Tissue Specific CLK Binding and Expression of Isoforms in Brains and Heads of Drosophila

Master’s Thesis

Presented to

The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Department of Biology
Michael Rosbash, Advisor

In Partial Fulfillment
of the Requirement for

Master’s Degree

by
Xiao Chen

May, 2011
All animals have circadian rhythms which are driven by a core molecular clock. CLOCK (CLK) is one of the core clock components which binds to genes to activate transcription. In this study, we performed chromatin immunoprecipitation (ChIP) of CLK, and RNA extraction from both Drosophila heads and brains to see whether CLK binding is tissue specific and to examine the expression levels of the mRNA isoforms controlled by CLK.

There are around 1,500 CLK direct targets discovered in Drosophila, but less than 5% of these genes produce cycling mRNAs. One possible explanation is that CLK could bind and drive the transcription on different genes in different tissues. To test this question, we tried to develop a chromatin IP assay from brains. Although we could generate fragmented chromatin of approximately the correct size, we were unable to detect a CLK ChIP signal above background. To circumvent this problem, we ablated the eyes using GMR-hid and performed whole head chromatin IPs. These experiments revealed potential tissue specific CLK transcriptional targets.

To validate these and test the possibility of isoform specific CLK transcriptional activation, I isolated RNA from brains and heads and used qPCR to measure the
expression levels of different isoforms in these tissues at six timepoints throughout the
day. We then further modified the experiment by using GMR-hid CLK-V5 flies
instead of brains to reduce the workload. We found that several genes that are direct
targets in the eye are not only expressed in the eye but also in other tissues in the
head.
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1. Introduction

The word circadian comes from Latin. Circa means “approximately” and dium or dies means “day”. Thus, all together the word means “about a day”. Organisms, from cyanobacteria to human, function following an intrinsic rhythm that has a period of around 24 hours. Light, food and temperature are cues that drive the rhythmic outputs. The molecular mechanism of how organisms keep this rhythm has been studied extensively. Even though both the components and the molecular mechanisms of the core clock within the organisms vary among kingdoms [1]-[6], the core clocks are conserved between Drosophila and mammals [5], [7], [8]. Thus it makes Drosophila one of the ideal model organisms for studying circadian rhythm.

Previous studies have shown that circadian rhythms in Drosophila are driven primarily by two interlocked negative feedback transcriptional loops. One feedback loop includes two repressors, PERIOD (PER) and TIMELESS (TIM). When the CLOCK-CYCLE (Clk-Cyc) heterodimeric transcription factor binds to the E-box, which is a DNA regulatory element, from mid-day through early night, Per and Tim are expressed [9]-[12]. The peak Per and Tim mRNAs appear early in the night, while the peaks of proteins do not appear until late night [13]-[19]. Per/Tim heterodimers then feedback and repress Clk-Cyc dependent transcription by binding to Clk and thus preventing Clk-Cyc heterodimers from binding to activate other genes [20]. In the Clk feedback loop, Clk-Cyc heterodimers also activate high expression of Vri and Pdp1ε through binding to E-boxes [21]-[23]. Vri accumulates and binds to Vri/Pdp1ε box regulatory elements, inhibiting Clk transcription [21]-[23]. As a result, Clk mRNA
and those mRNAs regulated by Clk cycle in opposite phases.

These two feedback loops are not operated separately, but instead are interlocked. Per and Tim act as repressors in both loops [13], [21], [23], [24], and consequently, cycling of both Tim and Per is required for maintaining the clock. However, surprisingly, Clk mRNA cycling may not be necessary for circadian rhythm, given that driving Clk mRNA to the antiphase does not affect the rhythms significantly [25]. Post-transcriptional and post-translational modification also play role in regulation of circadian rhythm.

Organisms can entrain the clock according to the daily environmental cycle, including light, temperature, and food and so on. However, light is considered as the strongest factor. A light pulse can shift the oscillator phase in Drosophila. For example, when a fly is in a light-dark (LD) condition, and it is given a light pulse instead of turning the light on at ZT0, which is the time lights are turned on, there is no or little effect on the oscillator phase. However, phase changes are observed when a light pulse is given at an abnormal time such as either ZT12 (the phase will delay) or ZT20 (the phase will advance). It is thought that this phase shift is driven by the activation of the photoreceptor CRY which in turn causes the ubiquitin-proteasome dependent degradation of Tim through tyrosine phosphorylation [26]. Whether the phase is shifted forward or backward depends on the levels of Tim mRNA [27]-[30]. During early night, loss of Tim is induced by light and causes a delay in Per nuclear localization and phosphorylation. Yet an advanced degradation of Per is induced during the late night [30].
Clock gene expression is tissue specific, and the expression pattern of the core clock genes indicates the presence of circadian oscillators. Many clock genes are only expressed in certain subset of cells in Drosophila. For instance, about 75% of Per in fly heads is expressed in photoreceptors of the compound eye, and the other 25% is distributed in antennae, the proboscis, ocelli, the esophagus, fat bodies, brain glia and six clusters of brain neurons [31]-[34]. All these tissues with Per expression have circadian rhythms. In fact, the definition of cells with circadian oscillators has been defined as cells with Per expression. Usually, other core clock components are also expressed in these cells [35]-[38]. Yet Tim and Per are expressed exclusively in cells with oscillators, and Clk is also expressed in both oscillator cells and non-oscillator cells [37].

