Dependence of timing of mitotic events on the rate of protein synthesis and DNA replication in sea urchin early cleavages

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Abstract. To understand what processes affect the cell-cycle timing of mitotic events in early cleavage cycles of sea urchin embryos, a study was made on the effects of (a) reducing protein synthesis with emetine and (b) DNA replication with aphidicolin, on the timing of nuclear envelope breakdown, anaphase onset and cytokinesis. When protein synthesis was slightly inhibited by administration of emetine, the delay in the mitotic events increased, with an increase in the delay in accumulation of proteins up to the levels to which cells must synthesize the proteins to execute the cleavage. This indicated that protein synthesis affects the timing of mitotic events. The delay in cleavage cycles caused by a slight inhibition of DNA replication with aphidicolin was in proportion to the concentration of aphidicolin administered, suggesting that DNA replication also affects the timing of mitotic events. Furthermore, it was confirmed that accumulation of the proteins to the levels required for execution of the first cleavage precedes completion of DNA replication as a requirement for execution of the first cleavage. These results imply the existence of process(es) affected by protein synthesis that are included in a feedback control system which prevents the initiation of mitosis until after the completion of DNA replication; it is the characteristic of a cell-cycle control system that has been predicted theoretically.

Cells must maintain a proper temporal order among cell-cycle events to ensure the accurate transmission of their genetic information to their daughter cells. There has long been an interest in the timing controls for cell-cycle events (Nurse 1990, Murray & Hunt 1993), and many processes that participate in cell-cycle progression have been found in recent years (reviewed by Nurse 1994, Nasmyth 1996). Various investigations of the cell cycle have demonstrated that the processes which constitute the eukaryotic cell cycle are divided into two classes: those which constitute a cell-cycle engine and those which constitute its 'downstream' events (reviewed by Murray 1992, Murray & Hunt 1993). The cell-cycle engine consists of enzymes that control the downstream events and reactions that autonomously produce a cyclic change in the activity of these enzymes. In early embryonic cells, which possess a primary cell-cycle engine, mitosis and interphase are regarded as the results of a

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change in the activity of M-phase promoting factor (MPF), a complex of p34^{cdc2} and cyclin B. The activation of MPF, which is accomplished by post-translational modifications of the complex, is indispensable for entry into mitosis: active MPF is a prerequisite for nuclear envelope breakdown (NEB) (for a review see Nigg 1993). The active MPF then leads to its own inactivation by activating cyclin degradation machinery (reviewed by King *et al.* 1996). This degradation machinery triggers the separation of sister chromatids (namely anaphase onset), and the inactivation of MPF induces nuclear envelope re-formation and cytokinesis. This cell-cycle engine is driven by the synthesis of cyclin B: accumulation of cyclin B beyond a critical threshold is both necessary (Minshull, Blow & Hunt 1989) and sufficient (Murray & Kirschner 1989) for triggering mitosis in early *Xenopus* embryos.

In contrast to early frog embryos, blocking some downstream events arrests the progress of the cell-cycle engine in most cell cycles by a 'checkpoint', which uses a feedback control that arrests the engine until the downstream event is complete (reviewed by Hartwell & Weinert 1989, Murray & Hunt 1993). For example, the checkpoint for entry into mitosis keeps cells from entering mitosis until DNA replication is complete, using the feedback control that prevents the activation of MPF through post-translational modifications (Meijer, Azzi & Wang 1991, Smythe & Newport 1992, Kumagai & Dunphy 1995). Therefore, the timing of cell-cycle events can be influenced by not only the progression rate of the cell-cycle engine, which is driven by cyclin synthesis, but also the timing of the completion of such downstream events (e.g. DNA replication) which restrain the cell-cycle engine by feedback control.

Following genetic studies, which revealed the logical relationships of genes, previous researchers on the cell cycle assumed simply that the timing of cell-cycle events was determined by a 'simplified dependent relationship' as a logical relationship of necessity; i.e. if initiation of some late process requires the completion of early processes that act in parallel with each other and the late process is initiated only when all the requirements are fulfilled, the most dilatory of the early processes determines the timing of initiation of the late process, and none of the other processes affect the timing (for an example of such a view, see Fig. 3 in discussion about processes that determine the cell-cycle timing of mitosis by Nurse 1990).

