The Nitrate Reductase Circadian System. The Central Clock Dogma Contra Multiple Oscillatory Feedback Loops

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The ability of plants and other organisms to show endogenous circadian rhythms and to adapt to daily and photoperiodic events is often associated with a central molecular clock (Bünning, 1973; Edmunds, 1988). Using the oscillatory nitrate reductase (NR) system as an example, we argue that circadian rhythms and their functionality can be perceived without postulating a central molecular chronometer.

The molecular biology of circadian rhythmicity in the model organisms *Neurospora* and *Drosophila* and also in mammals and cyanobacteria shows striking homologies (Dunlap, 1999). Negative feedback loops, where "clock proteins" inhibit their own transcription/translation together with the (positive) transcription and translation processes, are central elements in defining the core mechanisms of these rhythms (Fig. 1A). Simulation calculations with negative feedback models (Goodwin, 1965; Leloup et al., 1999; Ruoff et al., 1999) not only confirm the basic understanding of these rhythms, but also provide predictions on important properties; for example, the relationship between the homeostasis of the circadian period and the stability of clock proteins (Ruoff et al., 1996; Iwasaki and Dunlap, 2000; Liu et al., 2000).

In higher plants the picture is not as clear as it is in the organisms referred to above, but here experiments point toward the importance of negative feedback regulation. In plants and algae, examples of uncorrelated circadian "clocks" have been found that require interpretations other than the "central clock concept" (McClung, 2000). For instance, in *Gonyaulax*, bioluminescence and cell aggregation rhythms were desynchronized under special light conditions (Roenneberg and Morse, 1993), and in bean plants, CO_2 assimilation and leaf movements were shown to have different period length (Hennessey and Field, 1992). In Arabidopsis, the *AtGRP7* (or *CCR2*) mRNA and *At*GRP7 protein undergo circadian oscillations in constant light, and the data are consistent with *AtGRP7* and *At*GRP7 being part of a negative feedback loop (Heintzen et al., 1997). When the oscillations of endogenous *AtGRP7* transcripts were depressed by introducing overexpression of AtGRP7 in transgenic Arabidopsis, other circadian rhythms like transcripts in *CAB* or catalase mRNA were not influenced. The *AtGRP7* and AtGRP7 oscillations were conceived as part of an autonomous feedback oscillator, or alternatively viewed as still governed by a central oscillator because in the Arabidopsis toc1 mutant the period lengths of several circadian rhythms, including the *AtGRP7* transcript, were shortened. Another interesting example is the oscillatory NR system.

CHARACTERIZATION OF THE NR OSCILLATORY SYSTEM

Circadian oscillations of NR mRNA have been demonstrated in, for instance, maize (*Zea mays*; Lillo and Ruoff, 1989), *Nicotiana plumbaginifolia* (Deng et al., 1990), Arabidopsis (McClung and Kay, 1994), and tomato (*Lycopersicon esulentum*; Galangau et al., 1988; Jones et al., 1998). Translation of NR mRNA into active NR enzyme is necessary to obtain the negative feedback in this oscillating system (Fig. 1B). This is demonstrated by feeding plants with tungstate, which inactivates NR by replacing molybdate in the cofactor at the nitrate-reducing site of NR (Deng et al., 1989), and by studying various mutants of *N. plumbaginifolia* mutated in the *Nia* gene (gene coding for the NR apoenzyme) or *Cnx* genes (genes involved in synthesis of the molybdate-binding cofactor of NR; Pouteau et al., 1989). In these tungstate-fed or mutated plants the NR enzyme is inactive, the NR mRNA usually stays at a higher level, and no oscillations are seen. It can be concluded that the catalytic activity of NR is necessary for repression of the NR mRNA level (Pouteau et al., 1989). During nitrate assimilation, nitrate is reduced by NR to nitrite and further by nitrite reductase to ammonium, which is assimilated into Gln by Gln synthetase. Gln is a candidate for being involved in exerting the negative feedback on NR expression because Gln was found to oscillate in *N. plumbaginifolia* in reverse phase to NR mRNA (Deng et al., 1991). Increased Gln concentration correlating with decreased NR gene transcription, and vice versa, was also established for several tobacco mutants (Scheible et al., 1997). However, the exact mechanism of Gln inhibition has not been revealed and certainly Gln itself may not necessarily be

