12,000 g, the supernatant was stored as the chromatin at −80°C until analysis.

Chromatin was diluted at 1/10 in a buffer solution containing 50 mM Tris·HCl (pH 8.0), 167 mM NaCl, and 1.1% Triton X-100, mixed with anti-H3K4me3 (39159; Active Motif, Carlsbad, CA), anti-pan-acetyl H3 (39139; Active Motif), or anti-RNA polymerase II (Pol II 102660; Active Motif) at 1:50 concentration and incubated with rotation overnight at 4°C. The level of input DNA contained in chromatin used for ChIP analysis was estimated without any reactions. Protein G agarose beads (9007, 20 μL for each reaction; Cell Signaling Technology, Danvers, MA) for ChIP-qPCR or protein G agarose beads (9007, 20 μL for each reaction; Cell Signaling Technology, Danvers, MA) for ChIP-seq were added, and the samples were incubated with rotation overnight at 4°C. After centrifugation at 12,000 g, the supernatant was stored as the chromatin at −80°C until analysis.

Fig. 3. Gene expression and histone modifications in typical slow genes. A: relative expression levels of Myh7 and Hspb1 in the plantaris (PL, open bars) and soleus (Sol, closed bars), normalized by Rp121 (internal control). The distribution of active histone markers H3K4me3 (B) and H3 acetylation (C) at the sites from the transcription start site (TSS) to 200 bp downstream and from 200 to 400 bp downstream, normalized by the amount of DNA used for the reaction, are shown. The values of ChIP-qPCR data are expressed as % input. Values are means ± SE. *P < 0.05.

Fig. 4. Absolute expression levels of fast- and slow-specific genes in plantaris and soleus. The distribution of the signal intensity of each gene obtained from microarray (Agilent) analysis in plantaris (PL, top) and soleus (Sol, bottom) muscles was plotted. Gray lines show the distribution of all genes (58,717 probes) with various signal intensities. Levels of signal intensity (expression) of four target fast-specific (Actn3, Cd24, Gpt2, and Mstn), slow-specific (Bdh1, Lgl1, Pacrg, and Slc16a7), and typical slow (Myh7 and Hspb1) genes are indicated.
sepharose beads (17061801; GE Healthcare, Little Chalfont, UK) for ChIP-seq were added in the chromatin-antibody mixture and incubated with rotation for 4 h at 4°C. The beads were then washed five times in 1 ml of RIPA buffer (50 mM Tris·HCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 8.0) containing 150 mM NaCl followed by second wash, also five times, in 500 μl of RIPA buffer containing 500 mM NaCl. The beads were resuspended in 250 μl of elution buffer (10 mM Tris·HCl, 300 mM NaCl, 5 mM EDTA, and 0.5% SDS, pH 8.0). RNase A was added up to a final concentration at 50 mM and incubated for 15 min at room temperature to ensure that the PCR product was not amplified from the remaining RNA. Subsequently, proteinase K (Takara Bio, Shiga, Japan) was added up to a final concentration of 0.6 mg/ml and incubated for 1 h at 65°C. DNA was extracted by adding phenol-chloroform-isoamyl alcohol solution (25:24:1) and by centrifugation at 12,000 g for 10 min at 20°C. The supernatant was sampled and ethanol precipitation was performed.

Fig. 5. Enhanced expression of fast-specific genes is independent of the beginning of muscular activity after birth. The postnatal changes in the expression of fast-specific (top) and slow-specific (bottom) genes were determined in the plantaris (PL, open bars) and soleus (Sol, closed bars) muscles on postnatal days 2 (P2), 7 (P7), 14 (P14), and 21 (P21). The expression level was represented as relative values (log2 ratio) to one of the values in the soleus P2 group. Note that fast genes were upregulated in the plantaris during the first week after birth, although the transcription of slow genes, except for Lgi1, was upregulated after the beginning of voluntary locomotion, which started the third week after birth. Values are means ± SE. *P < 0.05 vs. plantaris.

0.5% SDS, pH 8.0). RNase A was added up to a final concentration at 50 mM and incubated for 15 min at room temperature to ensure that the PCR product was not amplified from the remaining RNA. Subsequently, proteinase K (Takara Bio, Shiga, Japan) was added up to a final concentration of 0.6 mg/ml and incubated for 1 h at 65°C. DNA was extracted by adding phenol-chloroform-isoamyl alcohol solution (25:24:1) and by centrifugation at 12,000 g for 10 min at 20°C. The supernatant was sampled and ethanol precipitation was performed.

