The E-Box is a widely used DNA control element. Despite its brevity and broad distribution the E-Box is a remarkably versatile sequence that affects many different genetic programs, including proliferation, differentiation, tissue-specific responses, and cell death. The circadian clock is one of the latest pathways shown to employ this element. In this context, E-Boxes are likely to play a key role in establishing the robust waves of gene expression characteristic of circadian transcription. The regulatory flexibility of the E-Box hinges on the sequence ambiguity allowed at its core, the strong influence of the surrounding sequences, and the recruitment of spatially and temporally regulated E-Box-binding factors. Therefore, understanding how a particular E-Box can accomplish a specific task entails the identification and systematic analysis of these cis- and trans-acting E-Box modifiers. In the present study we compared the E-Box-containing minimal promoters of vasopressin and cyclin B1, two genes that can respond to the transcriptional oscillators driving the circadian clock and cell cycle, respectively. Results of this comparison will help elucidate the manner in which discreet DNA modules associate to either augment or restrain the activation of potential circadian E-Boxes in response to competing regulatory signals.

Homographs are words that share the same spelling but differ in sound, origin, or meaning. By extension, an enhancer (E)1-Box can be defined as a “transcriptional homograph”: a short DNA element (word) that, depending upon the features of the sequence and cellular environment (context), can dispense very different transcriptional outputs (messages). By recruiting the proto-oncogene c-myc, for example, an E-Box can drive cells to become growth factor independent, to speed through G1 of the cell cycle, to avoid differentiation, or, paradoxically, to undergo apoptotic death (1, 2). The ability of an E-Box to support myogenic factor-mediated muscle differentiation, even in many non-muscle cell types (3), is another example of the regulatory plasticity provided by this multifunctional site.

In fact, since its identification in 1985 as a control element in the immunoglobulin heavy chain gene promoter (4), the E-box has been found to influence the expression of a large number of genes that share no additional obvious relationships. The list includes genes such as actin (5), ornithine decarboxylase (6), prothymosin (7), vasopressin (8, 9), TGF-β (10), BRCA2 (11), cyclin B1 (12), glycophorin-B (13), and myosin (14), among many others.

The perplexing diversity of E-Box-dependent processes has fueled an intense search for trans-acting factors regulating a given E-Box. Predictably, such efforts have resulted in an ever-expanding list of transcription factors that can recognize E-Box consensus sequences. Virtually every such factor uses a basic helix-loop-helix (bHLH) (15) motif to interact specifically with promoters and other proteins. A partial catalog of E-Box-binding factors includes Myc (5), Arnt (16), Max (17), MyoD (18), Mad (19), upstream stimulatory factor (USF) (20), Mxi1 (21), E47 (22), TFE3 (23), TAL1 (24), and the more recently described BMAL1 (25) and CLOCK (26, 27) proteins.

Obviously, the sequence context surrounding an E-Box must also play a role in ushering different promoters toward different patterns of expression, an issue that has been the subject of extensive studies. The vast amount of data collected as a result of these efforts revealed a complex picture in which the position and number of E-Boxes (28, 29), their flanking sequences (30–33), and the specific proteins that interact with them (31, 34), all combine to achieve a particular effect.

The involvement of an E-Box in the regulation of gene expression by the circadian clock was first recognized during the analysis of rhythmic expression of the period gene in Drosophila (35). Several additional examples have been discovered since (9, 26, 36–38), and the E-Box element is now considered a central integrator within the transcription-translation loop (TTL) that constitutes the circadian clock, even though the contextual features required to place an E-Box under circadian control are yet to be defined. One important characteristic shared by known circadian E-Boxes is their ability to specifically recruit a BMAL1-CLOCK (B/C) complex (9, 25–27). This fact, however, should immediately shift our attention to the still unknown features that form the basis for a productive interaction between a circadian E-Box and the B/C heterodimer.

Efforts to characterize the E-Box in the context of clock-controlled genes (CCGs) have not yet provided a clear understanding of the sequence constraints imposed upon the region flanking a circadian E-Box (39–43). It is clear that in most cases reported so far, multiple and seemingly randomly spaced E-Boxes appear to be needed to support circadian transcription (36, 40). This apparent requirement, however, is certainly not sufficient, because many non-circadian promoters feature multiple E-Boxes. Thus, clock and non-clock controlled gene promoters must enforce additional rules for E-Box utilization to attract or deflect a circadian input. It is also likely that specific
bases must be present in the flanking sequence to support high affinity binding of a B/C complex, as suggested by a consensus derived through in vitro studies (25). However, it is not presently clear how this flanking consensus might affect the circadian competence of a perfect E-Box in its natural environment.

