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ORCHESTRATION OF OXIDATIVE STRESS RESPONSES IN *DROSOPHILA MELANOGASTER*: A PROMOTER ANALYSIS STUDY OF CIRCADIAN REGULATORY MOTIFS

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23.1 INTRODUCTION

Circadian clocks are endogenous molecular regulators that coordinate daily changes in gene expression and cellular and physiological functions with external day/night cycles. The core circadian clock in the fruit fly *Drosophila melanogaster*, consists of interacting molecular feedback loops. In the main loop, transcriptional activators (called positive clock elements), encoded by genes *Clock* (*Clk*) and *cycle* (*cyc*), stimulate the expression of two other clock genes, *period* (*per*) and *timeless* (*tim*), in the early night [1]. This leads to periodic increases in the levels of *per*/*tim* mRNA and PER/TIM proteins. These proteins (called negative elements of the clock) accumulate in cell nuclei late at night and act as inhibitors of the CLK-CYC complexes, resulting in the suppression of *per* and *tim* transcription (Fig. 23.1) [2]. In the second feedback loop, daily oscillations of *Clk* mRNA are achieved via a rhythmically active transcriptional repressor, encoded by *vrille* (*vri*), and transcriptional activator, encoded by *Pdp1*ε [3–6]. Rhythmic expression of *vri* and *Pdp1*ε is also activated by CLK-CYC complexes, via a similar mechanism as *per* and *tim* (Fig. 23.1).

CLK is a basic helix-loop-helix (bHLH) transcription factor that has a PER-ARNT-SIM (PAS) protein interaction domain. An approximately 69-base (b) enhancer sequence is situated ~500 b upstream of the *per* transcription start site (TSS). Within this enhancer, a consensus "E-box" bHLH transcription factor binding site having the sequence CACGTG is required for transcriptional activation [7]. Likewise, a consensus CACGTG E-box, approximately 2.5 kilobases (kb) upstream of *tim*, has been known to be essential for its transcriptional activation [8, 9]. Recent studies have shown strong rhythmic binding of CLK to the E-box upstream of *per* [10, 11]. Also. there was weak rhythmic binding of CLK to the intronic E-box of *tim* [11].

The circadian rhythms generated by these clocks are responsible for 24-h oscillations in diverse biological processes. While the central genes governing circadian pacemaker rhythmicity have mostly been identified, clock-controlled output molecules responsible for regulating rhythmic behaviors remain largely unknown. Hence, identification of genes controlled by the central clock would be important in providing an avenue for understanding circadian oscillations in diverse biological processes including locomotor activity, feeding behavior, hormone secretion, digestion, and the onset of sleep. Genomewide studies of circadian gene expression revealed rhythms in the expression of multiple genes involved in various metabolic pathways and stress response in *D. melanogaster* [12–14]. A number of genes involved

Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling, First Edition.
Edited by Tahira Farooqui and Akhlaq A. Farooqui.

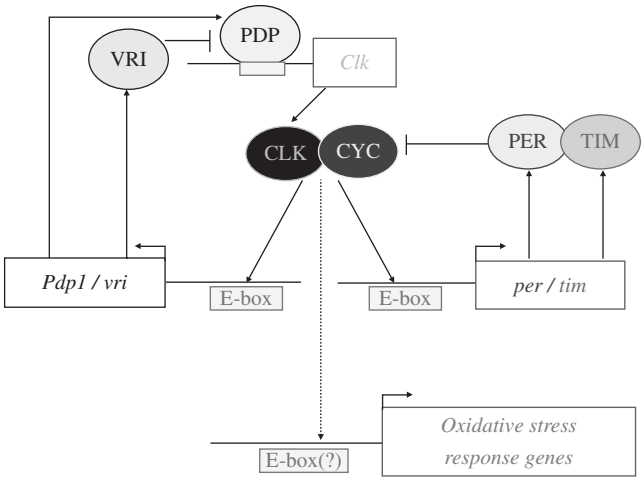

Fig. 23.1 Model of the *Drosophila* circadian clock. CLK and CYC heterodimers bind to E-boxes on *per* and *tim* promoters and activate their transcription during the day and early evening. PER and TIM proteins accumulate and translocate into the nucleus to repress their own activators CLK/CYC. During the day, PER and TIM are degraded, allowing a new cycle of transcription to start. In another loop, CLK/CYC activate transcription of *vri* and *Pdp1ε*, as VRI and PDP1ε proteins accumulate and translocate into the nucleus to inhibit and activate *Clk* transcription, respectively. We hypothesize that genes coding for various oxidative stress response genes could be under the control of the circadian clock (dotted arrow).