Fig. 6. Effects of overloading on fast- and slow-specific gene expression and histone modifications in plantaris muscle. A: changes in gene expressions of fast-specific (top) and slow-specific (bottom) genes between normal (control, open bars) and overloaded (closed bars) plantaris. Distribution of H3K4me3 (B) and H3 acetylation (C) in fast- and slow-specific genes. Data from eight sites (four genes including two sites for each gene) are individually plotted and compared between normal (control, open circles) and overloaded (closed circles) plantaris. Values for ChIP-qPCR data are expressed as % input. Values are means ± SE in A. The mean value of each site in each group (n = 6) was plotted in B and C. *P < 0.05.
using Ethachinmate (Nippon Gene). The final pellet was resuspended in Tris-EDTA buffer and stored at −20°C. A negative control was also tested via the same process, but normal rabbit immunoglobulin (2729; Cell Signaling Technology) was used for the ChIP reaction.

**Sequencing analysis.** Libraries of ChIP samples for DNA sequencing were constructed using the SOLiD Fragment Library Barcoding Kit (Life Technologies, Waltham, MA), according to the manufacturer’s instructions. AMPure (Beckman Coulter, Brea, CA) was used for size-selection of sequencing libraries. SOLiD4 was used to sequence these libraries. The reads we obtained were mapped to the rat reference genome (rn4) by Bioscope (Life Technologies). Mapped reads were normalized using the Homer software suite for ChIP-seq analysis (9). Occupancies of mapped reads around the transcription start site (TSS) were calculated using CEAS (34) and visualized using the free software known as R (https://www.r-project.org).

**Quantitative PCR.** Quantitative PCR analysis was performed using the QuantiStudio 7 Flex Real-Time PCR system (Applied Biosystems). To analyze mRNA expression, the specific Taqman probes (Applied Biosystems) were purchased and used according to the manufacturer’s recommended dilution procedures. For ChIP-qPCR analysis, primer pairs were designed at 0–200 bp and 200–400 bp regions from the TSS of fast (Actn3, Cd24, Gpt2, and Mstn) and slow (Bdh1, Lgi1, Pacrg, Slc16a7, Myh7, and Hspb1) genes (see Table 1 for the sequences). Quantitative PCR was performed using a Power SYBR master mix (Applied Biosystems). A mixture of cDNA or DNA combined with all samples and the particular dilutions (1/5, 1/25, 1/125, and 1/625) were tested to evaluate the standard curve in each assay and the values were used to quantify the relative differences among experimental samples. The quantitative values of samples were further normalized by the expression level of Rpl21 gene as the internal control for mRNA expression, and by the level of individual input for ChIP-qPCR (% input).

**Western blot analysis.** Chromatin-enriched extract was obtained as stated above for protein expression analysis but without fixation. The chromatin extract was dissolved in an equal amount of x2 loading buffer (20% glycerol, 12% 2-mercaptoethanol, 4% SDS, 100 mM Tris-HCl, and 0.05% bromophenol blue, pH 6.7). SDS-PAGE was carried out on a 12.5% polyacrylamide slab gel (1.5 mm thickness) at a constant current of 20 mA per gel for ~2 h at 4°C. Equal amounts (10 μl) were loaded onto each lane. After SDS-PAGE, the proteins were transferred to polyvinylidene-fluoride membranes (Bio-Rad) by using trans-blot cell (Bio-Rad) at a constant voltage of 60 V for 2 h at 4°C. After the transfer of protein, the membranes were blocked in blocking buffer (5% nonfat dry milk, diluted in 0.1% Tween-20 in Tris-buffered saline) for 1 h. The membranes were incubated overnight at 4°C with anti-pan-acetyl H3 (39139; Active Motif) or anti-total H3 (4620; Cell Signaling Technology) diluted 1:2,000 in 0.1% Tween-20 in Tris-buffered saline containing 5% BSA. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 30 min. The antibody-bound protein was detected by a chemiluminescence method using an enhanced chemiluminescence plus kit (GE Healthcare).

