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Circadian clocks and phosphorylation: Insights from computational modeling

Review Article

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Abstract: Circadian clocks are based on a molecular mechanism regulated at the transcriptional, translational and post-translational levels. Recent experimental data unravel a complex role of the phosphorylations in these clocks. In mammals, several kinases play differential roles in the regulation of circadian rhythmicity. A dysfunction in the phosphorylation of one clock protein could lead to sleep disorders such as the Familial Advanced Sleep Phase Disorder, FASPS. Moreover, several drugs are targeting kinases of the circadian clocks and can be used in cancer chronotherapy or to treat mood disorders. In *Drosophila*, recent experimental observations also revealed a complex role of the phosphorylations. Because of its high degree of homology with mammals, the *Drosophila* system is of particular interest. In the circadian clock of cyanobacteria, an atypical regulatory mechanism is based only on three clock proteins (KaiA, KaiB, KaiC) and ATP and is sufficient to produce robust temperature-compensated circadian oscillations of KaiC phosphorylation. This review will show how computational modeling has become a powerful and useful tool in investigating the regulatory mechanism of circadian clocks, but also how models can give rise to testable predictions or reveal unexpected results.

Keywords: *Circadian rhythm • Computational modeling • Phosphorylation • Mammals • Drosophila • Cyanobacteria*

1. Introduction

Since the pioneering work of JJ D'ortous de Mairan in 1729 on the rhythmic leaf movement in plants [1], circadian rhythms (from Latin *circa* about and *dies* day) have been studied in many organisms such as cyanobacteria [2], fungi [3], fruit flies [4], plants [5], mammals and humans [6]. These rhythms are observed at the physiological level and originate from a regulatory molecular mechanism already present at the level of a single cell. Based mainly on a negative autoregulatory feedback loop, clock proteins repress the transcription of their own gene [7-9]. The mechanism of the regulatory network of circadian clocks turned out to be more complex than previously understood [10,11]. An increasing number of genes and proteins are known to be involved in the generation of circadian rhythms as well as different transcriptional, translational and post-translational regulations. In this latter case, phosphorylation [12], acetylation [13], sumoylation [14], ubiquitination [15] or proteasomal degradation [16] are amongst the main processes.

Very recently, particular attention has been paid to the role of the phosphorylations in circadian clocks for at least three main reasons. Firstly, several physiological disorders and in particular sleep disorders such as FASPS (Familial Advanced Sleep Phase Syndrome) have been linked with a problem of phosphorylation in one of the main proteins of the human circadian clock [17]. Secondly, the knowledge of the mechanism involving the phosphorylations in circadian clocks has rapidly increased in complexity these last years with the discoveries of several kinases involved in the circadian clock machinery and with the differential functions associated with these kinases, controlling the degradation and stabilization of clock proteins or their nuclear entry [18-20]. Finally, kinases are enzymes that are found not only in circadian clocks but also in

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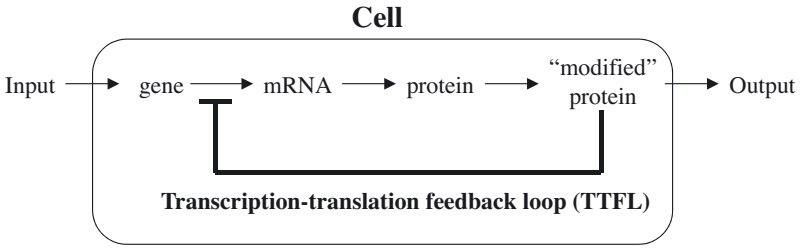

Figure 1. The Transcription-Translation Feedback Loop (TTFL). A clock gene is transcribed into mRNA, then translated into a protein that can be modified by kinase/phosphatase, or by the formation of a complex with other proteins. This „modified" protein then regulates directly or indirectly the transcription of its gene.

many other systems and they are under extensive investigation as targets for drug development such as in cancer chronotherapy [21,22].

Because of the sheer complexity discovered recently in the role of phosphorylations in circadian clocks, computational modeling has become a very powerful and useful tool to better understand the mechanism of action of these phosphorylations but also to unravel the counterintuitive or unexpected results [18].

This review will first focus on modeling circadian rhythms and will show the roles and advantages of a theoretical approach. Then, the review will focus on the mammalian circadian clock showing the role and effect of several kinases involved in the generation of circadian rhythms as well as the link between these kinases and sleep phase disorders or mood disorders. In a second section, a comparative study between mammals and *Drosophila* will show the similarities and differences between these two organisms. In a third section, the atypical circadian clock of cyanobateria will highlight how phosphorylation without any transcriptional negative autoregulatory loop can be sufficient to produce sustained oscillations. Finally, in the last section, several other post-translational regulations will be examined such as the role of the phosphatases or of acetylation/deacetylation.

