Expression of the orphan nuclear receptor *ERR α* is under circadian regulation in estrogen-responsive tissues

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Abstract

Circadian gene expression has been demonstrated in many tissues and involves both positive and negative regulatory loops. The potential interferences of circadian rhythmicity with other well-known biologic rhythms, such as the ovarian cycle, at least in part controlled by estrogens, has not been questioned. The estrogen receptor-related receptor (*ERR*) α is an orphan nuclear receptor that is widely expressed in estrogen-responsive tissues such as liver, uterus and bone. In addition, expression of the *ERR α* gene has been proposed to be transcriptionally controlled by estrogens in the uterus. Here we show that the expression of *ERR α* displays a circadian rhythmicity in liver, bone and uterus. This is in contrast to other uterine estrogen-regulated genes. Analysis of clock/clock mutant mice shows that *ERR α* is an output gene of the circadian clock oscillator. The expression of clock-control genes, such as *Bmal1* and *Rev-erba*, also displays diurnal oscillations in the uterus, but not in bone. In this tissue, however, *Per2* displayed a rhythmic expression, altogether suggesting unconventional loops in the regulation of circadian rhythm in bone.

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Introduction

Circadian regulation of gene expression is a general phenomenon that has been demonstrated in many organisms ranging from plants to animals (Albrecht & Eichele 2003, Schultz & Kay 2003). It is thought to help organisms anticipate the cyclic changes in the ambient environment by promoting adequate responses in terms of physiological and behavioral processes. In mammals, daily light signals are conveyed from the retina to the suprachiasmatic nuclei (SCN), where they reset a molecular clock. The SCN then signals to peripheral organs, such as the liver, where autonomous circadian clocks have been demonstrated. The peripheral clock present in the liver can also be reset by humoral signals connected to food availability (Damiola *et al.* 2000, Stokkan *et al.* 2001). The molecular clock itself comprises a positive limb involving CLOCK and

BMAL proteins that promote transcriptional activation of the members of the negative limb such as *Per* and *Cry* genes (reviewed in Schibler & Sassone-Corsi 2002, Albrecht & Eichele 2003, Fu & Lee 2003, Van Gelder *et al.* 2003). In turn, the products of these genes repress the activation driven by the CLOCK–BMAL heterodimeric complex. This feedback loop also regulates the rhythmic expression of the *Rev-erba* orphan nuclear receptor, which downregulates the expression of *Bmal1* and thereby contributes to the robustness of the oscillation (Preitner *et al.* 2002). The deficiency in one or several of these core genes in natural or engineered mouse mutants results in dramatic consequences concerning the rhythmicity of circadian activity and/or rhythmic gene expression (for examples, see van der Horst *et al.* 1999, Bunger *et al.* 2000, Oishi *et al.* 2000, Preitner *et al.* 2002, Oster *et al.* 2003).

The mechanisms by which the molecular clock cycles has thus recently gained understanding, and output genes (that is, genes that are regulated by but are not regulating the central clock) have been identified by cDNA array technology (for example, see Grundschober *et al.* 2001, Panda *et al.* 2002, Ueda *et al.* 2002; reviewed in Delaunay & Laudet 2002). However, very few pathways that precisely connect circadian oscillators to physiological outputs have been established.

