April, 2004

Expression of haPer1 and haBmal1 in Syrian Hamsters: Heterogeneity of Transcripts and Oscillations in the Periphery

Eric L Bittman, *University of Massachusetts - Amherst*
Yanhong Tong
Hongnian Guo
Judy McKinley Brewer
Alexamder S Bois

Expression of haPer1 and haBmal1 in Syrian Hamsters: Heterogeneity of Transcripts and Oscillations in the Periphery

Yanhong Tong, Hongnian Guo, Judy McKinley Brewer, Han Lee, Michael N. Lehman and Eric L. Bittman

J Biol Rhythms 2004 19: 113
DOI: 10.1177/0748730403262871

Expression of *haPer1* and *haBmal1* in Syrian Hamsters: Heterogeneity of Transcripts and Oscillations in the Periphery

Yanhong Tong,[*] Hongnian Guo,[*] Judy McKinley Brewer,[*]
Han Lee,[†] Michael N. Lehman,[†] and Eric L. Bittman[*,1]

[*]*Center for Neuroendocrine Studies, Program in Neuroscience and Behavior, and Department of Biology, University of Massachusetts, Amherst, MA 01003;*
[†]*Department of Anatomy and Cell Biology, University of Cincinnati, Cincinnati, OH 45267*

Abstract The molecular biology of circadian rhythms has been extensively studied in mice, and the widespread expression of canonical circadian clock genes in peripheral organs is well established in this species. In contrast, much less information about the peripheral expression of *haPer1*, *haPer2*, and *haBmal1* is available in Syrian hamsters despite the fact that this species is widely used for studies of circadian organization and photoperiodic responses. Furthermore, examination of oscillating expression of these genes in mouse testis has generated discrepant results, and little is known about gonadal expression of *haPer1* and *haBmal1* or their environmental control. To address these questions, the authors examined the pattern of *haPer1* and *haBmal1* in heart, kidney, liver, muscle, spleen, and testis of hamsters exposed to DD. In most organs, Northern blots suggested the existence of single transcripts of each of these messenger RNAs (mRNAs). *haPer1* peaked in late subjective day and *haBmal1* during the late subjective night. Closer inspection of SCN and muscle *haPer1*, however, revealed the existence of two major transcripts of similar size, as well as minor transcripts that varied in the 3′–untranslated region. In hamster testis, two *haPer1* transcripts were found, both of which are truncated relative to the corresponding mouse transcript and both of which contain a sequence homologous to intron 18 of *mPer1*. Neither testis transcript contains a nuclear localization signal, and *haPer1* transcripts lacked the putative C-terminal CRY1-binding domain. Furthermore, the testis deviated from the general pattern in that *haPer1* and *haBmal1* both peaked in the subjective night. In situ hybridization revealed that *haPer1*, but not *haBmal1*, showed a heterogeneous distribution among seminiferous tubules. Hamster testis also expresses 2 *haPer2* transcripts, but no circadian variation is evident. In a second experiment, long-term exposure to DD sufficient to induce gonadal regression was found to eliminate circadian oscillations of both testicular *haPer1* transcripts. In contrast, gonadal regression was accompanied by a more robust rhythm of *haBmal1*.

Key words circadian rhythms, *haPer1*, *haBmal1*, canonical clock genes

1. To whom all correspondence should be addressed: Dr. Eric L. Bittman, Department of Biology, 221 Morrill Science Center, University of Massachusetts, Amherst, MA 01003; e-mail: elb@bio.umass.edu.

JOURNAL OF BIOLOGICAL RHYTHMS, Vol. 19 No. 2, April 2004 113-125
DOI: 10.1177/0748730403262871

The molecular basis of circadian rhythms in rodents and other mammals has been clarified in recent years through investigation of the roles of core clock-related genes, including *Per1*, *Per2*, *Clock*, *Bmal1*, and *Cryptochromes 1* and *2* (Reppert and Weaver, 2002). Investigation of the effects of mutations or knockouts of these genes indicates that their expression plays a critical role in determining whether circadian rhythms are expressed and, if so, with what period. Furthermore, the regulation by light of *Per* expression within the central pacemaker, localized to the suprachiasmatic nucleus of the hypothalamus (SCN), may be essential in entrainment. Critical to the model is the existence of transcriptional-translational feedback loops whereby the CLOCK:BMAL1 dimer provides a positive regulation of *Per* and *Cry* expression through interaction with E-box motifs within their promoter regions. This stimulation of transcription is in turn inhibited by PERIOD:CRYPTOCHROME dimers. Essential to this model is the finding that *Per* and *Bmal1* gene expression peaks at opposite phases in the SCN, with the former occurring in the subjective day and the latter in the subjective night.

Not only are these canonical clock genes expressed in the SCN, but their messenger RNAs (mRNAs) and protein products oscillate in peripheral organs of mice and rats. Levels of *Per1* and *Bmal1* expression in liver, muscle, kidney, and other organs are typically found to lag their peak in the SCN by 3 to 9 h (Oishi et al., 1998a, 1998b; Oishi et al., 2000; Zylka et al., 1998). Nevertheless, peak levels of *Per* and *Bmal1* expression in the periphery occur at opposite circadian phases, as they do in the central pacemaker. These findings suggest that the pacemaker regulates oscillations of the expression of canonical clock genes in the periphery. The discovery that electrolytic lesions of the SCN blunt or eliminate oscillations of *rPer2* in rat liver and lung (Sakamoto, et al., 1998), as well as *mPer1* and *mBmal1* in mouse liver (Akhtar et al., 2002), supports this model. The relationships between the canonical clock genes and their products may differ between the pacemaker and the periphery, however. For example, the *Clock* mutation eliminates *Bmal1* oscillations in the SCN but has only a blunting effect in kidney and heart (Oishi et al., 2000). Furthermore, not all organs show similar patterns of expression of canonical clock genes. The testis is particularly unusual, in that most studies have failed to find oscillations of *mPer1* in this tissue, and it appears that *mPer2* is not expressed there (Zylka et al., 1998; Miyamoto and Sancar, 1999; Morse et al., 2003; Bittman et al., 2003; Alvarez et al., 2003). Furthermore, the possible existence of multiple *Per1* transcripts within and between the SCN and peripheral organs has received little attention.

The Syrian hamster has been widely used in studies of circadian rhythms, and studies of regional differences in *period* gene expression within the SCN of both free-running and light-exposed hamsters have recently provided insight into differences in functional compartments of the central pacemaker (Hamada et al., 2001). In contrast, the patterns of peripheral *Per1* and *Bmal1* expression have not been explored systematically in the Syrian hamster. The presence of *haPer1* mRNA was demonstrated in the kidney and testis of hamsters killed at ZT4 (Yamamoto et al., 2001), but oscillations of *haPer1* or *haBmal1* expression were not explored, nor were differences in transcripts present in particular organs investigated. The availability of the *tau* mutant hamster (Ralph et al., 1990) has proven useful in studies of the role of the SCN as a central pacemaker, and the striking seasonal changes in reproduction in this species (Goldman, 2001) have made it a useful model in which to study the effects of day length and the role of melatonin. Thus, it is desirable to complement our understanding of clock gene expression in the hamster central pacemaker with characterization of oscillations of these gene products in the periphery.

To exploit the advantages of the Syrian hamster as a model for studies of the effects of the *tau* mutation, SCN grafts, and photoperiodic responses on peripheral clock gene expression, we sought to characterize the patterns of *haPer1* and *haBmal1* expression in a variety of peripheral organs in constant darkness. In light of the discrepancies between the results of different groups that have examined gonadal expression of *Period* gene expression in the mouse, we were particularly interested in the pattern of clock gene expression in the testis of this species. Our initial findings indicated that the patterns of *haPer1* and *haBmal1* expression in hamster testis depart from the mouse model in unexpected ways and led us to characterize a novel pattern of expression and cleavage of *haPer1* transcripts in testes, skeletal muscle, and SCN. We were stimulated by these findings to explore the relationship between gonadal regression and expression of *haPer1* and *haBmal1* in the testis of this photoperiodic species.