Here we probed the nature of these rules by systematically assessing the contribution of the sequence context toward the dramatic differences in B/C responsiveness displayed by vasoressin (AVP) and cyclin B1 (CYC) gene promoters. Despite the presence of a perfect E-Box in both promoters and at very similar locations, these two genes are subject to significantly different modes of transcriptional regulation. The AVP promoter has been shown conclusively to be under circadian regulation by the B/C heterodimer in the rodent suprachiasmatic nucleus (SCN) (9, 26). Elsewhere in the hypothalamus, transcription of the AVP gene is also modulated by systemic hypotony and hypotension (44). On the other hand, the CYC promoter responds to cell cycle (45) and USF transcriptional activity (46). Interestingly, it has been suggested that cyclin B1 expression might also be affected by clock-related mechanisms (47). Our results highlight how different informational units along the DNA can combines to modify the circadian performance of a perfect E-Box.

EXPERIMENTAL PROCEDURES

Collection and Preparation of Reporter and Expression Plasmid Vectors—A mouse AVP-luciferase reporter (AA) was constructed by splicing a PCR-generated fragment (positions 1265–1460 in GenBankTM accession number M88354) between the Nhel and BgII sites in pGL3-Basic (Promega, Madison, WI). The human CYC-luciferase reporter (CC) was produced by placing a PvuII to BgII human cyclin B1 promoter fragment (positions 671–873 in GenBankTM accession number U38638), derived from pGL2-upB (a gift from Ruth Muschel, University of Pennsylvania), between the SmoI and BgII sites in pGL3 Basic. The mouse CLOCK and human BMAL1 mammalian expression vectors (cytomegalovirus promoter-driven) were generously provided by Drs. N. Gekakis and C. Weitz (Harvard University). Mammalian expression vectors for human USF-1 and mouse USF-2 (SV40 promoter-driven) were a gift from Dr. M. Sawadogo (University of Texas). Mutant versions of the Luciferase reporter constructs were generated by site-directed mutagenesis with GeneEditor (Promega) as the template. PCR was performed using PfuTurbo DNA polymerase (Stratagene) and the appropriate primers. The PCRamplified DNA techniques using the following strategies. The PmlI restriction sites at the center of the E-boxes in the AA and CC reporters were used to target the 40-bp-long sequences (centered around each E-Box) of the opposite promoter to generate the AC/A and CA/C constructs. These PmlI restriction sites were also used as transition points to generate the chimeric promoter constructs AC and CA. The following changes were introduced to mutagenize various cis-acting elements in the AVP promoter (core E-Box sequence in bold): E-box1, CACCTG toCACACCTG; E-box2, CAGATG to CAGAAATG; AP-1, TGAATCA to TCGAGCA; CRE, CTGAGGTGTG to CTGAGGTCGTG: AA6T, GCCCACG to TCCACCGT: AA12C, CACTGACACGCC to CACGTGACACACC; AA6C, CAGGGCCACCGT to GGAGGGCCACCGT: AA6T, GCCCACG to TCCACCGT: AA12C, CACTGACACGCC to CACGTGACACACC; AA6C, CAGGGCCACCGT to GGAGGGCCACCGT. For the construction of the CA reporter the first 31 bp of the CC promoter were placed immediately upstream of the AA promoter. CC+3/4 and AC+3/4 were generated by inserting a 43-bp-long double-stranded (ds) fragment containing the third and fourth E-boxes of the AA promoter into the BstAPI site in the CC promoter, located 87 bp downstream of the perfect E-box. Transected AVP promoter vectors −27AA, −22AA, −12AA, and −8AA were constructed by replacing a Smal/HindIII fragment in the original AA vector, which starts at position −48 relative to the center of the E-box, with a high fidelity PCR-generated fragment containing the 24, 19, 9, or 5 bp immediately upstream of the E-box, respectively. For construction of pGL3P/AVP-E and pGL3P/CYC-E, the same 40-mer sequences mentioned above were inserted into the Smal site of a pGL3Promoter luciferase reporter vector (Promega).

