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Relationships between circadian rhythms and modulation of gene expression by glucocorticoids in skeletal muscle

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Departments of ¹Biological Sciences and ²Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, New York; ³New York State Center of Excellence in Bioinformatics and Life Sciences; ⁴Biomedical Engineering Department, Rutgers University Piscataway, New Jersey; and ⁵Children's National Medical Center, Washington, DC

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Almon RR, Yang E, Lai W, Androulakis IP, Ghimbovski S, Hoffman EP, Jusko WJ, DuBois DC. Relationships between circadian rhythms and modulation of gene expression by glucocorticoids in skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 295: R1031–R1047, 2008. First published July 30, 2008; doi:10.1152/ajpregu.90399.2008.—The existence and maintenance of biological rhythms linked to the 24-h light-dark cycle are essential to the health and functioning of an organism. Although much is known concerning central clock mechanisms, much less is known about control in peripheral tissues. In this study, circadian regulation of gene expression was examined in rat skeletal muscle. A rich time series involving 54 animals euthanized at 18 distinct time points within the 24-h cycle was performed, and mRNA expression in gastrocnemius muscles was examined using Affymetrix gene arrays. Data mining identified 109 genes that were expressed rhythmically, which could be grouped into eight distinct temporal clusters within the 24-h cycle. These genes were placed into 11 functional categories, which were examined within the context of temporal expression. Transcription factors involved in the regulation of central rhythms were examined, and eight were found to be rhythmically expressed in muscle. Because endogenous glucocorticoids are a major effector of circadian rhythms, genes identified here were compared with those identified in previous studies as glucocorticoid regulated. Of the 109 genes identified here as circadian rhythm regulated, only 55 were also glucocorticoid regulated. Examination of transcription factors involved in circadian control suggests that corticosterone may be the initiator of their rhythmic expression patterns in skeletal muscle.

corticosteroids; clock genes; gene arrays; expression profiling; transcriptome

VIRTUALLY ALL ORGANISMS HAVE biological rhythms associated with the light-dark cycle (4, 24, 25, 34). Although a good deal is known about the central control of such rhythms by the brain, less is known about how that control is translated to peripheral tissues. The central core of the mammalian circadian clock is located in the suprachiasmatic nucleus (SCN) in the anterior part of the hypothalamus, which receives direct input by way of the retinohypothalamic tract. Outputs from the SCN are directed to other parts of the hypothalamus that control both anterior and posterior pituitary hormones, as well as to the area

of the hypothalamus and medulla that control the autonomic nervous system. It is these hormonal and autonomic outputs that to a large degree convey light-dark rhythmicity to the rest of the body. Besides receiving inputs from the retina, the SCN also receives inputs from forebrain areas and from the locus ceruleus that modulate the influence of the SCN. The nonretinal inputs to the SCN provide the flexibility for the existence of diurnal and nocturnal animals, as well as for animals being able, when necessary, to shift wake-sleep periods from night to day and vice versa (5, 6).

Simplistically, the central clock mechanism involves an autoregulatory negative feedback loop with a periodicity of approximately 24 h (5, 6, 34). The elements of this basic feedback loop are several transcription factors, including CLOCK and BMAL1, which heterodimerize and enhance the expression of Period (Per) and CRYPTOCHROME (CRY). These two transcription factors heterodimerize and repress the expression of CLOCK and BMAL1. The core system is entrained to the light-dark cycle with CLOCK:BMAL being high during the light period and Per:CRY being high during the dark period. However, as many as 17 transcription factors have been defined as also being involved in the central clock mechanism (10). These include within the basic mechanism several isoforms of Per and CRY, as well as neuronal PAS domain protein 2 (Npas2, Mop4), which has been reported as an alternative to CLOCK as a heterodimerizing partner with BMAL (7). The precise role of the other transcription factors in the central clock is still a subject of investigation.

The input from the SCN to the regulation of both pituitary hormones and the autonomic nervous system impart rhythmicity to peripheral tissues. However, rhythmicity in peripheral tissues is further complicated by more diffuse behavior-related factors such as sleep-wake patterns, eating, and physical activity, which alter systemic energy demands. Many of the transcription factors involved in regulating the central clock are also expressed in peripheral tissues (10). However, their regulation is complicated by variations in ancillary factors, such as hormonal patterns, autonomic output, and behavior that orchestrate peripheral rhythmicity (23). The existence of both diurnal

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and nocturnal mammals and the phenomena of phase shifting by food restriction illustrate both the complexity and flexibility in peripheral rhythmicity. Perhaps one of the best illustrations of the flexibility of rhythmic behavior outside the SCN is the observation that rhythmic behavior with a periodicity of ~24 h can be induced in a variety of cells in culture (34).

The central clock anticipates the change in photoperiod and prepares the animal for the upcoming period of activity and feeding, regardless of whether that period is in the light or dark. The hypothalamus-pituitary-adrenal (HPA) axis is of particular importance to the active feeding period, as illustrated by the fact that the effector hormones, glucocorticoids, are high during the light period in diurnal animals and high during the dark period in nocturnal animals (6). The generally accepted mechanism for most glucocorticoid effects involves binding of free steroid to a cytoplasmically localized receptor, translocation of ligand-bound receptor into the nucleus, binding of a ligand receptor dimer to specific DNA sites (GREs, glucocorticoid response elements), and modulation of the amount of selective mRNA (13). Although some effects on mRNA stability have been noted, the common mechanism involves increasing or decreasing the rate of transcription of particular genes. Even though more recent evidence suggests that this mechanistic view may be overly simplistic, it is clear that changes in the amount of specific mRNAs are the basis of the vast majority of glucocorticoid actions (2, 3). By virtue of their circadian rhythmicity, glucocorticoids themselves are effectors of many but not all circadian changes in gene expression.

Skeletal muscle represents about 40% of the body mass of most mammals. From a systems point of view, the musculature is central to not only movement and posture, but also to energy metabolism. The musculature uses a very large amount of energy, most of which is in the form of lipid fuels, to maintain posture against gravity and body temperature relative to the environment. The musculature is also responsible for about 75% of the insulin-directed glucose disposal, which supports the phasic mechanical functions of type 2 fibers (15). In addition, under the influence of glucocorticoids, the musculature is shifted into net degradation of proteins, which provide amino acid carbon for gluconeogenesis in the liver and kidney (2). Energy expenditure by muscle should vary as a function of circadian rhythms with the highest expenditure during the animal's active feeding period.

Previously, we profiled the time-dependent response of skeletal muscle from adrenalectomized rats to two different dosing regimens of the synthetic glucocorticoid, methylprednisolone (MPL) (3). By using such animals, we removed not only the endogenous source of glucocorticoids, but also major aspects of the systemic influence of the sympathetic nervous system. One time series involved giving a single bolus dose of MPL (50 mg/kg) and euthanizing animals at 16 time points over a 72-h period. The second time series involved delivering MPL ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) via Alzet pump and euthanizing animals at 10 times over a 168-h period. In the present report, we describe the use of Affymetrix arrays to analyze skeletal muscles from intact rats maintained on a strict light-dark regimen consisting of 12:12-h light-dark cycle with three animals killed at 18 time points during the 24-h period. This rich time series allowed us to identify genes whose expression was rhythmically modulated and to group those genes into eight relatively

discrete temporal circadian clusters. We grouped the genes with circadian rhythms into 11 functional categories to examine cellular activity as a function of temporal cluster. In addition, comparisons with our previous work allowed us to evaluate the response profiles of genes with circadian rhythms to the two dosing regimens of exogenous corticosteroid, providing insight into the role of glucocorticoids in regulating rhythms of gene expression in skeletal muscle.

MATERIALS AND METHODS

Animals. Fifty-four normal (150–175 g) male Wistar rats were purchased in two separate batches of 27 from Harlan Sprague Dawley (Indianapolis, IN), and experiments were initiated at body weights between 225 and 275 g. Animals were housed and allowed to acclimatize in a constant-temperature environment (22°C) equipped with a 12:12-h light-dark cycle. Twenty-seven rats (*group 1*) were acclimatized for 2 wk before study to a normal light-dark cycle, in which lights went on at 8 AM and off at 8 PM. The onset of light cycle was considered as *time 0*. The other 27 rats (*group 2*) were acclimatized for 2 wk before study to a reversed light-dark cycle, where lights went on at 8 PM and off at 8 AM. Two weeks of acclimation was sufficient time to reestablish circadian cycles, as indicated by blood corticosterone concentrations in the two groups. Rats in *group 1* were killed on three successive days at 0.25, 1, 2, 4, 6, 8, 10, 11, and 11.75 h after lights on to capture the light period. Rats in *group 2* were killed on three successive days at 12.25, 13, 14, 16, 18, 20, 22, 23, and 23.75 h after lights on to capture the dark period. Animals killed at the same time on successive days were treated as triplicate measurements. Because normal rats were used, minimal animal handling with the least possible environmental disturbances was used to minimize stress. Night vision goggles were used to carry out animal procedures conducted in the dark period. At death, rats were weighed, anesthetized by ketamine:xylazine (80:10 mg/kg), and killed by aortic exsanguination. Blood was drawn from the abdominal aortic artery into syringes using EDTA (4 mM final concentration) as anticoagulant. Plasma was harvested from blood by centrifugation (2,000 g, 15 min, 4°C) and frozen at minus 80°C until analyzed for corticosterone. Gastrocnemius muscles were excised and frozen in liquid nitrogen immediately after death and stored at minus 80°C until RNA preparation. Both acute and chronic MPL dosing experiments have been previously published (26, 32). In brief, populations of adrenalectomized male Wistar rats were given doses of the synthetic glucocorticoid, MPL. In the acute experiment, the animals were given a single bolus dose (50 mg/kg) of MPL and were killed at 16 times over a 72-h period following dosing. In the chronic experiment, the animals were administered $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ MPL via Alzet osmotic pumps and were killed at 10 time points over a 168-h period. All rats had free access to rat chow and 0.9% saline drinking water. Our research protocol adheres to the "Principles of Laboratory Animal Care" (NIH publication 85–23, revised in 1985) and was approved by the University at Buffalo Institutional Animal Care and Use Committee.

Plasma steroid assays. Plasma corticosterone concentrations were determined by a sensitive normal-phase HPLC method, as previously described (11). The limit of quantitation was 10 ng/ml. The interday and intraday coefficients of variation (CV) were less than 10%.

Microarrays. Muscle samples from each animal were ground into a fine powder in a mortar cooled by liquid nitrogen, and 100 mg was added to 1 ml of prechilled TriZOL Reagent (Invitrogen, Carlsbad CA). Total RNA extractions were carried out according to manufacturer's directions and were further purified by passage through RNeasy mini-columns (Qiagen, Valencia, CA) according to manufacturer's protocols for RNA cleanup. Final RNA preparations were suspended in RNase-free water and stored at minus 80°C. The RNAs were quantified spectrophotometrically, and purity and integrity were assessed by agarose gel electrophoresis. All samples exhibited 260/

280 absorbance ratios of ~ 2.0 , and all showed intact ribosomal 28S and 18S RNA bands in an approximate ratio of 2:1, as visualized by ethidium bromide staining. Isolated RNA from each muscle sample was used to prepare target according to manufacturer's protocols. The biotinylated cRNAs were hybridized to 54 individual Affymetrix GeneChips Rat Genome 230A (Affymetrix, Santa Clara, CA), which contained 15,967 probe sets. The 230A chip was used in the chronic infusion experiment as well, allowing direct comparison between the two experiments. The 230A gene chips contain over 7,000 more probe sets than the ones used (U34A) in our earlier muscle bolus dose MPL study. The high reproducibility of in situ synthesis of oligonucleotide chips allows accurate comparison of signals generated by samples hybridized to separate arrays. This data set has been submitted to GEO (GSE8989).