in oxidative stress responses were found to be expressed in a daily rhythm. We have previously reported that there is a circadian regulation in the response to oxidative stress [15], suggesting a concerted circadian expression in the antioxidant or cytoprotective genes in response to reactive oxygen species (ROS) or redox stress. We also reported a significant accumulation of oxidative damage to proteins (protein carbonyls) in *period* null (per^{01}) mutants compared to wild-type flies [15, 16], similar to a previous study [17]. Increase in the susceptibility of clock-disrupted flies to exogenous oxidative stressor would imply that these flies have either impaired antioxidant and/or reduced repair systems. However, in flies with a functional clock, these systems are apparently unaffected [15, 16].

Against this backdrop, we hypothesize that the genes coding for different oxidative stress responses may be regulated by the circadian clock directly or indirectly. If this hypothesis is true, such genes should have binding sites for clock elements in their promoter regions, upstream of the TSS. We conducted a bioinformatics-based promoter analysis of major oxidative stress responsive systems in *Drosophila* with two main objectives: (i) to identify the putative conserved circadian regulatory sequence (E-box) and (ii) to identify novel conserved motifs in the promoter regions and predict their function with online bioinformatics tools (Fig. 23.2).

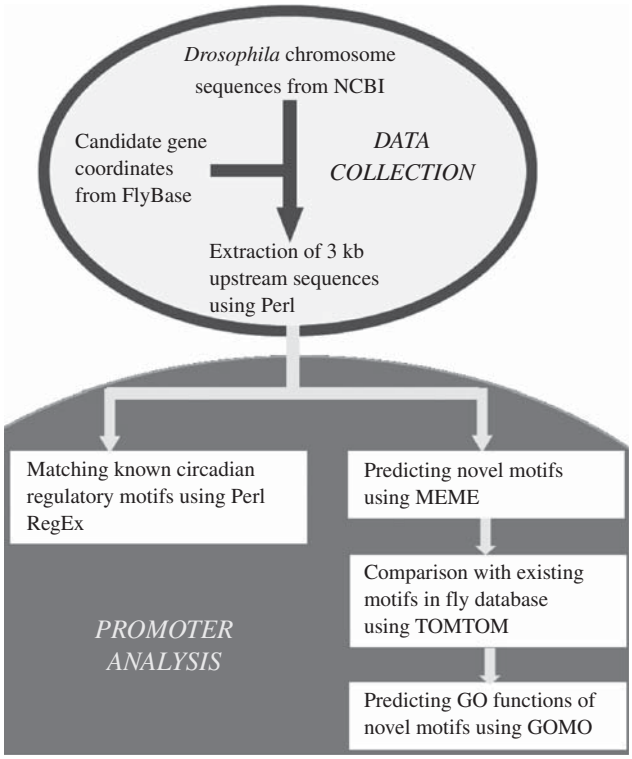

Fig. 23.2 Sequential procedure of the study. Data collection consisted of obtaining *Drosophila* chromosome sequences and coordinates of candidate genes and extraction of 3-kb upstream sequences. Promoter analysis comprised a search for known circadian regulatory motifs and prediction of novel putative motifs with online bioinformatics tools.

23.2 PROMOTOR ANALYSIS

23.2.1 Chromosome Sequences and Gene Coordinates

The *D. melanogaster* chromosome sequences (NT 004354, NT 033777-79, NT 037436) were downloaded from the National Center for Biotechnology Information (NCBI) genome database (http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&term=Drosophila%20melanogaster[orgn]). The coordinates and strand information for every candidate gene were obtained from FlyBase (http://flybase.org/) (Table 23.1).

23.2.2 Extraction of Promoter Sequences

Two-kilobase upstream sequences for each gene coding region were extracted with the substring function of Perl, a high-level, general-purpose UNIX scripting interpreted programming language.

23.2.3 Verification of Promoter Regions

The promoter sequences were subjected to BLASTn in FlyBase (http://flybase.org/) to verify their correct locations.

