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## Period gene expression in mouse endocrine tissues

Eric L. Bittman, Leo Doherty, Liyue Huang, and Allison Paroskie

Department of Biology, Program in Neuroscience, and Center for Neuroendocrine Studies, University of Massachusetts, Amherst, Massachusetts 01003

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**Bittman, Eric L., Leo Doherty, Liyue Huang, and Allison Paroskie.** *Period* gene expression in mouse endocrine tissues. *Am J Physiol Regul Integr Comp Physiol* 285: R561–R569, 2003. First published May 29, 2003; 10.1152/ajpregu.00783.2002.—Circadian rhythms are generated by the oscillating expression of the *Per1* and *Per2* genes, which are expressed not only in the central brain pacemaker but also in peripheral tissues. Hormones are likely to coordinate physiological function in time. We performed in situ hybridization to localize *mPer1* and *mPer2* mRNA to particular cell types and tissue compartments in adrenal, thyroid, and testis. BALB/c mice maintained in a 12:12-h light-dark cycle expressed *mPer1* in adrenal medulla, particularly in late afternoon and early night. *mPer2* mRNA was more intensely expressed in adrenal cortex, especially in afternoon and evening. *mPer1* mRNA was detected in thyroid. *mPer1* was found in some but not all seminiferous tubules of each mouse at all times of day. Quantitation in C57BL/6 mice revealed a significant increase in the number of heavily labeled seminiferous tubules early in the night. Consistent with in situ hybridization, immunocytochemistry showed PER1 protein in spermatocytes and spermatids (spermatogenic stages VII–XII). Staining in spermatogonia and interstitial cells was inconsistent. Double labeling with 5'-bromodeoxyuridine showed PER1 expression first occurring 5 days after DNA replication. We conclude that *mPeriod* genes are expressed in peripheral endocrine glands. Central regulation, adenyphyseal control, and functional importance of expression and phase remain to be elucidated.

peripheral endocrine glands; seminiferous tubules; circadian rhythms; C57BL/6 mice; testis

MANY PHYSIOLOGICAL AND BEHAVIORAL functions occur at specific times of day and continue to oscillate with a period of ~24 h after transfer of organisms to constant environmental conditions. Such circadian rhythms characterize a variety of endocrine functions, including those that regulate reproduction, metabolism, thermoregulation, and responses to stress (3, 32). A central pacemaker governing mammalian circadian rhythms has been localized to the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (23). In recent years, these anatomical studies have been complemented by increased understanding of the molecular basis of the mammalian circadian clock (25). In mice, the rhythmic transcription of two orthologs of the *Drosophila Period* gene appears to be essential to circadian rhythms (4,

37). *mPer1* and *mPer2* are transcribed during the daytime in the central pacemaker under the positive regulation of a dimer composed of BMAL1 and CLOCK, which bind to E-box motifs in the promoter regions of these genes. PER1 and PER2 proteins dimerize with each other, or with cryptochromes-1 and -2. These protein complexes interact with BMAL1 and CLOCK to remove the positive drive on *Period* gene transcription during the subjective night (25). Evidence has been presented that *Per2* expression stimulates *Bmal1* transcription during the night. Light signals may entrain circadian rhythms by inducing *Per1* and *Per2* transcription in the SCN. Outputs of the SCN may control both endocrine and nervous signals that govern rhythmic functions in many peripheral organs.

In addition to their striking rhythmicity of expression in the central pacemaker in the SCN, high-amplitude oscillations of *mPer1–3* also occur in peripheral tissues, including liver, heart, spleen, and skeletal muscle (37, 38). The rhythmic expression of core clock genes in primary culture of hepatocytes and fibroblast cell lines indicates not only that circadian clocks may exist in individual cells of differentiated organs but also that blood-borne factors may set circadian phase in the periphery (6). Further evidence that coordination of circadian rhythms of gene expression throughout the body may be dependent on endocrine signals is provided by evidence that glucocorticoids can entrain clock gene expression not only in cultured fibroblasts but also in liver, heart, and kidney (5). Nevertheless, the expression of circadian clock genes in pituitary-dependent endocrine organs has not been addressed systematically. Among such organs, only the testis has been examined for *Per* expression, and the results are controversial. Northern blot analysis indicated a high amplitude rhythm of *mPer1* and *mPer3* in the testis of C57BL/6 mice, although *mPer2* was reported to be absent or expressed only at low levels (38). In studies employing other methods, however, no such rhythms of *mPer1* expression were found in mouse testis (2, 20, 21).

To understand the possible physiological role of *period* gene expression in peripheral tissues, it is necessary to identify the cell types in which it is expressed. In light of the important role of the adrenal, testis, and thyroid in coordination of physiological function, we set

Address for reprint requests and other correspondence: E. L. Bittman, Dept. of Biology, Program in Neuroscience, and Center for Neuroendocrine Studies, Univ. of Massachusetts, Amherst, MA 01003 (E-mail: elb@bio.umass.edu).

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out to describe the timing and distribution of *Period* gene expression using in situ hybridization in BALB/c mice. Our failure to find evidence of rhythmic *mPer1* expression in testis of this strain prompted us to extend our studies to the C57BL/6 strain in which such rhythmicity was originally reported in studies utilizing Northern blots (38). The distribution of *mPer1* mRNA in mouse testis revealed by our in situ hybridization studies led us to perform immunocytochemistry to characterize the appearance of PER1 protein in testis. This enabled us to confirm that *mPer1* expression occurs predominantly in seminiferous tubules and to determine the stages of spermatogenesis to which it is restricted.

## METHODS

**Studies in BALB/c mice.** All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts at Amherst. In the first experiment, 14 adult male BALB/c mice were maintained in a 12:12-h light-dark cycle (12L:12D; lights on 0700) until they were anesthetized by CO<sub>2</sub> inhalation and rapidly killed by decapitation at *zeitgeber time 3.5* (ZT3.5; 1030, *n* = 4), ZT9.5 (1630, *n* = 3), ZT14.5 (2130, *n* = 4), or ZT19.5 (0230, *n* = 3). Brain, testis, and adrenal were removed and rapidly frozen on dry ice at each time of day. Thyroid was removed only from mice killed at 1030. Expression of *mPer1* and *mPer2* was examined in sections prepared from each of these tissues at each time of day.

