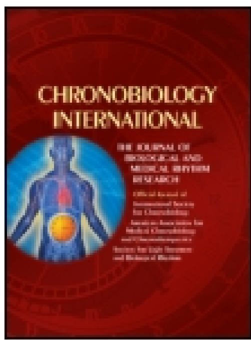

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REVIEW

# The Circadian E-Box: When Perfect Is Not Good Enough

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## ABSTRACT

Life on earth has evolved on a photic carousel, spinning through alternating periods of light and darkness. This playful image belies the fact that only those organisms that learned how to benefit from the recurring features in their environment were allowed to ride on. This selection process has engendered many daily rhythms in our biosphere, most of which rely on the anticipatory power of an endogenously generated marker of phase: the biological clock. The basic mechanisms driving this remarkable device have been really tough to decode but are finally beginning to unravel as chronobiologists probe deeper and wider in and around the recently discovered gears of the clock. Like its chemical predecessors, biological circadian oscillators are characterized by interlaced positive and negative feedback loops, but with constants and variables carefully balanced to achieve an approximately 24h period. The loops at the heart of these biological oscillators are sustained by specific patterns of gene expression and precisely tuned posttranscriptional modifications. It follows that a molecular understanding of the biological clock hinges, in no small measure, on a better understanding of the cis-acting elements that bestow a given gene with its circadian properties. The present review summarizes what is known about these elements and what remains to be elucidated.

*Key Words:* Review; Circadian transcription; E-box.

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## MOLECULAR CHRONOBIOLOGY: A PRIMER

Understanding how the circadian pacemaker works has been a serious challenge for many years. More recently, however, the decades-old knowledge base, a few breathtaking technological advances, and the imaginative tenacity of many investigators in the field have finally converged to generate a successful model. This conceptual framework can explain many of the observed properties of known clocks and guide us in the design of experiments to predict and test new ones.

Many excellent reviews have been written about the basic gears of the clock and how they are assembled to establish self-sustained and entrainable 24h rhythms (Reppert and Weaver, 2002; Takahashi, 1995; Weaver, 1998). For the purpose of this review it should suffice to describe a simplified version of the current (vertebrate) model. The BMAL and CLOCK (Bunger et al., 2000; Gekakis et al., 1998) helix-loop-helix (HLH) proteins can heterodimerize to transactivate circadian promoters featuring the right kind of regulatory elements (to be discussed later in finer detail). The negative arm is currently populated by proteins, such as the Periods (Tei et al., 1997; Zylka et al., 1998) the Chryptochromes (Griffin et al., 1999; Kume et al., 1999) and the Decs (Honma et al., 2002), which show time-dependent patterns of accumulation, interaction and posttranslational modifications and can suppress BMAL/CLOCK transcriptional activity (Honma et al., 2002; Kume et al., 1999; Lee et al., 2001). In addition, Per2 and the Cry proteins can enhance, directly or indirectly, the rhythmic expression of BMAL in mammals, closing a second positive feedback loop necessary to perpetuate the clock cycle (Shearman et al., 2000; Yu et al., 2002).

## THE APPEARANCE OF THE E-BOX IN CIRCADIAN TRANSCRIPTION

Modulation of gene expression has been typically regarded as a key event in the establishment of circadian rhythmicity. After all, many clock genes (CG) and clock-controlled genes (CCG) display robust oscillations in steady-state mRNA levels (Takahashi, 1995). These observations led naturally to the concept of a circadian *cis*-acting regulatory element, originally coined "circadian clock-responsive element" [CCRE; (Takahashi, 1995), or "time-box" (Ishida, 1995)]. Surprisingly, most of the ensuing evidence pointed to a commonly found hexameric sequence (CACGTG; the perfect E-box) as the target of the circadian clock effectors BMAL and CLOCK.

Hao et al. (1997) identified the first transcriptional circadian regulatory sequence (CRS) in an animal system. It consisted of a 69-base pair (bp) enhancer in the promoter region of the *period* gene in *Drosophila* and contained a perfect E-box. The CRS conferred high-amplitude mRNA cycling to a heterologous reporter gene in both light–dark cycles and constant-darkness conditions. The CRS was also able to rescue behavioral rhythms and drive rhythmic *per* transgenic expression in lateral neurons (the site of the *Drosophila* clock) of *per* null flies (Hao et al., 1997; 1999). Higher resolution analysis, via the use of discrete CRS deletions showed that extra E-box sequences could still mediate detectable cycling. These early results suggested that the CRS-embedded E-box operates in the context of an extended sequence that functions as a circadian element, and tissue-specific transcriptional enhancer.