TABLE 23.1 Location of consensus E-box (CACGTG) sequences upstream of the transcription start site (TSS) in the candidate genes*

Gene ID	Gene Name	Chromosome: Coordinates (Strand)	E-Box Location
CG2647	*period (per)*	X: 2579613.2586813 (+)	525, 1399, 1477
CG17888	*Par domain protein 1 (Pdp1)*	3L: 7807437.7860472 (+)	1848
CG3962	*Kelch-like ECH-associated protein 1 (Keap1)*	3R: 12899661.12905665 (−)	1735
CG17894	*cap-n-collar (cnc)*	3R: 19011300.19047683 (−)	1618
CG11793	*Cu/Zn Superoxide dismutase (Sod)*	3L: 11105381.11106838 (−)	
CG8905	*Mn Superoxide dismutase 2 (Sod2)*	2R: 12660421.12661413 (−)	
CG6871	*Catalase (Cat)*	3L: 18815706.18821294 (+)	
CG2151	*Thioredoxin reductase 1 (Trxr1)*	X: 8136517.8142072 (+)	
CG11401	*Thioredoxin reductase 2 (Trxr2)*	3L: 22543326.22545122 (+)	
CG31884	*Thioredoxin 2 (Trx2)*	2L: 9613165.9616048 (+)	
CG3315	*Thioredoxin-T (TrxT)*	X: 5204614.5205921 (−)	
CG10964	*sniffer (sni)*	X: 8135531.8137337 (−)	
CG6835	*Glutathione Synthetase (GS)*	X: 17783580.17792636 (+)	901
CG2259	*Glutamate-cysteine ligase catalytic subunit (Gclc)*	X: 7996613.8010177 (−)	89, 1436
CG4919	*Glutamate-cysteine ligase modifier subunit (Gclm)*	3R: 18511022.18512249 (−)	2749
CG10045	*Glutathione S transferase D1 (GstD1)*	3R: 8193269.8194987 (−)	
CG5164	*Glutathione S transferase E1 (GstE1)*	2R: 14285898.14286728 (+)	
CG8938	*Glutathione S-transferase S1 (GstS1)*	2R: 12980758.12984935 (−)	
CG7266	*Ecdysone-induced protein Eip71CD (msrA)*	3L: 15504153.15506302 (+)	2544
CG6584	*SelR (msrB)*	3R: 6689071.6694379 (−)	

*The *period* and the *Pdp1* gene were used as controls to verify the Perl RegEx script.

23.2.4 Matching E-Box Elements with Perl Regular Expression

The presence and location of the consensus circadian regulatory sequence (E-box) were detected with Perl Regular Expression (RegEx).

23.2.5 Identification of Novel Conserved Motifs and Comparison with the Fly Database

Novel conserved motifs (6–10 bases) in the promoter regions were identified with the motif discovery tool Multiple Elm for Motif Elicitation (MEME) http://meme.sdsc.edu/meme4_3_0/intro.html [18]. Putative novel motifs were compared to a database of fruit fly motifs, FLYREG v2, with the motif comparison tool TOMTOM. The Sandelin–Wasserman similarity function was chosen for motif column comparison with a significance threshold q value of 0.5. The q value is the estimated false discovery rate if the occurrence is accepted as significant [19].

23.2.6 Predicting Functions of Novel Motifs

Gene Ontology (GO) terms and functions associated with the identified novel motifs in the promoter regions were searched with the software Gene Ontology for Motifs (GOMO v4.3.0) http://meme.sdsc.edu/meme4_3_0/cgi-bin/gomo.cgi [20].

23.3 RESULTS

23.3.1 Characterization of Circadian Regulatory Sequences

The consensus E-box sequence (CACGTG) was found in 6 of the 18 candidate genes. Three consensus E-box elements were identified in the *per* promoter 525, 1399, and 1477 bases upstream of the TSS (Table 23.1, Fig. 23.3). The E-box element in *per* has been previously characterized and identified by functional studies [7, 10, 11, 21]; thus this gene served as our control to test the successful functioning of the Perl RegEx. A single E-box was identified in the promoter regions of *Pdp1*, *Kelch-like ECH-associated protein 1 (Keap1)*, *cap-n-collar* (*cnc*), *Glutamate cysteine ligase modulatory (Gclm)* subunit, and *methione sulfoxide reductase (msrA)* genes, whereas two E-boxes were found in *Glutamate cysteine ligase catalytic (Gclc)* subunit gene.