One-cell sea urchin embryos, for example, must fulfill two requirements to initiate mitosis: synthesis of proteins up to their required levels (Wagenaar 1983) and completion of DNA replication (Ikegami *et al.* 1978, Meijer *et al.* 1991). The process of protein synthesis and that of DNA replication act in parallel with each other and are mutually independent (Ikegami *et al.* 1979, Zhang & Ruderman 1993, Genevière-Garrigues *et al.* 1995). Adding protein synthesis inhibitor early in interphase blocks entry into mitosis, but adding it in S-phase does not (Wagenaar 1983, Genevière-Garrigues *et al.* 1995), demonstrating that the requirement for the proteins for execution of mitosis is fulfilled before the completion of DNA replication. Assuming the simplified dependent relationship, it is therefore expected that the timing of mitotic events will depend on the time of the completion of DNA replication, and not on the time at which the proteins have accumulated to the required levels.

Because few studies have been carried out on the cell cycle in terms of the timing control itself for cell-cycle events, a comprehensive understanding of which processes influence the timing of cell-cycle events in intact dividing cells is still lacking. Basic approaches, such as mutating a gene whose product operates some process or blocking that process with sufficient concentration of specific inhibitors, are too imprecise for investigation of whether the process controls the timing of some cell-cycle event in intact cells, because any necessary process that is greatly limited acts as a control step in the event.

By contrast, the response of the timing of the cell-cycle event to small perturbations in a process with low concentration of a specific inhibitor for the process will reveal whether the process influences the timing of the cell-cycle event in the normal cell cycle: a delay in the cell-cycle event will be proportional to a delay in the process if the process influences the timing of the cell-cycle event in the normal cell cycle; a delay in the cell-cycle event will not increase with an increase in the delay in the process due to lower concentration of the inhibitor if the process does not influence it.

This study was undertaken to understand which processes, among those necessary for cell-cycle progression, influence the cell-cycle timing *in vivo*, by examining the effects of low concentrations of emetine, a protein synthesis inhibitor that inhibits EF2-dependent polysome translocation (Jiménez, Carrasco & Vázquez 1977), and aphidicolin, an inhibitor of DNA polymerase α (Ikegami *et al.* 1978, 1979), on the timing of mitotic events, using highly synchronous early sea urchin embryos. Unfertilized sea urchin eggs are arrested in post-meiotic interphase and cyclin is undetectable in them (Edgecombe, Patel & Whitaker 1991). The rate of cyclin synthesis is strongly activated after fertilization and remains essentially the same thereafter (Evans *et al.* 1983).

As had been expected, it was shown that DNA replication affects the timing of mitotic events in early cleavage cycles of intact sea urchin eggs. However, contrary to expectation, protein synthesis was also found to affect the timing of mitotic events, though it was confirmed that the accumulation of proteins to their required levels long precedes the completion of DNA replication. Possible explanations for these results are discussed.

MATERIALS AND METHODS

Handling of eggs

Gametes of the sea urchin *Hemicentrotus pulcherrimus* were obtained by injection of 1 mM acetylcholine chloride into the body cavity. Semen was collected 'dry' and stored at 4°C until use. The eggs were washed several times with artificial sea water (ASW) and stored as a gently stirred suspension at 5°C. In each experiment, gametes obtained from the same parent were used throughout. In the following experiments, fertilized eggs were cultured at 19.2°C.

Cytological procedures

To examine the effects of reducing protein synthesis or retarding DNA replication, a determined concentration of emetine (Fluka Chemie AG, Buchs, Switzerland) or aphidicolin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) was administered to a batch of unfertilized eggs prior to fertilization. (Aphidicolin was dissolved in dimethyl sulphoxide at a concentration of 10 mg/ml, and dispersed in ASW to give a final concentration of dimethyl sulphoxide less than 0.1%.) Then the egg suspension was inseminated in ASW containing the inhibitor and suspended in a test tube in a thermostat bath until further procedure. In these experiments another batch of eggs that did not contain the inhibitor was prepared concurrently for use as a control.

