Minireview

Human molecular chronotyping in sight?

Urs Albrecht

Address: Department of Medicine, Division of Biochemistry, University of Fribourg, Rue du Museé 5, 1700 Fribourg, Switzerland.
E-mail: urs.albrecht@unifr.ch

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Abstract

Recent research on mouse models has taken us closer to deciphering the molecular clock mechanism that defines an individual's 'body time'. How feasible will it be to create a molecular timetable that allows determination of individual body time from tissue harvested at a single time point?

All human beings have in common the characteristic of going to sleep at night and waking up in the morning automatically. As we stumble into a new day, the body prepares itself for the new tasks ahead and increases heart rate, blood pressure and temperature; conversely, these parameters decrease in the evening. Such daily occurring rhythms with a period of about 24 hours are termed circadian rhythms, because they manifest themselves even under constant conditions - that is, even in the absence of stimuli that recur with a 24 hour periodicity, such as daylight. The length of the circadian period is relatively unaffected by changes in temperature or nutritional state. Its phase, however, can be synchronized, or 'entrained', to the environment by light, food or temperature.

It is thought that the circadian timing system provides a benefit to the organism by providing a temporal structure across the body so as to modulate and synchronize biological function and to prevent activation of biochemical pathways that have adverse effects on each other. During the day catabolic processes facilitate engagement with the environment, whereas at night anabolic functions of growth, repair and consolidation predominate (reviewed in [1]). It is evident that medical treatment elicits responses from the body that are dependent on its internal time. Hence, medical treatment can have adverse effects on the patient when it is given at the 'wrong' hour of the day [2,3]. This makes it essential to determine body time if we are to maximize the benefits of medical treatment. It is of great interest to understand how the circadian clock works at the molecular level and how to use this information to generate a molecular timetable that allows profiling of an individual's biochemical pathways in time. Over the past few years significant progress towards this end has been made using animal models. In this article I briefly describe the molecular mechanism of the clock in mammals and then discuss a microarray-based method, proposed by Ueda *et al*. [4] for determining body time.

The molecular circadian oscillator and some of its targets

The molecular mechanism of the circadian clock has been unraveled by means of genetic analysis in *Drosophila* and mammals (reviewed in [5], see Figure 1). This mechanism seems also to be applicable to humans. An autosomal dominant mutation in the human *Per2* gene that inactivates a binding site for casein kinase I ϵ (CKIϵ) results in familial advanced sleep phase syndrome (FASPS) [6]. Because hypophosphorylated Per proteins seem to have a higher metabolic stability than their hyperphosphorylated counterparts, the mutant protein accumulates and the threshold levels of Per complexes required for feedback repression are reached faster than in individuals without the mutation. As a consequence, the period length of the circadian clock shortens and hence, the inner wake-up call of FASPS patients is advanced and falls in the early morning hours. It should be pointed out that the mechanism illustrated in Figure 1 represents a working hypothesis, because many issues remain