23.3.2 Identification of Novel Conserved Motifs

The promoter sequences of candidate genes were further subjected to predictive analysis using MEME for searching potential novel putative motifs that were common to them. Four such motifs, 6–10 bases long with a log likelihood ratio of 185 and highly significant expect (e) value, were identified (Fig. 23.4). Furthermore, these novel motifs were subjected to a comparison with the

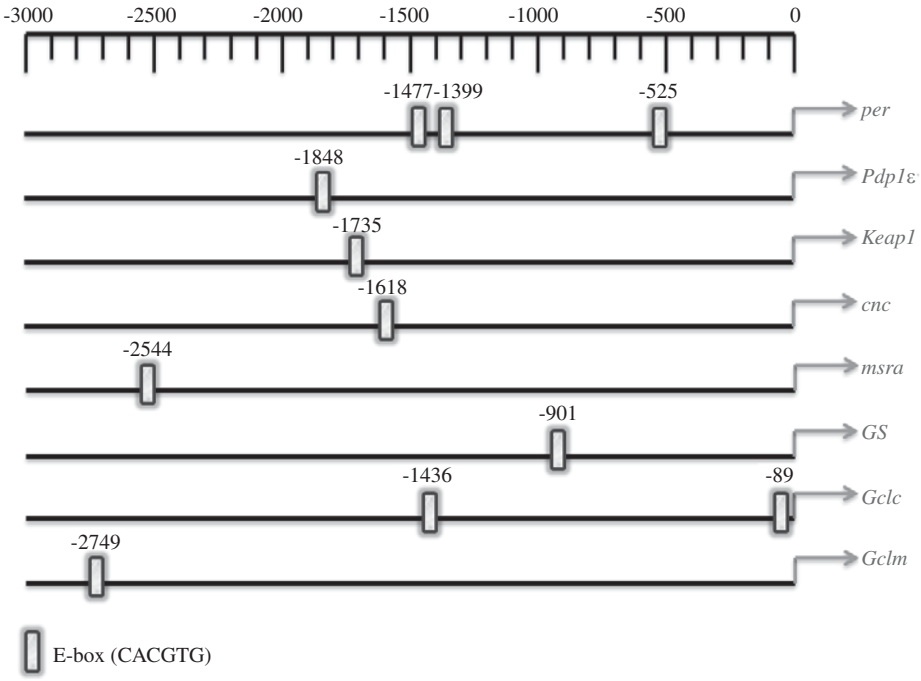

Fig. 23.3 Block diagram showing the relative location of consensus circadian regulatory sequences (colored boxes) upstream of the transcription start site (green arrows) in different candidate genes. The canonical E-box, 2822 bases upstream of *tim*, has not been depicted in this figure.

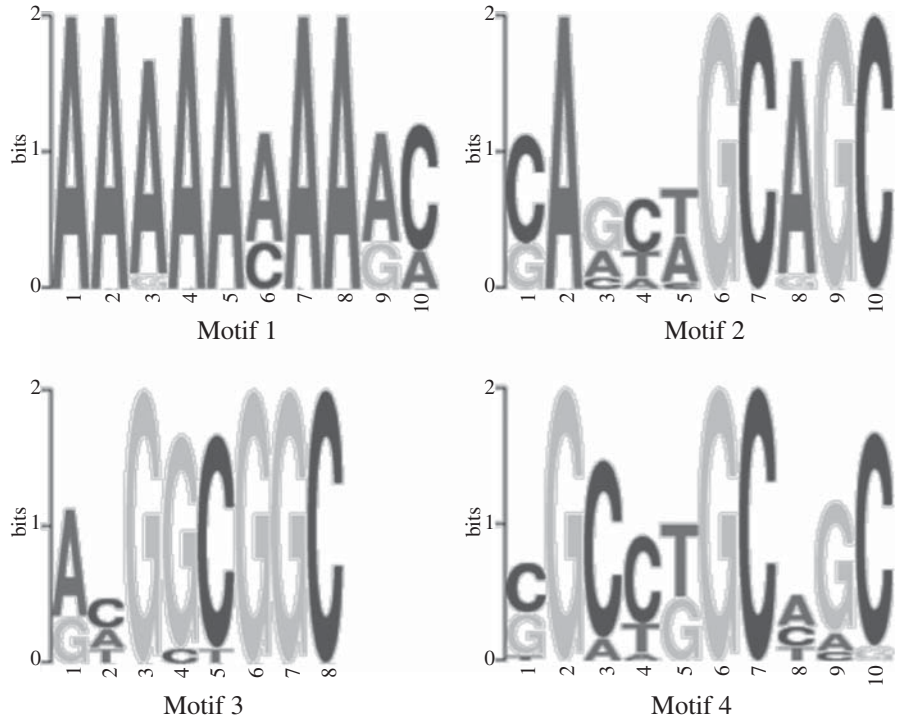

Fig. 23.4 Sequence LOGOS of the MEME motifs 1–4 displaying the probability of each base appearing at every possible position in the motif. The total height of the stack is the information content of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack. *(See color insert.)*

