Enhanced Phenotyping of Complex Traits with a Circadian Clock Model

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Abstract

Models of biological systems are increasingly used to generate and test predictions in silico. This article explores the basic workings of a multi-feedback network model of a circadian clock. In a series of in silico experiments, we investigated the influence of the number of feedbacks by adding and removing one or more. We further explore the possibilities of testing in silico models in classic “circadian” protocols. In addition, we performed an in silico mutagenesis screen (by altering parameters throughout the network), creating a library of mutants (based on “phenotype,” not “genotype”), and subjected them to a variety of straightforward “circadian” protocols. The results of this mutant “taxonomy” are surprising. While most mutants can be identified (separated) using a limited set of experimental protocols, some resist such a separation, even when “mutations” are at vastly different locations within the complex model. Furthermore, some protocols distinguish similar alleles of the same component, which would be counterproductive. The described taxonomy invites experimental verification, in vivo, and may ultimately streamline genotyping of complex traits, which may have been based previously on imprecise phenotypes.

Introduction

One approach in modeling biology is to mimic cellular processes as close to “reality” as possible, with precise kinetics of known reactions being converted into mathematical algorithms. This approach has been successful in modeling circadian properties, such as free running rhythm in virtual constant darkness (DD) and constant light (LL), and temperature compensation (Gonze et al., 2001; Leloup et al., 1997; Rensing et al., 1997). As new genetic components are discovered, models are accordingly amended (Gonze et al., 2001). High and low “levels of abstraction” can also be combined, effectively moving from phenotype, such as behavior, to mathematical principles. Traditional engineering uses models in this way. In the biological sciences, research on chemotaxis works back and forth, between the overt physiology, physical properties, genetics, and mathematics to understand the system as well as modules within it (Iglesias et al.,
Description of biological phenomena with physical terms can result in identification of critical parameters that must be described at the upper level (Elowitz et al., 2000; Zak et al., 2003).

An alternative approach is to move up in levels of abstraction and model concepts rather than data. This top-down approach can, for example, be driven by a given phenotype: algorithms of the model are tuned to match the phenotype as closely as possible. The circadian system is ideal for this type of modeling, as a set of common features has been described in organisms from single cells to mammals. Combining high and low levels of abstraction has resulted in creative experiments involving, for example, the construction of artificial transcriptional regulatory networks in bacteria (Elowitz et al., 2000). We have chosen to primarily model concepts or global features rather than modelling the specific kinetics of known components.

So what are some of the common circadian features that a clock model should mimic? This list will depend on one’s concept of the clock and will influence how and to what end the model is used. Because circadian systems have evolved in a world of daily changes, systematic entrainment should be considered the single most important function of the clock (Roenneberg et al., 2003a). This means that the phase of entrainment, although ultimately controlled by zeitgeber cycles, is clearly not driven, for example, by the cyclical transitions from light to dark or warm to cold. Depending on the length and/or the strength of the zeitgeber cycle or on its relative proportions between day and night (e.g., photoperiod or thermoperiod), the phase of entrainment will be different and not simply locked to one of the transitions. A good example is entrainment by light/dark cycles of asexual spore formation in Neurospora: onsets of this developmental process occur at around midnight in all 24-h photoperiod cycles (Tan et al., 2004). Another example is hamsters that entrain earlier or later in the day depending on zeitgeber strength (Pittendrigh et al., 1976). It is the phase of entrainment that is reflected in the chronotype distribution in humans (Roenneberg et al., 2003c). We have much information regarding the internal phase of entrainment of hormones, such as melatonin in various conditions, but when one looks at individuals, this phase is different according to chronotype (Bailey et al., 1991, 2001; Duffy et al., 1999).

A second feature of circadian systems is a ca. 24-h free running period in constant conditions. It should be noted that the ability to free run in constant conditions is, most likely, as much a consequence as a prerequisite of how evolution has solved the “problem” of entrainment (Roenneberg et al., 2002)—a triggered hourglass mechanism would not develop a free running rhythm. Other features, such as Aschoff’s rule (the dependence of the free running period on the intensity of the constant light) or
temperature compensation (Aschoff, 1979; Pittendrigh, 1960), can also be included in a list of “phenotypic” demands posed on a model.