Transient Transfection Assays—NIH-3T3 cells (ATCC, CRL-1658) were plated at a density of 3 × 10^4 cells per well in a 24-well plate (Costar, Cambridge, MA) and transfected 24 h later with a mixture containing LipofectAMINE/Plus (1.25 μg/2.5 μl) reagents (Invitrogen), 5 ng of reporter plasmid DNA, and 0.5 μg of a 1:1 expression vector mixture (BMAL1/CLOCK or USF1/2) or carrier pcDNA in 50 μl of Vitacell Dulbecco’s modified Eagle’s medium (ATCC, no. 30–2002) without fetal bovine serum. On the following day, 0.5 ml of culture medium (Vitacell Dulbecco’s modified Eagle’s medium, supplemented 10% fetal bovine serum) was added to the cells, which were harvested 24 h later. Transient transfection efficiencies were determined by measuring the enzyme activity generated by a co-transfected thymidine kinase promoter-driven Renilla luciferase (RL) plasmid (0.5 ng). Plasmid DNA for transfection was prepared using the Qiagen kit (Qiagen, Valencia, CA). We were able to corroborate high levels of expression for Clock, USF1, and USF2 proteins by Western blot analysis using the following antibodies: anti-Clock (Novus Biologicals, Littleton, CO), anti-USF1 SC-8983, and anti-USF2 SC-862 (Santa Cruz Biotechnology, Santa Cruz, CA). Expressed levels of BMAL1 appeared significantly lower, either due to the intrinsic instability of this protein or the weakness of the various available anti-BMAL1 antibodies that we used (anti-BMAL1, Affinity Bioreagents, Inc., Golden, CO; SC-8614 and SC-8550, Santa Cruz Biotechnology). Expression of functional BMAL1 (and CLOCK) proteins was confirmed in preliminary experiments in which the simultaneous transfection of both expression vectors was an absolute requirement for transactivation of the AVP promoter through its perfect E-Box. Firefly luciferase (FL) and Renilla luciferase enzyme activities were measured using the Stop and Glo kit (Promega) following the manufacturer’s recommendations. Unless otherwise indicated, representative singe-experiment data are at least two independent experiments performed in triplicates. Statistical analysis was performed by a Student’s t test for unpaired samples.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as previously described (38) with some modifications. To prepare nuclear extracts, 1 × 10^6 NIH-3T3 cells were seeded in 10-cm plates and harvested when they reached 100% confluency. Nuclear extracts were prepared with the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce) following the manufacturer’s recommendations, except that the extraction buffers were supplemented with aprotinin, pepstatin, leupeptin (at 1 μg/ml each), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM NaF. Synthetic, staggered-ended oligonucleotides (Integrated DNA Technologies, Coralville, IA) ranged from 30 to 70 nucleotide length. Probes were radiolabeled by incubation with Klenow enzyme (Roche) and [α-32P]dCTP (Fig. 2, A and B) and gel purified on an 8% polyacrylamide gel before use. For supershifting experiments, 1 μl of affinity-purified rabbit polyclonal antiserum (Santa Cruz Biotechnology) against either USF2 (sc-6822 X) or c-Fos (sc-7202 X) was added to the binding assay reaction mixture 30 min before the addition of the radiolabeled probe (100,000 cpm). For blocking experiments, 1 μl of the appropriate antiserum was preincubated for 2 h at 4 °C in a vertical gel. For the pre-blocked antibody was then added to the binding assay reaction as described above. For competitive EMSA, 1 μl of an unlabeled double-stranded oligonucleotide stock solution was added to the pre-incubation mixture to achieve final concentrations of between 10- and 2000-fold molar excess. 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. After the run the gel was removed, dried, and exposed to a PhosphoImager screen (Amersham Biosciences) for later quantitation using the ImageQuant™ software package.