Quantitative real-time RT-PCR. The quantity of muscle glutamine synthetase along with gene-specific in vitro transcribed cRNA standards were determined by quantitative real-time RT-PCR using TaqMan probes. Briefly, primer and probe sequences were designed using PrimerExpress software (Applied Biosystems, Foster City, CA) and custom synthesized by Bioscience Technologies, (Novato, CA). The RT-PCR was performed using Brilliant QRT-PCR Core Reagent Kit, 1-Step (Stratagene, La Jolla, CA) in a Stratagene MX3005P thermocycler, according to manufacturer instructions. A standard curve was generated using the in vitro transcribed sense cRNA standards. Primer and probe sequences are as follows: forward primer (5'-CGCCCGCCGTCTGA-3'), reverse primer (5'-TCTCCTGGC-CGACAATCC-3'), and probe (5-FAM-TCCACGAAACCTCCAA-CATCAAACGACTTT-BHQ-3'). Intraday and interday CVs were 18% and 10%, respectively.

Data set construction. As discussed above, animals were killed at precise times on three successive days to obtain data points for the light period and three successive days to obtain data points for the dark period. Animals killed at the same time on different days were treated as three replicates for that time to construct a 24-h light-dark cycle. To obtain a clear picture of an entire cycle, two 24-h periods were concatenated to obtain a 48-h period, which allowed visualization of rhythms that spanned the dark-light and light-dark transitions.

Data mining. A nonlinear curve fit using MATLAB was conducted, which fitted a sinusoid function $[A \cdot \sin(Bt + c)]$ to the data, including the replicates. Genes that could be curve fitted with a R^2 correlation of greater than 0.8 were kept. This curve-fitting approach enabled use of replicate information instead of depending on the ensemble average necessary with Fourier transforms or Lomb-Scargle methods. This approach is viable due to our relatively large number of time samples. This data set was then loaded into a data mining program, GeneSpring 7.0 (Silicon Genetics, Redwood City, CA), and we normalized the value of each probe set on each chip to the average of that probe set on all chips. To identify genes with similar patterns of oscillation within the daily cycle, we applied Quality Threshold Clustering (QT) in GeneSpring using Pearson's correlation as the similarity measurement.

RESULTS

Data mining. The assumption used to process this data set is that genes whose expression levels are part of the circadian rhythm will show one full oscillation every 24 h. To exploit this assumption, a nonlinear curve fit was conducted, which fitted a sinusoid to the data, including the replicates. We identified 122 probe sets which fit the model $[A \cdot \sin(Bt + c)]$ with a R^2 correlation greater than 0.8. Because of probe set redundancy (more than one probe set for a single gene), the 122 probe sets represent 109 genes. Using GeneSpring, we normalized the value of each probe set on each chip to the average of that probe set on all chips, such that the expression pattern of all probe sets oscillated approximately around 1. There

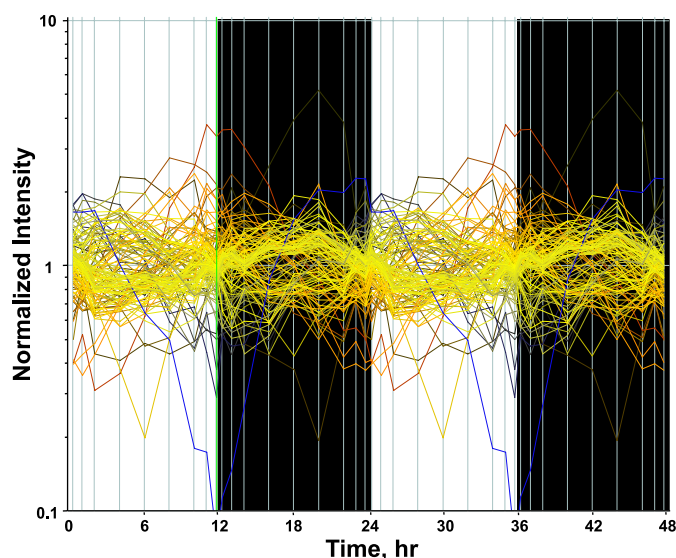


Fig. 1. A nonlinear curve fit using MATLAB was conducted which fitted a sinusoid function $[A \cdot \sin(Bt + c)]$ to the data, including the replicates. Genes that could be curve fitted with a R^2 correlation of greater than 0.8 were kept.

appear to be two major patterns, as illustrated in Figure 1, which presents the expression pattern of all 122 probe sets. Some probe sets (represented by individual lines in Fig. 1) show expression patterns greater than one during the light period but less than one during the dark period, reflecting maximum expression during the animal's light-inactive period. Conversely, other probe sets reach a maximum (values greater than one) during the dark/active period. However, because of the richness of the data set, we were able to identify more discrete relationships between oscillations in expression and the light-dark periods. To group genes with similar patterns within the daily cycle, we applied Quality Threshold Clustering (QT clustering), yielding eight clusters. Figure 2 shows these eight clusters with the centroid (average of all of the genes in that cluster) highlighted by a white line. Fifty-seven percent of the genes (62 of 109) are in clusters 6, 7, or 8 with maximum expression during the dark/active period. Thirty-one percent of the genes (34 of 109) are in clusters 1, 2, 3, or 4, with maximum expression during the light/inactive period, and the remaining 12% (13 genes) are in cluster 5 with a maximum at hour 12, the transition between light and dark. Corticosterone reaches its maximum plasma concentration at hour 13.3 (Fig. 3). Table 1 provides a detailed list of all genes in each cluster, including probe set ID, accession number, Pearson's correlation coefficient with the centroid, gene symbol, gene name, and gene function.

Regulation. We examined the chip for probe sets for genes previously identified as involved in regulation of circadian patterns (34). The 230A chip contained probe sets for 17 of these transcription factors. However, only eight (*Per1*, *Per2*, *BMAL1b*, *Bhlhb3*, *DBP*, *Nfil3*, *Nr1d1*, and *Nr1d2*) showed distinct circadian oscillation. Figure 4 provides profiles for these eight transcription factors with circadian rhythms and their respective clusters. The chip did contain probe sets for *Per3*, *Bhlhb2*, *RORB*, *RORC*, *CRY2*, and *CLOCK*. On visual inspection, the signals for *Per3*, *RORB*, and *CRY2* were very low, which may prevent identification of rhythmicity. However, the signals for *Bhlhb2*, *RORC*, and *CLOCK* were reasonably intense but did not show any indication of oscillation.

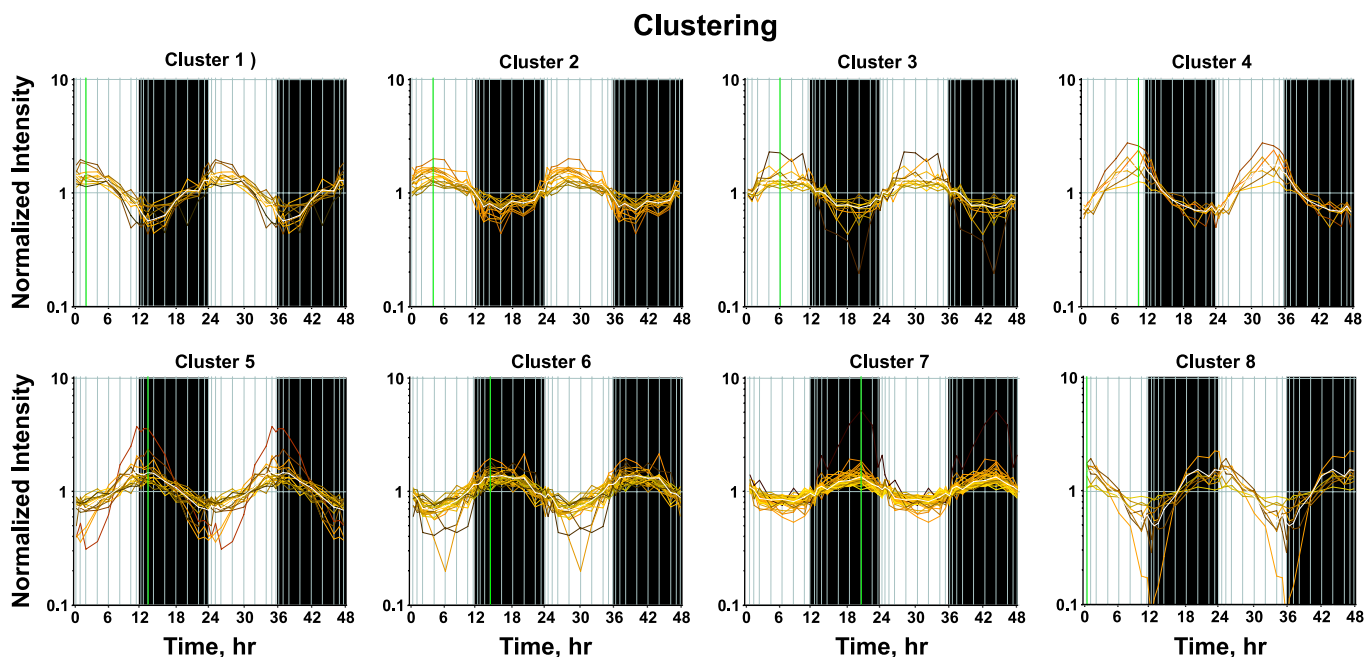


Fig. 2. QT clustering of genes in muscle measured over 24 h. Each probe set has greater than a 0.75 Pearson's correlation with the centroid of the cluster.

Previously, we conducted two time series experiments in which cohorts of adrenalectomized rats were given MPL either as a single bolus dose or chronic infusion, and the muscles were analyzed by gene arrays. Because a major regulator of circadian rhythms is the HPA axis, these arrays were examined for clock genes. Figure 5 shows the acute and chronic profiles for *Per2*, *Bhlhb3*, *DBP*, *Nr1d1*, and *Nr1d2*. In the acute profile, all five genes respond to the single-dose with a transient oscillation. *Per2* began the oscillation with increased expression while *DBP*, *Nr1d1*, *Bhlhb3*, and *Nr1d2* begin the oscillation with down-regulation. With chronic infusion, *Per2* appears to oscillate throughout the 168-h infusion period beginning with enhanced expression. Consistent with the response to

acute dosing *DBP*, *Bhlhb3*, *Nr1d1*, and *Nr1d2* all show initial downregulation in response to infusion. *DBP* recovers baseline by 36 h and starts a slow decline throughout the remainder of the infusion. *Nr1d1* recovers baseline by 24 h, shows a period of dampened oscillation for an additional 36 to 48 h, then begins a period of slow decline similar to *DBP*. *Bhlhb3* looks almost like an inverse of *Per2*, in that it recovers baseline by about 48 h, after which it goes down again, and then shallowly starts back up again. *Nr1d2* almost recovers baseline by 36 h, then maintains a new expression level slightly lower than baseline throughout the remainder of the infusion period. Because of their fluctuating behavior, for the most part, these five genes did not meet our criteria for corticosteroid regulation when the acute and chronic treatment data sets were initially mined (3). With acute dosing, *BMAL1b* oscillated with a pattern similar to *Per2*. However, with chronic infusion *BMAL1b* showed a strong peak of enhanced expression followed by a lower level of enhanced expression (Fig. 6). This figure also shows just the chronic profiles of *Per1* and *Nfil3* as the U34A chip did not contain probe sets for these genes. Both showed enhanced expression throughout the infusion period.

