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REVIEW

BIOLOGICAL TIMING AND THE CLOCK METAPHOR: OSCILLATORY AND HOURGLASS MECHANISMS

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ABSTRACT

Living organisms have developed a multitude of timing mechanisms—"biological clocks." Their mechanisms are based on either oscillations (oscillatory clocks) or unidirectional processes (hourglass clocks). Oscillatory clocks comprise circatidal, circalunidian, circadian, circalunar, and circannual oscillations—which keep time with environmental periodicities—as well as ultradian oscillations, ovarian cycles, and oscillations in development and in the brain, which keep time with biological timescales. These clocks mainly determine time points at specific phases of their oscillations. Hourglass clocks are predominantly found in development and aging and also in the brain. They determine time intervals (duration). More complex timing systems combine oscillatory and hourglass mechanisms, such as the case for cell cycle, sleep initiation, or brain clocks, whereas others combine external and internal periodicities (photoperiodism, seasonal reproduction). A definition of a *biological clock* may be derived from its control of functions external to its own processes and its use in determining temporal order (sequences of events) or durations. Biological and chemical oscillators are characterized by positive and negative feedback (or feedforward) mechanisms. During evolution, living organisms made use of the many existing oscillations for signal

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transmission, movement, and pump mechanisms, as well as for clocks. Some clocks, such as the circadian clock, that time with environmental periodicities are usually compensated (stabilized) against temperature, whereas other clocks, such as the cell cycle, that keep time with an organismic timescale are not compensated. This difference may be related to the predominance of negative feedback in the first class of clocks and a predominance of positive feedback (autocatalytic amplification) in the second class. The present knowledge of a compensated clock (the circadian oscillator) and an uncompensated clock (the cell cycle), as well as relevant models, are briefly reviewed. Hourglass clocks are based on linear or exponential unidirectional processes that trigger events mainly in the course of development and aging. An important hourglass mechanism within the aging process is the limitation of cell division capacity by the length of telomeres. The mechanism of this clock is briefly reviewed. In all clock mechanisms, thresholds at which "dependent variables" are triggered play an important role. (*Chronobiology International, 18(3), 329–369, 2001*)

Key Words: Aging; Biological clocks; Biological time-keeping; Cell cycle clocks; Circadian clocks; Hourglass clocks; Review; Telomere

BIOLOGICAL CLOCKS: AN OVERVIEW

The multiplicity and significance of timing mechanisms in living systems is becoming better appreciated. Timing may be established by oscillators, hourglass devices, or dominolike chains. Such oscillators, along with hourglass systems, are often called *biological clocks* in analogy to the functioning of mechanical/electrical clocks or other technical time-measuring devices in human societies. Generally, clocks determine time points, temporal sequences of events, and durations of time intervals. This clock metaphor has been frequently applied to various biological phenomena, especially to the basic rhythmic processes controlling circadian rhythms (1–5) and cell cycle events (6–8), ultradian (9,10), endogenous tidal (11), lunar (12–14), and annual (15,16) periodicities (Table 1). The ovarian cycle (17,18) also represents highly coordinated and recurrent sequences of events that are controlled by one or more clock system(s). In vertebrate development, furthermore, the sequential development and differentiation of somites are attributed to the functioning of an oscillatory clock (19); this is true also for numerous periodic spatial structures in fungi, plants, and animals (20).

The clock metaphor has also been extended to mechanisms that are based on nonperiodic phenomena (Table 2), such as the accumulation or degradation of substances that serve to determine the duration of time intervals and that can trigger processes at defined threshold concentrations (hourglass mechanisms). Such mechanisms are known to be involved in developmental timing in plants (27), in *Caenorhabditis elegans* [as determined by the concentrations of hetero-

Table 1. Biological Clocks: Oscillatory Clocks

Oscillator	Controlled Functions	Significance	Reference
Ultradian oscillators in microorganisms	Cell cycle, respiration, locomotor activity	Coordination (?), unknown	10
Ultradian oscillators in plants	Leaf movement	Unknown	21
Ultradian oscillators in vertebrates	Locomotor activity	Unknown	22
Circatidal, circalunidian oscillators in crabs	Locomotor activity	Anticipation of and adaptation to environmental changes	11, but see also 23
Oscillations in vertebrate embryo	Ca^{2+} content and other variables	Developmental decisions	24
Circadian oscillator in almost all organisms	Few to numerous	Anticipation of and adaptation to environmental changes, coordination	3, 4, 5
(Semi) circalunar oscillator in insects	Mating	Anticipation of and adaptation to environmental changes, coordination	12–14
Ovarian and neuroendocrine cycles in humans/mammals	Numerous	Coordination	17, 18
Circannual oscillator in plants and animals	Numerous; sexual development, flowering	Anticipation of and adaptation to environmental changes, coordination, periodic	15, 16, 25
Oscillating developmental gene activity in vertebrates	Somite development	Order in space	19, 26

chronic gene products (28–30)] in the vertebrate embryo (24), oligodendrocyte differentiation (31), and placental timing of gestation (32,33).

The *C. elegans* development in particular has been intensively analyzed, revealing inhibitory mechanisms in the control of heterochronic gene expression. The transition from the first to the second larval stage state requires the inhibitory action of a small RNA (lin-4 RNA), and the transition from late larval to adult cell state requires another inhibitory small RNA (let-7 RNA), which binds to sequences in the 3′ untranslated region of heterochronic genes (28,34). The temporally controlled small RNAs have recently been detected in a wide range of animal species, including vertebrate, ascidian, hemichordate, mollusc, annelid, and arthropod RNAs (35). However, the mechanisms underlying their temporal appearance are not yet clear.

The aging process itself is often considered as an accumulation of damage in DNA and other cellular structures, mainly due to the mitochondrial generation of reactive oxygen species (36–38), which eventually leads to a complete breakdown of cells—often by means of programmed cell death (apoptosis), and ulti-

Table 2. Biological Clocks: Hourglass Clocks

Timer	Controlled Functions	Significance	Reference
Unknown timer in plants	Length of juvenile state, flowering	Coordination, control of morphogenesis	27, 45
lin 14 and other gene products in *Caenorhabditis elegans*	Length of juvenile state, unequal cell divisions	Control of morphogenesis	28, 29
clk-1—product in *C. elegans*	Physiological rates	Developmental time, aging, oscillations	30
N/C-ratio (?), cyclin E/Cdk2 (?) in early vertebrate embryo	Midblastula transition	Developmental decisions	24
p27 (?) in mammalian cells	Onset of differentiation	Developmental decision	31
Mitochondrial/nuclear DNA damage in multicellular organisms	Aging, apoptosis, life span	Death of organism	37, 38, 46
Female biological clock in humans	Oocyte apoptosis	Functional life span of gonads (menopause)	47
Telomere length in multicellular organisms	Proliferation capacity	Death of organisms	39, 41
Feto-placental clock in humans	Duration of gestation	Initiation of birth	32, 33
Evolutionary (molecular) clock = mutation rate of organisms	Diversity	Generation of species, orders, etc.	42, 43, 48

mately to the death of the organism. This process is aided by a limited capacity of somatic cells to proliferate (39), a limitation set by the length of the telomeres and their shortening by each round of replication (40,41).

Different mutation rates in certain genes during evolution are taken as a measure for the time elapsed between a common ancestor and recent species (evolutionary or molecular clock; 42–44).

In more complex timing systems, several oscillatory and hourglass signals may act together to determine a "time point" or "duration," sometimes together with external (abiotic) signals (Table 3). In the human (mammalian) brain, several timing phenomena can be distinguished: the perception of simultaneity, successiveness, temporal order, subjective present, and duration (49), as well as the control of periodic processes such as breathing, movement, rapid eye movement (REM) sleep, circadian rhythms, and the like, and the duration of sleep, for example. Recent evidence suggests that two subcortical structures, the cerebellum and basal ganglia, play a critical role in both perception of temporal order and movement (50,51). It is, however, not yet clear whether oscillatory clocks, oscillation-counter devices, or multiple interval (hourglass) timers are responsible for the various timing processes.

Table 3. Biological Clocks: Multiple Clocks

Devices	Controlled Functions	Significance	Reference
Oscillations + hourglass (?), neuronal timer in the brain of mammals		Perception and control of sequential events, time interval estimation	49, 65
Circadian + hourglass in humans	Initiation of sleep	Coordination (?), recovery (?)	53, 54
Oscillator + hourglass (?) in the cell cycle of eukaryotes	Replication, mitosis	Coordination, directionality	7, 8, 56
Circadian clock(s) + light-dark cycle, "photoperiodism" in plants and animals	Reproduction, flowering	Adaptation to seasonal changes	57–60
Circadian clock(s) + different day length + annual period of low temperature + developmental timer in plants	Flowering	Adaptation to seasonal changes	25
Annual + lunar + tidal + circadian clocks or external influences in the grunion fish	Reproduction	Coordination of males and females for reproduction + adaptation to environmental changes	63, 64
Annual + lunar + circadian clocks or external factors in the Palolo worm	Reproduction	Coordination of gametes-bearing epitoc ends + adaptation to environmental changes	61, 62

Hippocampal GABA-ergic inhibitory interneurons play a major role in the generation of large-scale network γ-oscillations and function as a clock that dictates when principal cells fire during suprathreshold excitation (52). Sleep is initiated by both types of clocks, by an oscillatory (circadian) clock and a homeostatic (hourglass) mechanism (53–55). The cell cycle consists of oscillatory elements (particularly evident in cells of early embryos) and relaxation oscillators or hourglass mechanisms in adult growing cells (8; see below). An additional oscillator has been described in the G1 phase of yeast (56).

A complex clock system was developed in animals and plants that makes possible the determination of day lengths over the year (photoperiodism). Changes in day length need to exceed a species-specific critical length to trigger certain functions, such as mammalian reproduction (57), insect diapause (58), or plant flowering (59,60). The underlying mechanism consists of a circadian clock that controls phase-dependent light sensitivity: Only when the duration of the daily light span is long (or short) enough to interact with (or avoid) this phase

can the organism react to it by inducing the respective function. Photoperiodism is thus based on the coincidence of a light signal with a certain clock phase, which determines a certain time point, not the actual duration of the day length. Photoperiodic mechanisms may be combined with external annual signals, such as low temperature in the winter, and developmental (hourglass) timers (25).