METHODS

Animals

In the first experiment, adult male Syrian hamsters ($n = 20$) were maintained in cages equipped with running wheels for ease of assessment of circadian phase. Animals were released from 14L:10D into constant darkness (DD) for a period of 3 weeks. Hamsters were killed in groups of 5 by rapid decapitation at CT3, CT9, CT15, or CT21. Brains and peripheral organs were immediately removed, frozen on dry ice, and stored at –80 °C.

In a second experiment, we examined effects of photoperiod and gonadal regression on gene expression in testis. To determine whether gonadal status affects *haPer1* and/or *haBmal1* expression, 20 hamsters were group housed in DD for 11 to 13 weeks. For the last 3 to 5 days, they were placed in individual cages equipped with running wheels for assessment of circadian phase. Another group of 20 hamsters remained in 14L:10D until 3 to 5 days prior to sacrifice, at which time they were transferred to individual running wheel cages in DD. Groups of 5 animals from each treatment were rapidly decapitated in darkness at CT3, CT9, CT15, or CT21. Testes and other organs were rapidly removed, frozen on dry ice, and stored at –80 °C. RNA was extracted and analyzed by Northern blotting as described below.

Northern Blots

Total RNA was extracted from peripheral tissues using guanidinium isothiocyanate (Ultraspec reagent, Biotex Labs, Friendswood, TX). Approximately 10 µg of total RNA was run on 1% agarose formaldehyde gels. RNA was blotted onto a Zeta-Probe GT membrane (Bio-Rad Inc) and hybridized to cDNA probes labeled to a specific activity of 2×10^6 cpm/mL by random priming (Stratagene Prime-It II kit). The following probes were used: *haPer1* (homologous to *mPer1* nt 950-1586, accession number AF022992, in SK(+) vector, digested with *Hind*III and *Spe*I, provided by Dr. Toshi Hamada, Columbia University), *haBmal1* (homologous to *mBmal1*, nt 300-1770 of accession number AF07917, in pCDNA3 vector, digested with *BamH*I and *Kpn*I), and rat GAPDH (about 1.25 kb in pIBI30 vector, digested with *Pst*I) (Fort et al., 1985). Blots were hybridized using ULTRAhyb hybridization buffer (Ambion, Inc.) following the manufacturer's protocol and exposed at –80 °C to Kodak BioMax MS film in a cassette containing two intensifying screens. Quantitative densitometry was used to determine the abundance of *haPer1*, *haBmal1*, and *GAPDH*. Individual blots were repeatedly probed and stripped so that the abundance of transcripts of clock-related genes could be expressed relative to *GAPDH* on the same blot. Ratios between each transcript and *GAPDH* were calculated for normalization purposes. Samples from individual hamsters were run in separate lanes, and all time points were run on the same gel.

In Situ Hybridization

To verify the pattern of testicular *haPer1* expression and to resolve the distribution of *Per1* mRNA between compartments and among seminiferous tubules, frozen cryostat sections (11 µm thick) were prepared from 8 hamsters sacrificed at CT3, CT9, CT15, or CT21 on the 3rd day of DD. Frozen sections were mounted onto slides and stored at –80 °C until the time of hybridization, at which point they were air-dried and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 30 min. Slides were hybridized as previously described (Bittman et al., 2003), except that salmon sperm DNA was omitted from the hybridization buffer and a 4×10^4 cpm/µL probe was used on each slide. The *haPer1* antisense probe was a 637-bp segment homologous to nt 950-2586 of *mPer1* (AF02292), including exons 6 through 12. The *haBmal1* probe was a 284-bp subclone (nt 1-294, AF070917). Sense probe was used as a control to evaluate nonspecific hybridization. After posthybridization washes (2 × 20 min 50% formamide, in 1× SSC (standard sodium citrate) followed by RNase at 50 µg/mL), slides were exposed to film for up to 1 week. Slides were dipped in NTBIII emulsion and exposed for appropriate durations. Following development of emulsion-dipped autoradiograms, slides were counterstained with hematoxylin and eosin and imaged at 200× using a Zeiss Axioskop and NIH Image.

Cloning of *haPer1* Transcripts

Northern blotting revealed the presence of *haPer1* transcripts with a length of about 3.2 kb in each hamster peripheral organ, as well as a second transcript of 2.8 kb in the testis. As a first step in obtaining the sequence of the 5′-end of both *haPer1* transcripts from testis, total RNA was reverse transcribed using random hexamer primers and TaqMan reverse transcription reagents (Applied Biosystems). The product was

amplified using primers based on Genbank data (accession number NM011065) and *mPer1* sequences (see Table 1 for primer sequences and Fig. 2 for their position in the cDNA). Total RNA was reverse transcribed by SuperScript™ II reverse transcriptase, amplified by platinum PCR supermix (Invitrogen), and cloned into a pGEM T-easy vector (Promega Corp.). The 3′- and 5′-RACE were performed to complete the sequence of each *haPer1* transcript. Then, 100 ng total RNA was reverse transcribed using primer AP for 3′ RACE. First-strand cDNA was then amplified sequentially, first using primer pair AUAP and F1550 and then with primer pair AUAP and F1700. The second product was cloned into the pGEM T-easy vector. To sequence the 5′-end of each testis *haPer1* transcript, 5′ RACE was performed following the manufacturer's protocol (5′-RACE kit, Invitrogen) using nested primers R1200 and R880. Primer pair F280 and R1900 were used for overlap cloning. All of the clones were sequenced at either the University of California (Davis) or the University of Massachusetts (Amherst) facilities. The CLUSTALW program for multiple-sequence alignment (Biology WorkBench) (Thompson et al., 1994) was used to compare transcripts.

To clone *haPer1* from hamster skeletal muscle and SCN, similar protocols were performed on total RNA from each of these tissues, taken from an adult male that was sacrificed by rapid decapitation at ZT3 of a 14L:10D cycle. The 3′-RACE was performed with the primers AP, AUAP, F1550, and F2200. Then, 100 ng total RNA was reverse transcribed with AP. The product was first amplified with primer pair F1550/AUAP and then amplified with primer pair F2200/AUAP (F1550 and F2200 are nested primers). The second-run PCR amplification product was cloned into the pGEM T-easy vector and sequenced. The nested primers for 5′-RACE were R380 and R280. Primer pairs F1550/R2350 and F1550/R2660 were used for overlap cloning for different transcripts.

Table 1. PCR primers used in cloning *haPer1* transcripts

	Primer Sequence
F280	5′ TGGAATTCACTGAGAGCAGCAAGAGTACAA 3′
R1900	5′ AGGAAGCTTTCCAGGCAGTTGATCTGCTGG 3′
AP	5′GGCCACGCGTCGACTAGTACT$_{17}$3′
AUAP	5′GGCCACGCGTCGACTAGTAC3′
F1550	5′GTGGGATTCGCCCTCTGATGTCTCCTGGTC3′
F1700	5′ATTGAGTCCCGGGCCAAGCCTCCACCT3′
R1080	5′TCTTGTCAGGAGGGATGCG3′
R880	5′GGTGAATTCCTTGAGGCCTGAACCTGCAGA3′
F2220	5′AGTCGGACATCATCATGATGGAGGACCTGC 3′
R380	5′TGAGAATTCACTACTGCAGCCACTGGTAGA3′
R280	5′TTGTACTCTTGCTGCTCTCAGTGG3′
R2350	5′GACAGCACAGCCTTGGTCAGA3′
R2600	5′GCCGAATTCCAGAAGAGGGCACAGGTGAAG3′

Statistical Analysis

The Kruskal-Wallis test was used to evaluate Northern blot results to assess effects of time of day on mean values of *haPer1* and *haBmal1* (both normalized to GAPDH), using samples from 5 animals per circadian time point. ANOVA was used to evaluate effects of photoperiod and time of day on *haPer1* and *haBmal1* in the second experiment. Outcomes were considered statistically significant at $p < 0.05$. To analyze in situ autoradiograms, the NIH Image program was used to estimate mRNA abundance by measurement of the mean integrated density of silver grains over 100 randomly selected seminiferous tubules from each hamster sacrificed at each circadian time. The Kolmogorov-Smirnoff test (McCabe et al., 1989; Bittman et al., 2003) was used to evaluate effects of time of day on the distribution of autoradiographic grain densities over seminiferous tubules.