Finally, when putting pen to paper, some unifying features of circadian physiology might be incorporated into the formal model. The signature of our models (Roenneberg et al., 1998a, 1999, 2002) has been to designate an input feedback, acknowledging the important regulation of sensory input (especially light) pathways by the clock (Fleissner et al., 1988). We call this a Zeitnehmer (German for “time taker,” Roenneberg et al., 1998b), and it represents the endogenous, clock regulation on the input system to the clock itself.

At its simplest, the original Zeitnehmer model looks like two coupled feedback loops—one representing the input pathway and the other the rhythm generator (Roenneberg et al., 1998a) (but both are important for the circadian system to function normally). Simulations show that this model has a free running period of ca. 24 h in constant conditions and that it entrains systematically to different zeitgeber cycles (i.e., it has a generic phase–response curve, PRC). The state variables in both oscillators are rhythmically expressed. As constructed, and as a consequence of the interlocked oscillators, the downstream oscillator is arrhythmic in the absence of the rhythmic, clock-controlled input pathway. The components of both oscillators would, in practice, be called “clock genes” or “clock proteins.”

What does the Zeitnehmer model imply for biological circadian systems? It presents several dilemmas: how would one distinguish between clock genes that are on the input feedback versus the rhythm generator? How is the coupled oscillator model compatible with the simple transcription/translation loop model? Facing these questions, we used traditional circadian protocols, namely T cycles, on Neurospora wild-type and clock mutant strains (Merrow et al., 1999). Using entrainment with temperature cycles, a systematic series of phase angles shows the existence of additional ca. 24-h oscillatory mechanisms in arrhythmic clock mutant strains. Several clock mutant strains also display rhythmic conidiation in the circadian range, even though the rhythm lacks the precision of the wild type and some of the compensation to changes in temperature or nutrition (Lakin-Thomas et al., 2000; Loros et al., 1986). Furthermore, we and others found that the clock gene frequency (frq) in Neurospora is placed squarely on the light input pathway regulating the sporulation rhythm. When there is no FRQ, there is no light-regulated conidiation (Chang et al., 1997; Lakin-Thomas et al., 2000; Merrow et al., 1999). This clock gene would, therefore, qualify for placement in the input oscillator. Thus, a simple eukaryotic model system experimentally fulfills hypotheses predicted by computational modeling.
In the meantime, the molecular mechanism of the circadian clock has grown increasingly complex (Young et al., 2001). The mechanistic backbone of the molecular loop remains, however, transcription and translation based. Any of these central feedback reactions could be completed in a few hours (Merrow et al., 1997) and, indeed, feedbacks in relatives of clock genes have been timed with very short periods (Hirata et al., 2002)! Furthermore, we still lack a satisfactory explanation for the precision in circadian rhythms. We, therefore, revisited the coupled oscillator model to function as a network of several, interconnected feedback loops, thereby increasing its complexity.

The Network Model

The Model

The basic model (Fig. 1) is a network of five feedback loops, all of which oscillate with periods of far under 24 h when uncoupled from each other (Ronneberg et al., 2002). Individual feedbacks are constructed around two state variables (S1 and S2), resembling the transcription/translation loop idea, although they equally could represent any other negative feedback in the metabolism of the cell (e.g., enzyme-product feedbacks). S1 is regulated by production and degradation rates, by negative feedback from S2, and by a coupling factor that connects it to the next feedback in the network. All feedbacks (FB1 through FB5) damp rapidly when not connected within the network. Although basically constructed in the same way, each of the feedbacks has different rates and

![Fig. 1. The network model. Each feedback loop (FB) is a short-period, damping oscillator with two state variables. FBs are coupled in a head-to-tail fashion. In addition, each feeds back onto the input oscillator, FB1, a so-called Zeitnehmer effect. The network is entrained through FB1.](image-url)
coupling factors. Only when the system is connected in a network do the oscillations of all components remain self-sustained with a period in the circadian range.