The Affymetrix 230A chip was used for chronic infusion experiments, while the acute dosing experiment used the older U34A chip, which contained fewer probe sets. Because both this circadian experiment and the chronic infusion experiment used the same chip, we were able to make a direct comparison between corticosteroid-responsive genes and those with circadian rhythms. Data mining identified over 2,000 genes in the chronic infusion data set that were responsive to the infusion of $0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ MPL via Alzet osmotic pumps (3). Of the 109 genes with circadian rhythms, only 55 were responsive to MPL infusion, while 67 were not. The circadian rhythm-regulated probe sets that are also responsive to MPL infusion are highlighted in bold in Table 1. Just as the 109 genes are not equally distributed among the eight clusters, the percentage of genes in each cluster that respond to MPL is not the same. For

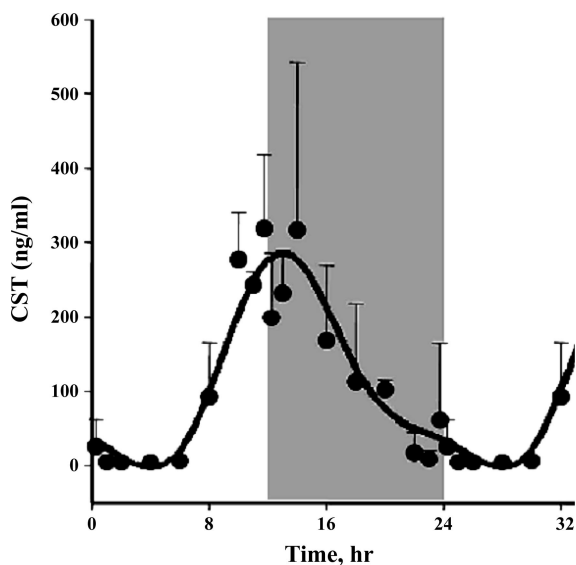


Fig. 3. Plasma corticosterone (CST) as a function of circadian time as measured by HPLC. Unshaded areas indicate light period and shaded areas indicate dark period.

Table 1. *Circadian-regulated genes arranged by cluster*

Probe ID	Accession No.	Correlation No.	Ontology	Symbol	Gene Name	Gene Function
Cluster 1						
1398767_at	NM_017314	0.996	protein degradation	<i>Ubb, ubc</i>	Ubiquitin B, C	protein catabolism
1372280_at	BI295982	0.985	protein degradation	<i>Asb2</i>	Ankyrin repeat and SOCS box-containing protein 2	E3 ubiquitin ligase complexes
1370510_a_at	AB012600	0.963	transcription	<i>Arntl, BMAL1</i>	Aryl hydrocarbon receptor nuclear translocator-like	circadian rhythm, transcription regulation
1388426_at	BF398848	0.936	lipid metabolism	<i>srebfl</i>	sterol regulatory element-binding transcription factor 1	regulation of transcription, sterol metabolism
1370847_at	AA801238	0.927	immune	<i>Spon2</i>	Spondin 2	innate immune response
1374204_at	BM388946	0.902	protein degradation	<i>Wsb1</i>	WD repeat and SOCS box-containing 1	E3 ubiquitin ligase complexes
1376963_at	BM388710	0.899	transcription	<i>Dyrk2</i>	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	chromatin remodeling
1376944_at	AI407163	0.794	signaling	<i>Prlr</i>	Prolactin receptor	signaling, JANUS KINASE 2, GHR and SOCS
Cluster 2						
1387294_at	NM_054011	0.977	mitochondrial	<i>Sh3bp5</i>	SH3-domain binding protein 5	mitochondrial JNK cascade
1385243_at	AI180340	0.972	transcription	<i>Maf</i>	V-maf musculoaponeurotic fibrosarcoma oncogene	transcription factor, DNA binding
1390028_at	BE096356	0.971	transcription	<i>Dyrk2</i>	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	also in cluster 1; see function
1399005_at	BG673380	0.965	signaling	<i>ppp2r5a</i>	Protein phosphatase 2, regulatory subunit B (B56), alpha	signal transduction
1392939_at	BM388933	0.962	transport	<i>Slc41a3</i>	Solute carrier family 41, member 3	inorganic cation transport (Mg)
1372624_at	BF551377	0.955		<i>EST</i>	Unknown	unknown function
1371602_at	BI274110	0.933	signaling	<i>Tspan9 NET-5</i>	Tetraspanin 9	signal transduction
1367979_s_at	NM_012941	0.931	lipid metabolism	<i>Cyp51</i>	Cytochrome P-450, subfamily 51	sterol 14-alpha-demethylase cholesterol synthesis
1378423_at	AI639060	0.901	signaling	<i>Atcay</i>	Caytaxin	associated with Cayman ataxia
1379550_a_at	BF290483	0.889	transcription	<i>Gtf2ird1</i>	GTF2I repeat domain-containing protein 1	regulates muscle fiber-type-specific promoters
1370166_at	AI169682	0.848	cytoskeleton	<i>Sdc2</i>	Syndecan 2	transmembrane proteoglycan, cytoskeletal binding protein
1375378_at	BE108882	0.827	transcription	<i>Qki</i>	Quaking homolog, KH domain RNA binding	Transcription, RNA-binding protein
1370835_at	AF267197	0.755	transcription	<i>Tox4</i>	Epidermal Langerhans cell protein LCP1	transcription regulation, DNA-dependent
1387321_at	NM_053381	0.753	transport	<i>Atp1b4</i>	ATPase, (Na ⁺)/K ⁺ transporting, beta 4 polypeptide	sodium:potassium -exchanging ATPase activity, Na ⁺ -K ⁺ transport
Cluster 3						
1368277_at	NM_017041	0.961	cell cycle	<i>Ppp3ca</i>	Protein phosphatase 3, catalytic subunit alpha isoform, calcineurin A	G1/S transition of mitotic cell cycle
1374636_at	BI298596	0.951	cell cycle	<i>Phf17</i>	PHD finger protein 17	proapoptotic barrier to proliferation
1376089_at	BI294974	0.947	lipid metabolism	<i>Ldlr</i>	Low-density lipoprotein receptor	cholesterol homeostasis
1375534_at	BE118698	0.923	signaling	<i>Pum2</i>	Pumilio homolog 2	proliferation and self-renewal of stem cells
1388924_at	AA818262	0.921	lipid metabolism	<i>angptl4</i>	Anigopoietin-like 4	negative regulation of lipoprotein lipase
1389618_at	BI285035	0.890		<i>EST</i>	Unknown	unknown function
1398913_at	AI599394	0.886	cell cycle	<i>numa1</i>	Nuclear mitotic apparatus protein 1	cycle-related protein
1387172_a_at	NM_031131	0.870	signaling	<i>Tgfb2</i>	Transforming growth factor, beta-2	negative regulation of apoptosis, regulation of cell proliferation
1375984_at	BE103689	0.842	transcription	<i>Zfxh4</i>	Zinc finger homeodomain 4	DNA-dependent transcription, muscle differentiation
1367894_at	NM_022392	0.831	lipid metabolism	<i>insig1</i>	Insulin induced gene 1	retention of the SCAP/SREBP complex in the ER

Continued

Table 1.—Continued

Probe ID	Accession No.	Correlation No.	Ontology	Symbol	Gene Name	Gene Function
1374166_at	AW252076	0.798		<i>EST</i>	Unknown	unknown function
Cluster 4 1370816_at	M25804	0.988	transcription	<i>nr1d1</i>	Nuclear receptor subfamily 1, group D, Member 1	steroid hormone receptor-circadian rhythm
1367602_at	AI013390	0.984	transcription	<i>Cited2</i>	Cbp/p300-interacting transactivator Glu/Asp-rich carboxy-term Dom 2	positive transcription regulation
1367601_at	NM_053698	0.965	transcription	<i>Cited2</i>	Cbp/p300-interacting transactivator Glu/Asp-rich carboxy-term Dom 2	positive transcription regulation
1388471_at	AA800197	0.954	signaling	<i>tcp11l2</i>	T-Complex 11 (mouse) like 2	cell communication
1369150_at	NM_053551	0.953	mitochondrial	<i>Pdk4</i>	Pyruvate dehydrogenase kinase, isoenzyme 4	acetyl-CoA biosynthesis from pyruvate
1386946_at	NM_031559	0.922	mitochondrial	<i>cpt1a</i>	Carnitine palmitoyltransferase I	transfer long chain fatty acids across mitochondrial inner membrane
1368511_at	NM_133303	0.900	transcription	<i>Bhlhb3</i>	Basic helix-loop-helix domain containing, class B, 3	transcription regulation
Cluster 5 1390430_at	BF284190	0.971	transcription	<i>nr1d2, RVR</i>	Nuclear receptor subfamily 1, group D, member 2	DNA-dependent transcription regulation
1387874_at	AI230048	0.963	transcription	<i>Dbp</i>	D site of albumin promoter-binding protein	transcription regulation, circadian rhythm
1370541_at	U20796	0.959	transcription	<i>nr1d2, RVR</i>	Nuclear receptor subfamily 1, group D, member 2	DNA-dependent transcription regulation
1388525_at	BE112999	0.944	signaling	<i>PIK3IP1</i>	Phosphoinositide-3-kinase interacting protein 1	binds to the p110 catalytic domain modulates PI3K
1371864_at	AW524563	0.943	transcription	<i>Klf9</i>	Kruppel-like factor 9	mediates expression of growth-associated genes
1372452_at	BG666882	0.938	mitochondrial	<i>Gpam</i>	Glycerol-3-phosphate acyltransferase	fatty acid metabolism
1373866_at	AI228596	0.930	mitochondrial	<i>Coq10b</i>	Coenzyme Q10 homolog B	oligonucleotide cyclase/lipid transport protein
1371583_at	AI598399	0.927	transcription	<i>Rbm3</i>	RNA binding motif protein 3	RNA processing, miRNA-mediated gene silencing
1374574_at	AI547611	0.915		<i>EST</i>	Unknown	unknown function
1369050_at	NM_053923	0.907	signaling	<i>Pik3c2 g</i>	Phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	Ras pathway, vesicle trafficking, secretion, and apoptosis
1387669_a_at	NM_012844	0.904	small molecule metabolism	<i>ephx1</i>	Epoxide hydrolase 1, microsomal	small molecule metabolism
1389632_at	AA799294	0.878	signaling	<i>Rhobtb1</i>	Rho-related BTB domain-containing protein 1	small GTPase-mediated signal transduction
1368249_at	NM_053536	0.868	carbohydrate metabolism	<i>Klf15</i>	Kruppel-like factor 15	regulates GLUT4 expression
1388384_at	AI407618	0.866	cytoskeleton	<i>Dynl1l1</i>	Dynein light chain LC8-type 1	molecular motor
1374855_at	BI279017	0.856	transcription	<i>Per1</i>	Period 1	transcription regulation, circadian rhythm
1368283_at	NM_133606	0.823	mitochondrial	<i>Ehhadh</i>	Enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	acyl-CoA metabolism, fatty acid beta-oxidation
Cluster 6 1387703_a_at	AF106659	0.964	protein degradation	<i>usp2, ubp69</i>	Ubiquitin specific peptidase 2	deubiquitinating enzyme, muscle cell differentiation
1386935_at	NM_024388	0.927	carbohydrate metabolism	<i>nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1	modulates regulators of skeletal muscle energy homeostasis