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Thus, we begin to suspect that "circadian" E-boxes in general might perform a specific and complex function in concert with additional information modules along the promoter (Kyriacou and Rosato, 2000). A particularly well-described example can be found around the circadian *timeless* E-box in *Drosophila* (Darlington et al., 1998; Wang et al., 2001). In this promoter system, mutagenesis of non-canonical E-boxes (the TER sites) as well as an unrelated, novel element (the PERR site), around the canonical *timeless* E-box can prevent reporter gene transactivation by a dBMAL/CLOCK complex in S2 cells (McDonald et al., 2001).

Additional examples of BMAL/CLOCK responsive E-boxes in circadian genes have been steadily piling up and include *vrille* (Blau and Young, 1999), *D-element binding protein* [*dbp*] (Ripperger et al., 2000), *vasopressin* [AVP] (Jin et al., 1999), *serotonin N-acetyltransferase* [AA-NAT] (Chen and Baler, 2000), *prokineticin 2* [PK2] (Cheng et al., 2002) and the three mammalian *periods* (Hida et al., 2000; Travnickova-Bendova et al., 2002).

Consolidation of an E-box as the apparent transcriptional fulcrum of the circadian clock is one of the most intriguing aspects of the current model. This hexameric consensus sequence is in fact a very promiscuous *cis*-acting DNA element and a member of an even more ubiquitous E-box family of transcription regulatory sites (CANNTG), which has been implicated in a bewildering array of transcriptional processes. As pointed out in the first commentary on "circadian" E-boxes (Kyriacou and Rosato, 2000), we would like to understand not only why certain E-boxes become circadian, but also why other E-box containing promoters, such as those in the *vasoactive intestinal peptide* [VIP], *cholecystokinin* [CCK] or *Substance P* [SP] genes for example (Silver et al., 1999), remain non-circadian.

Virtually every transcription factor that interacts with an E-box belongs to the HLH family. Their number is so large (currently in excess of 240) that at least two competing cataloging systems have been proposed to group them in different classes based either on protein properties and tissue distribution (Massari and Murre, 2000) or evolutionary relationships among the sequences (Atchley and Fitch, 1997). HLH proteins bind as homo- or heterodimers and can activate and/or repress gene expression. Proteins such as upstream stimulatory factor USF (Potter et al., 1991), TFE3 (Artandi et al., 1994), c-Myc (Biben et al., 1994), E47 (Ellenberger et al., 1994), TAL1 (Hsu et al., 1994), Arnt (Antonsson et al., 1995), Myo D (Huang et al., 1996), Max (Zhang et al., 1997), Mad (Hurlin et al., 1995), Mxi1 (Lee and Ziff, 1999), as well as the circadian activators BMAL (Bunger et al., 2000; Hogenesch et al., 1998) and CLOCK (Darlington et al., 1998; Gekakis et al., 1998) belong to an expanding list of factors capable of using an E-box to advance their various transcriptional agendas.

Since the first description of an E-box-dependent transcriptional event in 1985 (Church et al., 1985; Ephrussi et al., 1985) this regulatory element has stuck out its palindromic head many times showing positive and negative influences on the expression of an ever-increasing number of genes. As mentioned above, the affected genes mediate a diverse assortment of biological processes, such as cell proliferation, differentiation and transformation, apoptosis, and now, the circadian transcription loop. A partial catalogue of E-box-regulated genes includes *actin* (Biben et al., 1994), *ornithine decarboxilase* (Walhout et al., 1997), *prothymosin* $\alpha$ (Gaubatz et al., 1994), *TGF*-$\beta$2 (Scholtz et al., 1996), *BRCA*-2 (Davis et al., 1999), *cyclin* B1 (Farina et al., 1996), *glycophorin* B

