Egg timers: how is developmental time measured in the early vertebrate embryo?

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Summary

Eggs and early embryos appear to be programmed to undertake particular developmental decisions at characteristic times, although precisely how these decisions are timed is unknown. We discuss the possible roles and interactions during early vertebrate development of two broad categories of timers: 1) those that involve cyclic or sequential mechanisms, referred to as clocks; and 2) those that require an increase or decrease in some factor to a threshold level for progression of time, referred to as hourglass timers. It is concluded that both clock-like timers linked to various features of the cell cycle and hourglass timers are involved in early developmental timing. The possible involvement of elements of circadian clock timers is also considered. ***BioEssays*** **22: 57–63, 2000.**

Introduction

The development of a vertebrate egg involves spatial and temporal organisation. The temporal organisation has two distinct elements: the relative serial ordering of developmental choices confronted by cells and the rate of development. Changing the sequence of different developmental choices could have profound effects on subsequent morphology and may have been an important element in evolutionary change. The rate of development might also be important for ultimate morphology. For example, if different developmental rates are applied regionally within an embryo, disproportionate growth and/or differentiation of some areas might occur. These regional variations in rate of development might themselves lead to changes of sequence, by altering the spatial signalling patterns between adjacent parts of the embryo.[1] Despite the importance of timing for development, the nature of its organisation remains elusive.

Biological timers, unlike atomic decay clocks, use chemical reactions which are prey to disturbance by fluctuations in temperature, pH, ionic concentration, etc. They are, thus, likely to be less accurate but potentially more responsive to inputs and adaptable in their outputs. Biological timers that are truly clocks "manage" the effects of environmental fluctuation through these responsive and adaptive properties via oscillatory feedback interactions (Fig. 1). An example of such a clock is the endogenous cellular circadian rhythm, and possibly also the cell cycle. A developmental clock might incorporate elements of, or be closely related to, either of these. However, a second category of timer is nonoscillatory, involving the activation of a cell autonomous molecular memory by a specific developmental event such as fertilisation (Fig. 1). The decay or accumulation of a product to a threshold level might then provide a measure of time elapsed since this event, regardless of cell cycle progression or circadian rhythmicity. This sort of timer would express itself more like passage of sand through an hourglass than movement of the hand of a watch. It is of course possible that hourglass-type timers might function as elements in a clock, since the running down or building up of a cellular component might provide either the trigger or checkpoint for progression to the next part of the cycle. What separates clock from hourglass timing mechanisms is the feedback component that gives cyclicity and directionality to the clock, and which the simple hourglass lacks. Building compensatory mechanisms (for temperature fluctuations, etc.) into these feedback systems gives a measure of stability to a dynamic system, an important property of developmental systems which need to be able to (and to a limited extent do) progress adaptively, despite environmental fluctuations.

There is no reason why more than one type of timer should not coexist either within the early embryo as a whole or within any one region of an embryo. Moreover, given the unique nature of early developmental cell cycles, it is possible that as the egg moves from a unicellular to a multicellular organisation, different timers might interact increasingly.[2] Indeed, when considering conceptually how eggs and early embryos might measure time, there are different organisational possibilities (Fig. 2). The timing mechanism might be envisaged as either hierarchical, with a single master developmental timer driving a cascade of sub-timers, or as a multiple system, in which several potentially independent timers run either in series or in parallel, in the latter case with the possibility of variable degrees of interconnectivity.[3,4] In this article, we review current evidence for the nature of timers in the earliest stages of development of vertebrates, with occasional reference to other de-

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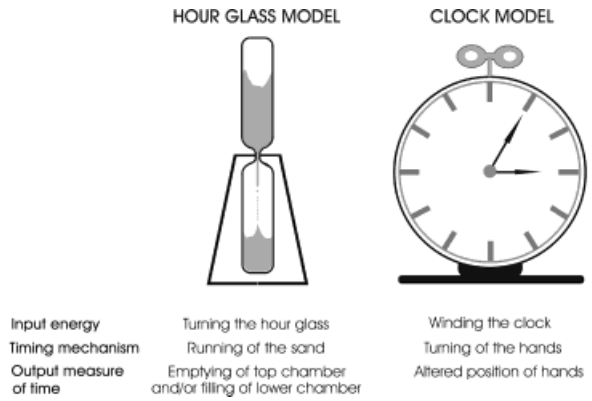

Figure 1. Two models of possible timing mechanisms for early development in which the timing mechanism itself is distinguished from input and output components. The essential difference between the two models is that the clock has a cyclical mechanism, while the hourglass is driven by a linear flow.

velopmental systems. The review is not intended to be comprehensive, but to identify pertinent evidence for mechanisms and their operations and to identify possibilities for further work. It is too early in the study of timers to make definitive general statements about how the timing of early development is controlled.