2. Modeling circadian rhythms

Computational models have early been proposed as a useful tool to study the properties and characteristics of circadian rhythms. Before experimental data on the underlying molecular mechanism were available, abstract physical models such as the Van der Pol limit cycle oscillator were used to probe properties of circadian oscillations. This model has proved highly useful in studying many features about circadian rhythms: entrainment by light-dark cycles, phase shifts by light pulses, response of the human circadian clock to perturbations by light pulses [23-25], and the possibility of suppressing circadian rhythms by a pulse of light [26-29]. Moreover, simulations using the Van der Pol model have provided insights into the evolutionary significance of circadian rhythms in cyanobacteria [30,31], and the possible dynamics of the suprachiasmatic nuclei that can be seen as a population of coupled oscillators [32].

Another class of models relies on the Goodwin model proposed in the 60's [33]. This model was not originally designed to study circadian rhythms but the structure that underlies this model is based on a Transcription-Translation Feedback Loop (TTFL; see Figure 1) where a protein inhibits the transcription of its own gene. At the beginning of the 90's, the same negative feedback loop structure was discovered in the *Drosophila* circadian system [7]. Later on, this mechanism was also discovered in other circadian systems, from *Neurospora* [34] to mammals [35], and even in cyanobacteria [36] or plants [37]. Although this TTFL is commonly admitted amongst experimentalists and modelers, there is actually no proof that it is the core oscillatory mechanism for sustained 24 hour oscillations in all organisms. As a matter of fact, there are some evidences pointing to other complementary mechanisms such as the FLO, *i.e.* the Frequency-Less Oscillator, in *Neurospora* [38] or the oscillator based merely on phosphorylations in cyanobacteria [39] (see also section 5 below). Similarly, in *Drosophila* and mammalian cells, the TTFL could not be the only mechanism generating circadian rhythms.

With the development of molecular biology, it appeared that many genes and proteins are involved in circadian clocks. To account for these observations, it became necessary to resort to computational models based on molecular mechanisms. The Goodwin model was the master choice for these studies. Such Goodwin based models were thus proposed for *Drosophila* [40-45], *Neurospora* [42,44,46] and mammals [47,48]. Initially based on few variables, these models progressively gained in complexity with the increasing number of genes and regulations (positive or negative) involved in the circadian regulatory mechanism (see for example Figure 4 in [49]).