Even more complex is the control of swarming of epitoque ends of the Palolo worm (containing eggs and sperm) at a well-defined time of the year, the lunar month, and the day (61,62). The same applies, with the inclusion of a tidal signal, to the timing of egg laying and fertilization in the grunion fish (63,64). The various periodicities (for which the endogenous nature has not always been demonstrated) act together with developmental hourglass timers to trigger temporally coordinated actions very much the same way as calendars and clocks function in coordinating the activity in factories and human societies.

The term *clock* was introduced to characterize a mechanism that times (or perceives in the case of brain clocks) the temporal order or duration of one or many events (66); that is, the clock mechanism not only self-organizes its own processes, but also coordinates the sequence or duration of other (dependent) processes by means of signals. Several authors restrict the use of the term clock to oscillating devices and distinguish them from hourglass mechanisms. However, in view of the common usage of the term clock for both mechanisms (hourglass = "Sanduhr" in German, for example) and their involvement in timing mechanisms, we also included hourglass processes.

We begin this review by briefly discussing the definitions of terms such as timing, time, clock, rhythm, and cycle as found in dictionaries and their relevance for biological timing. We then focus on oscillatory clocks, on the principal mechanisms of oscillations, and on their significance for the organism.

Among the numerous oscillatory clocks, we selected the circadian and the cell cycle clock for close analysis. These two clocks are well analyzed with respect to their underlying mechanisms, their phase or frequency control, and triggering signals. They differ considerably in their purpose and properties. The circadian clock controls the rhythmicity of a few (in fungi) up to many hundreds (in mammals) of dependent variables and thus generates a temporal order of events in synchrony with and anticipation of the recurrent environmental day-night cycle. To this end, the clock mechanism is phase modulated by periodic environmental signals such as light and temperature, but is insensitive to different constant temperatures (temperature compensation) and several other parameters.

The cell cycle clock, on the other hand, triggers the temporal sequence of cell cycle events such as DNA synthesis and mitosis. Its period length, however, strongly depends on external factors such as temperature, nutrition, and growth factors. This is necessary to allow the sequence of events to occur only when essential requirements are met. The cell cycle clock may include hourglass mechanisms such as certain threshold values (cell size, attachment of kinetochores to microtubuli), as well as oscillatory devices (7,56) and dominolike mechanisms by which the completion of one step triggers the next one. The cell cycle clock(s)

is not, or is only indirectly, designed to keep time with environmental periodicities; rather, it is designed to time a sequence of events in relation to a developmental timescale.

For both (circadian and cell cycle) clock types, several models have been developed to simulate some of their main properties and underlying mechanisms, such as various feedback systems. Some of these models are briefly summarized here with the aim of highlighting the essential features of the two clock systems.

Hourglass clocks, on the other hand, mainly determine intervals of time (duration). Their usage and significance also evolved from the group of unidirectional (linear or exponential) processes.

In both oscillatory and hourglass clocks, events not belonging to the clock processes themselves are triggered by signals or actions of the clock. This triggering often occurs at defined threshold concentrations of the oscillatory or hourglass process, which thus determines the timing of events at a certain time point (phase of oscillator) or after a certain time interval (duration).

As an example of an hourglass clock, we chose the telomere-dependent restriction of the number of cycles in a somatic cell, also called the "Hayflick" limit after the author of many earlier experiments (39). The role of decreasing telomere length in determining the life span of cells has recently received increasing basic and applied interest and is subject to intensive analysis (41,67).

DEFINITIONS

Timing

The term *Timing* is the focus of this article. The meaning of this term is (1) "to arrange or set the time for (an event or occasion): schedule"; (2) "to adjust (a time piece) to keep accurate time"; (3) "to adjust or control so that movements or events occur in proper sequence"; (4) "to set, maintain or record the pace or duration of," (5) "to cause to keep time with something" (68,69). All these definitions can be applied to the functioning of biological clocks: Biological clocks generally control the succession of events (triggering them at certain time points). Biological clocks are often regulated to keep correct time, for example, by light and temperature signals in the case of circadian clocks. Biological clocks may control the speed and duration of dependent variables, for example, the rate of enzyme or gene activities (30). Biological clocks eventually keep time with the solar day or with the development of an organism.

Time is (together with the three dimensions in space) a dimension about a vast amount of literature exists in the fields of philosophy, physics, astronomy, biology, psychology, and others. Among the different meanings of this term listed in dictionaries, two are particularly relevant to the discussed issues: (1) "the measured or measurable period during which an action, process or condition exists or continues: *duration*"; and (2) "the point or period when something occurs: *occasion*" (68). The latter *time point* may be defined by a "moment, hour,

day or year as indicated by a clock or a calendar" (68). A *time point* is a mathematical construct analogous to a point in Euclidean geometry, that is, strictly speaking, it has no duration, whereas a *time span* (duration) or interval is analogous to a line segment.

The sequence of events (or system changes), on the other hand, creates the perception of time (or time itself). Astronomical, mechanical, biological, psychological, and historical changes can be used to deduce a timescale for the temporal ordering of events. The respective times are then called astronomical time, mechanical time, biological time, and so on. Even though the standard units of time were derived first from astronomical time (year, day, hour, minute, second, etc.) and were later based on atomic oscillations, these time units are ubiquitously used in science in general and in biology in particular. However, it may be useful in biology also to refer to biological time, for example, to the number of cell cycles between certain developmental events or to the degree of mitochondrial DNA damage relative to the aging process.

Clock

The term *clock* (late Latin *clocca* = bell) is a "device for indicating or measuring time by means of hands moving on a dial" (68). Another device is the *hourglass*, "an instrument for measuring time" (68). Both devices can mark an event (trigger an alarm or ringing of a bell), that is, determine a time point either at a certain time of day or after a certain length of time. Both can also be used to measure the *duration*: The hands of the clock indicate an interval by two different positions in their circular movement; the hourglass indicates an interval by reaching a certain quantity (of sand, water, number of oscillations).

It is important to note that the definition of a biological clock relies on its analogy to human clocks and their purposes, that is, that the organism "uses" an oscillation or a unidirectional process as a clock. It is also important to emphasize that oscillatory clocks often do not "measure time" (duration), but rather determine a time point at which an event is to occur. This is often misunderstood.

Rhythm, Periodicity, Oscillation, and Cycle

For periodic processes in biological systems, the terms *rhythm*, *periodicity*, *oscillation*, and *cycle* have been used more or less synonymously; however, there has been some preferences in the use of terms for different phenomena. The terms *rhythm* or *perodicity* are usually applied to circadian, tidal, lunar, or annual changes and to heart beat, breathing, body movements (swimming, running, flying), muscle contractions, ciliar beats, leaf movements, and so on. The term *oscillation* (or *wave*) is used more often in the context of electrical waves in the brain or action potentials of neurons. The term *oscillator* has been used in refer-

ence to a primarily rhythmic system—the circadian oscillator, for example. *Cycle* is used in the context of cell cycle, reproduction cycle, ovarian cycle, seasonal cycle, population cycle. These slightly differing applications of the terms may be attributed to a slightly different meaning that takes into account the constancy of the period length. The term *oscillation* is applied to periodic changes of a rather exact period length, which seems to be true also for *rhythm* and *periodicity*, but less so for the term *cycle*. The cell cycle is an example of a regular recurrent sequence of events, however, with largely varying period lengths. The terms *fluctuation* and *variation* are used for rhythmic, as well as for stochastic, changes and may thus be misleading.

OSCILLATORY CLOCKS

General Mechanisms of Oscillators

On the basis of the fundamental work of Nicolis and Prigogine (70) and Noyes (71), our understanding of the thermodynamic and kinetic requirements of chemical oscillatory processes has increased enormously. Today, the mechanisms of many chemical oscillatory reactions are well understood (72). No matter how complex a physiological or chemical system may be, it is now generally agreed that such a system can be understood in terms of the underlying elementary processes and their interactions. Elementary processes occur in simple steps and involve in general two (in rare cases, three) reactants or products (71).

When can we expect that a certain set of chemical or physiological processes will show oscillations? Franck (73) noticed the interesting fact that all known physicochemical oscillators exhibit positive and negative feedback simultaneously, a situation Franck described as "antagonistic feedback" (Fig. 1). The positive feedback is the destabilizing element that drives the system from its steady state. The negative feedback is opposed to destabilization and directs the system back to its steady state. As shown in Fig. 1, the positive destabilizing element in the antagonistic feedback has two possible states (positive backward and negative forward steps) and also two states for the negative component, leading to the four (I–IV) possibilities. Suitable rate constants and timing between processes within the antagonistic feedback provide sufficient conditions for oscillations to occur and also to determine the period length of the oscillator. In most chemical oscillators, as in the Belousov-Zhabotinsky reaction (74), the destabilizing positive feedback is due to autocatalysis, which produces an explosionlike increase in one of the reaction intermediates. As discussed below, autocatalysis is important, for example, to cell cycle oscillators and to many other biological oscillators.

