Circadian Programs of Transcriptional Activation, Signaling, and Protein Turnover Revealed by Microarray Analysis of Mammalian Cells

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Summary

Many aspects of physiology and behavior are temporally organized into daily 24 hr rhythms, driven by an endogenous circadian clock. Studies in eukaryotes have identified a network of interacting genes forming interlocked autoregulatory feedback loops which underlie overt circadian organization in single cells [1, 2]. While in mammals the master oscillator resides in the suprachiasmatic nuclei of the hypothalamus [2], semiautonomous circadian oscillators also exist in peripheral tissues [3–5] and in immortalized fibroblasts, where rhythmicity is induced following a serum shock [6, 7]. We used this model system in combination with high-density cDNA microarrays to examine the magnitude and quality of clock control of gene expression in mammalian cells. Supported by application of novel bioinformatics tools, we find ~2% of genes, including expected canonical clock genes, to show consistent rhythmic circadian expression across five independent experiments. Rhythmicity in most of these genes is novel, and they fall into diverse functional groups, highlighted by a predominance of transcription factors, ubiquitin-associated factors, proteasome components, and Ras/MAPK signaling pathway components. When grouped according to phase, 68% of the genes were found to peak during estimated subjective day, 32% during estimated subjective night, with a tendency to peak at a phase corresponding to anticipation of dawn or dusk.

Results and Discussion

To examine circadian expression of mammalian genes, we used immortalized rat-1 fibroblasts as a model system of the peripheral clock, with the expectation of identifying genes that might be elements of the central oscillator and of its input or output pathways, i.e., clock-controlled genes (ccgs). This in vitro system behaves surprisingly like the suprachiasmatic nucleus (SCN), which contains the major mammalian circadian oscillator, and peripheral tissues such as the liver, in that known clock genes and ccgs exhibit similar rhythmic (e.g., *per2*) and nonrhythmic (e.g., *Clock*) expression profiles, adopt normal phase relationships among one another, and display typical temporal kinetics in response to light/serum/pharmacological treatment [1, 3–7]. Five biologically independent experiments were conducted in which confluent and quiescent cells were serum treated at time = 0 hr to initiate rhythmicity. RNA was subsequently harvested every 4 hr over a period of 2 days and used for Northern and RT-PCR analysis and for RT labeling as targets for microarray analysis. In control studies, immediate early gene (IEG) expression and subsequent rhythmicity of previously characterized genes [6, 7] *rPer1* (data not shown) and *rPer2* (Figure 1A) were confirmed. IEG expression following serum shock has been well characterized [8]; 12 well characterized IEGs and another 40 genes were identified on our arrays by their increased expression levels at time = 1 hr (see Supplementary Material including Figure S1 and Table S1, available with this article online). The excellent correspondence between expected and identified IEGs and rhythmic genes confirms the rhythmicity of the cell cultures and supports the validity of the microarray assay.

Having established the rhythmic behavior of gene expression of these RNA collections, cDNA microarrays were used to investigate the temporal expression profiles of RNA from the five experiments. Cy3-labeled DNA targets were constructed from the time-specific RNA samples and hybridized to duplicate microarrays, each consisting of 2147 cDNAs representing 2124 distinct genes (see Experimental Procedures). Our experimental design effectively yielded ten individual circadian cycles of expression data, with five immediately following the synchronizing serum treatment, and 5 days in free-run. The CORRCOS algorithm was used to objectively identify rhythmically expressed genes exhibiting a circadian periodicity (20–28 hr) [9]. This was applied to individual experiments and averaged group data (see Experimental Procedures). Table 1 lists the 41 genes, derived from 44 cDNA probes, that were identified as oscillating, using the CORRCOS analyses. The range of fluorescent intensity values, as a measure of transcript abundance, covers the range of values on the array. The mean ± SD period length of the identified circadian rhythms was 24.1 ± 1.3 hr. This compares well with the observations of Balsalobre et al. [6] of 22.5 ± 1.7 hr and a range of 20–27 hr.

As further validation that rhythmicity was being accurately reported, three of the genes identified in the microarray screen (*triose phosphate isomerase*, *ATP synthase subunit d*, and *rPer2*) were examined using quantitative real-time RT-PCR for validation purposes (Figure S2). All three genes showed matching temporal profiles but with 2-fold greater amplitudes than that found by microarray analysis. The molecular mechanism of the circadian clock is comprised of a series of coupled transcriptional-translational regulatory feedback loops: three *period* genes (*per1-3*) and two *cryptochrome*