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(Camara-Clayette et al., 1999), *myosin* (Navankasattusas et al., 1994), as well as clock and clock-controlled genes such as *cryptochromes* (Kume et al., 1999) and *vasopressin* (Blackwell et al., 1993; Coulson et al., 1999; Jin et al., 1999). The wide variety of genes taking advantage of the E-box sequence presents, in and of itself, convincing evidence for the existence of E-box modifying elements. Their identification and characterization has been attempted in many systems, and has revealed several strategies used to functionally discriminate among E-boxes. We can count among these the sequence composition at the core (-NN-) of the E-box (Blackwell et al., 1993) or its modification via, for example, methylation (Suetake et al., 1993), the influence of close flanking bases (Blackwell and Weintraub, 1990; Desbarats et al., 1996), neighboring E-boxes (Coulson et al., 1999; 2002; Muñoz et al., 2002) or *cis*-acting elements beyond the E-box (Coulson et al., 2002; Davis et al., 1999; McDonald et al., 2001) the presence of certain amino acid residues in the bHLH domain of E-box binding proteins (Kophengnavong et al., 2000), different combinations or structural conformations of dimeric proteins (Vervoorts and Luscher, 1999; Walhout et al., 1997), and the effect of $Mg^{+2}$ ions (Bendall and Molloy, 1994) on binding specificity. We are only beginning to investigate how these and other mechanisms may apply to the discriminatory capacity of a BMAL/CLOCK complex toward its circadian utilization of an E-box.

In the ongoing efforts to define a circadian E-box, we could benefit from the lessons learned by other researchers during their attempts to identify the particular characteristics of their favorite E-boxes. The crystal structures of E-box-bound factors, for example, reveal key elements used to modify the meaning of these core sequences. Homo- and heterodimers containing the Max protein, which can compete for a common E-box target, provide a good example. Analysis of E-box-bound Max homodimer crystals revealed that the C-terminal domains in both chains were disordered (Brownlie et al., 1997) suggesting the existence of additional stabilizing interactions with unidentified proteins. The structural and binding characteristics of a protein named MASH-1 offer another glimpse at the diversity of mechanisms employed to differentiate one E-box pathway from the next. Circular dichroism analysis showed that MASH-1 homodimers undergo a major conformational change upon binding which is, surprisingly, independent of the specific DNA sequence (Meierhan et al., 1995). In fact, a MASH-1 dimer can bind its target with very high affinity but the affinity for a completely unrelated sequence was only 10-fold lower. The implications of such a combination of properties is that, in some cases, target gene specificities will not be based on differential binding affinities. Taking all this information into account, we can only dream and speculate on what future crystals of a circadian E-box-bound BMAL/CLOCK complex might tell us about the strategies that make a circadian E-box special. For example, it has been reported that the ratio of oxidized to reduced cofactors can alter the E-box DNA binding properties of a BMAL/CLOCK dimer (Rutter et al., 2001). Because stimuli such as food intake and locomotor activity can so dramatically affect the clock, the notion that the circadian transcription complex might function as a metabolic rate sensor provides an attractive bridge between energy consumption and circadian output (Schibler et al., 2001). It would be very exciting indeed to visualize the effects of changing the redox potential upon the BMAL/CLOCK complex at the structural level.

Short of crystal structure analysis but in the footsteps of classical E-box inquiry, the circadian attributes of a perfect E-box have been probed by various methods

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(Darlington et al., 2000; Hogenesch et al., 1998; Lyons et al., 2000; McDonald et al., 2001; Muñoz et al., 2002). In spite of a fair amount of information that points to the E-box as a likely transcriptional necessity for clock-controlled promoters, the regulatory information contained within it is clearly insufficient to recreate proper circadian expression profiles. Again, a multiplicity of strategies must exist, perhaps as assorted as the CCGs themselves.

## CIRCADIAN E-BOX: A MATTER OF CONTEXT?

There is a vast literature dealing with the fact that bases inside and immediately outside of the E-box can restrict the range of transcription factors capable of binding to this element. Hogenesch et al. (1998) identified two consensus sequences ($\underline{\mathbf{C}}_{-3}A_{-2}C_{-1}G_{+1}$-$T_{+2}G_{+3}A_{+4}$ and $\underline{\mathbf{T}}_{-3}$ $A_{-2}C_{-1}G_{+1}T_{+2}G_{+3}A_{+4}$), via an in vitro DNA selection protocol, that can bind recombinant BMAL-containing complexes. The presence of a C or a T at $-3$, relative to the E-box axis of symmetry, influenced partner selection by BMAL (called MOP3 in that study). Specifically, while a BMAL/CLOCK complex bound an E-box with a C at $-3$, BMAL/HIF1α (hypoxia-inducible factor 1α) and BMAL/HIF2α heterodimers preferred a T at that position. This study further suggested to the authors that BMAL brings to the complex the recognition and high affinity binding to half of an E-box site when it is flanked by an A on its $3'$ side (GTGA). In fact, a $3'$ A is a somewhat recurring, albeit by no means universal, theme in strongly circadian E-boxes. It will be interesting to analyze the target range of complexes between CLOCK and potential non-BMAL partners, which could reveal other gene sets regulated by CLOCK in central and peripheral oscillators.

