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Timing of Events in Mitosis

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Summary

Background: Regulation of the major transitions in the cell cycle, such as G1/S, G2/M, and metaphase to anaphase, are increasingly well understood. However, we have a poor understanding of the timing of events within each phase of the cell cycle, such as S phase or early mitosis. Two extreme models of regulation are possible. A "regulator-controlled model" in which the order of events is governed by the activation of a series of cytoplasmic regulators, such as kinases, phosphatases, or proteases; or a "substrate-controlled model" in which temporal regulation is determined by the differential responses of the cellular machinery to a common set of activators.

Results: We have tried to distinguish between these two models by examining the timing of both biochemical and morphological events in *Xenopus* egg extracts during mitosis. Several proteins respond with different delays to the activation of Cdc2. We have found that the timing of phosphorylation is largely unchanged when these proteins are exposed to extracts that have been in mitosis for various periods of time. Similarly, when *Xenopus* interphase nuclei are added to extracts at different times after the G2/M transition, they undergo all the expected morphological changes in the proper sequence and with very similar kinetics.

Conclusions: Our results suggest that during early mitosis (from prophase to metaphase) the timing of biochemical events (such as phosphorylation) and morphological events (such as structural changes in the nucleus) is at least partly controlled by the responses of the substrates themselves to a common set of signals.

Introduction

The exquisite temporal sequence of events in mitosis was appreciated by histologists before the turn of the 20th century [1, 2]. More recently, the biochemical order of events in cell division has been the subject of intense study, and in the last 15 years there has been significant progress in our understanding. We now have a good appreciation of how entry into S phase and entry into and exit from mitosis are controlled by the oscillation in abundance of a family of regulators called the cyclin-dependent kinases (Cdks) and how these and other events are controlled by specific proteolysis reactions [3, 4]. Although entry into and exit from S phase and mitosis are now better understood, the control of the temporal order of events within these stages of the cell cycle remains unclear. For example, during mitosis, cells undergo ordered structural changes in the nucleus and cytoskeleton that can be observed readily by light microscopy. Centrosome separation, chromosome condensation, nuclear envelope breakdown, spindle formation, and chromosome congression onto the metaphase plate all must occur in a strict temporal order within the mitotic timeframe. In addition, the stages of anaphase and telophase must each be coordinated with cell division. While the entry into mitotic prophase can be understood in terms of Cdc2/cyclin B activation and the passage from metaphase into anaphase can be understood in terms of ubiquitin-dependent proteolysis mediated by the anaphase-promoting complex (APC), we have only a fragmentary picture of the timing of events between these transitions.

Two different models to describe the general temporal regulation of events in the cell cycle were proposed in the 1970s and 1980s. One, based on the concept of a biochemical oscillator, was termed the "clock" theory [5]. The second, termed the "domino" model, was based on the view of the cell cycle as a set of dependent reactions [6, 7]. Today these two extreme views have been unified. The major cell cycle transitions (G1/S, G2/M, and anaphase/metaphase) are controlled by regulators, such as Cdks or APC and SCFs (a complex of Skp1, cullin, and F box proteins), that are not part of basic cell cycle processes, for instance, DNA replication or mitosis. Checkpoints and feedback controls on the regulators, which monitor the events of the cell cycle, ensure the completion of specific processes. While this model describes the regulation of the major transitions, regulation of the timing of events between the transitions is not as well understood. Again, two extreme models are possible (Figure 1). In the first model, which we have termed "regulator-controlled timing," a series of miniregulators, such as a temporal cascade of kinases, would be activated in succession after the G2/M transition. As shown in Figure 1, in this model a cascade of kinases would initiate different events at different times. In the alternative model, termed "substrate-controlled timing," all of the kinases (and phosphatases) are activated nearly simultaneously and would remain stable until the next transition point at metaphase/anaphase. The timing of events between G2/M and metaphase/anaphase would have to be controlled by the individual responses of the particular substrates, such as by the kinetics of multiple phosphorylations or by slow conformational changes. The postulate of two extreme models is heuristic, and we might expect that in reality both would contribute. The question we have raised in this

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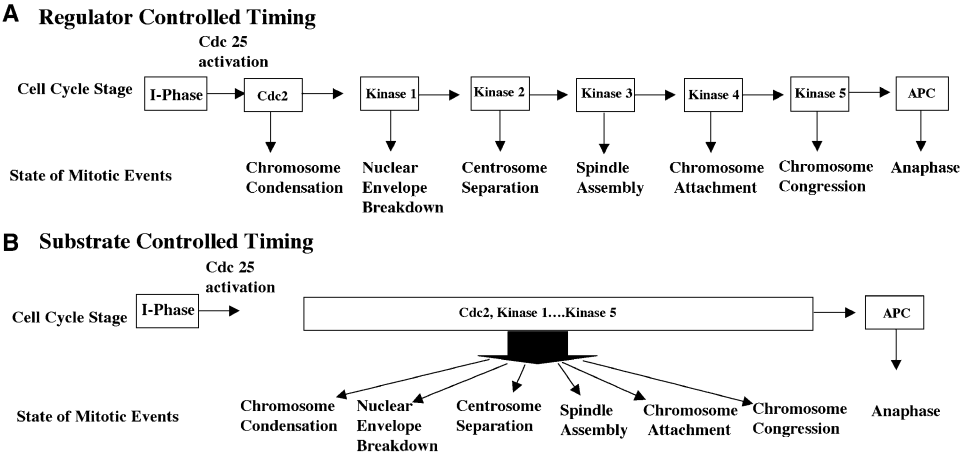

Figure 1. Alternative Models for Regulation of Mitotic Progression

(A) Regulator-controlled timing: the temporal regulation of mitotic events is controlled by sequential activation and inactivation of regulators in the extract. In this model, mitotic events are governed wholly by sequential regulation of activators such as kinases and phosphatases (here shown as kinases).

