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A Molecular Switch for Photoperiod Responsiveness in Mammals

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Summary

Seasonal synchronization based on day length (photoperiod) allows organisms to anticipate environmental change. Photoperiodic decoding relies on circadian clocks, but the underlying molecular pathways have remained elusive [1]. In mammals and birds, photoperiodic responses depend crucially on expression of thyrotrophin (β subunit RNA (TSHβ)) in the pars tuberalis (PT) of the pituitary gland [2–4]. Now, using our well-characterized Soay sheep model [2], we describe a molecular switch governing TSHβ transcription through the circadian clock. Central to this is a conserved D element in the TSHβ promoter, controlled by the circadian transcription factor thyrotroph embryonic factor (Tef). In the PT, long-day exposure rapidly induces expression of the coactivator eyes absent 3 (Eya3), which synergizes with Tef to maximize TSHβ transcription. The pineal hormone melatonin, secreted nocturnally, sets the phase of rhythmic Eya3 expression in the PT to peak 12 hr after nightfall. Additionally, nocturnal melatonin levels directly suppress Eya3 expression. Together, these effects form a switch triggering a strong morning peak of Eya3 expression under long days. Species variability in the TSHβ D element influences sensitivity to TEF, reflecting species variability in photoperiodic responsiveness. Our findings define a molecular pathway linking the circadian clock to the evolution of seasonal timing in mammals.

Results and Discussion

Circadian and Photoperiodic Influences on Pars Tuberalis TSHβ Expression

Recently it has become clear that the pars tuberalis (PT) region of the anterior pituitary gland is a master controller of seasonal biology in mammals and birds [5]. Cells in the PT produce thyroid-stimulating hormone (TSH, also known as thyrotrophin) at levels that are increased by exposure to long days and suppressed by short days [2–4]. This photoperiodic control is mediated through changes in TSHβ subunit RNA (TSHβ) expression. PT-derived TSH acts by a retrograde mechanism on TSH receptor-expressing cells in the neighboring basal hypothalamus, which are the principal sites of type 2 thyroid hormone deiodinase (Dio2) expression in the hypothalamus [6]. This enzyme is a gatekeeper for the effects of thyroid hormone in the hypothalamus, controlling the availability of the active form of thyroid hormone, triiodothyronine, and dictating the expression of summer phenotypes [7, 8].

A unique feature of mammalian seasonal biology [2–5] is that these effects of day length on PT function depend on the pineal hormone melatonin, which acts via a high density of melatonin receptors localized in the PT [9, 10]. Melatonin is a circadian signal, with a nocturnal waveform proportional to the length of the night, and consequently, in the PT, melatonin controls the rhythmical expression of multiple transcription factors implicated in circadian function [11–15]. Hence, we hypothesized that photoperiodic effects on TSHβ expression in the PT are initiated through melatonin’s effects on circadian transcription factors in this tissue.

Circadian gene expression depends on three main classes of canonical circadian response element: E boxes (CANNTG), D elements (RTTAYGTAAY), and retinoid-related response elements (ROREs; WAWNTRGGTCA), each of which are regulated by cognate transcription factors [16]. Examination of the TSHβ promoter revealed a highly conserved D element located a short distance from the transcription start site (Figure 1A; see also Figure S1A available online), potentially sensitive to the PAR-bZIP family members thyrotroph embryonic factor (TEF), D element-binding protein (DBP), and hepatic leukemia factor (HLF), which are considered important outputs of the circadian clock [17]. In luciferase reporter assays, using −3 kb of the sheep TSHβ promoter (TSHβ-luc), we observed transcriptional sensitivity to each of these factors (Figure 1B), and consistent with the original assignment of TEF as a trans-activator of the TSHβ promoter [18], the order of potency of these effects was TEF > HLF > DBP. Moreover, in electrophoretic mobility shift assays, TEF bound to a 35 bp oligonucleotide centered on the TSHβ D box more strongly than either HLF or DBP (Figure 1C). Although several E boxes are also present in the proximal 3 kb of the TSHβ promoter region, these are poorly conserved between species and do not respond to the core circadian transcription factors CLOCK and BMAL1 in the sheep (Figure S1B). No consensus RORE could be found.

We next considered whether Tef gene expression is photoperiodically regulated in the PT and, if so, whether these changes precede photoperiodic induction of TSHβ expression. We used a long-day induction protocol developed previously to study seasonal neuroendocrine changes in Soay sheep [19]. This involves acclimating sheep to short photoperiod (SP, 8 hr light/16 hr dark) and then switching them to long photoperiod (LP, 16 hr light/8 hr dark) by delaying lights off by 8 hr (Figure 1D). This acutely shortens the melatonin signal, delaying its evening rise by 8 hr [19]. Stimulation of increased TSHβ immunoreactivity in the PT, hypothalamic Dio2 expression, and plasma prolactin levels ensues during the subsequent 15 days (Figures S1C–S1E). The expression of TSHβ RNA increases rapidly (p < 0.001 by two-way analysis of variance [ANOVA]), so that levels across the 24 hr cycle (area-under-the-curve estimate) are some 2.5-fold above baseline by the third day of LP (LP3), and nearly 6-fold increased by
gene expression in the PT can be found in Figure S1.