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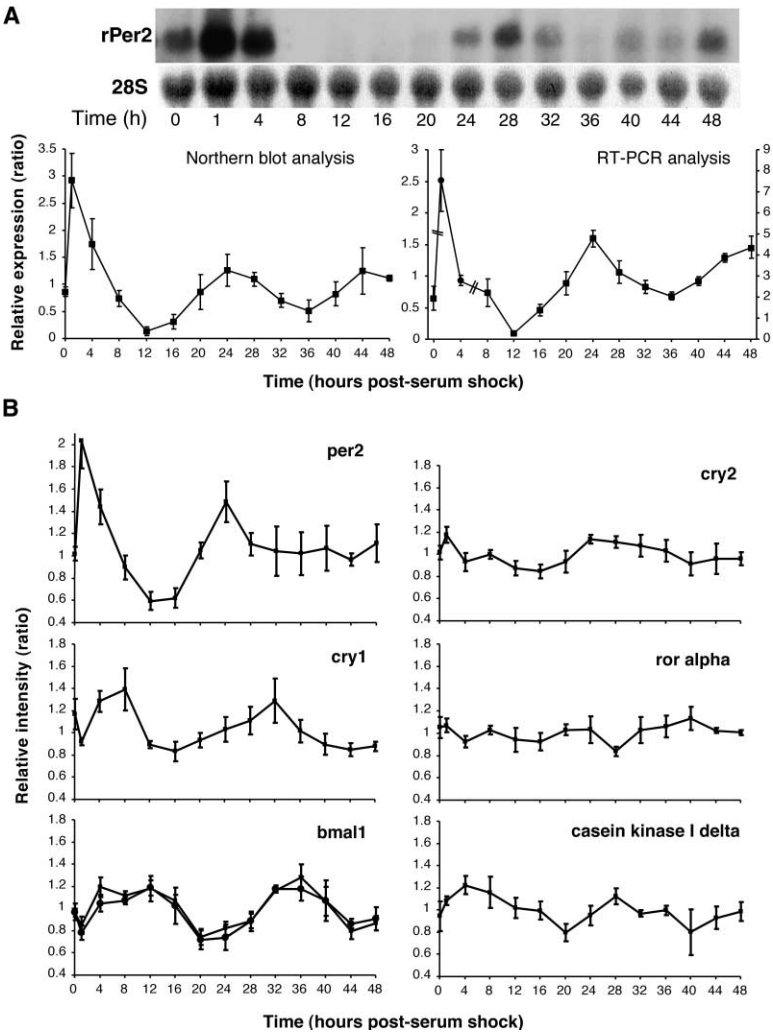

Figure 1. Validation of Microarray Analysis of Circadian Rhythmic Gene Expression in the Fibroblast Cell Culture Model

(A) (Top) Representative Northern blot of rat-1 fibroblast RNA hybridized with a probe for *rPer2* and showing a rhythmic pattern of expression (6.2 kb band). Samples were collected at time = 0, 1 hr, and every 4 hr through to 48 hr. Note large induction of *rPer2* at 1 hr and 4 hr and peak of rhythm at ~24 hr and 48 hr. (Bottom left) Densitometric analysis of the above Northern blot is plotted after normalization using ethidium bromide-stained 28S ribosomal RNA. Values are mean and SEMs ratios of median values of three Northern blots from experiments 1, 2, and 5. (Bottom right) RNA from time courses in experiments 3 and 4 were examined using quantitative RT-PCR and show acute serum response and rhythmic pattern in *rPer2* transcript. Values are mean ± SEM ratio of expression relative to medial expression value from three repeat samples from the same cDNA source for each of the two experiments (n = 6).

(B) Canonical clock genes in rat-1 cells examined using microarray analysis show expectant rhythmic and nonrhythmic patterns of behavior. *per2*, *bmal1* (rat cDNA probe, circle; mouse cDNA probe, square), and *cry1* (left) show reproducible significant rhythmicity ($p < 0.05$, CORRCOS) in group-averaged data and in all four experiments tested with PPs of expression predicated from observations using Northern blot analysis of rat-1 and mouse NIH3T3 cells ([6, 7]; Duffield et al., 2000, SRBR abstract). Consistent with these other studies, the expression of the clock gene *cry2*, the nuclear orphan receptor, *ROR* α, and *casein kinase I*δ were not found to be rhythmic in rat-1 cells. The immediate early gene response of *per2* is as expected from the RT-PCR and Northern blot data, with a clear peak at 1 hr and drop below t = 0 hr levels at 8 hr. Values are mean ± SEM ratio of time-specific expression relative to medial expression value from the five independent experiments (n = 3 to 5 each time point).

genes (*cry1* and *cry2*) comprise the negative loop, and their transcription, being driven at consensus E-box elements by CLOCK:BMAL1 heterodimers, forms the positive loop. Casein kinase I ϵ (CKIϵ) and δ (CKIδ) phosphorylate PER1 and PER2 and in turn regulate their stability [1, 2, 10]. cDNA probes on our microarray yielded clear confirmation of expected rhythmicity among controls: *per2*, *bmal1*, and *cry1* showed reproducible significant rhythmicity with appropriate phasing in all experiments tested ($p < 0.05$, CORRCOS; Table 1 and Figure 1B), while *cry2*, *CKI*δ, and the nuclear orphan receptor *ror*α were not found to be rhythmic on the microarrays (Figure 1B), this being consistent with other studies ([6, 7]; G.E.D. et al., 2000, Society for Research on Biological Rhythms, SRBR, abstract). Overall, the confirmatory RT-PCR studies and the rhythmic expression of canonical clock genes *per2*, *cry1*, and *bmal1* indicate that the other 38 mRNA species are also differentially expressed in a circadian manner.