The in vitro binding selection assay (Hogenesch et al., 1998) has also suggested that a BMAL/CLOCK complex could bind with high affinity to the extended sequence (G/T)G(A/G)A$\underline{\text{CACGTG}}$ACCC, an imperfect palindrome containing a perfect E-box. This type of studies represents a useful general approach to probe potential sequence preferences by a transcription factor. It is important to note, however, that in vitro-translated proteins may not carry the proper post-translational modifications (e.g., phosphorylation) capable of modulating conformation, affinity and specificity. In spite of this, the preference toward the proposed extended sequence was partially corroborated recently in the context of a natural promoter (Muñoz et al., 2002). Point mutations at $-4$ and $-6$ in the E-box region of the circadian *vasopressin* gene promoter revealed that the presence of the sequence GNA$\underline{\text{CACGTG}}$ did in fact contribute to high responsiveness to the BMAL/CLOCK complex.

Disappointingly, global studies of circadian gene expression appear to negate the existence of simple preference rules when it comes to BMAL/CLOCK binding. A recent analysis of the circadian transcriptome in the mouse liver (Panda et al., 2002) netted nine rhythmic genes, with high expression during the approximate BMAL/CLOCK peak (Young and Kay, 2001), that were both, affected in the CLOCK mutant strain and carriers of potential circadian E-boxes. These extended E-box sequences constitute a sort of in vivo binding selection sampling. As such, these elements represent potential predictors for the general sequence preferred by the BMAL/CLOCK complex in vivo, at least in this peripheral oscillator system and assuming a direct role of such E-boxes. While all the sites were followed by the expected A at $+4$ (although predictably, since that position was part

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of the query string), the −4, −5 and −6 positions displayed no sweeping preference. Furthermore, a rodent vs. human alignment of these selected upstream sequences revealed essentially no conservation across species either (Panda et al., 2002). These observations are inconsistent with a major role for 5′ extended consensus extracted from the in vitro binding selection assay (Hogenesch et al., 1998).

On closer examination, even well-established BMAL/CLOCK responsive E-boxes such as those present in the *period 1* (Hida et al., 2000), *dbp* (Ripperger et al., 2000), *avp* (Jin et al., 1999) or *prokineticin 2* (Cheng et al., 2002) genes exhibit, at best, a very modest inclination to adhere to the proposed 5′ extension of the consensus sequence (Fig.1). It is true that some sites display partially better fits (particularly the PK2 sites) but the results of the sequential disruption of the five mPer1 E-boxes do not suggest that any one site is better than the others (Hida et al., 2000). One should also consider, however, that many circadian promoters (like *period* and *dbp*) contain multiple E-boxes located upstream and/or downstream of the transcription start point (TSP). Intriguingly, while m*per* 1 contains five perfect E-box sites along its upstream region, all twelve identifiable E-boxes along the 1.7 kb proximal region of the closely related m*per* 2 promoter are non-canonical (Travnickova-Bendova et al., 2002). Perhaps, a multiplicity of "well"-positioned E-boxes can compensate for significant deviations from the in vitro-selected core and/or extended binding site, as has been previously suggested (Lyons et al., 2000). The additive effect on

| | | −7 | −6 | −5 | −4 | | +4 | +5 | +6 | +7 |
|---|---|---|---|---|---|---|---|---|---|---|
| **PER1** | | | | | | | | | | |
| | 1 | C | C | T | G | **CACGTG** | T | T | **C** | **C** |
| | 2 | C | C | C | T | **CACGTG** | G | **C** | T | **C** |
| | 3 | **T** | A | **G** | C | **CACGTG** | **A** | **C** | A | G |
| | 4 | A | C | **G** | **A** | **CACGTG** | G | G | **C** | **C** |
| | 5 | A | **G** | T | C | **CACGTG** | C | A | G | G |
| **DBP** | | | | | | | | | | |
| | 1 | **T** | C | **G** | C | **CACGTG** | **A** | G | T | **C** |
| | 2 | **G** | C | C | **A** | **CACGTG** | **A** | T | G | **C** |
| **AVP** | | | | | | | | | | |
| | 1 | C | **G** | C | C | **CACGTG** | T | G | T | **C** |
| **PK2** | | | | | | | | | | |
| | 1 | **G** | A | **G** | C | **CACGTG** | G | A | A | **C** |
| | 2 | C | T | **G** | T | **CACGTG** | **A** | **C** | **C** | **C** |
| | 3 | C | C | **A** | G | **CACGTG** | T | A | T | G |
| | 4 | A | A | C | G | **CACGTG** | **A** | G | A | G |
| | 5 | **T** | **G** | **A** | G | **CACGTG** | **A** | G | G | C |
| | 6 | **G** | **G** | **G** | G | **CACGTG** | C | G | C | G |
| **CONSENSUS** | | **(G/T)** | **G** | **(A/G)** | **A** | CACGTG | **A** | **C** | **C** | **C** |

