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Modulation of BMAL/CLOCK/E-Box complex activity by a CT-rich *cis*-acting element

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Abstract

The interaction between the BMAL1/CLOCK transcription factor and the *cis*-acting element known as the E-Box is a key event in the regulation of clock and clock-controlled gene expression. However, the fact that the ubiquitous E-Box element sits at the center of a presumably highly discriminating control system generates a certain level of puzzlement. Widely spread E-Boxes with a generic sequence CANNTG have been associated with expression of genes involved in a host of disparate biological processes, including the orchestration of circadian physiology. The intriguing specificity of this short DNA consensus element begs the hypothesis that its actual circadian properties might be encoded elsewhere, e.g., other factors or adjacent sequences. In a previous study, we found evidence that a short sequence in the mouse arginine vasopressin (AVP) proximal promoter has the ability to confer robust BMAL1/CLOCK responsiveness onto an adjacent E-Box. Here, we report the systematic analysis of this element. Our findings further define the determining features and sequence boundaries of this element, establish the effect of the photoperiod upon its interacting protein(s), and suggest that its cognate binding activity might be modulated by Zn^{2+} in a peripheral oscillator.

Keywords: E-Box; Circadian; Clock; BMAL; Pineal gland; Zinc; Poly-pyrimidine track

1. Introduction

It is widely accepted that positive and negative interlocking transcriptional–translational loops (TTL) are behind the generation and maintenance of rhythmic mRNA expression in both central and peripheral clocks in mammals (Reppert and Weaver, 2002). In the last two decades, several components of the clock machinery have been identified. Today, there are many variable factors, along the emerging circadian signaling pathway, known to modulate the default phase of the clock as well as its sensitivity to the resetting effects of physiological time cues (zeitgebers). Phosphorylation/dephosphorylation cascades, targeted degradation, nuclear translocation and the assembly of macromolecular complexes at circadian E-Boxes are some of these regulatory key points (Tamanini et al., 2005).

Because of the robust rhythms displayed by mRNA levels of both clock genes (CG) and clock-controlled genes (CCG), rhythmic modulation of transcription has been one of the most exciting playgrounds for chronobiologists. In the context of circadian transcriptional control the two bHLH-PAS transcriptional factors, BMAL1 and CLOCK, are considered key integrators at the core of the clock (Bunger et al., 2000; Gekakis et al., 1998; Ikeda and Nomura, 1997; Vitaterna et al., 1994). The transcriptional activity of this complex ebbs and flows in carefully choreographed synchrony with the expression and post-translational modifications of key regulatory proteins in the nucleus (Etchegaray et al., 2003); throughout this cycle, however, the complex remains tightly bound to its cognate sites, the perfect DNA E-Boxes (CACGTG) in the regulatory regions of CG and CCG (Appelbaum et al., 2004; Blau and Young, 1999; Chen and Baler, 2000; Darlington et al., 1998; Hao et al., 1997; Hogenesch et al., 1998; Jin et al., 1999; Kume et al., 1999; Okada et al., 2001; Ripperger et al., 2000).

Several studies have attempted to address the question of what turns this promiscuous DNA element into such a discriminating site, by systematically searching for potential *cis*- and *trans*-acting factors that might be able to influence a BMAL/CLOCK/E-Box macromolecular complex. For example, a computer analysis of many rhythmically expressed genes revealed that a substantial number of circadian promoters fea-

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tures, in addition to their standard E-Box, ROR and cyclic AMP responsive (CRE) elements (Ueda et al., 2002). In addition, a recent study revealed that the phase and light responsiveness governed by four E-Boxes in the zebrafish *per* gene promoter, depend on promoter context (Vallone et al., 2004). Following a similar strategy, we had found in an earlier comparative study of two minimal E-Box-containing promoters, the arginine-vasopressin (AVP) and the cyclin B1 (CYC), that ablation of a short sequence in the AVP promoter could abolish the robust response of the promoter to BMAL/CLOCK transactivation (Muñoz et al., 2002). Those results suggested that the DNA region upstream of the AVP E-Box could play a permissive role and contribute to the strong and differential responsiveness of the AVP gene to BMAL/CLOCK (Muñoz et al., 2002). The goal of the present study was to characterize this AVP upstream region, in order to identify the putative *cis*- and *trans*-acting factors responsible for making the AVP E-Box strongly circadian.

2. Materials and methods

2.1. Reporter and expression plasmid vectors

A minimal mouse AVP promoter (positions 1263–1460 in GenBank™ accession number M88354) was cloned between the *Nhe*I and *Bgl*II sites into pGL3-Basic (Promega, Madison, WI) carrying Firefly luciferase (FL) reporter gene. The mouse CLOCK (C) and human BMAL1 (B) mammalian expression vectors (cytomegalovirus (CMV) promoter-driven) were generously provided by Drs. N. Gekakis and C. Weitz (Harvard University). SV40 promoter-driven expression vectors for the upstream stimulatory factors 1 and 2 (USF-1/2, human and mouse, respectively) were a gift from Dr. M. Sawadogo (University of Texas). Mutant version of the Luciferase reporter construct was generated by site-directed mutagenesis (GeneEditor, Promega). To mutagenize the CT-rich region (CTRR) in the wild type (WT) AVP promoter, the sequence CTGGCTCCCCTCCTCCACC was changed to CTCGAGAAAAGAAGAAACC (CTRR-).