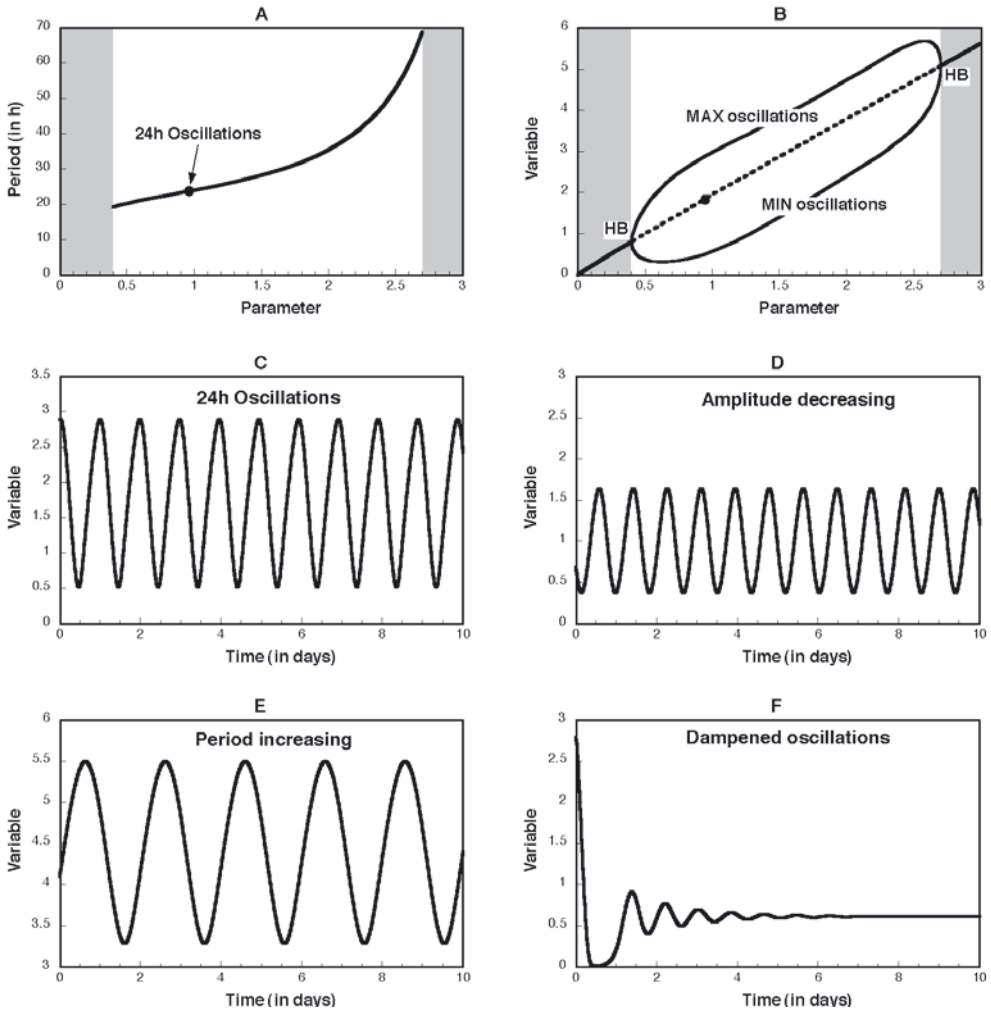

Figure 2. Influence of one parameter on the amplitude and the period of the oscillations. (A-B) Bifurcation diagrams showing the effect of a parameter on the period (A) or the amplitude (B). HB represents the „Hopf bifurcation" that separates the oscillatory domain from the stable steady state. The areas in gray correspond to a stable steady state, *i.e.* no oscillations, while areas in white correspond to limit cycle oscillations. (C) Circadian oscillations obtained after the appropriate selection of the parameter values (black dot in panels A-B). (D-E) Influence of a change in the value of this parameter on the amplitude (D; the amplitude decreased by 50%) or on the period (E; period doubled). Such a change in parameter value is often observed in short- or long-period clock mutants showing a decrease or an increase in the period. (F) When the system exits the oscillatory domain, oscillations disappear after some dampening. Such phenomenon is observed for arrhythmic knockout mutants losing their rhythmicity after several days. The results shown in Figure 2 were obtained by numerical integration of the equations of a simple 5-variable circadian model for *Drosophila* [40]. The parameter and variable considered here are v_d (the rate of PER degradation) and M (the *per* mRNA), respectively. The value of the parameters are the same as in ref. [40], except for panels D, E, F where v_d equals 0.5, 2.4, and 0.3 μM/h, respectively.

The models of the Goodwin type predict that in a certain range of parameter values the genetic regulatory network undergoes circadian oscillations of the limit cycle type, whereas outside this range the gene network operates in a stable steady state (Figure 2). These models can assess the role of some specific variables (e.g. the mRNA or the protein levels) or some biological processes (e.g. the phosphorylation or the entry into the nucleus). In the model, a mutation is viewed as a change in a parameter value. Thus, the effect on the period, the amplitude or the phase of the oscillations can be easily tested for short- or long-period mutants or for arrhythmic knockout mutants (Figure 2). These models were also used to study some general physiological phenomena such as temperature compensation [50-52], phase shifts of the clock [41,42,53,54], and the possibility of suppressing circadian rhythmicity with a pulse of light [55].

The goals of such models are multiple [54]. One of the primary roles is to provide a realistic and quantitative framework to account for the molecular mechanism underlying circadian clocks. Theoretical conceptualization often leads to clarification of hypotheses. Computational modeling also offers the possibility to analyze complex

situations involving multiple, coupled variables, where sheer intuition became much less reliable. Moreover, models show that certain types of behavior only occur under precise conditions, *i.e.* in a domain bounded by critical parameter values (see Figure 2A-B). They also allow determination of the qualitative and quantitative effects of each parameter (Figure 2C-D) and identification of key parameters in the circadian clock. Another useful aspect of modeling pertains to the rapid exploration of different regulatory mechanisms and their large ranges of conditions as well as the possibility to ask questions which may be inaccessible or hard to address experimentally. Finally, last but not least, computational models give rise to testable predictions and suggestion of experiments, which will either validate the underlying mechanism or call for its modification.

Amongst the above mentioned advantages of a computational approach, here are two concrete examples. Firstly, a model for the mammalian circadian clock [47] reveals the existence of multiple sources of oscillatory behavior. In the mammalian circadian clock, several positive and negative loops are interconnected. On one hand, PER and CRY form a TTFL where PER inhibits its own transcription through BMAL1, and on the other hand, BMAL1 forms another TTFL by inhibiting its own transcription through REV-ERBα. From this structure we see that even in the absence of the first TTFL pertaining to PER, the second TTFL pertaining to BMAL1 is theoretically enough to generate oscillations in the absence of any PER proteins. This result was obtained theoretically (See Figure 4 in [47]), and was then confirmed experimentally several years later for the triple PER knockout mice showing some remaining rhythmicity [56]. Secondly, during the exploration of the effect of the rate of phosphorylation of the PER proteins on the period of the oscillations as well as on their phase during light-dark entrainment, this same model reveals some interesting counterintuitive results. It is generally assumed that a shorter endogenous period gives rise to an earlier phase and a longer period to a later phase, such as for FASPS in humans [17] or the *per* short or long mutants in *Drosophila* [57]. Nevertheless, the model reveals that for some region in the parameter space, the opposite phenomenon appears, *i.e.* an increase in the period leads to an early phase (see Figure 3 in [47]). Computational insights for such result are particularly useful in understanding the mechanism underlying phase advances or delays and give clues in comprehending experimental observations that do not fit the common short period / early phase or long period / late phase views.