Cell cycle oscillators as egg timers

Early developmental cell cycles

There is a large body of evidence implicating some sort of counting mechanism based on the cell cycle in the measurement of early developmental time. It is important, therefore, to note that the cell cycles of the early embryo have a number of unique features. During the "cleavage" divisions of the early embryo there is no accompanying cell growth. Instead, the nucleo-cytoplasmic (N/C) ratio increases progressively with each cell division after fertilisation to proportions found in most adult cells. In addition, early cleavage divisions may be unequal in both volume and informational content, G1 and G2 phases are generally absent or much reduced in duration, and these early developmental cell cycles are controlled entirely at post-transcriptional levels via proteins and mRNAs laid down during oogenesis. The activation of transcription from the new embryonic template is one developmental feature that seems to mark a change in cell cycle properties. These general features of early development are characteristic of all multicellular organisms that have been studied in any detail, even though the absolute time scales and the numbers of developmental cell cycles involved differ considerably among organisms.

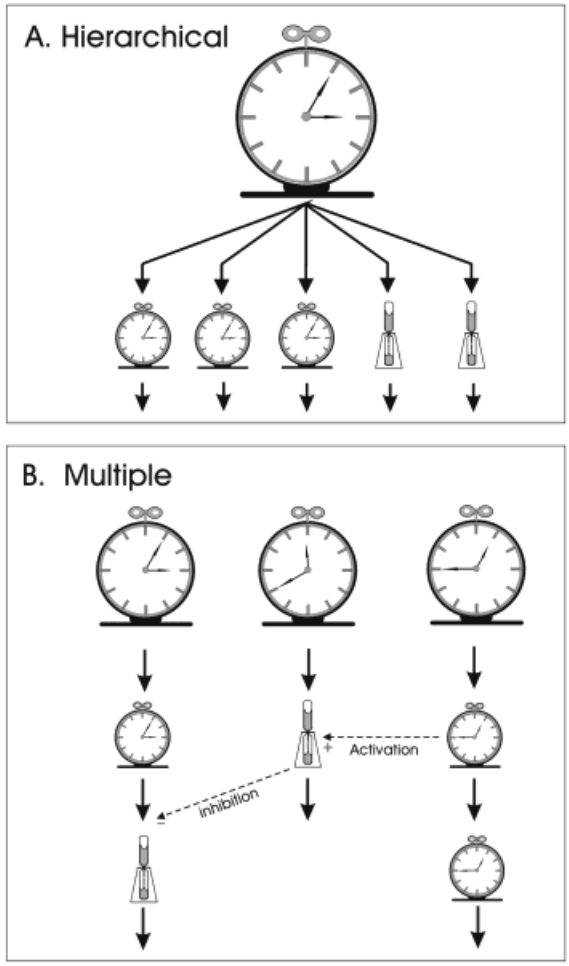

Figure 2. Models for the possible organisation of timing mechanisms in embryonic cells. A hierarchical model implies a single master timer regulates several downstream mechanisms. An alternative model has multiple parallel timers, each with their own inputs, mechanisms, and outputs. Parallel timing cascades might interact positively or negatively, thereby phase linking with each other, so as to coordinate cell function (shown by dotted lines).

Developmental transitions occur at specific developmental cell cycles

The existence of a cell cycle-dependent developmental timing mechanism is supported by the observation that certain key developmental transitions always occur during certain developmental cell cycles. In mouse embryos, for example, some transcription from the embryonic genome occurs at the end of the first cell cycle,[5,6] general transcriptional activation occurs in the second cell cycle,[7] polarisation occurs in the fourth cell cycle to initiate cell diversification,[8] and blastocoel cavitation occurs in the sixth cell cycle.[9] In

Xenopus embryos, the mid-blastula transition (MBT) at the 13th developmental cell cycle (stage 8.5) is characterised by initiation of transcription, lengthening of the cell cycle from 15 minutes to several hours, asynchrony of cell cleavage, increased cell motility[10] and degradation of cyclin E1.[11] Three cell cycles later (stage 10) the early gastrulation transition (EGT) occurs. This is coincident with degradation of cyclins A1 and A2 and the maternal mRNAs encoding them, and with the activation of a potentially apoptotic programme in the absence of prior zygotic activation.[12–14] In *Drosophila* embryos, transcription occurs in the 10th cell cycle and cellularization in the 14th cell cycle.[15,16]

These lines of evidence have been taken to suggest that early developmental timing might use one or more measures of cell cycles traversed to record time passing. However, the evidence is purely correlative and so could also mean that an underlying timing mechanism drives both developmental transitions and cell cycles.