A Clock for All Times of Day

All phases of the circadian cycle are represented among the rhythmic genes (Figure 2A), suggesting diverse pathways are used to connect the core oscillatory system with output. When the genes were grouped according to phase, the majority (68%) were found to peak between 36 and 44 hr post serum treatment (predicted day, Figures 2B and 2C), 32% between 24 and 32 hr (predicted night, Figures 2B and 2C). Within these two groups, there was a tendency to peak at a phase in the middle of the day or night or at a phase anticipating onset of dawn or dusk. This finding is surprisingly similar to that observed in an analysis of ccg expression in *Arabidopsis* [9]. Consistent with this, the only other known ccgs examined in rat-1 fibroblasts are *rev-erb* α and *D-box binding protein* (*dbp*), and these also fall within this dominant group of genes that peak at 16–20 hr and 40–44 hr post serum shock and that precede the *per2* peak by 4–8 hr [6, 7]. It is noteworthy that the peak in expression of

Table 1. Genes Expressed in rat-1 Cells with a Circadian Period Revealed by Microarray Analysis

Clone ID[a]	Gene I.D./Description[b]	Functional Group[c]	GenBank Accession No.	Mean ± SEM Intensity[d]	Mean ± SEM Period Estimate[e]	Mean Amplitude[f]	Phase Estimate (h)[g]
Dartmouth M	mouse period 2 (mPer2)	A	NM_011066/AF036893	46 ± 7	22.6 ± 1.5d	2.9 ± 0.25	24.0 ± 1.5
Dartmouth M	mouse Crypotochrome 1 (mCry1)	A	AF156986	22 ± 2	25.8 ± 0.9a	1.9 ± 0.16	32.4 ± 0.5
UI-R-C1-la-b-09-0-UI R	rat bmal1/TIC/MOP3	A	AF015953/AI059871	35 ± 5	24.6 ± 1.4a	2.3 ± 0.36	35.5 ± 1.2
Dartmouth M	mouse bmal1/MOP3	A	AB012602	41 ± 2	24.4 ± 1.4a	2.0 ± 0.14	35.4 ± 0.9
Image 1092823 M	H. sapiens basic-leucine zipper transcription factor MafG (MAFG)	B	AF059195	57 ± 10	24.8 ± 1.1b	1.8 ± 0.21	27.2 ± 0.9
UI-R-C3-tp-e-01-0-UI R	ETF-related factor-2, transcriptional enhancer factor (ETFR-2) e-139	B	D87965/D87966	59 ± 1	27.1 ± 0.3b	2.1 ± 0.37	28.0 ± 1.4
UI-R-C1-kc-d-10-0-UI R	M. musculus Upstream transcription factor 1 (USF1) 2e-74	B	U41741	73 ± 13	22.2 ± 1.5c	2.6 ± 0.37	30.0 ± 1.6
Image 669616 M	mouse promyelocytic leukaemia protein (PML) isoform 1	B	U89412	736 ± 124	22.9 ± 0.9a	2.5 ± 0.43	40.9 ± 0.8
Image 463999 M	M. musculus mitochondrial transcription factor A (mtTFA)	B	U57939/U63858	798 ± 122	24.3 ± 0.9a	2.4 ± 0.65	40.8 ± 1.6
UI-R-AA1-aac-d-06-0-UI R	CLIM-2, M. musculus LIM domain binding protein 1 (Ldb1)	B	U89488	200 ± 25	22.8 ± 0.5a	2.1 ± 0.33	42.6 ± 1.1
PRI_95795 R	R. norvegicus mRNA for muscle LIM protein/Cysteine-rich protein 3 (Csrp3) 0.0	B	X81193	42 ± 5	24.0 ± 0.6c	2.1 ± 0.26	30.7 ± 1.4
IMAGE:475889 5′ R	PBX2 homeodomain protein	B	AF020198	146 ± 16	24.1 ± 1.2b	2.0 ± 0.16	42.5 ± 1.0
Image 518683 M	human siah binding protein 1 (SiahBP1)/FUSE-binding protein (FBP) 4.8e-100	B	U51586	447 ± 84	23.5 ± 1.2a	2.1 ± 0.4	42.0 ± 1.5
PRI_100748 R	transcriptional regulator RPD3 homolog, R.norvegicus histone deacetylase 2 (Hdac2) e-137	C	U31758	125 ± 20	26.3 ± 0.7c	2.0 ± 0.09	42.8 ± 1.8
UI-R-C0-hs-g-07-0-UI R	signalase (eukaryote), microsomal signal peptidase 18kd subunit	D	R11105	180 ± 11	24.9 ± 0.7b	2.0 ± 0.11	42.2 ± 1.4