RESULTS

A single *haPer1* band of about 3.2 kb was found in Northern blots prepared from RNA extracts of liver, heart, kidney, and skeletal muscle of hamsters maintained in DD for 3 weeks prior to sacrifice. Peak expression was consistently found in each of these organs at CT9 (Fig. 1). Statistical analysis revealed significant effects of time of day on the abundance of *haPer1* (relative to GAPDH) in hamster heart ($p < 0.01$), kidney ($p < 0.005$), and skeletal muscle ($p < 0.05$, Kruskal-Wallis test). A similar trend was evident in liver but did not attain statistical significance ($p = 0.12$). A single *haBmal1* transcript of about 2.8 kb was also detected in these tissues, with peak abundance at CT21 (180° out of phase with *haPer1*). The relative abundance of *haBmal1* varied significantly with time of day in kidney, heart, and liver ($p < 0.005$, Kruskal-Wallis test). A similar trend in skeletal muscle was not statistically significant ($p = 0.14$). In spleen, we detected *haBmal1* transcript of similar size and temporal pattern of expression, which peaked at CT3 ($p = 0.03$). We were unable to resolve a clear *haPer1* band in spleen.

The pattern of *haPer1* expression in testis differed from that in other organs in 2 striking ways. First, 2

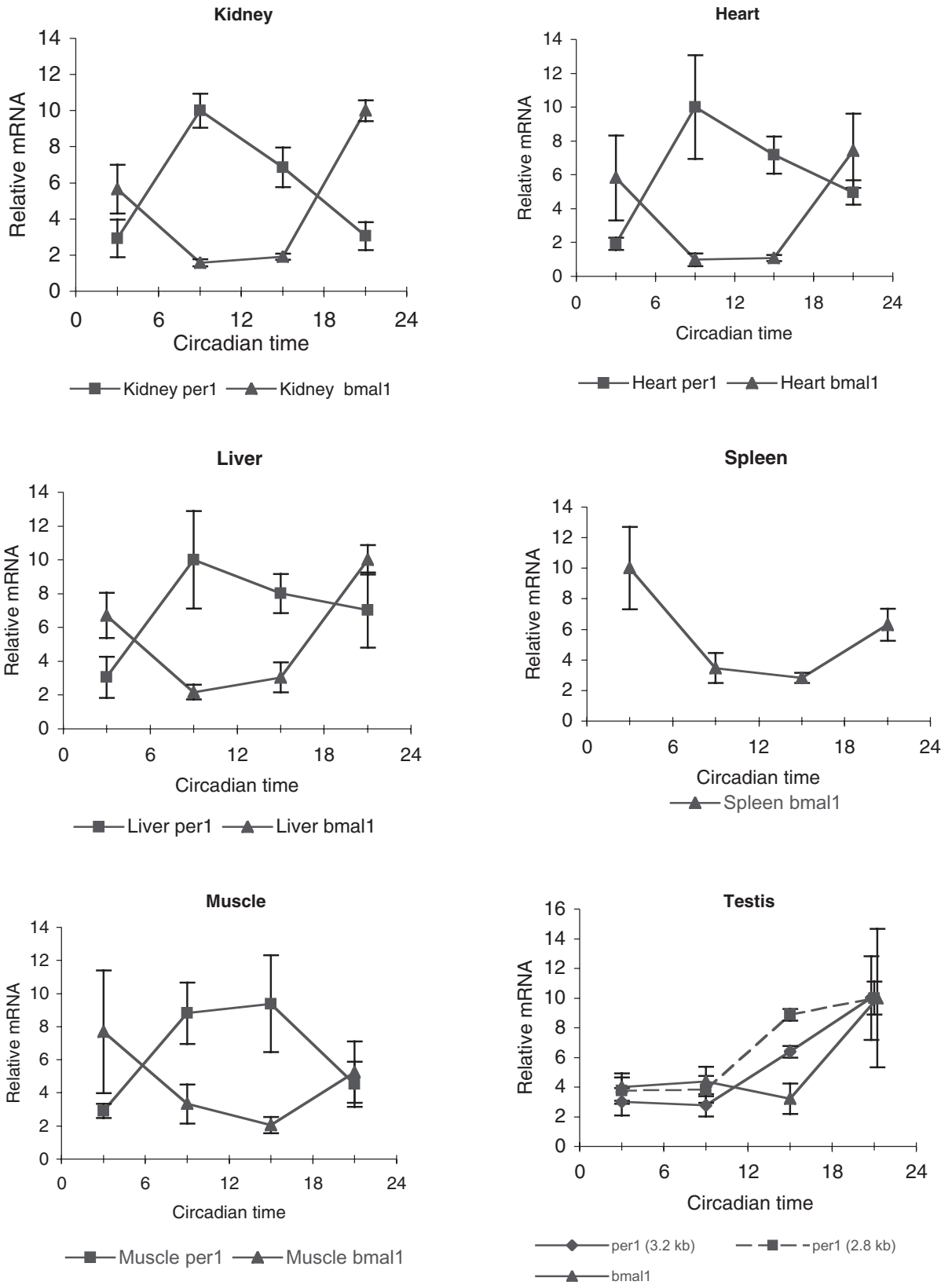

Figure 1. Abundance of *haPer1* and *haBmal1* messenger RNAs (mRNAs) (relative to *GAPDH*) in peripheral organs of hamsters maintained in DD for 3 weeks prior to sacrifice. Animals were sacrificed at CT3, CT9, CT15, or CT21, as defined by locomotor activity records, and RNA was quantified by Northern blotting. Note that 2 *haPer1* transcripts are found in testis and that they peak in phase with *haBmal1* during subjective night. Each point represents mean ± SEM of 4 to 5 animals.

transcripts of about 2.8 kb (testes short transcript) and 3.2 kb (testes long transcript) were consistently observed. Northern blots indicated that the relative abundance of both transcripts varied significantly with time of day ($p < 0.03$ and 0.003, respectively; Fig. 1). Second, in contrast to the other organs we studied, both testicular *haPer1* transcripts peaked during the late subjective night. In situ hybridization revealed that *haPer1* mRNA is confined to seminiferous tubules (Fig. 2). The labeling showed a heterogeneous distribution: at each CT, some tubules but not others are labeled above background (as defined by hybridization using sense control probe). Statistical evaluation of autoradiograms confirmed the results of Northern blotting: analysis of the distribution of labeling using the Kolmogorov-Smirnov test showed that significantly fewer tubules were heavily labeled at CT3 than at CT9, CT15, or CT21 ($p < 0.001$ in each comparison; Fig. 2). Although Northern blots indicated that the abundance of *haBmal1* in testis also rose during subjective night, in situ hybridization did not indicate a significant effect of time of day on expression of this gene in hamsters maintained in DD. The *haBmal1* autoradiographic grain distribution pattern was more homogeneous among seminiferous tubules than that of *haPer1* at all times of day.