RESULTS

The vasopressin and cyclin Gene Minimal Promoters as Models Of Differential E-Box Utilization—To expose the features that distinguish a “circadian” E-Box we compared the mouse AVP (48) and human CYC (12) promoters. These upstream regulatory regions contain a single perfect E-Box at similar positions relative to the transcription start points (Fig. 1A). First we assessed the responsiveness of these two E-box-containing minimal promoters toward BMAL1/CLOCK- and USF2-USP2-mediated transactivation by transient transfection of an AVP or CYC-driven reporter gene into NIH-3T3 cells (Fig. 1B; compare AA versus CC). Because E-boxes can affect many transcription processes the data is presented as basal versus induced (corrected) levels of reporter gene activity (horizontal bars) as well as stimulation index (numerical values).

Consistent with previous reports (9, 38), the AA construct was strongly stimulated by B/C, an effect that was dependent
upon the presence of both transcription factors (data not shown). In sharp contrast, the CC construct was completely refractory to the co-expression of the circadian heterodimer. This clear difference forms the basis for the model we used in this study to investigate the defining features of a representative circadian E-Box. The consistently higher level of basal activity observed in the CC construct reflects the fact that this promoter is strongly induced during the G2/M phase of the cell cycle (45). Thus, cells traversing this mitotic point in an asynchronous culture can support significant levels of transcription.

The differential response of the AVP and CYC E-Box-containing sequences to the overexpression of B/C could result from different affinities for various DNA-binding factors. To test this notion we compared the ability of two double-stranded oligonucleotides (underlined sequences in Fig. 1A), centered around the AVP and CYC E-Boxes, to recruit DNA-binding factors in an electrophoretic mobility shift assay (Fig. 2A). As expected, both sites were able to generate robust and specific nucleoprotein complexes when mixed with NIH-3T3 nuclear extracts as the source of E-Box-binding factors (Fig. 2A, first lane in both panels). A preliminary test with several antibodies revealed that the vast majority of nucleoprotein complexes formed between the AVP or CYC E-Box probes and NIH-3T3 nuclear extracts contained a USF-2 related moiety (Fig. 2A, only the results of anti-c-Fos and anti-USF-2 antibodies are shown). Despite the overall similarity in the patterns generated by the two probes, we noticed that the AVP E-Box-dependent nucleoprotein complex was consistently weaker. This suggested a relatively lower affinity of the AVP E-Box for the dominant binding factor in the extract (i.e. USF-2-related) because the specific activities of the two probes were identical, and the free probe was present in large excess. Consistent with this interpretation, a cold CYC E-Box extended sequence was approximately twice as efficient as a cold AVP E-Box competitor at displacing either the AVP E-Box or CYC E-Box probe from the USF-2-related complex (Fig. 2B).

As shown above, the extended AVP and CYC E-Boxes can bind USF-2-like factors in vitro with apparently different affinities and respond very differently to B/C stimulation when operating from within their natural environments. These
Characterization of a Circadian E-Box

Fig. 2. DNA binding and transactivation competence of AVP and CYC E-Box-containing sequences outside of their natural context. A, EMSA analysis of NIH-3T3 nuclear extracts and double-stranded oligonucleotide probes carrying the AVP or CYC extended E-Box sequences (underlined sequences in Fig. 1A). Supershifting reactions were performed in the presence of either control (−), anti USF-2, or anti c-FOS antibodies (A arrowhead). Specificity was tested by blocking the antibodies with the immunizing USF-2 (U) or c-FOS (C) peptides (P arrowhead) prior to the binding assay. S arrowhead: supershifted complex; E arrowhead: E-Box complex; F arrowhead: free probe. B, cold competition EMSA (upper panel) and quantitative densitometric (lower panel) analysis of the AVP or CYC E-Box nucleoprotein complexes in the presence of increasing amounts of cold double-stranded competitor (C arrowhead) oligonucleotides carrying either the AVP (●) or CYC (○) E-Box extended sequences shown in Fig. 1A. C, performance of 40-mer sequences centered around the AVP and CYC E-Boxes when placed in a heterologous promoter. NIH-3T3 cells were transiently transfected with luciferase reporter vectors driven by either an SV40 promoter (pGL3P) or the same promoter supplemented with one copy of either the AVP (pGL3P/AVP-E) or CYC (pGL3P/CYC-E) E-Box containing 40-mers. Cells were co-transfected with either pCDNA (open bars) or BMAL1/CLOCK (solid bars). D, performance of the AVP and CYC E-Box 40-mers when placed at the center of the E-Box in the opposite promoter. Experiments were performed essentially as in C. Results are representative of two (C) or four (D) independent experiments and are expressed as relative firefly luciferase activity and stimulation index.