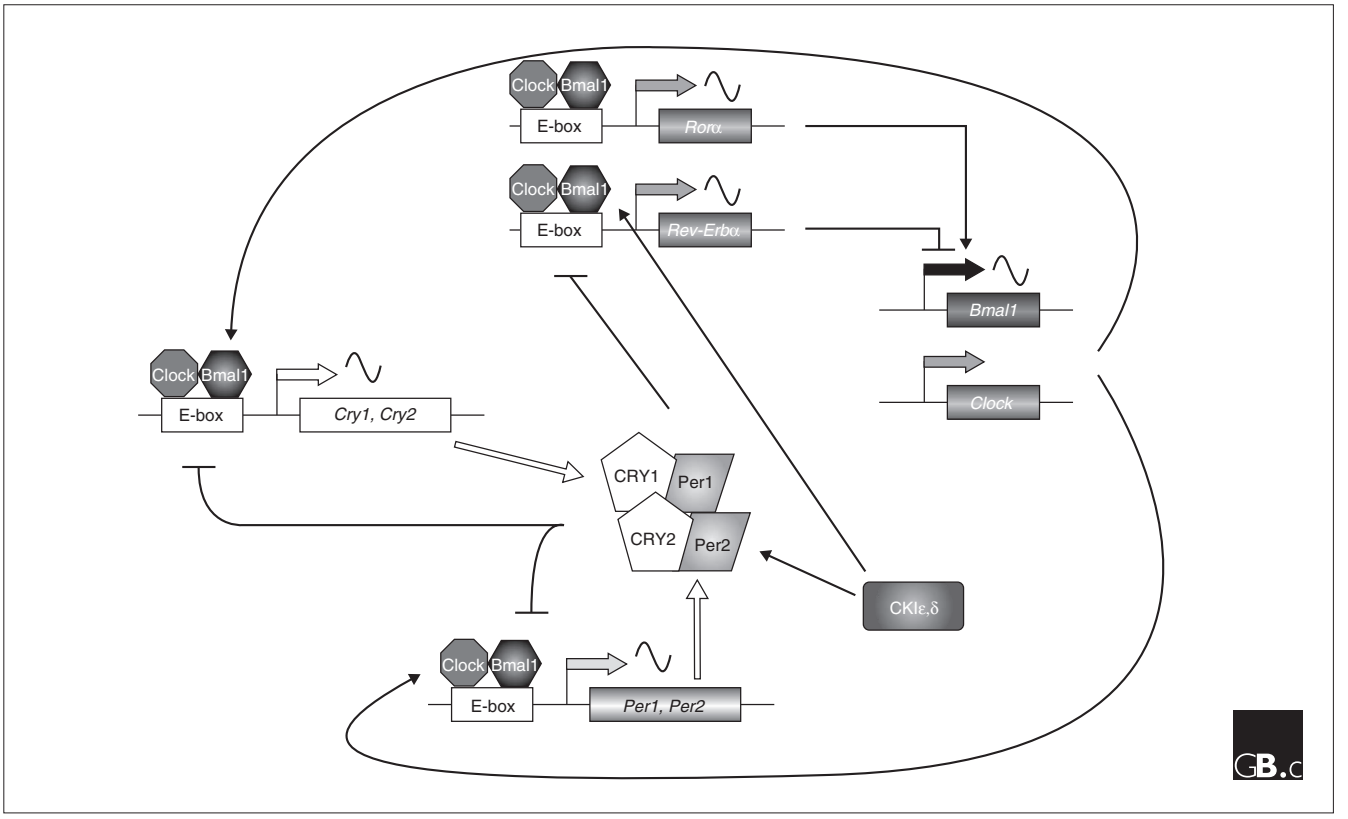

Figure 1
A simplified model of the mammalian circadian clock mechanism. Bmal1 and Clock proteins bind at E-box enhancer elements present in the promoters of *Rorα*, *Rev-Erbα*, *Cry* and *Per* genes and drive their expression in the nucleus. Complexes of Per and Cry proteins in the nucleus inhibit Clock/Bmal1 action by an unknown mechanism, thereby down-regulating their own expression and that of *Rorα* and *Rev-Erbα*. Absence of Rev-Erbα protein derepresses *Bmal1* and possibly also *Clock*, and their proteins reinitiate a new circadian cycle. How the timing between Rev-Erbα and RORα proteins is established is not understood. An essential feature of the clock is posttranslational modification. Casein kinase I (CKI) ϵ and δ isoforms phosphorylate Per, Cry and Bmal1 proteins, decreasing their stability and therefore critically regulating the time of action of clock proteins. Periodic gene expression is indicted by a single wave.

unanswered in this simplified scheme. For example the phase of *Rev-erbα* expression should be in phase with the expression of *Per* and *Cry* genes according to the model, but in fact its mRNA accumulation peak differs from theirs by about 9-11 hours.

The genes involved in the molecular feedback-loop described in Figure 1 could be viewed as analogous to the cogwheels of a wristwatch. Without hands such a clock would not be of great use. Similarly, the circadian clock must somehow interact with biochemical pathways that produce physiological circadian rhythms in the organism. The obvious assumption is that some of the molecular clock components interact or activate genes involved in the relevant biochemical pathways. The search for E-box enhancer elements in the promoters of potentially important proteins, and examination of their transcriptional regulation by Clock and Bmal1, has led to the identification of several clock-controlled genes. Among these are the arginine vasopressin gene (*Avp*), D-element-binding protein (Dbp), type 1 adenylyl cyclase (AC1) and the cell-cycle regulator *wee1*. Of note is that Dbp can regulate expression of members of the cytochrome P450 family, such as cholesterol 7α-hydroxylase, and can impose a circadian activation pattern on these genes as a result of its own circadian regulation by Clock/Bmal1 [7]. Hence, such genes are regulated indirectly by Clock/Bmal1 via Dbp. A change in clock parameters will therefore not affect all circadian-expressed genes in the same manner, and this complicates the idea of creating a unifying time-course gene-expression map for different individuals within a population.