These unexpected findings led us to examine *haPer2* and *haClock* expression in muscle and testis. The results indicate that *haPer2* is expressed in both muscle and testis of hamsters. Northern blots indicate the existence of two major transcripts of approximately 4.3 and 7.9 kb. Neither of the *haPer2* transcripts, nor the *haClock* transcript, showed significant variation with time of day (data not shown).

To test the hypothesis that the two *haPer1* transcripts in testis represent alternative splice variants and to examine differences between transcripts in different organs, we performed reverse transcriptase PCR (RT-PCR). The sequence of the testis transcripts is schematically illustrated in Figure 3. The first 2.45 kb of the coding sequence of each testis transcript is 92% homologous to the corresponding portion of *mPer1* (accession NM011065). The 3′-terminal 300 nt of each testis transcript contains more than 87% homology to intron 18 of *mPer1* (nt 12130 to 12410 of accession AB030818), with the longer transcript containing a longer sequence homologous to this mouse intron. Both testis *haPer1* transcripts include the coding sequences of both PAS domains and the putative CK1ε binding domain (Vielhaber et al., 2000). However, neither testis *haPer1* transcript contained the coding sequences of consensus nuclear localization sequence (NLS) or its masking domain described by Vielhaber et al. (2000). The putative CRY1 C-terminal domain described by Eide et al. (2002) was also absent. The 3′-end of both transcripts contains an AAUAAA short sequence. 5′-RACE experiments on both hamster testes *haPer1* transcripts revealed a 5′–untranslated region (UTR), which is more than 90% homologous to mouse *Per* but is 30 nt longer.

For comparative purposes, we also analyzed RNA extracted from SCN and muscle using RT-PCR, and amplicons were cloned and sequenced. Although the muscle transcript and the long transcript found in testis are approximately the same size in Northern blots, we discovered striking differences in their sequences, which indicate interorgan variation in splicing patterns (Fig. 3). The 5′-end and middle part of *Per1* transcripts isolated from hamster muscle and SCN are very similar to both transcripts from testes. However, there are several isoforms of the 3′-end in both muscle and SCN. Of the two major transcripts isolated from muscle and SCN, one is very similar to testes long transcript, with the first 2.45 kb of the coding sequence homologous to *mPer1* and with the sequence corresponding to intron 18. We refer to this variant as a testes-like transcript. This transcript contains an AAUAAA sequence before the polyA tail. The second transcript is homologous to *mPer1* over the first 2.6 kb of the coding sequence and contains the CK1ε binding domain, NLS, and part of the masking domain. We refer to this as a mouse-like transcript, in that it lacks the sequence corresponding to mouse intron 18. At the 3′-end of the mouse-like transcript, there are exact ARE and 15-LOX-DICE sites. However, there is no AAUAAA short sequence before the polyA tail. None of these transcripts contains the coding sequences of the CRY1 binding domain identified by Eide et al. (2002).

In contrast to SCN and muscle, in which direct cloning of *haPer1* via 3′-RACE repeatedly generated a mouse-like transcript, multiple attempts to generate such a transcript from testis were without success. It is our interpretation that the mouse-like transcript represents at most a minor transcript in testis. Nevertheless, we were able to amplify such a sequence by RT-PCR using an appropriate primer pair (F1550/R2600). Several additional minor transcripts were also isolated from testes, muscle, and SCN; these differed at the 3′-end with extensive truncation around and after the stop codon (Fig. 3). The existence of such variant transcripts may explain why the *haPer1* transcripts

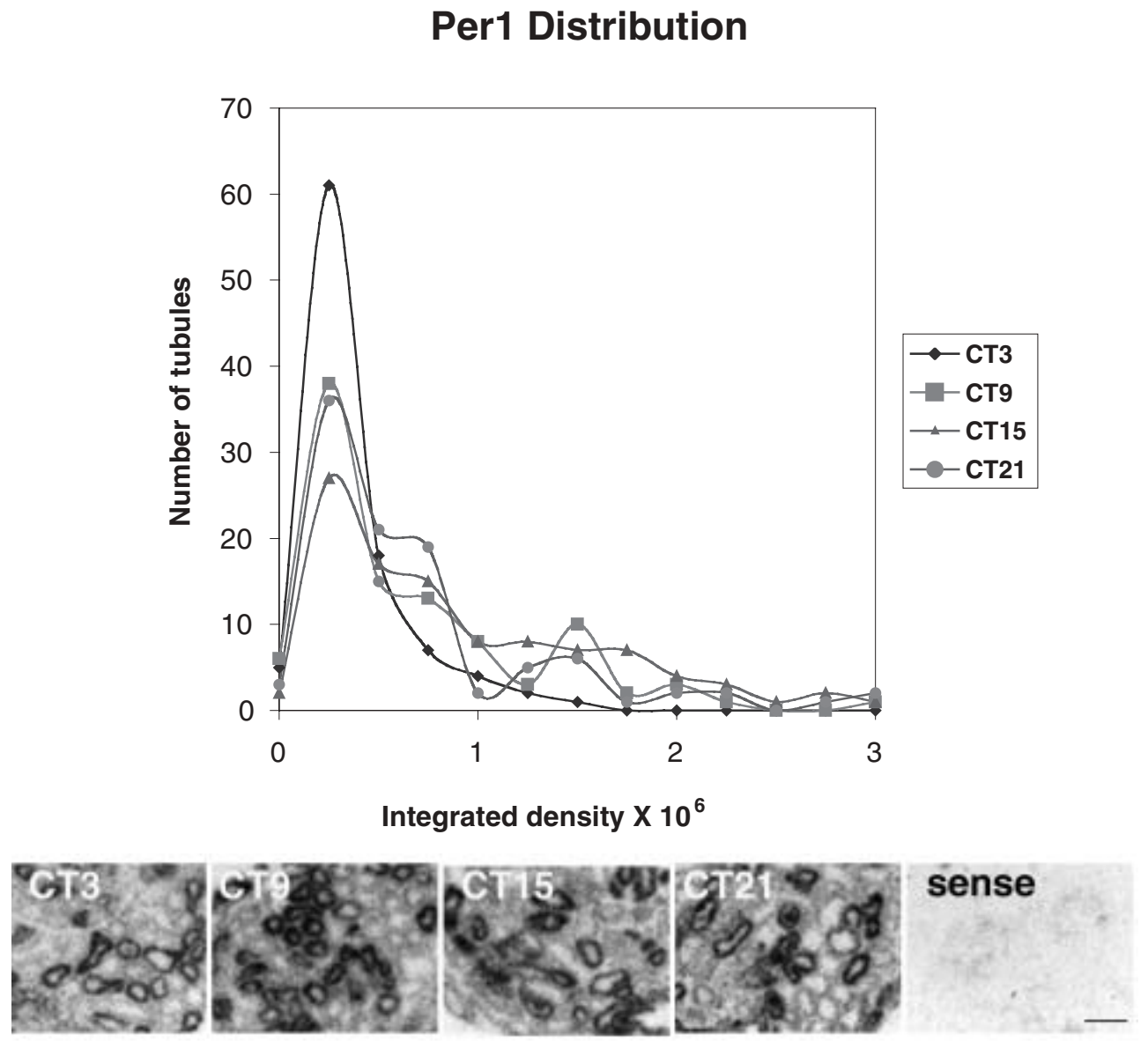

Figure 2. Top, histogram illustrating the distribution of *haPer1* messenger RNA (mRNA) among seminiferous tubules in emulsion autoradiograms prepared after in situ hybridization. Autoradiograms were analyzed using NIH Image to calculate integrated densities reflecting the extent of hybridization over 100 randomly selected tubules at each of four circadian times (CT3, CT9, CT15, CT21). Histograms were evaluated using the Kolmogorov-Smirnov test. At CT3, the distribution of tubule labeling was shifted significantly to the left relative to other times ($p < 0.001$), indicating a nadir of *haPer1* expression. This result confirms the outcome of Northern blot analysis (see Fig. 1). Bottom, representative film autoradiograms of testis collected from hamsters at CT3, CT9, CT15, and CT21 and hybridized to antisense probe. Sense probe control autoradiogram shown at far right. Scale bar, 500 μm.

gave more diffuse bands on our Northern blots. The GenBank accession numbers for the short and long *haPer1* transcripts obtained from testis have been entered as AF543842 and AF543843, respectively. The testis-like SCN transcript has been entered as AY423767 and two mouse-like SCN transcripts as AY423770 and AY423771. Muscle mouse-like and testis-like *haPer1* transcripts are entered in the GenBank as AY423768 and AY423769, respectively.