***Figure 1.*** Perfect E-boxes found in the *PER1*, *DBP*, *AVP* and *PK2* regulatory regions. Extended E-box sequences drawn from promoters known to respond strongly to the action of the BMAL/CLOCK heterodimer have been overlaid on top of the proposed BMAL/CLOCK preferred binding site derived by an in vitro binding selection assay (Hogenesch et al., 1998). Matches are in bold. The alignment suggests that, if a preferred consensus exists, its influence on E-box choice might be weak or, at least, less than overwhelming.

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BMAL/CLOCK transactivation of the five E-boxes present in the m*period* 1 promoter alluded to above (Hida et al., 2000) is certainly consistent with this hypothesis.

On the other hand, it seems obvious yet worth pointing out that the presumed preference for a particular flanking sequence can also be used to achieve the opposite goal of preventing the circadian activation of non-circadian E-boxes. The presence of specific nucleotides immediately outside of a perfect E-box might keep the motif occupied by constitutive and abundant factors, which might render an E-box refractory to other, more rare or discriminating regulators such as BMAL/CLOCK. For example, upstream stimulatory factors 1 and 2 (USF1/2) are ubiquitous bHLH nuclear proteins involved in the regulation of many genes such as the cell cycle-dependent *cyclin B1* gene (Cogswell et al., 1995; Hwang et al., 1995). Bendall and Molloy (1994) had defined an optimum USF binding sequence: $R_{-5}Y_{-4}C_{-3}A_{-2}C_{-1}G_{+1}T_{+2}G_{+3}R_{+4}Y_{+5}$, where R and Y are purine and pyrimidine bases, respectively. A look at two E-box-containing promoters, *vasopressin* (oscillatory and CCG) vs. *cyclin B1* (oscillatory but not directly CCG), reveals that the former had a 25% match to this flanking consensus while the latter (which is regulated by USF) displayed a 75% match (Muñoz et al., 2002). It seems reasonable to propose that this strategy could be one of the mechanisms responsible to render certain E-boxes non-responsive to BMAL/CLOCK. This notion is consistent with our own observations that recombinant BMAL and NPAS2 (a CLOCK ortholog) complex cannot distinguish between 40-mer double stranded oligonucleotide DNA probes centered on CCG or non-CCG E-boxes in vitro (unpublished). In addition, this scenario could also apply to the differential use of the same E-box in different tissues. Thus, the presence of different competitors for the *AA-NAT* E-box could explain the contrast between its responsiveness to BMAL/CLOCK in retina cells and its refractoriness in a pineal environment (Chen and Baler, 2000).

The existence of E-box modifiers that might impart particular gene-specific profiles of expression is an intriguing possibility. Upstream of the *vasopressin* E-box, for example, we have recently detected a putative switch of circadian transcription (Muñoz et al., 2002). Located a mere turn of a helix away, this CT-rich region (CTRR) can interact with DNA binding proteins, in a tissue-specific fashion, to apparently modulate BMAL/CLOCK-mediated activation. Its placement in front of a non-circadian E-box such as the one in the *cyclin B1* gene promoter partially rescues this site from BMAL/CLOCK refractoriness in NIH-3T3 cells (Muñoz et al., 2002). Neither the mechanism of action of this site nor its general applicability is currently known. By the same token, the D-Box, an element bound by the D-Box binding protein (DBP) and found in the *per 1* promoter, has been shown to contribute to the maximal responsiveness of adjacent E-boxes to BMAL/CLOCK through a cooperative mechanism (Ripperger et al., 2000; Yamaguchi et al., 2000). Unlike the DBP enhancer, however, the *vasopressin* CTRR appears to require the presence of BMAL/CLOCK in order to reveal its effect. This behavior points to a different, rather specific layer of control that could modulate the action of the BMAL/CLOCK complex on a gene and tissue-specific basis.