It has long been recognized that the circulating levels of diverse hormones vary according to a day-to-night rhythm. For instance, mammalian blood concentrations of testosterone and estrogens are maximum during the late part of the night, whereas follicle-stimulating hormone (FSH)/luteinizing hormone (LH) levels peak in the afternoon (Krajnak *et al.* 1998, Ankarberg & Norjavaara 1999, Mitamura *et al.* 2000). Conversely, estrogens and testosterone, as shown by heterochronic exposure, influence the circadian rhythm in rodents by modulating their physical activities (Morin *et al.* 1977, Jechura *et al.* 2000). However, the circulating levels of various hormones are also regulated according to the ovarian cycle (lasting 4 days in mice). Superimposition of circadian and ovarian cycles has been shown for the expression of pituitary-expressed FSH/LH, which is maximum in the afternoon of the day of proestrus under the influence of SCN-produced vasoactive intestinal polypeptide (Krajnak *et al.* 1998). The situation is less clear in the uterus, which is a typical estrogen-responsive tissue. Indeed, sex hormones, particularly estrogens, exert profound cyclic influences on this organ, in which they are responsible for proliferation and differential gene expression, in preparation for the conceptus implantation. The expression of clock controlling genes has been demonstrated in the uterus (Johnson *et al.* 2002), but it is not known whether their mRNA oscillates, as it does in other peripheral tissues. The existence of a peripheral clock in the uterus has therefore not been formally demonstrated. This remark also holds for bone, another tissue that is highly sensitive to estrogens, although it does not undergo cyclic proliferation and differentiation. Nevertheless, bone supports an equilibrium between formation and resorption, the rate of which has been shown to be more elevated during the night (Srivastava *et al.* 2001, Siebel *et al.* 2002 and references therein).

The estrogen receptor-related receptors (ERR) belong to the nuclear receptor superfamily of ligand-dependent transcription factors (Giguère *et al.* 1988, Hong *et al.* 1999; for reviews, see Giguère 2002, Laudet & Gronemeyer 2002). In contrast to the estrogen receptors to which they are structurally related (hence their names), their transcriptional activities are not regulated by estrogens nor by any identified natural ligand (see Horard & Vanacker 2003 for a review). However, the expression of *ERRα* has been suggested to be regulated by estrogens in mouse uterus (Liu *et al.* 2003). *ERRα* is expressed in a variety of tissues, during both embryonic development and adult life. For instance, its mRNA has been detected in liver, uterus and bone, both in the embryo and in the adult (Giguère *et al.* 1988, Bonnelye *et al.* 1997a). In the latter tissue, *ERRα* has been suggested to exert a role in controlling proliferation and differentiation *ex vivo* (Bonnelye *et al.* 2001), and it activates the expression of osteopontin, an osteoblastic differentiation marker (Vanacker *et al.* 1998). Furthermore, *ERRα* might control bone mineral density *in vivo* (B Horard and J-M Vanacker, unpublished).

In this report, we show that *ERRα* mRNA expression oscillates in liver. The circadian nature of the variations of *ERRα* expression was assessed using *Clock* mutant mice, which display constant *ERRα* mRNA levels over time. The expression of *ERRα* in the uterus was also found to be circadian, as was that of *Bmal1* and *Rev-erba*. Surprisingly, these two genes were not cyclically expressed in bone. However, oscillations of *ERRα* and *Per2* expression demonstrate the existence of a circadian rhythm at the level of mRNA expression in this tissue.

Materials and methods

Animals

Female OF1 mice aged 6 weeks (Charles River Laboratory, Lyon, France) were entrained on a L13/D11 cycle for 1 week before the day of experiment. For light-dark (LD) experiments, animals were housed in the same conditions. For constant darkness (DD) conditions, animals were transferred to DD at the start of the normal photoperiod (corresponding to CT0). Animals were killed by cervical disruption at 4h intervals ($n = 3-4$).

Hind limb long bones were flushed with PBS to eliminate the bone marrow. Tissues were immediately frozen in liquid nitrogen after collection. Organs were mechanically minced in liquid nitrogen, and RNAs were purified by guanidinium thiocyanate/phenol/chloroform extraction. RNA samples extracted from male clock mutants and wild-type littermates were a generous gift of Joseph S Takahashi (Northwestern University, Evanston, IL, USA).

DNA microarray experiments

Livers from male C57Bl/6 mice kept on an LD 12/12 cycle were dissected at 4h intervals from zeitgeber time (ZT) 0 to ZT20. For each time point, liver samples from ten animals were pooled, and total RNA was prepared. For each sample, cDNA synthesis, biotin labeling of cRNA and hybridization to murine U74Av2 Genechips were performed in duplicate according to the recommendations of the manufacturer (Affymetrix, High Wycombe, UK). Gel files were condensed by using MAS4 algorithm (Affymetrix). Genes exhibiting an average differential intensity above 2 and a temporal variation in expression were identified after ANOVA with a significance threshold set at $P < 0.01$, using the GenAnova software. *ERRα* was represented by two probe sets (102145_f_at and 103964_at) that gave similar expression profiles.