existing motifs in the fruit fly motif database FLYREG database-Bergman and Pollard v2 with TOMTOM. The motifs were compared by using the Sandelin–Wasserman similarity function, and only statistically significant matches ($P < 0.001$) with a low false discovery rate (q value) less than 50% were obtained.

Three of the four novel motifs had significant alignments with existing motifs in the database. Motif 1 aligned with the existing *hb* motif (P value = 0.00012, q value = 0.018) (Fig. 23.5A). No significant match was obtained for motif 2. Motif 3 produced significant alignments with three existing motifs in the database, *Med* (P value = 0.0038, q value = 0.021), *Mad* (P value = 0.0038, q value = 0.021), and *brk* (P value = 0.0096, q value = 0.035) (Fig. 23.5B). Motif 4 aligned to the *Med* (P value = 0.0014, q value = 0.041) and *Mad* (P value = 0.0021, q value = 0.3) motifs (Fig. 23.5C).

23.3.3 Predicting Functions of Novel Motifs

GO terms and functions associated with the novel motifs in the promoter regions were searched with GOMO v4.3.0. Interestingly, motif 4 was predicted to be a transcription factor binding site for the regulation of transcription, locomotor rhythms, and the entrainment of the circadian clock (Table 23.2). Further, motif 2 was identified to be a potential site for the binding of casein kinase 2 (CK2), a serine/threonine protein kinase that is known to phosphorylate clock proteins, resulting in their circadian rhythmicity [22, 23]. Motifs 1 and 3 were predicted to have general housekeeping functions like triplet codon-amino acid adaptor activity and regulation of biosynthetic processes, respectively (Table 23.2).

23.4 DISCUSSION

The consensus E-box bHLH transcription factor binding site has been shown to mediate rhythmic expression of several core clock genes [7, 11]. The presence of this consensus sequence in the promoter regions of six antioxidant genes in *Drosophila* suggests likely circadian control in their regulation.

Keap1 and *cnc* both revealed the presence of the typical E-box in their promoters. Members of the CNC (cap 'n' collar)-basic leucine zipper family of transcription factors are principal mediators of defensive responses to redox stress [24]. The *cncC* isoform transcribed off the *Drosophila cnc* locus has been suggested to be the counterpart of mammalian *Nrf2* (NF-E2-related factor 2), which contains the KEAP1 binding ETGE motif and an upstream hydrophobic region [25, 26]. The *Drosophila* genome also harbors a homolog of vertebrate *Keap1* genes. This gene (CG3962) and its protein product (dKeap1) have been shown to have striking similarity with mammalian and zebrafish homologs. The physical association between dKeap1 and CncC has previously been suggested by a genomewide yeast two-hybrid (Y2H) experiment [27]. This interaction predicts that dKeap1 should act as a negative regulator of CncC in vivo. It has been demonstrated that dKeap1/CncC signaling regulates oxidative stress responses in *Drosophila* [26] and could also function in xenobiotic stress responses. Circadian expression profiling of drug-processing genes and transcription factors in mouse has revealed that both *Keap1* and *Nrf2* are rhythmic with a 50% increase in *Nrf2* expression at 2 P.M., whereas the expression of its cytoplasmic repressor *Keap1* was shown to be higher (40%) during the dark phase than during the light phase [28]. A similar rhythmic pattern of these genes remains to be identified in the case of *Drosophila* because of circadian binding elements in their promoters.