Tissues were processed for in situ hybridization as previously described (7). Briefly, sections were cut at 11  $\mu$ m on a cryostat and immediately mounted onto polylysine-coated slides (Superfrost Plus, Fisher Scientific) that were stored at  $-75^{\circ}\text{C}$  until the time of hybridization. Slides were then thawed and dried for 10 min. Sections were deaminated in acetic anhydride (0.25%)-triethanolamine (0.1 M in 0.9% NaCl), dehydrated and delipidated through a series of graded ethanols and CHCl<sub>3</sub>, and rehydrated and air dried. Hybridization was performed with the use of sense and antisense cRNA probes for *mPer1* and *mPer2* prepared from templates provided by Dr. Steven M. Reppert (Harvard Univ. Medical School). Antisense probes for both *mPer1* (corresponding to nucleotides 340–761, GenBank sequence AF02292) and *mPer2* (corresponding to nucleotides 9–489, GenBank sequence AF035830) were transcribed with the use of SP6 RNA polymerase after linearization with *NotI*. Sense probes for control hybridizations were prepared with the use of T7 RNA polymerase after linearization with *BamHI*. Slides were incubated overnight at  $57^{\circ}\text{C}$  under glass coverslips in a humidified chamber with hybridization buffer 2 $\times$  standard sodium citrate (SSC); 50% (vol/vol) formamide; 10% (wt/vol) dextran sulfate; 250  $\mu$ g/ml yeast transfer RNA; 500  $\mu$ g/ml sheared, single-stranded salmon sperm DNA; 50 mM DTT; and 1 $\times$  Denhardt's solution (0.002% Ficoll, 0.002% polyvinylpyrrolidone, and 0.02% bovine serum albumin) containing  $\sim 7.5 \times 10^5$  counts $\cdot$ min<sup>-1</sup> (cpm) $\cdot$  $\mu$ l of <sup>35</sup>S-labeled cRNA probe<sup>-1</sup>. Posthybridization washes consisted of rinses in 1 $\times$  SSC at room temperature (RT), 2 $\times$  SSC-50% formamide for 5 min and then 20 min at  $52^{\circ}\text{C}$ , and 2 $\times$  SSC at RT, followed by incubation in an RNase buffer consisting of RNase (100  $\mu$ g/ml, Boehringer Mannheim) in 5 M NaCl, 1 M Tris, and 0.5 M EDTA. Slides were rinsed in 2 $\times$ SSC at RT, incubated in 2 $\times$  SSC-50% formamide for 5 min at  $52^{\circ}\text{C}$ , rinsed again in 2 $\times$  SSC, and air dried. Slides were apposed to X-ray film for 3–14 days to provide film images, after which they were dipped in Kodak NTB2 emulsion, developed after suitable

intervals, and counterstained with toluidine blue (brain), hematoxylin and eosin (testes and thyroid), or Giemsa (adrenal). Film autoradiograms were quantified using NIH Image; integrated density measurements were obtained as a measure of abundance of targeted mRNA after subtraction of the integrated density of equivalent sections from the same individual mice that had been subjected to in situ hybridization using sense (control) probe of the same specific activity. Emulsion-dipped, stained sections were used to match localization of signals on films and microscopically examined to establish distribution of autoradiographic signal within each organ.

**Studies in C57BL/6 mice.** In the second experiment, adult males were housed in 12L:12D (lights on 0715). One hundred ten C57BL/6 mice (3- to 6-mo old) were used in these studies. Thirty-four of these mice were injected with the thymidine analog 5'-bromodeoxyuridine (BrdU, 50 mg/kg ip; Sigma, St. Louis, MO) to mark cell birth at intervals ranging from 4 h to 21 days before death. Mice were anesthetized with ketamine-xylazine (120:16 mg/kg sc) at ZT6.75 (1400), ZT11.25 (1830), ZT15.25 (2230), ZT19.25 (0230), or ZT23.75 (0700). Some tissue from these animals was used for further in situ hybridization studies. In most cases, the thyroid complex, one testis, and one adrenal were removed and rapidly frozen on dry ice. These tissues were stored at  $-75^{\circ}\text{C}$  until sectioning and in situ hybridization as described above. The numbers of grains over seminiferous tubules were quantified in emulsion-dipped slides by use of NIH Image.

The remaining tissues were used for immunocytochemical studies and staging of spermatogenesis with the use of established criteria (27). In a few instances, one testis was decapsulated and immersed in Bouin's fixative before transcardial perfusion to minimize pressure-induced disruption of tubular structure. Animals were transcardially perfused with 4% paraformaldehyde or Bouin's fixative before collection of remaining tissues, which were postfixed overnight, rinsed, dehydrated in graded ethanols, cleared in ethanol and xylene, and infiltrated and embedded in paraffin. Tissues were sectioned at 6  $\mu$ m and mounted onto subbed slides. Sections were deparaffinized in xylene, rehydrated in graded ethanols, and incubated in 0.3% H<sub>2</sub>O<sub>2</sub> to neutralize peroxidases. After rinsing in Tris-buffered saline (TBS) and TBS containing 0.3% Triton X-100 (TBS-T), sections were blocked with 5% normal goat and/or normal donkey serum as appropriate. When testes were to be examined for colocalization of BrdU and PER1, sections were denatured in 2 N HCl for 30 min at  $37^{\circ}\text{C}$ .