fences notwithstanding, both the AVP and CYC E-Box-containing fragments were weakly responsive to B/C stimulation when placed in the context of the SV40 promoter (Fig. 2C). This result indicates that high affinity of an extended E-Box site for an abundant factor (such as USF-2) may not be sufficient to preclude a low level of B/C responsiveness by a perfect E-Box, although it might contribute to the establishment of refractoriness in the proper context. Such an extended sequence, in isolation, may be unable to support robust stimulation of a true circadian E-Box (such as AVP) or prevent the marginal but significant level of transactivation latent in a non-circadian E-Box (CYC).

Moreover, when the 40-mer E-Box containing sequences derived from AA and CC were wedged in the middle of the opposite E-Box, the resulting CA/C and A(C)A constructs responded in the same general direction as the original CC and AA promoters, respectively (Fig. 2D). Specifically, A(C)A displayed a robust level of induction even though the stimulation index was lower than AA due to increased basal activity. In contrast, CA/C was unable to mount a response to B/C. This result suggests that most of the observed sensitivity to B/C is either granted or blunted by DNA elements located beyond the inserted fragments. The following experiments were designed to systematically search for these cis-acting modifiers of E-Box activity.

E-Box Modifiers Upstream of the Perfect AVP E-Box—First, we focused on the 5′ flanking region by converting different portions of the AA promoter into the equivalent CC sequences. We found that CA, which carries a CYC-derived 5′ flanking region, displays a lower level of basal activity relative to CC but a CC-like refractoriness toward B/C (Fig. 3A). The dramatic effect observed after switching the 5′ region was reversed when the AVP-5′ sequence was used to push the CYC 5′ sequence away from the E-Box (CAA). This result makes the existence of a potent B/C repressor in the 5′ CC region less likely. On the other hand, when increasing amounts of CYC 5′ sequences were replaced by AVP 5′ sequences we observed a slight reduction in basal activity as well as a gradual recovery in B/C stimulation (Fig. 3, AA12C and AA6C). The substitutions in CA, AA12C, and AA6C all include a change to a purine-pyrimidine (Pu-Py) dinucleotide immediately preceding the CYC E-Box, which has been previously shown to confer high affinity binding toward a USF complex (32). Despite this the AA6C construct regained a significant level of B/C stimulation. Taken together, these results suggest the existence of a B/C enhancer element upstream of the AVP E-Box rather than a negative site upstream of the CYC E-Box. Consistent with this hypothesis, deletion of the first 40 bp in the AA minimal promoter had a dramatic negative impact on the ability of the remaining sequence to respond to B/C (Fig. 3A, −8AA) without affecting USF-mediated transactivation (not shown). Thus, a previously unidentified upstream cis-acting (non-E-Box-like) element appears to be required to sustain the response of the AVP E-Box to B/C.

To narrow down the possible location of this B/C enhancer, we generated an additional series of 5′ deletions between the AVP E-Box and the 5′-end of the minimal promoter. All vectors in this series (−8AA through −27AA) responded equally well to
the action of USF (data not shown). In contrast, the poor response of these reporters to B/C overexpression suggests that sequences between positions H11002 and H11002 in this minimal AVP promoter might play an important and specific role in E-Box-dependent B/C transactivation (Fig. 3 B). This 21-bp sequence, which includes a pyrimidine run, contains significant matches to known cis-acting elements (identifiable through the MatInspector V2.2 transcription factor search engine of the Transfac 5.0 database) (50). Chief among these are putative I H9260/H9252, MZF1, and NF-1 DNA-binding sites.

Next, we probed the influence of adjacent 5' positions on the response of the E-Box to B/C. It had been previously proposed that a guanine at position H11002 and an adenine at position H11002 are part of an extended preferred consensus for the B/C-related MOP3/MOP4 complex in vitro (25). According to those results, the AVP E-Box is preceded by an optimal glycine at H11002 and a suboptimal cysteine at H11002. To assess the effect of modifying the presumed binding affinity for the B/C complex, three additional mutants were generated in which the cysteine and glycine bases at positions H11002 and H11002, relative to the center of the palindromic E-Box, were targeted. The performance of three constructs with substitutions at these positions was consistent with the proposed role of these contact points toward B/C responsiveness. The glycine to threonine transversion at position H11002 (Fig. 4, AA6T) resulted in a substantial reduction in B/C stimulation without affecting the response to USF (not shown). On the other hand the predicted positive effect of the cysteine to alanine transversion at position H11002 (AA4A6T). Interestingly, the CYC E-Box is also preceded by a glycine at position H11002, which is consistent with the good response of the A(C)A construct to B/C.

