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Functional Molecular Analysis of a Circadian Clock Gene *timeless* Promoter from the Drosophilid Fly *Chymomyza costata*

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Abstract The circadian transcription of the *tim* gene is tightly regulated by the protein complex dCLK/CYC, which directly interacts with a series of closely spaced E-box and E-box–like elements in the *Drosophila timeless* promoter. The *tim* promoter from *D. melanogaster* has been studied in detail both in tissue cultures and in living flies yet has never been investigated in other species. This article presents a detailed functional analysis of the *tim* promoter from the drosophilid fly, *Chymomyza costata*, in *Drosophila* tissue cultures. A comparison of *tim* promoters from *wt* and *npd*-mutants confirmed that the 1855 bp deletion in the latter removes crucial regulatory *cis*-elements as well as the minimal promoter, being subsequently responsible for the lack of *tim* mRNA expression. Deletion and substitution mutations of the *wt tim* promoter showed that the region containing the canonical E-box, TER-box, and 2 incomplete E-box sequences is essential for CLK/CYC-mediated expression, while the PERR element appears to be a repressor in S2 cells. Furthermore, the expression of the circadian genes *timeless*, *period*, *vrille*, and *doubletime* was quantified in *C. costata* adults. Striking differences were found in expression profiles for *tim*, *per*, and *vri* between *wild-type* and *npd*-mutant individuals.

Key words *timeless* promoter, E-box, *Chymomyza costata*, circadian and photoperiodic clock, *Drosophila* S2 cells, *timeless* null mutation

According to the current model, the *Drosophila* circadian clock system relies on the existence of 3 interlocked feedback loops (reviewed by Tomioka and Matsumoto, 2010; Hardin, 2005; Hall, 2003). The central loop consists of the genes *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*). Transcription of *per* and *tim* genes is activated by the protein complex dCLK/CYC. PER and TIM proteins then repress transcription of their own genes by binding to the dCLK/CYC dimer. The temporal delay between the transcription peak of *per* and *tim* mRNA and the accumulation of PER and TIM proteins in the cytoplasm and their subsequent entry into the nucleus is ensured by the action of 2 kinases, DOUBLETIME and SHAGGY. The second loop comprises the *Clk*, *cyc* genes, and 2 other transcription factors, *vrille* (*vri*) and *Par Domain Protein 1* (*Pdp1*). The dCLK/CYC complex activates the expression of both *vri* and *Pdp1* genes, and the resulting VRI and PDP1 proteins in turn regulate the rhythmic transcription of *dClk*. The

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third loop involves the *Clk*, *cyc*, and *clockwork orange* (*cwo*) genes. The rhythmic expression of *cwo* is also directly regulated by dCLK/CYC. The CWO protein then functions as a transcriptional repressor that suppresses not only its own expression but also that of *per*, *tim*, *vri*, and *Pdp1*.

All the 3 loops within the *Drosophila* circadian clock mechanism involve the dCLK/CYC protein dimer, which serves as the main positive regulator of the circadian transcription. dCLK (Allada et al., 1998) and CYC (Rutila et al, 1998) are helix-loop-helix PAS (Period-Arnt-Sim) domain proteins that bind to the E-box sequence (CACGTG) in *Drosophila* promoters. The overall length and structure of *per*, *tim*, *vri*, and *cwo* promoters vary, but all of them possess 1 or more canonical E-boxes (Hao et al., 1997; McDonald et al., 2001; Blau and Young, 1999; Matsumoto et al., 2007). The function of both *per* and *tim* promoters was studied in *Drosophila* tissue cultures as well as in living flies (Hao et al., 1997; Stanewsky et al., 2002; McDonald et al., 2001; Okada et al., 2001). A detailed analysis of the *tim* promoter in *D. melanogaster* suggested an important role in the regulation of *timeless* transcription for the following elements: 1 canonical E-box (CACGTG), 2 Tim-E-box–like sequences (TER1, TER2; GCGGCACGTTG), and a PERR-box (GTTCG CACAA) (McDonald et al., 2001).

A recent study on the drosophilid fly, *Chymomyza costata*, revealed a mutation in the *timeless* promoter region responsible for the mutant phenotype of the *npd* (non–photoperiodic-diapause) fly strain (Stehlík et al., 2008). The *npd*-mutant of *C. costata* was originally identified by Riihimaa and Kimura in 1988. The *npd*-mutant larvae do not respond to short photoperiods and continue to develop into pupae and adults, while the wild-type strain responds to subcritically short days by entering larval diapause. Subsequent experiments confirmed that this autosomal recessive mutation is also responsible for circadian defects in adult eclosion (Kostál and Shimada, 2001). Polymorphism associated with the *tim* gene segregated with the *npd* phenotype, and *tim* mRNA expression was reduced to undetectable levels in *npd*-mutants (Pavelka et al., 2003). These results indicated that the locus *npd* could code for the *tim* gene. An ensuing study was focused on detailed analysis of *tim* gene in *wt* and *npd*-mutant larvae (Stehlík et al., 2008). In contrast to the *wt* strain, the *npd*-mutant produces insignificantly low levels of *tim* mRNA that do not show any daily oscillations. Furthermore, the TIM protein was never at any time detected in the CNS. It was shown that the *npd*-mutant carries (a) 37 amino acid substitutions in the putative TIM protein and (b) a 1855 bp deletion in the *tim* promoter. As the amino acid substitutions were interpreted as irrelevant, the authors assumed the deletion to be the primary cause of the lack of *tim* transcription in the *npd*-mutant.