**Quantification of bands was performed using ImageJ image analyzing software.** The protein level was expressed as the integrated density of the band, which was calculated as the mean density multiplied by the band area. The intensity of the band was further normalized as the levels relative to the individual total H3.

**Statistical analysis.** All values are expressed as means ± SE. Significant differences were examined by unpaired t-test if the comparison was performed between two groups (normal plantaris vs. soleus and normal vs. overloaded plantaris). For data that were obtained from the experiments in neonatal rats, denervation, and VPA administration models, significant differences were examined by two-way ANOVA followed by a Scheffe’s post hoc test. Differences were considered significant at the 0.05 level of confidence.

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**RESULTS**

**Differences in histone modifications between fast- and slow-twitch skeletal muscles.** The distribution of the transcriptionally active histone marker H3K4me3 and H3 acetylation were compared between the fast-twitch plantaris and slow-twitch soleus muscles of adult rats by using ChIP-seq. Figure 1 shows the average modification profile near the TSS. The data were further separated into gene sets specifically expressed in the plantaris (fast genes) or soleus (slow genes), which were selected on the basis of Agilent Microarray data. In the plantaris muscle, the epigenome around the region close to the TSS of fast genes, including the promoters and 5′ ends of gene bodies, showed greater histone modifications (both H3K4me3 and H3 acetylation) than that of all or slow genes (Fig. 1, A and C). However, in the soleus, no relationship was observed between expression and the transcriptionally active histone markers (Fig. 1, B and D).

**Selection of type-specific genes.** For a more thorough investigation, four fast (Actn3, Cd24, Gpt2, and Mstn) and four slow (Bdh1, Lgi1, Pacrg, and Slc16a7) genes that exhibited the greatest differences in their expression between plantaris and soleus muscles were selected in a nonbiased manner from microarray data (see MATERIALS AND METHODS for selection criteria) and analyzed by ChIP-qPCR. Fast genes showed 35-fold to 7,383-fold greater expression in plantaris than in soleus muscles, whereas expression of slow genes was 3-fold to 9-fold greater in soleus than in plantaris muscles (Fig. 2A). Levels of histone modification were measured at the 5′ ends of gene bodies (0–200 and 200–400 bp from the TSS, Fig. 2B).

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Similar to the ChIP-seq results, H3K4me3 and H3 acetylation were significantly (2-fold to 8-fold) greater in all plantaris fast genes (Fig. 2, C–F). However, the results for these markers of transcriptional activity in slow genes were similar between the plantaris and soleus muscles, with the exception that H3K4me3 and H3 acetylation was higher for Lgi1 in the soleus than in the plantaris (Fig. 2, C–F).

We also compared the gene expression and histone modifications in Myh7 and Hspb1 genes, known active genes in slow-twitch muscles. The level of gene expression of the Myh7 and Hspb1 genes in the soleus was 7.8- and 2.7-fold greater, respectively, than that in the plantaris muscle (P < 0.05, Fig. 3A). Levels of H3 acetylation at the 5' ends of gene bodies were significantly greater (2-fold to 75-fold) in both Myh7 and Hspb1 genes in the soleus than in the plantaris, although the level of H3K4me3 was similar between the plantaris and soleus (Fig. 3, B and C).

Figure 4 demonstrates the distribution of all genes on the basis of signal intensities and shows dual peaks in both the plantaris and soleus muscles. Genes in the lower peak might be transcriptionally inactive, show low expression in skeletal muscle fibers, or be expressed in nonmuscle cells; some slow genes are located in this area in the plantaris. However, the type-specific genes showed relatively high expression levels in both the plantaris and soleus.

**Type-specific gene expression in neonatal rats.** To check whether these type-specific genes are upregulated due to muscular activity or are prenatally regulated, changes in their expressions were investigated during the postnatal period in neonatal rats. The expression of all fast genes (Actn3, Cd24, Gpt2, and Mstn) was significantly higher (3-fold to 22-fold) in the plantaris muscles than in soleus muscles within 7 days after birth (Fig. 5). These differences became even larger (7-fold to 1,004-fold) on postnatal day 21 (P < 0.05). A similar difference was observed for the slow gene, Lgi1, which was significantly upregulated (approximately twofold) on postnatal days 2 and 7. This increase rose to approximately 13-fold on postnatal day 21 in the soleus muscle vs. plantaris muscles. Expression levels of the other slow genes (Bdh1, Pacrg, and Slc16a7) were similar between the plantaris and soleus muscles through postnatal day 7. Expression of these slow genes increased thereafter and reached to 2-fold to 3.5-fold higher in soleus muscles compared with plantaris muscles on postnatal day 21.