While diurnal rhythms in the activity of various antioxidant enzymes have been described from various phylogenetically distant organisms [29], not many studies have actually been able to demonstrate rhythms at the mRNA level. In this study, the canonical E-box was not detected in any of the classical antioxidant enzyme genes. The circadian clock has been reported to gate the expression of two *Catalase* genes (*CAT2* and *CAT3*) in *Arabidopsis* to distinct opposite circadian phases [30]. Rhythms in catalase activity has been reported in mouse brain, kidney, and liver [31], while epigenetic inactivation of circadian clock gene *BMAL1* in hematologic malignancies in human cell lines has been reported to disrupt circadian expression pattern of *catalase* [32]. However, we have not discerned any circadian expression pattern in *Cat* gene expression in *Drosophila* [15]. Neither *Cu/Zn Superoxide dismutase* (*Sod*) nor Mn *Superoxide dismutase* (*Sod2*) revealed any of the putative circadian binding elements in their promoter regions.

Sniffer protein (encoded by *sni*) is an NADPH-dependent carbonyl reductase belonging to the enzyme family of short-chain dehydrogenases/reductases (SDRs). *sni* has been directly implicated in the cellular defense mechanism against oxidative stress in *Drosophila* [33], and not much is known about its circadian regulation. *Trxr1* has been reported to substitute for the glutathione reductase system in *Drosophila* and plays a crucial role in the thiol-based glutathione recycling system [34], which is essential to combat oxidative stress. Similarly, *Trx2*, *msrA*, *msrB*. and *GstS1* have also been reported to participate in oxidative stress responses in *Drosophila* [35–37]. While some of these genes participate directly in elaborating an antioxidant defense response, there are others such as methione sulfoxide reductases (*msr*) that perform a role in repair of oxidatively damaged proteins.