The testes collected from hamsters maintained in DD for 3 weeks in the foregoing studies were large and appeared grossly functional, but we wondered whether the early stages of gonadal regression may have affected *haPer1* expression and contributed to

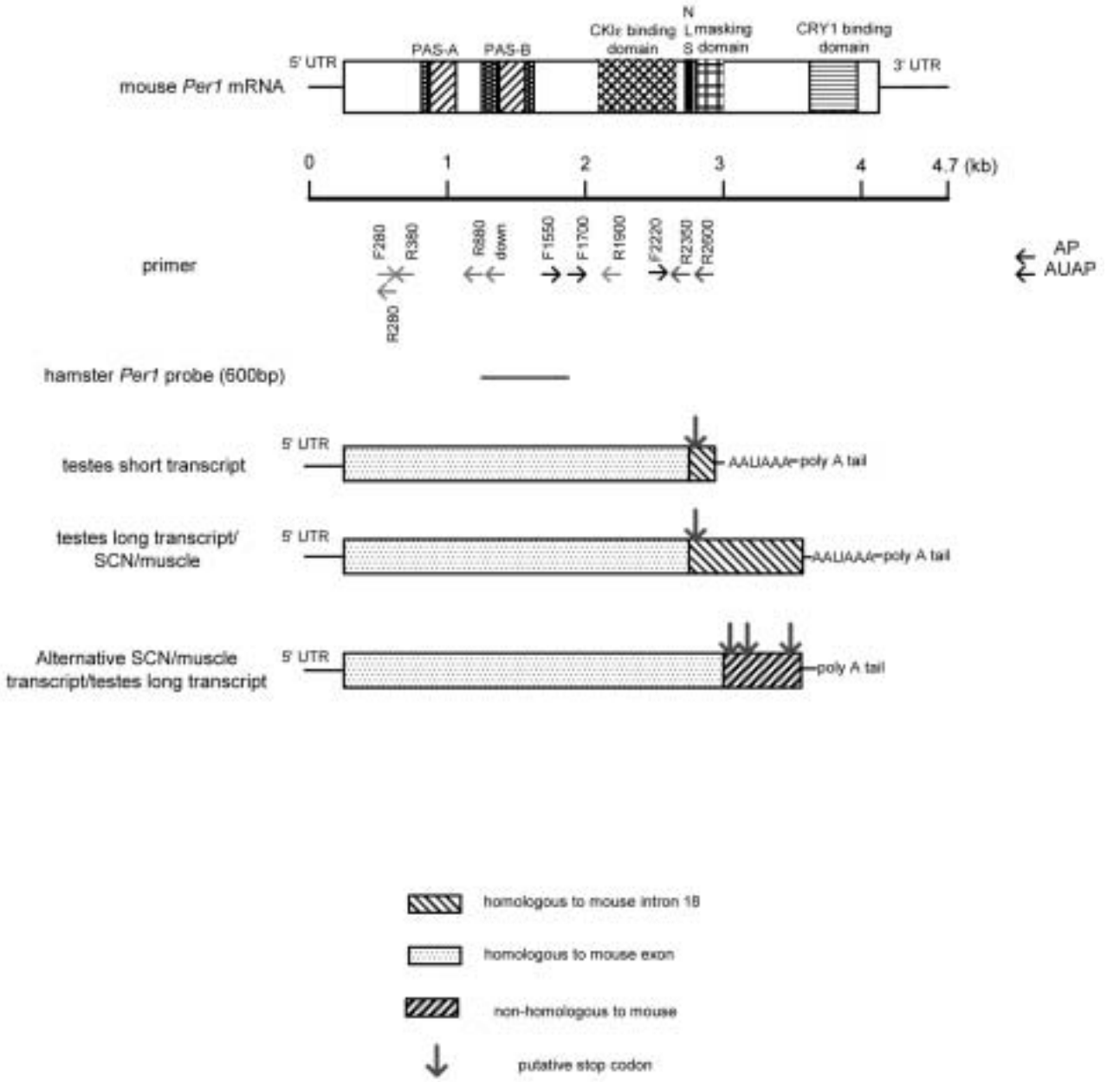

Figure 3. Top, schematic diagram illustrating functional coding domains of mouse *mPer1* mRNA protein and the corresponding nucleotide sequence used to design primers (middle) for amplification and cloning of hamster testis, muscle, and SCN *Per1* transcripts (bottom). All hamster transcripts lack the 3′ sequence corresponding to the mouse CRY1 binding domain. Unlike *haPer1* cloned from muscle and SCN, both the short and the long major transcripts in testis also lack the mouse nuclear localization and masking domains.

some of our unexpected findings. As expected, the testes of hamsters exposed to DD for 11 to 13 weeks prior to sacrifice in the second experiment were much smaller than those of animals sacrificed after only 3 to 5 days of constant darkness (0.40 ± 0.05 g vs. 3.13 ± 0.14 g, respectively; $p < 0.001$). As shown in Figures 4 and 5, photoperiod had profound effects on canonical clock gene expression in hamster testis. Analysis of variance revealed significant effects of circadian time (as determined by phase of locomotor activity) and of photoperiod, as well as a significant interaction between photoperiod and circadian time, on *haPer1* abundance relative to GAPDH (each $p = 0.0001$). Similarly, relative *Bmal1* abundance was significantly