Fixed sections were stained with the use of either a previously validated rabbit anti-PER1 antibody obtained from Dr. S. M. Reppert (1:4,000; see Refs. 11 and 15) or anti-PER1 obtained from Affinity Bioreagents (1:500–1:4,000). These antibodies were raised against residues 6–21 and 39–51 of mouse PER1, respectively. Antibody was detected with the use of fluorescent (Cy2-, Cy3-, or FITC-conjugated) donkey anti-rabbit (Jackson Immunochemicals, 1:100). A Western blot indicated that the Affinity Bioreagents anti-PER1 labeled two bands of  $\sim 32$  and 55 kDa in protein extracted from mouse testis. Absorption of the Affinity Bioreagents anti-PER1 with the peptide against which it was raised eliminated all staining in tissue sections and eliminated the 32-kDa band and greatly reduced intensity of the 55-kDa band in the Western blot. Omission of primary antibodies eliminated fluorescent staining in tissue sections. Tissue from animals given BrdU was also incubated with rat anti-BrdU (Caltag; 1:500). In these experiments, PER1 was detected with the use of Cy2- or FITC-conjugated goat or donkey anti-rabbit, and BrdU was detected using Cy3-conjugated

goat anti-rat (1:100; Jackson Immunochemicals) as secondary antibody. Cellular colocalization of PER1 and BrdU was assessed by confocal microscopy by use of a Bio-Rad microscope; only when Cy2 or FITC and Cy3 staining were found within the same 1- to 2- $\mu\text{m}$  optical plane of focus were cells scored as colocalizing PER1 and BrdU.

**Statistical analyses.** In film autoradiograms, the NIH Image program was used to estimate mRNA abundance by measurement of the mean integrated density over equivalent regions of SCN, adrenal cortex, adrenal medulla, or testis; this was recorded for each animal. This measure takes into account the number of autoradiographic grains over anatomically defined regions of interest. Group means were calculated and used for comparisons. In emulsion autoradiograms, the mean integrated density of silver grains over 100 seminiferous tubules was measured for each mouse, and the results were evaluated by one-way analysis of variance to evaluate the main effect of time of day. To evaluate possible effects of time of day on the distribution of grain densities

over seminiferous tubules, Kolmogorov-Smirnoff two-sample tests were conducted (7, 12, 18). Only tubules with integrated densities in excess of background (as defined by hybridization signal generated by sense control cRNA probes) were included in the Kolmogorov-Smirnoff analysis. In each case, statistical significance was assessed at  $P \leq 0.05$ .

## RESULTS

As previously reported in other strains (21, 24), both *mPer1* and *mPer2* mRNA were detected in the SCN of BALB/c mice during the light phase, with little or no signal during the night. In the adrenal glands, *mPer1* was most prominently expressed in the medulla. Labeled cells were detected at all times of day, but labeling was highest at ZT9.5 and ZT14.5 (Fig. 1, A–E). *mPer1* labeling was less obvious in adrenal cortex but also increased during the afternoon and evening.