Continued

Table 1.—Continued

Probe ID	Accession No.	Correlation No.	Ontology	Symbol	Gene Name	Gene Function
1372011_at	BI292028	0.925	small molecule metabolism	<i>Gda</i>	Guanine deaminase	purine metabolism
1389251_at	AA944380	0.923	small molecule metab.	<i>Nudt7</i>	Nucleoside diphosphate-linked moiety X Motif 7	peroxisomal, regulates CoA and acyl-CoA levels
1370019_at	AF394783	0.916	small molecule metab.	<i>Sult1a1</i>	Sulfotransferase family 1A, phenol-preferring, member 1	transfer sulfate group 3ϵ-phosphoadenosine 5ϵ-phosphosulfate
1387053_at	NM_012792	0.916	small molecule metab.	<i>Fmo1</i>	Flavin-containing monooxygenase 1	NADPH-dependent oxidative metabolism of many drugs
1371615_at	BI279069	0.911	lipid metabolism	<i>Dgat2</i>	Diacylglycerol O-acyltransferase homolog 2	triglyceride synthesis, glycerol, lipid metabolism
1368375_a_at	AF015718	0.908	immune	<i>Il15</i>	Interleukin 15	positive regulation of T cell proliferation
1388804_at	BI300986	0.901	cell cycle	<i>Mn1</i>	Meningioma 1	negative regulation of progression through cell cycle
1390171_at	AA924717	0.901	transcription	<i>FAM76A</i>	Family with sequence similarity 76, member A	nuclear inhibitor of protein phosphatase-1
1370663_at	D31838	0.899	cell cycle	<i>wee1</i>	Wee 1 homolog	DNA replication checkpoint, G2/M transition
1369625_at	AA891661	0.877	transport	<i>Aqp1</i>	Aquaporin 1	major water transport molecule I vascular
1372178_at	BM385981	0.875	transport	<i>Abcc1</i>	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	transporter of glutathione-linked compounds from cells
1373092_at	BE109587	0.874	signaling	<i>Tgfb3, BETA-GLYCAN</i>	Transforming growth factor, beta receptor III	modulates several tissue development and repair processes
1368182_at	NM_130739	0.849	lipid metabolism	<i>acs16, facl6</i>	Acyl-CoA synthetase long-chain family member 6	ligation of long-chain fatty acids with coenzyme A
1371479_at	AA946026	0.847	transcription	<i>Mettl7a</i>	Methyltransferase like 7A	methyltransferase activity, metabolic activity
1373818_at	AI408351	0.847	transport	<i>kctd12</i>	Potassium channel tetramerisation domain containing 12	voltage-gated potassium channel
1368303_at	NM_031678	0.844	transcription	<i>Per2</i>	Period homolog 2	circadian rhythm, protein binding, signal transduction
1386987_at	NM_017020	0.840	immune	<i>Il6r, Il6ra, IL6RI</i>	Interleukin 6 receptor	energy metabolism
1373282_at	AI406494	0.824	mitochondrial	<i>slc25a33</i>	Solute carrier family 25, member 33	mitochondrial carrier proteins, transport
1368431_at	NM_017112	0.762	cytoskeleton	<i>Hpn</i>	Hepsin	membrane bound serine protease
Cluster 7						
1388113_at	BG673321	0.969	mitochondrial	<i>cox8a</i>	Cytochrome <i>c</i> oxidase, subunit VIIIa	electron transport chain
1368004_at	NM_022529	0.969	mitochondrial	<i>mrpl23</i>	Mitochondrial ribosomal protein L23	protein biosynthesis, translation
1386887_at	NM_053586	0.966	mitochondrial	<i>Cox5b</i>	Cytochrome <i>c</i> oxidase, subunit Vb	electron transport chain
1367609_at	NM_031051	0.965	immune	<i>Mif</i>	Macrophage migration inhibitory factor	regulation of macrophage activation
1375197_at	BG665384	0.954	mitochondrial	<i>LOC686442</i>	Ubiquinol-cytochrome <i>c</i> reductase subunit	electron transport chain
1371912_at	AI231358	0.952	mitochondrial	<i>Ndufs7</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 7	electron transport chain
1369590_a_at	NM_024134	0.951	transcription	<i>Ddit3, GADD153</i>	DNA-damage inducible transcript 3	dominant-negative inhibitor of the transcription factors C/EBP
1387015_at	NM_030873	0.950	cytoskeleton	<i>Pfn2</i>	Profilin 2	regulation of actin polymerization and/or depolymerization
1388364_at	BG381650	0.949	mitochondrial	<i>Ndufs3</i>	NADH-ubiquinone oxidoreductase Fe-S protein 3	electron transport chain
1388361_at	BI303856	0.940	mitochondrial	<i>PDSW</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	electron transport chain
1371351_at	AI233192	0.938	transcription	<i>Rpo 1-3</i>	RNA polymerase 1-3	transcription regulation

Continued

Table 1.—Continued

Probe ID	Accession No.	Correlation No.	Ontology	Symbol	Gene Name	Gene Function
1367568_a_at	NM_012862	0.928	cytoskeleton	<i>Mgp</i>	Matrix gamma-carboxyglutamic acid protein	inhibitor of soft-tissue mineralization
1398868_at	AI408157	0.927	mitochondrial	<i>timm13, sirt6</i>	Translocase of inner mitochondrial membrane 13	protein folding and targeting
1388683_at	AI411174	0.924	transcription	<i>Lsmd1</i>	LSM domain containing 1	RNA processing
1367481_at	BI278301	0.920	protein degradation	<i>vps28</i>	Vacuolar protein sorting 28	ubiquitin-dependent protein catabolism
1389288_at	BI279838	0.917	mitochondrial	<i>Ndufa2</i>	NADH-ubiquinone oxidoreductase 1 Alpha subcomplex, 2	electron transport chain
1379243_at	AA819547	0.917	mitochondrial	<i>Ndufa6</i>	NADH-ubiquinone oxidoreductase 1 Alpha subcomplex, 6	electron transport chain
1367735_at	NM_012819	0.897	mitochondrial	<i>Acadl</i>	Acetyl-coenzyme A dehydrogenase, long-chain	lipid, fatty acid metabolism, electron transport
1373850_at	AW251315	0.894	extracellular	<i>Smpd13b</i>	Sphingomyelin phosphodiesterase, acid-like 3B	hydrolase activity, acting on glycosyl bonds, secreted
1388767_at	BI296393	0.892	cell cycle	<i>Pcd6</i>	Programmed cell death 6-interacting protein	caspase activation, induction of apoptosis by extracellular signals
1367632_at	NM_017073	0.892	protein degradation	<i>Glul, Glns</i>	Glutamate-ammonia ligase glutamine synthetase	glutamine synthesis, muscle atrophy
1372091_at	BI275959	0.889	cytoskeleton	<i>Mig12</i>	MID1 interacting G12-like protein	inhibition of microtubule depolymerization, microtubule stabilization
1389230_at	AA818910	0.886	signaling	<i>arrdc3</i>	Arrestin domain containing 3	thioredoxin interacting protein
1370428_x_at	AJ249701	0.885	immune	<i>RT1-Aw2, RT1-A2, RT1-A2q</i>	RT1 class Ib, locus Aw2	antigen processing of peptide antigen via MHC class I
1367506_at	BF283488	0.879	mitochondrial	<i>mrpl11</i>	Mitochondrial ribosomal protein L11	protein biosynthesis, translation
1371324_at	BG673668	0.876	transcription	<i>SF3B5</i>	Splicing factor 3B subunit 5	RNA splicing, mRNA processing
1367670_at	NM_017005	0.867	mitochondrial	<i>Fh1, Fh</i>	Fumarate hydratase 1	conversion of fumarate to malate
1375971_at	BM386028	0.861	protein degradation	<i>Cln5</i>	Ceroid-lipofuscinosis, neuronal 5	lysosome organization and biogenesis
1369973_at	NM_017154	0.858	immune	<i>Xdh</i>	Xanthine dehydrogenase	inflammation, vascular, muscle damage
1371397_at	AI104068	0.858	signaling	<i>Nosip</i>	Nitric oxide synthase interacting protein	negative regulation of nitric oxide synthase activity
1375211_at	BM391506	0.846	extracellular	<i>Rnaset2</i>	Ribonuclease T2	secreted RNases
1371649_at	BI289411	0.846	mitochondrial	<i>mrps24</i>	Mitochondrial ribosomal protein S24	protein biosynthesis, translation
1386857_at	NM_017166	0.842	signaling	<i>Stmn1</i>	Stathmin 1	signaling relay, phosphoprotein
1369467_a_at	NM_012621	0.800	carbohydrate metabolism	<i>Pfkfb1</i>	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	glycolysis
1369545_at	NM_017086	0.782	transcription	<i>Egr3</i>	Early growth response 3	regulation of myotube fate
Cluster 8 1373108_at	BM390827	0.989	carbohydrate metabolism	<i>ppp1r3c</i>	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	glycogen metabolic process
1388395_at	AI406939	0.973	cell cycle	<i>G0 s2</i>	G0/G1 switch gene 2	regulation of progression through cell cycle
1398246_s_at	NM_053843	0.957	immune	<i>Fcgr3</i>	Fc receptor, IgG, low affinity III	antigen processing and presentation
1372964_at	BI294751	0.954	transcription	<i>arid5b</i>	AT rich interactive domain 5B	negative regulation of transcription
1368136_at	NM_012887	0.923	transcription	<i>Tmpo, Slc25a3</i>	Thymopoietin	nuclear membrane protein
1367850_at	NM_053843	0.922	immune	<i>Fcgr3</i>	Fc receptor, IgG, low affinity III	antigen processing and presentation
1368488_at	NM_053727	0.899	immune	<i>Nfil3</i>	Nuclear factor, interleukin 3 regulated	DNA-dependent transcription regulation
1374029_at	AI229596	0.869		<i>EST</i>	Unknown	unknown function

Probe sets exhibiting a circadian pattern in rat skeletal muscle arranged by cluster. Probe ID refers to identification number used by Affymetrix, which is distinct from GenBank accession numbers. Correlation no. represents the Pearson's correlation following QT clustering. Probe sets highlighted in bold are genes that are also MPL responsive.