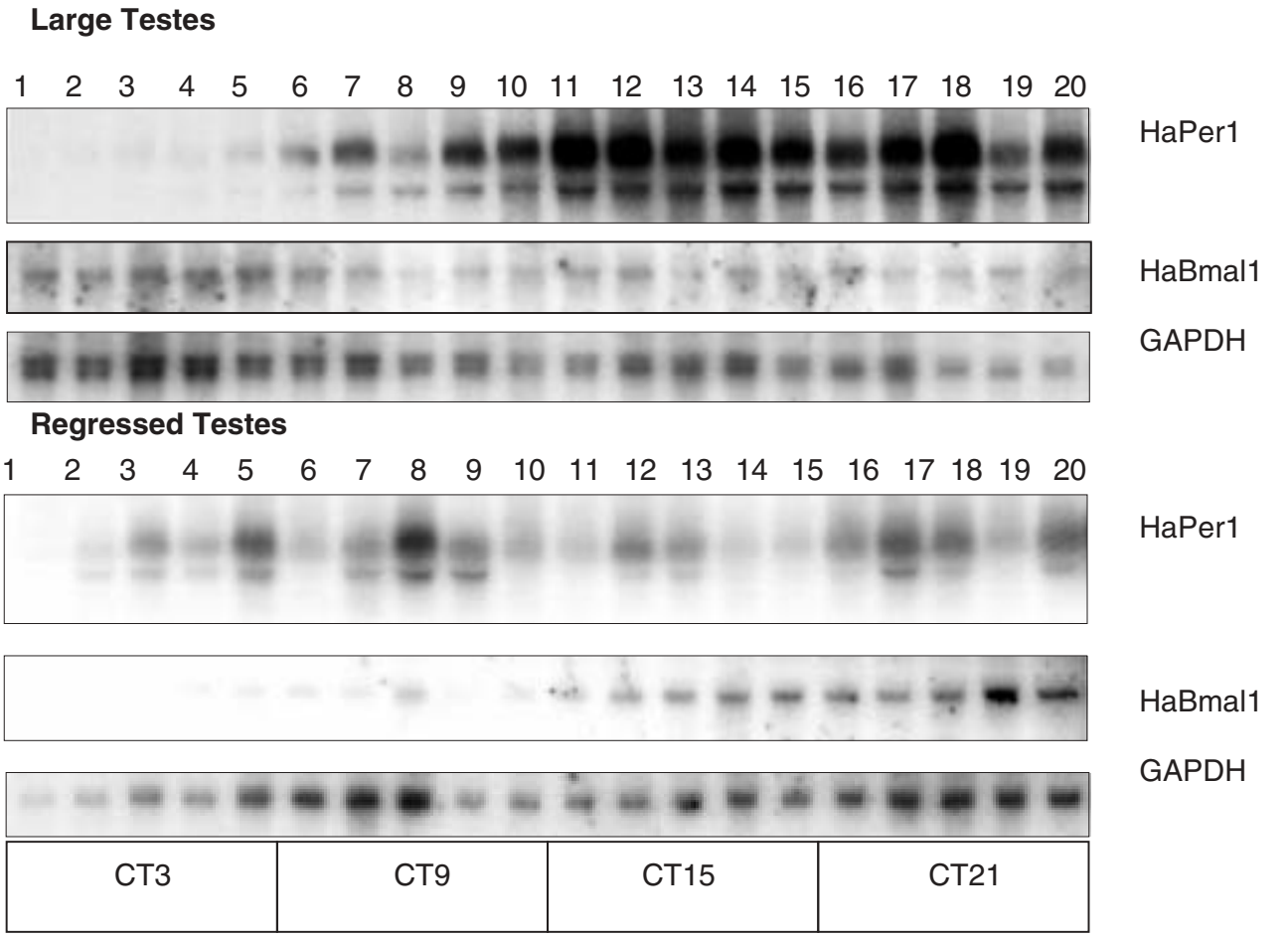

Figure 4. Northern blots illustrating testicular *haPer1*, *haBmal1*, and *GAPDH* in hamsters maintained in long days until transfer to DD either 3 to 5 days (top) or 11 to 13 weeks (bottom) before sacrifice. Blots were sequentially probed and stripped; relevant portions of autoradiograms are shown in each case. Note the presence of 2 major *haPer1* transcripts and reduction in *haPer1* expression in hamsters with regressed testes.

affected by circadian time ($p < 0.001$) and by photoperiod ($p = 0.01$), and a significant interaction occurred between photoperiod and circadian time ($p < 0.01$). In the testes of hamsters acutely transferred from 14L:10D to DD, both the short and long transcripts exhibited the striking rise during the subjective night, which we had previously observed in hamsters exposed to DD for 3 weeks. In contrast, prolonged exposure to DD eliminated the rhythmic pattern of relative *haPer1* expression in regressed testes. The *short* transcript was not found in most regressed testes, although the *long* transcript was consistently present. In contrast, the *haBmal1* rhythm was striking in regressed testes of hamsters maintained chronically in DD, but *Bmal1* expression did not vary with circadian time in animals that had been in 14L:10D until 3 to 5 days prior to sacrifice.

DISCUSSION

The present results establish that peripheral organs of the Syrian hamster, like those of other rodents, generally express *Per1* and *Bmal1* in antiphase and with peaks that lag the SCN. However, the pattern of splicing of *haPer1* transcripts varies between and even within organs; the testis differs from other organs in expressing two *Per1* transcripts, both of which are spliced differently than the forms found in other organs. Particular domains identified in *mPer1*, including the NLS and the CRY1 binding domain, are absent in at least some of the *haPer1* transcripts. Furthermore, *haPer1* abundance peaks during subjective night in the testis, rather than during subjective day as in other organs. Finally, our data indicate that photoperiodic signals that profoundly influence

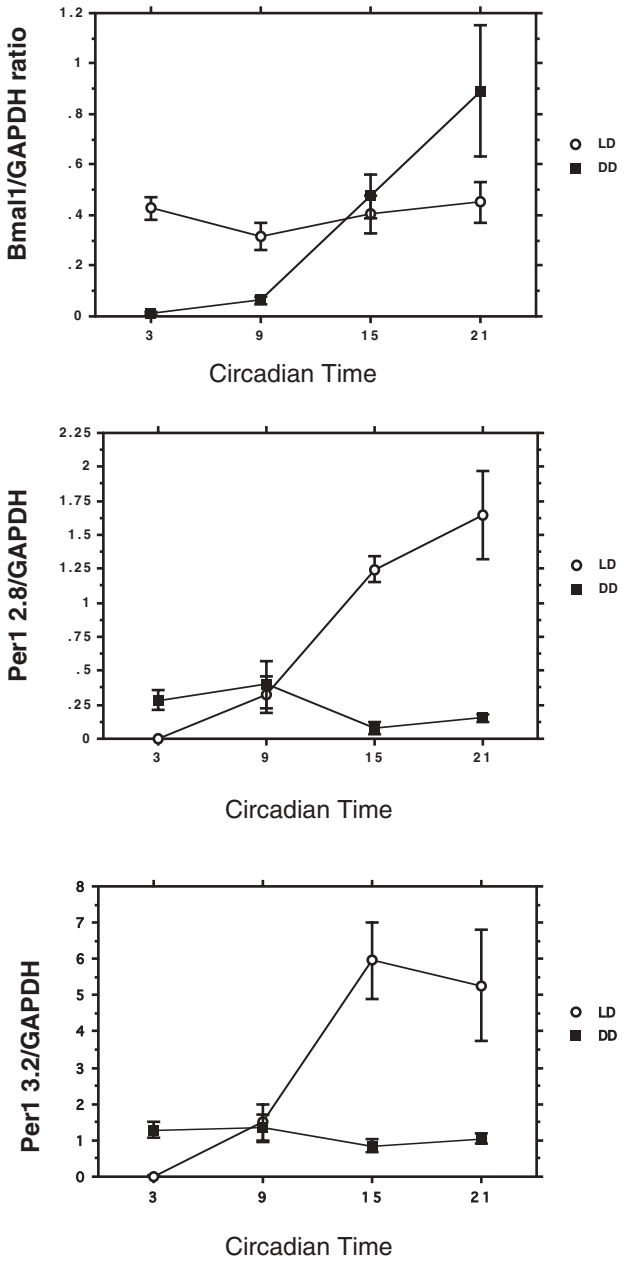

Figure 5. Quantification of Northern blots showing temporal patterns of *haBmal1* transcript (top) and of *haPer1* transcripts (short middle, long bottom), relative to *GAPDH* in testis of hamsters transferred from 14L:10D 3 to 5 days (LD, open circles) or 11 to 13 weeks (DD, filled squares) prior to sacrifice at the indicated circadian times. Each point represents mean ± SEM of 5 hamsters.

gonadal function exert a striking effect on rhythms of canonical clock gene expression in hamster testis.

