Circadian genes in a blind subterranean mammal II: Conservation and uniqueness of the three *Period* homologs in the blind subterranean mole rat, *Spalax ehrenbergi* superspecies

Aaron Avivi†§, Henrik Oster†§, Alma Joel*, Avigdor Beiles*, Urs Albrecht†, and Eviatar Nevo

*Laboratory of Animal Molecular Evolution, Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel; †Max-Planck-Institute for Experimental Endocrinology, Feodor-Lynen-Strasse 7, 30625 Hannover, Germany; and ‡Department of Medicine, Division of Biochemistry, University of Fribourg, Rue du Musée 5, 1700 Fribourg, Switzerland

Contributed by Eviatar Nevo, July 10, 2002

We demonstrated that a subterranean, visually blind mammal has a functional set of three *Per* genes that are important components of the circadian clockwork in mammals. The mole rat superspecies *Spalax ehrenbergi* is a blind subterranean animal that lives its entire life underground in darkness. It has degenerated eyes, but the retina and highly hypertrophic hardner gland are involved in photoperiodic perception. All three *Per* genes oscillate with a periodicity of 24 h in the suprachiasmatic nuclei, eye, and hardener gland and are expressed in peripheral organs. This oscillation is maintained under constant conditions. The light inducibility of *sPer1* and *sPer2*, which are similar in structure to those of other mammals, indicates the role of these genes in clock resetting. However, *sPer3* is unique in mammals and has two truncated isoforms, and its expression analysis leaves its function unresolved. *Per*’s expression analysis in the hardener gland suggests an important participation of this organ in the stabilization and resetting mechanism of the central pacemaker in the suprachiasmatic nuclei and in unique adaptation to life underground.

Life on Earth is adapted to cyclical phenomena imposed by the external environment (1). Most organisms have circadian systems that synchronize physiological events to the external 24-h cycle (2). The underlying molecular-genetic mechanisms of these clocks exhibit an extraordinary evolutionary conservation from cyanobacteria through plants, fruit flies, and mammals. All of these clock systems consist of autoregulatory transcriptional/translation feedback loops with positive/negative regulatory elements and similar genetic machinery (3, 4).

Two basic helix–loop–helix PAS (PER-ARNT-SIM) transcription factors, CLOCK and MOP3 (BMAL1), form the positive elements of the system and drive transcription of three *Period* (*Per* 1, 2, 3) and two Cryptochrome (*Cry 1, 2*) genes. The protein products of these genes are thought to be components of a negative feedback complex that inhibits the CLOCK/MOP3 heterodimer, thereby closing the circadian loop.

The enigma of circadian rhythms in a blind subterranean mammal is intriguing (5–7). We have already shown that a CLOCK/MOP3-driven clock exists in *Spalax* (8). Here we continue to decipher its circadian machinery.

The Evolutionary Model of Blind Subterranean Mammals

The blind subterranean mammals, mole rats of the *Spalax ehrenbergi* superspecies in Israel, consist of four species that have been studied multidisciplinarily as an evolutionary model of speciation and adaptation (5–7). *Spalax* lives in total darkness, yet it perceives the daily and seasonal temporal cycles underground (9). Behaviorally, *Spalax* displays polyphasic and polytypic day-night activity patterns (10, 11) coupled with polyphoric (12) and seasonal (13) variation, supported by a unique photoperiodic perception mechanism (9). *Spalax* has a degenerated s.c. functional eye (13, 14), which, together with the hardener gland, participates in photoperiodic perception (9, 15–18). The retina harbors *Rhodopsin* (19, 20) and *Coneopsin* (21), adaptively effective in photoperiodic perception (22, 23), and expresses *alpha-B-crystallin* (24). The photic signals entrain *c-fos* in the suprachiasmatic nuclei (SCN) Zeitgeber (25) and can possibly activate circadian genes.

Evolutionarily, *Spalax*’s perceptive brain structures (SCN and striatum) were expanded and sight pathways were drastically (>90%) reduced. The visual cortex was replaced by somatosensory cortex (26–28), *Per* homologous ACNGGN-repeats cycle in the hypothalamus (29) and melatonin precursors occur in the hardnerian and pineal glands and retina (30).