RT-PCR

An amount of 2 µg total RNA was DNaseI-digested and retrotranscribed in a final volume of 80 µl with MMTV retrotranscriptase (Fermentas, Souffelweyersheim, France) under the conditions recommended by the supplier. A volume of 2 µl of the reaction was submitted to quantitative PCR, using specific primers and the SYBR-Green PCR kit (Qiagen). Reactions were performed several times in duplicate on an Opticon apparatus (VWR, Illkirch, France). Most results were also confirmed on a Light Cycler apparatus (Roche).

The PCR cycle used was as follows: enzyme activation – 95 °C, 15 min; cycles – 95 °C, 15 s; 50 °C, 25 s; 72 °C, 18 s (45 times).

The specificity of the amplification was controlled by the fusion temperature of the amplicons. The size of the products was checked on agarose gels.

Oligonucleotides were designed with the Primer3 program available at the Whitehead Institute web site (www-genome.wi.mit.edu). Each oligonucleotide within a pair hybridizes on a different exon. The sequences of the oligonucleotides were as follows:

ERRα: CAAACGCCTCTGCCTGGTCT and ACTCGATGCTCCCCTGGATG

36B4: ACCTCCTTCTTCCAGGCTTT and CCCACCTTGTCTCCAGTCTTT

Rev-erba: TGGAAGACAGCAGCCGAGTGT and CATAGTGGAAGCCTGAGGCCA

Bmal1: CAGAGCCGGAGCAGGAAAAA and ACAGCTGGAAGGCGATGACC

Per2: ATCAACCCGTGGAGCAGGAA and GGGAGCTGCGAACACATCCT

Opn: TCTCCTTGCGCCACAGAATG and TCGTCCATGTGGTCATGGCT

Statistical analysis

Relative cDNA amounts in each sample were determined by the $\Delta\Delta CT$ method (using the constant 36B4 gene as an internal reference). Average values within a time group were plotted, and S.E.M. values were calculated. ANOVA analyses were used to compare each set of data. Variations were significant with at least $P < 0.05$.

Results

Genome-wide analysis of rhythmic gene expression in male mouse liver using high-density oligonucleotide DNA microarrays identified the orphan nuclear receptor *ERRα* as a putative target of the circadian clock. *ERRα* showed a rhythmic expression pattern with trough and peak levels at ZT4 and ZT12–16 respectively (Fig. 1A). This cyclic expression was confirmed by quantitative PCR (QPCR) experiments performed on RNA extracted from an independent set of female animals bred in a 13h light:11h dark cycle (LD13:11; Fig. 1B left panels). As a marker of circadian clock activity, we used the *Rev-erba* gene that displays a robust oscillation in liver (Pineda-Torra *et al.* 2000, Preitner *et al.* 2002), and for which an expression peak was observed at the expected ZT8. To determine whether the cyclic expression of *ERRα* was dependent on an endogenous clock, animals kept in DD conditions (Fig 1B, right panel) were

Figure 1 Rhythmic expression of *ERRα* in mouse liver. (A) cDNA array experiment. Male animals ($n=10$) set in light/dark (LD) conditions were killed every 4 h. RNA extracted from livers was pooled and submitted to microarray experiments. Shown is the expression of *ERRα* as a function of zeitgeber time (ZT). Error bars indicate the variations between duplicate experiments. (B) QPCR experiments. Female animals ($n=3-4$) set in LD (left panels) or DD, (right panels) conditions were killed every 4 h. Q PCR was performed on individually retrotranscribed liver RNA to amplify the indicated genes. Expression levels, relative to the expression of the 36B4 gene, are plotted as a function of ZT or circadian time (CT), and expressed $\pm$ S.E.M. White, shaded and black bars at the top of the graphs indicate objective day, subjective day and night respectively.