The rhythmic expression of *Per1* and *Bmal1* in peripheral tissues, including liver, muscle, and kidney, is well established in mice (Albrecht et al., 1997; Sun et al., 1997; Zylka et al., 1998; Oishi et al., 1998a, 1998b; Balsalobre et al., 2000) and rats (Sakamoto et al., 1998). The capacity of serum to induce damping rhythms of *Per1* expression in cultured fibroblasts (Balsalobre et al., 2000; Yagita et al., 2001), as well as *Per1* promoter activity to persist for several cycles in organ culture (Yamazaki et al., 2001), indicates that peripheral tissue contains a damping oscillator. Destruction of the SCN reduces or eliminates peripheral oscillations of *rPer2*, *mPer2*, and *mBmal1* (Sakamoto et al., 1998; Ahktar et al., 2002). Peaks of *Per1* and *Bmal1* expression in peripheral organs occur in late subjective day and late subjective night, respectively. This represents a phase lag relative to their expression in SCN, which is consistent with a master-slave relationship. Although humoral signals appear capable of manipulating the phase of canonical clock gene expression in peripheral tissues (McNamara et al., 2001; Oishi et al., 1998a, 1998b; Nonaka et al., 2001; Balsalobre et al., 2000), the relative importance of endocrine versus neural signals is not well established. The role of the SCN as a master pacemaker is best established in hamsters (Ralph et al., 1990) through use of the *tau* mutant strain, which permits transplantation studies in which a period difference marks the influence of the donor clock. The present findings, which establish and characterize rhythms of *Per1* and *Bmal1* expression in hamster peripheral organs, will be useful in future studies that exploit this model in studying the relationship between pacemaker and slave oscillators.

Studies of *Per1* expression in rodent testis have yielded discrepant results. Although Zylka et al. (1998) concluded from Northern blots that *mPer1* and *mPer3* expression oscillates in mouse testis, these findings have not been replicated by others using Northern blots (Miyamoto and Sancar, 1999), RNase protection assays (Alvarez et al., 2003; Morse et al., 2003), or in situ hybridization (Morse et al., 2003; Bittman et al., 2003) to study this tissue. Western blotting and immunocytochemical analyses show that PER1 protein is expressed in spermatocytes and spermatids and indicate low and temporally invariant levels of *mBmal1* in mouse testis (Morse et al., 2003). The present data, which indicate that *haPer1* is expressed rhythmically in hamster testis, demonstrate that the mouse pattern cannot be generalized to other rodents. The heterogeneity of spermatogenic assemblies along the length of the tubule argues for the importance of a distributional analysis at different circadian times, and such an approach has provided a more sensitive analysis in our mouse studies (Bittman et al., 2003).

The Kolmogorov-Smirnov tests confirmed and extended the results of our Northern blot analyses in the present experiments. Localization of *haPer1* and/or PER1 protein to particular cell types in hamster testis may help to explain apparent species differences and suggest a physiological role of *period* gene expression in testicular function. Syrian hamsters exhibit significant (twofold) daily rhythms of testosterone secretion (Vaughan et al., 1994), whereas the expression of such rhythms in mice varies with strain (Lucas and Eleftheriou, 1980). Despite the fact that *Per1* expression is concentrated in the seminiferous tubules rather than the interstitial tissue, it may be functionally related to androgen production. This suggests that high-amplitude rhythms of *Per1* may be found in Siberian hamsters, which exhibit dramatic circadian rhythms of serum testosterone (Hoffmann and Nieschlag, 1977). Although rhythmicity of *haBmal1* appears less consistent, we observed a peak during the late subjective night in testes collected from hamsters kept in DD for 3 to 11 weeks. Thus, testicular *haPer1* transcripts peak in phase with *haBmal1*, rather than in antiphase as in other organs. This association is surprising in light of the current molecular model in which a CLOCK:BMAL1 dimer promotes *per* gene expression, but PERIOD:CRYPTOCHROME dimers block this excitatory drive. This may reflect a difference between testis and other tissues in the functions of these proteins.

The significance of splice variants of *haPer1* in testis is unknown but also points to physiological differences with mouse and perhaps other species. Of particular interest is the truncation of both transcripts and inclusion of sequences that are found in intron 18 in the mouse. The major *haPer1* transcripts found in testis lack the nuclear localization sequence but contain both PAS domains. This suggests that their protein products may dimerize with other PAS proteins in testis but be less readily translocated to the nucleus. This hypothesis could be tested in immunocytochemical studies. The existence of differentially spliced *hPer1* transcripts was inferred from cloning of a human heart muscle cDNA library (Sun et al., 1997), but the significance of this finding for the operation of transcriptional-translational circadian feedback loops has not been explored. Alternative splicing of the 3′-untranslated region of *Drosophila Per* has been reported and may have a functional role in regulating seasonal responses (Majercak et al., 1999).

The variation of the 3′-UTR of *haPer1* most likely affects the stability, localization, and translational efficiency of mRNA (Kojima et al., 2003). The mouse-like transcript contains an exact ARE sequence, which is believed to regulate mRNA degradation, and a 15-LOX-DICE site, which is believed to regulate the stability and translation of mRNA. The finding of alternative transcripts in SCN is especially provocative. It will be important to determine whether the splicing of the 3′-UTR region varies with circadian phase or is influenced by exposure to light. It remains to be determined whether the unusual circadian pattern of *haPer1* abundance in testis, including the peak during subjective night, is related to its unique splicing in this tissue (i.e., inclusion of sequence that corresponds to mouse intron 18). We can only speculate that the splicing pattern may affect the half-life of the transcripts, which in turn may increase their abundance at CT15 and CT21. Future studies that employ transcript-specific probes will be useful in assessing the relative abundance of various splice variants.

The isoforms of *haPer1*, which we cloned from hamster SCN, muscle, and testis, are expected to lack the C-terminal region identified by Eide et al. (2002) as the site at which mCRY1 interacts with mPER. Studies in transfected cells indicate that this interaction is necessary for CKIε to be translocated to the nucleus. Yet rhythmic phosphorylation of haPER is evident in both central and peripheral tissues of wild-type and *tau* mutant hamsters (Lee et al., 2001). Other kinases may regulate PER phosphorylation, and CKIε may interact with different sites in hamster PER than in mouse PER. Alternative splicing of members of the *Bmal* family has been reported, (Ikeda and Nomura, 1997; Yu et al., 1999; Okano et al., 2001; Schoenhard et al., 2002).

The predominance of *Bmal2* splice variants differs between organs and appears to influence the ability of their protein products to regulate *Per* transcription through interactions with CLOCK (Schoenhard et al., 2002). In contrast to the present findings, these variants affect the N-terminal sequence of *Bmal2*. Although we did not set out to investigate the existence of BMAL splice variants in hamsters, we would not be surprised to find that these exist within and between tissues and that they may be photoperiodically regulated. Further investigation of the consequences and tissue distribution of shortened isoforms of PER1, BMAL1, and other core circadian gene products is clearly warranted.

A physiological role for expression of clock-related genes in the testis is supported by the finding that gonadal regression is correlated with profound changes in levels of *haPer1* and *haBmal1*. Exposure to

short days or constant darkness (as in the present study) induces a precipitous decline in the secretion of gonadotropins and prolactin, which results in the arrest of spermatogenesis and steroidogenesis. Although evidence for hormonal regulation of clock gene expression has been gathered for liver and vascular smooth muscle, it remains to be determined whether pituitary hormones regulate *haPer1* or *haBmal1* in testis. Furthermore, the possible role of testicular innervation remains to be explored. The disappearance of *haPer1* coincident with gonadal regression, however, is consistent with localization in germ cells (Miyamoto and Sancar, 1999; Morse et al., 2003; Alvarez et al., 2003; Bittman et al., 2003). The persistence of the *haBmal1* rhythm in regressed testis is more surprising, however, and suggests that this gene may play a different role or perhaps be expressed in a different cell type in the testis. Our in situ hybridization study in long-day testes supports this speculation in that the pattern of *haBmal1* expression, while localized to seminiferous tubules, was far more uniform than the strikingly patchy pattern of *haPer1* mRNA. Our finding that *haPer2* is expressed in hamster testis, albeit without significant circadian rhythmicity, presents a further contrast with the situation in mice (Zylka et al., 1998). It will also be useful to examine *haPer3*, *haCry1*, and *haCry2* expression patterns to gain a fuller understanding of circadian oscillations in the hamster testis and other peripheral organs. Nevertheless, our findings demonstrate striking species differences among rodents in this regard, as well as a level of photoperiodic control. Both findings expand our understanding of circadian gene expression in the periphery.