What is the genetic basis of circadian rhythmicity in *Spalax*? We cloned, sequenced, and unraveled the expression of the circadian *Clock* and *MOP3* cDNAs of three species of the *S. ehrenbergi* superspecies in Israel (8). Both genes are relatively conserved, yet Clock displays a unique Q-rich area as compared with other mammals, assumed to function in circadian rhythmicity, and *Spalax* CLOCK/MOP3 dimer is less potent than its human counterpart in driving transcription.

Here we describe the cloning, sequencing, and expression of the three *Period* cDNAs of *Spalax*. Its three *Per* cDNAs are conserved, yet they show features unique to *Spalax* especially in *Per3, Per1*, and *Per2* cycles in the SCN, eye, and hardener gland. *Per3* is structurally unique among studied sighted mammals and awaits functional elucidation.

Materials and Methods

Animals. We analyzed adults (100–150 g), belonging to *Spalax judaei*, (2n = 60) from Anza, Samaria (7). Field-trapped animals were kept at 22–24°C with seasonal photoperiod. We selected diurnal animals that were kept under a 12-h light/12-h dark cycle. For analysis of *Per* transcriptional activity in constant darkness, light was turned off at Zeitgeber time (ZT) 12, and animals were kept in the dark for at least 2 days before being killed under dim illumination (15-W safety red light). Light inducibility experiments were done on animals kept in light/dark for a week with a short light pulse (15 min, >200 Lx) at specified ZT followed by release into constant darkness. For gene induction analysis brains were taken 1 h after illumination. Each experiment was done on three sets of animals.

Abbreviations: SCN, suprachiasmatic nuclei; ZT, Zeitgeber time; RT-PCR, reverse transcription–PCR; SDH, in situ hybridization; CK1ε, casein kinase 1ε.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AJ345059–AJ345062).

1 A.A. and H.O. contributed equally to this work.

2 To whom reprint requests should be addressed. E-mail: aaron@esti.haifa.ac.il.

Cloning of Spalax Per cDNAs. We cloned the three Spalax Per cDNAs by reverse transcription–PCR (RT-PCR) (31). Oligos were synthesized according to the ORF of the known human and mouse homologous sequences (GenBank accession nos. AF022991, AB002370, and AB047686 for human Per1, Per2, and Per3, respectively, and AF022992, AF036893, and AB013605 for mouse Per1, Per2, and Per3, respectively). Whole brain total RNA was prepared by using the TriReagent RNA isolation reagent (Molecular Research Center, Cincinnati). First-strand cDNA was synthesized with oligo(dT) as a primer and Super-reagent (Molecular Research Center, Cincinnati). The cDNA product was taken for PCR by using TaqDNA polymerase (Appligene, Strasbourg, France). The annealing temperature, elongation time, and MgCl2 concentration were adjusted for each specific PCR. In the case of sPer3 isolation, we verified our RT-PCR results by also cloning through cDNA library screening (32). Spalax brain cDNA library in Lambda-TripleEx was screened by using a partial mPer3 cDNA as a probe. Sequencing was determined by thermocycling sequencing using di-deoxy nucleotide terminators (3700 DNA Analyzer, Perkin–Elmer/ Applied Biosystems) at the sequencing unit of the Weizmann Institute of Science (Rehovot, Israel).

Evolutionary Analysis. The evolutionary analysis of the Per cDNAs presented here is based on distances and divergence calculations (Wisconsin package version 10, GCG). Evolutionary Analysis of the Spalax Per Genes. Protein trees of PER1, PER2, and PER3 in Spalax, mice, rats, and humans appear in Fig. 1. The Drosophila Per (GenBank accession no. X103656) was also compared, but it was very different from the three mammalian Per proteins. The computer program used (GCG/10) estimated the distance of the dPer from its mammalian counterparts as maximal and beyond the accuracy of the method. The estimated divergence time between Spalax and other rodents is much shorter than the divergence between humans and rodents. Therefore, we expected that the genetic distance between Spalax and mice or rats would be considerably smaller than between humans and these two rodents. We also expected that the distance between humans and Spalax and between humans and mice or rats would be similar. Any divergence from these expectations can suggest that additional factor(s) influence the rate of evolution of the Per genes of Spalax and, therefore, deviate from the phylogenetic divergence time.