3. Role of the phosphorylations in the mammalian circadian clock

3.1 Several kinases with multiple functions

The regulatory mechanism of the mammalian circadian clock is based on negative and positive feedback loops [6,11]. In the negative limb, the PER and CRY proteins form a PER-CRY complex that inhibits indirectly the transcription of their own genes. In the positive limb, the CLOCK and BMAL1 proteins form a CLOCK-BMAL1 complex that activates many clock genes such as the *Per* and *Cry* genes. Other feedback loops involve (i) the activation by CLOCK-BMAL1 of *Rev-Erbα* and *Rora* genes whose protein products inhibit or activate the transcription of *Bmal1*, respectively, and (ii) the inhibition of *Bmal1* by its own protein, BMAL1. Gene duplication is also observed in mammals leading to several copies of clock genes or clock proteins, *i.e.* PER1, PER2, PER3, and CRY1, CRY2. Besides the transcriptional regulation, several translational and post-translational regulations are involved in the generation of circadian rhythms in mammals. Amongst them, the mechanism of action of the phosphorylations has been the focus of attention of many scientists because of its high degree of complexity involving several kinases with multiple functions. The main kinases known to play a key role in the generation of circadian rhythms [58] are Casein Kinase I (CKI), Glycogen Synthase Kinase 3 (GSK3), and Mitogen-Activated Protein Kinase (MAPK).

CKI phosphorylates the PER proteins and triggers them for proteasomal degradation [16,18]. Recent experimental data also unraveled a second and opposite role for the phosphorylation of PER2 by one form of CKI, Casein Kinase Iε [19,59]. The function of this phosphorylation is to stabilize the PER2 protein either through increased nuclear retention, and hence decreased protein degradation [19], or through increased *Per2* transcription [59]. Finally, when CKI is coexpressed with the PER proteins, the phosphorylation induced by the kinase can either mask the nuclear localization domain of PER1 leading to its cytoplasmic retention or, conversely, induces the nuclear translocation of PER3, while nuclear translocation of PER2 seems to be unaffected by CKI [20,60,61]. On the other hand, GSK3 promotes the nuclear entry of PER2 but not PER1 or PER3 [62].

The PER proteins are not the only substrates for the CKI and GSK3 kinases. Indeed, CKI phosphorylates the CRY proteins when they are in a complex with PER, the physiological significance of this phosphorylation is not clear though [63]. This kinase can also phosphorylate the BMAL1 proteins leading to a positive regulation of the

transcriptional activity of the CLOCK-BMAL1 complex [63]. Moreover, besides promoting the nuclear entry of PER2 [62], GSK3 also increases the proteasomal degradation of CRY2 [64] and leads to the stabilization of REV-ERBα [65]. Finally, MAPK kinases are involved in the attenuation of CRY's ability to inhibit CLOCK-BMAL1-mediated transcription [66], as well as in the inhibition of the transcriptional activity of BMAL1 [67].

Thus, the role of the kinases in the circadian clock appears more and more complex. Computational models could be very useful in this case to investigate the multiple effects of the kinases in the circadian clock. They could help to test different hypotheses, select more plausible mechanisms of action of the kinases and also highlight essential steps in the multiple phosphorylations of clock proteins. Another advantage of computational modeling is its power of prediction. An excellent example was recently given with the *Tau* mutant in hamsters. Mathematical modeling [18] has revealed that this *Tau* mutant, which was initially characterized by a loss of function *in vitro* and a shortening of the period [68], should in fact be associated with a gain of function. Experimental data further confirm that PER is more phosphorylated and more prone to degradation in the *Tau* mutant [18,69]. The effect of this mutation is highly dependent on the substrate and show only a gain of function for PER while for other substrates a loss of function is observed [18,69].