This model was constructed in consideration of the ubiquity of feedbacks within molecular, biochemical systems, the commonly described short periods of their (damped) oscillations, and the lack of explanation to date of the biochemical basis of the ca. 24-h period. It is likely that prior to a clock with properties such as entrainability by light, temperature, compensation, robustness, and precision, the metabolism of the cell already contained many such feedbacks that would need to be coordinated so that cellular biochemistry remains controllable and predictable in a cyclic world (Roenneberg et al., 2002). Construction of this model showed us that the most common result of stringing together multiple feedbacks was chaotic behavior, which would be incompatible with efficient cellular function. Recently described oscillations in yeast may be a real example of this sort of precircadian clock metabolic programming (Adams et al., 2003; Klevecz et al., 2004). Although we generally use the model as a representation of the molecular clock, it could just as well be used to explore coupling of numerous peripheral oscillators (e.g., liver and brain) or even interacting circadian systems of ecologically interdependent organisms.

In constructing the model, incorporation of complexity, specifically using sub-24-h feedbacks, and entrainability of the system was all that was demanded. The individual feedbacks were systematically assigned increasing rates (rates 1 through 5 to 100 through 500, respectively, in steps of 100). These values govern both production and destruction of the $S_1$ component of each feedback. Tuning the model involved simply adjusting the coupling factors between the feedbacks. What continues to surprise us is that when the model is probed in silico with circadian protocols, it commonly “behaves” like real biological circadian systems (Roenneberg et al., 2002). Originally we also explored the effect of zeitgeber and Zeitnehmer strength on the system and its entrainment. The Zeitnehmer is necessary for reliable and precise entrainment. The model shows systematic phases of entrainment with different zeitgeber strengths. The impact of coupling within the network was examined, and in this model it is the key to self-sustained rhythms. It also determines how susceptible or resistant the system is to entraining with a given zeitgeber strength (Roenneberg et al., 2002).

**How Many Feedbacks?**

The model is based on an assumed evolution of a circadian clock by assembling existing cellular components (feedbacks) in a network rather than creating circadian components de novo. The actual molecular mechanism
will certainly be very different in configuration and will differ widely from organism to organism or even between different tissues in the same organism (Roenneberg et al., 2003b). The head-to-tail configuration, for instance, should look more like lacework, with coupling between all or at least several feedbacks. In an attempt to understand what model features contribute which circadian properties, or which ones are critical, we explored the effect of changing the number of oscillator components within the network.

A six-feedback model was constructed by duplicating each of the existing oscillators and systematically placing it, in turn, between all feedbacks (yielding, by permutation, 25 individual experiments). The general structure was maintained, with the connections disrupted for the insertion and then repaired to incorporate the new feedback. The results were surprising, in that, except for one constellation, none of the resulting models exhibited a self-sustained rhythm with either a constant “zeitgeber” influx (in silico LL) or in the absence of a zeitgeber (in silico DD, Table I). Almost half of the new networks entrained in zeitgeber cycles. When the length of the zeitgeber phase was titrated (like changing photoperiods), some of the six-feedback models entrained in fewer cycle protocols, others in more, relative to the wild type. In all cases where entrainment was observed, the entrained phase changed with the zeitgeber cycle, indicating entrainment and not driven synchronization. The loss of the free run was thus not a complete loss of the clock. Rather, in about half of the six-oscillator models, the hallmark of the clock in nature—entrainment—is intact.

We increased the number of feedbacks in the model further, making a module of duplicated FB2 and FB4, coupled as though in tandem. This unit was systematically placed between all of the original five FBs. This time, all configurations showed all of the basic properties that are found in the five oscillator model (Table II). When the model was decreased to three oscillators, similarly, it behaved like a circadian system, whereas a