Below we present the evolutionary analysis for each Per gene separately.

Per1. There is agreement between the above evolutionary expectations and the PER1 proteins (Fig. 1 Top) and PER1 nucleotide sequence (tree not shown). The relative distances in protein and nucleotides between Spalax and mice are 75% (6.85 vs. 9.12) and 80% (10.51 vs. 13.18) of the distances between Spalax and humans, respectively. The distances between rodents (Spalax and mice) and humans are similar: 9.12 and 8.04 in the protein and 13.18 and 13.26 in the nucleotide sequence.

Per2. Rat PER2 (GenBank accession no. MN031678) was also included and expected to be similar to mouse PER2. As in Per1, PER2 results were in agreement with the phylogenetic expectations (Fig. 1 Middle). The relative distances between Spalax and mice were 42% (11.55 vs. 27.54) or 59% (13.07 vs. 22.09) of the distances between Spalax and humans in protein and nucleotides, respectively. The distances between Spalax and rats were 46% (12.69 vs. 27.54) or 66% (14.6 vs. 22.09) of the distances between Spalax and humans in protein and nucleotides, respectively. The distances between rodents (mice, rats, and Spalax)
and humans were also similar: 27.00, 27.70, and 27.54 for protein and 22.56, 23.58, and 22.09 for nucleotides, respectively.

**Per3**. As mentioned, we cloned two truncated clones of *Spalax* **Per3** (named a and b). Both clones start at the equivalent of mouse 468 bp (110 bp 3’ to the mouse ATG initiation codon). We could not isolate any further 5’ sequences either through RT-PCR or cDNA library screening. Both clones contain an insertion of 198 bp at position 1211 bp of mice that interrupts the ORF. Furthermore, s**Per3**a has two deletions, the prominent one is 432 bp in length starting at position 1478 bp of the mouse sequence. At the starting point of this deletion in s**Per3**a, s**Per3**b has a cluster of termination codons at any of the three reading frames. The apparent initiation ATG is located immediately after the deletion in the s**Per3**a or these termination codons in s**Per3**b. Omitting the changes in the s**Per3**b clone yielded a *Spalax* **Per3** ORF, which is similar to that of mice and humans. It should be emphasized that similar products have never been obtained in negative control amplifications with templates generated without reverse transcriptase enzyme, eliminating the possibility of genomic DNA contamination. Furthermore, the s**Per3** clones that were isolated from the *Spalax* brain cDNA library contain a shorter 3’ untranslated region than those of mice and humans, and in contrast to them, contain an adenylation site 940 bp 3’ to the termination TAA codon. The published 3’ untranslated regions of mice (1,164 bp) and humans (2,421 bp) do not reach the adenylation site. Southern blot analyses suggest that the *Spalax* s**Per3** is probably a pseudogene (results not shown).

The nucleotide distances between *Spalax* **Per3** and that of mice or humans are similar. The protein distances (Fig. 1 **Bottom**) between the two *Spalax* **Per3** and mice or humans were 56.21 vs. 57.20 for s**Per3**a, respectively and 60.41 vs. 57.74 for s**Per3**b, respectively. Kimura’s two-parameter nucleotide distance analysis (33) gave more than two substitutions per bp. Thus, the exact calculated value is meaningless and depends heavily on the assumptions of the correction factors. Nevertheless, the calculated distance of *Spalax* vs. mice is even larger than the calculated distance of *Spalax* vs. humans. The same is true for the distances calculated separately for synonymous and nonsynonymous substitutions.

**Synonymous vs. Nonsynonymous Substitutions in the Per Family.** Our calculations show that **Per2** has a ratio around 0.2, indicating that it attained optimum function before the divergence of the species. **Per1** and **Per3** have a ratio of 0.43 to 0.75, a relatively high ratio suggesting adaptive evolution. The *Drosophila* **Per** showed a ratio >1.0, indicating positive selection for a functional change.