One important question scientists have about circadian rhythm is how circadian oscillators regulate rhythmic behaviors. One high throughput way to identify genes under circadian control is to use microarrays to identify oscillating mRNAs. 150 cycling transcripts have been discovered [40]-[44] in several different studies. These transcripts stop cycling in ClkJrk, which means they are under Clk control. Also, some transcripts in non-oscillators cells are increased or decreased in ClkJrk. Therefore, perhaps Clk not only regulate gene expressions that are cycling but also some that are not cycling [45]-[46].

In an effort to identify genes controlled by the Clk/Cyc transcription factors, our lab performed CLK chromatin immunoprecipitations and identified CLK binding sites using tiling arrays. Interestingly, more than 1,500 targets of Clk/Cyc were identified –
many more than previously thought. In addition, very few of these CLK direct targets have cycling mRNAs. And we are interested in testing several possible explanations. First, we want to see whether the CLK binding sites are the same in both heads and brains by doing ChIP. Second, we would like to learn whether the expression of these CLK direct targets is of the same levels in heads and brains by qPCR measuring the amount of mRNAs.

2. Results and Discussion

I. Tissue Specific CLK Binding patterns in Heads and Brains of Drosophila

Since compound eyes and a brain are two main components of a head, and there is strong signal of CLK binding to more than 1,500 genes in heads, we wanted to see whether we can detect CLK-DNA interactions in brains.

A. GMR-hid flies ChIP

One simple way to look at the contribution of eyes to ChIP is to use genetic tools to remove the eyes. We can do this by performing CLK-ChIP in GMR-hid flies with ablated eyes. By comparing CLK binding sites in GMR-hid versus wt control, we found that approximately 40% of genes have dramatically altered CLK binding patterns when eyes are ablated, suggesting a large portion of CLK binding occurs in eyes. In addition, about 20% of genes show unchanged or increased CLK in flies with eyes ablated. The results to some extent prove our tissue specific hypothesis, so we decided to go on with brain ChIP.

B. Brain ChIP

Because a brain is one of the major components of a head, and it has many neurons in
it controlling the whole fly, so it is logical to deduce that the CLK binding pattern in brain may be different from the whole head. We modified the protocol we used for head ChIP of Drosophila in order to isolate chromatin from dissected fly brains. We used flies that have been merged for one day for entrainment in a 25°C incubator with LD condition for at least 3 days, and anaesthetized flies with CO₂ and collected flies heads into PBS in the dark room at ZT14. Then we switched the heads to 70% EtOH for 30 seconds to enable formaldehyde to better penetrate the heads in the later step. After that, the heads are washed with PBS and put into 1% formaldehyde for 20 minutes for fixation. Later we used glycine to stop the reaction and washed heads in PBS for three times to make sure all the reagents are gone. Afterwards, we dissected flies in PBS and kept them in 1.5ml eppendorf tube with PBS, spun down and froze with liquid nitrogen for further uses. Once we had isolated fly brains, we performed a slightly modified version of the Fly Head ChIP protocol (see Methods for detailed protocol). In short, we sonicated whole brains rather than isolating nuclei prior to sonication and added more material to the IP to try to boost signal.

To make sure the DNA fragments after sonication are of an appropriate size that can be immunoprecipitated, we checked the input samples amplified with four pairs of primers respectively using electrophoresis gel (Figure.1). According to the experience we had for doing head ChIP, 200bp-500bp appears to be the best DNA length for immunoprecipitation. It turned out that sonication of 15 seconds for 5 times with 15 seconds interval on ice worked best with these samples. Once we knew that the chromatin was sheared adequately and could be isolated from the samples, we
continued with the IP. The resulting DNA fragments were analyzed using qPCR with primers specific to tim, and primers specific to rpl32 are used as a negative control. We have done the experiment for three times, but the enrichment at the tim promoter is not significant above background levels.