Liver; D/D

Figure 2 Circadian expression of *ERRα* in the liver is clock-dependent. Details same as for Fig. 1B, using livers from male wild-type (squares) or *clock*^{-/-} animals (triangles) set in DD conditions.

analyzed. For both *ERRα* and *Rev-erba*, we observed the same temporal expression pattern as in LD animals, indicating that *ERRα* is a likely target of the circadian clock in liver.

The mutation of the *Clock* gene in mice results in an arrhythmic molecular and behavioral phenotype (King *et al.* 1997). To determine genetically whether *ERRα* is a clock-controlled gene, we analyzed its expression in livers of *Clock*-deficient mice set in DD conditions (Fig. 2). In these animals, *ERRα* expression did not vary as a function of time, whereas wild-type littermates displayed the expected rhythmicity. As a positive control, the circadian expression of *Rev-erba* observed in wild-type animals was abolished in *Clock*-deficient mice. These data indicate that *ERRα* is downstream of CLOCK. However, the CLOCK-BMAL heterodimer did not exert any regulatory activity on mouse *ERRα* promoter when assayed in transient transfections (data not shown), suggesting an indirect control.

The complex pattern of expression displayed by mouse *ERRα* includes the uterus (Bonnelye *et al.* 1997a), an organ that is highly sensitive to the cyclic hormonal status. The circadian expression of *ERRα* in male and female liver therefore offers a means to study the rhythmicity of gene expression in the uterus. We analyzed the expression of *ERRα*, using RNAs extracted from mice set in LD or DD

conditions (Fig. 3). In both cases, *ERRα* expression was found to vary over time. Expression in DD uterus reaches its maximum at ZT12 (identical to the one observed in the liver), whereas LD animals surprisingly displayed an expression peak at ZT16 (that is, 4 h later than in the liver). In contrast, expressions of both *Rev-erba* and *Bmal1* were found to peak at identical ZT (8 and 4 respectively), independently of light conditions.

ERRα has been shown to be expressed in both embryonic and adult bones, and hypothesized to play an important role in the development and/or homeostasis of this tissue (Bonnelye *et al.* 1997b, 2001). Bone is also a hormone-responsive tissue that excretes degradation products in a circadian manner (Srivastava *et al.* 2001). However, circadian regulation of gene expression in bone remains to be demonstrated. We observed that *Rev-erba* or *Bmal1*, two robust markers of circadian clock activity, did not display any cyclic expression in this tissue (data not shown). In contrast, *ERRα* exhibited a circadian expression pattern in bone with a peak at ZT12 in mice kept under LD or DD conditions (Fig. 4). *Osteopontin* (*opn*, encoding an extracellular matrix protein involved in bone remodeling) has been proposed as an *ERRα*-target gene in bone (Vanacker *et al.* 1998). We therefore hypothesized that *opn* expression should follow that of *ERRα*. Analysis of *opn* expression indeed revealed a

Uterus; L/D

Uterus; D/D

Figure 3 Circadian gene expression in mouse uterus. Details same as for Fig. 1B, using RNA extracted from uterus.

rhythmic pattern, with a maximum at ZT16. This finding suggests that *ERRα* is a mediator of *opn* circadian expression in bone. The rhythmicity of *ERRα* expression in bone also suggests the existence

of a molecular clock in this tissue. *Per2* was indeed found to be expressed in a circadian manner, peaking at ZT12, and thus revealing the existence of a peripheral clock in bone.

Figure 4 Circadian expression of *ERRα* in mouse bones. Details same as for Fig. 1B, using RNA extracted from bone-marrow-free long bones.