**sPer Genes Oscillate in the Spalax SCN.** ISH with antisense riboprobes in the brain revealed a rhythmic pattern of expression for s**Per1** and s**Per2**, mainly in the SCN but s**Per3** is widely spread in the brain (Fig. 2). Maximal expression for s**Per1** was at ZT6 and for s**Per2** and s**Per3** at ZT12. The amplitude of s**Per3** expression was markedly lower (P < 0.05) than that of s**Per1** and s**Per2**. The sense (control) riboprobes of the three s**Per** had a reproducible background hybridization that did not overlap with the antisense probe. No rhythmic expression with the sense ribobprobe hybridization intensity was noted.

**sPer Genes Exhibit a Diurnal Oscillation in Spalax Peripheral Tissues.** Significant expression of the three s**Per** genes was noted, using RT-PCR, in lung, intestine, liver, harderian gland, eye, brain, and skeletal muscle (data not shown).
A: sPer(s) expression in the EYE

For all three Per genes of Spalax are present in the photoperiodic retina, the site of light detection.

The Spalax Harderian Gland. Expression maxima in the harderian gland could be observed by quantitative RT-PCR (Fig. 3B) and ISH (data not shown) at the following ZTs: sPer1 at ZT6 and sPer2 and sPer3 at ZT12. Quantitative RT-PCR analysis in the liver (Fig. 3B) revealed rhythmic expression of sPer genes with maxima of sPer1 at ZT12 and of sPer2 and sPer3 at ZT18. The oscillation in the Spalax liver, as in its eye, shows a 6-h delay compared with the Spalax SCN. However, the circadian rhythm in the Spalax harderian gland is synchronous with the expression pattern in the Spalax SCN.

The Circadian Oscillation of sPer Genes Is Maintained in Constant Darkness. sPer gene RNA levels in the SCN, eye, and harderian gland were studied at four time points over a 24-h period, on the second day in constant darkness (not shown). ISH revealed that RNA levels of all three sPer genes were rhythmic and the oscillation pattern under constant darkness was similar to that under 12-h light/12-h dark conditions. Highest levels were observed during the subjective day in the SCN at circadian time 6 for sPer1 and circadian time 12 for sPer2 and sPer3. The peak levels of mRNA in the eye were 6 h later than in the SCN, but were synchronized with the SCN in the harderian gland. The amplitude of sPer3 rhythmicity was markedly lower than that of sPer1 and sPer2 in all three tissues studied and only nearly significant (P > 0.05).

Differential Light Regulation of Spalax Per Genes. Previous studies have shown that mPer1 and mPer2 expression in the SCN is induced by exposure to light at night (39, 40), whereas mPer3 is unaffected (41). We examined inducibility of the sPer genes in the SCN, eye, and harderian gland by nocturnal light pulses at ZT14 and ZT22 (Fig. 4). These time points were chosen for study as light pulses at these times produce phase delays and advances in locomotor activity.

Quantitation of the in situ results showed that 1 h after a light pulse at ZT14, sPer1 and sPer2 were significantly induced in all three tissues. Remarkably, the level of sPer1 induction in the harderian gland was significantly (P < 0.05) higher than in the SCN or the eye, reinforcing its great importance for the Spalax clock. One hour after the light pulse at ZT22 only sPer1 was significantly induced in the three tissues examined. Like the sClock gene, sPer3 gene was not light inducible either at ZT14 or ZT22 in the three tissues tested.

Discussion

Adaptive Selection on Per Genes in Spalax to Life Underground in Total Darkness. Like other mammals, the subterranean blind Spalax has three Per genes.