**Effects of overloading in plantaris muscle on gene expression and histone modifications.** We further investigated whether characteristic slow-type histone modifications are specific to the soleus or are acquired by adaptive regulation. We determined the effects of enhanced muscular activity in the plantaris by the transection of synergists’ distal tendons on the...
expression of type-specific genes and histone modifications. The expression level of slow genes was significantly (1.7-fold to 2.3-fold) greater in the overloaded plantaris than in the normal plantaris, whereas the expression of fast genes was unaffected (Fig. 6A). No changes were observed in H3K4me3 levels, however, H3 acetylation was significantly decreased in both fast and slow genes within the plantaris muscles (Fig. 6, B and C).

**Effects of silenced muscle activity.** Denervation, which silences muscle activity, triggers the wasting of skeletal muscles via the inhibition of histone deacetylase (HDAC) activity and subsequent acetylation of muscle-specific proteins (1, 22). We therefore sought to determine the effects of denervation on gene expression and H3 acetylation in type-specific genes of the plantaris and soleus. The expression level of pan-acetyl H3 was measured in sonication-extracted proteins of the plantaris and soleus muscles by Western blotting. The level of pan-acetyl H3 was significantly upregulated in denervated muscles (2.5-fold and 2.7-fold in plantaris and soleus vs. normal muscles, respectively) (Fig. 7). The results showed that denervation successfully induced the hyperacetylation of H3 in skeletal muscles.

The expression levels of fast genes were downregulated by denervation, although similar effects were not observed in slow genes (Fig. 8A). Contrary to the hyperacetylation of H3 in the extracted protein, the levels of H3 acetylation bound to fast genes became less in denervated plantaris (P < 0.05, Fig. 8C). We further determined the level of Pol II in these sites by ChIP-qPCR (Fig. 8D). Denervation caused a drastic displacement of Pol II (≤ 97% of normal levels in all genes). In plantaris fast genes, denervation resulted in the transcriptional inactivation via loss of mapped H3 acetylation and Pol II. The lowered Pol II level in the slow genes of plantaris did not affect gene expression.

In the soleus, gene expression levels were upregulated in fast genes (Actn3, Cd24, and Mstn) and downregulated in slow genes (Bdh1, Lgi1, and Slc16a7) (Fig. 8B). The results of ChIP-qPCR in H3 acetylation and Pol II recruitment showed minor effects in both fast and slow genes after denervation (Fig. 8D). However, we also observed that levels of Pol II recruitment in the normal soleus muscle were significantly lower than those in the normal plantaris muscle.

**Effects of forced histone acetylation.** Denervation-related effects might be caused by the change in the transcriptional program due to muscle inactivation and to enhanced histone acetylation. We further determined the responses of plantaris and soleus muscles to the pharmacological induction of histone acetylation by administration of the HDAC inhibitor, VPA.

VPA treatment significantly upregulated H3 acetylation in the protein extract of both the plantaris (1.6-fold) and soleus (2.4-fold) muscles (Fig. 9). Increased acetylation of H3, determined by ChIP, was noted in fast and slow genes of the plantaris (P < 0.05, Fig. 10C). However, the level of H3 acetylation was not affected by VPA treatment in the soleus. Distribution of Pol II significantly increased in accordance with the enhanced H3 acetylation by VPA treatment in the plantaris (Fig. 10D). In the saline-treated group, the level of Pol II recruitment was significantly less in slow genes of the soleus than in the plantaris. However, no effects on Pol II recruitment were observed in either fast or slow genes of the soleus after VPA treatment. The VPA-induced acetylation of H3 did not generally affect the expression of fast or slow genes in the plantaris or in the soleus in which H3 acetylation and Pol II recruitment were unaffected (Fig. 10, A and B).