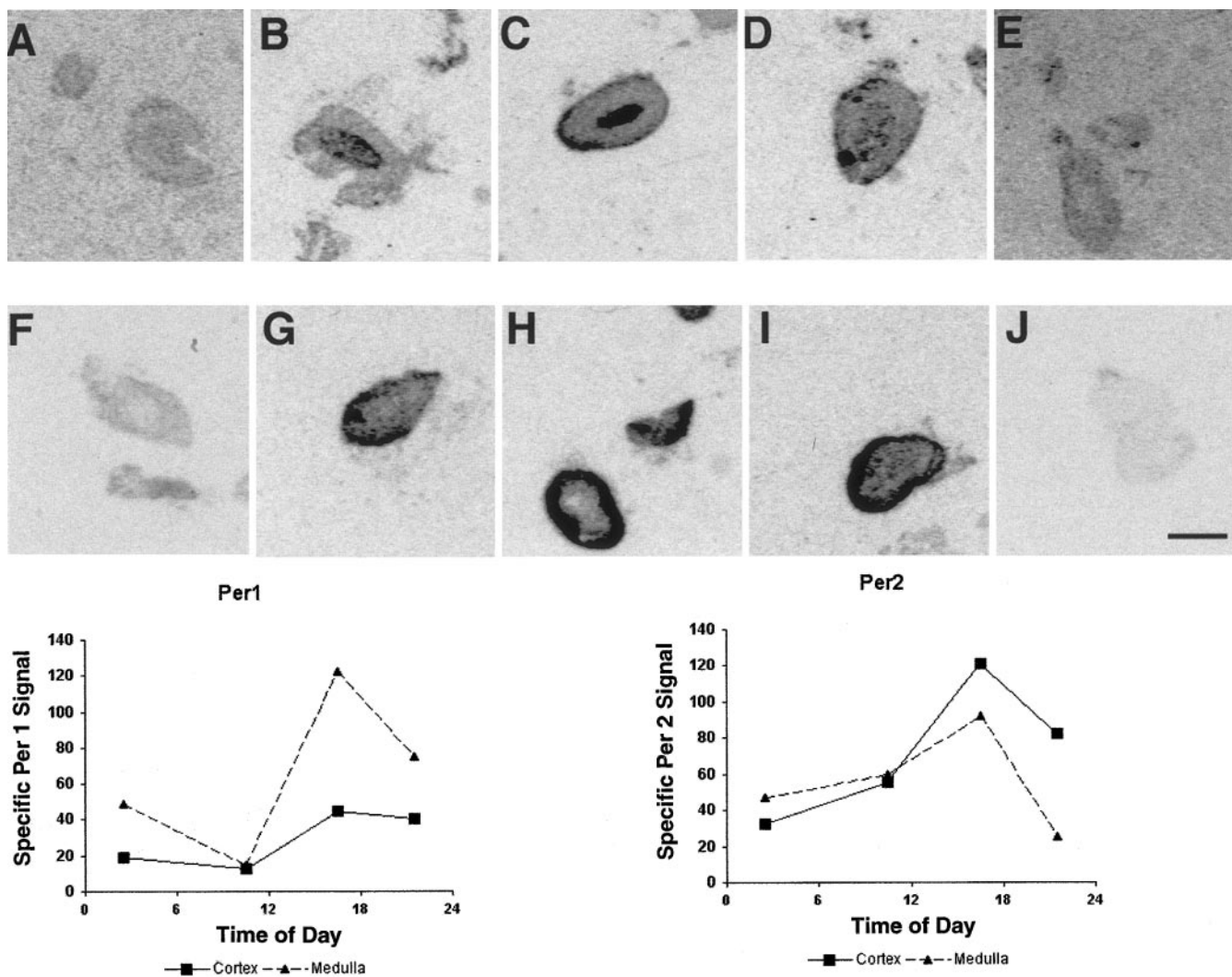


Fig. 1. *Top*: expression of *mPer1* (A–D) and *mPer2* (F–I) mRNAs in representative film autoradiograms of adrenal glands of BALB/c mice. Tissue was collected at 1030 (A and F), 1630 (B and G), 2130 (C and H), or 0230 (D and I) from mice maintained on 12:12-h light-dark cycle (12L:12D; lights on 0700). Nonspecific hybridization, as determined by use of  $^{35}\text{S}$ -labeled sense cRNA on tissues collected at 1030, is shown for *mPer1* (E) and *mPer2* (J). Scale bar, 100  $\mu\text{m}$ . *Bottom*: mean levels of specific in situ signal, obtained from quantitative densitometry and after subtraction of nonspecific from total hybridization, are illustrated below the autoradiograms. *mPer1* values are shown at *left*; *mPer2* values are shown at *right*. Dashed line and  $\blacktriangle$ , adrenal medulla; solid line and  $\blacksquare$ , adrenal cortex.

*mPer2* mRNA was most abundant in adrenal cortex at ZT9.5 and ZT14.5; intermediate levels of labeling were evident at ZT19.5, and little specific signal was found at ZT3.5 (Fig. 1, F–J). *mPer2* labeling was less consistently found in adrenal medulla but was more abundant at ZT9.5 than during the morning.

In contrast to both the SCN and the adrenal glands, autoradiographic films of testis revealed significant but heterogeneous expression of *mPer1* without clear differences between the four ZTs at which we sampled. Inspection of emulsion-dipped slides revealed that *mPer1* was abundantly expressed in some but not all of the seminiferous tubules of each mouse, regardless of the time of day (Fig. 2). At ZT9.5 but not at other times of day a few interstitial cells were also labeled. Autoradiographic grains were concentrated within the seminiferous epithelium, intermediate between the basement membrane and the tubular lumen. Also, in contrast to autoradiograms of *mPer2* hybridization in the SCN of the same mice, no *mPer2* signal was evident in the testis at any of the times of day or night sampled. Sense probes for *mPer1* or *mPer2* produced no signal in testis, indicating the lack of nonspecific hybridization.

Our finding that *mPer1* expression did not vary dramatically with time of day in the testes of BALB/c mice was inconsistent with the report based on Northern blots that testicular *mPer1* mRNA vary 100-fold over the circadian cycle in the C57BL/6 strain (37). To evaluate the possibility of a strain difference, we performed further in situ hybridization and immunostaining experiments in C57BL/6 mice. Our autoradiograms do not support the interpretation that this discrepancy arises from a strain difference: testicular *mPer1* expression in C57BL/6 mice was similar in pattern to

that found in BALB/c males. Grains were almost exclusively found over the seminiferous epithelium, and at any time of day label was observed over some but not all of the tubules. Analysis of variance revealed no main effect of time on the mean integrated density of labeled seminiferous tubules ( $F_{4,22} = 1.384$ ;  $P > 0.20$ ). Analysis of the distribution of grain densities over seminiferous tubules by use of the Kolmogorov-Smirnov test, however, indicated significant effects of time of day. Specifically, testes collected at ZT15.25 had slightly but significantly more seminiferous tubules with high grain counts than those obtained at ZT11.25 ( $P < 0.05$ ) or ZT19.25 ( $P < 0.02$ ; Fig. 3).

The restricted distribution of *mPer1* mRNA within seminiferous tubules of C57BL/6 mice and the presence of labeling in some tubules but not others within the same testis prompted us to stain for PER1 protein in fixed tissue obtained from the same mice in which the in situ hybridizations were performed. In agreement with the in situ hybridization studies, immunocytochemical staining utilizing two different antibodies to PER1 predominantly stained cells within the seminiferous tubules (Fig. 4). The Affinity Bioreagents antibody generated more extensive intratubular staining than did the Reppert antibody. Interstitial cell staining was occasionally seen as well but was much less consistent. Staining was sometimes confined to the basal compartment but was also often extensive and more intense in spermatocytes and was occasionally found in round and elongate spermatids (Fig. 4C). Immunostaining was often present but diffuse in the cytoplasmic compartment and intense in the cell nuclei. Not all seminiferous tubules were labeled, indicating that PER1 expression might be confined to particular cell

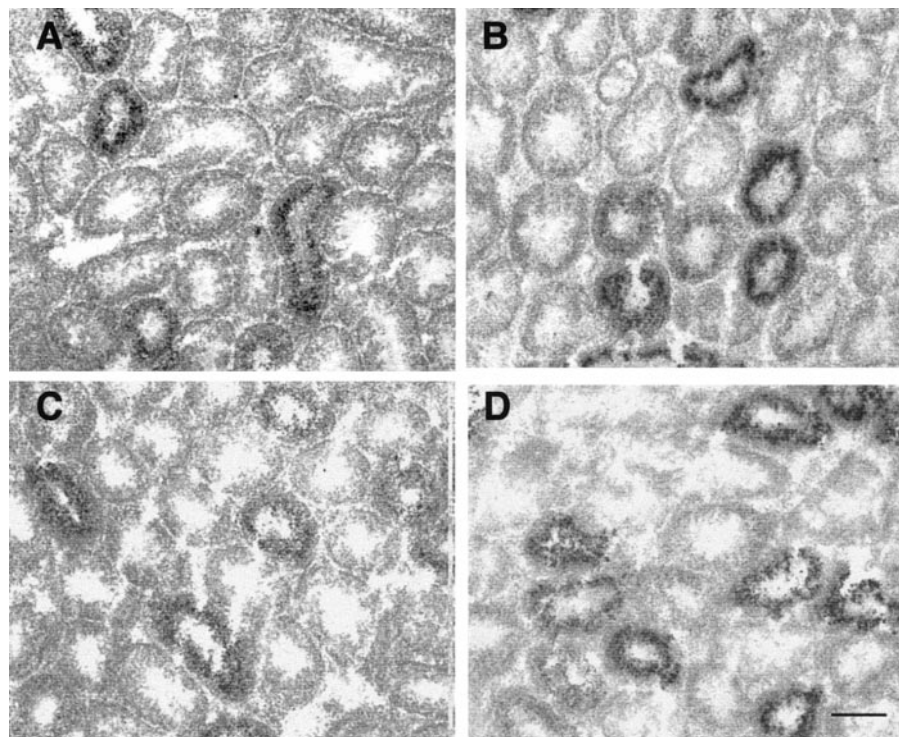


Fig. 2. Emulsion-dipped autoradiogram illustrating presence of *mPer1* mRNA by in situ hybridization in some but not all seminiferous tubules at 1030 (A), 1630 (B), 2130 (C), and 0230 (D) of BALB/c mice on 12L:12D (lights on 0700). Scale bar, 200  $\mu$ m.