This study aims to elucidate (1) whether the 1855 bp deletion is truly responsible for the null phenotype in the *npd*-mutant, (2) how the individual *cis*-acting elements in the *tim* promoter (the E-box, TER-box, and PERR-box) contribute to the transcription of *tim* in *C. costata*, and (3) the impact of this *tim* null mutation on the circadian clock of *C. costata*. To fulfill these goals, the expression of the circadian clock genes *tim*, *per*, *vri*, and *dbt* was quantified in both in *wt* and *npd*-strains.

METHODS

Fly Strains

Two strains of *Chymomyza costata* (Diptera: Drosophilidae) were used: wild-type strain (*wt*, originally collected in Sapporo, Japan, in 1983) and non–photoperiodic-diapause (*npd*) mutant strain (Riihimaa and Kimura, 1988). Larvae, pupae, and adults were fed an artificial diet (Lakovaara, 1969) and reared at a constant temperature of 19 °C. The *wt* strain was kept in long-day (LD) conditions (16 h light/8 h dark), during which all larvae developed into pupae and adults. The *npd*-strain was kept in short-day (SD) conditions (12 h light/12 h dark). If needed for gene expression studies, the newly eclosed *wt* adults were entrained to SD conditions for 4 to 5 days before they were collected, and likewise *npd* adults were entrained to LD conditions.

Partial Cloning of *per* gDNA and *vri* and *dbt* cDNA of *C. costata*

Total RNA was isolated from 20 to 30 adult *C. costata* heads using TriReagent (Molecular Research Center, Cincinnati, OH, USA). cDNA was synthesized from 1 μg of total RNA using a $dT_{(16)}$ primer and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Genomic DNA from *C. costata*, *D. melanogaster*, and *T. castaneum* was isolated using DNeasy Blood & Tissue Kit (Qiagen).

Specific *per* primers (see Table S1 in Online Supplement S1) were designed according to *wt* (AB014476) and *npd* (AB014477) *per* sequences. This primer pair was used to isolate a 65 bp intron from

genomic DNA. We then designed intron-overlapping *per* primers (Supplement S1, Table S2) for quantitative gene expression (Q-PCR). Degenerate primers were designed using published sequences of *D. melanogaster*, *Anopheles gambiae*, and *Mus musculus* and were used to isolate suitable fragments of *vri* and *dbt* cDNA (Supplement S1, Table S1). Successfully cloned fragments of *vri*, and *dbt* cDNA were used to design primers for Q-PCR (Supplement S1, Table S2).

Temporal Gene Expression Studies

wt and *npd* flies were collected every 4 h in both LD and SD regimes. Total RNA from 10 to 20 heads at each time point was isolated using TriReagent (Molecular Research Center). Total RNA was treated with Turbo DNase according to standard protocol (Turbo DNA-free Kit, Applied Biosystems, Foster City, CA, USA) and successful DNA removal was confirmed using PCR. cDNA was synthesized from 1 μg of DNase-treated RNA using a $dT_{(16)}$ primer and SuperScript III Reverse Transcriptase (Invitrogen). Q-PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a C1000 Thermal Cycler (Bio-Rad Laboratories). The Q-PCR conditions were as follows: initial denaturation for 3 min at 95 °C, followed by 10 sec at 95 °C, 20 sec at 50 °C, 20 sec at 72 °C, 40 cycles. *RP49* (syn. *RpL32*) was used as an endogenous reference gene. Primer sequences are available in Supplement S1, Table S2.

Constructs

The promoter reporter plasmids were constructed using the pBluescript II KS⁻ phagemid vector (Stratagene, La Jolla, CA, USA). The SV40 polyadenylation signal region was amplified using PCR from the pAc5.1/V5-HisA Vector (Invitrogen) and cloned into the EcoRI-KpnI sites of the pBS II KS⁻ vector. The AscI site was introduced into the 5′ end of the SV40 simultaneously. Firefly luciferase was amplified using PCR and cloned into the SpeI-AscI sites of the pBS II KS⁻/SV40 vector, and the Kozak sequence (AGTATGG) was introduced onto the 5′ end of luciferase (underlined sequence corresponds to the translated region). The *wt tim* and *npd tim* promoter fragments (Fig. 1A) were amplified from *C. costata* genomic DNA using PCR (primers in Supplement S1, Table S3). These fragments were cloned into NotI-SpeI sites of the pBS II KS⁻/*luc*/SV40 vector (Fig. 2A, constructs *wt*, *npd*). The *wt tim* promoter with 3′ or 5′ truncated ends (Fig. 2A, 2, 3, 4, 6) and full-length *wt tim* promoters with either deleted PERR-box or E-box regions (Fig. 2A, 1, 5) were amplified using PCR and cloned into NotI-SpeI sites of the pBS II KS⁻/*luc*/SV40 vector.

Promoter modifications in E-box region (Fig. 2B, 7-20) were generated by PCR (details are available in Supplement S1), and resulting constructs were verified by sequencing. pActin (pAc) Clk V5, pAc per V5, and the *Renilla* luciferase reporter under the control of the *copia* promoter were generously provided by P. Nawathean, previously described in Nawathean and Rosbash (2004) and McDonald et al. (2001).