3.2 Sleep disorders

Dysfunctions of the circadian clock in humans were recently associated with physiological disorders of the sleep-wake cycle such as the familial advanced sleep phase syndrome (FASPS) or its mirror case, the delayed sleep phase syndrome (DSPS) [17,70,71]. People affected by these syndromes have generally a period of sleep advanced or delayed by several hours, respectively. The FASPS syndrome was initially associated with hypophosphorylation of the human PER2 protein by casein kinase Iε, CKIε [17]. The missense mutation affects the CKI-binding domain of PER2. This syndrome was also linked with a mutation in the kinase CKIδ causing a decrease in its activity [72]. On the other hand, the origin of DSPS is less clear but a change of activity of Casein Kinase I could also be responsible for this syndrome [70]. A polymorphism in human CKIε (S408N) - leading to a more active form of the enzyme - is less frequent in individuals with DSPS. An increase of activity of CKIε could play a protective role in the development of the syndrome. However, while this observation seems to be true for the Japanese population [70], the polymorphism has no influence on this sleep disorder in the Brazilian population [73]. Finally, CKI can phosphorylate at least two distinct sites of PER leading to opposite effects on the period [18,19,59]. Phosphorylation at one site, Serine 659, is responsible for FASPS and induces nuclear retention and stabilization of PER2, while phosphorylation at another site leads to the degradation of this protein, as observed for the *Tau* mutant.

Several computational models tried to simulate either the FASPS or the *Tau* mutant [18,19,47,74]. In the 16-variable model of Leloup and Goldbeter [47], only one site of phosphorylation of the PER proteins is considered, *i.e.* a phosphorylation leading to the degradation by the proteasome. Although only one phosphorylation is taken into account in this model, it affects all the different forms of the PER proteins, free or in complexes, as well as in the cytosol or in the nucleus. This allows the model to produce a dependence of the rate of phosphorylation on the period that is not purely linear but goes through several maxima and minima [47]. The advantage of such a curve is to reproduce different experimental results (a period either decreasing or increasing with the level of phosphorylation) when choosing the appropriate region of parameter values for the model. In the 73-variable model of Forger and Peskin [18,48], decreasing the rate of phosphorylation always leads to a longer period. This theoretical result was in contradiction with the assumption that in the *Tau* mutant, hypophosphorylation of PER was responsible for the shorter period. After additional experimental observations [18,69], the *Tau* mutant was finally found to be a gain of function specific for the PER proteins, as it was predicted by the model. The „simple" 5-variable model of Vanselow *et al.* [19] based on a Goodwin oscillator [33] was also used to test different hypotheses about FASPS. In this model, multiple phosphorylations of PER lead to several forms of this protein with different affinities to enter and/or exit the nucleus. This theoretical approach helps to understand the mechanism involved in FASPS but also shows that different phosphorylations can lead to opposite effects on the period. Finally, integrating multiple phosphorylation steps for PER into a detailed computational model will probably be one of the future challenges for modelers.

3.3 Kinases as drug targets

Kinases are enzymes not only found in the circadian clock but also in many other systems and are privileged targets for many drugs. In cancer chronotherapy, several drugs tested so far are kinase inhibitors such as Seliciclib, a cyclin-dependent kinase (CDK) inhibitor (CDKI) which also targets CKIε [21]. In a recent experimental study [21], Seliciclib was administrated in mice at the same time for 5 consecutive days. Twenty four hours after

the last administration, samples were collected in order to examine the level of different clock proteins both in healthy liver and in tumor cells. The most striking result shows that administration of Seliciclib in tumor cells (where no clear rhythms are normally detected) can restore rhythmicity while in healthy tissue the opposite effect is sometimes observed, *i.e.* a suppression of the existing rhythmicity. Moreover, in tumor tissues, the phase and the amplitude of the oscillations is strongly dependent on the time of administration of the drug.

While some explanations seem difficult to find in order to account for these experimental results, computational modeling can offer some hints. Numerical simulations show that a system can give rise to either sustained oscillations or a stable steady state (no oscillations) (Figure 2). The frontier between these two states is defined by a „point of bifurcation", also called „Hopf bifurcation" (HB in Figure 2B). Interestingly, when the system is outside the domain of oscillations but close to this point of bifurcation, oscillations are dampened (Figure 2F). The closer to this threshold, the slower the dampening of the oscillations is obtained. In the case of the repetitive administration of an inhibitor of CKIε, even if the system is originally in a steady state (corresponding to a tumor cell with no rhythmicity), it can nevertheless be entrained by this administration and show dampened oscillations for several days. The oscillations can even look very similar to sustained oscillations (as in a healthy tissue) if the system is close enough to the point of bifurcation. Conversely, in the case of the administration of the inhibitor in healthy tissue, Seliciclib could be perceived by the system as another strong signal as compared to light and leads to counter-balancing effects resulting in some desynchronization characterized by a loss of rhythmicity.

Besides cancer therapy, drugs such as kinase inhibitors are also widely used to treat several disorders such as mood or bipolar disorders and depression. In this case, lithium is very often administrated and acts as a mood stabilizer. Lithium is now known to be a potent inhibitor of GSK3 [75,76] and to lengthen the period of circadian rhythms [76,77]. Up to now, GSK-3 has never been incorporated explicitly into a computational model but with the recent discoveries of its multiple effects on the circadian clock [62,64,65] and the impact of lithium in treating several disorders, future modeling work will surely take into account this kinase.