**DISCUSSION**

**Muscle type-specific histone modifications.** We have demonstrated the presence of several histone modifications near muscle type-specific genes that are activated in the fast-twitch plantaris or slow-twitch soleus muscles of adult rats. Previous studies (3, 37, 38) reported that H3K4me3 and H3 acetylation were closely related to the activation of gene transcription. The activation of fast genes in the plantaris agreed with these results. However, according to the genome-wide analysis of histone modifications, the transcription of slow genes was independent of H3K4me3 and H3 acetylation in the soleus. Differences in the expressions of type-specific genes were magnified in the plantaris, indicating that epigenetic histone modifications allowed significant gene upregulation. Furthermore, magnitudes of expression of slow genes in the soleus muscle were not necessarily lower than those of all genes (Fig. 4), so that less histone modification did not simply indicate a weak transcription of slow genes in soleus muscle.

Generally, DNA that is upstream from the TSS lacks contact with nucleosomes, which enables transcriptional factors and Pol II binding and the onset of transcription (11, 29, 39). Nucleosomes that are downstream of the TSS play an important role in gene transcription, with histone modifications such as H3K4me3 (33) and H3 acetylation (29) allowing for transcription to occur. The present study performed ChIP-qPCR at these sites. Similar to the ChIP-seq results, we observed no

![Fig. 9. Effects of administration of valproic acid (VPA) on the level of H3 acetylation. Top: typical blot patterns of pan-acetyl H3 and total H3 in normal and experimental plantaris and soleus. Bottom: graphs show expression levels of pan-acetyl H3 relative to the total H3 with (+) or without (−) the administration of VPA in plantaris (PL, left) or soleus (Sol, right) muscles. Values are means ± SE. *P < 0.05 vs. the respective control group.](http://jap.physiology.org/)
differences in H3K4me3 or H3 acetylation between plantaris and soleus muscles for the three slow genes, Bdh1, Pacrg, and Slc16a7. The distribution of histone modifications was higher than what we found in the background levels tested with normal IgG, indicating that histones were modified at these sites in both muscles. Only one slow gene, Lgi1, showed histone modifications that are typical of fast-twitch-specific genes within the soleus, and these modifications corresponded with higher gene expression. This demonstrates that some genes specific to slow-twitch muscles, such as the soleus, are associated with H3K4me3 and H3 acetylation.

We further checked the histone modifications of typical slow genes such as Myh7 (type I MHC) and Hspb1 (25-kDa heat shock protein). These genes had enhanced H3 acetylation and upregulated gene expression in the soleus, whereas H3K4me3 results for these genes did not differ between plantaris and soleus muscles (Fig. 3, B and C). Although the gene expression of Myh7 and Hspb1 was significantly upregulated in the soleus compared with that in the plantaris (Fig. 3A), the magnitude of the expression was high in both plantaris and soleus muscles (Fig. 4), indicating that these genes are transcriptionally active in both muscles but that the physiological stimuli leading to their transcriptional upregulation might be stronger in the soleus. The previous study (27) also reported that the down-regulation of type I MHC gene expression was associated with decreased H3 acetylation. Taken together, the genes with high transcription rates are associated with H3 acetylation and might be regulated by transcriptional factors.

The postnatal expression of specific genes illustrates the distinct expression patterns between genes with and without associated histone modifications. For instance, fast genes and Lgi1, in which H3K4me3 and H3 acetylation are mapped in transcriptionally active adult muscles, are already activated at birth, independent of muscle activity. Conversely, activation of other slow genes starts after the beginning of voluntary locomotion, although the magnitude of the upregulation is less pronounced compared with that of fast-twitch muscles. These results indicate that the genes associated with activating histone modifications are activated during prenatal development. Jerkovic et al. (10) reported that regenerating fibers spontaneously express the slow MHC gene during the early phase of recovery after injury in the rat soleus muscle. However, this regeneration-related transcription of slow MHC gene were never observed in the absence of the nerve. With these observations we suggest that neuromuscular innervation enables slow vs. fast phenotype differentiation, which may associate with the type-specific pattern of histone modifications.