<table>
<thead>
<tr>
<th>In silico protocol</th>
<th>Result</th>
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<tbody>
<tr>
<td>No zeitgeber (“DD”)</td>
<td>One is self-sustained&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Constant zeitgeber (“LL”)</td>
<td>None are self-sustained</td>
</tr>
<tr>
<td>Zeitgeber cycle</td>
<td>About half entrain&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Changing phase in various zeitgeber cycles</td>
<td>All that entrain</td>
</tr>
</tbody>
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<sup>a</sup> When FB3 is placed between FB4 and FB5.  
<sup>b</sup> When FBs are placed between FB3 and FB4, FB4 and FB5, or FB5 and FB1.
four-oscillator network was like the six-feedback model, arrhythmic but entrainable (data not shown). Thus, for the specifications of this network, an odd number of feedbacks is essential for yielding the full spectrum of experimentally probed circadian properties. We may be able to exploit this observation further to understand dynamic aspects of coupling feedbacks. For instance, if interactions between the feedbacks (in the coupling factor) were manipulated, then circadian properties might be recovered. If coupling were imposed between all feedbacks (as described earlier), the odd number of feedbacks might not be critical. Furthermore, if a model with an even number of feedbacks had initially been tuned to optimally mimic a circadian system, it might have been the odd-number models that showed arrhythmicity. This possibility still has to be investigated.

**Additional Properties of the Network Model:**

**In Silico Clock Experiments**

As published, the five-feedback model shows some basic circadian properties, namely free running rhythm and systematic entrainment. We ran additional simulations to probe the model using some of the many protocols that have described specific clock properties.

Aschoff’s rule [the systematic modification of frequency in constant light of increasing fluence (Aschoff, 1979)] was mimicked by running the model in constant (i.e., noncyclical) zeitgeber strengths from 0 (DD) to 50. The wild-type model typically uses a zeitgeber strength of 40 and becomes arrhythmic with a constant zeitgeber input (LL) of 50. However, at all lower fluence rates, period correlates positively with the strength of the input signal (Fig. 2A).

In its entrainment, the model is similar to biological systems—it approaches a steady state over several days rather than locking onto a given phase immediately. Furthermore, in different photoperiods, it has phase
angles that shift relative to the zeitgeber transitions, as has been shown in mammals, flies, and fungi (Aschoff et al., 1978; Pittendrigh et al., 1976; Tan et al., 2004). Thus alterations of the zeitgeber quantity (either strength or duration) result in changes in entrained state. By changing the rate of FB$_1$ (affecting production and destruction of state variable 1), a stable entrainment in a light:dark cycle of 12 h in each condition (LD 12:12) becomes a free run, which occasionally shows relative coordination to zeitgeber cycles (bottom 2 panels, Fig. 2B) (Wever, 1979). In the top two panels of Fig. 2B,
the system entrains, showing an advancing phase relationship to the zeitgeber with increasing rate (top panel, Fig. 2B). The bottom two panels of Fig. 2B show an increasing tendency to free run as the rate is increased further. The amount of increase in the FB1 rate is less than 20%, with 100% (i.e., the value chosen in the original, “wild-type” model) in the top panel and 118% in the bottom one.

Note that the model was initially not tuned for these specifically circadian response characteristics. This still does not mean that the network model resembles a circadian system, but it does give us a tool with which to probe the elements of the model that confer or impact various circadian responses.

**In Silico Clock Mutants**

**Evolution of a Circadian System**

Circadian systems produce distinct, quantifiable phenotypes (free running periods in constant conditions or phases of entrainment) that reflect the activity of many genes (Young et al., 2001). As our network model is designed, it lends itself to “genetic” and “evolutionary” descriptions that can be compared to, for example, the mammalian circadian system (Roenneberg et al., 2003b). The model is based on the assumption that the intact system, with all of its properties (self-sustainment, compensation, precision, etc.), evolved through successive mutation, which would effectively tune the network for optimal (circadian) function (Roenneberg et al., 2002). Some polymorphisms within the network system so that it may have an altered free running rhythm and/or so that it entrains to zeitgeber cycles with a different phase relationship. Polymorphisms have been associated with individually different circadian response characteristics (Ebisawa et al., 2001; Ralph et al., 1988; Toh et al., 2001). Figure 2B demonstrates that in silico “polymorphisms” (by changing parameters) in our model excellently mimic the behavior of real circadian clocks by inducing altered phases of entrainment or even loss of entrainability. We have, therefore, used the model to further probe the consequences of mutations/polymorphisms placed in various locations within the network.