![Figure 1. PCR results of Input fragments](image)

The first and last lanes are log2 ladders. Lane 1-4 are samples of chromatin from fly heads used as a positive control; lane 5-8 are input samples of CLK-V5 flies; lane 9-12 are input samples of Canton-S flies. Each sample is PCR with four pairs of primers. The primers span 1300bp, 1900bp, 200bp and 99bp respectively.
The inconsistent results we got with qPCR may be due to low yield with ChIP. To increase DNA yield, we amplified the DNA from brain ChIP using ChIP-seq amplification protocol. Using this protocol, we were able to amplify input sample but not IP possibly due to low levels of starting material.

The lack of enough starting material becomes a big problem for the ChIP and ChIP-seq experiments. Either improvement on ChIP efficiency or amplification efficiency needs to be done in order to get valid data.

II. Identification of Tissue Specific CLK Direct Targets

As mentioned in the introduction, although many CLK targets are found in heads, only a small amount of them have cycling. Since fly eyes have contributed a large portion of head tissue, it is possible that there are many eye specific transcripts activated independent of CLK binding. Even if transcripts don’t cycle in the eyes but
they could hide cycling in brains. In order to test this hypothesis, a straight forward method to use is RNA extraction followed by qPCR. Since the majority of RNAs in heads come from eyes and brains, we wanted to measure RNA extracted from heads and brains and find out isoforms that are expressed differently between the two tissues. A less labor intensive option is to isolate RNA from GMR-hid flies instead of fly brains.

A. RNA Isolation from Heads and Brains of Drosophila

We extracted RNA from heads and brains from CLK-V5 flies. All the flies are collected at ZT14, which is a timepoint that most of the genes are expressed at the highest level. During the first trial, we extracted RNA from 15 heads and brains but got very little amount. We got 7.27ug mRNA from heads and 4.12ug mRNA from brains. Therefore about 60% of whole head RNA comes from the brains. We later performed qPCR with Lilli specific primers (Figure.3). Compared to the whole fly heads, around 56% and 26% of mRNAs are detected for Lilli long isoform and short isoform respectively when we only take the brains. The rest of the mRNAs may exist in other tissues of the heads, most probably in the eyes, so transcripts are gone when we get rid of the other tissues. In Lilli’s CLK binding patterns, CLK binding is gone when eyes are removed in GMR-hid flies, suggesting that the major CLK binding occurs in eyes. So the RNAs expressed in brains may be controlled by other activation factors or by lower levels of CLK binding that are not detectable in ChIP.

The graph also demonstrates that the two isoforms are not expressed at a same level no matter in the whole fly heads or the brains only, and Lilli long isoform seems to be
expressed at the same level in eyes and brains, whereas most of the Lilli short isoform is not expressed in the brains.

Figure 3. qPCR Results of Brain ChIP

In order to test more isoforms, we repeated this experiment with a larger number of brains and heads. A decent amount of DNA was isolated but it was of poor quality (Brain: 260/230 = 0.29; Heads: 260/230 = 2.35). It is probably because of phenol and salt contamination that the value of 260/230 of brains is extremely low which might impact amplification in later steps. People in our lab used to have the similar problem when extracting RNA from brains. Thus, an extra step is required to purify RNA for RNA extraction from brains.

CLK binding occurs mostly in the eyes for all the transcripts tested here. Also previous data has proved that ninaA and norpA are highly enriched in eyes. Therefore we wanted to see whether the other genes look similar to norpA and ninaA and have enrichment of RNA expression in eyes.

Although not ideal, we made cDNA from these samples. We used rpl32 to normalize
all the samples (Figure 4). As shown by previous studies, ninaA and norpA are expressed more highly in heads than in brains. GoI seems to be highly expressed in both brains and heads with some isoform variability. Most of the other transcripts tested are mainly expressed outside brains. This is consistent with the fact that these genes are CLK direct targets in the eyes. However, the experiment still needs to be repeated to verify the data due to the poor quality of mRNA we used.

**Figure 4. qPCR Results of Heads and Brains**

**B. RNA Isolation from Heads of GMR-hid CLK-V5 flies**

Due to the fact that isolation of RNA from brains is both labor-intensive and not working very well, we took advantage of a fly line in the lab in which the eyes are ablated using a GMR-HID transgene. By isolating RNA from GMR-hid CLK-V5 flies and CLK-V5 flies (as a control) we can eliminate any RNA from the eyes, however, it
is not as clean as RNA from brains, and other head tissues such as the fat body, salivary gland etc. remain. Thus, by comparing the expression levels of RNA from CLK-V5 flies and GMR-hid CLK-V5 flies, we can achieve something close to what we wanted to do with brains in a much more efficient way.