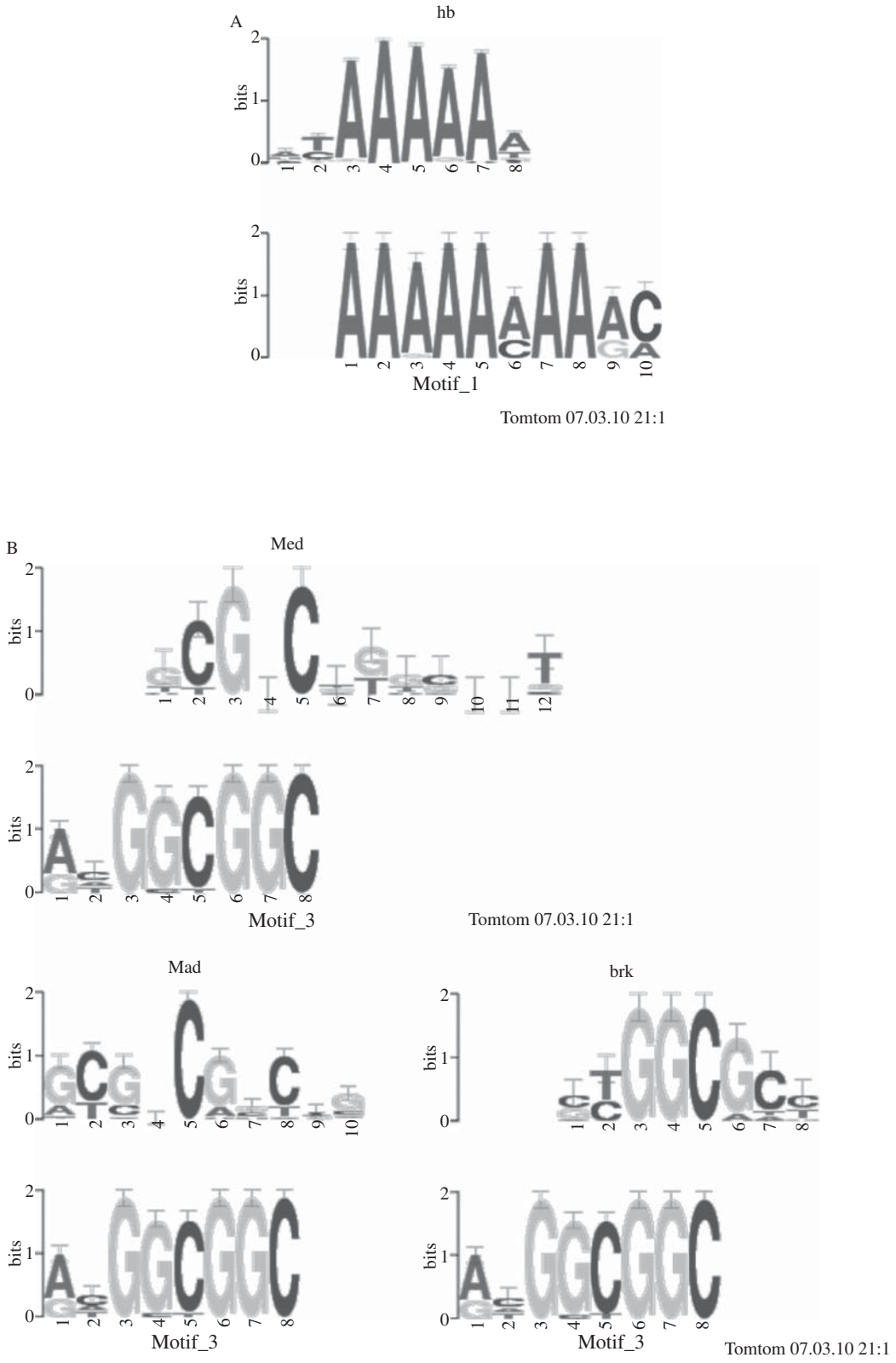

Fig. 23.5 TOMTOM output of the comparison of MEME motifs with existing motifs in the *Drosophila* database (FLYREG; Bergman and Pollard v2). Only statistically significant matches are displayed ($P < 0.001$) with a low false discovery rate ($q < 0.5$). (A) Motif 1. (B) Motif 3. (C) Motif 4. *(See color insert.)*

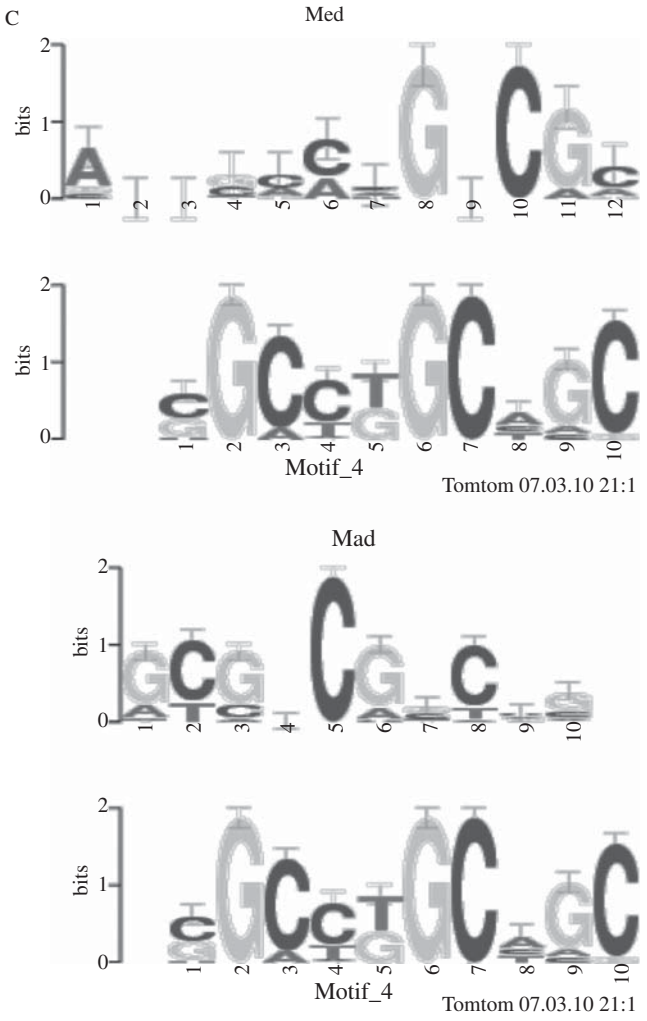

Fig. 23.5 (*Continued*)

Additionally, the tripeptide L-γ-glutamyl-L-cysteinyl-glycine, that is, glutathione (GSH), is the most abundant cellular non-protein thiol and has multiple physiological roles in vivo, acting as a cellular redox buffer, a potent nucleophile, and an antioxidant against ROS, and recent evidence also suggests that intracellular GSH is a key regulator of stress-activated signal transduction pathways [38]. GSH is synthesized by glutamylcysteine ligase (GCL), which is the primary rate-limiting reaction in the pathway. GCL is heterodimeric, having a catalytic subunit (*Gclc*) and the modulatory subunit (*Gclm*). Both of these genes showed the canonical E-box consensus sequence.