ACKNOWLEDGMENTS

This research was supported by NIMH RO1 59166 to ELB. We thank Alison Miller and Lynn Wright for expert animal care. We are especially grateful to Drs. Deborah Good and Lawrence M. Schwartz for advice and assistance with Northern blotting methods.

REFERENCES

Ahktar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, and Kyriacou CP (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 12:540-550.

Albrecht U, Sun ZS, Eichele G, and Lee CC (1997) A differential response of two putative mammalian circadian regulators, *mPer1* and *mPer2*, to light. Cell 91:1055-1064.

Alvarez JD, Chen D, Storer E, and Sehgal A (2003) Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. Biol Reprod 69:81-91.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellndonk C, Reichardt HM, Schutz G, and Schibler U (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289:2344-2347.

Bittman EL, Doherty L, Huang L, and Paroskie A (2003) *Period* gene expression in mouse endocrine tissues. Am J Physiol Regul Integr Comp Physiol 285:R561-R569.

Eide E, Vielhaber EL, Hinz WA, and Virshup DM (2002) The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase 1 epsilon. J Biol Chem 277:17248-17254.

Fort P, Marty L, Piechaczyk M, el Sabrouty S, Dani C, Jeanteur P, and Blanchard JM (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res 13:1431-1442.

Goldman BD (2001) Mammalian photoperiodic system: formal properties and neuroendocrine mechanisms of photoperiodic time measurement. J Biol Rhythms 16:283-301.

Hamada T, LeSauter J, Venuti JM, and Silver R (2001) Expression of *Period* genes: Rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker. J Neurosci 21:7742-7750.

Hoffmann K and Nieschlag E (1977) Circadian rhythm of plasma testosterone in the male Djungarian hamster (*Phodopus sungorus*). Acta Endocrinol 8:193-199.

Ikeda M and Nomura M (1997) CDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. Biochem Biophys Res Commun 233:258-264.

Kojima S, Hirose M, Tokunaga K, Sakaki Y, and Tei H (2003) Structural and functional analysis of 3′ untranslated region of mouse *Period1* mRNA. Biochem Biophys Res Commun 301:1-7.

Lee C, Etchegaray JP, Cagampang FR, Loudon AS, and Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. Cell 107:855-867.

Lucas LA and Eleftheriou BE (1980) Circadian variation in concentrations of testosterone in the plasma of male mice: A difference between BALB/cBy and C57BL/6By inbred strains. J Endocrinol 87:37-46.

Majercak J, Sidote D, Hardin PE, and Edery I (1999) How a circadian clock adapts to seasonal decreases in temperature and day length. Neuron 24:219-230.

McCabe JT, Desharnais RA, and Pfaff DW (1989) Graphical and statistical approaches to data analysis for in situ hybridization. Methods Enzymol 168:822-848.

McNamara P, Seo S, Rudic RD, Sehgal A, Chakravarti D, and FitzGerald GA (2001) Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: A humoral mechanism to reset a peripheral clock. Cell 105:877-889.

Miyamoto and Sancar A (1999) Circadian regulation of cryptochrome genes in the mouse. Mol Brain Res 71:238-243.

Morse D, Cermakian N, Brancorsini S, Parvinen M, and Sassone-Corsi P (2003) No circadian rhythms in testis: *Period1* expression is *Clock* independent and developmentally regulated in the mouse. Mol Endocrinol 17:141-151.

Nonaka H, Emoto N, Ikeda K, Fukuya H, Rohman MS, Raharjo SB, Yagita K, Okamura H, and Yokoyama M (2001) Angiotensin II induces circadian gene expression of clock genes in cultured vascular smooth muscle cells. Circulation 104:1746-1748.

Oishi K, Fukui H, and Ishida N (2000) Rhythmic expression of BMAL1 mRNA is altered in *Clock* mutant mice: Differential regulation in the suprachiasmatic nucleus and peripheral tissues. Biochem Biophys Res Commun 268:164-171.

Oishi K, Sakamoto K, Okada T, Nagase T, and Ishida N (1998a) Antiphase circadian expression between *Bmal1* and period homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. Biochem Biophys Res Commun 253:199-203.

Oishi K, Sakamoto K, Okada T, Nagase T, and Ishida N (1998b) Humoral signals mediate the circadian expression of rat period homologue (*rPer2*) mRNA peripheral tissues. Neurosci Lett 256:117-119.

Okano T, Sasaki M, and Fukada Y (2001) Cloning of mouse BMAL2 and its daily expression profile in the suprachiasmatic nucleus: A remarkable acceleration of *Bmal2* sequence divergence after *Bmal* gene duplication. Neurosci Lett 300:11-114.

Ralph MR, Foster RG, Davis FC, and Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 247:975-978.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935-941.

Sakamoto K, Nagase T, Fukui H, Horikawa K, Okada T, Tanaka H, Sato K, Miyake O, Ohara O, Dado K, et al. (1998) Multisense circadian expression of rat period (*rPer2*) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus of the brain. J Biol Chem 273:27039-27042.

Schoenhard JA, Eren M, Johnson CH, and Vaughan DE (2002) Alternative splicing yields novel BMAL2 variants: Tissue distribution and functional characterization. Am J Physiol Cell Physiol 283:C103-C114.

Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, and Lee CC (1997) RIGUI, a putative mammalian ortholog of the *Drosophila* period gene. Cell 90:1003-1011.

Thompson JD, Higgins DG, and Givson TJ (1994) CLUSATL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

Vaughan MK, Menendez-Pelaez A, Buzzell R, Baughan GM, Little JC, and Reiter RJ (1994) Circadian rhythms in reproduction and thyroid hormones in gonadally regressed male hamsters exposed to natural autumn photoperiod and temperature conditions. Neuroendocrinology 60:96-104.

Vielhaber EL, Eide E, Rivers A, Gao ZH, and Virshup DM (2000) Nuclear entry of the circadian regulator *mPer1* is controlled by mammalian casein kinase 1 epsilon. Mol Cell Biol 20:4888-4899.

Yagita K, Tamanini F, vanderHorst GT, and Okamura H (2001) Molecular mechanisms of the biological clock in cultured fibroblasts. Science 292:278-281.

Yamamoto S, Shigeyoshi Y, Ishida Y, Fukuyama T, Yamaguchi S, Yagita K, Moriya T, Shibata S, Takahina N, and Okamura H (2001) Expression of the *Per1* gene in the hamster: Brain atlas and circadian characteristics in the suprachiasmatic nucleus. J Comp Neurol 430:518-532.

Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, and Tei H (2001) Resetting central and peripheral circadian oscillators in transgenic rats. Science 288:682-685.

Yu W, Ikeda M, Abe H, Honma S, Ebisawa T, Yamauchi T, Honma K, and Nomura M (1999) Characterization of three splice variants and genomic organization of the mouse BMAL1 gene. Biochem Biophys Res Commun 260:760-767.

Zylka MJ, Shearman LP, Weaver DR, and Reppert SM (1998) Three period homologs in mammals: Differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20:1103-1110.