4. The *Drosophila* circadian clock

Since the discovery of the first *Drosophila* gene homologs in mammals in 1998 [78], many similarities were discovered between both systems [79]. Most of the clock genes and their protein products are present in both organisms, e.g. *per*, *tim*, *cry*, *clock*, ... There are however some discrepancies since some proteins are replaced by others although their function remains identical: e.g. PER binds to TIM in *Drosophila* but to CRY in mammals, the inhibitor VRI and the activator PDP1 of the CLOCK/CYC complex in *Drosophila* is replaced by the inhibitor REV-ERBα and the activator RORa of the CLOCK/BMAL1 complex in mammals. While gene duplication is observed in mammals, only a single copy of the clock genes is present in *Drosophila*: e.g. *Per1*, *Per2*, *Per3*, or *Cry1*, *Cry2* genes in mammals but *per* and *cry* genes in *Drosophila*. Several kinases are also present in *Drosophila*: Doubletime or DBT [80,81] and SHAGGY [82] which are the homologs of CKI and GSK-3 in mammals, respectively, and CKII [83]. Identical negative and positive autoregulatory feedback loops are also encountered in both organisms: CLOCK/CYC in *Drosophila* and CLOCK/BMAL1 in mammals activate the transcription of many genes while PER/TIM in *Drosophila* and PER/CRY in mammals inhibit this activity. Finally, one of the main differences between these two organisms appears through the action of light which leads to the degradation of the TIM protein in *Drosophila* and induces the transcription of the *Per* genes in mammals.

Each new experimental observation made in one system is often rapidly tested in the other one. The understanding of the circadian system in *Drosophila* could help to explain observed data in mammals. Conversely, the differential roles of the phosphorylations discovered in mammals can be investigated in *Drosophila*.

In *Drosophila*, PER is phosphorylated by DBT and is then recognized by the SLIMB protein which marks PER for proteasomal degradation [15]. Mutations of the *dbt* gene can lead to a shorter period (dbt^S mutant), a longer period (dbt^L) or arrhythmicity (dbt^{AR}) but in all these mutants, the activity of the kinase DBT is decreased although with different strength [84]. The stronger effect is observed in the dbt^{AR} mutant where only 10% of the wild-type level of activity is present. The dbt^L and dbt^S mutants show a reduction of the activity by 30% or 15%, respectively [85].

Recently another role of the phosphorylations of PER by DBT was linked to the activity of PER as repressor on CLK [85]. Three distinct regions of the PER protein in *Drosophila* are known to interact with

DBT, the N-terminal (a.a. 149-170), C-terminal domain (a.a. 1136-1224) and a central region (a.a. 580-645) but only the latter one shows an effect on the repressor activity of PER [85]. This region is subdivided into a per-short (per-S) and a per-short downstream domain (per-SD). Interestingly, the per-SD domain looks very similar to the mammalian domain of PER that has been correlated with FASPS [85]. When both domains are close together and unphosphorylated, the PER protein is stable and has no repressor activity. When the per-S domain is phosphorylated at one site, PER is hypophosphorylated, has an intermediate stability and a low repressor activity. On the other hand, when the per-SD is phosphorylated at several sites, the PER protein is then hyperphosphorylated and becomes very unstable but has a high repressor activity.

Phosphorylations in these two domains of PER thus lead to different stabilities of the protein and different levels of repressor activity that can explain the origin of several mutants with either long or short periods. This observation could also account for the result showing that a missense mutation (T44A) in the human CKIδ gene shortens the period in mammals while transgenic *Drosophila* carrying the same CKIδ-T44A gene shows a longer period [72]. Recent observations [86] suggest however that despite a high degree of similarity in primary sequence and kinase function between CKIε and DBT, these two kinases could have very different activity in *Drosophila*.

Most of the early models for the *Drosophila* circadian clock took into consideration the phosphorylation of the PER proteins [40,41,44]. The initial aim of this modeling approach was generally to introduce in the system a delay that favors the appearance of oscillations. Now many recent models pay more attention to the transcriptional or other post-translational regulations without integrating explicitly any phosphorylation [87,88]. The recent discovery of the differential functions of CKI or DBT will certainly incline modelers to reconsider the importance of the phosphorylations in the regulatory mechanism of the *Drosophila* circadian clock and lead them to integrate multiple roles of these phosphorylations in their models.

5. The Cyanobacteria circadian clock

While for many years the paradigm for circadian clocks of most living organisms was the transcriptional negative autoregulatory feedback loop, recent data about cyanobacteria unravel a new mechanism based merely upon phosphorylation. Robust temperature-compensated circadian cycling of KaiC phosphorylation was shown to persist in the presence of a transcription or translation inhibitor [89]. Even more amazing results were obtained by putting only the three main circadian clock proteins (KaiA, KaiB, KaiC) and ATP in a test tube. This experiment shows that a minimal system based only on phosphorylations without any transcription is sufficient to reproduce circadian rhythms [39].