E-Box Modifiers Downstream of the Perfect AVP E-Box—It is clear from the previous results that the 3' CYC sequence is strongly linked to the high basal level of expression of a perfect E-Box (Fig. 3A, compare CA versus CC), and that the 5' AVP sequence is likely to contain a B/C enhancer (Fig. 3B). To test whether the upstream enhancer region in AA is sufficient to confer robust B/C responsiveness onto the CC promoter, we designed the construct referred to as AC. The importance of this promoter construct is 2-fold. First, AC displays a B/C stimulation index that is approximately double the one observed in CC (Fig. 5A; 2.1 ± 0.3 versus 0.8 ± 0.2, p < 0.0001, n = 4). Second, this improved level of stimulation over CC still fell far below the robust 20- to 30-fold increase that we observe consistently with the AA promoter. The higher basal level of expression conferred by downstream CC elements can partially account for this difference. It is conceivable, however, that additional AVP 3' cis-acting elements are required to achieve or approach a full-fledged B/C response. This auxiliary function could be provided by one or more of the previously identified consensus sequences located between the perfect AVP E-Box and the start of transcription (Fig. 5A) (51). To test this hypothesis several candidate sites were mutated, either individually or in selected combinations, and the resulting constructs were tested for B/C responsiveness. As predicted, the perfect E-Box was absolutely required for B/C responsiveness because its mutation completely abrogated stimulation (Fig. 5A, AA4M). We found that alteration of any of the three down-
stream E-Box-like sequences in the AVP promoter (AA2M, AA3M, and AA4M) compromised its ability to respond to B/C. It is important to point out that some of these constructs also displayed a reduced response to USF (data not shown), suggesting that the downstream, imperfect E-Boxes may also serve more promiscuous roles, responding perhaps to multiple signaling pathways. As a consequence, it is not possible to assign a weight to their specific contribution to clock-mediated phenomena versus their possible roles in unrelated (e.g. metabolic) or basal transcription-related events. In a related set of constructs we found that mutation of the downstream AP-1 element had no significant effect on the ability of this reporter to respond to B/C (Fig. 5B, AP1/H11002). Mutation of the CRE-like element, either by itself or in combination with the AP1 mutation, had a modest negative effect that did not reach statistical significance but could suggest a weak enhancer role (Fig. 5B, CRE− and AC−).

It is possible that the positive contribution of the AVP E-Box-like sequences emerges from cooperative interactions between the perfect E-Box and the upstream sites. We reasoned that the unavailability of suitable accessory elements might render the perfect E-Box incapable of mounting a strong response to B/C, just like in the AC construct. To test this hypothesis, we inserted a 43-bp-long region encompassing the third and fourth AVP E-Box-like sequences downstream of the perfect E-Box in the CC and AC constructs (Fig. 4C). As predicted, this sequence had a significant positive effect on B/C transactivation, which was absolutely dependent upon the presence of the upstream E-Box enhancer (Fig. 5C, compare CC+E to AC+E). This synergistic configuration between elements aligned upstream and downstream of the E-Box appeared to be B/C-specific because the USF response was virtually unaffected (not shown).

**DISCUSSION**

The perfect E-Box is one of the DNA elements most widely used for controlling transcription. The recent discovery of its involvement in circadian gene expression prompted us to investigate the molecular basis for the differential use of an E-Box in circadian versus non-circadian environments. A few prior studies that have addressed this issue have focused on the E-Box containing the circadian regulatory sequence (CRS) of the *Drosophila period* gene promoter (40–42).
studies present clear evidence that sequences beyond the 6-bp E-Box element ought to provide important information for the efficient activation by the circadian machinery through the dCLK/CYC heterodimer, the Drosophila equivalent of mammalian B/C.