The E-box is a widely used DNA control element. It affects many different genetic programs, including proliferation, differentiation, tissue specific responses, and cell death. The circadian clock is also one among many pathways that employ the E-box for establishing the robust waves of gene expression characteristic of circadian transcription. However, presence or absence of E-box alone in the promoter regions of genes may not be sufficient to guarantee control by the clock. 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 [39]. However, lack of an E-box in the promoter region of a gene does not necessarily mean that the gene may not be under circadian control. Hence, other motifs that are conserved in the promoter regions of genes under likely circadian control may serve as binding sites for clock transcription factors (TFs).

TF binding sites in the promoters of clock genes and oxidative stress responsive genes used in this study revealed novel conserved motifs that had significant matches with existing *Drosophila* motifs in the database (Fig. 23.4, Fig. 23.5). One of these motifs (motif 4) had a predicted functional role in the entrainment of circadian rhythms and locomotor function, while another (motif 2) was a predicted binding site for casein kinase 2 (CK2), known to phosphorylate clock proteins [23, 40], playing an important role in their stability and function (Table 23.2). However, the presence of a variety of circadian elements that suggest control of promoter activity is not the only manner of control of rhythmic activity. In

TABLE 23.2 Gene Ontology (GO) functions associated with the novel MEME motifs in the promoter regions of candidate genes using GOMO v4.3.0

Motif	GOMO Score	*P* Value	*Q* Value	Accession No.	GO Definition
1	2.09e-10	1.42e-06	1.73e-03	GO:0000499	*molecular_function:* base pairing with mRNA
	2.09e-10	1.42e-06	1.73e-03	GO:0030533	*molecular_function:* triplet codon-amino acid adaptor activity
2	2.39e-05	1.42e-06	9.44e-03	GO:0005956	*cellular_component:* protein kinase CK2 complex
3	5.16e-04	1.37e-04	2.57e-02	GO:0034984	*biological_process:* cellular response to DNA damage stimulus
	5.26e-04	1.42e-04	2.57e-02	GO:0009889	*biological_process:* regulation of biosynthetic process
4	9.82e-05	3.13e-05	4.47e-02	GO:0045449	*biological_process:* regulation of transcription
	2.02e-04	5.55e-05	4.47e-02	GO:0045475	*biological_process:* locomotor rhythm
	3.58e-04	1.10e-04	4.70e-02	GO:0009649	*biological_process:* entrainment of circadian clock

some instances, nonrhythmic mRNA expression can result in rhythmic protein expression; for example, in the dinoflagellate *Gonyaulax polyedra*, luciferin binding protein (LBP) mRNA is not rhythmic, but protein expression (and bioluminescence) exhibits clear circadian oscillation mediated by the rhythmic binding activity of the RNA binding protein CCTR to the *LBP* 3′ UTR [41]. Similarly, rhythmic expression of the deadenylase nocturnin in *Xenopus* retina may potentially regulate steady-state downstream target mRNAs to generate protein cycles [42, 43]. Circadian oscillations at the protein level but not in the mRNA level have also been reported in mouse liver [44]. Thus, although the present analysis indicates that different antioxidant systems may have binding sites for circadian clock TFs and may be directly or indirectly controlled by the circadian clock, it is still largely predictive in nature. Further functional studies are needed to substantiate the results obtained in this study. This may include chromatin immunoprecipitation (ChIP) to find potential TFs binding to the novel motifs and the use of Y2H screens with reporter genes.

23.5 SUMMARY AND CONCLUSION

The regulation of gene expression (specifically in response to oxidative stress) by the circadian clock has emerged as a novel subdiscipline in molecular biology and has promising therapeutic implications. While the identification of genes controlled by the central clock would be important to understand the diverse biological processes under circadian control, most studies have stagnated at the level of microarray data. Our study moves a step beyond and identifies candidate genes likely to be under circadian transcriptional control. While these studies are no doubt important, further characterization of the genes would be required for a greater understanding of how the clock orchestrates a concerted protective function in an organism in response to oxidative stress. Thus a more detailed investigation is to be undertaken to validate their control by the circadian clock. Many of these genes are not only likely to prove integral to the fly but could also lead to the identification of homologous genes in mammalian systems.

ACKNOWLEDGMENTS

We thank Dr. Todd Mockler, Department of Botany and Plant Pathology and Center for Genome Research and Biocomputing, OSU, for his guidance and help with Perl programming.

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