Due to the positive and negative feedbacks, chemical or physiological oscillators contain, contrary to the hourglass mechanisms, stabilization elements as an integral part of the oscillator; these are able to provide homeostasis of the period against environmental influences like temperature (75; see also the section

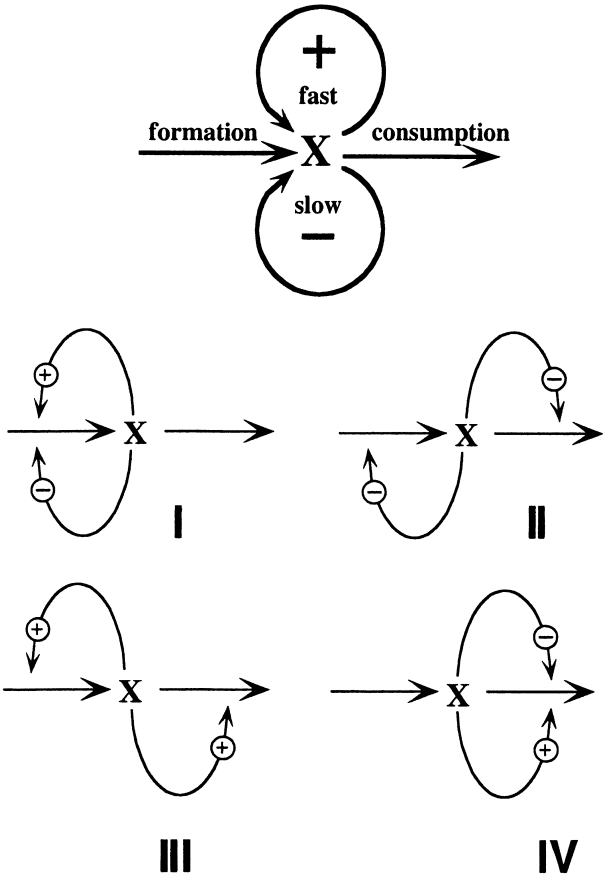


Figure 1. Antagonistic feedback, that is the simultaneous (?) action of positive and negative feedback on a given variable *X* as a necessary condition to obtain chemical and physiological oscillations. The four schemes represent the possibilities of how positive and negative feedback may be realized by backward and forward activation/inhibition. (After Ref. 73.)

below, Modeling of Circadian Clocks) and pH (76) in circadian rhythms, for example. In hourglass clocks, however, only one reaction (the rate-determining one; see General Mechanisms of Hourglass Processes, below) determines the time interval between certain events; therefore, hourglass clocks (like most chemical processes) are generally not compensated against changes in temperature or other factors.

Significance of Oscillations for Organisms

A vast number of oscillations of different frequencies exist in living organisms, as reviewed previously in part by Sollberger (77), Rensing (78), and Ed-

munds (3). In contrast to chemical oscillations, biological oscillations can be classified according to their significance to the living organism (Fig. 2):

- no or unknown significance
- signal transduction and processing
- transport and movement
- clocks related to environmental timescales
- clocks related to organismic timescales

The first class, no or unknown significance, comprises the glycolytic oscillator (79), the pathologic oscillatory states such as Parkinson tremor (80), neutropenia (81), malaria attacks (82), depressive states (83), and prey-predator periodicities (84), for example.

The second class of oscillators is involved in the transport, pumping, or movement of materials within the organism or cell or the movement of organisms and cells themselves. The principle underlying most transport, pump, or movement processes is a contraction-relaxation cycle of muscle cells based on high-frequency actin-myosin interactions during contraction (85). This contraction-relaxation cycle can travel as a wave along the digestive tract, the urinary tract, arteries, and other tubular structures and thus transport the material inside. Other

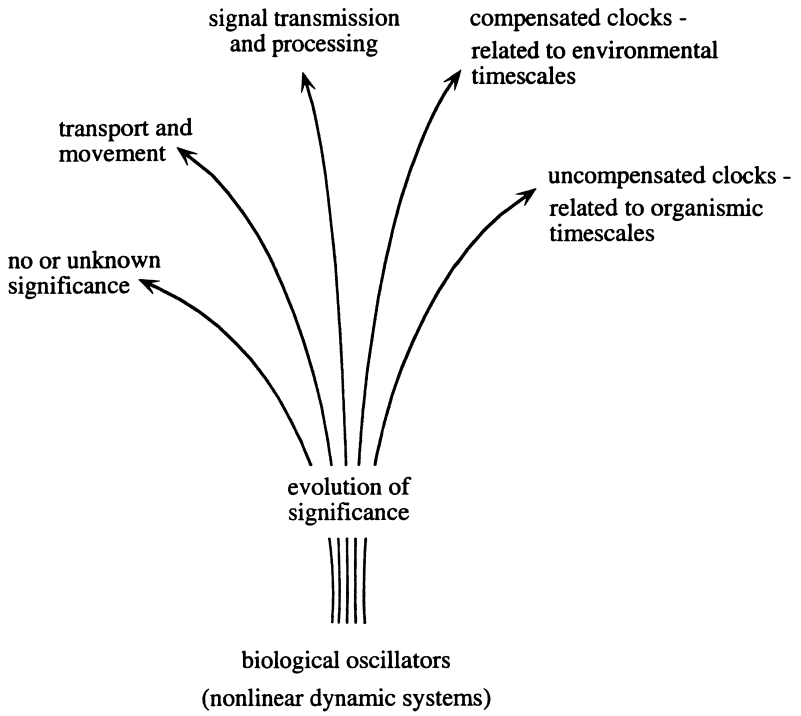


Figure 2. Evolution of timing mechanisms (clocks) and other significant functions in oscillatory systems. For details, see text.

contraction cycles of tubular systems occur as stationary processes and work like a pump, in the heart, respiratory organs, or medusas, for example, which pump blood, air, and water, respectively.

The movement of animals in space is generally based on rhythmic movements of legs, wings, or fins or on rhythmic movements of the whole body, for example, in nematodes, insect larvae, fish, and snakes. Similar mechanisms of movement are observed at the cellular level. In *Physarum*, a rhythmic flow of the cytoplasm is generated by actin-myosin contraction waves (86,87). A water pump (contractile vacuole) works in many ciliates (88), and movement of cells can be achieved by rhythmic beating of cilia or flagella(e).

The third class comprises frequency-coded signal transductions along excitable membranes and within cells. In animals, waves of action potentials are observed in the membranes of sense, nerve, and muscle cells (excitable systems), which consist of high-frequency changes of the membrane potential progressing in one direction. The different frequencies (or bursts with different intervals) transmit information about graded inputs (85). Synchronous oscillations of various frequencies in the brain have been associated with cognition (encoding of new information), as well as with search and retrieval processes in long-term memory (89–91), whereas pathologic synchronization leads to seizures (92). Within cells, calcium waves are observed after a hormonal stimulus that may equally serve a frequency-coded signal transmission system (93). Periodic signaling is also observed, for example, between *Dictyostelium* using propagating waves of cAMP, in pulsatile hormones (gonadotropin-releasing hormone, growth hormone) (94,95), or in fireflies, which use intermittent light flashes (96).

The fourth and fifth classes of endogenous biological oscillators are termed *clocks* (see above; Table 1; Fig. 2). We propose that "compensated" and "uncompensated" clocks be distinguished. The first group is designed to keep time with environmental periodicities and needs to be compensated for temperature and other variables, whereas the second group keeps time with biological timescales, such as developmental steps that need not be stabilized against environmental influences. In case of the endogenous circadian compensated clocks, the adaptive value resides in the synchronization of a sequence of processes with reference to the recurrent 24h day-night changes. This allows the anticipatory adaptation of the organism to predictable future changes in the environment. The body temperature in humans, for example, rises before dawn, that is, before the actual environmental change occurs, and thus allows the organism to be exactly "on time" with respect to its physiological adaptation. In addition, the circadian clock probably serves to coordinate a sequential order of processes within a cell and an organism. Apart from these coordinating functions for internal processes and synchronization with external changes, the circadian clock also coordinates interactions between individuals of the same (or different) species, for example, the courtship behavior of *Drosophila* males and mating (97).

Coordinative functions are also generated by uncompensated biological clocks such as the cell cycle or ovarian cycle. This class of clocks controls pro-

cesses related to an organismic time that is dependent on internal and external conditions. In both classes, the main functional principle of oscillatory clocks is the determination of time points (not time intervals). Oscillatory clocks trigger certain processes at defined phases of the oscillator, independent of the duration of time elapsed beforehand.

Preliminary Definition of an Oscillatory Clock

How can one distinguish an oscillatory system that has been termed a circadian clock or cell cycle clock from another oscillatory system, such as leg movement? Constant frequency, temperature compensation, or the generation of temporal order are not specific criteria for clocks. A possible distinction of an oscillatory clock may be that it times events that are "external" to the clock system by means of signal pathways or other actions, and that the organism "uses" the oscillator (clock) to create a higher degree of temporal order. Clocks, by definition, should produce output signals, but they often also receive input signals to set the phase or frequency appropriately. A dominolike chain of events is not considered a clock because each event directly causes the next one.

Circadian Clocks

Endogenous rhythms with a period length of about 24h are called *circadian* rhythms (98,99; Latin *circa* = about, *dies* = day). They are observable also under constant environmental conditions and usually deviate from a period length of exactly 24h. Apart from the endogenous 24h rhythms, rhythmic changes can be induced in organisms by the daily changes of light and dark or high and low temperatures. These rhythmicities and those of uncertain origin (exogenous or endogenous) are usually termed *daily* rhythmicities or *24h periodicities*. The distinction between circadian (= endogenous) and daily rhythm (= exogenous or uncertain origin) has a long and controversial history, and the terms are often used synonymously in the literature. Yet, efforts should be made to maintain or strengthen this distinction.

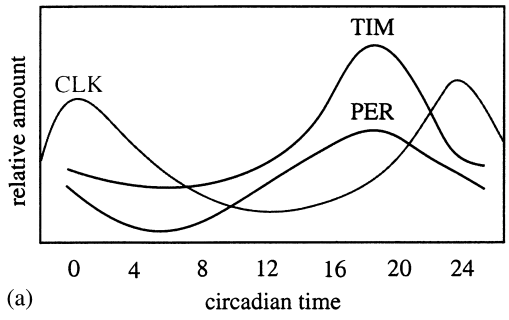
In the last decades, the intracellular pacemaker(s) that drives the numerous circadian rhythms is more commonly referred to as the *circadian clock* (1–3,100). This clock metaphor suggests that the oscillation has evolved to function as a clock (see definition and significance of clocks above). The functions of the circadian clock require that the clock mechanism can be reset (or synchronized) by means of external signals ("zeitgeber") and that it be able to produce internal signals to time the numerous driven processes ("hands"). Since circadian clocks act to synchronize organismic processes with the daily astrophysical changes, they also developed a mechanism that makes the clock rather independent of environmental temperature conditions (temperature compensation), although the

molecular mechanisms of such compensations are still unclear (76,101,102). The clock mechanism has recently been unraveled, at least partly, in organisms as diverse as a cyanobacterium (*Synechococcus*) (103,104), a fungus (*Neurospora*) (4,105), an insect (*Drosophila*) (106), and a mammal (mouse) (107–109). This does not exclude a more complex structure of the mechanisms as those presently known. The successful analysis of the basic circadian clock mechanisms in these four organisms, particularly during the last decade, has been reviewed frequently and is subject to intensive investigations. We refer here mainly to two most recent reviews that summarize the main features of the circadian clocks as revealed by the study of these four organisms (4,5).