In cyanobacteria, KaiC monomers possess two sites of phosphorylation [90] and are able to bind ATP and form a stable hexamer [91-93]. KaiC also shows both autokinase and autophosphatase activities [89,94,95]. The balance between these two processes varies across the day giving rise to hypophosphorylated forms during the day and hyperphosphosphorylated forms during the night. A second clock protein, KaiA, forms dimers and induces the phosphorylation of KaiC [36,96,97] while a third clock protein, KaiB, forms dimers or tetramers and inhibits the activity of KaiA [95,96,98]. The three Kai proteins can bind together and form multimeric complexes [98,99]. Although the mechanism only based upon these three proteins is sufficient to drive oscillation of KaiC phosphorylation, it is also coupled with a more „classical" transcriptional/translational oscillator in order to keep a 24 h rhythm [100,101].

After the discovery in 2005 [39,89] that a phosphorylation loop is sufficient to produce sustained oscillations, several computational models appeared in 2006 based either on the sole regulation of the phosphorylations of the Kai proteins [102-104] or with its coupling with a transcriptional feedback loop [105]. Interestingly, because of the lack of detailed information about the molecular mechanism of the cyanobacteria circadian clock, many different approaches were tested by computational modeling. Different hypotheses were tested which requires KaiC hexamers binding together and forming complexes [102,103], KaiA and KaiB present under different forms [105], or exchange of monomers among KaiC hexamers [102]. While some hypotheses of these early models such as the formation of KaiC multicomplexes [102,103] contradict with new experimental data [99], other hypotheses such as the monomer shuffling among KaiC hexamers [102] are now supported by experimental data [99,106].

In 2007, several computational models incorporated the monomer shuffling [106,107]. Some models are also based on the allosteric transition of KaiC hexamer giving rise to a system where isolated KaiC hexamer naturally presents cyclical oscillation of the phosphorylated and dephosphorylated states of their monomers [106-108]. This oscillation observed in the hexamers can then be synchronized by different ways [107,108]. While all the models presented so far do not differentiate the two phosphorylation sites of KaiC monomer, recent

experimental data show major differences between these two sites [109,110]. Thus, more recently, a simple model incorporated this observation and reproduced circadian oscillation of KaiC phosphorylation [109]. Finally, the effect of temperature was also investigated by means of a theoretical model [106] that shows how temperature transitions can induce phase shifts without losing temperature compensation of the period. Such effect of temperature was recently further investigated [111].

In two years (2006-2007) many computational models emerged from the original experimental observation of a circadian mechanism based merely on phosphorylation rather than on a more classical transcriptional feedback loop [39,89]. As described above, these models are based on many different experimental hypotheses. The mathematical structures are also completely different from one model to another. Ranging from „simple" to more complex models, positive to negative feedback loops, coupling between oscillators to synchronization among KaiC hexamers and stabilizing or destabilizing the system, all these different approaches were useful in understanding the regulatory molecular network underlying the circadian oscillations of KaiC phosphorylation but also offered testable experimental predictions.

Although a lot of attention was paid recently to the KaiC phosphorylation as a possible core mechanism for the circadian clock of cyanobacteria, it is important to remember that the transcriptional negative feedback loop is also another possible mechanism for the generation of a circadian rhythmicity. Interestingly, recent data shows that even without the phosphorylation rhythm, the cells show oscillations in the transcriptional machinery [101]. These results point to the fact that multiple coupled oscillatory systems are necessary to maintain circadian rhythms in cyanobacteria [110]. The two oscillating systems, *i.e.* the autonomous post-translational oscillator (PTO) and the „classical" transcription-translation feedback loop (TTFL), are closely connected and could either be both self-sustained and provide additional robustness when coupled [101] or the PTO could be the core oscillator and be embedded within a dampened TTFL oscillator [112].

6. Other post-translational regulations

While this review mainly focuses on the role of kinases in different circadian clocks, phosphatases are also important elements in the regulatory mechanism [113-117]. Several phosphatases are involved in circadian clocks such as PP1 [113,114], PP2A and PP2B [115,116], or PP5 [117]. The effect of these phosphatases on clock proteins can sometimes be quite complex. For example, phosphatase PP5 influences the level of phosphorylation of the protein PER in two opposite directions. On one hand, PP5 is directly targeting the PER proteins being responsible for its dephosphorylation and leading to lower levels of phosphorylation of PER. On the other hand, kinase CKIε is submitted to multiple phosphorylations (up to 8 sites) and its level of activity increases when the level of phosphorylations decreases [118]. As PP5 can also target CKIε for its dephosphorylation, the level of activity of the kinase is then increased. Thus PP5 leads now indirectly to higher levels of phosphorylation of PER. Up to now, no computational models incorporate either the different steps of phosphorylation of CKIε leading to different levels of activity of the kinase, or the explicit role of phosphatases in the molecular mechanism of circadian rhythms.