The location of circadian E-boxes relative to the TSP is another important issue that has not been addressed systematically so far. As is the case for non-circadian promoters, perfect E-boxes have been identified not only upstream but also downstream of the +1 position, usually in an intronic region. For example, an intronic E-box in the *AA-NAT* gene

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has been shown to support BMAL/CLOCK activation in transfected retina cells (Chen and Baler, 2000). Similarly, the CLOCK complex can activate transcription from intronic E-boxes in the *dbp* gene (Ripperger et al., 2000). Neither one of these examples, however, have been formally demonstrated to be essential for cycling in vivo. In contrast, the intronic E-box in the *timeless* gene in *Drosophila* failed to mediate dCLK/dBMAL activation in a cell culture system, using rather its upstream E-boxes to achieve circadian rhythmicity (McDonald et al., 2001; Wang et al., 2001).

There are many examples of significant effects stemming from the action of intronic sites on transcription. In many cases these sites are sensitive to increases in cAMP (Baler et al., 1999; Susini et al., 2000) or Ca++ (van Haasteren et al., 2000), for example. Therefore, we should be alert to the presence of sites such as the downstream regulatory element [DRE; (Carrion et al., 1999)] that could function as a second messenger-sensitive elongation block in circadian promoters, or the intronic elements in the *period* gene in *Drosophila* that contribute to the correct phasing of mRNA expression (Stanewsky et al., 1997; 2002). In practical terms, the existence of intronic regulatory sequences raises an important issue regarding the functional analysis of promoter regions. Such analyses typically involve the placing of upstream sequences in front of the TSP of a heterologous reporter gene, e.g., luciferase (LUC) or green fluorescent protein (GFP). At present, and in light of mounting evidence showing the importance of downstream regions (both exonic and intronic) in the complex regulation of stimulus-driven (Finkbeiner, 2001) as well as tissue-specific (Bornstein et al., 1987; Burke et al., 1999; Xu and Saunders, 1998) gene expression, it might be critical (albeit tedious) to also consider downstream regions as essential contributors to the proper temporal regulation of gene expression.

## THE CIRCADIAN E-BOX: A MEETING PLACE

As a focal point of the clock machinery the circadian E-box serves as the meeting place for many of the known key players. A series of recent elegant biochemical studies let us imagine a carefully staged set of interactions at the circadian site. Both BMAL and CLOCK were shown to interact with histone-acetyltransferases p300 (Etchegaray et al., 2002) and CBP (Takahata et al., 2000). Then, following a standard sequence of events, binding of a BMAL/CLOCK complex upon a competent E-box leads to chromatin remodeling and recruitment of the RNA polymerase II machinery (Struhl, 2001). In vertebrates, large macromolecular complexes assemble and disassemble on top of these occupied E-boxes, on a circadian basis, to uncouple the DNA bound BMAL/CLOCK heterodimer from the interacting acetyltransferases (Etchegaray et al., 2002). These complexes contain Period and Chryptochrome proteins as well as casein kinase Iε (Lee et al., 2001), which can regulate the activity/stability of Period (Camacho et al., 2001; Keesler et al., 2000; Lee et al., 2001), BMAL and CRY proteins (Eide et al., 2002) through site-specific phosphorylation. The repressive event is likely to occur without disruption of the interaction between BMAL/CLOCK and the E-box (Ishikawa et al., 2002; Lee et al., 2001), a mechanism differing from the reported repression of *Drosophila* BMAL/CLOCK by the Per/Tim complex, which does inhibit DNA binding activity (Lee et al., 1999). Accordingly, it has been proposed that, except for

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the natural decay due to its rates of dissociation and turn over, the vertebrate BMAL/CLOCK complex is mostly "on" the E-box (Lee et al., 2001). This is an interesting concept since having the site constitutively bound would mean that CCGs are transcriptionally active by default. The tonic expression of *per* 1 and 2 genes in cryptochrome-deficient mice (Okamura et al., 1999) supports this hypothesis. Such an arrangement would endow CCGs with particularly high sensitivity and the ability to mount an immediate response following the disappearance of the repressor complex. It will be interesting to assess whether BMAL and, CLOCK., either in their known or alternative heterodimeric configurations, can also regulate transcription from E-boxes on other (non-circadian) promoters using different rules of engagement.