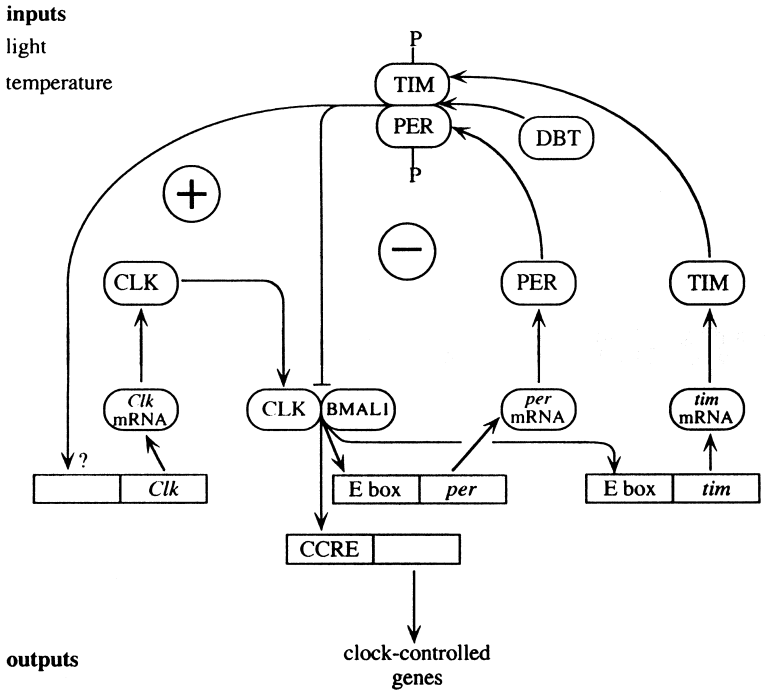
In short, the clock mechanisms of these organisms consist of circadian oscillation(s) in the expression of a few clock genes. The products of the clock genes, that is, clock mRNA(s) and clock protein(s), undergo circadian concentration changes, as demonstrated for example by the *Drosophila* clock (Fig. 3a). The rising limb of the PER and TIM oscillations is brought about by positively acting transcription factors (CLK and BMAL1 = CYC), which often act as dimers (by means of PAS domains), which bind to E boxes in clock gene promoters and initiate the clock gene expression. The falling limb is caused by the clock protein(s) themselves, which act as inhibitors of their own gene activity by a negative feedback action on CLK/BMAL1 (Fig. 3b). This inhibitory action of the clock proteins PER and TIM is then relieved by their degradation, which allows a new round of clock gene activation. Phosphorylation of the clock proteins PER and TIM by kinase(s), for example, by double time (DBT), plays an important role in the nuclear translocation, as well as in the degradation dynamics. In *Drosophila*, a dimerization of the two clock proteins and threshold concentrations in the cytoplasm are also important for the oscillation (for a review, see Ref. 106). Whether this basic mechanism contains thresholds of concentrations (for example, for the inhibitory action of the clock protein) that need to be reached before the next process can be initiated is suggestive, but not proven. A positive feedback of PER/TIM may be created by its stimulating activity on the positively acting *clk* gene (110) (Fig. 3b). Such positive loops have been described also in the *Neurospora* clock.

In *Neurospora*, *Drosophila*, and the mouse, the rising and falling portions of the clock mRNA(s) and clock protein(s) are roughly symmetrically distributed (lasting about 12h each); this is evidence against the existence of a relaxation-type oscillator. However, it does not exclude threshold phenomena. A change in FRQ degradation by mutation, therefore, may lead to stronger changes in the frequency as compared to changes in the synthesis rate (see model below). Frequency changes of the circadian clocks, however, generally are small, much smaller than those of the heart (111) or of the cell cycle (see below).

The phase-shifting effects of light pulses are roughly symmetrically distributed between advancing and delaying portions of the phase-response curve (PRC), which allows symmetric phase control, that is, adjustment of the endogenous phase to the environmental periodicity by advance, as well as delay, phase



(a)



(b)

Figure 3. The circadian clock of *Drosophila*. (a) Oscillatory clock elements: PER and TIM proteins reach their maxima in the middle of the subjective night about 4h after the maximum of their mRNA species (not shown). The CLK maximum is observed at about 0h circadian time (beginning of the subjective day) at the same time as its mRNA (not shown). BMAL1 seems not to oscillate. (After Ref. 4.) (b) Basic structure of the oscillator: CLK (Clock protein) and BMAL1 (or Cycle protein) form a heterodimer and act positively on the *period* (*per*) and *timless* (*tim*) genes (and on clock-controlled genes, ccgs). PER and TIM proteins also form a heterodimer that is phosphorylated, for example, by Double time (DBT) and is subsequently transported into the nucleus, in which it inhibits CLK/BMAL1. These interactions form a negative feedback loop (–). PER/TIM, furthermore, seem to act positively on the *clk* gene and thus form a positive feedback loop (+).

shifts (see Ref. 112). The effects of light may be mediated by induction of clock gene transcription (*Neurospora*) or by enhancing clock protein (TIM) degradation (113) and are mediated in part by the highly conserved cryptochrome (114,115).

The earmarking of clock proteins for degradation and thus the degradation rate seem to depend on clock protein phosphorylation. External signals—light, temperature, hormones (?)—may therefore transmit their phase-shifting effects by acting on the activity of kinases (casein kinase I, see Ref. 116; *double time* in *Drosophila*, see Ref. 117), phosphatases, as well as on other signal pathways (118).

The clock function (i.e., the triggering) of various secondary events is not clearly known. In *Neurospora*, several genes are rhythmically activated (CCG, circadian controlled genes), which among other processes, causes the circadian rhythm of conidiation (119). It is assumed that these genes are activated by the same mechanism as the clock gene(s). In multicellular animal organisms, pacemaker oscillators are found in the brain (insects, see Ref. 120; mammals, see Ref. 121) and eyes (122). In the pacemaker region of the mammalian brain (SCN, suprachiasmatic nucleus), the clock mechanism gives off signals (123) and subsequently influences many functions—such as the sleep-wake cycle—and, interestingly, the periodic activity of clock genes in various tissues (124). Both hormonal and neuronal signal-transmitting systems act to coordinate the large number of physiological variables (125).

In conclusion, the mechanism of the circadian clock is rather stable with respect to effects on its frequency, particularly regarding the influence of temperature. The stability seems to be based on a strong negative feedback and minor positive feedback loops. This is useful for a clock designed to function at the same frequency as the daily environmental changes. On the other hand, the circadian clock needs to be synchronized with these external changes, a need that is accomplished by strong differential effects of light and temperature signals on clock processes that lead to synchronizing phase shifts (phase control).

Modeling of Circadian Clocks

The goal of models is to understand the oscillatory physiology in terms of chemical component processes. Especially, reaction kinetic models can provide quantitative descriptions of the individual components of the oscillator and provide predictions of still unknown aspects. In this way, kinetic models are useful in the design of further experiments.

The Goodwin Model

The use of model organisms like *Neurospora* and *Drosophila* and their clock mutants in combination with molecular analyses made it possible to gain

insights into the essential mechanistic elements of circadian rhythms. The basic element of the circadian pacemaker in *Neurospora*, *Drosophila*, mammals, and cyanobacteria (4) is a negative feedback regulation of certain clock genes, by which the corresponding proteins inhibit their own transcription or translation. Interestingly, more than 30 years ago, Goodwin (126–128) proposed a simple three-dimensional model that contains this element of the circadian clock. The Goodwin model (Fig. 4a) is a *minimal model* because it contains only the most

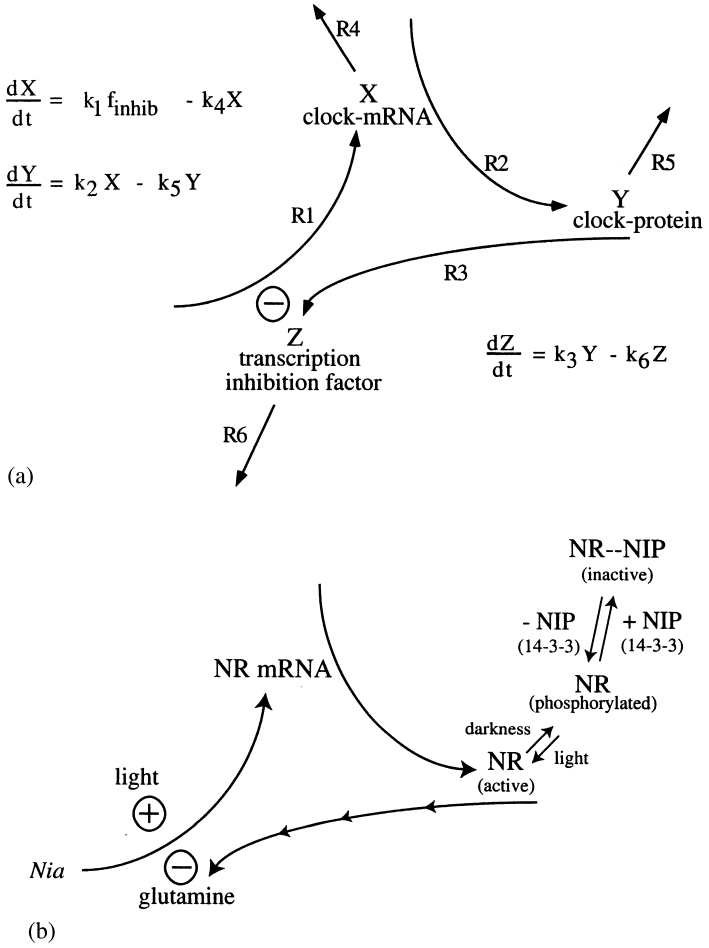


Figure 4. (a) The Goodwin model. Reaction R1 is the formation of clock mRNA *X*; reaction R2 is the synthesis of clock protein *Y*, and R3 is the production of a transcription inhibitor *Z*. R4, R5, and R6 represent degradation reactions. The inhibition factor is given by $f_{\text{inhib}} = 1/(1 + Z^9)$. In case of threshold inhibition (see discussion of the Goodwin model in text), $f_{\text{inhib}} = 0$ when $Z < Z_{\max}$, while $f_{\text{inhib}} = 1$ when $Z \leq Z_{\min}$. (b) Negative feedback in the nitrate reductase (NR) control of higher plants as a putative circadian oscillator. The assumed repressor is glutamine, which is a product of active NR. However, little is known about the mechanisms of inhibition. Darkness induces phosphorylation of NR, which then can bind a nitrate reductase inhibition protein (NIP) of the 14-3-3 protein family, leading to an inactive NR-NIP protein complex. (From Ref. 181.)

essential features, while details concerning other feedback regulation and protein-protein interactions are not included.