Beyond phosphorylation/dephosphorylation, other post-translational regulations are also important in the regulatory mechanism generating circadian clocks. The main processes include acetylation [13], sumoylation [14], ubiquitination [15] or proteasomal degradation [16]. Amongst these processes acetylation and deacetylation have gained much attention in recent years. One of the main elements of the circadian machinery in mammals, CLOCK, was shown to be an histone acetyltransferase that is required for the transcription of many genes [13,119]. When CLOCK-BMAL1 binds to E-Box promoter elements, CLOCK targets histones H3 and H4 for acetylation [119] as well as BMAL1 [120] and PER2 [121]. When acetylated, PER2 and BMAL1 seems more stable [121,122]. On the other hand, the NAD+-dependent enzyme SIRT1 is a histone deacetylase that has the opposite effect of CLOCK [121,122]. Few computational models [123] have already included acetylation steps in their equations and because of its novelty, the detailed acetylation mechanism involving SIRT1 has never been modeled. Nevertheless, future models will certainly incorporate acetylation/deacetylation as one of their main components.

7. Conclusions and perspectives

Phosphorylation is a key element in the generation of circadian rhythms in all living organisms. The discovery of many kinases with multiple functions revealed a high degree of complexity in this process. It becomes more and more difficult to define exactly the impact of each phosphorylation in the circadian clock. Therefore, computational models can substantially contribute

to a better understanding of the multiple roles of the phosphorylations. Computational modeling has become a powerful and useful tool in investigating the regulatory mechanism of circadian clocks in many organisms. Models have given rise to testable predictions such as in the case of the *Tau* mutant [18] or have revealed unexpected results such as the possibility of oscillations in PER knockout mice [47]. Theoretical approaches have also lead to a wide exploration of the possible oscillatory mechanisms in the cyanobacteria clock [102-111]. Future models will certainly investigate in details the complex role of phosphorylation in mammals and *Drosophila*.

Due to the vast amount of new experimental data published every year on circadian clocks, recent models incorporate an increasing number of elements corresponding to newly discovered genes, mRNAs, proteins, interactions, or positive and negative feedback loops. Models with large numbers of variables and parameters – although attractive by their level of details – generate however new problems. One problem, linked to the robustness of these systems, raises the following question : does the increased number of positive and negative feedback loops lead to a more robust system? Theoretical studies [124] show that this is not always the case and question the role or usefulness of all the regulatory loops involved in circadian clocks. Another problem of models containing a large number of parameters pertains to the reliability of the predictions as the values of most of these parameters remain unknown. With an increasing number of parameters, it can become difficult to find the same quantitative results, independently of the choice of the set of parameter values. This disadvantage could however turn into an advantage for modelers as the predictions tested experimentally can help them to specify the most appropriate choice of parameter sets and lead to more quantitative results.

Although a large number of variables could be problematic, it is nevertheless often necessary to take them into account for describing some particular mutants or regulatory mechanisms. For example, it is not possible to consider the difference between PER1 and PER2 (or CRY1 and CRY2) mutants if only one variable of the model (PER or CRY) describes both proteins. Thus, some models [48] incorporate all the different forms of the proteins and allow the investigation of both PER1 and PER2 mutants. Other models [47] just consider a single form of the PER protein and study for example the global effect of phosphorylation or degradation of all the forms of the protein. As an another example, the roles of REV-ERBα, RORa or SIRT1 cannot be investigated if these elements are not explicitly incorporated in the model. One possibility however is to compare a "core" model and an extended version of the model that incorporates these elements. Such an example was shown in ref. [47] where, on one hand, the negative feedback loop of BMAL1 on its own transcription was modeled directly while, on the other hand, the feedback loop was described indirectly by incorporating the effect of REV-ERBα. Interestingly, the theoretical results showed that most of the conclusions drawn from the full model with REV-ERBα remained similar to the "core" model without REB-ERBα, suggesting the possibility to work with a "simplified" model if REV-ERBα is not directly assessed.

Models will continue to evolve but not necessarily tend to be more and more detailed descriptions of circadian clocks. As shown recently [19,125], even very basic models can highlight interesting principles or give rise to testable predictions. We could indeed focus on only one step (*i.e.* the sole effect of the phosphorylation, the role of acetylation, or the function of some transcriptional or translational regulations) and describe these processes in a detailed way, while simplifying other steps of the circadian clock. In other words, model can be adapted to the specific questions under investigation. Finally, models are like geographical maps, everything depends on the level of details we are looking for. Whatever the scale may be, they all offer different useful perspectives.

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