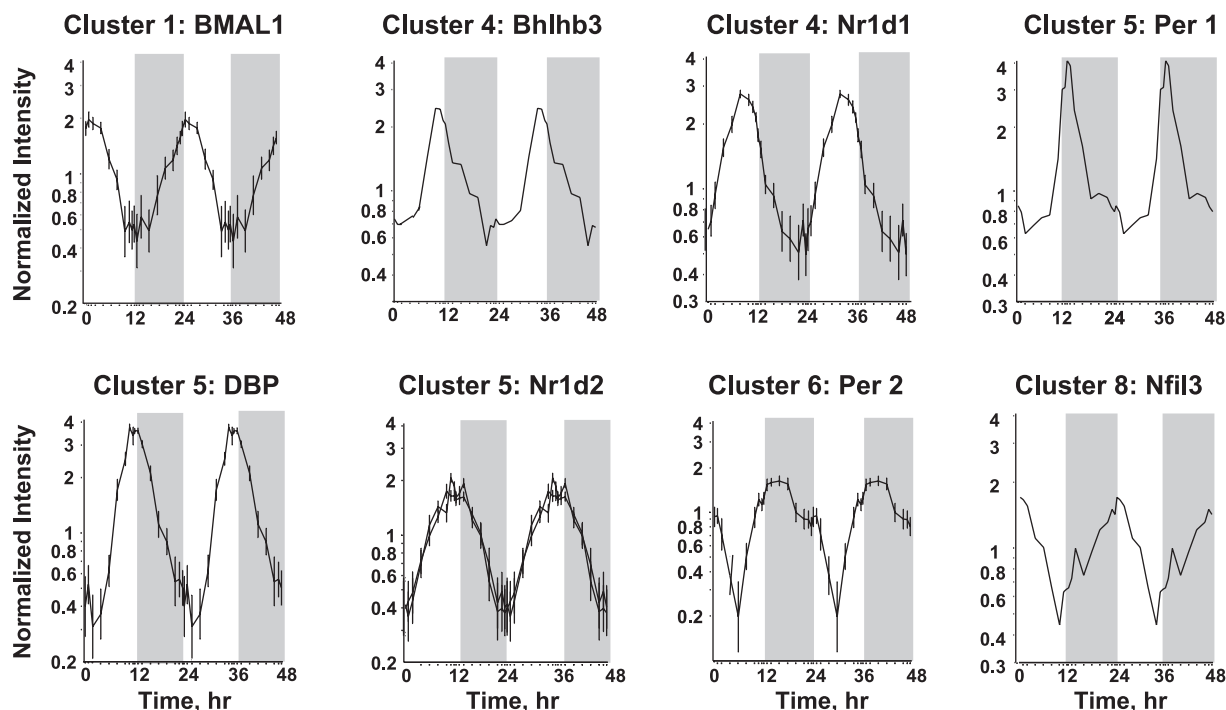


Fig. 4. Expression patterns of 8 clock-related transcription factors in skeletal muscle as a function of circadian time. Unshaded areas indicate light periods, and shaded areas indicate dark periods.

example, only 25% of the genes in *cluster 1* are responsive, while 71% of the genes in *cluster 6* are responsive. *Cluster 6* is the first cluster to reach a maximum after plasma corticosterone reaches its maximum. The reverse is also true; not all genes that are responsive to MPL infusion have circadian rhythms. For example, Fig. 7 shows the response profiles of two genes to MPL infusion (*bottom*) together with their response profiles over the 24-h circadian time series (*top*). Both of these genes are likely involved in the atrophy caused by MPL infusion. The first is glutamine synthetase (GS), which is involved in the export of amino acid carbon from the musculature (22). The second is eukaryotic translation initiation factor 4e binding protein (*Eif4ebp*), which binds to the initiation factor and prevents the initiation of translation (33). Both genes are responsive to MPL. GS has a strong circadian rhythm, which has also been verified by quantitative RT PCR (Fig. 8). In contrast, *Eif4ebp* does not exhibit a circadian rhythm.

Functional groupings. The accession numbers for all 122 probe sets were analyzed using the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) to both definitively identify the gene and to obtain alternative names and symbols. Using this information, we extensively researched each gene to identify and categorize its function. The genes were then separated into the following 11 functional groupings: carbohydrate metabolism, cell cycle, cytoskeleton/extracellular, immune, lipid metabolism, mitochondrial, protein degradation, signaling, small molecule metabolism, transcription, and transport. In several cases, we needed to decide on the most appropriate category because some genes could fit into more than one group. For example, the transcription factor, sterol regulatory element-binding transcription factor 1 (*SREBP1*), was placed in the category of lipid

metabolism because it is critical to this function, while alternative placement in transcription would also have been valid (8). Similarly, two genes, WD repeat and suppressor of cytokine signaling (SOCS) box-containing 1 and ankyrin repeat and SOCS box-containing protein 2, were placed in the immune functional category rather than signaling. Both of these genes are adaptor/regulatory modules that contain a SOCS box that is essential for the physiological inhibition of interferon signaling (28). Table 2 provides the grouping of genes into functional categories.

DISCUSSION

This report describes a gene array analysis of circadian oscillation of mRNA expression in the skeletal muscle of adult male Wistar rats. Animals were killed at nine time points during a 12-h light period and nine corresponding time points during a 12-h dark period ($n = 3$ per time point). Muscle RNAs from each of the 54 animals were applied to individual Affymetrix GeneChips Rat Genome 230A, which contained 15,967 probe sets. Analysis yielded 122 probe sets (representing 109 genes) with a 24-h periodicity. Because of the richness of this data set, we were able to apply QT clustering and identified eight clusters with maxima at different times during the cycle. Four clusters peaked during the light period and contained 31% of the genes, three clusters peaked during the dark period and contained 57% of the genes, and one peaked very close to the light to dark transition (12% of the genes). In 13 cases, there were two probe sets on the chip for a gene. In 12 of these cases, both probe sets were in the same cluster with similar correlations to the centroid. However, in one case, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (*Dyrk2*), one probe set was in *cluster 1* with a maximum at

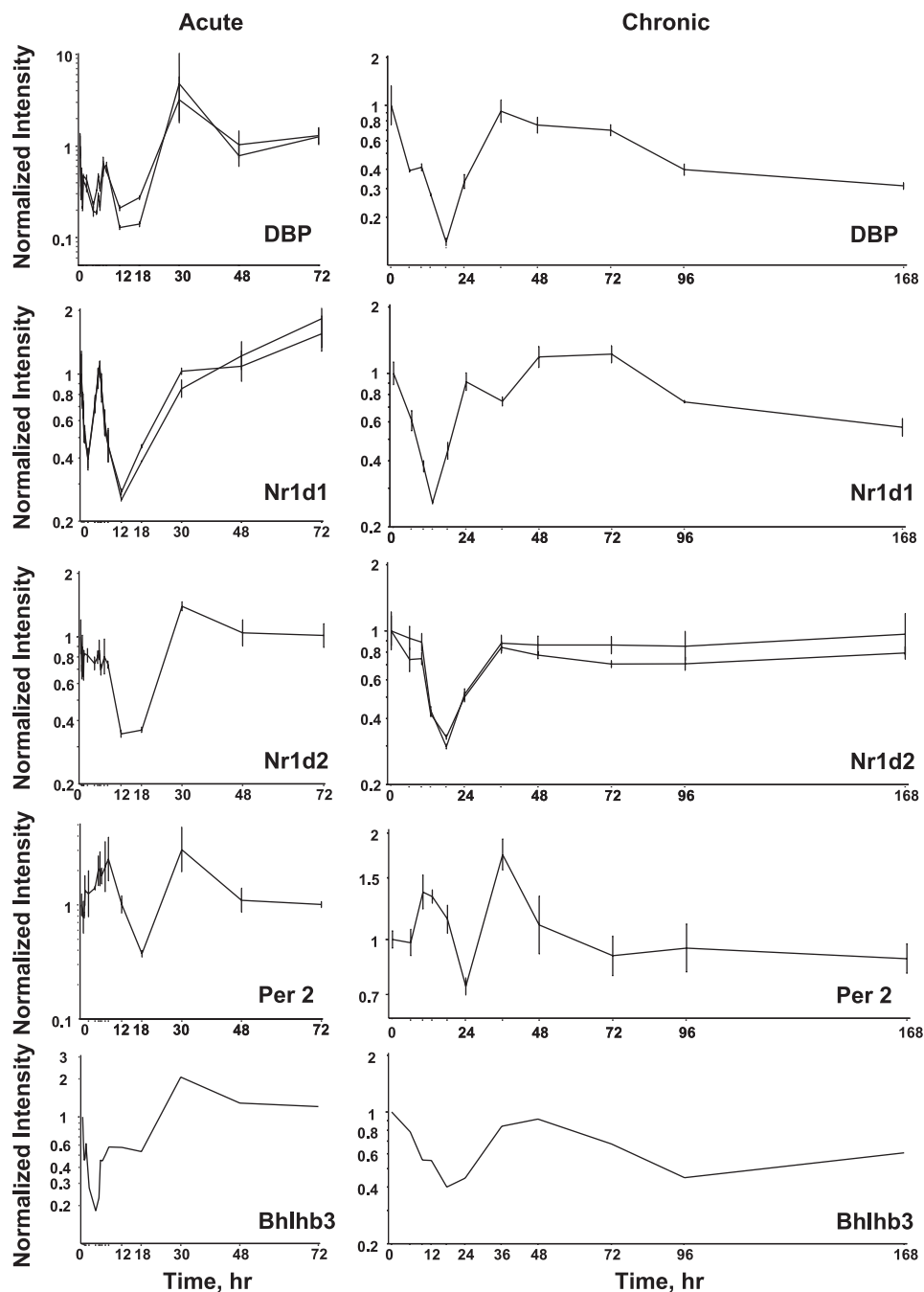


Fig. 5. Expression patterns of 5 clock-related transcription factors in skeletal muscle as a function of time after methylprednisolone (MPL) administration to adrenalectomized animals. *Left*: data from acute (bolus 50 mg/kg) MPL dosing. *Right*: data from chronic ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) MPL infusion.

2 h, and the other was in *cluster 2* with a maximum at 4 h. Both probe sets BLAST to the consensus sequence for this gene (NM_001108100.1). Both have similar scores around 1,000, indicating a very good match with the consensus sequence. Visual inspection of the expression patterns confirms that they are different in both time and intensity.