Image 407454 M	Human putative ubiquitin C-terminal hydrolase (UHX1)	D	U44839	555 ± 52	21.5 ± 0.5a	1.9 ± 0.14	39.4 ± 1.2
Image 2158975 M	H. sapiens UEV1As (UBE2V) conjugating enzyme, alternatively spliced.	D	U97281	649 ± 115	21.6 ± 0.9c	1.9 ± 0.09	39.7 ± 2.6
UI-R-E0-cq-c-04-0-UI R	H. sapiens mRNA for ubiquitin-conjugating enzyme UbcH7	D	AJ000519	171 ± 10	24.2 ± 0.8b	1.9 ± 0.31	40.6 ± 1.2
PRI100836 R	rat proteasomes C2 component e-138	E	M29859	114 ± 13	23.1 ± 0.9c	2.5 ± 0.41	39.8 ± 1.0
UI-R-C2-my-c-11-0-UI 3′ R	proteasome alpha 6 subunit	E	X59417	280 ± 58	23.8 ± 1.1a	1.9 ± 0.19	41.4 ± 1.0
Image 1096884 M	ECE-2 Related 5′ sequence, endothelin converting enzyme-2	F	AA871868	51 ± 7	25.8 ± 1.2b	1.6 ± 0.04	28.6 ± 1.5
PRI_87509 R	R. norvegicus cathepsin K 0.0	E	AF010306	43 ± 3	24.6 ± 0.9a	1.7 ± 0.18	28.1 ± 1.6
PRI_100757 R	rat ATP synthase subunit d. 4.0e-103	G	D13120	561 ± 71	24.0 ± 0.5a	2.7 ± 0.33	42.0 ± 0.9
PRI_105941 R	rat ATP synthase subunit d. 1.7e-103	G	D13120	606 ± 37	24.0 ± 0.5b	2.7 ± 0.33	42.3 ± 1.2
PRI_80025 R	rat ATP synthase subunit d. 1.7e-102	G	D13120	647 ± 69	24.0 ± 0.5b	2.7 ± 0.33	42.5 ± 1.1
PRI_109000 R	R. norvegicus (clone 71) triosephosphate isomerase 6.8e-82	H	L36250	460 ± 115	24.5 ± 0.7a	3.5 ± 0.33	41.6 ± 0.5
PRI_113284 R	rat mitochondrial proton/phosphate symporter 2.7e-174	I	M23984	909 ± 128	25.3 ± 1.0b	2.2. ± 0.07	42.9 ± 1.3
UI-R-A0-aw-e-04-0-UI R	human heparin-binding vascular endothelial growth factor (VEGF)	J	M32977	38 ± 8	23.4 ± 0.8a	1.8 ± 0.25	31.5 ± 1.4
Image 850418 M	Mouse G alpha 11 (G-protein) subunit	K	M55411	33 ± 4	24.4 ± 1.2a	1.8 ± 0.22	30.9 ± 2.0
UI-R-Y0-acc-e-12-0-UI R	M. musculus G protein-coupled receptor GPCR8 (Orphan)	K	U46923	210 ± 12	24.6 ± 0.8b	2.6 ± 0.35	43.6 ± 1.9
UI-R-A1-dq-e-02-0-UI R	cdc42	K	U37720	589 ± 61	23.5 ± 0.9a	2.1 ± 0.43	40.2 ± 2.4
UI-R-E0-da-b-11-0-UI R	human ras-related C3 botulinum toxin substrate (rac)	K	M29870	340 ± 35	24.8 ± 1.3b	2.7 ± 0.51	44.3 ± 2.4
PRI_82462 R	Rat annexin V, lipocortin 5 7.7e-110	L	D42137	282 ± 26	23.0 ± 0.7c	2.3 ± 0.19	41.0 ± 0.9
Image 1480899 M	intercellular adhesion molecule (ICAM-1)	M	M90551	52 ± 5	25.4 ± 0.8b	3.4 ± 0.98	27.3 ± 0.6
PRI_101143 R	sequence 4 from patent US 5527884/Rat allograft inflammatory factor 1 (Aif)/ ionized calcium binding adapter molecule 1 (iba1)/microglial response factor MRF-1 3e-31	N	I22424/NM_017196 D82069/AB000818	51 ± 3	23.0 ± 1.1c	3.2 ± 0.98	28.5 ± 1.1
PRI_82516 R	rat 70 kd heat-shock-like protien 0.0	O	M11942	2484 ± 239	23.8 ± 1.1a	2.2 ± 0.52	39.8 ± 1.8
UI-R-A0-bn-g-02-0-UI R	3′ similar to rat leukotriene A4 hydrolase	P	J02959	164 ± 17	24.2 ± 0.8a	2.4 ± 0.33	41.1 ± 1.8
PRI_95416 R	rat non-muscle myosin alkali light chain e-127	Q	S77858	1095 ± 161	26.3 ± 0.7b	2.3 ± 0.24	42.8 ± 1.3
PRI_95418 R	rat non-muscle myosin alkali light chain 1e-89	Q	S77858	1069 ± 103	26.6 ± 0.8b	2.4 ± 0.32	43.0 ± 1.1
PRI_100845 R	R. norvegicus pervin/advillin 0.0	Q	AF099929	90 ± 8	24.0 ± 1.2a	2.3 ± 0.31	39.0 ± 2.2
UI-R-Y0-acq-c-06-0-UI R	human ubiquitin-like protein (GdX). Similar to hormonogenic site of thyroglobulin	R	J03589	63 ± 4	21.4 ± 0.6c	1.7 ± 0.07	38.2 ± 0.6

(*continued*)

Table 1. Continued.