However, it may be instructive to review briefly some features of the Goodwin model. The three intermediates in the Goodwin model are the clock mRNA *X*, the clock protein *Y*, and the inhibitory factor *Z*, leading to a set of three coupled ordinary differential equations that are solved numerically. It might be interesting to note that the clock protein *Y* can be considered either as an enzyme that catalyzes the production of inhibitor *Z* or as a noncatalytic protein that serves as a precursor for *Z*. In fact, both possibilities are possible. A good example for a catalytic "clock protein" is the nitrate reductase (NR) enzyme in the higher plant (NR is a multidomain enzyme containing heme, flavin, and a molybdenum pterin as cofactors). NR exhibits light-induced circadian rhythms (129), and the inhibitor of the *nia* transcription (the gene coding for the NR apoprotein) is considered to be glutamine (130), which is an end product of the nitrate assimilation pathway (Fig. 4b). In this pathway, NR catalyzes the rate-determining step, that is, the reduction of nitrate to nitrite. In case NR is catalytically inactive (which can be accomplished by feeding plants with tungstate instead of molybdenum salts), no rhythm is observed, and *nia*-mRNA remains at a high steady-state level (for review, see Ref. 131). Examples for which the clock protein *Y* serves as a precursor for the inhibitory factor *Z* are found in *Neurospora*, *Drosophila*, and mammals (Ref. 4; see also Fig. 3).

Interesting are the predictions of the Goodwin model concerning the circadian period length and its relationship with the clock protein's stability (degradation rate). The Goodwin model predicts an increased circadian period in case the clock protein becomes more stable, that is, when it is less easily degraded compared to the wild type. In addition, an increase in activation energy leads also to a loss of temperature compensation, as observed in the long period *Neurospora crassa* mutant frq^7 (132). This predicted relationship between period length and clock protein stability has recently been tested for *Neurospora.* Liu et al. (133) mutated several phosphorylation sites in FRQ. At one site (Ser 513), this led to a dramatic reduction in the rate of FRQ degradation and, as expected, to a very long period (≈31h).

Threshold Type of Inhibition in the Goodwin Model

The inhibition factor $1/(1 + Z^9)$ of the original Goodwin model (Fig. 4a) is often criticized because of its unrealistic high exponent (Hill coefficient). The reason for this large exponent is related to the low number of intermediates in the model. Once the number of intermediates is increased, the exponent in the inhibition factor can be decreased, and realistic values are obtained. The large Hill coefficient is simply the price one pays when dealing with minimal models.

A consequence of the Hill-type inhibition is a soft resetting behavior of the oscillator; the oscillator exhibits many transients (changes in the period) after a

perturbation (101). The occurrence of soft resetting in the Goodwin model is very similar to the circadian resetting behavior observed for *Drosophila* (134) and humans (135). On the other hand, this (soft) resetting behavior is fundamentally different from that observed in *Neurospora,* for which transients have not been found (136). To model the hard *Neurospora* resetting, the observed phase-response curves and the kinetics of the *frq*-mRNA and FRQ-protein time curves, Ruoff et al. (137) introduced a threshold-type inhibition as an alternative to the $1/(Z + Z^9)$ factor. In the case of threshold inhibition, inhibition of transcription occurs only after a certain upper concentration threshold Z_{max} has been exceeded. Beyond that, Z decreases eventually, and transcription can start again when Z falls below a lower threshold Z_{min} (137,138). As a result, relaxation oscillations are obtained with a hard (immediate) resetting, which describes *Neurospora's* light phase-response curves very well (138).

Why are some resettings, like that of *Drosophila*, soft, while in *Neurospora* is it hard—[which does not correspond to the difference between type 1 and type 0 phase responses as discussed by Winfree (139)]? The reason for these differences appears to be related to the advantages an organism may gain when adaptation to environmental changes is achieved in one step (1 day) or bit by bit over several days. It is presently unknown if a clear difference exists between organisms that reset hard and those that have soft resetting. Viewing organisms from the point of their complexity, one might expect soft resetting in more complex organisms because they might need more time for their adaptation processes to environmental changes. On the other hand, this does not exclude the possibility that also less complex organisms show soft resetting, and one may wonder whether soft resetting can also be observed in *Neurospora* under certain conditions. The advantage of soft (or even chaotic) resetting may lie in a greater "adaptiveness" of organisms or organs to environmental changes.

Other Models

Several other reaction kinetic models have been proposed to improve the performance of the simple Goodwin model. These models have mostly been based on the *Drosophila* circadian rhythm.

Olde-Scheper at al. (140) were able to reduce the Hill coefficient from 9 to 2 by considering a two-variable model with an increased reaction order of the protein synthesis process. This favors the positive element of transcription, but the kinetic implications of such an increase in reaction order have not been investigated.

Rather detailed models have recently been proposed by Hong and Tyson (141), Leloup and Goldbeter (142), and Gonze et al. (143). These models are of considerable complexity, and the number of adjustable parameters (rate constants) is rather large. The Goldbeter model and its modifications (143) increase the number of clock intermediates by considering explicitly two phosphorylated

forms of the clock protein PER. This allows, as mentioned above, a decrease of the Hill coefficient in the inhibition term to more realistic values. The Hong and Tyson (141) model stresses the importance of PER-PER dimerization in the Goldbeter model to achieve temperature compensation.

However, using models with a large number of adjustable parameters is problematic: Although such models in general better fit experimental data, the danger is that their dynamic structure may not reflect the important processes within the biological oscillator. Recently, Tyson et al. (144) proposed a minimal two-equation model for the *Drosophila* circadian rhythm; this model is based on dimerization and proteolysis of PER and TIM and has the advantage that it can be easily analyzed and provides interesting predictions. The model exhibits a remarkable insensitivity of the oscillatory period to certain crucial parameters, that is, it can exhibit temperature compensation, and its dynamic behavior is consistent with the phenotypes of mutations at the *per*, *tim*, and *dbt* loci. Another interesting aspect of this model is that it predicts hysteresis between clock protein synthesis and degradation as a function of light intensity. In general, predictions are an important aspect of all models and play an essential part in the design of new experiments to verify or refute theories and models.

Modeling Temperature Compensation

The important property of temperature compensation (see, for example, the *Chronobiology International* special issue, Ref. 75) ensures that oscillators become chronometers such that their period is compensated toward environmental temperature changes. Since most chemical and biochemical reactions increase the reaction rate by a factor of two or three when temperature is increased by 10°C, certain compensating mechanisms must be operative to ensure the same period. A recent theory (145) of temperature compensation points toward the existence of an activation energy weight balanced between period-increasing and period-decreasing component processes. This has been applied in the Goodwin model (132) and in the two-equation model by Tyson et al. (144). A similar behavior to obtain temperature compensation was also observed by Leloup and Goldbeter (142) in their *Drosophila* model. A first example of a temperature-compensated chemical oscillator has recently been reported (146). It will be interesting to see the above theory tested in both chemical and physiological oscillators.

Cell Cycle Clocks

The cell cycle consists of DNA replication (S phase) and separation of the replicated chromosomes into two new nuclei (mitosis, M phase) and daughter cells (cytokinesis). Preparatory and control processes take place mainly at the

end of G1 and G2 phases, that is, before entry into the S and the M phase, respectively, but also during mitosis (8,147). The control processes recognize the completion of preparatory steps (for example, amount and activity of phase-specific proteins, size of the cell, absence of DNA damage, completion of replication, exact binding of chromosomes to spindle fibers) and allow or disallow the transition to the next step. These control processes are called *checkpoints* (for review, see Ref. 148).

Cyclins and cyclin-dependent kinases (Cdks) play a decisive role in most checkpoints. Cdks program and trigger the unidirectional sequence of events of cell cycle progression by means of oscillatory changes of their activity (for review, see Ref. 7). Higher eukaryotes show phase-specific cyclins and Cdks. The G1 cyclin D1 associates with Cdks 4 and 6, increases during G1, and is strongly dependent on signals such as growth factors (Fig. 5a). The G1 cyclin E associates with Cdk2 and increases drastically, due to autocatalytic processes, at the G1 checkpoint and then decreases by means of degradation. The S cyclin A combines with Cdk2, increases slowly during the S and G2 phases, and is degraded quickly during M. Finally, G2/M cyclin B combines with Cdk1 (= Cdc2), increases during S and G2 phases, and is also degraded quickly during the M phase. The Cdk activity seems to follow relaxation kinetics, particularly in the course of G2 and M. In G1 and G2, the increasing Cdk activity apparently needs to reach critical thresholds during the checkpoints in G1 and G2/M. Cdk activity is strongly determined by the amount of the phase-specific cyclins, that is, the regulatory subunits of Cdks.