Discussion

Circadian regulation of *ERRα* expression

Circadian regulation of *ERRα* was observed by two different methods, namely, cDNA arrays and QPCR. Moreover, independent sets of animals, bred in different laboratories, displayed the same temporal expression, at least in liver. No sexual dimorphism was observed in the *ERRα* liver expression pattern. Further evidence in favor of *ERRα* as a clock-controlled gene is adduced (i) by the study of animals kept under DD in which *ERRα* oscillations persist (therefore demonstrating that *ERRα* does not respond to light *per se*) and (ii) by the use of *Clock* mutant mice, in which expression of this gene is constant through time (demonstrating that *ERRα* oscillations are molecular-clock-dependent). Our data are consistent with the results published by Ueda *et al.* (2002), in which *ERRα* expression was also found to peak in both LD and DD livers at ZT and circadian time (CT) 13 respectively. Cyclic expression is not a general property of ERR subfamily members, since, for instance, *ERRγ* expression in liver does not vary with time (data not shown). *ERRα* expression also cycles in the uterus and in long bones. cDNA arrays studies on SCN, liver and heart have shown that, although about 10% of genes were found to oscillate in a given organ, only a subset of them are cyclically regulated in different tissues (Albrecht & Eichele 2003 and references therein). Cyclicity of *ERRα* uterine expression can be observed in mice kept under both LD and DD conditions. However, the time of maximal expression of this gene shows a 4 h delay in the LD uterus compared with liver and bone, a difference abolished in DD animals. Despite this unexplained difference between LD and DD mice, *ERRα* clearly displays a circadian regulation of expression.

Rat1 fibroblasts have been proposed as a cell-culture model for the study of circadian rhythm, since these cells exhibit oscillations of clock-control gene expression upon high-serum shock (Balsalobre *et al.* 1998, Rosbach 1998, Yagita *et al.* 2001). Under these conditions, we could detect such a pattern for *Rev-erba*, but not for *ERRα* (data not shown). However, only 2% of total genes are estimated to oscillate in Rat1 cells, whereas this number reaches about 10% *in vivo* (Panda *et al.* 2002, Albrecht & Eichele 2003).

Transient cotransfection experiments in COS cells did not reveal any regulation of a 1.3 kb

mouse *ERRα* promoter fragment by PER2, REV-ERBα or CLOCK-BMAL heterodimer, used either alone or in combination (data not shown). In contrast, the mouse *Rev-erba* promoter was regulated by CLOCK-BMAL (positively), REV-ERBα itself and PER2 (negatively), as expected (Preitner *et al.* 2002, Triqueneaux & Laudet, unpublished). Although an output gene of the molecular clock, *ERRα* is therefore not directly controlled by circadian proteins (in contrast to *Rev-erba*). The existence of a yet unknown intermediate or intermediates between the molecular clock and *ERRα* expression must be hypothesized.

Circadian rhythm and hormone-responsive tissues

Circadian oscillations of hormone levels in mammalian blood have long been recognized and have been linked to behavioral processes. For instance, the concentrations of various steroid hormones, such as testosterone, estrogens and glucocorticoids, peak in human blood during the later part of the night (Ankarberg & Norjavaara 1999, Gudmundsson *et al.* 1999, Mitamura *et al.* 2000). These elevations facilitate the renewal of physical activity during the early wake phase. Another example is the peak FSH/LH expression that occurs every afternoon in female mice, independently of the phase of the ovarian cycle (Krajnak *et al.* 1998). FSH/LH excretion is also upregulated during proestrus. Thus, on the afternoon of this particular day, maximum concentrations of gonadotropins are achieved that allow synchronization of ovulation (that is, the efficiency of the egg release from the ovary that occurs a few hours after the FSH/LH surge) with behavior (that is, mating in the middle of the night). The converse phenomenon, that is, hormones influencing circadian rhythm, has also been documented. For instance, in rodents, inappropriate treatment with estrogens or testosterone can perturbate the circadian rhythm of physical activity (Morin *et al.* 1977, Jechura *et al.* 2000). However, no explanation of this phenomenon has been proposed in terms of gene-expression regulation, although steroid hormones act as transcription modulators through their cognate receptor.