Regulation of the central SCN clock involves as many as 17 genes (34). The 230A array contained probe sets for 14 of these. Eight (*Per1*, *Per2*, *BMAL1b*, *Bhlhb3*, *DBP*, *Nfil3*, *Nr1d1*, and *Nr1d2*) showed distinct circadian oscillations in skeletal muscle. However, six (*Per3*, *Bhlhb2*, *RORB*, *RORC*, *CRY2*, and *CLOCK*) did not demonstrate oscillations in our study. In the cases of *Per3*, *RORB*, and *CRY2*, this may be due to very

low signal intensities. However, the signal intensities of *CLOCK*, *Bhlhb2*, and *RORC* were reasonable. Zamboni et al. (40), using a much sparser data set and statistical analysis, did report a circadian rhythm for *CRY1* in human skeletal muscle (40). However, visual inspection of their data does not suggest an oscillation with a 24 h periodicity, which was the criterion that we applied here. While the 230A chip used here did not contain a probe set for *CRY1*, it did contain a probe set for *CRY2*. McCarthy et al. (20) reported circadian oscillation of *CRY2* in mouse skeletal muscle using RTPCR. This suggests that our result may be due to the inability of the *CRY2* probe set on the 230A array to measure the signal. In contrast, *CLOCK* exhibited a strong signal but no circadian pattern in our studies.

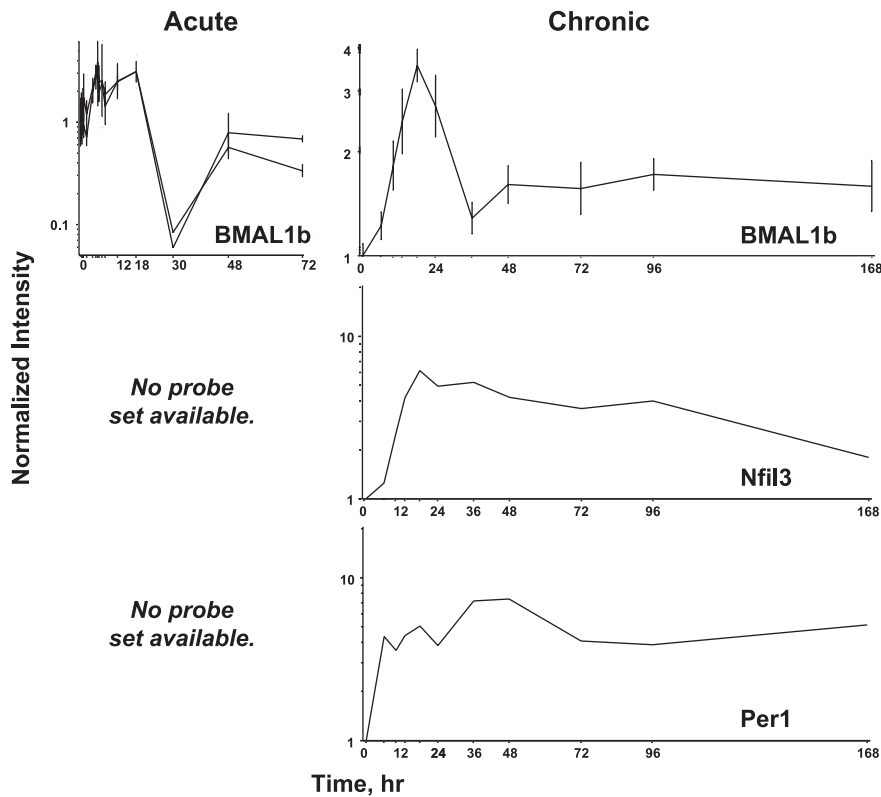


Fig. 6. Expression patterns of 3 clock-related transcription factors in skeletal muscle as a function of time after MPL administration to adrenalectomized animals. *Left*: data from acute (bolus 50 mg/kg) MPL dosing. *Right*: data from chronic ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) MPL infusion.

It has been reported by others that at least in some tissues, *CLOCK* is expressed at tonic levels and that cycling is due to the rhythmicity of its heterodimeric partner *BMAL* (27). Steeves et al. (30), using Northern blot hybridization, did report the expression of *CLOCK* in skeletal muscle (30). However, they only measured one time point, so oscillation was not measured. Our result confirms their observation that *CLOCK* is expressed in skeletal muscle and indicates that it does not have circadian rhythmicity. In addition, in our chronic infusion studies *CLOCK* does not respond significantly to MPL.

Because skeletal muscle represents about 40% of the mass of a normal healthy mammal, it is a major target of endogenous

glucocorticoids. The large protein mass of the musculature provides a major underpinning for systemic glucose homeostasis. Glucocorticoids shift muscle into a net negative nitrogen balance, and much of the resultant amino acid carbon is used to synthesize glutamine. Glucocorticoids also enhance the expression of enzymes in the liver and kidney that use the amino acid carbon released from the musculature for gluconeogenesis. We also used kinetic-based RT PCR to measure the expression of mRNA for glutamine synthesis in the same muscle used for this array experiment. As expected, RT PCR and array data exhibit virtually identical circadian patterns (Fig. 8). The array results presented here put that observation within the broader

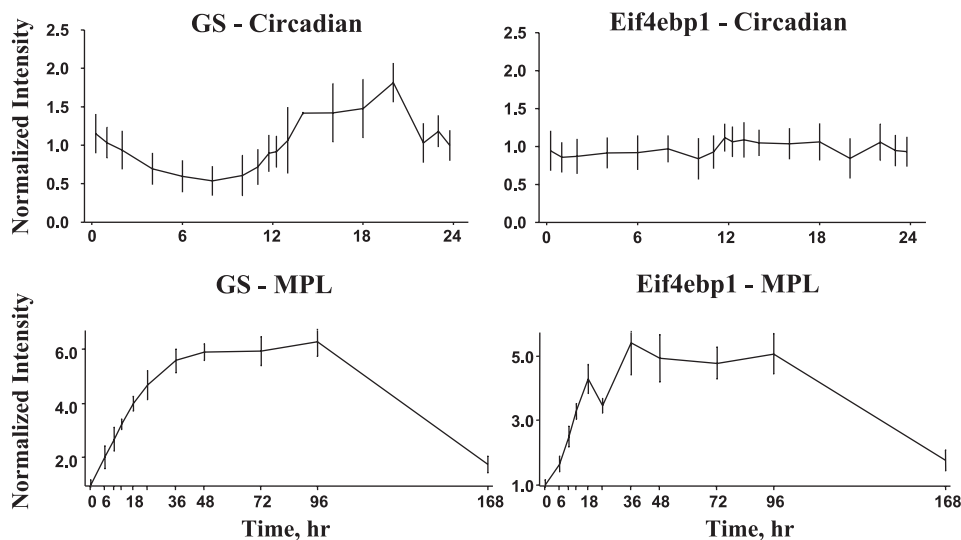


Fig. 7. Expression patterns of GS (*left*) and *Eif4ebp1* (*right*) following chronic infusion of MPL ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) over 168 h (*top*), and within the 24 h circadian cycle (*bottom*).

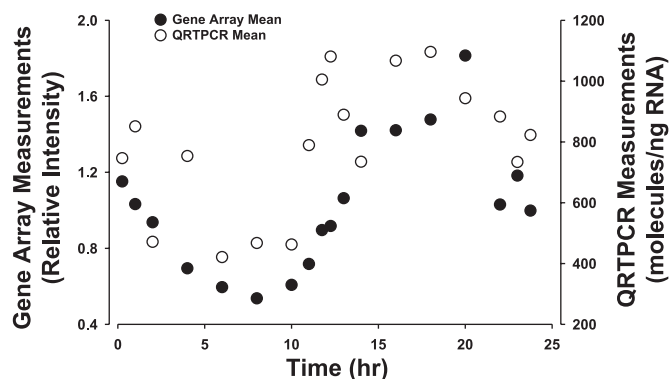


Fig. 8. Circadian pattern of expression of glutamine synthetase measured by gene arrays (●) and by kinetic-based quantitative RT PCR (○).

context of all gene expression changes and demonstrates that this enzyme is in *cluster 7*, which reaches a maximum about 5 h after the maximum of the corticosterone rhythm. Synthetic glucocorticoids, corticosteroids, are widely used therapeutic agents for modulation of immune/inflammatory responses. Previously, we measured the time course of the responses of skeletal muscle to both a single bolus dose and continuous infusion of the corticosteroid methylprednisolone (3). Because those experiments were conducted with adrenalectomized animals, corticosterone circadian rhythmicity was absent. Although corticosteroids are widely used agents to modulate immune/inflammatory responses, they have a low therapeutic index. The effect of these agents on the musculature contributes substantially to the side effects of corticosteroids. Not only does the musculature atrophy to provide amino acid carbon for increased gluconeogenesis by the liver and kidney but also the musculature becomes insulin resistant. The result is a condition known as steroid diabetes.

The effects of glucocorticoids/corticosteroids on skeletal muscle are quite complex. Some, like the enhanced expression of glutamine synthetase, appear to be due to the interaction of the hormone receptor complex with a GRE 5' to the coding region. Others are less direct and more complex due to the effects of corticosteroids on, among other things, the expression of other transcription factors. For example, corticosteroids enhance the expression of myostatin, a muscle-specific transcription factor (18). We examined our previously obtained acute and chronic data sets to ascertain the effect of chronic MPL infusion and where possible acute dosing on the eight transcription factors identified in the current data set as having circadian rhythms. In the six cases for which acute profiles were available, the response was that the drug caused the expression to begin oscillation, which dampened with time. The first phase of this oscillation was up for *BMAL1b* and *Per2*, while the first phase of the oscillation was down for *Bhlhb3*, *DBP*, *Nr1d1*, and *Nr1d2*. With chronic infusion, the initial effect was in the same direction as the acute dosing. *Per2*, *Bhlhb3*, *DBP*, and *Nr1d1* continued oscillating with different periodicities throughout the infusion period, while *Nr1d2* made a significant initial decline and almost recovered baseline. However, three genes *BMAL1b*, *Nfil3*, and *Per1* showed very significant prolonged changes in expression. *BMAL1b* showed a very sharp increase in expression that dropped by about 50% by 36 h to a state of continuous

enhanced expression over baseline throughout the infusion period. *Per1* quickly went up 5- to 6-fold and remained at this level throughout the infusion period. *Nfil3* also quickly went up about 5- to 6-fold and slowly began to decline over the infusion period but was still about twice the baseline at the end (168 h). Taken together, the results suggest that the expression of some of these transcription factors, *BMAL1b*, *Nfil3*, and *Per1*, are more closely linked to the corticosterone circadian rhythm than others (*Per2*, *Bhlhb3*, *DBP*, *Nr1d1*, and *Nr1d2*). However, the fact that all of the measurable transcripts in our acute bolus dosing study begin to oscillate suggest that the corticosterone rhythm may be the initiator of their rhythmic pattern of gene expression. This is consistent with the observation by McCarthy that some genes in muscle maintain rhythmic expression in *CLOCK* mutant mice and that diurnal and nocturnal animals can phase shift not only their glucocorticoid rhythm but also the rhythms of gene expression in peripheral tissues (20).

We also compared circadian-regulated genes with those identified as regulated by the chronic infusion of MPL. Of the more than 2,000 probe sets identified as responsive to MPL infusion, only 57 probe sets (55 genes) with circadian rhythms were among the glucocorticoid-regulated group. However, these data sets together allowed us to address two basic but related questions. The first is: do all genes that respond to corticosteroids have circadian rhythms? The second is: do all genes with circadian rhythms respond to corticosteroid? The answer to both questions is "No." Only 55 of the genes identified were both circadian and MPL-responsive. The facts that all genes that respond to MPL are not circadian and that all genes with circadian rhythms do not respond to MPL suggest that there is some diversity in mediating mechanisms. This result is consistent with both the previously described observations comparing our acute and chronic profiles and the current observation that the genes can be separated into eight clusters (3).