In the present study we compared the arginine vasopressin and cyclin B1 E-Box-containing promoters, two prototypic control regions, only the first of which is regarded as a well established circadian clock-controlled region. Despite the apparent inability of the cyclin B1 promoter to support B/C transactivation (Fig. 1B), cyclin B1 mRNA displays a robust ~50-fold oscillatory pattern in its accumulation as a function of cell cycle-related events (45), some of which are thought to involve the perfect E-Box at position -170 (12, 46). Thus, even though the AVP and CYC genes contain a perfect E-Box in their promoters, and their transcripts rise and wane with a period of ~24h, their rhythms are the result of fundamentally different regulatory processes. We reasoned that a comparative analysis of these minimal promoters could help identify novel DNA elements that either permit or forbid a perfect E-Box from being activated by the circadian clock.

The basic premise in this study is that the CYC E-Box is sequestered in an environment that is not conducive to B/C transactivation, at least not in asynchronous NIH-3T3 cells (Fig. 1, compare AA to CC). Therefore, flanking and/or accessory sites can be presumed to play a key role in the differential response to B/C displayed by the two promoters. The absence of such sequences is likely to explain the fact that 40-bp-long DNA fragments, centered around the two perfect E-Boxes, display a similar, albeit weak, response to the action of B/C when placed in the context of a heterologous SV40 promoter (Fig. 2C). From a regulatory standpoint, this result posits a potentially unacceptable situation. On one hand, every E-Box-containing promoter should be at least marginally responsive to the circadian clock. Truly circadian promoters, on the other hand, usually display a dramatically more robust induction during their rhythmic cycles of expression.

Our initial analysis of the flanking sequences confirmed the existence of short-range effects exerted by the region immediately adjacent to the E-Box that can either improve the binding affinity of the core site for B/C and/or increase the basal level of expression by conferring high affinity binding toward constitutive or abundant transcription factors. These effects could explain the higher basal level of the AC/CAT construct when compared with the parental AA promoter (Fig. 2D), as well as the significant increase in the response of AC to B/C, relative to CC (Fig. 5A, 2.6 ± 0.9 versus 0.8 ± 0.2; S.I. ± S.D., n = 4). Also consistent with this model is the complete abrogation of AVP responsiveness to B/C seen in the CA construct. Formally, these results are also compatible with the existence of a positive regulatory element upstream of the AVP E-Box. This hypothesis is consistent with the results obtained with a series of AA 5’ deletion mutants, which allowed us to narrow down the location of this potentially novel B/C enhancer to a site between positions -27 and -48 upstream of the AVP E-Box (Fig. 3B). The contribution of such a site was also revealed by the fact that the addition of the third and fourth E-Boxes into AC, but not into CC, significantly enhanced the capacity of this chimera to respond to B/C (Fig. 5C). Taken together, these results suggest that we have uncovered, in the AVP promoter, the first example of a non E-Box-like element working to achieve maximal responsiveness to B/C. The molecular basis for the influence of this 21-bp sequence upon B/C activity is currently under investigation.

It is not surprising that the bases immediately adjacent to an E-Box help restrict the universe of factors that could interact productively with the site. In fact, there is a vast literature dealing with the base preferences, inside and outside of the core E-Box, displayed by various E-Box factors (see Refs. 28, 32, and 33). Furthermore, Bmal1 appears to select different target sequences as a function of the partner that is provided in vitro (25). Ours is the first study to address the issue of extended context requirement by a B/C complex from the perspective of a natural mammalian promoter.

The systematic, albeit partial, comparison of the apparent relative strength of these sites with regards to B/C stimulation revealed potentially important information about the contact points around the E-Box that are critical for efficient B/C stimulation in vivo. The extended base preference of a related complex containing MOP3 (Bmal1) and MOP4, a brain-specific homologue of CLOCK, had been previously proposed to be (G/T)G(A/G)ACACGTGACCC, using an in vitro binding site selection protocol (25). Our study corroborated this extended consensus, at least on the 5’-side of the perfect AVP E-Box. We have focused on positions −6 and −4 because they appear to be conserved among a collection of strong circadian E-Boxes.2 First, transversion of a threonine for the glycine at position −6 (relative to the central symmetry axis) resulted in a dramatic reduction in responsiveness that was restricted to the B/C pathway. This defect was completely reversed when the original cysteine at position −4 was substituted by a presumably optimal alanine (Fig. 4, AA4A67T).