Transfection Assay

S2 cells were cultured in HyClone SFX-Insect medium (HyClone Laboratories, Cache County, UT, USA) supplemented with 10% FBS (Gibco) at 25 °C. S2 cells were plated onto 24-well tissue culture plates at 75% to 80% confluence. The following day, HyClone SFX-serum medium was replaced with HyClone SFX-serum free medium and incubated for 2 h. The medium was aspirated afterward, and S2 cells were transfected with 400 μL of transfection mix consisting of HyClone SFX-serum free medium, 4% Cellfectin Reagent (Invitrogen), and 30 ng of promoter reporter construct. As an internal control for transfection efficiency, 200 ng of the *Renilla* luciferase reporter under control of the *copia* promoter (McDonald et al., 2001) was added to each well. Some wells were supplemented with 5 ng pAc Clk V5 and 50 ng pAc per V5. After 6 to 8 h, 400 μL of HyClone SFX medium supplemented with 20% FBS was added to the transfected S2 cells, and they were incubated for the next 40 to 48 h at 25 °C. The transfected cells were harvested and processed with the Dual-Luciferase Reporter Assay System (Promega , Fitchburg, WI, USA) according to the manufacturer's recommendations and measured with an Orion II luminometer (Berthold, Bad Wildbad, Germany). Promoter luciferase activity was normalized to *Renilla* luciferase activity. Then *wt tim -luc*/CLK promoter activity (Figs. 1B and 2A, construct *wt*) was taken as the reference value (100%), and activities of the remaining constructs were normalized accordingly by relative percentage.

Sequences and Statistical Analysis

Alignment of the conserved *tim* promoter regulatory region from different insect species (accession

numbers are in Supplement S1) was performed using MegAlign program (DNASTAR, Lasergene, WI, USA).

Transfection assay data and Q-PCR data were analyzed by 1-way ANOVA and Tukey's multiple-comparison test (Statistica 9.1, StatSoft Inc., Tulsa, OK, USA; www.statsoft.com).

For analysis of the circadian rhythm characteristics, the expression profiles of *per*, *tim*, *vri*, and *dbt* were fitted with single cosine curves, defined by the equation Y = mesor + (amplitude*cos(2*π*(X-acrophase)/wavelength)) with a constant wavelength of 24 h. Amplitude, acrophase, mesor, and coefficient of determination R^2 (i.e., goodness of fit) were calculated (Prism 5 software, Graphpad, La Jolla, CA).

RESULTS

The *tim* Promoter of the *npd*-Mutant Exhibits No Transcription Activity in S2 Cells

To demonstrate the connection between a deletion in the *tim* promoter and the dramatic reduction in *tim* transcription observed in *npd*-mutant larvae of *C. costata* (Stehlík et al., 2008), we generated *wt* and *npd tim* promoter-luciferase constructs (Fig. 1A) and measured the level of luciferase activity in S2 cells.

Transfection of the cells with the promoter constructs alone resulted in baseline luciferase activities. Cotransfection of p(Ac) Clk with *wt tim-luc* strongly induced luciferase transcription, which was used as a reference and is plotted as 100% (Fig. 1B). However, *npd tim-luc* showed low levels of transcription activity independent of the presence of *dClk* (0.06%, Fig. 1B). As PER is a known negative regulator of dCLK-CYC activity (Darlington et al., 1998), we used a p(Ac) per plasmid to ensure that our experimental design was effective. The addition of p(Ac) per reduced luciferase transcription activity by 40% compared to the value observed for *wt tim-luc*/CLK (Fig. 1B; $p < 0.05$).

We conclude that the 1855 bp deletion contains crucial elements for the proper functioning of the *tim* promoter since its deletion in *npd tim-luc* completely abolished luciferase activity in S2 tissue culture.

Interspecific Comparison of *tim* Promoters in S2 Cells

As previously described, the 1855 bp deletion in the *tim* promoter of the *C. costata npd*-mutant contains 1 canonical E-box (CACGTG), 1 TER-box (Tim-E-box-like repeats; GCAGCACGTTG), 1 PERR-box

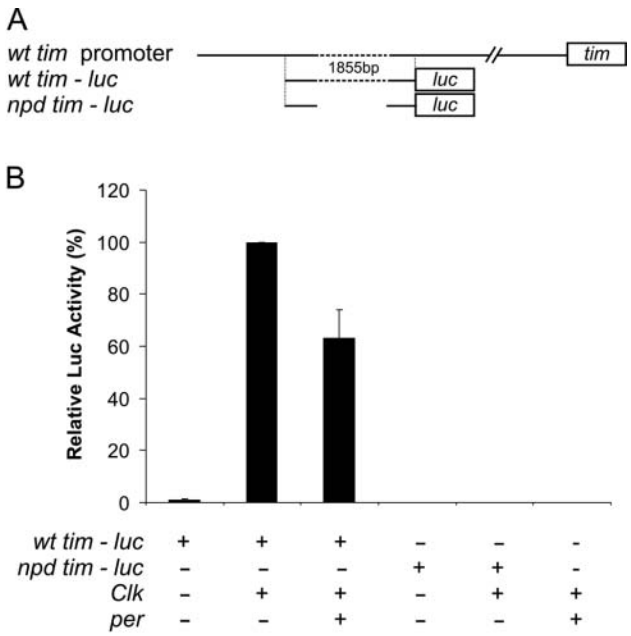

Figure 1. In vitro comparison of *wt* and *npd* promoters. (A) Schematic illustration of the *C. costata timeless* promoter region is shown by the first line. The broken line indicates a portion of the promoter that was not sequenced; the dashed line corresponds to the 1855 bp deletion in the *npd* strain (see Supplement S2 for a detailed promoter illustration). *wt tim-luc* (second line) and *npd tim-luc* (third line) represent constructs used to transfect S2 cells. (B) Relative luciferase activities of *wt tim-luc* and *npd tim-luc* constructs. Promoter constructs were cotransfected with pAc Clk V5 (Clk) and pAc per V5 (per) (the plasmid combination is indicated below each bar). Luciferase activities were normalized to *copia:Renilla* activity, and the *wt tim-luc*/CLK promoter activity was taken as the 100% reference value.