Clone ID[a]	Gene I.D./Description[b]	Functional Group[c]	GenBank Accession No.	Mean ± SEM Intensity[d]	Mean±SEM Period Estimate[e]	Mean Amplitude[f]	Phase Estimate (h)[g]
UI-R-E0-ct-g-08-0-UI R	H. sapiens ubiquitin-like protein e-173	R	AF095740/D23662	282 ± 29	23.3 ± 1.0b	2.2 ± 0.26	39.4 ± 0.3
PRI_101142 R	H. sapiens hypothetical protein MGC3222 0.0	R	NM_024334	146 ± 13	25.3 ± 0.9b	2.7 ± 0.5	42.1 ± 1.1
PRI_108611 R	mouse, sim. to putative mitochondrial outer membrane protein import receptor 1e-76	R	BC005801	131 ± 10	24.0 ± 1.1a	2.1 ± 30	41.9 ± 1.7
PRI_81887 R	novel	R		47 ± 6	22.7 ± 1.4b	1.9 ± 0.3	30.4 ± .0

[a] Identity of clone cDNA spotted on microarray, including species (rat, R; mouse, M).

[b] Gene identity and BLAST E value are based on the most up-to-date public GenBank BLAST search of sequence information.

[c] Function assigned based on literature searches and according to classification scheme utilized by the Expressed Gene Anatomy Database (EGAD, http://www.tigr.org/docs/tigr-scripts/egad_scripts/role_report.spl) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.com). A, gene/protein expression: RNA synthesis: transcription factor: clock gene; B, gene/protein expression: RNA synthesis: transcription factor; C, gene/protein expression: RNA synthesis: transcription regulation; D, protein synthesis: posttranslational and modification/targeting; E, protein synthesis: posttranslational and modification/targeting: protein turnover; F, protein synthesis: protein turnover: metalloprotease; G, metabolism: energy/TCA cycle: H + transporting, vacuolar; H, metabolism: sugar/glycolysis; I, metabolism: transport; J, cell signaling/cell communication: hormone/growth factors; K, cell signaling/cell communication: intracellular transducer; L, cell signaling/cell communication: intracellular transducer/apoptosis; M, cell/organism defense: immunoglobulin superfamily: inflammatory response; N, cell/organism defense: immunology; O, chaperone/cell/organism defense: homeostasis; P, cell/organism defense: inflammatory response; Q, cell structure/motility: cytoskeletal; R, unknown.

[d] Array fluorescence intensity values calculated from data from five individual experiments. Note mean ± SD fluorescence value for all cDNA probe sets on arrays was 161 ± 456, range 11 to 7573.

[e] Mean period estimates calculated from data from CORRCOS (n = 3 to 8). a, rhythmic and in phase in all four experiments; b, rhythmic and in phase in three experiments and suboptimal rhythm with 20–28 hr period and in phase in one experiment; c, not rhythmic or rhythmic but out of phase in one experiment—data excluded from the generation of mean profile; d, rhythmic and in phase in all four experiments but two of which have a suboptimal 20–28 hr period.

[f] Maximum peak-to-trough amplitude calculated from data from three to four individual experiments. Calculation does not include data that might pertain to an acute serum induction of expression (values t = 0 hr to t = 4 hr).

[g] Phase estimate of second peak, in hours post serum shock, assigned from mean phase data (n = 3 to 4) calculated by CORRCOS.

two of the few described ccgs in the SCN, *dbp* and *vasopressin* (*AVP*), also precedes *per2* in mouse SCN by ~3–4 hr [11], perhaps suggesting that this phase is the most represented by ccgs in both central and peripheral tissues. The 24 hr and 36 hr phases were represented only by the canonical clock genes *per2* and *bmal1*, respectively. In any case, the functional significances of circadian gene expression should be at the protein level, which, if rhythmic at all, will phase lag the peak in RNA by a period of time reflecting the lag to translation and the stability of the protein. Precedents from *dbp* and *AVP* within the SCN reveal a short lag between peak mRNA and peak protein of up to 2 hr [11–13], whereas canonical clock genes *per1*, *per2* and *cry1* show a longer delay of 4–6 hr [14, 15]. From these limited observations, we might expect to observe peak protein levels 2–6 hr after peak mRNA levels. In this case, the major expression group (with mRNA peaking at 40–44 hr) would yield protein levels peaking around 42 and 46 hr post serum shock (predicted late day/dusk). Taken altogether, these data suggest that a majority of clock-controlled processes are most active at the transition between day and night and that the clock facilitates anticipation of this external environmental event. In vivo, in a nocturnal rodent, such as the rat, this coincides with the onset of the active phase of the 24 hr day, as measured by metabolism and behavior.