**Making Mutants**

To simulate genetic mutations, we changed the following parameters: the rates of each feedback, their coupling factor, and the strength of the negative feedback on the production of $S_1$. For each “mutation,” only a
single parameter in a given FB was altered. Values were changed within a range between half and double of the original ("wild type") value by running series of simulations. When no mutant phenotype emerged, the value was changed more. The *Zeitnehmer* function and zeitgeber strengths were also targeted in this *in silico* “mutagenesis.” This latter would be comparable to mutations in sensitivity to light (i.e., light signal reception or transduction). It could, however, also simulate nongenetic effects such as shielding clocks from light by living predominantly inside. The resultant polymorphic population of models was “screened” in a LD cycle of 12:12 h in each condition, and the resulting time series were evaluated in CHRONO (*Roenneberg et al.*, 2000) for their phase of entrainment.

The described virtual mutant screen reveals network components that are more or less sensitive or tolerant to changing the phenotype. The input feedback loop is generally the most susceptible in terms of yielding an early or late chronotype upon alteration of its components (perhaps this is not surprising, as the screen was phase of entrainment in 12:12, and this is the input feedback). In contrast, the oscillator symmetrically furthest away from the input feedback loop (FB3, see Fig. 1) proved most resistant to showing a mutant phenotype. Because the system as a whole relies on every one of the oscillators to be part of the network for rhythmity with normal circadian properties, these observations suggest that the clock genes discovered in mutant screens to date would disproportionately represent the input feedback.

We grouped the mutants (many showed a “wild-type” phenotype) according to phase of entrainment. They included some that entrain early (larks) and some that do so late (owls). In this respect, the *in silico* mutants approximately resemble the chrono-type distribution in a human population (*Roenneberg et al.*, 2003c), an excellent example of a complex genetic trait. However, our understanding of the genetic basis of human chrono-types is limited. Only a minority of early or late chronotypes have the same genetic polymorphisms, and very few have, so far, been identified. This could reflect the relatively crude phenotyping tools that we have for humans. Until recently, a subjective questionnaire was the primary instrument in this regard (*Horne et al.*, 1976). We now prefer one that asks, although still subjectively, for bedtime and wakeup time (*Roenneberg et al.*, 2003c) for both work and free days. With better, more quantitative information, one might design simple protocols or tests to enhance phenotyping. We propose to use the model to try to uncover which of the circadian protocols (or a combination of different circadian protocols) is most powerful to separate apparently similar phenotypes, in this case similar phase of entrainment in LD 12:12.
We now concentrate on mutants with a delayed phase of entrainment in an in silico LD 12:12 (Table III). The mutants are named as though they were genetic mutants. The mutants sensitivity 1-1 and sensitivity 1-2 (sen) are two alleles (i.e., altering the same parameter), in this case, zeitgeber strength. no1-1 and 1-2 represent changes in the strength of negative feedback in FB1. no5-1 has a decreased feedback in FB5. Rate mutations (rate1-1 and 2-1) represent rate decreases in FB1 and FB2, respectively. Coupling factor changes in FB3 and FB4 are described by cf3-1 and cf4-1. Finally, the Zeitnehmer function was decreased to yield zn1-1 and zn1-2.

