Involvement of clock genes in seasonal, circadian and ultradian rhythms of Nasonia vitripennis
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Identification of alternative splicing of period in *Nasonia vitripennis*

Elena Dalla Benetta
Background

Circadian clocks are endogenous timing mechanisms that generate daily rhythms in various organisms, ranging from cyanobacteria to humans. Although rhythms controlled by a circadian clock are self-sustained and persist robustly with a period close to 24 hours under conditions of constant darkness and temperature, they are entrained by environmental cues (zeitgebers), such as light, temperature, and food intake (Rusak et al., 1993; Soriano, 1981). Transcription is considered the prime mechanism driving daily rhythms in gene expression. The control of gene expression is a complex process, mRNAs and proteins can undergo many processing and regulatory steps that influence their expression. These post-transcriptional and post-translational regulations, such as alternative splicing, phosphorylation and dephosphorization, modulate sub-cellular localization, protein interactions and protein functions, and thus are very important to shape biological rhythms (Virshup et al., 2007; Zheng & Sehgal, 2012).

The period (per) gene has been demonstrated to play a crucial role in circadian rhythm generation in many organisms, including mammals (Chen et al., 2009; Lee et al., 2011) and insects (Hardin et al., 1990; Konopka & Benzer, 1971; Sauman & Reppert, 1996). Different per isoforms have been described in many insects. For example, in Drosophila melanogaster alternative splicing of per is induced by low temperature and results in two different isoforms (Colot et al., 2005; Diernfellner et al., 2005; Majercak et al., 1999). The ratio of the two isoforms is crucial to phase mRNA and protein expression of per. Alternative splicing of per has also been reported from the Silk Moth Bombyx mori, in which the two isoforms differ in 5 amino acids (GTQEK) on the PAS A domain (Takeda et al., 2004). A similar set of PERIOD isoforms has been reported for honeybees, Apis mellifera and Apis cerana, but in this case for a deletion/insertion of eight amino acid residues near the so-called perS mutation site (Minamoto et al., 2012; Shimizu et al., 2001).

In Nasonia vitripennis, the per gene shows a latitudinal cline in allele frequencies that correlate with a latitudinal cline in photoperiodic diapause response (Paolucci et al., 2016). In chapter 3, expression profiles of per mRNA were investigated and revealed a different expression pattern between southern and northern lines and between photoperiods. Here, I address the question whether N. vitripennis also expresses different per isoforms, whether southern and northern lines show differences in the expression of those isoforms, and what function could be associated to these per isoforms.
Results and discussion

Different *Nasonia per* transcripts have been found in the NCBI (National Centre for Biotechnology Information) (NCBI Resource Coordinators, 2017) database. They have been aligned with the homologous *per* of *Apis mellifera* and *Apis cerana japonica*, indicating an amino acid identity of about 46%. Two of these *per Nasonia* transcripts correspond to the alpha and beta versions described from the honeybee (Shimizu et al., 2001) (Fig. 1). We confirmed the presence of the two *per* transcripts in *Nasonia* by reverse transcription (RT)-PCR, and by cloning and sequencing of the variants (Fig. 2B, C). The alternative splicing interest the last 24bp (8nt) of the intronic region between exon 9 and 10 (Fig. 2A; 3). The cDNA structure of *per* clearly showed the existence of splice consensus sites (Maniatis & Tasic, 2002; Norton, 1994), i.e. GU (GT in cDNA) at the 5’ side and AG at the 3’ side of the specific 24 bp sequence. This strongly suggests that the isoforms with the 24 bp sequence difference were produced by alternative splicing from an intron adjacent to this splicing site (Fig. 3).