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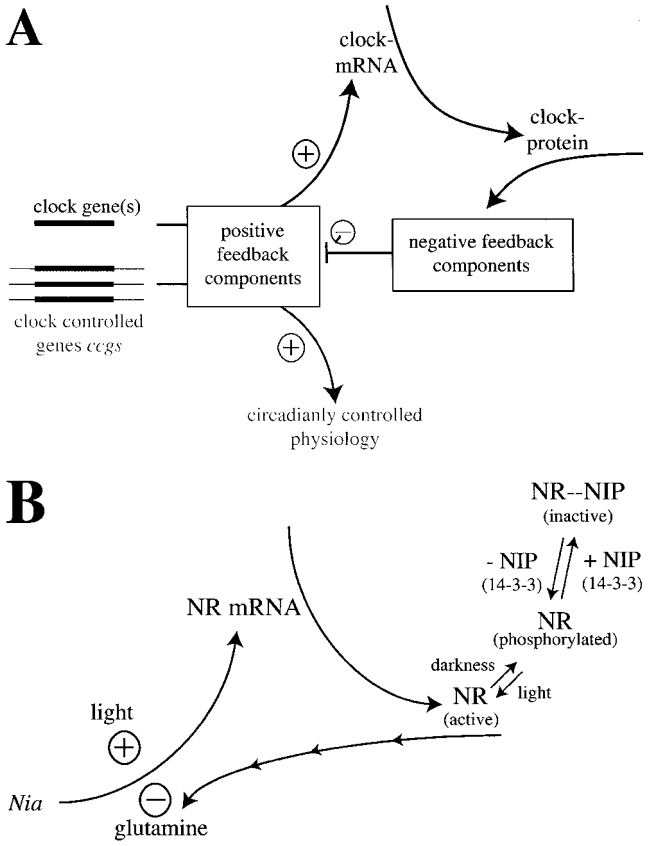

Figure 1. A, Basic elements of what is known at present about the assumed circadian clock mechanisms (black) and output rhythms (gray) in *Synechococcus*, *Neurospora*, *Drosophila*, and in mammals (Dunlap, 1999). In plants this picture of a (master) clock appears not to be generally valid. B, Negative feedback defining the NR circadian oscillator in higher plants. The assumed repressor is Gln, which is a product of active NR. However, little is known about the mechanisms of inhibition.

the effector, but possibly some product derived from, or dependent on, Gln may act as an effector.

Photosynthetic active light and nitrate are well-known factors necessary for high expression of NR (Lillo, 1994). Photosynthetic active light is apparently an important factor driving the positive feed forward (transcription) in the NR rhythms. Rhythms in NR activity or NR mRNA were shown to persist only in continuous light but not in darkness for many plant species. For instance, this was observed in barley (*Hordeum vulgare*; Lillo, 1984), *N. plumbaginifolia*, (Deng et al., 1990), maize (Lillo and Ruoff, 1989), Arabidopsis (McClung and Kay, 1994), and *Khalanchoë fedtschenkoi* (Lillo et al., 1996).

Experiments with several transgenic *N. plumbaginifolia* nicely support the theory of NR as a self-sustained oscillating system. In the homozygous *nia* mutant E23, where the *Nia* gene is disrupted by a retrotransposon insertion (C. Meyer, unpublished data), the most abundant form of NR mRNA is a truncated and hybrid transcript ending in the 5′ end of the inserted retrotransposon (Vaucheret et al., 1992). This mutant is devoid of any NR protein or activity. Measurement of the truncated NR mRNA level showed that this mRNA was no longer oscillating. However, when the E23 mutant was crossed with the wild type so that the progeny had one wild-type gene (*Nia* promoter linked to *Nia* structural gene) and one mutated gene (*Nia* promoter linked to mutated structural *nia* gene), the NR mRNA oscillated as expected, and quite interestingly the mutated nia mRNA also showed oscillations. The mutated *nia* gene obeys the wild-type *Nia* gene because the negative feedback loop is now reconstituted due to the active NR enzyme of the wild-type oscillator. Therefore, in analogy with discussions on central oscillators that may drive subordinate non-self-sustained oscillators, the mutated, non-self-sustained NR oscillator is driven by the fully functioning wild-type NR oscillator; a nice example showing that a feature usually mentioned in relation to a central oscillator may hold also for other oscillators. In other transgenic *N. plumbaginifolia* plants, the *Nia* structural gene was driven by the constitutive 35S promoter and this was shown to abolish oscillations of NR mRNA, apparently because the 35S promoter could no longer recognize the negative feedback signal (Vincentz et al., 1993). Therefore, the experiments confirmed that the inducible/repressible wild-type NR promoter is necessary for oscillations of NR expression, and that the oscillations evolve on the transcriptional level. Arabidopsis mutants are now available with shortened (Strayer et al., 2000) or lengthened (Somers et al., 2000) periods in several circadian rhythms. It would be interesting to test if the NR rhythms would be altered in such a background.