If an animal is diurnal, increased mRNA expression near the end of the dark period begins to prepare the animal for activity and feeding. Similarly, downward changes during the end of the light period prepare the diurnal animal for inactivity and rest. For example nocturnin, a deadenylase that has peak expression in the early dark period in rodents, has been implicated in the regulation of genes important to feeding and energy metabolism (9). Rats are nocturnal, and cycling of gene expression in peripheral tissues like the muscle is reversed relative to humans who are essentially diurnal. On the basis of extensive literature searching, we placed each of the 109 genes into functional groupings as follows: carbohydrate metabolism, cell cycle, cytoskeleton/extracellular, immune, lipid metabolism, mitochondrial, protein degradation, signaling, small molecule metabolism, transcription, and transport. The objective of this analysis was to ascertain which functions are most influenced by circadian patterns and when they are occurring. The most populated functional group is transcription (Table 2). In addition to the 25 genes that appear on this table, *Nfil3* (which we placed in the immune grouping), *Klf15* and *nr4a1* (in the carbohydrate grouping), and *srebpf1* (in the lipid grouping) are also transcription factors. Thus about one-fourth of the genes with circadian rhythms are involved in the regulation of transcription/translation. It is also important to note that genes in this grouping occur in all eight clusters, which illustrate the complex nature of circadian rhythms on gene expression.

Table 2. Functional categorization of circadian regulated genes

Probe ID	Cluster	Symbol	Gene Name	Gene Function
Carbohydrate Metabolism				
1369467_a_at	7	<i>Pfkfb1</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	glycolysis
1373108_at	8	<i>ppp1r3c</i>	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	glycogen metabolic process
1368249_at	5	<i>Klf15</i>	Krueppel-like factor 15	regulates GLUT4 expression
1386935_at	6	<i>nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1	regulator of muscle energy homeostasis
Cell Cycle				
1398913_at	3	<i>numa1</i>	Nuclear mitotic apparatus protein 1	cycle-related protein
1374636_at	3	<i>Phf17</i>	PHD finger protein 17	proapoptotic barrier to proliferation
1368277_at	3	<i>Ppp3ca</i>	Protein phosphatase 3, catalytic subunit, alpha isoform, calcineurin A	G1/S transition of mitotic cell cycle
1388804_at	6	<i>Mn1</i>	Meningioma 1	negative regulation of progression through cell cycle
1370663_at	6	<i>wee1</i>	Wee 1 homolog	DNA replication checkpoint, G2/M transition
1388767_at	7	<i>Pdcd6</i>	Programmed cell death 6-interacting protein	caspace activation, induction of apoptosis by extracellular signals
1388395_at	8	<i>G0 s2</i>	G0/G1 switch gene 2	regulation of progression through cell cycle
Cytoskeleton-Extracellular				
1370166_at	2	<i>Sdc2</i>	Syndecan 2	transmembrane proteoglycan, cytoskeletal binding protein
1388384_at	5	<i>Dynll1</i>	Dynein light chain LC8-type 1	molecular motor
1368431_at	6	<i>Hpn</i>	Hepsin	Membrane-bound serine protease
1367568_a_at	7	<i>Mgp</i>	Matrix gamma-carboxyglutamic acid protein	inhibitor of soft-tissue mineralization
1372091_at	7	<i>Mig12</i>	MID1 interacting G12-like protein	inhibition of microtubule depolymerization, microtubule stabilization
1387015_at	7	<i>Pfn2</i>	Profilin 2	regulation of actin polymerization and/or depolymerization
1373850_at	7	<i>Smpd3b</i>	Sphingomyelin phosphodiesterase, acid-like 3B	hydrolase activity, acting on glycosyl bonds, secreted
1375211_at	7	<i>Rnaset2</i>	Ribonuclease T2	secreted RNase
Immune				
1370847_at	1	<i>Spon2</i>	Spondin 2	innate immune response
1368375_a_at	6	<i>Il15</i>	Interleukin 15	positive regulation of T cell proliferation
1386987_at	6	<i>Il6r, Il6ra, IL6R1</i>	Interleukin 6 receptor	energy metabolism
1367609_at	7	<i>Mif</i>	Macrophage migration inhibitory factor	regulation of macrophage activation
1370428_x_at	7	<i>RT1-Aw2, RT1-A2, RT1-A2q</i>	RT1 class Ib, locus Aw2	antigen processing and presentation via MHC class I
1369973_at	7	<i>Xdh</i>	Xanthine dehydrogenase	inflammation, vascular, muscle damage
1398246_s_at	8	<i>Fcgr3</i>	Fc receptor, IgG, low-affinity III	antigen processing and presentation
1367850_at	8	<i>Fcgr3</i>	Fc receptor, IgG, low-affinity III	antigen processing and presentation
1368488_at	8	<i>Nfil3</i>	Nuclear factor, interleukin 3 regulated	DNA-dependent transcription regulation
Lipid Metabolism				
1388426_at	1	<i>srebf1</i>	Sterol regulatory element-binding transcription factor 1	regulation of transcription, sterol metabolism
1367979_s_at	2	<i>Cyp51</i>	Cytochrome P-450, subfamily 51	sterol 14-alpha-demethylase cholesterol synthesis
1376089_at	3	<i>Ldlr</i>	Low-density lipoprotein receptor	cholesterol homeostasis
1388924_at	3	<i>angptl4</i>	Anigopietin-like 4	negative regulation of lipoprotein lipase
1367894_at	3	<i>insig1</i>	Insulin-induced gene 1	retention of the SCAP/SREBP complex in the ER
1368182_at	6	<i>acsl6, facl6</i>	Acyl-CoA synthetase long-chain family member 6	ligation of long-chain fatty acids with coenzyme A
1371615_at	6	<i>Dgat2</i>	Diacylglycerol O-acyltransferase homolog 2	triglyceride synthesis, glycerol, lipid metabolism
Mitochondrial				
1387294_at	2	<i>Sh3bp5</i>	SH3-domain binding protein 5	mitochondrial JNK cascade
1369150_at	4	<i>Pdk4</i>	Pyruvate dehydrogenase kinase, isoenzyme 4	acetyl-CoA biosynthesis from pyruvate
1386946_at	4	<i>cpt1a</i>	Carnitine palmitoyltransferase I	transfer long-chain fatty acids across the mitochondrial inner membrane
1368283_at	5	<i>Ehhadh</i>	Enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	acyl-CoA metabolism, fatty acid beta-oxidation
1373866_at	5	<i>Coq10b</i>	Coenzyme Q10 homolog B	oligonucleoxide cyclase/lipid transport protein
1372452_at	5	<i>Gpam</i>	Glycerol-3-phosphate acyltransferase	fatty acid metabolism
1373282_at	6	<i>slc25a33</i>	Solute carrier family 25, member 33	mitochondrial carrier proteins, transport

Continued

Table 2.—Continued

Probe ID	Cluster	Symbol	Gene Name	Gene Function
1367735_at	7	<i>Acadl</i>	Acetyl-coenzyme A dehydrogenase, long-chain	lipid, fatty acid metabolism, electron transport
1386887_at	7	<i>Cox5b</i>	Cytochrome <i>c</i> oxidase, subunit Vb	electron transport chain
1388113_at	7	<i>cox8a</i>	Cytochrome <i>c</i> oxidase, subunit VIIIa	electron transport chain
1388361_at	7	<i>PDSW</i>	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10	electron transport chain
1389288_at	7	<i>Ndufa2</i>	NADH-ubiquinone oxidoreductase 1 Alpha subcomplex, 2	electron transport chain
1379243_at	7	<i>Ndufa6</i>	NADH-ubiquinone oxidoreductase 1 Alpha subcomplex, 6	electron transport chain
1388364_at	7	<i>Ndufs3</i>	NADH-ubiquinone oxidoreductase Fe-S protein 3	electron transport chain
1371912_at	7	<i>Ndufs7</i>	NADH dehydrogenase (ubiquinone) Fe-S protein 7	electron transport chain
1375197_at	7	<i>LOC686442</i>	Ubiquinol-cytochrome <i>c</i> reductase subunit	electron transport chain
1367506_at	7	<i>mrpl11</i>	Mitochondrial ribosomal protein L11	protein biosynthesis, translation
1368004_at	7	<i>mrpl23</i>	Mitochondrial ribosomal protein L23	protein biosynthesis, translation
1371649_at	7	<i>mrps24</i>	Mitochondrial ribosomal protein S24	protein biosynthesis, translation
1398868_at	7	<i>timm13, sirt6</i>	Translocase of inner mitochondrial membrane 13	protein folding and targeting
1367670_at	7	<i>Fh1, Fh</i>	Fumarate hydratase 1	conversion of fumarate to malate
Protein Degradation				
1398767_at	1	<i>Ubb, ubc</i>	Ubiquitin B, C	protein catabolism
1374204_at	1	<i>Wsb1</i>	WD repeat and SOCS box-containing 1	E3 ubiquitin ligase complexes
1372280_at	1	<i>Asb2</i>	Ankyrin repeat and SOCS box-containing protein 2	E3 ubiquitin ligase complexes
1387703_a_at	6	<i>usp2, ubp69</i>	Ubiquitin-specific peptidase 2	deubiquitinating enzyme, muscle cell differentiation
1375971_at	7	<i>Cln5</i>	Ceroid-lipofuscinosis, neuronal 5	lysosome organization and biogenesis
1367632_at	7	<i>Glul, Glns</i>	Glutamine synthetase	glutamine synthesis, muscle atrophy
1367481_at	7	<i>vps28</i>	Vacuolar protein sorting 28	ubiquitin-dependent protein catabolism
Signaling				
1376944_at	1	<i>Prlr</i>	Prolactin receptor	signaling, JANUS KINASE 2, GHR and SOCS
1378423_at	2	<i>Atcay</i>	Caytaxin	associated with Cayman ataxia
1399005_at	2	<i>ppp2r5a</i>	Protein phosphatase 2, regulatory subunit B (B56), alpha	signal transduction
1371602_at	2	<i>Tspan9 NET-5</i>	Tetraspanin 9	signal transduction
1387172_a_at	3	<i>tgfb2</i>	Transforming growth factor, beta-2	negative regulation of apoptosis, regulation of cell proliferation
1388471_at	4	<i>tcp112</i>	T-complex 11 like 2	cell communication
1369050_at	5	<i>Pik3c2 g</i>	Phosphatidylinositol 3-kinase, C2 domain containing, gamma	Ras pathway, vesicle trafficking, secretion, and apoptosis
1388525_at	5	<i>PIK3IP1</i>	Phosphoinositide-3-kinase interacting protein 1	binds to the p110 catalytic down modulates PI3K
1389632_at	5	<i>Rhobtb1</i>	Rho-related BTB domain-containing protein 1	small GTPase-mediated signal transduction
1373092_at	6	<i>Tgfb3, BETA-GLYCAN</i>	Transforming growth factor, Beta receptor III	modulates several tissue development and repair processes
1386857_at	7	<i>Stmn1</i>	Stathmin 1	signaling relay phosphoprotein
1371397_at	7	<i>Nosip</i>	Nitric oxide synthase interacting protein	negative regulation of nitric oxide synthase activity
1389230_at	7	<i>arrdc3</i>	Arrestin domain containing 3	thioredoxin interacting protein
Small Molecule Metabolism				
1387669_a_at	5	<i>ephx1</i>	Epoxide hydrolase 1, microsomal	small molecule metabolism
1389251_at	6	<i>Nudt7</i>	Nucleoside diphosphate-linked moiety X motif 7	peroxisomal, regulates CoA and acyl-CoA levels
1370019_at	6	<i>Sult1a1</i>	Sulfotransferase family 1A, phenol-preferring, member 1	transfer of a sulfate group from 3 ϵ -phosphoadenosine 5 ϵ -phosphosulfate
1387053_at	6	<i>Fmo1</i>	Flavin-containing monooxygenase 1	NADPH-dependent oxidative metabolism of many drugs
1372011_at	6	<i>Gda</i>	Guanine deaminase	purine metabolism
Transcription				
1375378_at	2	<i>Qki</i>	Quaking homolog, KH domain RNA binding	transcription, RNA-binding protein
1375984_at	3	<i>Zfx4</i>	Zinc finger homeodomain 4	DNA-dependent transcription, muscle differentiation