Both alpha and beta variants were detected in both heads and bodies of southern and northern lines (Fig. 2B). Unfortunately qPCR methods failed to quantify the two mRNAs due to lack of good sequence for designing specific primers. A semi-quantitative analysis following the procedure described by Shimizu et al., (2001) also failed to give reliable information about expression levels of the two variants. Although the functional difference between PERIOD α and β is unknown, it is necessary to quantify the ratio of isoform α and isoform β in PERIOD protein or period mRNA forms. So far, we have not succeeded in such quantifications in spite of attempts to differentiate the ratio between period α and period β mRNAs. As shown in Fig. 2, the amount of period α mRNA was judged to be larger than that of period β mRNA. To quantify this difference more firmly, we may need an elite system to amplify each mRNA isoform accurately and quantitatively. Moreover, it would be very interesting to investigate the temporal pattern of expression of the two isoforms through the day, in southern and northern wasps. As reported in chapter 3 per expression profile is affected differently by photoperiod in southern and northern lines of *N. vitripennis*, it is thus necessary to investigate whether light or temperature affect the splicing efficiency in favour of one or the other forms in the two lines.

Comparative analysis of the *Nasonia* and *Drosophila per* gene confirmed the high similarity with the *Drosophila* region containing the perS mutation site as in *Apis* (Shimizu et al., 2001; Minamoto et al., 2012) (Fig. 4). Interestingly, in *Drosophila* this region is very important for phosphorylation regulation (Garbe et al., 2013) and mutations in this area lead to different PER phosphorylation that leads to different protein stability (Garbe et al., 2013) (Fig. 5, 6). Comparing the residuals, targeted by phosphorylation in *Drosophila*, we could predict a differential phosphorylation between the α and β isoforms present in *Nasonia* that could lead to different protein stability and subsequently different protein timing.
Therefore, it is also necessary to investigate the stability of the two isoforms at the protein level. Isoforms of the PERIOD proteins can be detected by sophisticated monoclonal antibodies that can differentiate the insertion/deletion of the 8 amino acids, so that immunocytochemical study could then clarify the phosphorylation pattern, stability and neuronal localization of the two isoforms.

**Fig. 1** mRNA alignment of *period* from *Apis cerana japonica* and *Nasonia vitripennis*

*Per* variants alignment revealed the presence of both *alpha* and *beta* mRNA in *Nasonia vitripennis*.

**Fig. 2** Detection of *alpha* and *beta* variant of *per* in *Nasonia vitripennis*

(A) Gene structure of *per* from exon 8 to exon 12. Grey boxes indicate exons, grey lines introns and black lines the alternative splicing. Small black arrows indicate respectively the location of forward (F) and reverse (R) primers to amplify the region of the alternative splicing. (B) RT-PCR amplification of the splicing region detects the presence of both *alpha* and *beta* variants. (C) Alignment of the sequenced α and β fragments using either the forward (F) or the reverse (R) primer.
Identification of alternative splicing of period in *Nasonia vitripennis*.

**Fig. 3 Structure of the *per* splicing site**

cDNA structure of the splicing site revealed the presence of the splice consensus sites GT at the 5’ side and AG at the 3’ side of the specific 24 bp sequence.

**Fig. 4 Protein alignment for *per***

PER sequences alignment revealed conservation of the *per* S region of *Drosophila* (red arrows) and of the phosphorylated residues (green boxes).
Fig. 5 Potential regulation of *Drosophila* PER via phosphorylation
In scenario (A), the PER-SD domain regulates phosphorylation of residues within the PER-short domain, while scenario (B) proposes that phosphorylation of the PER-Short and PER-SD domains control clock speed in parallel. In both cases the degradation process is regulated.

Fig. 6 Predicted phosphorylation site in *Nasonia* alpha and beta per variants.
The two bars represent the two PER variants, in blue the longer one (α-per) and in green the shorter one (β-per). In red is represented the acceptor splicing site that in the α sequence includes the first 24bp of the exon 10. Black bars represent the conserved predicted phosphorylation sites and orange bars the predicted phosphorylation sites that differ between the two variants.
Conclusion and open questions

To maintain approximate 24-hour cycles at the molecular level, clocks must be tightly regulated at several steps to maintain the correct period, phase and amplitude of the rhythms of thousands of proteins that generate the wide range of rhythmic biological processes. As described above, there is now abundant evidence that post-transcriptional mechanisms play an important role in shaping these rhythms (Kojima et al., 2011). However there are many open questions that need to be addressed to understand the function of different per isoforms. For the future it will be very important not only to specify the functions of these two per isoforms, but also investigate their temporal and spatial expression pattern. Especially it would be very interesting to explore functional differences in southern and northern lines of Nasonia in order to contribute to understand the mechanism of circadian rhythms and photoperiodism in this species.