(PER repeat; GTACGCACGA), and the putative transcription initiation site (Stehlík et al., 2008). Apart from these elements, 7 noncanonical E-boxes (CANNTG other than CACGTG) and 2 incomplete E-boxes (ACGTG) were found (see Supplement S2 for a detailed promoter illustration).

To gain a better understanding of the evolution of the *tim* promoter, we compared the sequences of 12 *Drosophila* species, *Chymomyza costata*, the mosquitoes *Aedes aegypti* and *Anopheles gambie*, and the beetle *Tribolium castaneum*. These sequences vary in length, and extensive sections of the *tim* promoter sequences are mutually inconsistent. Only 1 region within all of the examined sequences was conserved enough to perform an alignment (Fig. 2D). A highly conserved canonical E-box and an adjacent CACGTTG motif are the only common features within this alignment. A previous study of *D. melanogaster* (McDonald

et al., 2001) showed that the W-box, TER1, canonical E-box, PERR-box, and TER2 are all functionally relevant *cis*-acting elements. As we examine organisms that are more distantly related to *D. melanogaster*, we observe a reduction in the number or length of these elements, in addition to variation in their arrangement. The W-box, PERR-box, and TER2 are only present within the *melanogaster* subgroup (Fig. 2D, *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, and *D. erecta*). A partially conserved TER2 sequence is present within *D. pseudoobscura* and *D. persimilis*, but within other *Drosophila* species only the canonical E-box, TER1, and the overlapping incomplete E-box (ACGTG) are conserved. TER1 and the incomplete E-box are further reduced to the core TER sequence CACGTTG within *A. aegypti*, *A. gambie*, and *T. castaneum*. Thorough examination of the *C. costata tim* promoter revealed 1 PERR-box sequence located 330 bp upstream of the canonical E-box (Supplement 2, Fig. 2A). Unlike the *D. melanogaster* PERR-box, there are 2 base substitutions in the sequence (*C. costata* PERR-box: GTACGCACGA).

The conservation of the elements described above suggests their role in expression control. We prepared a set of truncated and differently modified *wt tim-luc* constructs (Fig. 2A, B) to test this hypothesis.

The *npd* Deletion Removes the Minimal Promoter Sequence

Luciferase transcription of the 1237 bp 3′ truncated end of the *wt tim* promoter is completely abolished even when all other putative regulation elements are present (the PERR-box, incomplete and canonical E-boxes, and TER-box; Fig. 2A, 6; *wt* vs. 6: $p < 0.01$). The 3′ end of the *wt tim* promoter apparently contains the transcription initiation site and the minimal promoter required for proper transcription. This hypothesis is further supported by (1) the identification of the transcription initiation site by 5′ RACE (see Supplements S1 and S3), and (2) high levels of luciferase expression in constructs where the 1096 bp or 1179 bp 3′ to the transcription initiation site were removed (see Supplement S3).

PERR-Box Has a Repressive Role in the *Cc tim* Promoter

A 863 bp long 5′ end truncation of *wt tim-luc* by did not affect transcriptional activity (Fig. 2A, 3; *wt* vs. 3; $p > 0.05$). However, a more extensive 1172 bp truncation, which removes the PERR-box (Fig. 2A, 2; Fig. 2B, 7; construct 7 has extra NotI site) showed a 38% increase in transcriptional activity as compared to the *wt tim-luc* construct (*wt* vs. 2, 7; $p < 0.01$). Similarly, deletion of the PERR-box in an otherwise full-length *wt tim-luc* construct caused a 47% increase in transcriptional activity (Fig. 2A, 1; *wt* vs. 1; $p < 0.01$), suggesting that *C. costata* PERR-box has a repressive role in S2 cells.

To test whether *C. costata* PERR-box has also a repressive role in the context of the *D. melanogaster* promoter (McDonald et al., 2001), we introduced this *C. costata* element into *Drosophila* E-box region (see Supplement S4, 25). Nevertheless, this modification led to decrement comparable with complete PERR-box removal (Supplement S4, 26), indicating that *C. costata* PERR has no function when placed into *D. melanogaster* position.

The Synergistic Cooperation of the Canonical E-Box and Adjacent Enhancer Elements

We progressively deleted all 4 motifs within the E-box region (Fig. 2A, 14, 13, 11, 8), and to simplify cloning we generated these mutations in a construct lacking the first 1172 bp including repressive PERR-box element. Therefore, the nonmutated construct 7 leads to 140% activity as compared to that of the full *tim wt* promoter. Deletion of the TER-box reduced transcriptional activity by ~35% (13; 7 vs. 13; $p > 0.05$), while deletion of the canonical E-box reduced transcriptional activity by ~20% (11; 7 vs. 11; $p > 0.05$) relative to the reference (construct 7). Eliminating the incomplete E-boxI decreased transcriptional activity significantly by 37% (14; 7 vs. 14; $p < 0.01$), while deletion of incomplete E-boxII did not result in any significant changes (8; 7 vs. 8; $p > 0.05$).