**Eya3 Is a Photoperiodically Induced Coactivator for TEF-Induced TSHβ Transcription**

The absence of a photoperiodic effect on the amplitude of Tef expression in the PT suggested to us that additional factors interact with TEF to control TSHβ expression in this tissue. Two recent studies highlight the transcriptional coactivator Eya3 as being strongly induced by increasing photoperiod in the PT [4, 20]. Eya3 is of particular interest because it is a member of a developmental regulatory network including the Pax and sine oculis (Six) gene families believed to be critical for the formation of structures including the eyes and pineal and pituitary glands [21–23]. Intriguingly, in the PT of the Japanese quail, expression of Eya3 rises in parallel with that of TSHβ following exposure to long days [4], hinting at a role early in the photoperiodic induction process.

We therefore compared the daily profiles of Eya3 and TSHβ expression in the sheep PT under SP and at LP3 and LP15 (Figure 2A). Under all three conditions, Eya3 showed a transient peak in expression approximately 12 hr following dark onset, with an amplitude that increased 3-fold by LP15 (p < 0.001 by two-way ANOVA for photoperiod × Zeitgeber Time (ZT) interaction). Under LP, peak Eya3 expression preceded that for TSHβ by some 4 hr, supporting the concept that EYA3 may be involved in transcriptional activation of TSHβ.

EYA3 is thought to act by dimerizing with SIX-family proteins, forming a transcriptional coactivator complex [21–23]. We therefore analyzed tissue expression patterns for the sheep orthologs of various Six family members by reverse transcriptase-polymerase chain reaction and in situ hybridization (Figures S2A and S2B). Whereas Six1 had a highly PT-enriched pattern of expression, other members were ubiquitously expressed (Six4), enriched in neighboring hypothalamic sites (Six6), or undetectable (Six2). Although Six1 expression in the PT was not rhythmic (Figure S2B), it increased slightly with LP exposure, so that mean levels were some 50% higher at LP15 than in SP controls (p < 0.05).
TEF/EYA3 Synergy and Seasonal TSHβ Expression

Returning to the TSHβ promoter, we identified a putative MEF3 site, predicted to mediate EYA3/SIX1 actions [24, 25], downstream of the D element (see Figure 1A; Figure S1A); this is also conserved across mammals. In further reporter assays, SIX1 had a mild potentiating effect on the actions of this is also conserved across mammals. In further reporter assays, SIX1 had a mild potentiating effect on the actions of wild-type (WT) EYA3 for potentiating TEF/SIX1 action (Figure 2C). No independent effects of EYA3 or SIX1 on promoter activity, increasing the response by an order of magnitude compared to that seen for TEF alone (Figure 2B). This effect of EYA3 appeared to be independent of its reported tyrosine phosphatase activity, because a phosphatase-dead EYA3 (pdEYA3) in which a single point mutation disrupts the catalytic domain (D263A; see [25]) showed a potency similar to wild-type (WT) EYA3 for potentiating TEF/SIX1 action (Figure 2C). No independent effects of EYA3 or SIX1 on TSHβ-luc activity were observed (Figure S2C).

Mutations of the putative MEF3 site present in the TSHβ promoter had a negligible effect on the combined TEF/SIX1 response (Figure S2D), supporting the concept that EYA3 and SIX1 act as coactivators of TEF rather than as an independently transcriptionally active heterodimer. In line with this, we found that mutating the D element to destroy the palindromic half-site organization suppressed both the direct effects of TEF and the potentiating effects of SIX1 (Figure 2D), suggesting that this element is crucial for photoperiodic regulation of TSHβ expression.

Circadian and Melatonin-Dependent Control of Eya3 Transcription

We next considered the mechanism whereby Eya3 expression is increased by the transfer to LP. Exploration of the Eya3 promoter demonstrated that it is controlled through three conserved E boxes sensitive to CLOCK and BMAL1 and through a conserved D element sensitive to TEF and SIX/EYA3, all located with 500 bp of the transcription start site (Figures 3A–3C; Figure S2E). This promoter organization accounts for the rhythmical expression of Eya3, which, like other rhythmic genes in the PT, is controlled by melatonin [14]. Melatonin onset at lights off is the major phase-resetting signal in this tissue, so that long days generate waves of gene expression in the PT peaking later relative to dawn than do short days [14]. We have shown previously that, in addition to setting the phase of gene expression rhythms in the PT, melatonin directly suppresses the expression of a range of E box-controlled genes [15]. Similarly, melatonin implants given to sheep acclimated to LP and then exposed to constant light suppress Eya3 expression (Figure 3D). These effects probably stem from melatonin’s suppression of cAMP signaling, because derepression of cAMP-dependent pathways following melatonin withdrawal at dawn is critical for the morning peak of Per1 expression seen in the PT [13]. We are currently investigating possible cAMP-dependent regulation of the Eya3 promoter.