It is interesting to note that when we compared the nucleoprotein complexes generated between NIH-3T3 nuclear extracts and either the AVP or CYC E-Box-containing oligonucleotides, the most reproducible difference was the significantly more intense signal generated when the CYC probe was used (Fig. 2A). This result suggested that the CYC E-Box can bind constitutively expressed B/C-binding factors, such as the USF complex, with higher affinity than the AVP probe. Indeed, EMSA analysis supports this notion (Fig. 2, B and C). This finding is consistent with the previously reported flanking base preferences of USF (RYCACGTGKY) (32) in which there exists a 3 versus 1 of 4 possible matches between CYC and AVP, among the four flanking bases. It is tempting to propose that E-Boxes with high affinity for such binding factors may normally be occupied and not available for interaction with the more discriminating B/C complex. Thus, high affinity for a constitutive and abundant E-Box-binding factor (such as USF2) might render a site less available for occupancy by relatively rare and/or highly controlled factors. This strategy could explain, at least in part, the refractoriness of the CYC E-Box toward B/C and could be in general use by perfect E-Boxes to reduce or avoid circadian fluctuations. Naturally, high levels of basal activity will reduce the stimulation index. This characteristic, however, need not preclude a promoter from being controlled by the clock. In fact, the AVP gene itself displays high levels of constitutive activity in vivo, in both the suprachiasmatic nucleus and supraoptic nucleus (SON) (9), where the appropriate signaling pathways can still trigger a robust increase above background.

**Effect of Sequences Downstream of the Perfect AVP E-Box**—In addition to the strong positive effect of the newly identified AVP upstream sequence, we have also found that each of the three downstream E-Box-like sequences could cooperate in the response to B/C (Fig. 5A). The importance of secondary E-Box sites had been predicted following the isolation and characterization of several circadian regulated gene promoters, like period or dbp, which include several E-Box elements. In addition to the E-Box-like elements we also inves-

2 R. Baler, unpublished observation.
tigated the possible influence of the AP-1- and CRE-like elements located downstream of the perfect AVP E-Box. Mutation of the AP-1 site had no detectable effect on the level of B/C stimulation. Inactivation of the CRE site, however, led to a decrease in promoter activity in response to B/C, suggesting that this site could potentially act as a weak enhancer (Fig. 5B).

The Circadian E-(Tool)Box?—The endowment of the promoters used in this study with E-Box modifiers allows the transcriptional machinery to modulate the circadian strength of a perfect E-Box over a wide range of amplitudes. As a consequence, the potential of a perfect E-Box to respond to B/C stimulation can manifest itself through many values, from total refractoriness to high sensitivity. The non-circadian end of the spectrum can be reached with a combination of high affinity binding for constitutive factors (e.g. USF-like) and/or the presence of cis-acting binding sites that mediate high levels of basal expression (Fig. 6A). Circadian rhythmicity, on the other hand, could be obtained in a stepwise fashion by a shift in the binding affinity away from USF-like factors (B) and toward the B/C complex (C) and then by the acquisition of specific E-Box-like accessory elements (D). Finally, our results with several promoter constructs (AC, AC+3/4, A(C)A, and −24AA) strongly suggest that an upstream, non-E-Box-like element provides a significant contribution to achieve maximal B/C transactivation (E). These and other factors are likely to combine in different promoters to generate a continuum of circadian amplitudes. Because these accessory sites could mediate temporally controlled activities, it is conceivable that such a configuration also provides for some degree of flexibility in the spectrum of phase angles in rhythmic gene expression.

With regards to the promoter context, it might be possible now to predict and test the circadian response of other perfect E-Box-containing promoters using the basic guiding principles outlined herein. This is becoming an important issue as high-density array analysis yields ever increasing numbers of circadian regulated genes (52, 53). The present study also has significant implications regarding the effect of the cellular environment in maintaining proper circadian and non-circadian regulation of transcription. For example, based on the high affinity of the non-circadian CYC E-Box for the abundant USF2-containing complex, we would predict that the absence of USF2 protein could result to some extent in the “circadian release” of this promoter, which might now display a weak level of clock-dependent regulation. The possible role of USF2 in preventing circadian fluctuations in the transcription of perfect E-Box-containing promoters is a testable hypothesis because two independent USF2 knockout mouse strains are now available (54, 55).

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