Deletion of both incomplete E-boxes did not significantly change luciferase transcriptional activity (Fig. 2A, 9; 7 vs. 9; $p > 0.05$). However, after deletion of both the TER-box and canonical E-box (Fig. 2A, 19), incomplete E-boxI and E-boxII were able to maintain 37% of the reference transcriptional activity (7 vs. 19; $p < 0.01$). Deleting the canonical E-box and either the incomplete E-boxI or E-boxII (Fig. 2A, 17, 16) resulted in a decrease by 62% and 42% in transcriptional activity, respectively (7 vs. 17, 16; $p < 0.01$). Likewise, deletion of the TER-box and incomplete E-boxII (Fig. 2A, 15) resulted in a 40% reduction in transcriptional activity (7 vs. 15; $p < 0.05$). Only when we removed the TER-box, canonical E-box, and incomplete E-box II did the transcriptional activity decrease

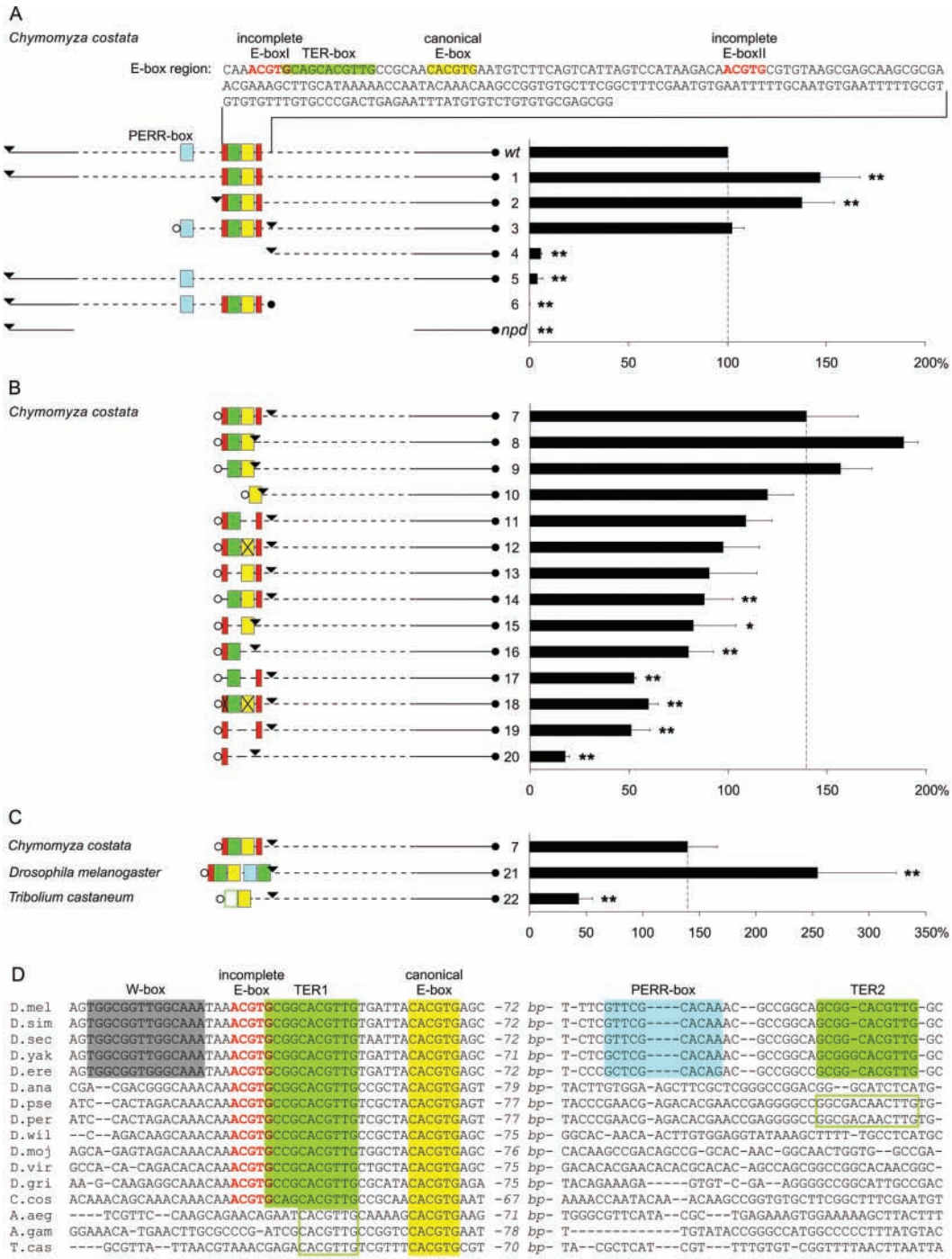

Figure 2. **In vitro detailed analysis of the *tim* promoter. Constructs are on the left side of panel, with a dashed line indicating the 1855 bp deletion in the *npd* promoter and with symbols illustrating the addition of restriction sites into constructs: NotI (▾), SpeI (●), SacII (○). The black chart indicates the relative expression induced by the *dCLK* transcription factor, where expression is measured relative to *copia:Renilla* activity. All constructs were normalized to *wt* expression (100%). * $p < 0.05$, ** $p < 0.01$ (1-way ANOVA followed by Tukey's test; construct *wt* was used as reference in A, construct 7 was used as reference in B, C). (A). *C. costata* deletion constructs. On top, the sequence of the 216 bp E-box region is shown. The *cis*-elements that were manipulated are highlighted, and their colors correspond to the schematic depictions shown below. (B) *C. costata* deletion and substitution constructs. Mutated E-boxes are crossed out, construct 12 contains an AGATCA substitution for the canonical E-box (CACGTG), and construct 18 contains 4 and 6 adenines, respectively, within the incomplete E-boxI (ACGTG) and canonical E-box. (C) Interspecific comparison of the E-box region from *D. melanogaster*, *C. costata*, and *Tribolium castaneum*. All 3 constructs are derived from *C. costata* construct 6 and differ only in species-specific E-box regions. (D) Alignment of the conserved *tim* promoter regulatory region from different insect species (the NCBI accession numbers are indicated in S1). The color coding corresponds to the simplified pictograms shown in A, B, and C.**