Material and methods

Experimental line and rearing conditions

For this study we used isogenic lines established from wasps collected from the field in 2009 (see for details Paolucci et al., 2013). The lines were maintained on Calliphora spp. pupae as hosts in mass culture vials under diapause-preventing conditions, LD16:08, and temperature of 20 ± 1 °C.

Bioinformatic search

Protein and cDNA sequences of Nasonia period (per) were found in NCBI (NCBI Resource Coordinators, 2017), orthologous sequences of Apis cerana Japponica, Drosophila. melanogaster and virilis, Camponotus floridanus and Ceratitis capitata have been found through BLAST (Altschul et al., 1990) search using the Nasonia per mRNA as input sequences. Accession numbers are reported in table 1. Sequence alignments of per from different insect species were performed with Geneious (Kearse et al., 2012).

Identification of the two isoforms using reverse transcription (RT)-PCR

Primers were design with primer3 (Untergasser et al., 2012) in the region surrounding the alternative splicing sites. Amplicon size was about 100bp in order to facilitate the visualization on gel after reverse transcription (RT)-PCT.
RNA was extracted from individual bodies and heads, separately for females and males, with Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. Each sample was subjected to a DNase treatment to eliminate any DNA contaminations, and about 1ug of RNA was used to synthesize cDNA with RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Scientific). cDNA was used as template for RT-PCR using the forward primer (5’-TGTGGTTGTGGACGACAGG-3’), that maps in exon 9, and the reverse primer (5’-CTGTCGAAGTACTCGTGTTG-3’), that maps in exon 10 (Fig. 2A). The RT-PCR profile was: 3 min of activation phase at 95°C, 35 cycles of 15 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C. In order to detect small size differences, PCR product was run on 3% TBE agarose gel containing ethidiumbromide. To confirm the two splicing variants, PCR products were ligated into PGEM-T vector (Promega, Madison, WI, USA) after purification using the GeneJET PCR purification kit (Fermentas, Hanover, MD, USA). Ligation reaction was used to transform competent JM-109 E. coli (promega, madison, WI, USA). Colony-PCR was conducted with PGEM-T primers (5’-GTAAAACGACGGCCAGT-3’ and 5’-GGAAACAGCTATGACCATG-3’) at 94°C for 3 min, 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 2min, with a final extension of 7 min at 72°C. Both strands were sequenced and fragments were aligned to one another and to period mRNA variants to inspect the splicing variation.

<table>
<thead>
<tr>
<th>Table 1. Sequences used for alignment</th>
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<tbody>
<tr>
<td>Sequence name</td>
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<tr>
<td>-------------------------------------</td>
</tr>
<tr>
<td>N. vitripennis_period x1</td>
</tr>
<tr>
<td>N. vitripennis_period x4</td>
</tr>
<tr>
<td>A. japonica_period α</td>
</tr>
<tr>
<td>A. japonica_period β</td>
</tr>
<tr>
<td>C. floridanus_period x1</td>
</tr>
<tr>
<td>C. floridanus_period x2</td>
</tr>
<tr>
<td>C. floridanus_period x3</td>
</tr>
<tr>
<td>C. capitata_period x1</td>
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<td>D. virilis_period A</td>
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<td>D. melanogaster_period A</td>
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Acknowledgements

I would like to thank Ties Ausma for his attempts to quantify the two splicing variants, Leo W. Beukeboom and Louis van de Zande for fruitful discussion and comments.