Diverse Daily Programs of Gene Expression

The cycling genes identified contribute to a wide range of diverse functions (Table 1), of which regulation of transcription, protein turnover, cell signaling, and cell motility are major functional groups. Seven novel ccgs identified in rat-1 fibroblasts in the current study were also identified in concurrent studies of mouse liver (*Csrp3*, *vegf*, *G protein α 11*, *Ubce7*) and brain hypothalamus (*triose phosphate isomerase*, *Annexin V*, *cdc42*, *Ubce7*), ([16]; C.P. Kyriacou and M.H. Hastings, personal communication). Specific gene groups were common among all three tissues: components of the Ras/MAPK signaling pathway, ubiquitin system, metalloproteases, proteasome complex, genes involved in mitochondrial transport, extracellular adhesion, carbohydrate metabolism, and members of the LIM homeodomain and Annexin/lipocortin gene families. Members of the Hsp70, upstream stimulatory factor transcription factor (*usf*), and promyelocytic leukemia protein transcription factor (*pbx*) families were also common between rat-1 cells and mouse liver. This strongly suggests the role of circadian regulation of certain genes (e.g., *Ubce7*) and the above mentioned gene groups in general cellular function. While our data were being analyzed, a similar study, Grundschober et al. (2001) [17], reported 2% of genes to be cyclically expressed in rat-1 cells, with periodicities between 16 and 52 hr, thus spanning the circadian range and beyond. Although there appeared to be little overlap in the sets of genes examined there and here, the control genes *per2* and *cry1* were identified as rhythmic in both studies and consistent was the diversity of cellular functional groups represented.

Transcriptional Regulation

The single largest group and the most common functional theme to emerge from the rhythmically expressed

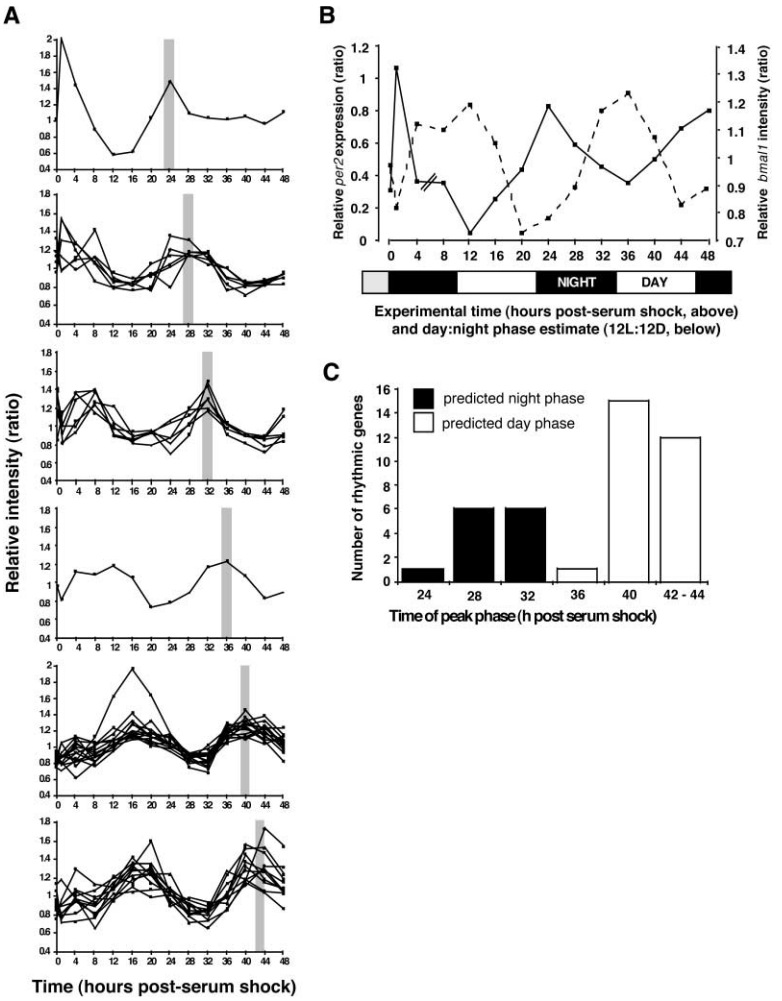

Figure 2. All Phases of the Circadian Cycle Are Represented by the Rhythmic Genes

(A) Six profiles of rhythmic gene expression were identified from the microarray analysis clustered according to phase of second peak, starting at t = 24 hr (top chart) through to t = 42–44 hr (bottom chart). Mean peak phases represent four experiments, estimated using CORRCOS algorithm (see Table 1). Values are mean ratio of time-specific expression relative to medial expression value from the five independent experiments. Gray bars indicate estimated peak phase of the second peak of rhythm.