A close connection between light-induced transcription and circadian rhythms of gene transcription has been pointed out for plant genes other than NR. For example, a fundamental and interesting problem in separating the circadian promoter elements from light-regulated promoter elements became apparent for the *CAB* (chlorophyll a/b-binding protein) genes (Fejes et al., 1990; Andersen and Kay, 1995; Millar, 1999). The light inducibility may be the essential factor of the promoter that together with a negative feedback result in circadian oscillations of transcription of the genes. A special "clock-perceptive" part of the promoter is not necessarily reality.

In crassulacean acid metabolism plants, phosphoenolpyruvate carboxylase (PEPc) activity and carbon dioxide uptake in the leaves show circadian rhythms (Nimmo et al., 1987). The correlation between these rhythms and the NR rhythm were investigated, and showed that when plants were transferred to darkness the NR rhythm is arrested in its "night state," whereas both the CO_2 exchange and PEPc activity rhythms were arrested in the "day state" (Lillo et al., 1996). The desynchronization of NR and PEPc rhythms is difficult to understand in

view of one master clock and points also to the existence of independent rhythms.

All known physicochemical oscillators (biological oscillators included) contain positive and negative feedback loops as a necessary (but not sufficient) condition for oscillations to occur (Franck, 1980). The appropriate timing between these positive and negative elements either in the form of suitable rate constant values or delays is what finally leads to oscillations. Certain biological oscillators (for example the cell cycle) contain autocatalytic (i.e. self-amplifying) loops (Goldbeter, 1996). It is interesting to note that the known molecular mechanisms of circadian oscillators are found not to include autocatalysis, but are based on the transcription/translation process and its inhibition by downstream products (Fig. 1A; Dunlap, 1999). In this respect, the essential elements required for sustained oscillations of NR expression are clearly present without postulating a link to a central clock (Fig. 1B). The question then arises: Why is there an approximately 24-h period of the rhythm under prolonged constant light? This is certainly a crucial question; however, it basically is not different from the question also to be asked concerning a central clock. Important factors for period length are degradation rates of the components involved. This has been demonstrated for the *Neurospora* clock protein FREQUENCY. This protein is more rapidly degraded when phosphorylated, and it was shown that mutation of the phosphorylation site (Ser 513) led to a dramatic reduction in degradation rate and a very long period of sporulation (more than 30 h; Liu et al., 2000). In fact, phosphorylation of clock components appears to be important in all the model organisms, i.e. also in *Drosophila*, mammals, and cyanobacteria (Dunlap, 1998; Nishiwaki et al., 2000). NR is known to be phosphorylated at a specific site, and this leads to binding of so-called 14-3-3 proteins and inhibition of NR activity (MacKintosh et al., 1995; Huber et al., 1996). In addition, phosphorylation is thought to influence degradation rate of the NR protein itself (Kaiser and Huber, 1997; Cotelle et al., 2000). More research is needed before the influence of phosphorylation on the circadian rhythms of NR expression can be predicted. However, phosphorylation is a universal way of controlling cell metabolism, and may also be nature's solution to adjusting period length of biological oscillations.

CONCLUSIONS

As long as only a handful model organisms and within them (except for prokaryotes) only a small number of different output rhythms are studied, a master clock based on a transcription-translation feedback loop may be satisfactory to explain the underlying mechanism for the rhythms known in each organism. However, even a transcription-translation feedback loop is not satisfactory for explaining all circadian rhythms. A striking example is the giant algae *Acetabularia acetabulum*, known to show a circadian rhythm in photosynthesis even when the nucleus is removed (Bünning, 1973). The huge amount of new data being gathered is changing our comprehension of how rhythms are created and influenced. For instance, the dogma that the output rhythms should not influence the underlying oscillating mechanism is on its way out. In vertebrates, there is clearly a feedback from the output behavior back to the clock and from the clock to the input photoreceptors (Dunlap, 1999). In plants, studies on photoreceptors add complexity to the picture concerning input to the oscillator (Bognár et al., 1999). The idea of a master clock in each organism is attractive, but likely an oversimplification. Although a common theme can be identified in the various oscillators, comparative analysis of the available experimental data suggest multiple independent origins of the intracellular oscillator (Dunlap, 1999). The selective pressure caused by the diurnal light/darkness shift is constantly present, and may have selected regulatory components to create circadian rhythms not once but many times within one organism. There is a preference in the scientific community to use a mode of expression implying that a central clock is a biological fact. However, as pointed out especially by many plant biologists, a central clock is not a fact of life, but an idea. In general, a neutral approach would be to analyze circadian rhythms as self-sustained phenomena as an alternative to the control by a central clock. Attempts to explain all circadian rhythms by a "hidden" underlying mechanism is not satisfactory, and the components previously considered as output and input to a clock may also be part of self-sustained rhythmic feedback loops. The central clock concept may lead to similar outcomes as the construction of epicycles in the description of planetary motion to preserve the notion of an earth in the center of the solar system.

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