To measure the time when sufficient protein had accumulated to allow execution of the *n*-th cleavage, 100 μ M emetine was applied to zygotes: at given time intervals before the *n*-th cleavage, 0.1 ml of the egg suspension was added to test tubes containing 0.8 ml of 112.5 μ M emetine dissolved in ASW (giving a final concentration of 100 μ M emetine), and these test tubes were shaken in a thermostat bath. After sufficient time (more than 3 h after insemina-

tion for the first cleavage and more than 4 h for the second cleavage), 0.1 ml of ASW containing 2% glutaraldehyde was added to the test tubes. From each fixed aliquot, 100 eggs were counted and the percentage of cleaved eggs was measured later. The time when 50% of the eggs could execute the n -th cleavage, in a plot of the percentage of the n -th cleavage as a function of the time when emetine was added, was defined as tp_n .

To determine the time of the n -th cleavage of a batch, aliquots from the batch were fixed in 1% glutaraldehyde (final concentration) at given time intervals around the n -th cleavage, then were later scored for the n -th cleavage by phase contrast microscopy. The time when 50% of eggs had started the n -th cleavage was defined as tc_n .

To determine the mitotic stages, aliquots were fixed in ethanol and acetic acid (3:1). To judge whether the nuclear envelope had broken down, supernatant of the fixed aliquots was removed and 25% (v/v) glycerol was added, followed by 50% glycerol, and the aliquots were observed by differential interference contrast microscopy. The time when 50% of the eggs had undergone the n -th nuclear envelope breakdown was defined as tn_n . To judge whether anaphase had started, the fixed aliquots were washed with 45% acetic acid and stained with 1% orcein in 45% acetic acid, and then observed by bright-field microscopy. The time when 50% of the eggs had undergone the n -th anaphase onset was defined as ta_n .

Time-lapse video microscopy

The temperature of the eggs on a microscope was regulated by a homemade thermo-control chamber as previously described (Yamada & Mihashi 1998). Briefly, a glass laboratory dish was set in the control chamber on the stage of an inverted phase contrast microscope. ASW flowed through the glass dish to maintain water temperature and other environmental parameters constant.

To measure the time of the first cleavage of eggs to which 100 μ M emetine or 10 μ g/ml aphidicolin had been applied after fertilization, gametes were inseminated in ASW and suspended in a test tube in a thermostat bath. At a given time, a suspension of the fertilized eggs was added to emetine ASW giving a final concentration of 100 μ M emetine or 10 μ g/ml aphidicolin, then the eggs were added to the glass dish filled with 100 μ M emetine or 10 μ g/ml aphidicolin ASW. The time course of early cleavages was recorded for groups of eggs (about 30 in each group) with a charge-coupled device (CDD) camera that was mounted on the microscope and attached to a time-lapse video cassette recorder. The videotape was played back and forth so that the time interval between insemination and the beginning of cleavages could be determined for each egg.

Two observation systems were used in these experiments. In one system, an egg sample was kept under a given concentration of an inhibitor and cleavage cycles were measured in the presence of the inhibitor. The other system ran concurrently without the inhibitor, and another egg sample in this system was used as a control.

RESULTS

Measurement of the time when proteins had accumulated sufficiently to allow execution of cleavage

The time when newly synthesized proteins had accumulated sufficiently to allow execution of a cleavage, hereafter referred to as the time of commitment to the cleavage, was used as an indicator of the progression of protein synthesis in the following experiments. To determine the time of commitment to a cleavage, the following measurements were conducted: A batch

of eggs was fertilized and aliquots were removed at given time intervals and placed into ASW containing a high concentration of emetine to stop further protein synthesis. The aliquots were fixed after a time long enough to allow cleavage. The percentages of cleaved eggs in the fixed aliquots were then measured and plotted against the time when emetine was added to the aliquots.