The distances between sPer1 and sPer2 and those of other rodents or humans are as expected from their divergence time, which is estimated to be 40 million years ago and 80 million years ago, respectively (6). The distances between Spalax or mice and humans are similar, as expected. Generally, the distances calculated for Per2 are larger than the distances for Per1. In our analysis of synonymous vs. nonsynonymous substitutions we relied on Liberles et al. (42), who suggested using this ratio to reveal selection for change in enzymatic function. Data of Mackalowski and Boguski (43) show that most rodents and human sequences have a ratio of 0.2. This finding indicates that such proteins, selected over millions of years, attained an optimum function before the divergence of rodents and primates and subsequent evolution was relatively conservative. They also considered ratios between 0.6 and 1.0 as suggesting a relaxation of functional constraint and selection. Our results show that sPer1 and sPer3 have a ratio of 0.4 to 0.6 and sPer2 has a ratio of about 0.2. Hence, presumably, sPer2 has not been changed to
function in a visually blind mammal living in a dark environment with negligible light cues. However, the figures obtained for sPer1 and sPer3 may suggest that molecular changes in these genes were necessary to fulfill their expected adaptive function in darkness. If we combine the calculated distances and the high ratio of nonsynonymous substitutions, the result supports the hypothesis of adaptive changes caused by natural selection, possibly in response to life in darkness underground.

sPer3 evinces a different situation. First, this locus in Spalax underwent major changes of deletions and insertions, resulting in two isoforms exhibiting truncated coding regions; somewhat similar results were reported for the hPer4 pseudogene (44). However, sPer3 is very different from hPer4. Its insertion could not be identified with any known sequence, in contrast to the fossil MER-2 mobile element that is within the hPer4 locus (44). When the changes in the sPer3 are omitted, an ORF similar to mPer3 is obtained. When the distances were calculated from the aligned shortened sequences the phylogenetic expectations were not met. The distances between sPer3 and mPer3 are similar or even larger than the distances between sPer3 and hPer3. This finding may support adaptive selective changes in the evolution of this gene and not just the neutral accumulation of substitutions over time. The comparison of sPer3 expression patterns with sPer1 and sPer2, which was described here and is discussed below, raises questions as to the role that sPer3 plays in the Spalax circadian system.

The Functional Circadian Domains of Spalax Per Genes. sPer1 and sPer2 contain all functionally relevant domains discovered in other mammalian PER proteins so far. The basic helix–loop–helix motif as well as the PAS A, PAS B (39, 45), and PAC CK1ε binding and phosphorylation sites (38) are conserved, suggesting their role as a CK1ε substrate in the central mechanism of the Spalax circadian clockwork. Remarkably, the whole putative CK1ε binding site as well as the N-terminal basic helix–loop–helix motif are missing in sPer3. This finding supports a speculation about its role in the biological clock (45).

Because Spalax is visually blind and lives entirely underground, hence denied outside Zeitgeber information (6), a robust and precise internal clock is necessary for the animal to keep track of time under negligible light cues. Indeed, in situ data revealed that Spalax Per genes’ expression is clearly rhythmic and maintained under constant conditions and in constant darkness. sPer1 and sPer2 expression in the Spalax brain is concentrated mainly in the SCN. However sPer3 is widely spread in different areas of the brain, similar to what has been reported for the mouse Per3 (41), and its oscillation levels are less prominent than those of sPer1 and sPer2. These findings raise a question as to the role of Per3 in the circadian system. The central pacemaker of the SCN signals time to the retina and peripheral clocks, as in the liver, where the circadian genes’ expression follows its rhythm with a delay of 4–6 h (41, 46). The blind Spalax Per genes’ expression is similar, and our results also show a lag of 6 h in the peak expression of the Per genes in the retina and liver. Although the exact role of Spalax Per3 in the maintenance of the circadian rhythm remains unresolved, it may prove substantial for time keeping underground.

The Harderian Gland: A Circadian Center in Spalax. Note worthy, mRNA levels in the Spalax hypertrophic harderian gland oscillate synchronously with the SCN. Similar synchronization in the peak levels of mRNA in the SCN and the harderian gland was seen also for Spalax MOP3 gene (8), the dimerization partner of the CLOCK protein (47) and an essential component of the circadian pacemaker in mammals (48). Furthermore, sPer1 inducibility in the harderian gland after light pulse during the dark phase of the circadian cycle is much higher than in the SCN. The harderian gland of Spalax is extraordinarily enlarged and occupies the entire eye socket, presumably as an adaptation to life underground. Previous studies (15) suggested that the harderian gland of Spalax is a possible photoreceptor and photoperiodic organ. Given its exposed position directly behind the atrophied minute eye, it seems likely that the harderian gland has a prominent role in the Spalax clock mechanism. This gland may demonstrate the extreme of evolutionary progression during the adaptive morphological and molecular reorganization for life underground (6).