Properties of epigenome in fast and slow muscles. Overloading enhances the level of neural activity (8) and the mechanical load (15, 16, 31). Enhanced muscular activity in the plantaris by the transection of synergists’ distal tendons caused the upregulation (i.e., 1.7-fold to 2.3-fold increase vs. normal...
plantaris muscle) of slow genes without additional H3K4me3, and with decreased H3 acetylation (Fig. 6). This was similar to the gene activation observed in the soleus muscle. Our results indicate that the histone modifications observed in slow-twitch muscles might be induced by an adaptive response to physiological stimuli and that they were not necessarily slow muscle-specific. Similar to our findings, Pandorf et al. (27) also reported that the downregulation of type I MHC gene expression was not associated with levels of H3K4me3 in the soleus following hindlimb unloading. Histone acetylation is closely related to H3K4me3 (5, 37). For instance, Crump et al. (5) demonstrated that histones modified to form H3K4me3 were targeted for acetylation by lysine acetyltransferase, p300, in mouse cells. H3K4me3 might be one of the main upstream factors in the cascade that mediates the muscle type-specific transcriptional machinery. Furthermore, the regulation of histone modification and gene expression in the soleus muscle was suggested to be closely related to the tonic neural activity present in the sedentary posture under gravity (15, 17). However, it is still unknown why the major slow genes, Myh7 and Hspb1, show enhanced H3 acetylation without an increase in H3K4me3.

Silenced activity by denervation and forced histone acetylation by VPA treatment affected the histone modification and Pol II recruitment, especially in the plantaris (Figs. 8 and 10). Furthermore, the recruitment of Pol II was higher in all genes of the normal plantaris compared with the soleus (Figs. 8D and 10D). Transcriptional basal activity in the soleus might be low due to lesser recruitment of Pol II. In fast muscle, factors related to histone modification may be accessible to genes due to their greater adaptability to physiological stimuli. Alternatively, a slow-type epigenome may play a protective role against stimuli. The presence of lesser active marks allows for moderate transcription, which may be activity-dependent in slow and overloaded fast skeletal muscles. These differences in the properties of muscle type-specific epigenome can explain in part how the phenotype transformation of skeletal muscle is mediated by the muscle type-specific transcriptional machinery. Furthermore, the expression of some genes was regulated in an H3 acetylation-related manner in the slow-twitch soleus.

**Histone acetylation and transcription.** Generally, the first several nucleosomes from TSS exhibit a similar positioning relative to the TSS and H3K4 methylation and acetylation (12). It is also known that acetylation of the first nucleosome results in further advantage for binding of the transcriptional complex (29). In the present study, we also observed that the level of Pol II at the 5′ end of the type-specific gene correlated with H3 acetylation (Fig. 11). However, the changes in gene expression were not similar to those of Pol II in denervation and VPA treatment models, further indicating that histone acetylation leads to the recruitment of Pol II at the TSS, although other factors might be necessary to move Pol II upon the gene with transcribing through the 3′ end. Greater transcription of slow genes was observed in the soleus, even though the level of Pol II recruitment was low. This suggests that the transcription of genes may be activated with a minimum, not zero, amount of Pol II. The phenomena that the level of Pol II recruitment did not change when the expression of slow genes decreased by denervation may be related to the lower baseline Pol II level. Slow-twitch muscle-specific genes were moderately activated by the slow muscle-specific mechanism, whereas the genes commonly expressed in both fast- and slow-twitch skeletal muscles might be regulated by histone modification.

**Conclusion.** The present study has demonstrated genome-wide differences in the map of transcriptionally active histone modifications in skeletal muscle. Genes activated in fast-twitch skeletal muscles showed magnified differences in gene expression compared with those in slow-twitch muscles in association with an increase in transcription-inducing histone modifications such as H3K4me3 and H3 acetylation. In contrast, slow-twitch muscle gene expression appeared to be independent of active histone modification. Fewer histone modifications allowed for only moderate Pol II recruitment in slow genes. We further found that the epigenome of fast-twitch muscle largely responded to physiological stimuli, whereas slow-twitch muscle was resistive. These results indicate that the epigenetic environment differs between fast and slow skeletal muscles. Although the mechanisms behind muscle type-specific histone modification are still unclear, muscular activity likely plays an important role in the regulation of histone modification.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS

F.K. and K. Nimura conception and design of research; F.K. performed experiments; F.K., K. Nimura, and S.I. analyzed data; F.K., K. Nimura, N.N., K. Nakata, and Y.O. interpreted results of experiments; F.K. prepared figures; F.K. drafted manuscript; K. Nimura, N.N., K. Nakata, and Y.O. edited and revised manuscript; F.K. and Y.O. approved final version of manuscript.

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