significantly (90%; Fig. 2A, 20; 7 vs. 20; $p < 0.01$), but the remaining incomplete E-boxI was still able to activate luciferase transcription 3 times higher than constructs where all motifs had been removed. The canonical E-box itself is able to fully recover luciferase activity (Fig. 2A, 10; 7 vs. 10; $p > 0.05$), but the presence of the TER-box and incomplete EboxI led to a further, albeit statistically not significant, increase in transcriptional efficiency (compare construct 10 with 9 and 8).

The above described modifications within the E-box region encompassed the complete removal of regulatory elements. We also synthesized a construct where the canonical E-box sequence CACGTG was replaced with AGATCA (Fig. 2B, 12), which does not correspond to any known regulatory motif. Alternatively, the sequences of the incomplete E-boxI and canonical E-box were replaced with 4 and 6 adenine bases, respectively (Fig. 2B, 18). When we compared these substitutive constructs to corresponding deletion constructs (Fig. 2B, compare 11 with 12 and 17 with 18), we did not find statistically significant differences in luciferase transcriptional activity (11 vs. 12; 17 vs. 18; $p > 0.05$).

E-Box Regions from *D. melanogaster*, *C. costata*, and *T. castaneum timeless* Promoters Have Different Activities In Vitro

We found remarkable differences in the *tim* promoter structure among the 12 *Drosophila* species, *C. costata*, *A. aegypti*, *A. gambiae*, and *T. castaneum*, especially in the region surrounding the canonical E-box (Fig. 2D). We chose 3 species, *D. melanogaster*, *C. costata*, and *T. castaneum*, that greatly differed in the structure of their E-box regions. We were particularly interested in how the different composition and arrangement of regulatory elements contribute to the luciferase transcriptional activity in S2 cells. Therefore, the *C. costata* E-box region was substituted with *D. melanogaster* and *T. castaneum* E-box regions.

The E-box region from *D. melanogaster* (construct 21) induced 80% greater luciferase transcriptional activity than the corresponding region from *C. costata* (Fig. 2C; 7 vs. 21; $p < 0.01$). In contrast, the luciferase transcriptional activity driven by the E-box region from the *T. castaneum tim* promoter (construct 22) showed a significant 70% decrease compared to the *C. costata tim* promoter (Fig. 2C; 7 vs. 22; $p < 0.01$). There is a clear relation between the number of *cis*-acting regulation elements within the E-box region and luciferase expression.

Partial Cloning of *vri* and *dbt* cDNAs and *per* Intron

cDNAs and genomic fragment of *per* were deposited in GeneBank under the following accession numbers: *per* HM447211, *vri* HM447212, *dbt* HM447210.

Daily Oscillations of *per*, *tim*, and *vri* in Adult Heads of *C. costata* Are Suppressed or Completely Abolished in the *npd*-Mutant

Riihimaa and Kimura (1988) first recognized the nondiapausing phenotype in the *C. costata* mutant strain. Further dissection of this *npd* mutation suggested that it also affects the circadian clock system (Kostál and Shimada, 2001; Pavelka et al., 2003), and later a disruption of the *tim* gene was shown to underlie the *npd*-mutant phenotype (Stehlík et al., 2008). To evaluate the impact of this *tim* mutation on the function of the whole circadian clock network in *C. costata,* we quantified the expressions of *tim*, *per*, *vri*, and *dbt* genes in both *wt* and *npd*-mutant strains reared under 2 different light regimes. Since gene expression in larvae is strongly affected by brain growth, we used adult flies, whose constant head size enables the assessment of the amplitude and acrophase of the expression curve more precisely.

A clear and robust cycling pattern was observed for *tim*, *per;* and *vri* mRNA transcripts in *wt* fly heads under both LD and SD conditions (Fig. 3, left column). The *tim*, *per*, and *vri* mRNAs peaked ~2 to 3 h earlier under SD conditions, which corresponds to an earlier photophase to scotophase transition in SD (acrophases are listed in Supplement S5). The *dbt* transcript did not possess rhythmic expression in *wt* under any of the used light regimes (Fig. 3, 1-way ANOVA; $F = 1.23$, $p > 0.05$ for LD; $F = 1.96$, $p > 0.05$ for SD).

The expression patterns of the circadian genes *tim*, *per*, and *vri* were strikingly different in the *npd*-mutant (Fig. 3, right column). The expression of *tim* was not detected in adult *npd*-mutants under either LD or SD regimes, which is consistent with published data (Pavelka et al., 2003; Stehlík et al., 2008). The *per* mRNA appeared to cycle in its abundance in the *npd*-mutant strain (1-way ANOVA; $F = 40.8$, $p < 0.01$ for LD; $F = 17.01$, $p < 0.01$ for SD), but the amplitude of its expression was reduced by 65% in LD and by 80% in SD relative to its maxima in the *wt* strain (see S5). In comparison with *wt*, the *vri* transcript levels in *npd* remain temporally level at an intermediate level in both LD and SD (1-way ANOVA; $F = 1.22$, $p > 0.05$ for LD; $F = 1.3$, $p > 0.05$ for SD). The *dbt* expression profiles did not change at all compared to *wt* strain.