2.2. Transient transfection assays

NIH-3T3 cells (ATCC, CRL-1658) were transfected with wild type and mutant versions of the AVP-driven firefly luciferase reporter vector following a protocol previously published (Muñoz et al., 2002). Briefly, cells were grown in Vitacell Dulbecco's modified Eagle's medium (ATCC, no. 30-2002) supplemented with 10% fetal bovine serum. A cocktail of LipofectAMINE/Plus reagents (Invitrogen) containing 5 ng of reporter plasmid DNA and 0.5 μg of a 1:1 expression vector mixture (BMAL1/CLOCK or USF-1/2) or carrier pcDNA in 50 μl of Vitacell Dulbecco's modified Eagle's medium without fetal bovine serum. Experiments were stopped 48 h post-transfection. The Thymidine Kinase (TK) promoter-driven Renilla luciferase (RL) reporter vector (0.5 ng) was used as internal control. Firefly and Renilla luciferase enzyme activities were measured using the Stop and Glo kit (Promega) following the manufacturer's recommendations. Results depict a single representative experiment of at least three independent experiments performed in triplicate. Statistical analysis was performed by a Student's *t*-test for unpaired samples.

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described (Muñoz et al., 2002). Whole extracts from bovine retina, cone-rod homeobox (CRX) knock-out mouse retina, rat cortex, suprachiasmatic nucleus (SCN), pineal gland, cerebellum, pituitary, hypothalamus, olfactory bulb, liver, spleen, kidney, heart, thymus and lung were prepared in high salt extraction buffer "C" (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA) supplemented with aprotinin, pepstatin, leupeptin (at 1 μg/ml each), 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride and 1 mM NaF. Synthetic, staggered-ended oligo-nucleotides (Integrated DNA Technologies, Coralville, IA) containing the AVP CTRR, with or without E-Box, were radiolabeled by a fill-in reaction with Klenow enzyme (Roche) and [alpha ^{32}P]-dCTP and gel-purified on an 8% polyacrylamide Tris-Acetate-EDTA gel before use. The sequences analyzed were 5′-CATGGCTGGCTCCCC-TCCTCCACCACCCTCTGCACTGACACGCC-CACGTG-TGTCCCCAGATG-3′ (long AVP) and 5′-CATGGCTGGCTCCCC-TCCTCCACCACCCTCTGCACTGACACGC-3′ (AVP Upstream Region-UR). For supershifting experiments, 1 μl of affinity-purified rabbit polyclonal antisera (Santa Cruz Biotechnology) against USF-2 (sc-862X) was added to the binding reaction mixture 30 min before the addition of the probe (100,000 cpm). For competitive assays, cold double-stranded oligonucleotides were pre-incubated for 30 min at a final concentration of 400-fold molar excess. Unlabeled sequences included: AVP E-Box: TGCACTGACACGCCCACGTGTGTCCCCAGATG (E1); CYC E-Box: AGGCGCAGAGGCAGACCACGTGAGAGCCTGGCCA-GGCCTT (E2) and CYC upstream region: CTGCCCGAGAGCGCAGGCGCA-GAGGCAGA (UR2). In addition, the following 5′- and 3′-end progressively truncated versions of AVP UR sequences were assayed:

1. CATGGCTGGCTCCCCTCCTCCACCACCCTCTGCACTGACACGCC;
2. CTGGCTCCCCTCCTCCACCACCCTCTGCACTGACACGCC;
3. TCCCCTCCTCCACCACCCTCTGCACTGACACGCC;
4. TCCTCCACCACCCTCTGCACTGACACGCC;
5. CACCACCCTCTGCACTGACACGCC;
6. CCCTCTGCACTGACACGCC;
7. TGCACTGACACGCC;
8. 2x(TGACACGCC);
9. 2x(CATGGCTGG);
10. CATGGCTGGCTCCC;
11. CATGGCTGGCTCCCCTCCT;
12. CATGGCTGGCTCCCCTCCTCCACC;
13. CATGGCTGGCTCCCCTCCTCCACCACCCT;
14. CATGGCTGGCTCCCCTCCTCCACCACCCTCTGCA;
15. CATGGCTGGCTCCCCTCCTCCACCACCCTCTGCACTGAC;
16. CATGGCTGGCTCCCCTCCTCCACCACCCTCTGCACTGACACGCC.

Electrophoresis was performed for 2 h at 4 °C in a vertical 5% TGE (40 mM Tris, 200 mM Glycine, 2.4 mM EDTA) native polyacrylamide gel. At the end of the run the gel was removed, dried and exposed to a PhosphorImager screen (Amersham Biosciences). BMAL1 (B) and NPAS2 (N) recombinant proteins were generously provided by Dr. Steve Mc Knight (University of Texas, Southwestern Medical Center). These proteins and bovine retina (BR) extracts were assayed in supershift reactions as described previously (Rutter et al., 2001), in the presence of 10 mM final concentration NADPH and 0.5× TBE running buffer. To study potential daily variations in DNA binding activity, EMSA assays were performed using whole protein extracts prepared from rat retina, SCN, or pineal gland, in buffer C. Animals were kept under 12 h light:12 h dark cycle for 3 weeks and sacrificed at ZT 2, 5, 8, 11, 14, 17, 21 and 23. Some animals were exposed to 15 min light pulse at ZT 14 and 22 (beginning and end of the dark period). When the effect of divalent cations on DNA binding activity was studied, the binding reaction was incubated for 1 h at 4 °C, with or without the indicated metals, before addition of the probe. Zinc, manganese, copper and magnesium chloride were used at 100 μM final concentration.

3. Results

A previous mutational analysis of a minimal AVP promoter (Muñoz et al., 2002) suggested that a DNA region immediately upstream of the AVP E-Box could modulate the strong responsiveness of this clock-controlled gene to the BMAL1/CLOCK complex. The identification of a 22-bases long 90% pyrimidine stretch within this region was the starting point for the present study. To test the hypothesis that this CT-rich upstream region (UR) plays a role in the observed phenomenon, we first performed an EMSA analysis using ^{32}P-labeled, double-stranded oligonucleotides, containing the AVP UR with (long probe) or