Simply increasing cell numbers does not function as a counting mechanism

The number of cells in an early embryo increases with each division. As a result, there are changes in intercellular contact patterns and this increases the possibility of differential signalling among cells. An increase in cell number, however, is not critical for early timing events. In the mouse, it has been shown that neither the number of rounds of cytokinesis nor the total number of cells in an individual conceptus are involved in the time-measuring mechanism. Altering cell number in preimplantation mouse embryos by blocking cytokinesis with cytochalasin,[17–20] by separating and culturing blastomeres individually or in smaller aggregates,[21] or by aggregating larger numbers of blastomeres[17] does not affect the timing of developmental events or transitions. Similar conclusions have been reached for other types of early embryo, including ascidians[22] and *Xenopus*.[23]

Some element of each cell cycle can function as a unitary counting device

In contrast, there is evidence to support the possibility that the number of cell cycles traversed functions as a unitary counting device in early embryos of several species. Obvious processes that may be an integral part of this timing mechanism are DNA replication and the increase in N/C ratio that occur with each cleavage division. Indeed, a critical N/C ratio is thought to be required for certain developmental events at the MBT in *Xenopus*, including transcription, division asynchrony, and increased cell motility.[10,23] An increase in N/C ratio is also responsible for the extended interphase in the loach,[24] and for division asynchrony in the zebrafish[25] and starfish embryos.[26] Thus, the timing of these events can be affected either by removing or adding cytoplasm[27] or by interfering transitorily with DNA replication, both of which influence N/C ratio. Conversely, in *Xenopus* the permanent inhibition of DNA replication blocks the MBT events, suggesting that an hourglass mechanism measuring time elapsed since fertilisation does not time the MBT. Neither transcription nor translation is required in *Xenopus* for the N/C ratio-dependent timer to operate.[23] Various mechanisms by which an increasing N/C ratio might achieve its effect have been proposed. The most common theory implicates the titration of cytoplasmic factors, possibly to release repression on chromosomal expression.[10,28,29] An alternative dilution hypothesis is that increasing numbers of mitotic apparati titrate out stocks of maternal cyclin.[15] The serial modification at division of replicating daughter strands of DNA,[30] or of their associated chromatin proteins,[31] has also been proposed as a means for counting cell cycles traversed.

There is also considerable evidence, however, for a different mechanism that persists in the absence of DNA replication, N/C ratio changes, or, indeed, in the complete absence of nuclei or mitotic apparati altogether. Thus, blocking DNA replication in one-cell mouse zygotes by treatment with a protein synthesis inhibitor or aphidicolin does not delay the oscillations in ion channel activity that normally parallel cell cycle progression[32,33] or prevent the activation of at least some elements of transcription.[34,35] Likewise, inhibition of DNA replication does not delay cavitation in mouse embryos.[21] Similarly, in *Xenopus* embryos, aphidicolin does not block the degradation of cyclin E1 that normally occurs at the MBT[11,13] and hydroxyurea suppresses DNA replication and halts the increase in N/C ratio, but not the degradation of maternal cyclin A at the EGT.[12]

Further evidence for a timing mechanism that does not depend on DNA replication comes from studies on enucleated embryos. Thus, enucleated newt,[36] *Xenopus*,[37] and sea urchin[38] eggs retain a sequence of surface waves of contraction that parallels cleavage divisions in intact eggs. Similarly, one-cell enucleated mouse eggs also show cortical contractile activity and changes in chromatin condensation activity,[39,40] as well as cyclic changes in the activity of a potassium channel that parallels cell cycle progression.[33] Furthermore, experimental modifications to the N/C ratio of mouse zygotes likewise do not affect the developmental programme of protein synthesis.[41]

These studies demonstrate that, while the number of rounds of DNA replication and/or a changing N/C ratio affects the timing of *some* developmental events, the timing of other events is unaffected. Some of these refractory events are non-oscillatory, and so may represent the activity of hourglass timers (see below), while others are oscillatory. These data suggest that DNA counting or measuring mechanisms alone cannot be responsible for measuring the passing of time in the early embryo and that there are other mechanisms that either

run in parallel with the DNA-counting timers or lie upstream of them and so might normally drive them.