Circadian rhythm is mostly studied in male animals for fear of interference with the sexual

rhythm, which is much more influential in females. In particular, the uterus, a paradigm of hormone-sensitive organs, undergoes profound remodeling, implicating cellular proliferation and differential gene expression according to the levels of circulating sex hormones which reflect the phase of the ovarian cycle. On the other hand, most clock genes are expressed in the uterus (Johnson *et al.* 2002), but their rhythmicity has not been demonstrated. Our data show that the mRNA of two of these genes, *Bmal1* and *Rev-erba*, oscillate in a manner comparable to what can be observed in the liver, demonstrating the existence of an autonomously functioning clock in the uterus.

It is unlikely that the expression peaks of these genes reflect the sex hormone status rather than autonomous circadian control. Indeed, QPCR experiments demonstrated that the expression of various genes regulated according to the stage of estrous cycle, such as *Wnt7a* (Miller *et al.* 1998) and *PR* (progesterone receptor) (Punyadeera *et al.* 2003 and references therein), does not significantly vary between the mice groups that we studied (data not shown). This is also the case for *ERα* (estrogen receptor alpha), in both uterus and bone. This suggests that no time group is a particular phase of the ovarian cycle. Bone expression of *ERα* is also not regulated in a circadian way (data not shown). *ERRα* has been suggested to be an estrogen-target gene (Liu *et al.* 2003). However, our unpublished experiments with immature or mature mice failed to detect any effect of estrogen treatment on *ERRα* expression in uterus and bone (data not shown). Furthermore, the timing of the estrogen peak (ZT 20–24; Mitamura *et al.* 2000), as opposed to that of *ERRα* expression (ZT12–16), renders it unlikely that *ERRα* oscillations are mostly controlled by estrogens. It could still be that circadian- and sex hormone-induced gene regulation of *ERRα* overlap. This has, for instance, been shown for FSH/LH expression, which is regulated both by the sex hormone status (maximum in proestrus) and by circadian rhythm through vasoactive intestinal polypeptide produced in the SCN (Krajnak *et al.* 1998).

Circadian rhythm in bone

Most tissues and organs contain an endogenous, autonomously functioning clock that is synchro-

nized by signals emanating from the SCN. To date, only the testis has been shown not to support any circadian clock, although contradictory results have been published (Zylka *et al.* 1998, Morse *et al.* 2003). In this respect, bone has never been studied, although indications exist in this direction. Indeed, the rates of bone formation and resorption are enhanced during the night (Seibel *et al.* 2002 and references therein). Moreover, the blood concentration of markers of bone formation and resorption, such as osteocalcin and alkaline phosphatase, varies according to the nycthemeral cycle. However, it is not known whether expression or excretion of these products is involved in this regulation. Again, no molecular study has been performed to demonstrate the existence of rhythmic gene expression. We found comparable profiles of temporal *ERRα* expression in both bone and liver, in which clock dependence clearly proves the circadian nature of *ERRα* gene expression. Moreover, the *opn* gene that has been suggested as an *ERRα*-target gene in bone (Vanacker *et al.* 1998) is also expressed in a circadian manner, and its expression peak immediately follows that of *ERRα*.

We also examined the temporal expression profiles of various clock components in bone. Our analysis revealed an absence of rhythmicity for *Bmal1* (which was barely detectable) and *Rev-erba* (although present in amounts comparable to those of the liver) (data not shown). Paralogs of these two genes, *Mop9/Bmal2* (Hogenesch *et al.* 2000, Okano *et al.* 2001) and *Rev-erbβ* respectively, were undetectable in our QPCR experiments (data not shown). This renders unlikely a functional compensation between paralogs, as has been found for NPAS2, which, in the forebrain, substitutes for its homolog CLOCK (active, for example, in the SCN) in dimerizing with the BMAL product, and fulfills equivalent functions (Rieck *et al.* 2001, Dudley *et al.* 2003). Essential components of the molecular clock are thus either not expressed or evenly expressed in bone. Yet an autonomous although unconventional clock oscillates in bone, as demonstrated by the oscillations of *Per2* (as a clock-controlling gene), *ERRα* (as an indirect output gene) and *opn* (as a possible consequence of *ERRα* rhythmic expression) mRNA levels. The study of the regulation of *ERRα* expression in bone might help to uncover the components of the circadian clock in this atypical tissue.

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