## THE CIRCADIAN E-BOX: THE BEGINNING OF A CASCADE

Because of its intimate relationship with the core components of the clock, activation of the circadian E-box can be viewed as the trigger of a cascade of rhythmic transcription. In the context of the temporal orchestration of gene expression, we should consider the possibility that additional unidentified circadian elements exist, which can initiate parallel or intersecting cascades of their own, either at the same or different phases. The E-box cascade starts at around ZT4 and ZT8 in central and peripheral oscillators respectively, and traverses different paths in different tissues (Akhtar et al., 2002; Ceriani et al., 2002; Panda et al., 2002). Hence, the transcriptional domino effect initiated by BMAL/CLOCK activation of selected E-boxes is responsible for the existence of many cycling genes that are indirectly affected by clock-impairing mutations at later, as well as preceding phases (Panda et al., 2002). Cluster analysis of the promoter of these genes will likely reveal important features of the tissue-specific chain of events that follow circadian E-box activation. Interestingly, some of the genes that belong to this category, such as the *Drosophila pdf* (Park et al., 2000) and *take-out* (So et al., 2000), have also been found to contain E-boxes which appear to be refractory to the positive action of the BMAL/CLOCK complex in transfection studies. It is conceivable that such non-responsive E-boxes, embedded in strongly circadian promoters, might be used as sites of repression by the BMAL/CLOCK complex. The reported inhibition of the c-Myc promoter by a BMAL/NPAS2 (or BMAL/CLOCK) heterodimer (Fu et al., 2002) demonstrates that such a mechanism is at least possible. Such sites could help to better define the strategies used by E-boxes to efficiently deflect the unwanted activation by a BMAL/CLOCK complex.

## THE BIGGER PICTURE

In order to create and maintain a balanced organism the transcription machinery has to make constant decisions on three basic fronts: the identity, the location and the timing of the genes it needs expressed. Thus, studies of the temporal aspect of gene regulation occupy a significant fraction of the transcription-related literature. It is noteworthy that up until the discovery of the, CRS., interest on E-boxes had been largely connected to their role in controlling the expression of cell-type specific genes (Massari and Murre, 2000), namely, control in *space* rather than *time*. Interestingly, another circadian renaissance of sorts

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(Alvarez and Sehgal, 2002), occurred recently when a Rev-Erbα binding site (Harding and Lazar, 1993) was found to play a role similar to that of the circadian E-box, although not essential for rhythm generation and affecting a different phase of the clock (Balsalobre et al., 1998; Preitner et al., 2002; Ueda et al., 2002). Again, the interest in Rev-Erbα had been previously directed elsewhere, specifically to its transcriptional repressive activity (Adelmant et al., 1996) and involvement, as an orphan nuclear receptor, in the regulation of adipogenesis and metabolism (McKenna and O'Malley, 2002). After Balsalobre et al. recognized its circadian rhythm of expression in the liver (Balsalobre et al., 1998), researchers probed, and eventually found a strong transcriptional link between Rev-Erbα and the fine regulation of the clock (Preitner et al., 2002). There is probably a lesson in the recurring discovery of a circadian use for previously known but (circadianly) "uninteresting" *cis*-acting elements.

The stories of the CRE and AP-1 elements could follow a similar path. During much of the last 20 years of transcriptional research, a large fraction of the research on transcriptional control of timing has been devoted to immediate early gene expression (IEG) and the biology of the activator protein-1 (AP-1) and cAMP responsive element (CRE), respectively. These DNA targets (and a large family of "like minded" sequences) mediate very precisely timed responses in gene expression. As a result of specific stimuli, the densely networked signal transduction cascades that end up hitting these sites lead to the controlled induction of specific sets of genes, with frequencies, amplitudes and durations that are characteristic for each individual case. It is not too bold to predict that the links between these well-established temporal regulators and the 24h transcription translation loop (TTL) will grow stronger with time.

The existence of an evolutionary relationship between AP-1 and CRE sites is virtually self-evident (Hai and Curran, 1991; Sloan and Schepartz, 1998). It is not impossible, and some have seriously considered this possibility (Kyriacou and Rosato, 2000; Liu and Green, 2002), that E-Boxes and CREs might also share an ancestor of time-sensitive *cis*-acting elements, perhaps containing a shared "ACGT" core (Fig. 2). Several examples of the capacity of CREs to generate circadian cycles of transcription have been reported (Obrietan et al., 1999; Tischkau et al., 2003) and reviewed previously (Kyriacou and Rosato, 2000). It is not surprising then that some investigators felt compelled to tinker with the potential relationships between the CRE and E-Box sites of dually regulated genes such as *per 1* (Travnickova-Bendova et al., 2002), *AA-NAT* (Chen and Baler, 2000) or *nocturnin* (Liu and Green, 2002).