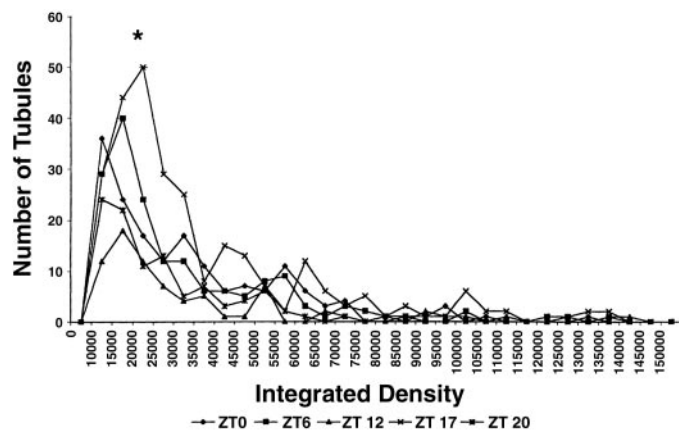


Fig. 3. Histograms illustrating effect of time of day on integrated density (proportional to nos. of autoradiographic grains indicating *mPer1* mRNA) in testis of C57BL/6 mice. Tissues were collected at zeitgeber time 23.75 (ZT0; ♦), ZT6.75 (■), ZT11.25 (▲), ZT15.25 (×), or ZT19.25 (★). \*Statistical analysis of distribution of integrated density of seminiferous tubules by Kolmogorov-Smirnov test indicated a statistically significant peak at ZT17 ( $P < 0.05$ ). ANOVA of mean integrated density, however, revealed no significant effect of time of day on *mPer1* expression.

associations. Histological analysis indicated that PER1 staining was present between stages VII and XII of spermatogenesis (27). PER1 immunoreactivity (PER1-ir) was apparent in spermatocytes of mice killed at each time of day, such that there was no obvious rhythm to the pattern of PER1 expression.

To determine more closely the maturational stage at which PER1 is expressed in maturing germ cells, mice were killed at various intervals after injection of BrdU. Colocalization of BrdU and PER1 was not observed by confocal microscopy in mice killed  $<5$  days after BrdU injection but was extensive in mice allowed to survive for 10–21 days, indicating that PER1 is not expressed until long after the completion of DNA replication in preleptotene spermatocytes (Fig. 5).

In the course of our in situ hybridization experiments, we made an effort to evaluate *mPer* expression in the thyroid gland. Specific *mPer1* label was detected at low levels in both the follicular and interstitial tissue of the thyroid of BALB/c mice killed at ZT3.5 in the first experiment. Although the primary purpose of the second experiment was to examine testicular expression of *mPer1* in the testis of C57BL/6 mice, thyroids collected from these animals were used to repeat and extend in situ hybridization studies of *mPer1* expression in this gland (Fig. 6). Extensive labeling of thyroid follicles was again found at all times of day, but we were unable to find consistent differences between the number of grains over thyroid follicles at the five different sampling times. We were unable to accurately quantify integrated densities reflecting *mPer1* mRNA levels in thyroid sections, however, due to variations in the thickness of follicles and edge artifacts.

## DISCUSSION

*Per1* plays a pivotal role in circadian rhythmicity. Mice deficient in this gene either fail to sustain circa-

dian oscillations of locomotor behavior or exhibit rhythms of aberrant period (4, 9, 37). *Per1* is expressed in a circadian pattern in many peripheral tissues, and deletion of this key circadian gene eliminates or alters the daily pattern of expression of a variety of clock-controlled genes in liver (37). The specific function of PER1 in peripheral tissues remains unclear, however, in part because its expression has not been localized to