To stop protein synthesis in sea urchin embryos, 100 μM emetine was applied in many experiments (Evans *et al.* 1983, Wagenaar 1983, Edgecombe *et al.* 1991, Meijer *et al.* 1991). It was demonstrated that this stopped incorporation of leucine into proteins within a few minutes after addition to sea urchin embryos (Hogan & Gross 1971, Wagenaar & Mazia 1978). For a start, the time course of the first cleavages on a group of eggs to which 100 μM emetine had been applied at a given time was measured (Figure 1a). As demonstrated by Wagenaar (1983), not all eggs cleaved when 100 μM emetine was applied at earlier times (from 15 to 20 min) after fertilization. Percentages of the first cleavage increased within 150 min after fertilization, and remained unchanged thereafter. In the following experiments, therefore, emetine-inhibited aliquots were fixed at more than 3 h after fertilization and the percentages of cleavage were measured as the final percentages of cleavage.

Figure 1b shows the final percentages of cleavage plotted against the time when 100 μM emetine was applied, for the data used in Figure 1a. This figure is an illustration of how tp_1 is determined. In a plot like Figure 1b, tp_1 is determined as the time when 50% of the eggs could execute their first cleavage. In this case, tp_1 was 17.5 min. The procedure for the measurements of tp_1 in the following experiments is summarized in Figure 2a.

Figure 1a also shows that the first cleavage of eggs was delayed compared to controls when 100 μM emetine was applied. A longer delay was observed when 100 μM emetine was applied at an earlier time after fertilization. (The delay relative to control increased almost in inverse proportion to the difference between tp_1 and the time at which 100 μM emetine was applied.) This observation indicates that the amount of protein synthesis after the time of commitment to a cleavage affects the timing of the cleavage.

Effect of reducing protein synthesis on tp and the timing of mitotic events

To evaluate whether protein synthesis affects the timing of cleavage and other mitotic events, the time of nuclear envelope breakdown (tn), the time of anaphase onset (ta), and the time of cleavage (tc) were measured, with a low concentration (order of μM) of emetine reducing protein synthesis; and the degree of protein synthesis inhibition was evaluated by measuring tp .

The time of commitment to the first cleavage increased as the emetine concentration increased (Figure 3a). The time of the first cleavage also increased as the emetine concentration increased (Figure 3b). Figure 3c shows the delay in tp_1 , tn_1 , ta_1 and tc_1 , plotted as a function of the concentration of emetine (tp_1 =time for 50% commitment to the first cleavage; tn_1 =time for 50% of the first nuclear envelope breakdown; ta_1 =time for 50% of the first anaphase onset; tc_1 =time for 50% beginning of the first cleavage.) The delays in tp_1 varied with emetine concentration, showing the inhibitory property of emetine on protein synthesis. The delay in the commitment to the first cleavage is in direct proportion to emetine concentration. The delay in the first cleavage (and the other mitotic events in the first cleavage cycle) is also in direct proportion to emetine concentration. This indicates that protein synthesis affects the timing of the first cleavage and the other first mitotic events.

In some cases, the increases in tp_1 as emetine concentration increased were not linear but had a positive curvature. Figure 3d shows an example of such a case, showing similar plots to Figure 3c. The time of commitment to the first cleavage increased linearly with increasing

Figure 1. (a) Time of the first cleavage when 100 μ M emetine was applied at various times after fertilization. Fertilized eggs were transferred to ASW containing 100 μ M emetine at 16 min (Δ), 20 min ($\bullet$), 30 min (Δ), or 40 min ($\square$) after fertilization, and added to a glass laboratory dish filled with 100 μ M emetine ASW. ($\circ$ indicate the control egg sample for the 40 min post-fertilization treatment group.) The time course of the first cleavage of eggs (the number of eggs contained in each sample is about 30) was monitored by time-lapse video microscopy. Each line shows the fraction of eggs that had cleaved at a given time along the abscissa. Further cleavage of non-cleaved eggs was not observed until 5 h after fertilization. No cleavage was observed when 100 μ M emetine was applied at 15 min after fertilization. (b) Plot for an illustration of determination of tp_1 . The ordinate is the final percentage of the first cleavage in each sample displayed in (a) and the abscissa is the time when 100 μ M emetine was applied. tp_1 is determined as the 50% cleavage time in a plot like this.

emetine concentration when relatively lower concentrations of emetine were administered, but the slope increased continuously as emetine concentration increased when higher concentrations of emetine were administered. (Note that the delay in cleavage does not always increase in direct proportion to increasing emetine concentration, but it always increases in similar proportion to the delay in the commitment.)