(B) Based on the peak and nadir phases of the *per2* and *bmal1* rhythms, it is possible to estimate circadian time and subjective night and day in rat-1 cells. Observations of rat and mouse peripheral tissues (heart, kidney, lung, liver, and skeletal muscle) in vivo (e.g., [3, 4]) reveal the *per2* rhythm to peak at ~ZT/CT14-15 (early night) and *bmal1* rhythm to peak at ~ZT23-2 (transition between night and day, dawn; ZT, Zeitgeber time; CT, circadian time). Since expression of *bmal1* and *per2* are appropriately rhythmic and phased with respect to one another and to other rhythmic genes, we can estimate that 24–32 hr post serum shock represents night phase and 36–44 hr, day phase. Values are mean ratio of time-specific expression relative to medial expression value from two experiments (*per2*, RT-PCR analysis, solid line) and from five experiments (*bmal1*, microarray analysis, dashed line).

(C) Histogram of the number of rhythmic genes at each of the six circadian phases and their predicted day:night assignment. Twenty-eight genes (68%) were found to peak during predicted subjective day, and 13 (32%) during estimated subjective night. Within these two groups, there was a tendency to peak at the phase toward the middle of the day or night and a phase anticipating onset of dawn or dusk.

genes is that of transcriptional regulators. Nine transcription factors in addition to the three transcription-associated clock genes were found to oscillate with a circadian period (Table 1). Expression of these genes was not all coincident, but, instead, all phases apart from 24 hr and 36 hr were represented by their mean peak expression values. Two of these genes, encoding basic-leucine zipper transcription factor (*maf-g*) and the transcriptional enhancer factor *ETF-related factor-2* (*etf2*), exhibit a combination of an immediate response to serum and a circadian rhythm with a peak phase (PP) of 28 hr, similar to that seen for *per1* and *per2*, suggestive of a possible influence on the core oscillatory mechanism of the circadian clock (Figure 3 and Table 1). Additional details of transcription factors can be found in the Supplementary Material and Table S2.

Chromatin modification by deacetylation of histones can also regulate transcription [18]. *Histone deacetylase 1* was found to oscillate in rat-1 cells with a PP of 40–44 hr (predicted late subjective day/dusk). A correlation has been observed in the SCN between clock resetting by light and light-induced histone phosphorylation [19], highlighting a potential role for chromatin modification in the signaling pathways to the circadian clock. It is also possible that histone regulation by acetylation/deacetylation and phosphorylation are major systems by which the clock might orchestrate differential expression of downstream ccgs.

Protein Turnover

Another major facet of cellular and circadian regulation is protein turnover. Five cycling genes have functions in cellular degradation; three encode components of the ubiquitin system, ubiquitin conjugating enzymes *Ube2V* and *UbcH7*, and *ubiquitin C-terminal hydrolase*; two are components of the proteasome, the *C2 component* and α *6 subunit*. Additionally, two ubiquitin-like proteins of unconfirmed function were also identified as circadianly regulated. All seven genes have similar PPs of ~40 hr in mRNA abundance (predicted mid-late day). Clearly, cellular degradation is an event that is under phase-specific circadian regulation; see Supplementary Material also.

Signaling: The Ras/MAPK Pathway, Cell Movement, and Cell-Cell Interaction

Two genes encoding components of the Ras/MAPK (mitogen-activated protein kinase) signaling pathway, the small Rho like GTPases *rac* and its activator *cdc42*, were identified as oscillating, with peaks in the late subjective

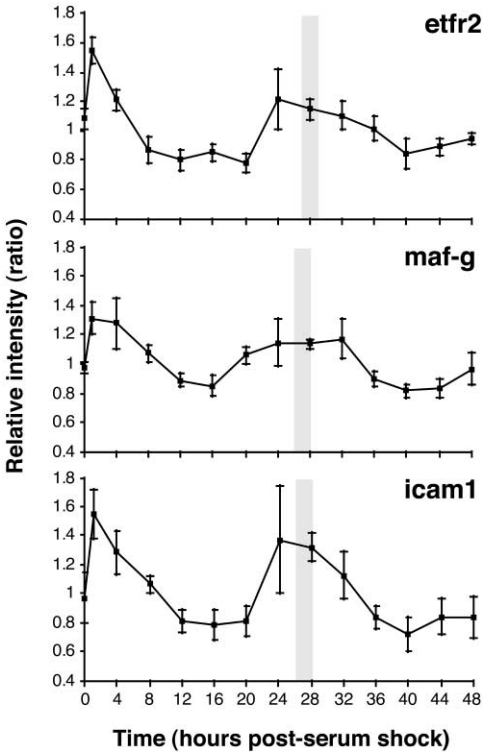

Figure 3. Microarray Analysis Identified Two Transcription Factors and an Intercellular Adhesion Protein that Show a *per1/per2*-like Pattern of Expression