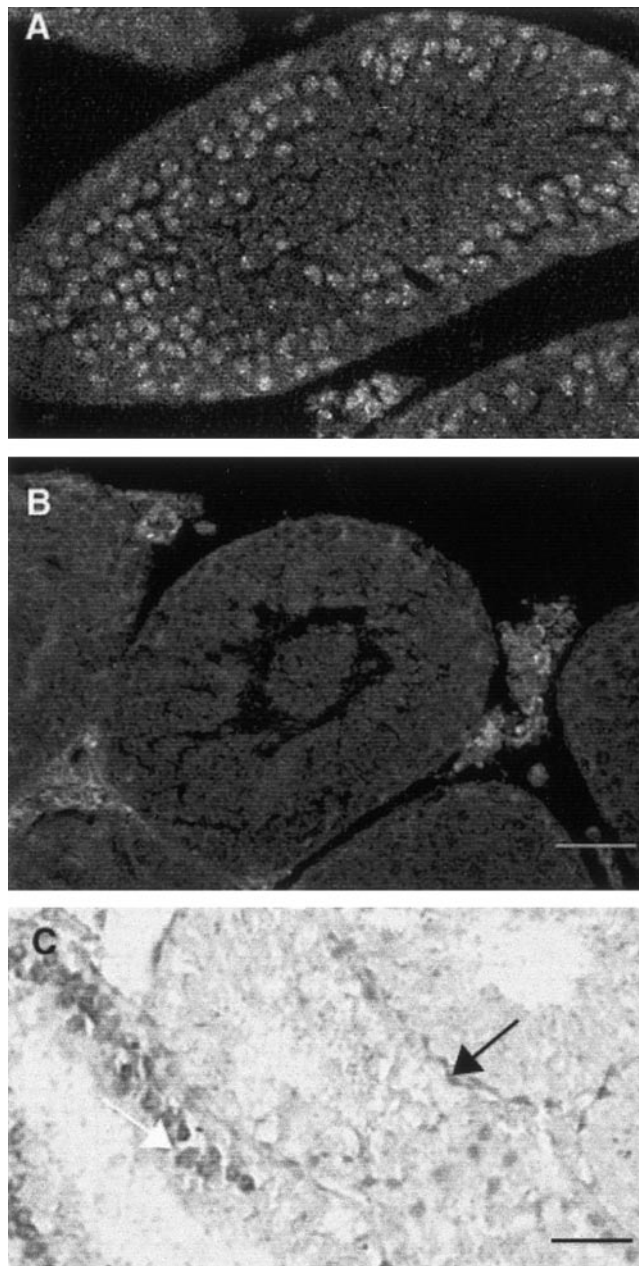


Fig. 4. PER1 immunostaining in mouse testis. A: dark-field image illustrating PER1 immunoreactivity (PER1-ir) detected by use of FITC-conjugated goat anti-rabbit. B: dark-field image illustrating nonspecific staining in the absence of primary antibody. Scale bar for A and B, 50  $\mu\text{m}$ . C: bright-field image illustrating PER1-ir detected by use of diaminobenzidine. Open arrow illustrates intense labeling observed in spermatocytes; solid arrow illustrates faint labeling seen in spermatogonia near basement membrane in some seminiferous tubules. Scale bar for C, 75  $\mu\text{m}$ .

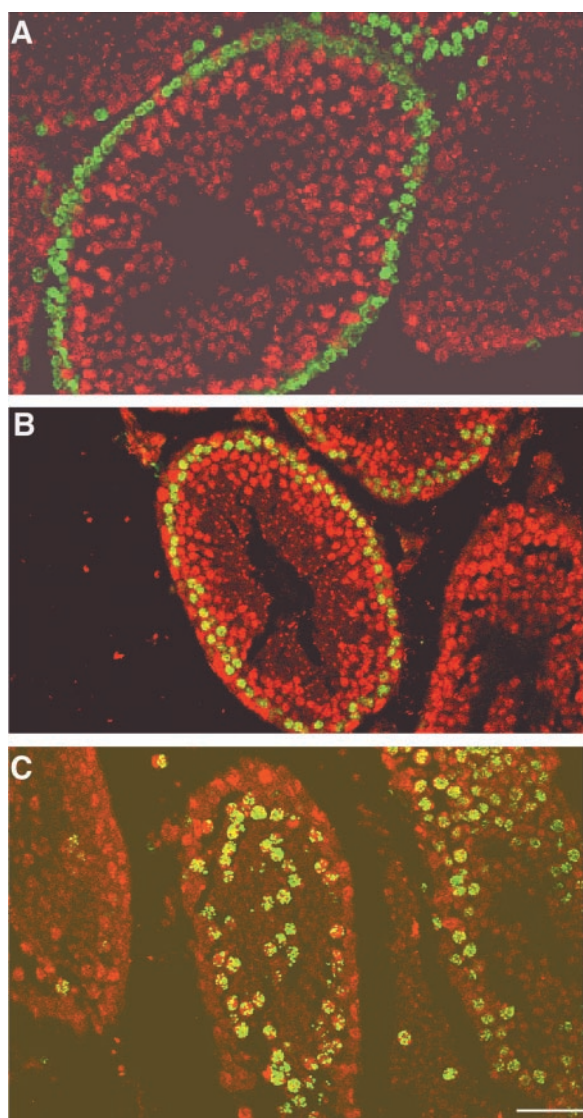


Fig. 5. Time course of colocalization of PER1-ir with DNA labeled during maturation of spermatocytes. Mice were injected with the cell birth marker bromodeoxyuridine (BrdU; green) 4 h (A), 4 days (B), or 10 days (C) before death. Sections were stained for PER1 (red) and analyzed by confocal microscopy. Only at survival times of 5 days or longer are spermatocytes containing BrdU-ir also found to contain PER1-ir, indicating that PER1 is expressed after completion of DNA replication in the preleptotene stage. Scale bar, 75  $\mu\text{m}$ .

specific cell types. Although endocrine tissues are of particular importance as sources of coordinating signals that may ensure appropriate phasing of a wide variety of physiological functions, the pattern or cell type of *Per* expression in these tissues has received relatively little study to date. The present experiments reveal that *mPer1* is expressed in both the adrenal cortex and adrenal medulla as well as the thyroid and testis. They call into question an earlier report (38) of a high-amplitude rhythm of *mPer1* expression in testis but clarify the cell types in which *mPer1* mRNA and PER1 protein are found. This should facilitate studies of the physiological function of this circadian gene.

The regulation of circadian gene expression in peripheral organs is poorly understood. Ablation of the

SCN has been reported to abolish rhythms of *rPer2* in rats (28) and to reduce or eliminate oscillating expression of a number of rhythmically expressed genes including *mPer2* and *mBmal1* in mouse liver (1). Circadian rhythms of expression of *mPer1* in mouse liver and of *rPer1* promoter activity in rat liver appear particularly responsive to cues arriving from food intake, the timing of which is in turn controlled by the SCN (5, 30). The synthetic glucocorticoid dexamethasone can shift the phase of *mPer1* mRNA in liver, kidney, and heart, and this action in liver requires glucocorticoid receptor (5). These findings underline the importance of understanding circadian rhythms in adrenal function. To our knowledge, the present study is the first to examine *Period* gene expression in the adrenal gland. Adrenal corticoids are secreted in anticipation of the active phase of the circadian cycle in a wide variety of both diurnal and nocturnal species under both entrained and free-running conditions. It is widely presumed that this rhythm is triggered by corticotropin-releasing hormone (CRH) and ACTH secretion, functions to prepare animals for activity, and coordinates physiological events appropriate for that