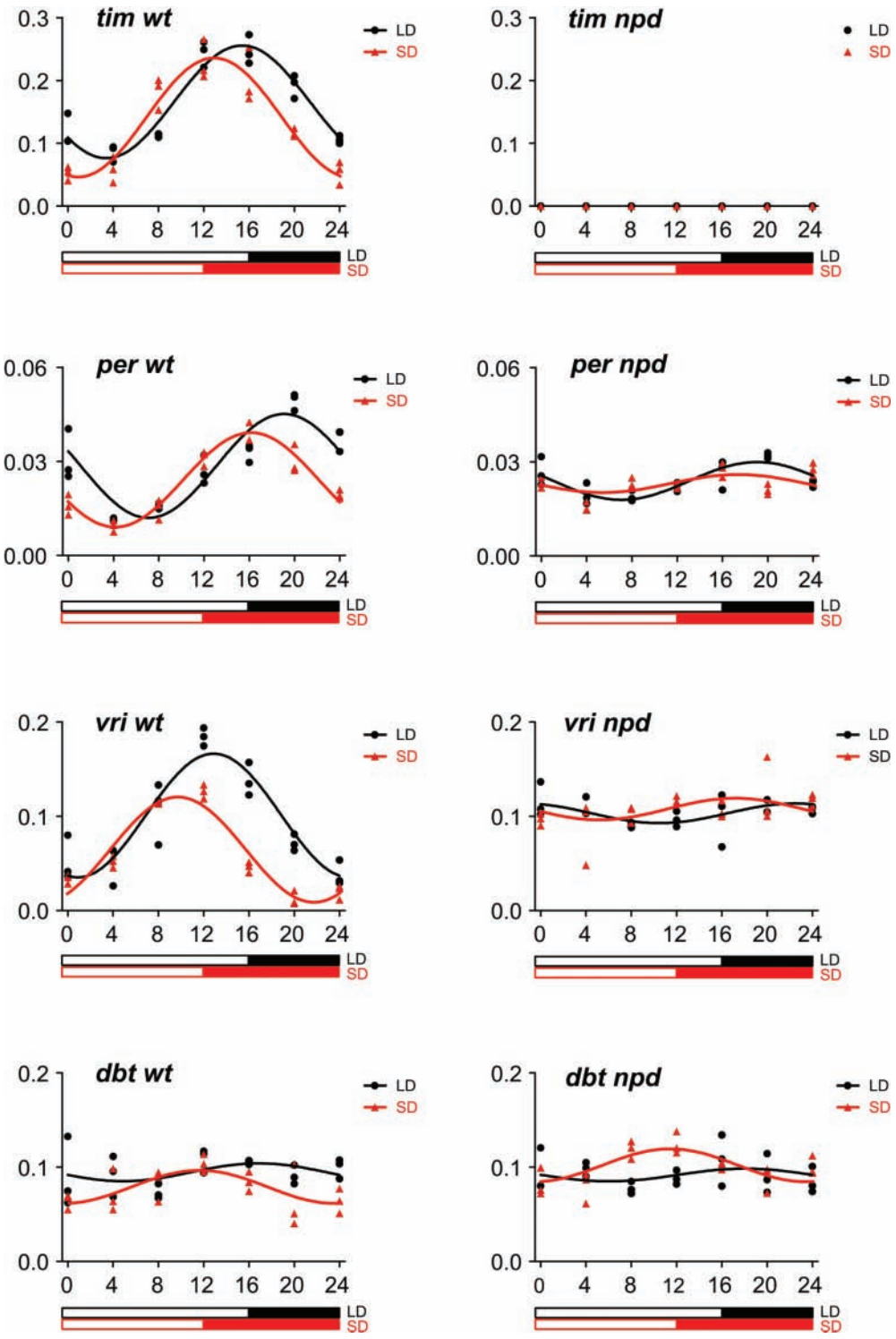

Figure 3. Temporal expression profiles of *per*, *tim*, *vri*, and *dbt* mRNA in *wt* (left column) and *npd*-mutant (right column) adult *C. costata* under LD (black circle) or SD (red rectangle) photoperiodic conditions. Levels of mRNA were determined by quantitative real-time PCR and were normalized to the levels of the housekeeping *Rp49* mRNA. Characteristics of the cosine curve (amplitude, acrophase, and coefficient of determination R^2) are available in Supplement S5. Empty and full bars under the x axis indicate the light and dark phases, respectively.

DISCUSSION

Since the subject of this study is a nonmodel organism, functional assays had to be performed in a heterologous system. In our case we used *Drosophila* S2 cells, a frequently used tool of insect chronobiology (McDonald et al., 2001; Chang et al., 2003; Nawathean and Rosbash 2004; Zhu et al., 2005; Matsumoto et al., 2007; Kadener et al., 2007).