We extracted RNA from heads of both CLK-V5 flies and GMR-hid CLK-V5 flies at each of the six time points, which are ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22, and did reverse transcription and qPCR with message specific primers. As a positive control, we first looked at tim expression in GMR-hid CLK-V5 flies (Figure 5). Tim is found to show robust rhythms from both of the type of fly heads. tim in CLK-V5 is roughly 4 folds of that in GMR-hid CLK-V5 at ZT14 which is the peak of the expression levels. Most of tim is expressed in eyes. The transcripts are likely to have cycling in both eyes and brains. Since the tim data looked good, we went on to look at the expression of two different isoforms of the CLK direct target Dlg1.

Figure 5. dlg1 CLK Binding Pattern in yellow white wt flies and GMR-hid flies.
The purple lanes represent CLK binding patterns in GMR-hid flies at ZT2 and ZT14 respectively.
The green lanes represent the wt flies.

Dlg1 is another gene that is found bound by CLK in wt flies but not in GMR-hid flies
(Figure.5; compare green and purple). In GMR-hid there is no detectable CLK binding on the dlg1 promoter suggesting a large portion of CLK binding is in the eyes. The transcripts of the longest dlg1 isoform are expressed in GMR-hid CLK-V5, and it is rhythmic in GMR-hid CLK-V5 but not in CLK-V5 (Figure. 6 and 7). dlg1 longest isoform expression is about 2-fold lower in GMR-hid CLK-V5 at ZT10 than in the CLK-V5 flies. The dlg1 second longest isoform shows rhythmicity in GMR-hid CLK-V5 but not in CLK-V5. Ablating the eyes causes a 27-fold reduction in the level of the second longest isoform of dlg1. This is also the isoform that appears to have CLK binding on its promoter in eyes.

Surprisingly we found both isoforms of dlg1 are cycling in GMR-hid CLK-V5 flies. Since no CLK was detected bound to these genes in GMR-hid flies previously, two possible explanations may be responsible for this observation. First, these transcripts could be controlled by other factors outside the eyes that drive cycling transcription. Another explanation is that CLK not only controls expression in the eyes, but also elsewhere in the head but this was not detectable via ChIP.
Figure 6. qPCR results of tim mRNA in GMR-hid CLK-V5 flies for six time points a day.

Figure 7. qPCR results of dlg1 longest in GMR-hid CLK-V5 flies for six time points a day.
Figure 8. qPCR results of dlg1 second longest in GMR-hid CLK-V5 flies for six time points a day.

The GMR-hid CLK-V5 binding patterns are the same in both figures, but the lower figure has an amplified Y axis.

3. Methods

Brain ChIP
Collecting Brains: Entrain young flies for 3-4 days and remove heads after CO₂ exposure. For dark timepoints, use red light scope in a dark room. After quickly removing heads, put them in a cell culture insert in a 24 well plate in Well 1. (25 heads per well.) Then switch them from well to well. 24-well plate should be set up as: Well 1 for PBS; Well 2 for cold 70% EtOH 30 seconds; Well 3 for cold PBS; Well 4 for 1% formaldehyde in PBS for 20 min; Well 5 for 125mM Glycine in PBS for 5 min. Well 6 and 7 and 8 are all for cold PBS wash. Dissect brains and place in eppendorf tube on ice. Quick spin, remove the PBS and quick freeze brain pellets on liquid nitrogen. Store at -80°C.

Making Chromatin: Thaw brains on ice, add 300ul Sonication buffer (20mM HEPES-Na (pH 7.5), 2mM EDTA (Ph 8.0), 1% SDS, 0.2% Triton X-100, 0.5mM Spermidine, 0.15mM Spermine (Sigma S3256) and water with mini-protease inhibitor tablets) and sonicate for 5 × 15s at setting 2 with 30s on ice between each pulse. Spin at 8,000g for 10 min to remove debris. Store supernatant at -80°C.

Immunoprecipitations: ChIPs were performed as previously described (Jerome et al. 2010).

Amplification of DNA samples for ChIP-seq: personal communication with Jerome Menet.

Brain RNA Isolation
Entrain flies like what is done in brain ChIP. Keep flies in dark until dissection. Dissect brains after CO₂ exposure and put them into eppendorf with RNase-free PBS. Spin and remove PBS. Extract RNA with Trizol according to manufacturers instructions. Treat samples with DNase (Turbo DNA Free; Ambion) and reverse transcribe RNA following manufacturer’s instructions (SuperscriptII, Invitrogen) to cDNA with random primers. Reactions were performed without reverse transcriptase as control for DNA contamination.

4. References