Figure 3e shows delay in tp_2 , tn_2 , ta_2 and tc_2 under various concentrations of emetine. The concentrations of emetine retard these second mitotic events to an extent similar to the commitment to the second cleavage. This indicates that protein synthesis also affects the timing of the second mitotic events.

Figure 2. Illustration of experiments to determine tp_1 . (a) Diagram of experiments to determine tp_1 . A batch of eggs was fertilized and aliquots were removed at given time intervals into ASW containing 10 μM emetine to stop further protein synthesis (filled arrowhead), then the aliquots were fixed at more than 3 h after fertilization and the percentages of the first cleavage in 100 eggs were measured later. The dark grey areas represent the presence of 100 μM emetine. tp_1 is defined as 50% cleavage time in a plot of the percentage vs. the time when 100- μM emetine was applied (i.e. a plot like figure 1b). (b) Diagram of experiments to determine tp_1 when the rate of protein synthesis is reduced with a low concentration (order of μM) of emetine. Unfertilized eggs were transferred to ASW containing the desired concentration of emetine (open arrowhead) then fertilized. The light grey area represents the presence of a low concentration of emetine. tp_1 is determined in the same way as in (a).

Figure 3. The time of commitment to cleavage and time of cleavage and the other mitotic events under various concentrations of emetine. (a) Measurement of tp_1 under various low concentrations of emetine. Unfertilized eggs were transferred to ASW containing 1 (●), 2 (■), or 3 (▲) μM of emetine and fertilized. (○ indicate the data from a control for the sample to which 1 μM emetine was administered.) Aliquots were then removed at predetermined time intervals into ASW containing 100 μM emetine, and the emetine-inhibited aliquots were fixed and the percentages of cleavage were measured. (b) Time of the first cleavage under various low concentrations of emetine. The aliquots from the same batch prepared above were fixed at predetermined time intervals and the percentages of cleavage were measured. Note that the abscissa is the time of fixation. tc_1 was determined with this plot as 50% cleavage time. (c) tp_1 , the time when 50% eggs had undergone the first nuclear envelope breakdown (tn_1), the time when 50% eggs had undergone the first anaphase onset (ta_1), and tc_1 under various low concentrations of emetine, from (a) and (b) (tn_1 and ta_1 were determined as described in Materials and Methods). The ordinate is the delay in tp_1 (□), tn_1 (△), ta_1 (◇), and tc_1 (○) relative to the control. (d) Another set of data on the time of commitment to the first cleavage and time of the first cleavage under various concentrations of emetine. The ordinate is the delay in tp_1 and tc_1 relative to control. From the batch that was incubated with 4 μM emetine, tn_1 and ta_1 were determined and the delay in tn_1 and ta_1 are also plotted. (e) tp_2 , tn_2 , ta_2 and tc_2 under various low concentrations of emetine. These data were from the same batches that were used in the experiment described in a–c.

Effects of retarding DNA replication on the timing of mitotic events

To confirm that DNA replication influences the timing of mitotic events as described in the Introduction, the effect of retarding DNA replication on the timing of mitotic events was measured, using aphidicolin. Figure 4a shows the delay in the first three cleavage cycles as a function of the concentration of aphidicolin. The delay in all the cleavage cycles is in

Figure 4. Time of early cleavages (and the other mitotic events) in the presence of aphidicolin. (a) The delay in the length of the first three cleavage cycles relative to control, as a function of the concentration of aphidicolin. The time course of the first three cleavage cycles of eggs was monitored by time-lapse video microscopy. Each error bar shows the 99% confidence interval of the difference in the mean for the length of a cleavage cycle, between the sample in the presence of a given concentration of aphidicolin and its control. (b) tn_1 , ta_1 and tc_1 in the presence or absence of 0.1 $\mu\text{g/ml}$ aphidicolin (using gametes that were obtained from a parent different from the parent that was used in (a)). The black area represents the time until NEB, the light grey area represents the time from NEB to anaphase onset, and the dark grey area represents the time from anaphase onset to cytokinesis. (c) Time course of the first cleavage when 10 $\mu\text{g/ml}$ aphidicolin was applied at various times after fertilization, monitored by time-lapse video microscopy. Fertilized eggs were transferred to ASW containing 10 $\mu\text{g/ml}$ aphidicolin at 40 min (▲), 42 min (■), 46 min (△), or 50 min (□) after fertilization, and added to a glass laboratory dish filled with 10 $\mu\text{g/ml}$ aphidicolin ASW. (○ indicate the egg sample that was a control for the sample to which 10 $\mu\text{g/ml}$ aphidicolin was applied at 50 min after fertilization.) Further cleavage of non-cleaved eggs was not observed till 10 h after fertilization.