The transcriptional enhancer factor (*ETF-related factor-2*), the basic-leucine zipper transcription factor (*maf-g*), and the intercellular adhesion molecule (*icam1*) show consistent peaks in expression between 1–4 hr and at 24–28 hr. This highlights both an early gene response and a circadian rhythm with a similar phase to that of the canonical clock genes *per1* and *per2*. Values are mean ± SEM ratio of time-specific expression relative to medial expression value from the five independent experiments (n = 3 to 5 each time point). Gray bars indicate CORRCOS estimated phase of the second peak of rhythm.

day (40–44 hr). The Ras/MAPK signaling pathway has been linked to both input to and output (ccgs) from the circadian oscillator. Ras/MAPK signaling is implicated in light-induced phase shifting of the SCN clock [20], in inducing rhythmicity in fibroblasts [21], and in circadian output in *Drosophila* [22, 23]. Indeed, the concurrent study of Akhtar et al. [16] of mouse liver also identified ccg expression of components of the Ras/MAPK pathway. Light during subjective day is unable to trigger the MAPK pathway in the SCN, nor phase shift the clock [20]. That is, light input is gated [24], and since both *cdc42* and *rac* expression profiles peak late in the day, a rhythm in components of the Ras pathway could enhance the sensitivity of the cell to extracellular changes at the transition from light to dark.

The activated Rho GTPases Rac and Cdc42 also regulate integrins, cell surface adhesion molecules, via effects on the actin cytoskeleton; and the gene product of *intercellular adhesion molecule 1* (*icam1*, PP 28 hr) interacts with integrins in regulation of cellular adhesion to the extracellular matrix [25]. Rho and Ras GTPases are pivotal regulators of cellular migration, through modifications of the actin cytoskeleton; movement is effected in part by myosin alkali light chain, whose mRNA is also circadianly regulated in rat-1 cells (PP 43 hr). Another related GTPase, Ras acting through MAPK, leads to phosphorylation of myosin light chain kinase, which in turn phosphorylates myosin, causing increased contraction of actin-myosin filaments [26]. *Advillin*, which is rhythmically expressed (PP 39 hr), shares 59% amino acid sequence homology with the actin binding protein Gelsolin, which is a downstream effector of Rac signaling [27]. The finding that *cdc42*, *rac*, *myosin alkali light chain*, and *advillin* mRNA all peak at a similar phase highlights a possible gating of migration in the fibroblasts. Genes encoding VEGF and Annexin V are also circadianly regulated: VEGF binds to its extracellular receptor VEGFR-2 to promote cell proliferation, migration, and permeability, and Annexin V interacts directly with the intracellular domain of the VEGFR-2 [28].

Circadian regulation of *icam1* (*CD54*) also bears significance in the context of another rhythmic adhesion protein, polysialylated neural cell adhesion molecule (PSA-NCAM, CD56), which peaks during the subjective day in the hamster SCN (J.D. Glass, personal communication), and has been implicated in circadian rhythm function in the SCN [29]. *icam1* expression is also striking, since it exhibits a combination of an immediate early response to serum and a circadian rhythm with a PP of 28 hr, similar to that seen for *per1* and *per2* (Figure 3 and Table 1, see also Transcriptional Regulation, above). Based on the observations of NCAM in the SCN, we speculate that ICAM1 and cell adhesion processes may have a role in the maintenance of circadian rhythms in fibroblasts. Note that two other cellular adhesion molecules, *Vcam1* and *Glycam1*, were identified as ccgs in mouse liver and hypothalamus, respectively ([16]; C.P. Kyriacou and M.H. Hastings, personal communication), making extracellular adhesion a common theme in different tissues.

Conclusions

The current study has identified a set of genes that are under clock control, and, while some fall into a small number of key functional groups, it is the diversity of functional groups that is perhaps most striking. We used a cell culture system to estimate the significance of circadian regulation of gene expression in these cells and in the organism. Despite sampling only a portion of the genome, the proportion of ccgs we found, ~2%, is quite consistent with other available analyses from whole organisms from a variety of taxa: 2% and 6% in *Arabidopsis* [9, 30], 2% in *Neurospora* (M. Nowrousian, J.J. Loros and J.C. Dunlap, unpublished data), 1%–4% in *Drosophila* [23, 31], and 9% in the mouse liver [16]. While there are small differences in these values due to methodological and analytical differences between studies and between species and tissue, the global similarities are apparent: circadian regulation is a notable but not a common aspect of gene expression. It is difficult to gauge how this proportion of circadian mRNA regulation (1%–9%) will impact regulation at the physiological level. Precedents clearly exist for robust posttranscriptional circadian regulation as in *Neurospora* WC-1 [32], and

circadian regulation of the cellular protein turnover apparatus (see above) provides an additional mechanism by which this could occur.

Supplementary Material

Supplementary Material including additional data, figures, tables, Results, Discussion, and the Experimental Procedures are available at http://images.cellpress.com/supmat/supmatin.htm.

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