Control Mechanisms at the G1 Checkpoint

One of the most important checkpoints is located at the G1/S border. This checkpoint is rather well known and may serve as an example for the feedback mechanisms involved in the cell cycle. One of the main clock functions (output signals) of the active G1 Cdks is to trigger DNA synthesis; this is achieved by phosphorylation of proteins in the prereplication complex (149). It is also achieved by phosphorylation of the retinoblastoma protein (pRb), which then releases the transcription factors (E2F, DP), which in turn activate S-phase-specific genes (150). These S-phase genes also comprise genes for cyclin E and E2F, which when activated, form a positive feedback for the cyclin E/Cdk2 complex (Fig. 5b).

The cyclin concentrations, including cyclin E, are in part controlled at the level of degradation (151), which is brought about by activating cyclin degradation through Cdk activity, thus forming a negative feedback or positive feedforward loop (Figs. 5b and 6). In addition, phase-specific phosphorylation changes of the Cdks play significant regulatory roles (7). Inhibitory phosphorylation of Cdk2 may be relieved by a phosphatase (Cdc25) with activity that seems to respond to internal and external signals and Cdk2 activity, thus forming a posi-

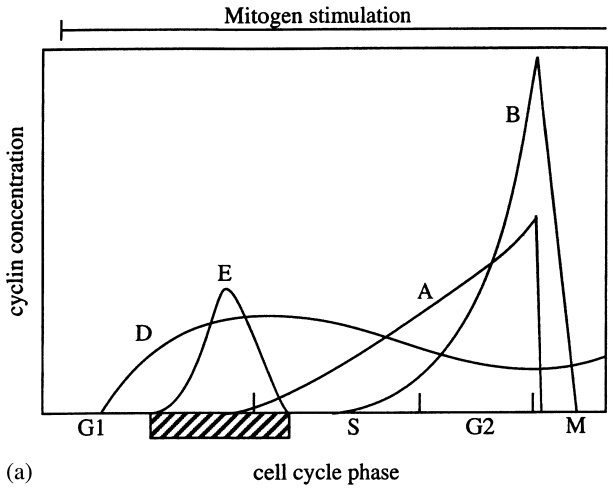


Figure 5. The cell cycle clock in higher eukaryotes. (a) Oscillatory (and hourglass?) clock elements: cyclin concentrations during mitogenic stimulation in the course of subsequent cell cycle phases; D, E, A, and B are different cyclins; G1, S, G2, and M are cell cycle phases; the hatched bar is the checkpoint at the G1/S transition, and its structure is illustrated in Fig. 5b. (After Ref. 6.) (b) Basic structure of the processes at the G1/S checkpoint. The cyclin E–dependent Cdk2 activates itself by positive feedback loops ⊕. One positive loop consists of phosphorylation of the retinoblastoma protein (pRb) by cyclin E/Cdk2. After phosphorylation of pRb, transcription factors E2F/DP are released and activate the transcription (Txn) of genes involved in S phase processes. These genes include the genes for cyclin E and E2F. The second positive loop consists of the phosporylation of an inhibitor (I) by Cdk2 and the subsequent degradation of I; the third positive loop may reside in the phosporylation and activation of a phosphatase that then removes an inhibitory phosphate group from Cdk2 (not shown). The negative feedback loop (–) consists of an autophosphorylation of cyclin E and its subsequent degradation (Deg). Another output signal is the phosphorylation of the prereplication complex proteins at the origin (ori) of replicons. TGFβ, transforming growth factor β.

tive loop (Figs. 5b, 6). A third regulatory level in the control of Cdk activity involves the inhibitory proteins of the Cip/Kip (p21, p27, p57) family (152). Their concentration is dependent on synthesis and degradation, which are both influenced by internal and external factors. The degradation of inhibitors (I) is also activated by the cyclin E/Cdk2 complex, thus forming another positive loop (Fig. 5b). These inhibitors mainly act on G1 Cdks, but recently p21-dependent cell cycle inhibition was also found for G2 Cdks. Thus, within the G1/S checkpoint, fast amplification of Cdk activity takes place by several positive feedback reactions (synthesis of cyclins, degradation of inhibitors, elimination of inhibitory phosphorylation; Fig. 5b).

The checkpoint(s) at the G2-M transition are of similar complexity (153) and contain strong positive feedbacks by means of activating a phosphatase that removes inhibitory phosphorylation and by activating a protein-degrading system that removes a protein inhibitor. A negative feedback (or positive feedforward)

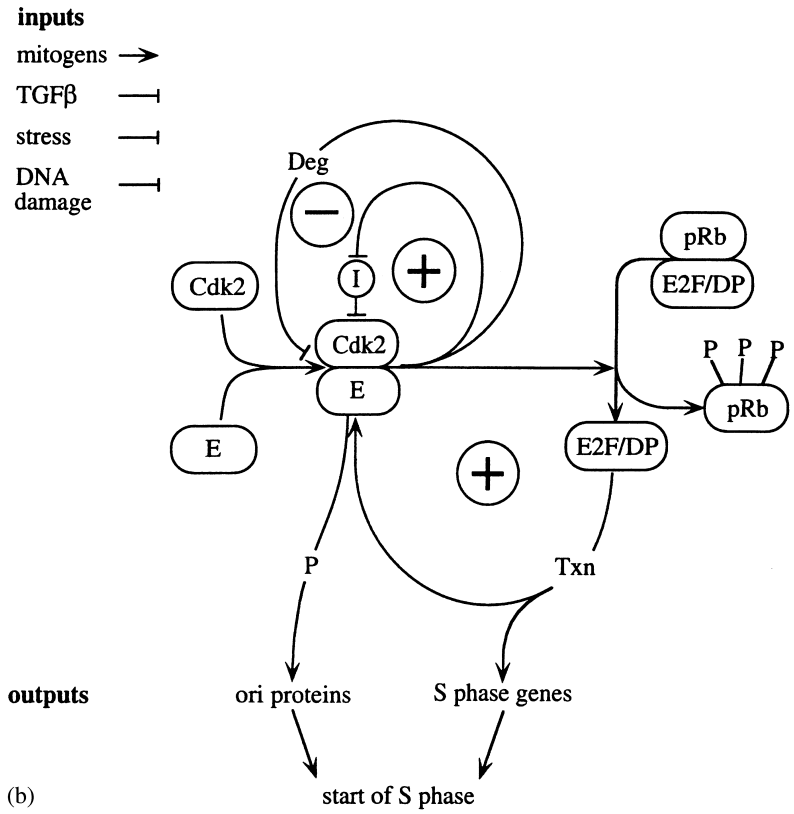


Figure 5. Continued.

process is realized by the same protein-degrading system that also degrades cyclin B. A still active cyclin B/Cdk1 complex would prevent further progress from M into G1 (8; see also Fig. 6).

The durations of G1 and G2 phases are especially subject to external control (nutrition, temperature, stress, as well as positively or negatively acting growth factors). Cell size control at the G1 checkpoint (in *S. pombe* also at G2) is a good example for these influences; the time to reach the necessary cell size that allows passage of the checkpoint is not only dependent on the above-mentioned factors, but also, particularly in the case of budding yeast, on the initial size of the mother or daughter cells after cytokinesis.

The frequency of the cell cycle is thus highly variable. The absence of inhibitory elements and in the presence of high concentrations of promoting elements, as in the case of fertilized vertebrate or *Drosophila* eggs, leads to cell cycle period lengths of only 10 minutes, whereas later in development, the period length of the cell cycle may last 10h–30h or more. Proliferation-promoting growth factors enhance differentiation; promoting factors lengthen the cell cycle.

The responses to inhibitory pulses have been observed particularly well

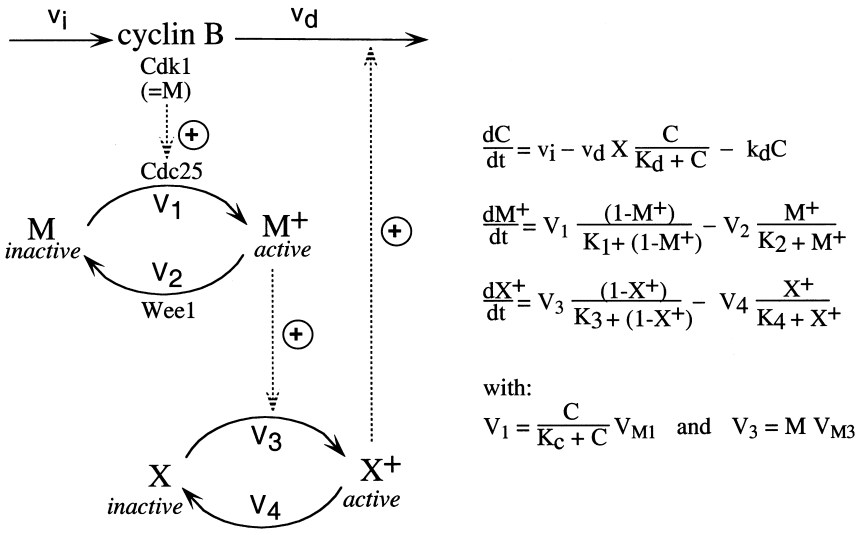


Figure 6. Minimal model of G2/M transition with a set of coupled differential equations for the mitotic oscillator. The control mechanism includes the activation of the inactive phosphorylated form of Cdk1 (+ cyclin B = M) to M^+ by the phosphatase Cdc25 (positive feedback), and the activation of a cyclin-degrading protease (anaphase-promoting complex, APC) from an inactive form *X* into its active form X^+, with the subsequent X^+-induced degradation of cyclin (as a positive feedforward). C, cyclin B; M, cyclin B/Cdk1 (= Cdc2); Wee1, inhibitory protein kinase. A description of this model and its properties can be found in Ref. 164.

with heat shock and cycloheximide pulses in *Physarum polycephalum* (154). Heat shock is also well known to arrest mammalian cells transiently in G1 and G2 (155,156).