Evidence for cytoplasmic oscillators

A clue to the nature of some of these mechanisms comes from experiments in which features of the cell cycle other than DNA replication, presence, or level relative to cytoplasmic volume have been perturbed. A central role for cdk/cyclin complexes in driving cells through the mitotic cycle has been established in recent years. The activity of these complexes is affected, with varying specificity, by a number of agents. Thus, inhibition of protein synthesis affects the periodic synthesis of cyclin B that is required to replenish degraded protein and so to reconstitute MPF, the cdc2/cyclin B complex necessary for passage through M-phase. In *Xenopus* embryos, inhibition of protein synthesis (or injection of antisense oligonucleotides to cyclin B)[42] blocks MPF activity and surface contractions,[43,44] but not cyclin E1 degradation at the MBT, nor, more significantly, the cyclic oscillations in cdk2/cyclinE activity that occur prior to this.[45] Likewise, the cyclic expression of *c-hairy1* mRNA in the developing chick presomitic mesoderm is refractory to inhibition of protein synthesis.[46]

In the preimplantation mouse embryo, evidence for a cytoplasmic oscillator comes from the observation that, as expected, inhibition of protein synthesis throughout fertilisation and the one-cell zygote stage blocks entry into the first mitosis together with the associated rises in MPF activity and cortical contractility.[40] It does not block another apparent manifestation of the cell cycle, however, namely, oscillations in K^+ and Ca^{2+} conductances.[33,47] Changes in ion channel activity and cytoplasmic ion concentrations that follow the cell cycle have been reported for early embryonic cell cycles of ascidians,[48,49] sea urchins,[50] loach,[51] and mice.[52,53] In mice, experiments on bisected one-cell zygotes demonstrate that absence of the nucleus also does not prevent cycling of channel activity in the anucleate fragment,[33] which suggests a cytoplasmic clock that is independent of transcription and of progress through the nuclear cycle. This cdk1/cyclin B-independent clock regulates both K^+ and Ca^{2+} channels, but does so with a different phase relationship to each.[47]

What is the nature of this putative cytoplasmic clock and its control in the mouse zygote? Although it functions in the absence of the nuclear cell cycle, the two cycles can interact via the activity of a tyrosine kinase at certain points.[33] Thus, arresting the nucleus in metaphase, by preventing the degradation of cyclin B,[54] or at the G1/S transition by inhibiting DNA synthesis, also halts the cytoplasmic clock. In addition, a MAP kinase-type pathway has been implicated as part of the signalling mechanism of the cytoplasmic clock. MAP kinase activity is high in the metaphase arrested oocyte and decreases after fertilisation in a manner that does not require degradation of cyclin B.[55] This decrease, like that in K^+ channel activity, is slow compared with inactivation of cdk1/cyclin B.[56] There is still a lag between the inactivation of MAP kinase and channel inactivation, however, suggesting an indirect action on the K^+ channel. Moreover, although activity of both Raf-1 and MEK increase during M phase of mitosis 1, this is accompanied not by MAP kinase activity but by myelin basic protein kinase (MBP kinase) activity. This suggests that novel kinase pathways are active at this time that are responsible for the phosphorylation and activation of MBP kinase and also, perhaps, ion channels.[56,57] It is possible that these signalling pathways form part of the cytoplasmic clock mechanism in mouse zygotes. It is unclear whether ion channel activities themselves form part of a clock mechanism or part of its output. It seems unlikely that the Ca^{2+} channel in the early mouse embryo is integral to the clock's mechanism since its inhibition pharmacologically does not prevent or delay progression of the cell cycle through mitosis.[47] This contrasts, however, to the observation that in sea urchin embryos Ca^{2+} channel inhibition does delay passage through the cell cycle.[50]