It might be useful to imagine a catalog of elements from which the transcriptional machinery can draw individual selections in order to pursue a specific temporal goal. The "alignment" presented in Fig. 2 was dreamt up in this spirit, and meant as a question rather than a formal speculation about the possibility that the obvious relatedness between the AP-1 and CRE sites could extend forward and backward to encompass other temporal modules such as the circadian E-box (through the nocturnin element (NE) as recently suggested (Liu and Green, 2002) and Rev-Erbα sites, respectively. Regardless of the actual merits of this alignment, this cartoon could be used to map out functional relationships between known "time boxes" acting along the circadian time frame. It can be argued, for example, that CREs and NEs are recruited to effect rapid induction in transcriptional output after sensing increases in cAMP and the levels of phospho-CREB, such as those seen during photic resetting of the clock (Gau et al., 2002). Circadian sites such as the E-box and Rev-Erbα elements are in charge of reading the phase of the clock

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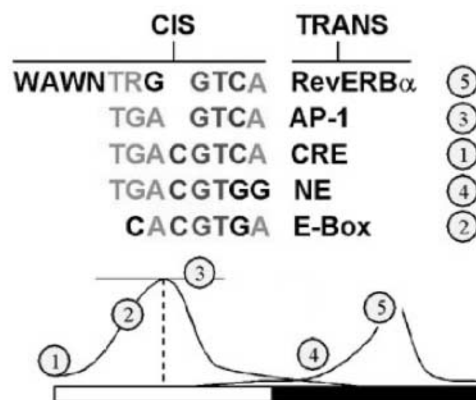


***Figure 2.*** The circadian E-box & Co. A collection of *cis*-acting regulatory elements has been recruited to perform specific functions within the circadian transcriptional machinery. Some, like the CRE (1) and NE (4) are engaged in sensing the activation of signal transduction cascades directly and link their state to the induction of genes. As such, these elements are ideally positioned to mediate temporal gating for photic and non-photic resetting. Other elements, such as circadian E-boxes (2) and Rev-Erbα (5) sites can operate as precise phase markers through positive and negative effects on transcription, particularly important for the control of clock genes. Secondary sites, such as AP-1 and AP-1-like elements could prove particularly well suited to modulate the amplitude of specific responses, with a more prominent role perhaps in the regulation of clock-controlled genes. The color alignment among the consensus sequences represents an admittedly fidgety attempt to find relationships among these five elements where there might be none. W = A or T; R = G or A; N = any base.

and activating or repressing specific genes accordingly. On the other hand, there is increasing evidence pointing to the ability of specific AP-1 complexes to modulate the strength (amplitude) of temporally defined gene responses (Okamura et al., 1999; Smith et al., 2001). It is likely that the over-simplified set of sites that includes the AP-1, CRE, E-box, NE, and Rev-Erbα sequences occupies a segment within a continuum of usable target elements, sometimes even with hard-to-define boundaries.

We have to face the fact that the difficulties in unambiguously defining a circadian E-box, first made explicit in Kyriacou's review (Kyriacou and Rosato, 2000), are still lingering three years later. By stepping back from the E-box tree, however, we might be able to appreciate various forests of elements whose interactions are the real key to specifying the "when," "where" and "how much" of circadian gene expression.

## ABBREVIATIONS

| | |
|---|---|
| AVP | vasopressin |
| bp | base pair |
| E-box | enhancer box |

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| | |
|---|---|
| CG | clock gene |
| CCG | clock-controlled gene |
| CCRE | circadian clock-responsive element |
| Cry | Cryptochrome |
| CRS | circadian regulatory sequence |
| CTRR | CT-rich region |
| HLH | helix loop helix |
| DBP | D-box binding protein |
| DRE | downstream regulatory element |
| NE | nocturnin element |
| NPAS2 | Neuronal PAS domain protein 2 |
| Per | Period |
| Tim | Timeless |
| TSP | transcription start point |
| TTL | transcription translation loop |
| USF | upstream stimulatory factor |

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