Determination of body time: chronotyping

Although the human body is regulated by a complex network of processes ordered along the timeline of a 24 hour day, the phase of the 24 hour oscillations can vary between individuals, as is evident from the observation that in every society we find some individuals behaving as larks and others as owls. This poses a problem for generalized medicine. Whereas a specific medical treatment schedule can have

positive effects for a 'lark' type of person, the very same therapy can have negative consequences for an 'owl' type of person [8]. This is not only an anecdotal issue: it is demonstrated by the finding that altering the timing of chemotherapy increases its efficiency [2]. To tailor the timing for a therapy such as chemotherapy for a single human being, it is of interest to determine the phase of the clock in the body relative to a reference time, such as the mean of the population's clock phase. Hence, an individual's internal body time reveals the chronotype (lark or owl) of that person. When referring to timings within the diurnal cycle of an individual's clock, researchers use the concept of zeitgeber time (ZT), in which ZT is 0 at the beginning of the light phase (day).

An elegant method for chronotyping individual mice has been proposed by Ueda *et al.*, [4]. Their method is based on a molecular timetable composed of a selection of genes that are supposed to indicate time. It is evident from the description of the molecular clock mechanism in Figure 1 that not all genes are similarly suited to being part of such a timetable. To select suitable genes Ueda *et al.* [4] applied two selection criteria for candidate genes analyzed using Affimetrix murine genome microarrays: circadian rhythmicity and high amplitude expression. Genes meeting these criteria were selected and assigned to a group according to the time of their maximal expression on the 24 hour scale. For example, a gene with maximal expression at ZT=0 is described as having a molecular peak time (MPT) of 0, while a gene with maximal expression at ZT=4 has an MPT of 4, and so on. Combining all these expression profiles gives a graph like that in Figure 2a. This molecular timetable allows us to define a relative body time given that all the genes in this timetable are normalized for similar maximal expression. For example, if we isolate tissue of an individual mouse at ZT=12 and determine the expression level of the genes selected for the molecular timetable (boxed in Figure 2a) we can represent the expression level of these genes as a function of their MPT (Figure 2b). If for all MPTs similar numbers of genes are present in the molecular timetable, a cosine curve can be fitted through the data points (Figure 2b) and an MPT curve is generated. The same procedure can be done with tissue isolated at other times (such as ZT=0, ZT=4 and ZT= 6). This illustrates that gene expression of a single tissue sample taken at a specific time can be visualized and can reveal temporal information. If this information is now compared to a standard cosine curve derived from the same tissue of a large number of individuals, deviations of an individual's MPT curve from the standard MPT can be derived (in Figure 2c an example of tissue collection at ZT=12 is shown). The peak of the standard MPT defines standard body time (BT_S), which is 12 for a tissue collected at ZT=12. If the MPT curve derived from an individual deviates from the standard MPT, this individual's body time is not ZT=12; as illustrated in Figure 2c, this is ZT=10 for individual 1 (BT_1) or ZT=14.5 for individual 2 (BT_2). If the difference in BT compared to the standard BT is larger than the error, the shift in internal timing of the body compared to a standard can be considered 'real'.

This method does not only allow determination of individual body time, it is also useful to reveal gross alterations in the functioning of the circadian clock. Ueda *et al.* [4] demonstrated this for mice mutant for the *Clock* gene. The expression levels of the genes from the molecular timetable plotted as a function of the MPT are scattered and no cosine curve can be fitted through the data points (Figure 2d), indicating a disrupted clock mechanism. This is consistent with the loss of circadian wheel-running behavior in constant darkness that is observed in these mice [9].

It appears that this method opens enormous possibilities for individual chronotyping, but for the moment we have to view this approach with a grain of salt. First, Ueda *et al.* [4] have shown the feasibility of this method in mice, where tissue is easily accessible and large quantities can be used for experimentation; will it work for humans? Second, the choice of tissue seems to be important. As revealed by a number of microarray studies, most tissues display phase-specific patterns of clock and clock-controlled gene expression (reviewed in [10]). For example in the heart most of the circadian-expressed genes peak around ZT=2 [11], whereas in fibroblast cells ZT=6 and ZT=22 are the most frequently observed peak times [12]. This makes it more difficult to select genes suitable for the molecular timetable, because it is important that the genes evaluated are evenly represented over time so that the cosine curve fitting is not biased towards a specific group of genes over-representing an MPT. Ueda *et al.* [4] used liver tissue in their proof-of-principle experiment, and this is the tissue with the most homogenous distribution of MPTs of different genes. But, they also applied the molecular timetable method to brain tissue and in particular to tissue from the suprachiasmatic nucleus (SCN), a structure in the ventral part of the hypothalamus that contains a circadian clock that can coordinate the clocks found in other tissues. MPTs in the SCN are not evenly distributed, but are clustered at dawn (ZT=22) and dusk (ZT=10) [13]. This clustering can also be seen in the data for SCN tissue provided by Ueda *et al.* [4] (in their supplemental Figure 8). In the cosine curve fitting, MPT 0 and MPT 14 are strongly under-represented, leading to a larger error in cosine curve fitting and hence the method is less precise for SCN tissue than for liver.