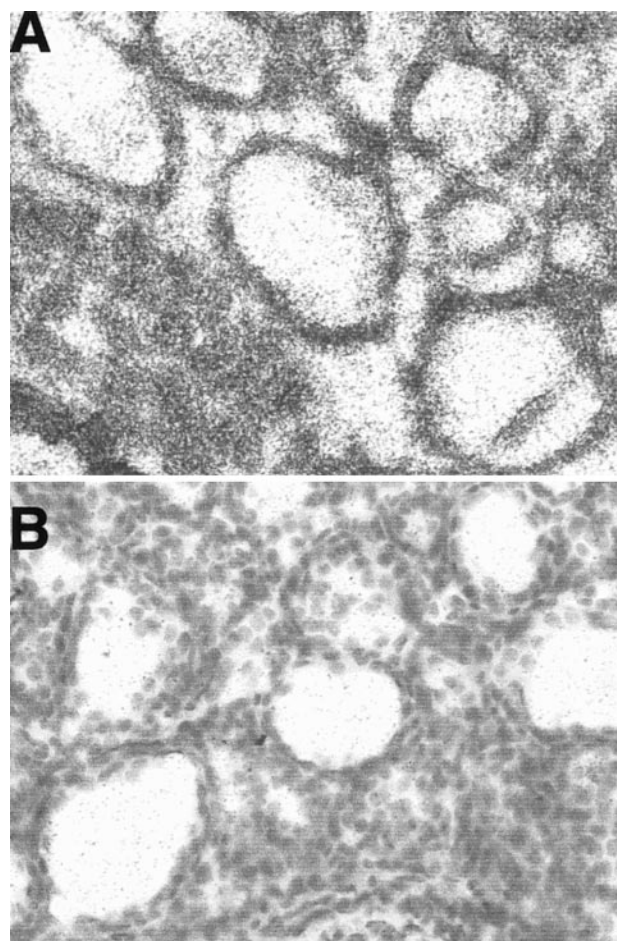


Fig. 6. Autoradiograms depicting in situ hybridization for *mPer1* in thyroid of C57/Bl6 mouse. A: follicular and interstitial labeling generated by use of  $^{35}\text{S}$ -labeled antisense cRNA probe. B: nonspecific labeling generated by use of sense probe.



phase of the circadian cycle (29). However, the role of adrenal expression of *mPer1*, *mPer2*, and/or other circadian clock genes in the local generation of glucocorticoid rhythms is unexplored. The precise pattern of adrenal period gene expression and its regulation by feeding cues, sympathetic input (8), or adenohipophysial signals require further investigation. Further studies, perhaps utilizing dual-label in situ hybridization, will be useful to determine whether *mPer* expression oscillates in specific zones of the adrenal cortex and whether this gene regulates secretion of specific glucocorticoid or mineralocorticoid hormones that may regulate other tissues.

Our finding that *mPer1* and *mPer2* are rhythmically expressed in the adrenal medulla may also have physiological significance. The role of these circadian genes in catecholamine production by chromaffin cells has not been explored but merits attention. Circadian rhythms of sympathetic activity are believed to coordinate oscillations of arousal, and catecholamine secretion rises at the onset of activity in both diurnal and nocturnal mammals. The appearance of *mPer1* and *mPer2* mRNAs in adrenal medulla during the late afternoon and early evening (Fig. 1) is consistent with a positive role of *Period* gene expression in the synthesis and/or secretion of adrenal catecholamines. Transneuronal retrograde tracing studies employing pseudorabies virus indicate that vasopressinergic cells of the SCN may control sympathetic preganglionic cells of the interomediolateral cell column that regulate the adrenal medulla (8, 33). The role of this input in the regulation of *mPer* expression in the adrenal is unknown, as are the roles of local *mPer1* and *mPer2* expression in adrenal hormone production. We cannot conclude from the relative abundance of *mPer1* and *mPer2* in the adrenal cortex or medulla that either of these mRNAs is more important for catecholamine or steroid synthesis. Examination of hormonogenesis in knockout strains or study of effects of selective neutralization of *Period* gene expression (for example, through use of small interfering RNA or antisense oligodeoxynucleotides) may prove useful in elucidating their roles in adrenal function.

The thyroid gland also plays a prominent role in metabolic regulation and may participate in circadian coordination of physiological functions. We found extensive *mPer1* mRNA in the thyroid gland of mice at all times of day. Autoradiographic grains were evident over both follicular cells and interstitial tissue. The histology of the thyroid made quantification of grain density difficult, however, because edge artifacts produce silver grains over the colloidal space of the follicles. Thus we were unable to determine whether a low amplitude diurnal rhythm of *mPer1* expression exists. This question should be explored further with the use of Northern blots, RNase protection assays, or other appropriate methods. Twenty-four-hour rhythms of thyroid-stimulating hormone, T<sub>3</sub>, and T<sub>4</sub> secretion have been described in humans and rats, and destruction of the SCN caused a reduction of the amplitude of thyroid hormone secretion under entrained conditions

(3, 14, 32). Furthermore, transneuronal retrograde tracing indicates that the thyroid receives sympathetic innervation that is ultimately regulated by vasopressinergic cells of the SCN (14). As is the case for the adrenal gland, the role of either autonomic or hypophysial signals in regulation of *mPer1* expression and the function of PER1 in hormonogenesis remain to be elucidated.

The regulation as well as the function of *mPer1* expression in the testis is also poorly understood. Expression of *mPer1* in the interstitial compartment of the testis was not consistent in either the in situ hybridization or the immunostaining experiments. Further histological studies, perhaps including additional times of day, will be necessary to evaluate fully the extent of clock gene expression in Leydig cells. Although daily fluctuations of plasma testosterone have been described, they vary between strains and species in consistency and amplitude, and their persistence under constant conditions is uncertain (3, 13, 17, 32). Although it may be worthwhile to extend such an analysis to *mPer* expression in the testes of species showing high-amplitude rhythms of androgen secretion (13), our findings of *mPer1* expression in mouse seminiferous tubules focus attention on the role of this gene in gametogenesis. The fact that mice deficient in *mPer1* expression are fertile (4, 9, 37), as are mice of other knockout strains that lack circadian rhythmicity (34, 36) and SCN-lesioned hamsters (26), indicates that neither the product of this gene nor circadian organization is critical to testicular function. More careful analysis of the efficiency of gametogenesis and the viability of sperm in these knockouts may, however, indicate a significant role for *mPer1*. The distribution of *mPer1* mRNA in our in situ hybridization studies was consistent with the appearance of PER1-ir in the immunocytochemical experiments. Autoradiograms of the fresh-frozen, postfixed testis sections used for in situ hybridization did not allow us to definitively determine the cell types or stages of gene expression, although very little labeling was found adjacent to the basement membrane. Our immunocytochemical study shows that PER1 staining is most intense between the leptotene spermatocyte and the spermatid stages. We observed less consistent, and less intense, immunostaining of spermatogonia. Our results are partly consistent with the finding of Alvarez et al. (2) who observed weak PER1 staining in seminiferous tubules of week-old mice, which contain only spermatogonia, and mPER1 immunoreactivity in primary spermatocytes at 14 days of age. Nevertheless, the results of our single-label immunostaining do not rule out the possibility of premeiotic PER1 expression, particularly in intermediate or type B spermatogonia. Our finding that BrdU is not colocalized with PER1-ir until several days after completion of DNA replication, however, is most consistent with a role for PER1 in maturation of spermatocytes after the leptotene stage and is less consistent with the observations of Alvarez et al. (2). We have not determined the half-life of PER1 in the testis, however, so it is not possible to determine whether the protein is synthesized continuously through these stages of sper-