These studies suggest that in *Xenopus* and mouse, and possibly early embryos of other species, there is an underlying cytoplasmic oscillator present which influences developmental events. The nature of this oscillator, however, remains obscure. Certain of the residual oscillatory features described above have the potential to profoundly influence a number of cellular processes. Thus, changes in ion channel conductance can function as highly effective amplifiers to convert cells rapidly from one metastable state to another, and there is already evidence linking changes in either global or local ion concentration with developmental or cell cycle transitions. The oscillations in potassium channel activity in mouse eggs are paralleled by cyclic changes in the cell resting membrane potential.[32] In sea urchin eggs oscillations in the intracellular concentration of IP_3, Ca^{2+}, and sulfhydryls are well placed to influence a multiplicity of cellular functions.[58–60] Furthermore, centrosomal replication cycles can continue in hamster and *Xenopus* eggs in which DNA and/or protein synthesis are blocked.[61,62] Any or each of these cytoplasmic oscillations could be close to a driving oscillator or even be part of its feedback loop.

Circadian rhythms and timing events: some speculations about the early embryo

Circadian rhythms are characterised by an approximate 24-hour period, temperature compensation, and the capacity to be entrained. They appear to be a universal feature of both pro- and eukaryotes and recent investigations into the molecular basis of the circadian clock indicate that the clock mechanism is highly conserved between species such as *Drosophila* and mouse.[63,64] Although most, if not all, cells express an endogenous circadian rhythm,[65] the majority of

molecular analyses have focused on neural tissues and the vast body of experimental data on a range of species and tissues has yet to be explained at the molecular level. The prevailing model emphasises a clock mechanism in which two distinct proteins, each with a protein–protein interaction site, called a PAS domain, form a heterodimer and act as a positive transcriptional regulator for two genes, *Per* and *Tim*. The products of these two genes accumulate within the cytoplasm and themselves form a heterodimer which is translocated into the nucleus, where it suppresses the activity of the PAS protein promoters, establishing a negative feedback loop which gives the clock its period.[64] Implicit in this model is a cycle of transcriptional, translational, and posttranslational events.

However, it is not at all clear that this model is universal for all cells, nor whether the same or homologous molecules might operate in distinctive oscillatory or nonoscillatory timing mechanisms. Thus, the literature on circadian rhythms is rich in examples that suggest independence from transcription and even translation, and there is evidence implicating posttranslational controls and signalling pathways involving Ca^{2+}, cAMP, and MAPK.[66] Moreover, evidence suggests that, in the same cell, more than one circadian oscillator can operate separately but coupled in parallel.[67] In many of the older studies it is difficult to distinguish whether the effects of experimental perturbations are functioning on the clock mechanism itself or via some input/output parameters (see Fig. 1). Nonetheless, the data do not preclude alternative mechanisms for the circadian clock. Certainly PER and TIM proteins have been implicated in diverse cellular functions,[68] including developmental processes.[69] For example, in *Drosophila* PER proteins expressed in the thorax are implicated in the control of ultradian rhythms (with a period of longer than 1 h but less than 24 h)[70] and *Per* mRNA in the ovary does not show circadian oscillations but, when mutated, does influence circannual rhythms (with a period of about 1 year).[71] Interestingly, in light of the earlier discussion, changes in ion channel activity and cytoplasmic ion concentrations have been shown to parallel the cellular circadian rhythm.[72–76]

There has been little analysis of circadian-implicated proteins or their homologues in early developmental stages to determine whether they are present, how they behave, or whether they contribute to either clock or hourglass type developmental timing mechanisms. In *Drosophila* larvae, PER protein is expressed at constitutively low and nonoscillatory levels.[77] In moth embryo neurons *Per* expression is also nonoscillatory and its neutralisation prevents embryo hatching.[78] The egg is a unicellular "organism" which, in many species, is released into an environment with diurnal variations that may be critical to its survival, fertilisation, and early development. Even in mammals, it is possible that the female reproductive tract might express a circadian rhythm that influences the timing of developmental events.

It may be, however, that the ancestral function of these proteins, now clearly implicated in circadian rhythmicity, is not restricted to circadian timing at these early developmental stages. Rather, their "ticking" may influence, for example, some of the oscillatory cytoplasmic features described earlier and in doing so, they may function in a variety of ways not restricted to the transcription-translation cycle described for *Drosophila* neurons nor to light:dark cycles of entrainment.