The triggering mechanism of cell cycle events by the cyclic activity changes of the Cdks is increasingly considered to be a clock (7), even though it is not certain whether the mechanism represents an oscillator (perhaps in the case of the high-frequency cycles early in the fertilized egg), an hourglass clock (in the case of G1/G2 checkpoints), or a domino system. In addition, the timing of cell cycle events is more complex; apart from the cyclic Cdk activity, other clocks (or oscillations) seem to be involved. One oscillator, for example, was detected in yeast (56). Circadian clocks often act on the cell cycle in unicellular organisms (3) or in multicellular organisms, as particularly well analyzed in the mouse, rats, and humans (157).

Models of Cell Cycle Clocks

In eukaryotes, the main regulatory system for the sequential order of cell cycle processes consists of one (in yeasts) or several (higher eukaryotes) protein kinases and their regulatory cyclin units. These cyclin-dependent kinases (Cdks)

phosphorylate proteins, particularly at the G1/S checkpoint, as well as at the beginning of the M phase. Cdks activate important regulatory steps that drive the cell into the next phase of the cell cycle. Activation of the Cdks itself is an autocatalytic process occurring in G1/S (see the preceding section) and G2/M (158). Thus, autocatalytic behavior is part of cell cycle kinetics and plays an important role in most models proposed for the cell cycle (Fig. 6). In the Kauffman-Wille model, the Brusselator (which is a theoretical chemical oscillator based on autocatalysis; 70) has been used as an example to explain mitotic oscillations. In later models, the autocatalytic activation of Cdc2 (= Cdk1) is taken explicitly into account and is described by several coupled differential equations (159,160). More detailed reaction schemes were proposed by Tyson (161), Novak and Tyson (162,163), while other theoretical models and refinements of cell cycle clock models can be found in Goldbeter (164), Gardner et al. (165), Obeyesekere et al. (166), and Novak et al. (153).

In the minimal cascade model of Goldbeter (167), the key element of the oscillation is not based on autocatalysis, but on the observed negative feedback between cyclin activation of Cdk1 and the Cdk1-induced degradation of cyclin (164 and references therein). The structure of this model (Fig. 6) is similar to that of the Goodwin model, but with the important property of threshold behavior. Also, the negative feedback regulation in Goldbeter's cascade model is realized by a positive feedforward activation (Fig. 1, class III in Franck's schemes; Fig. 6), instead of a backward negative feedback as in the Goodwin oscillator (Fig. 1, class I; Fig. 4).

From the considerations above, it is clear that cell cycle oscillations rest both on positive and negative feedback kinetics, but that either positive (autocatalysis) or negative feedback may become more dominant under certain conditions. According to Novak and Tyson (163), it appears that, in slowly growing cells, Cdk oscillations are based primarily on positive feedback, while in intact embryos, the mechanism may be primarily based on a negative feedback loop involving Cdk1-induced cyclin degradation. A more detailed discussion of these aspects can be found in Ref. 164.

HOURGLASS CLOCKS

General Mechanisms of Hourglass Processes

Since the discovery of the first chemical hourglass by Landolt in 1886 (72), clock mechanisms have been an interesting study object and are often used in lecture demonstrations. In all hourglass clocks, the "timer" (the reaction that controls the timing event) is the consumption of a critical substrate in a rate-determining step.

It may be illustrative to look closer at an hourglass mechanism, for example, at one of the existing variants of the so-called iodine clock. In this variant,

process R1, the oxidation of iodide ions I^- by peroxodisulfate $S_2O_8^{2-}$ is the rate-determining step. The slowly produced iodine I_2 is rapidly removed by process R2, regenerating I^- ions through the reduction of I_2 by thiosulfate ions $S_2O_3^{2-}$. Thus, the concentration of I_2 is always at a low level as long as thiosulfate ions are present.

$$S_2O_8^{2-} + 2I^- \rightarrow I_2 + 2SO_4^{2-} \quad \text{(slow)} \tag{R1}$$

$$I_2 + S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-} \quad \text{(rapid)} \tag{R2}$$

To work as a clock/timer, thiosulfate must be present in an amount less than peroxodisulfate $S_2O_8^{2-}$. In the beginning, I_2 is formed slowly, but is rapidly regenerated back to I^-. Thus, the I_2 concentration stays very low during this phase of the reaction, and the solution remains colorless. However, as soon as $S_2O_3^{2-}$ is consumed, the concentration of I_2 rises, which is visible by the appearance of a blue color in the presence of dissolved starch. Thus, this "chemical timer" is set by two components: (1) the rate at which reaction R1 proceeds (which is influenced by temperature, for example) and (2) the amount of thiosulfate, which can be considered as the "hand" of the clock.

Significance of Unidirectional Processes for Organisms

Development and differentiation are (usually) irreversible processes (Fig. 7). However, we do not consider them as hourglass processes, even though they may contain them, because development or differentiation do not serve as a timer for events other than their own. Aging may be regarded as both a destruction process and a timer for the death of the organism, which may be programmed to occur early or late. In the literature, aging is often considered as a timing mechanism.

Hourglass clocks play a predominant role in development and aging (Table 2). They determine the duration of developmental steps or the overall life expectancy of an organism. These durations are usually dependent on external variables such as temperature (if the temperature is not held constant, as in mammals and birds), nutrients, or cytotoxic agents, as well as internal variables such as metabolism and rate of generation of reactive oxygen species (ROS), to name only a few. This leads to a strong dependency of development and longevity on these external and internal variables, which in part may represent a mechanism in adapting to different environments.

The examples of hourglass clocks listed in Table 2 basically rely on the same (hourglass) principle: measurement of the duration of a time interval by means of the increase (or decrease) of a certain variable that triggers an event or a process after reaching a threshold value (Fig. 7).

The same difficulty as discussed in the section "Significance of Oscillations for Organisms" applies to the definition of an hourglass clock as compared to other unidirectional changes in the amount of substances, in the degree of dam-

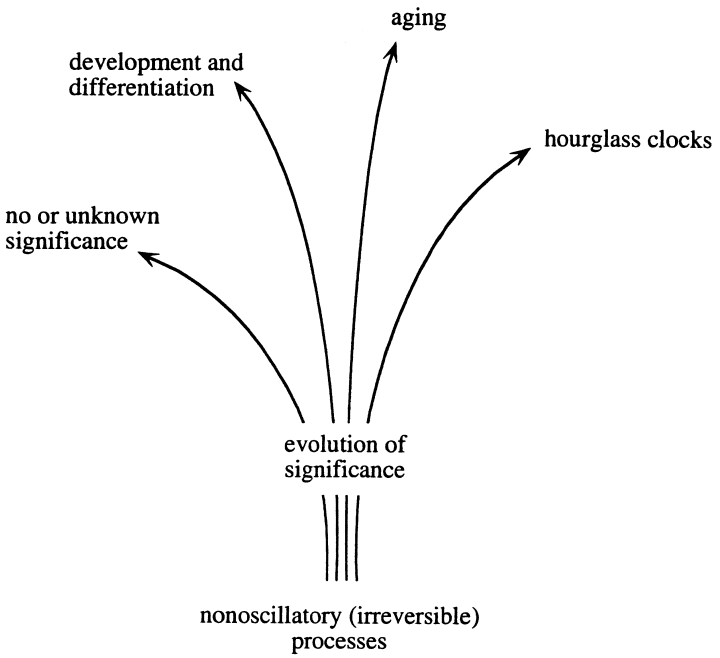


Figure 7. Evolution of timing mechanisms (hourglass clocks) and a few other functions of unidirectional processes. For details, see text.

age, or in the length of a molecule. Again, the usefulness of such a timing device that controls processes "external" to its own system may be the only valid criterion for a distinction. The slow linear decrease of the telomere length, for example, in the course of many replications reaches a threshold that limits the proliferation capacity of tissues (41).

The Telomere

In 1965, Hayflick was able to show that fibroblasts from embryonic lung tissues had the capacity to undergo about 50 population doublings in vitro, whereas cells taken from adult donors showed a fewer remaining cell divisions. This limited number of cell divisions found in somatic cells is called the *Hayflick limit*. Furthermore, embryonic fibroblasts that were frozen after 20 in vitro doublings only filled the remaining maximal population doublings (about 30 in this experiment) after thawing and continued culture. These findings suggested the existence of an internal cellular clock that "counts" the number of cell cycles. Further experiments indicated that this clock is located in the nucleus since a young cytoplast into which an aged nucleus was transplanted showed the same limit of replication as a normally aged cell (168).

It turned out later that the chromosome ends (telomeres) play a decisive role in limiting the replicative capacity, as deduced from the correlation between the declining capacity of cells to replicate and the shortening of telomeres (40). In humans and many other organisms, the telomeric DNA sequence is composed of hexameric repeats (5′-TTAGGG-3′) spanning about 10–12 kb in young human cells. During each round of replication, a loss of DNA at the ends of each chromosome takes place that most normal cells cannot avoid. This is due to the enzyme primase, which synthesizes short RNA primers toward the 3′ end of the lagging DNA strand in a stepwise fashion. This mechanism leaves the very 3′ end of a chromosome incompletely replicated and is known as the *end replication problem*. After each round of replication, about 50–200 nucleotides are lost at the 3′ ends of chromosomes (Fig. 8; 41,67,169–171). Recently, von Zglinicki et al. (172) reported that, in addition to telomeric shortening due to incomplete replication, a further decrease in length can be introduced through single-strand breaks caused by radicals. The cells in most adult human tissues are not able to replace the shortened telomeres. Loss of telomeric sequences results in severe consequences because telomeres play an important role in preventing end-to-end chromosome fusions (dicentrics) or nucleolytic digestion of chromosomes. It was shown that dicentric chromosomes and other chromosomal changes increase during the terminal passages of cells in culture (173).