matogenesis. The technical difficulties of maintaining germ cell cultures and the absence of suitable spermatogenic cell lines present obstacles to measuring the half-life of PER1. In addition, the function of PERIOD and other proteins critical to the circadian clock mechanism is affected by posttranslational modifications (15, 16, 35); our immunocytochemical techniques would not detect phosphorylation or other changes that could occur differentially at particular stages of spermatogenesis and might also vary with time of day. Although our immunoblot studies validate the use of these PER1 antibodies in testis immunostaining, our Western blot analysis indicates that PER1 protein is cleaved into smaller fragments in the testis than in the brain. Further analysis of tissue-specific processing of PER1 is warranted, and more detailed histological analysis, perhaps including a combination of immunostaining with electron microscopy, would be helpful to definitively identify not only the stages at which PER1 is expressed but also the association of this protein with chromatin.

Distributional analysis using the Kolmogorov-Smirnov test indicated that slightly more seminiferous tubules exhibited high mRNA levels during the early night than at other times of day in C57BL/6 mice. When the mean level of *mPer1* label (nos. of autoradiographic grains) was evaluated at various times of day, however, no statistically significant differences were found. The apparent lack of circadian rhythms of *mPer1* expression in our in situ hybridization and immunocytochemical studies is at variance with reports based on Northern blots of dramatic rhythms of *mPer1* mRNA in peripheral tissues (37, 38) but is consistent with recent reports (20, 21) of failure to find rhythms of *mPer1* mRNA in mouse testis by in situ hybridization and RNase protection assays. Our findings are also consistent with a recent RNase protection and immunostaining study finding expression of several circadian genes, including *mPer1*, but an absence of circadian rhythmicity in testes of C57BL/6 mice (2). Furthermore, our identification of peak mPER1 immunostaining in stages VII–XII of spermatogenesis is in good agreement with RNase protection analysis of *mPer1* transcript levels in dissected rat seminiferous tubules (21). Studies of *haPer1* expression in the testes of Syrian hamsters in our own laboratory support the existence of a relatively low-amplitude rhythm, as determined by Northern blot analysis and real-time quantitative PCR (31). These studies also indicate that two major transcripts may be expressed in hamster testes but show that the abundance of the two transcripts changes in parallel. Thus we feel it is unlikely that the absence of rhythmicity of *mPer1* mRNA in the present study reflects the masking of oscillation of a particular *mPer1* transcript by invariant levels of another transcript also detected by the same probe. The patterns of PER1 protein expression discovered in the present study are also consistent with the apparent lack of oscillation and heterogeneous pattern of *mPer1* mRNA in testis.

The absence of a high-amplitude rhythm of *mPer1* expression in testis distinguishes it from other peripheral organs. The spermatogenic cycle is characterized by a wave of meiotic division that propagates along the length of the tubule. Perhaps the complex coordination of the spermatogenic cycle would be compromised by circadian fluctuations of gene expression in maturing germ cells. Nevertheless, circadian or bidaily rhythms of meiosis have been reported in mouse testis under entrained and free-running conditions, respectively (24), and evidence has been presented that the numbers of spermatogonia and preleptotene spermatocytes peak in the early evening (10). Although this corresponds to our finding of a small but significant increase in the number of seminiferous tubules with high levels of *mPer1* mRNA at *ZT15.25* (Fig. 3), the lack of colocalization of PER1 protein with BrdU until several days after the injection of this thymidine analog (Fig. 5) makes it unlikely that expression of *mPer1* directly regulates the timing of DNA replication during spermatogenesis. It would be useful to carefully examine effects of mutations or knockouts that eliminate circadian rhythms (4, 30, 32, 33) on diurnal oscillations in the seminiferous epithelium.

Our in situ hybridization study indicates that *mPer2* is not expressed in testis, or is present in only low levels. This is another way in which the testis differs from other peripheral tissues: robust oscillations of *mPer2* have been described in liver, skeletal muscle, retina, and heart (38). In the central pacemaker, which is localized to the SCN, *mPer2* plays an essential role as a core component of the circadian clock (4, 37). Its absence in the testis may indicate that the role of *mPer1* also differs between the brain and the gonad, or that other clock constituents take over the role of *mPer2* in this organ. It is interesting to note that *mPer1* levels in the hamster testis, although rhythmic, vary in phase with *Bmal1* (31). This is consistent with a report that effects of *clock* gene mutations on genes that participate in the core circadian mechanism differ between the central pacemaker and peripheral organs (22).

### Perspectives

We have documented the expression of the circadian genes *mPer1* and *mPer2* in peripheral endocrine tissues. These genes are rhythmically but differentially expressed in adrenal cortex and medulla. *mPer1* is expressed in thyroid and testis, but clear circadian rhythmicity is not apparent. *mPer1* mRNA and PER1 protein are expressed predominantly in the seminiferous tubules and in spermatocytes and spermatids in stages VII–XII of spermatogenesis. Further work will be necessary to determine the control of *Period* gene expression by hypophyseal and autonomic inputs and the physiological role of the protein product of these genes in endocrine organs.

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## DISCLOSURES

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