Hourglass timers

Many lines of evidence support the idea that developmental timing may involve an hourglass type of memory that is driven by either an increase or decrease in protein levels to a threshold level. Much of this evidence comes from studies on early embryos of *Xenopus*, which will be used illustratively. As pointed out above, in *Xenopus* embryos, the timing of the selective degradation of cyclin E1 protein at the time of the MBT is not dependent on a cell cycle counting mechanism since it is not blocked by inhibitors of DNA or protein synthesis and so cannot rely on an increased N/C ratio.[11] It is suggested that an hourglass-type timing mechanism might be operating, which is initiated at fertilisation and runs for approximately 5–6 hours before activating a cyclin E protein degradation pathway. The possible molecular nature of the timing mechanism for this degradation has been investigated by increasing MAP kinase activity during early cleavage by injection of either c-mos or a constitutively active form of MAPK kinase. This treatment delayed the onset of cyclin E degradation, implicating the MAP kinase system in the timing process, possibly by acting to reset the timer to zero.[45] The possible involvement of MAP kinase is interesting, given its implication in the cytoplasmic oscillator of mouse zygotes described earlier.

Similarly, the timing of the increased turnover of cyclin A1 and A2 proteins in *Xenopus* embryos at the EGT is also independent of cell division, N/C ratio, DNA synthesis, and protein synthesis.[12] It does, however, involve the action of ICE-like caspases and inhibitors of this caspase activity, probably maternally encoded, are present in early *Xenopus* embryos.[13,14] It is the loss of this inhibitory activity which seems to determine the onset of the cyclin degradation and this inhibitor loss follows the loss of cyclin E by about 2–3 hours. These results suggest an overall timing mechanism that involves either: 1) two different hourglass timers that measure time in parallel (from fertilisation to MBT and from fertilisation to EGT); 2) two hourglass timers that operate in series (from fertilisation to MBT and then from MBT to EGT, although the second timer does not require the MBT for its activation); or 3) a single timer which is activated at fertilisation from which two independent readings are taken to determine first when cyclin E loss occurs and then when caspase inhibitor inactivation occurs.

The timing of other developmental events around *Xenopus* gastrulation are also suggested to result from an hourglass-like timing mechanism.[79] Thus, the first expression of the cardiac muscle actin gene in animal cells at the late gastrula stage depends upon an inductive interaction with vegetal cells. Its timing, however, is dependent on the developmental age of the responding cells and not on the time at which induction commences.[80] Moreover, the subsequent loss of competence to express this gene appears to be controlled in a cell-autonomous way, and is independent of protein synthesis and passage through the cell cycle.[81]

Studies on early *Xenopus* development suggest that cyclin E, as part of a cyclin E/cdk2 complex, may constitute an integral part of an early developmental hourglass timing mechanism.[45] As described earlier, cyclin E/cdk2 activity follows a cyclic pattern prior to the MBT. This pattern is not influenced by inhibition of DNA or protein synthesis or altered N/C ratio. Indeed, this activity constitutes an example of a cytoplasmic oscillator that is independent of the nuclear cycle of oscillation. The kinase activity of this complex can be moderated selectively by injection of a truncated form of the cdk inhibitor D34Xic1. D34Xic1 increases the period of the cyclin E/cdk2 kinase oscillations by about 20% and also extends the cell cycle by a corresponding proportion, probably due to extension of the S to M transition. In consequence, the timing of the MBT and the activation of transcription are delayed. Such an outcome is not surprising given the N/C ratio titration model for timing the MBT. In these experiments, however, degradation of both cyclin A and cyclin E, both putative hourglass-timed events, was also delayed. These observations seem to bring together the three types of timing mechanism explored so far in early *Xenopus* development. First, cyclin E/cdk2 activity is implicated (albeit indirectly) in the N/C ratio titration timer mechanism that involves DNA cycles. Second, it appears to influence the proposed hourglass timer mechanism. And third, it displays a cytoplasmic oscillatory cycle of its own. Does this mean that the oscillations of cyclin E constitute a component of the hourglass timer? This is unclear, it might do. Alternatively, there might be a downstream affected component. It will be important to identify an independent marker for the putative hourglass egg timer other than cyclin E degradation in order to verify the exact role of this mechanism.

Conclusions

We do not yet understand how early development is timed. There is evidence for a number of different timing mechanisms, some like clocks and others like hourglasses and different timing mechanisms may operate within the same cells at the same times. There is also evidence of crosstalk between timers. For some of the timers, elements of the component inputs, mechanism, and outputs are being identified. But, as the new millennial clock strikes, there remains much to challenge developmental biologists.

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