From our in vitro experiments we can summarize the following (Fig. 4): (1) The 1855 bp deletion in the *tim* promoter of the *npd C. costata* strain removes functional elements necessary for its transcription. (2) Within this region, 4 *cis*-acting elements (canonical E-box, TER-box, and 2 incomplete E-boxes) are essential for transcription activity, although each of these *cis*-elements contributes to different extend. (3) In general, deletion of an individual element had a smaller impact compared to multiple deletions. (4) In particular, the tandem arrangement of the TER-box and canonical E-box seems to be crucial for high levels of expression, while the incomplete E-boxI and E-boxII assist in maintaining transcriptional activity. These findings are

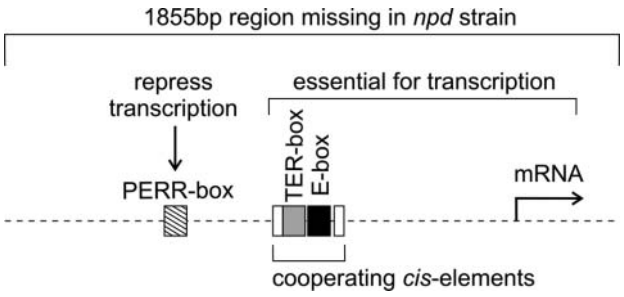

Figure 4. Schematic model of the *C. costata timeless* promoter. The transcription start site is marked by an arrow. *Cis*-elements relevant for transcription activity are depicted: PERR-box (dashed box), canonical E-box (black box), TER-box (gray box), and 2 incomplete E-boxes (open boxes). See Discussion for summary on contribution of each element.

consistent with results described for the *tim* and *per* circadian promoters in *D. melanogaster* (Hao et al., 1997; Okada et al., 2001; McDonald et al., 2001), which demonstrate the interaction of multiple E-boxes and/or E-box–like sequences as necessary for high expression and circadian oscillation.

Our experiments also point to a surprising difference between *D. melanogaster* and *C. costata* promoters. Previous analysis of the *tim* promoter from *D. melanogaster* identified the PERR-box as an enhancer of transcription in *Drosophila* S2 cells (McDonald et al., 2001). Surprisingly, our study shows that this element has a repressive role in the *C. costata* promoter. These data suggest that the location of PERR-box within promoter affects its function, at least in vitro. To explore the function of the PERR element in vivo (e.g., in neurons) will require efficient genetic transformation techniques that are not currently established in *C. costata*. However, certain transposons, such as piggyBac, have been effectively used in various insect species (Berghammer et al., 1999), opening the possibility that this type of transgenic manipulation will become available for *C. costata*.

Our in silico analysis of the upstream *tim* coding regions from different species revealed that while *tim* promoters vary dramatically in length and sequence, there is 1 region that contains the conserved canonical E-box and multiple E-box-like sequences (Fig. 2C). Even promoters from distantly related species (*T. castaneum*, *A. gambiae*, and *A. aegypti*) contain the highly homologous canonical E-box (CACGTG) and the TER-box core sequence (CACGTTG). Using a luciferase reporter assay, we have demonstrated a positive correlation between the number of canonical E-box and E-box–like sequences within the *tim* promoter and the level of expression. Accordingly, we found that the highest level of gene expression was obtained from the *D. melanogaster* construct, with lower expression being observed from the *C. costata* construct and the lowest levels of expression derived from that from *T. castaneum* sequences.

However, we cannot exclude the possibility that promoters from different organisms might be less efficient, per se, in *D. melanogaster* cells owing to potential differences in the design of the clock machineries. This prospect might be the case in *T. castaneum*, where protein sequences of the putative *tim* transcription factors tcCLK and tcBMAL are similar to the silk moth *Antheraea pernyii* homologues (data not shown). In *A. pernyii*, the transactivation domain lies in the C-terminus of apBMAL (Chang et al., 2003), whereas it is located in the C-terminus of dCLOCK in the *D. melanogaster* ortholog. Since CLK and BMAL transcription factors are conserved in protein sequence and domain structure between *D. melanogaster* and *Musca domestica* (Bazalova and Dolezel, unpublished data), we can reasonably expect that these genes are also conserved in *Chymomyza*, a genus much more closely related to *Drosophila*.

Our in vitro experiments confirmed the *npd* mutation as a *timeless* gene mutation that results in virtually no *tim* mRNA in vivo (Pavelka et al., 2003; Stehlík et al., 2008; and also this study). As a consequence, this mutation affects the expression profile of circadian genes *per* and *vri*, reducing their high-amplitude cycling to flat, intermediate expression levels. The expression pattern of *dbt* is neither cyclic nor affected by the *npd* mutation. All these findings are in accordance with the *D. melanogaster* data (Sehgal et al., 1994; Kloss et al., 1998; Blau and Young, 1999).

Photoperiod influences the phase of *per*, *tim*, and *vri* cycling in wild-type *C. costata* adults in a similar manner as in *D. melanogaster* (Qiu and Hardin, 1996; Majercak et al., 1999). In the case of *vri*, the amplitude is also significantly changed. The day length is a primer cue for diapause induction in *C. costata* (Riihimaa and Kimura, 1988), and it is tempting to speculate that the relative ratio of circadian gene products might be used in the photoperiodic time measurement (Tauber and Kyriacou, 2001). Since Pavelka et al. (2003) showed that double-stranded RNA interference is effective in *C. costata*, targeted knock-down seems to be a reasonable

approach to study circadian genes in this organism, especially those that are not essential for animal survival such as *period* (Konopka and Benzer, 1971) or *cryptochrome* (Doleželová et al., 2007).

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NOTE

Supplementary online material for this article is available on the *Journal of Biological Rhythms* Web site: http://jbr.sagepub.com/supplemental.

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