Differential Regulation of Spalax Per Genes. Light inducible experiments in Spalax reveal that sPer1 is light inducible both early (ZT14) and late (ZT22) at night, whereas sPer2 is light inducible only at the beginning of the night. This result is in accordance with the findings of Albrecht et al. (39). sPer3 levels are unresponsive to light pulses applied throughout the dark phase of the circadian cycle. This differential regulation among the three sPer genes suggests that each has a different regulatory function in the SCN. The behavioral effects of photic stimuli at these two time points (ZT14 and ZT22) have been characterized in mice (49, 50). Light pulse at ZT14 causes phase delays in behavioral rhythms and light pulse at ZT22 causes phase advances. Our results indicate a role of sPer1 in both phases and of sPer2 in the phase-delay mechanism. Our in situ data provide a molecular confirmation of previous
behavioral studies in Spalax (11) and link the activity pattern of these species with the cellular cycling in sighted animals. The poor sPer3 oscillation and the absence of light influence on sPer3 expression levels may suggest a role for sPer3 outside the central pacemaker. This finding is consistent with data from Per3-deficient mice (51, 52) but may prove an adaptation to life underground and deserves further critical studies.

All three Spalax Per genes are expressed in a wide variety of nonneural tissues as previously shown in Drosophila (53, 54) and mice (39, 41). In three of these tissues (liver, eye, and hardier gland) with the RNA levels for the three sPer genes oscillate. Oscillation of sPer genes in the eye, the target organ of light absorption, is rational. As we already suggested, the oscillation of the sPer genes in the Spalax hardier gland is in accordance with previous results, suggesting an important role of this tissue in circadian maintenance (15) and merits further intensive study. The oscillation in the liver and the widespread expression in other peripheral tissues that were examined suggest the existence of clocks outside the SCN.

Molecular-Genetic Tinkering of Circadian Genes in a Blind Mammal. This study substantiates the claim that the blind subterranean Spalax needs a photoperiodic system to perceive daily and seasonal cycles. It has retained a functional retina with effective visual pigment genes signaling to the SCN (14, 17–20, 23, 24) presumably induced by the small amount of photons that penetrate underground to an otherwise dark environment. Circadian genes in the retina, hardier gland, SCN, and other tissues, including Clock (8) and the three Per genes described here, process the light signals and translate them into the unique behavioral repertoire of Spalax, including polyphasic, polymorphic, and seasonal behavioral phenotypes. In this respect we should emphasize that Spalax exhibits naturally occurring predominantly either diurnal or nocturnal individuals. Currently we are studying the expression pattern of the Per genes in naturally occurring nocturnal animals or on diurnal animals after a phase shift of light. The mosaic evolution of the Spalax eye (17, 18), hardier gland (30), and brain (26–28) and its circadian genes provide a striking model of tinkering evolution at both the molecular and organismal levels. From an evolutionary perspective the genetic basis of circadian rhythms in the blind subterranean Spalax may be different from that of strictly diurnal or nocturnal and sighted mammals. Identification of the circadian genes of blind Spalax might advance insights into the structure and evolution of the circadian organization in mammals, including humans, at the molecular level and their ecological causation. Remarkably, the colonization of the subterranean dark ecological zone by Spalax did not obliterate the conservative circadian rhythmicity and its genetic basis of photoreceptiveness and clock genes. All of the circadian machinery was adaptively transformed to perceive light in darkness.

We thank Dr. Z. S. Sun for his initial help with the cloning of the Spalax Per1 probe. We are also grateful to Mr. Michael Margulis for his help with the computer graphic work. This work was supported by the Max Planck Society and Deutsche Forschungsgemeinschaft Grant AL549/1-1 (to U.A.) and the Ancell-Teicher Research Foundation for Genetics and Molecular Evolution (to E.N.).