proportion to the aphidicolin concentration. This suggests that DNA replication affects the timing of cleavages. In other words, feedback control that monitors the completion of DNA replication probably operates in a normal cell cycle. The effect of retarding DNA replication on the timing of the other mitotic events was also examined (Figure 4b). The low concentration of aphidicolin delayed each of the mitotic events to the same extent as the others, indicating that retarding DNA replication delays entry into mitosis and does not affect the time intervals between these mitotic events.

To understand when the feedback control becomes ineffective in preventing the first mitosis, the first round of DNA replication was inhibited by applying 10 $\mu\text{g/ml}$ aphidicolin, which inhibits DNA replication almost completely (Ikegami *et al.* 1978). Figure 4c shows the time course of the first cleavages on groups of eggs to which 10 $\mu\text{g/ml}$ aphidicolin was applied at various times. The application of 10 $\mu\text{g/ml}$ aphidicolin at 40 min after fertilization resulted in almost complete arrest of the cleavage cycle, showing the strong dependence of mitosis on the completion of DNA replication. The application of aphidicolin at 42 min after fertilization strongly delayed the first cleavage in all eggs. The application of aphidicolin at 46 min after fertilization delayed it in about half the eggs, and the application of aphidicolin at 50 min after fertilization delayed it in only small minority of the group of eggs. These results mean that the feedback control becomes too weak to retain their control over the timing of the mitotic events at nearly 50 min after fertilization (in half the population). In other words, the completion of DNA replication as a requirement for execution of the first cleavage is fulfilled at nearly 50 min after fertilization. Therefore, the commitment to the first cleavage, another requirement for execution of the first cleavage, precedes the completion of DNA replication by more than 20 min.

DISCUSSION

Meaning and measurements of the time of commitment

The time of commitment to a cleavage corresponds to the time when embryos have accumulated enough of that protein which requires the most time to accumulate an amount sufficient to allow execution of the cleavage. In the early sea urchin embryo, as well as in early *Xenopus* embryo, this protein is thought to be cyclin. The finding that MPF activity in fertilized eggs whose protein synthesis has been stopped just before 'the time of commitment' is similar to MPF activity in unfertilized eggs (Figure 4 in Genevière-Garrigues *et al.* 1995) supports this expectation. Namely, the time of commitment to the cleavage probably represents the time when cyclin has accumulated to its critical threshold to allow the MPF activation required to execute the cleavage.

In this study, t_p was measured instead of the amount of cyclin in order to understand the degree of protein (cyclin) synthesis inhibition and its effects on the cell-cycle engine. Since the rate of protein synthesis possibly changes during a cleavage cycle, especially when emetine is applied, it is difficult for the methods, which measure the amount of cyclin in eggs, to determine the influence of cyclin synthesis on the timing of mitotic events, unless a critical threshold of cyclin for execution of mitotic events is estimated. In contrast to these molecular biological methods, t_p can be directly compared with the timing of mitotic events using the same physical quantity, time, with similar precision.

Characteristics of inhibition of protein synthesis

The reciprocal of a rate of protein synthesis appears to have a linear relation to emetine concentration, up to at least 10 μM , both *in vivo* (Hogan & Gross 19971) and *in vitro*

(Grollman 1966, Jiménez *et al.* 1977). This property can be explained by the assumption, for example, that an enzyme which catalyses a rate-determining step in protein synthesis obeys Michaelis–Menten kinetics and is inhibited by emetine (Dixon 1953).