These two classes of effect of melatonin on Eya3 expression, phase synchronization and direct suppression, lead to a molecular model linking the circadian system to the photoperiodic response (Figure 4). The model postulates that the phase of Eya3 expression in the PT is critical for determining whether a strong peak does or does not occur. Independent of day length, Eya3 peaks some 12 hr after dark and melatonin onset. This means that under short days, the peak occurs during the night, while the melatonin level is high and exerting a suppressive effect, and so the peak is small. Contrastingly, under long days, the Eya3 peak occurs the following morning when the melatonin level is minimal, and so the peak is large. This classic “external coincidence timer” mechanism, in which a circadian oscillation interacts with a light-dependent stimulus [1], limits EYA3/TEF synergism to long days, controlling the onset of a summer phenotype.

In addition to this primarily circadian photoperiodic induction process, we suggest that the further amplification of the Eya3 peak, seen between LP3 and LP10, may also be due to amplification through transcriptional autoregulation at the Eya3 promoter via TEF/EYA3/SIX effects through the D element (see Figure 3C). The Tef gene also includes a promoter sensitive to E boxes and D elements in combination (Figure S3), and recent work has identified Tef-driven, D element-mediated effects as crucial for light entrainment of the E box-driven period gene rhythmicity at the core of the zebrafish circadian
D element (Figure 5); variability at positions 3 and 4 is seen in the sheep-like variant. The weakest response occurred with the mouse-like form, which is a perfect eight-base palindrome, followed by the human-like form, suggestive of attenuated photoperiodic sensitivity. An intermediate level of response was seen for the pig, related to photoperiodic sensitivity through the TEF-dependent pathway. In this regard, it is noteworthy that the seasonal melatonin response pathway has been implicated in seasonal affective disorder [28], and it will be of interest to explore genetic variability in the human TSHβ promoter locus.

Figure 3. Eya3 Is Transcriptionally Controlled through E Boxes and D Elements
(A) The proximal region of the Eya3 promoter harbors one conserved D element and three conserved E boxes. (B) CLOCK and BMAL1 (C+B) and TEF have additive effects on a 0.5 kb Eya3-luc reporter in COS7 cells. (C) SIX1 and EYA3 potentiate the effects of TEF on Eya3-luc activity. (D) Eya3 expression is suppressed in LP-acclimated sheep exposed to constant light prior to melatonin administration 16 hr after lights on and then sacrificed 3.5 hr later. Data are mean ± SEM from n = 6 animals in each group. ***p < 0.001, significantly reduced expression relative to sham-injected control animals by independent t test. Evidence for combined E box/D element control of Tef expression is given in Figure S3.

Variation in TSHβ D Element Function Is Correlated with Photoperiodic Sensitivity
Our observations implicate the proximal D element in the TSHβ promoter as a convergence point in the transcriptional network controlling the photoperiodic response. Across mammals, there is pronounced variability in the strength of photoperiodic sensitivity across primates and in pigs, among which the expression is given

Figure 5. Comparative Differences in TSHβ D Element Function in Relation to Photoperiodic Sensitivity
Upper panel: comparison of the core sequence in the TSHβ D element across mammals reveals several variant forms exemplified by the species shown. Lower panel: COS7 cell reporter assay of TEF responsiveness, using the sheep TSHβ-luc reporter mutated to give the variant forms shown in the upper panel. Data are shown as relative luciferase units (RLU), normalized to control values in the absence of TEF, and are mean ± SEM of triplicate observations.

Figure 4. Model for Photoperiodic Induction of Eya3 Expression in the Pars Tuberalis
See text for details.
Conclusion

In conclusion, we have defined a pathway linking day length to seasonal biology via a circadian, melatonin-dependent pathway. This pathway is the mammalian form of an ancestral photoperiodic timer, and we predict that a similar clock-controlled, TEF/Six1/Eya3-dependent mechanism will emerge as the conserved driver of seasonal change in birds and other vertebrate groups, even though the importance of melatonin for this process appears peculiarly mammalian [5]. Within the mammalian photoperiodic system, circadian properties are seen at multiple levels, notably the suprachiasmatic nucleus and pineal gland [1], as well as in the melatonin-dependent PT. What makes the PT remarkable is that it behaves like a circadianly controlled photoperiodic switch—a derived function that appears to depend crucially on promoter organization of clock-controlled genes lying upstream in the TSH-Dio2 pathway. Synthetic biology approaches highlight the capacity for combinations of the basic classes of circadianly controlled promoter elements to dictate phase of expression of rhythmical gene expression [16]. We believe that nature’s tinkerer with this principle has been the key to the evolution of light entrainment and photoperiodic response pathways.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.cub.2010.10.048.

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