Continued

Table 2.—Continued

Probe ID	Cluster	Symbol	Gene Name	Gene Function
1367602_at	4	<i>Cited2</i>	Cbp/p300-interacting transactivator, Glu/Asp-rich carboxy-terminal domain, 2	positive transcription regulation
1367601_at	4	<i>Cited2</i>	Cbp/p300-interacting transactivator, Glu/Asp-rich carboxy-terminal domain, 2	positive transcription regulation
1370816_at	4	<i>nr1d1</i>	Nuclear receptor subfamily 1, group D, member 1	steroid hormone receptor-circadian rhythm
1368511_at	4	<i>Bhlhb3</i>	Basic helix-loop-helix domain containing, class B, 3	transcription regulation
1387874_at	5	<i>Dbp</i>	D site of albumin promoter-binding protein	transcription regulation, circadian rhythm
1371864_at	5	<i>Klf9</i>	Kruppel-like factor 9	mediates expression of growth-associated genes
1390430_at	5	<i>nr1d2, RVR</i>	Nuclear receptor subfamily 1, group D, member 2	DNA-dependent transcription regulation
1370541_at	5	<i>nr1d2, RVR</i>	Nuclear receptor subfamily 1, group D, member 2	DNA-dependent transcription regulation
1371583_at	5	<i>Rbm3</i>	RNA binding motif protein 3	RNA processing, miRNA-mediated gene silencing
1368303_at	6	<i>Per2</i>	Period homolog 2	circadian rhythm, protein binding, signal transduction
1390171_at	6	<i>FAM76A</i>	Family with sequence similarity 76, member A	nuclear inhibitor of protein phosphatase-1
1371479_at	6	<i>Mettl7a</i>	Methyltransferase like 7A	methyltransferase activity, metabolic activity
1369545_at	7	<i>Egr3</i>	Early growth response 3	regulation of myotube fate
1371351_at	7	<i>Rpo1-3</i>	RNA polymerase 1-3	transcription regulation
1369590_a_at	7	<i>Ddit3, GADD153</i>	DNA-damage-inducible transcript 3	dominant-negative inhibitor of the transcription factors C/EBP
1388683_at	7	<i>Lsm1</i>	LSM domain containing 1	RNA processing
1371324_at	7	<i>SF3B5</i>	Splicing factor 3B subunit 5	RNA splicing; mRNA processing
1368136_at	8	<i>Tmpo, Slc25a3</i>	Thymopoietin	nuclear membrane protein
1372964_at	8	<i>arid5b</i>	AT rich interactive domain 5B	negative regulation of transcription
Transport				
1392939_at	2	<i>Slc41a3</i>	Solute carrier family 41, member 3	inorganic cation transport (Mg)
1372178_at	6	<i>Abcc1</i>	ATP-binding cassette, subfamily C (CFTR/MRP), member 1	transporter of glutathione-linked compounds from cells
1369625_at	6	<i>Aqp1</i>	Aquaporin 1	major water transport molecule I vascular
1373818_at	6	<i>kctd12</i>	Potassium channel tetramerization domain containing 12	voltage-gated potassium channel

Probe sets exhibiting a circadian pattern in rat skeletal muscle arranged by gene function. Probe ID refers to identification number used by Affymetrix, which is distinct from GenBank accession numbers. Cluster number refers to circadian expression patterns identified by QT clustering, as presented in Table 1.

Almost all of the genes in this grouping are involved in transcription. However, a notable exception is Pumilio homolog 2 (*Pum2*), which is in *cluster 3*. Cluster 3 also contains zinc finger homeodomain 4 (*Zfhx4*). *Pum2* is involved in mRNA degradation and has been shown to promote stem cell proliferation (37). Kostich and Sanes (16) observed that *Zfhx4* was high during proliferation but decreased with differentiation. These observations, together with the observation that transforming growth factor, beta-2 (*tgfb2*) in the signaling group, which is an inhibitor of differentiation (29), is also in *cluster 3* suggests that muscle stem cell proliferation may be initiated during the middle of the light/inactive period. Reinforcing this conclusion is the presence of nuclear mitotic apparatus protein 1 (*numa1*), PHD finger protein 17 (*Phf17*), and protein phosphatase 3, catalytic subunit, alpha isoform (*Ppp3ca*) from the cell cycle group in *cluster 3* as well.

The second most populated group is mitochondrial with 21 genes. In stark contrast to the transcription grouping, 14 of these genes are in *cluster 7*, which reached its maximum in the middle of the dark/active period of the animal. The gastrocnemius muscle is a mixed-fiber muscle, but because the animals are awake but relatively sedentary, the musculature in general is using primarily lipid fuels that require mitochondria. This

grouping also illustrates quite well that genes that work together are expressed together.

The third most populated group is signaling, with 14 genes. In addition to the cluster 3 gene, *tgfb2*, the signaling group also contains two probe sets for protein phosphatase 2, regulatory subunit B56 alpha (*ppp2r5a*), a cluster 2 gene that would be consistent with cell proliferation occurring during the light period (21). In contrast, the expression of genes such as *Stathmin 1* (35), along with the mitochondrial genes in *cluster 7*, suggests that the circadian expression is by differentiated cells. All genes in the functional immune group, except *Spondin 2* (*Spon2*), which is involved in innate immune responses (12), are expressed in *clusters 6, 7, and 8*, which reach maxima during the dark/active period.

Although there are only seven genes in the lipid group, several are central to the regulation of lipid metabolism in skeletal muscle. The first is *SREBP1* which is a master transcription factor that plays a role in the expression of many lipogenic genes (8). *SREBP1* is in *cluster 1* that reaches a maximum early in the light period. *Cluster 2* contains lanosterol 14-demethylase (*CYP51*), an enzyme important for cholesterol biosynthesis (31). *Cluster 3* contains three genes that are central to muscle lipid metabolism. The first is low-density

lipoprotein receptor (*LDLR*). The second is anigopietin-like 4 (*angptl4*), which inhibits lipoprotein lipase (39), and the last is insulin-induced gene 1 (*insig1*), which facilitates the retention of the SREBP cleavage-activated protein/SREBP complex in the endoplasmic reticulum (1). *Cluster 6*, which has a maximum during the dark period, contains two genes important for the synthesis of triglycerides. The first is acyl-CoA synthetase long-chain family member 6 (*acsl6*), which catalyzes the ligation of long-chain fatty acids with coenzyme A (19). The second is diacylglycerol O-acyltransferase homolog 2 (*Dgat2*), which catalyzes the covalent linking of diacylglycerol to long-chain fatty acyl-CoAs (17).

The functional group that we labeled cytoskeleton/extracellular contains eight genes. Only one of these, syndecan 2 (*Sdc2*) is in a cluster that has a maximum during the light period. For the most part, these genes seem to reflect muscle mechanical activity during the dark period. In contrast, the distribution of genes in the cell cycle group spans many clusters, which illustrates the progression of stem cells through the cell cycle with cell division beginning during the light period.

Surprisingly few genes with circadian rhythms are associated with protein degradation. Enhanced expression of GS, which is glucocorticoid responsive and is associated with several atrophy conditions is found in *cluster 7*, along with a few other relevant genes. In the case of GS, data suggest that the maximum expression of this gene is directly associated with the maximum of the corticosterone rhythm, which occurs several hours earlier (38). However, no such data are available for other *cluster 7* genes associated with protein degradation. What is surprising is that ubiquitin B, C (*Ubb, Ubc*) is in *cluster 1*. Enhanced expression of this gene is associated with several muscle atrophy conditions, including atrophy induced by glucocorticoids (14). The genes in the small molecule metabolism group concentrate in *cluster 6*, which probably reflects the increased activity of the animal during the dark period. Likewise, surprisingly few genes associated with carbohydrate metabolism have circadian rhythms. The fact that 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (*Pfkfb1*), along with most of the mitochondrial genes are in *cluster 7*, suggest that this is the period of highest energy demand by the musculature. However, these animals were housed in single cages and were relatively sedentary, which may influence energy demands by muscle fibers.

The last grouping was transport. Three of the four genes in this group were in *cluster 6*, which is rational, given the activity of the animals. The last, *Slc41a3*, is a Mg⁺ transporter found in *cluster 2* (36). Very little data are available on the function of this gene in skeletal muscle.

These microarray experiments only reflect changes in the amount of expressed message for a gene. Although we only identified 109 genes whose message had a rhythm with a 24-h periodicity, the nature of some of these genes will magnify the effect of the rhythmicity. For example, rhythmicity in *Pum2*, which mediates mRNA degradation, will likely change the amount of many other messages. However, these changes may not have a strict 24-h periodicity. Similarly, the fact that many phosphatases and kinases have rhythmicity will magnify oscillations at the functional level. Similar to secondary changes in the amount of message, rhythmic changes in function may not necessarily have a 24-h periodicity.

Perspectives and Significance

Although much progress has been made in understanding the control of circadian rhythms in the SCN, translation of control to peripheral tissues, which ultimately mediate systemic biological rhythms, is much less well understood. The present study identifies patterns of circadian gene expression changes in one peripheral tissue: skeletal muscle. It also identifies eight transcription factors that have been implicated in central clock mechanisms as having oscillating patterns in muscle as well. Extensions of such studies to other peripheral tissues such as liver and adipose tissue will address the question of what genes are regulated in a circadian pattern in multiple tissues of an organism and will allow a better understanding of how changes in multiple peripheral tissues can be integrated into systemic biological effects, such as rhythmic changes in energy metabolism. This study also demonstrates that although glucocorticoids serve as important signals for translating rhythms from central control centers in the brain to the periphery, factors other than glucocorticoids likely also play a role in this translation.

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