### Table III

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Description</th>
<th>Phenotype (phase, h after lights off)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>As described in text and in Roenneberg et al. (2002)</td>
<td>14.4</td>
</tr>
<tr>
<td>sen1-1</td>
<td>Zeitgeber at 60; 50% increased sensitivity to light</td>
<td>17.7</td>
</tr>
<tr>
<td>sen1-2</td>
<td>Zeitgeber at 80; 100% increased sensitivity to light</td>
<td>18.5</td>
</tr>
<tr>
<td>no1-1</td>
<td>40% decrease in negative feedback in FB1</td>
<td>18.0</td>
</tr>
<tr>
<td>no1-2</td>
<td>No negative feedback in FB1</td>
<td>19.8</td>
</tr>
<tr>
<td>no5-1</td>
<td>40% decrease in negative feedback in FB5</td>
<td>17.9</td>
</tr>
<tr>
<td>rate1-1</td>
<td>50% decrease in rate constant in FB1</td>
<td>18.8</td>
</tr>
<tr>
<td>rate2-1</td>
<td>95% decrease in rate constant in FB2</td>
<td>18.1</td>
</tr>
<tr>
<td>cf3-1</td>
<td>400% increase in coupling factor between FB3 and FB4</td>
<td>16.0</td>
</tr>
<tr>
<td>cf4-1</td>
<td>400% increase in coupling factor between FB4 and FB5</td>
<td>17.5</td>
</tr>
<tr>
<td>zn1-1</td>
<td>60% decreased Zeitnehmer strength</td>
<td>20.9</td>
</tr>
<tr>
<td>zn2-2</td>
<td>40% decreased Zeitnehmer strength</td>
<td>18.3</td>
</tr>
</tbody>
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We then asked the question of how we could use the model in combination with circadian protocols to distinguish the mutants from one another. Specifically, which circadian protocols are capable of identifying subgroups that correspond to mutations in different components? The mutants were first run through a constant condition routine, with no zeitgeber (simulating DD, Fig. 3). Two individuals retained a wild-type free running period, two became arrhythmic, six had long periods, and one was markedly long. Thus, with this first protocol, the arrhythmic mutant can be clearly isolated from the rest. Next, LL was simulated. This separated another two mutants from the group of six that had a long period in DD. In LL, one mutant in this group was arrhythmic and...
another showed a very long period compared with the rest within this group. The entrained phase in long photoperiods further distinguished the negative feedback mutants from each other and from a coupling factor and a \textit{Zeitnehmer} mutant. Interestingly, the mutants that were clustered together based on their wild-type period or arrhythmicity in the DD protocol still show no differences in their \textit{in silico} phenotype. They did show clear differences, however, when they were examined in short-photoperiod LD cycles. This protocol also separated two “alleles,” a result

Fig. 3. A phenotypic taxonomy. Eleven mutants with a delayed phase of entrainment were submitted to a series of circadian protocols to distinguish them from one another. Except for a coupling factor and a \textit{Zeitnehmer} mutant, all could be separated, using free running period (FRP) in DD, FRP in LL, entrained phase in a long and a short photoperiod, and temperature compensation.
that would be confounding in the absence of concrete genetic information. (In other words, in this case, enhanced phenotyping would create a cluster within a locus rather than clustering the mutants together.) Finally, a temperature compensation experiment was simulated (by uniformly increasing all rates with increasing “temperature”) to separate the remaining “mutants” (no5-1, cf4-1, and zn1-2). The negative feedback mutant showed decreased compensation relative to the coupling factor and the *Zeitnehmer* mutants, which were both compensated similarly compared with the wild-type model.

Conclusions

Our conclusion from this exercise is that enhanced phenotyping of delayed or advanced (data not shown) entrained phase can improve genotyping. Subtle clusters can be defined by exploring additional circadian properties with appropriate circadian protocols. For humans, this might include surveying them throughout the year with sleep logs or actimeters as they experience different photoperiods, a practical, nonclinical and cost-effective approach. For mice, the benefits are potentially greater, as they can indeed be submitted to most of these protocols relatively easily.

A caveat is that some mutations (i.e., cf4-1 and zn1-2, as described here) will be difficult, if not impossible, to distinguish phenotypically. Furthermore, in the process of making the mutant population, some mutation sites proved resistant to yielding a mutant phenotype (namely rates 3, 4, and 5). This highlights a critical role for biochemistry in following the path of oscillators, molecule by molecule, from input to core, to output, and back to input again. An additional implication is that the clock components discovered so far are predominantly involved in handling sensory input. Circadianly regulated input pathways are an integral part of the clock mechanism. This is also evident for our *in silico* clock model. The traditional distinction between input pathways and core clock mechanism is, therefore, conceptually unproductive because mutations in all relevant parts of the system are true clock mutants. The bottom line is that clock genes have been discovered for their profound effects on entrained phase or free running rhythm (Feldman *et al.*, 1971; Kondo *et al.*, 1994; Konopka *et al.*, 1971). While the input feedback has a large impact on the precise timing of phase, other loops can yield similar effects, and it is only an accurate timer in combination with the rest of the system to support it.

This exercise also addresses the question “why model?” or, more specifically, “Why model concepts without direct correlation to biochemical reactions?” As clearly shown here, the process allows one to
incorporate information collected from many systems and protocols into a cohesive system. Furthermore, the exercise produces many hypotheses that are experimentally testable.

Acknowledgments

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