It is evident that the reliability of the molecular timetable method depends crucially on the genes selected. One would expect that the clock components shown in Figure 1 are good candidates to be part of that timetable. But, comparing these genes with the table constructed by Ueda *et al.* [4] for mouse liver (162 genes) and mouse SCN (96 genes), only *Per2* and *Cry1* are included in the liver timetable, whereas *Per1*, *Per2*, *Cry1*, *Rorα* and *Dbp* are included in the SCN timetable. The reason for the higher representation of clock genes in the

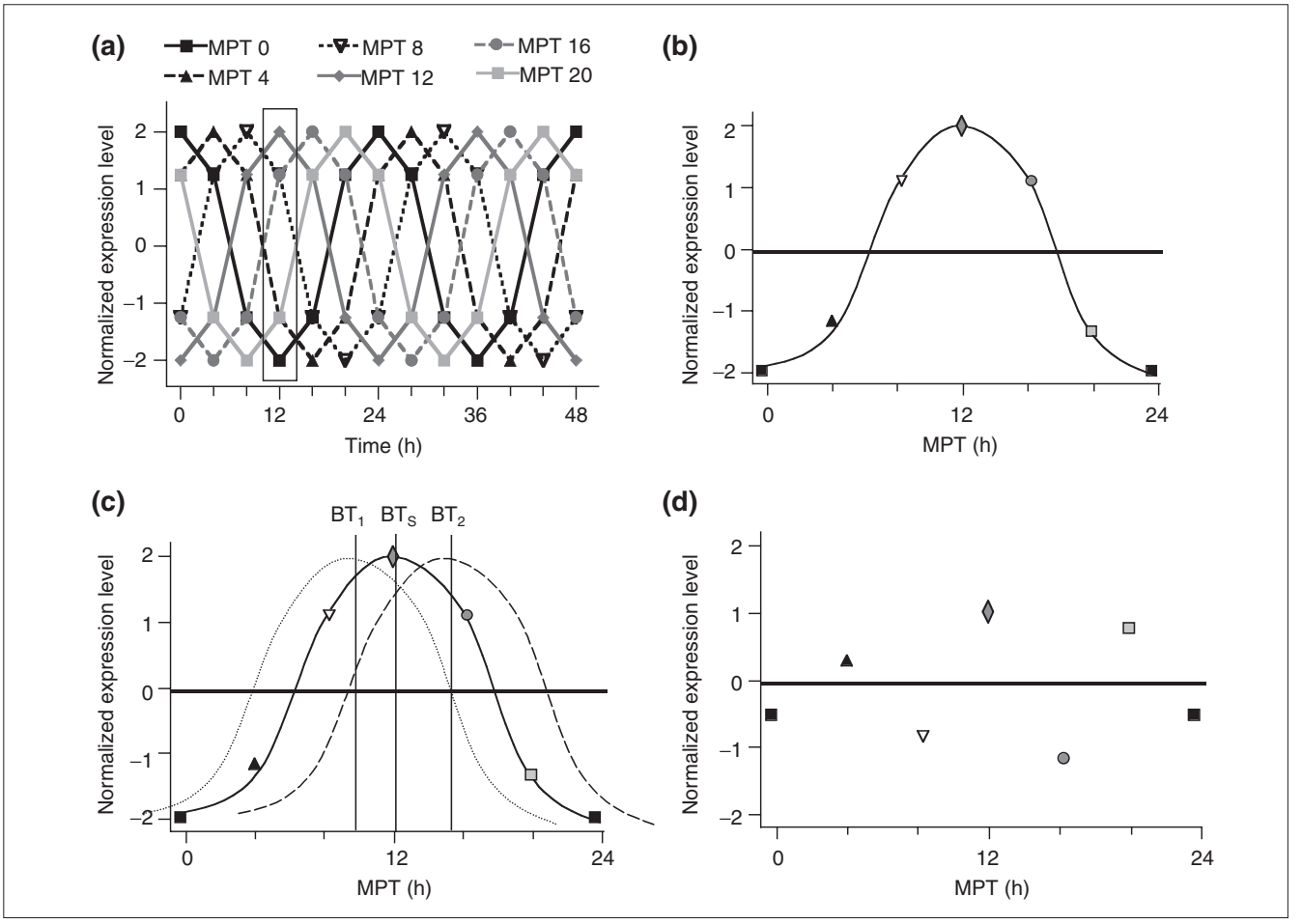

Figure 2
The molecular timetable method of Ueda *et al.* [4]. **(a)** The expression of genes that oscillate in a 24 hour fashion is characterized by their molecular peak time (MPT). Schematized genes with specific circadian patterns of expression are represented by different symbols. An overlay of all oscillating expression patterns should ideally render an even distribution of maximal gene expression over the 24 hour day. Note that the diagram is double plotted and displays two days (48 hours). This molecular timetable can be used to generate a diagram of MPT distribution at a single time point, such as ZT=12 (boxed). **(b)** Representation of the MPT at a single time point with a fitted cosine curve at ZT=12; genes with MPTs of 12 are maximally expressed. **(c)** A schematic diagram for detecting body time (BT). A standard cosine curve (solid line) for tissue harvested at ZT=12 is shown. The maximal normalized expression level indicates standard BT (BT_S). The cosine curve of tissue collected at ZT=12 of individual 1 (dotted line) reveals a BT_1 about 2 hours earlier than BT_S. Individual 2 displays a delayed cosine curve (hatched line, BT_2). **(d)** A plot of normalized gene expression of a clock-mutant individual. The scattered distribution does not allow fitting of a cosine curve.

SCN table might be the absence of strongly oscillating genes that peak at times other than those of the main clock components shown in Figure 1. Their maximal expression is observed at ZT=6-8 (*Per1*), ZT=8-12 (*Per2*), ZT=12 (*Cry1*), ZT=6 (*Ror*α) and ZT=12 (*Dbp*). In the liver, however, a large number of metabolic enzymes are part of the timetable, including alcohol dehydrogenase, several enzymes of the cytochrome P450 family, aminolaevulinate synthase 1, fatty acid synthase and hydroxymethylglutaryl-CoA synthase 1. Some of these genes are indirectly regulated by the clock (as discussed above), which might be a reason for a more even distribution of MPTs in the liver timetable. It is nevertheless astonishing that more clock genes are not included. One interpretation of this finding could be that transcription factors other than those shown in Figure 1 play a major role in driving clock gene expression in the liver. This idea is supported by the observation that several uncharacterized genes are part of the liver timetable.