If the critical threshold of cyclin, up to which cyclin must accumulate to allow execution of the first cleavage, is constant, it can be expected that the time of commitment to the first cleavage would increase linearly with the concentration of emetine which is administered to reduce protein synthesis. The results for tp_1 fit this expectation when a few μM emetine is applied, but the time increases more than expected when a higher concentration of emetine is applied (Figures 3c and d).

Degradation of cyclin can rationalize the deviation from a linear relation between tp_1 and concentration of emetine: with increasing emetine concentration, the rate of cyclin synthesis decreases and the degradation of cyclin becomes crucial. The arrest of the cleavage cycle by a finite concentration of emetine (Figure 1a) demonstrates the existence of the degradation since, if no degradation of cyclin occurred, the total amount of cyclin would rise above its threshold level—limited protein synthesis exists under high concentration of emetine (Grollman 1966, Hogan & Gross 1971)—and the following cleavages would be observed in time.

The cyclin-driven cell-cycle engine and its regulation by a feedback control that monitors the completion of DNA replication

The experimental results disagree with those expected from the assumption of ‘simplified dependent relationship’ described in the Introduction. This fact indicates that this assumption is too simplistic. The time of the first cleavage was much delayed relative to the control when 100 μM emetine was applied shortly after tp_1 (Figure 1), showing the inadequacy in assuming that none of the processes other than the most dilatory one of the early processes, affects the timing of initiation of a late process. In reality, this result suggests that the amount of cyclin *after* the accumulation of cyclin to its critical threshold affects the timing of mitosis (and that of MPF activation). This property has been predicted by mathematical modeling of the cell-cycle engine (Novak & Tyson 1993), in which positive feedback loops in MPF activation account for the existence of a critical threshold for the total amount of cyclin and a time lag in cyclin-induced activation of MPF. The long delay brought about by addition of 100 μM emetine shortly after tp_1 corresponds to the long lag time on cyclin levels close to the threshold in the model.

With low concentrations of emetine, the time of the mitotic events increases by the same (or less) extent as the increase in tp (Figure 3). One may consider that a delay in tp causes a similar delay in the following mitotic events because cyclin synthesis up to its threshold is an upstream event among mitotic events, but this is not the case. In the presence of emetine, the interval between tp and time of MPF activation should lengthen relative to the control, i.e. the delay in mitotic events should be longer than that in commitment, because the amount of cyclin is always suppressed (this effect seems non-negligible, shown in Figure 1a).

Protein synthesis affects the timing of mitotic events, whereas a feedback control that monitors the completion of DNA replication operates. Since protein synthesis does not affect the first round of DNA replication in sea urchin embryos (Wagenaar & Mazia 1978, Wagenaar 1983, Zhang & Ruderman 1993), this implies that the existence of process(es) affected by protein synthesis, among the processes that generate a signal informing the completion of DNA replication, respond to the signal, and then initiate mitosis.

The blockage of MPF activation by feedback control that monitors the completion of DNA replication can account for the experimental results. The timing of mitotic events is affected by the time of the completion of DNA replication, and furthermore, by the amount of cyclin after DNA replication as indicated above through the time lag in MPF activation after DNA replication. The delay in mitotic events by the application of a low concentration of emetine (Figure 3) is therefore attributable to a decrease in the amount of cyclin by a reduction of the rate of protein synthesis. Numerical simulations based on Novak and Tyson's model, which describes a cell-cycle engine with interference by unreplicated DNA (Novak & Tyson 1993), could fulfill the observed relationships (unpublished data).

As stated above, the timing of cell-cycle events is determined not only by a simplified dependent relationship, i.e. a relationship of necessity, but also by interaction between the processes after the necessity has disappeared: namely, the effect of the amount of cyclin on the timing of mitosis through the time lag between the completion of DNA replication and MPF activation. Therefore, it is important to conduct further studies that aim chiefly at understanding which processes influence the timing of cell-cycle events; for example, complementary experiments on the effect of a small acceleration instead of a small delay of a process, on the timing of mitotic events (although it may be impossible at present to perform such experiments by direct acceleration of the process on intact cells with high precision).

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