Several lines of experimental evidence support the hypothesis that telomere shortening leads to replicative cellular senescence:

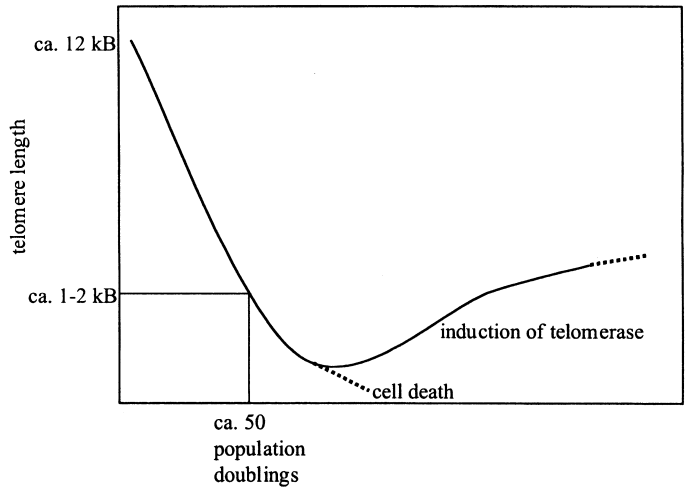


Figure 8. Decrease of telomere length due to replicative activity. After about 50 population doublings, cells stop dividing. Prior to the onset of cellular senescence, an accumulation of short telomeres occurs (1–2 kB) in human diploid fibroblasts (67). Most cells that continue dividing will die. The only cells that survive are those able to stabilize their shortened telomeres by activating telomerase, as is the case in most cancer cell lines. (Modified after Ref. 173.)

1. Fibroblasts from donors suffering from the progerioid Hutchinson-Gilford syndrome show diminished telomere length and can undergo fewer population doublings in vitro compared to fibroblasts derived from healthy donors of the same age (40).
2. The cellular senescence program can be restored in human renal carcinoma cells by reintroducing a normal chromosome, which leads to repression of the formerly active enzyme telomerase and thus to progressive telomere shortening (174).
3. The life span of human retinal pigment epithelial cells and foreskin fibroblasts was extended by introducing a plasmid, which enables the cells to express the catalytic subunit of the human telomerase (a reverse transcriptase). This subunit of the telomerase represents the limiting part for the activity and is not expressed in most tissues. Ectopic expression leads to telomerase activity and restoration of telomere sequences. Cells treated this way showed a normal karyotype and are able to exceed their normal life span by at least 20 population doublings (175).
4. Incubation with reverse transcriptase inhibitors suppressed telomerase activity, which leads to senescencelike processes in mouse fibroblasts (176).
5. An accumulation of short telomeres in human fibroblasts was found prior to the onset of replicative senescence, and this is significantly correlated with mean telomer length (67).
6. Expression of telomerase reverse transcriptase in fibroblasts from Werner syndrome (a progerioid syndrome) patients prevented accelerated cell aging normally displayed by cells from these donors (177).

For transformed cell lines or germ cell lines, it is necessary to elongate chromosome ends to overcome this end replication problem. Both cell forms do this by activating the transcription of the reverse transcriptase gene (*htert*) that encodes the protein subunit of the telomerase (178). This protein shows homologies to reverse transcriptases of viral origin (169,179,180). This enzyme is composed of at least two proteins and one RNA subunit and is able to synthesize DNA onto 3′ ends of DNA de novo using a small part of the enzyme's RNA subunit as a template (169), thus continuously restoring telomere length.

ROLE OF THRESHOLDS IN CLOCK DEVICES

In many and, perhaps all, clock devices, *thresholds* play an important role. Thresholds may be built into the oscillatory mechanism itself, such as in the degree of depolarization or hyperpolarization in the heart pacemaker (111) or concentration of clock proteins in the cytoplasm of *Drosophila*. Threshold val-

ues, furthermore, may serve to trigger dependent variables (Fig. 9). These dependent variables may be triggered at thresholds that are reached only for a short time, such as the maximal or minimal concentrations of the clock protein(s) (Fig. 9a, thresholds 1 and 3). This leads to "gating," that is, a short time window for processes such as mitosis that is gated by circadian rhythm (3,157). An intermediate threshold (Fig. 9a, threshold 2) may lead to activation of variables during the entire positive half-period (2+) or the entire negative half-period (2−), for example, as with the activity or rest cycle. In variables with built-in negative feedback loops, intermediate thresholds can also lead to short-term triggering, in this case, with a 12h period, which is often observed in locomotor activity. In all the instances discussed above, the variable is triggered at a certain *phase* of the oscillatory clock, that is, at a *time point*.

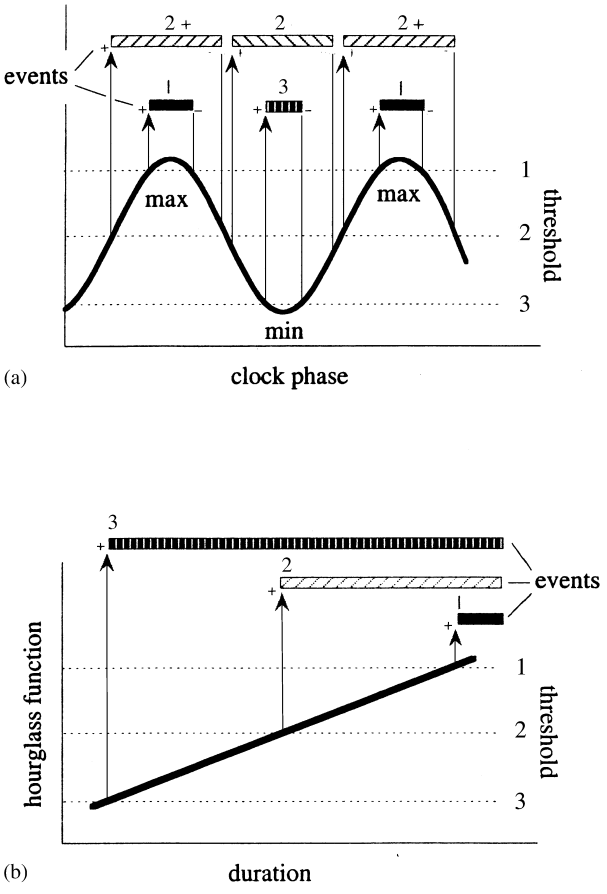


Figure 9. Triggering of events (a) by an oscillatory clock and (b) by an hourglass clock. Events can be triggered at different thresholds (1, 2, 3) at certain phases of the oscillatory clock or after a certain duration of the hourglass mechanism. The hourglass process is shown here as a linearly increasing process, but it may as well be realized as an exponentially increasing or a linearly or exponentially decreasing function.

In hourglass mechanisms, threshold values must be reached to trigger processes/events after a given duration of the hourglass process. Again, the triggered events can depend, negatively or positively, on an hourglass process and can last for a short or long interval of time (Fig. 9b). With this type of clock, the time at which the variable is triggered depends on the rate of the hourglass process, which determines the *duration* of the process until the threshold is reached.

CONCLUSIONS

Biological timing mechanisms (clocks) are increasingly detected in organisms. Two classes of clocks, oscillatory and hourglass, serve to determine events at certain time points (phases of the oscillator) or after certain durations (accumulation or depletion of a substance of an hourglass timer). Different thresholds (concentrations of substances, activity of proteins, or membrane potentials) of clock variables may trigger events in a defined sequence related either to external timescales (day, year) or endogenous times (development, differentiation, age).

Biological clocks are defined by their function as timers, similar to the various clock mechanisms used in human societies. This function of biological clocks has evolved during evolution, probably by attaching timing mechanisms to existing oscillatory or irreversible processes.

The timing of biological events can be related to periodic environmental changes (daily light-dark and temperature changes, tidal changes of mechanical agitation and pressure, lunar periodicities, annual changes of day length and temperature) either by direct responses to these changes or by endogenous oscillatory clocks of similar frequency that are synchronized with the environmental periodicities. Sometimes, a time point is determined by combined signals from exogenous and endogenous timers, for example, in photoperiodism. The endogenous oscillatory clocks in periodic environments serve to elicit anticipatory processes and are often compensated (stabilized) against the influence of external factors (temperature, pH, and metabolic substrates). These oscillatory clocks have adaptive (selective) value.

The timing of biological events can also be related to internal (biological) time, such as development and aging. Clocks for this timescale can be of the oscillatory type (cell cycle, ultradian rhythms, somite formation, annelid segments, leaf movement, ovarian cycle, etc.) or of the hourglass type (developmental steps in plants and animals, duration of pregnancy, apoptosis in egg and somatic cells, limits of cell division, etc.). Aging itself (i.e., triggering of cell death) may also be considered as an hourglass clock.

More than one clock mechanism is used, for example, in the cell cycle (hourglass plus oscillation[s]) or in the coordination of mating in the grunion fish or palolo worm (several external and endogenous periodicities). The sun and star compass orientation of bees and birds, respectively, require continuous daily time

determination based on a circadian clock. The continuous reading of the clock, however, is not yet understood.

The mechanism of the most well-known oscillatory clock, the circadian oscillator, consists of a predominant negative feedback loop in the expression and posttranslational activity of a few clock genes, together with a recently detected positive loop. How the frequency of this oscillatory mechanism is stabilized against external influences (such as temperature and pH) is not clear yet. Synchronization of this clock with daily environmental changes is based on strong phase-shifting effects of light and temperature signals (differential effects) transmitted by apparently highly conserved pathways.

The mechanism of the cell cycle clock contains strong positive and negative feedback (or positive feedforward) loops controlling the activity of cyclin-dependent kinases at the G1/S and G2/M checkpoints. The sequence of events within the whole cycle may be controlled by a combination of mechanisms described above with other oscillatory or hourglass devices or via domino effects. The period length of the cell cycle strongly depends on external conditions (growth factors, temperature, stress, and nutrition) and can be blocked by various stressors (DNA damage, heat shock, etc.).

Telomere length is a good example of an hourglass clock that determines the number of cell divisions (Hayflick limit). This mechanism shows a strong dependency on the rate of cell division; together with a defined threshold, they determine average life expectancy. Other hourglass timers determine the time of menopause in women (apoptosis of eggs), initiation of labor (i.e., the duration of pregnancy, possibly by an exponential increase of the corticotropin-releasing hormone), or death of multicellular organisms (increase of DNA damage, together with many accidental events).

The temporal order of an organism, that is, the "Gestalt" in time, as determined by these multiple timing mechanisms thus is as complex as is the spatial order or Gestalt in space. Both organizational structures develop and are maintained in tight cooperation and coordination.

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