In summary Ueda *et al.* [4] have demonstrated that the molecular timetable method is applicable for mice. Even in a mouse strain with a heterogenous genetic background the method allows the estimation of body time, albeit with less precision than in mice of homogenous genetic background. The greatest challenge for the future will be in tailoring the method for humans. Tissue collection will be a major constraint, given that it is practically not possible to get liver tissue (the best suited for the analysis) nor SCN tissue from

humans. More easily accessible tissues, such as buccal tissue or skin fibroblasts, must therefore be considered. The next step will be to select suitable genes in these tissues representing the whole 24 hours of a day in the heterogenous human population. Large numbers must be analyzed, so as to establish a standard to which individual samples can be compared. A complementary approach to body time estimation would be determination of an individual's clock period by culturing their fibroblasts. Because clock oscillation can be monitored in single cells [14] using marker genes indicated in Figure 1, the period length of an individual's clock can be derived. The phase of the clock relative to a standard - that is, the individual body time - can not be determined with this method, however. Real-time PCR offers another possibility not only for determining the clock period but also body time. Compared to the molecular timetable method, a PCR-based method for amplifying a few clock genes would need several tissue samples taken at different times. For example, buccal swabs taken every two to three hours from an individual would be suitable for constructing an individual's circadian expression curve for a particular clock gene. For all such methods a common crucial factor is standardization of tissue sampling. Human clock phase is strongly influenced not only by illumination, temperature and social factors but also, for example, by exercise [15]. To gain reproducible and comparable results external factors have to be excluded using constant routine protocols [16,17]: a person will have to be in isolation for a period of time. This illustrates the huge effort that will have to be made if we are to achieve precise individual chronotyping. But we can hope that characterization of human chronotypes using gene-expression-based methods will facilitate diagnosis of circadian rhythm disorders and might support the development of chronotherapy and personalized medicine, as long as the above-mentioned difficulties can be overcome.

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