(B) Substrate-controlled timing: the timing of events in mitosis is regulated solely by properties inherent in and interactions between the substrates and therefore is not dependent on a sequence of extrinsic regulatory steps. These two models are not mutually exclusive; therefore, a third possibility is that regulation of the mitotic clock is controlled by a combination of regulator- and substrate-controlled timing programs. I-Phase, interphase.

paper is whether we can find evidence for either of these mechanisms.

The *Xenopus* egg extract has proven to be a useful system for dissecting the biochemical steps underlying the simplified embryonic cell cycle. Previous experiments have shown that egg extracts will recapitulate both the biochemical oscillations of Cdc2/cyclin B activity and the morphological changes in the nucleus in the absence of the usual cell cycle targets, the nucleus, spindle, and centrosome [8, 9]. In addition, the requirement for cyclin synthesis and the role of various phosphorylation and dephosphorylation reactions in the egg extract have been studied extensively [8, 10–12]. Some phosphorylations specific to mitosis can be directly related to morphological changes, such as phosphorylation of the lamins [13–15], the Golgi protein GM130 [16], the microtubule regulators stathmin and Xmap [17–19], and the chromosomal protein histone H3 [20, 21]. Various components of the regulatory machinery, such as Cdc25 [22, 23], Cdc2 [24, 25], APC [26–28], separase, and securin [29], are regulated by phosphorylation during mitosis. These observations suggest that a study of the timing of phosphorylation reactions during mitosis can be used to follow many biochemical steps in mitosis.

Here we ask to what extent the temporal progression from G2/M to metaphase in *Xenopus* extracts is governed by regulator-controlled timing or substrate-controlled timing mechanisms. Our approach is to impose a temporal separation between the substrates and the regulators, not unlike the experimental design of the cell fusion experiments that helped establish the dominance of the mitotic state and the existence of checkpoint controls [30, 31]. We have initiated Cdc2 activation in extracts by the addition of cyclin B1 and then added various marker substrates after different delays to observe whether the characteristic phosphorylation of these proteins reflects the time the extract has progressed after Cdc2 activation or merely the time the marker proteins have been exposed to the mitotic extract. If the temporal sequence of events in mitosis is under simple regulator control, the timing of phosphorylations should be strongly affected by the state of the extract (i.e., length of time the extract has been incubated after Cdc2 activation). If, however, the timing of these events is under substrate control, then the phosphorylation of each substrate would occur at its own rate, regardless of whether the extract had just activated Cdc2 or whether the extract had been incubated for a longer time after Cdc2 activation. Finally, instead of adding individual proteins we added whole nuclei and examined the morphological steps leading to metaphase. In this case, we asked whether the timing of chromosome condensation, nuclear envelope breakdown, or metaphase congression depends on when the nuclei were added to the extract relative to Cdc2 activation. The results point to the importance of substrate control in this rather simplified cell cycle and suggest that if regulator control plays a part in other systems, it may sit on top of a fundamental process of substrate control to orchestrate the disparate events of mitosis.

Results

The Timing of Protein Phosphorylation during Mitosis

Previously, we identified 40 mitotic phosphoproteins in a small pool screen by comparing gel mobility patterns of cyclin-dependent protein phosphorylation in *Xenopus* interphase and mitotic egg extracts ([32] and A.B.G., P.T.S., and M.W.K., unpublished data). In this study, we followed the time course of phosphorylation during mitosis by the mobility (shift) for several of these proteins

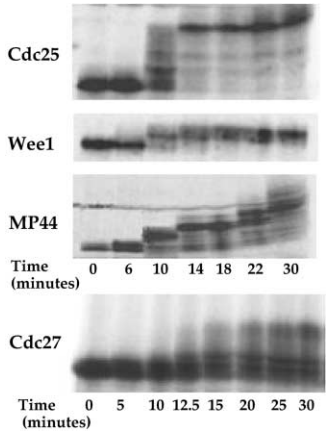

Figure 2. The Timing of Phosphorylation during Mitosis Occurs Later for Some Proteins

In vitro-translated, ^{35}S-labeled proteins and nondegradable GST-cyclin B1 were added to interphase extracts at time 0, and the reactions were incubated at room temperature. At each time point, 1 μl of the reaction was withdrawn and added to 7 μl of sample buffer for gel mobility analysis. The labels below the panel represent the time in minutes since GST-cyclin B addition to the extract.

(Figure 2) (see Table 1 for a more complete list). Neither the degree nor the timing of the shifts necessarily reflects the extent of phosphorylation. Instead, these gel shifts may reflect specific phosphorylation events. The timing of the shifts varies widely. Cdc25 and Wee1, two important activators of the G2/M transition, were phosphorylated completely, as measured by gel shift, between 8 and 12 min after addition of cyclin B to interphase extracts. The abrupt Cdc25 shift is coincident with H1 kinase activation signaling the transition into M phase (data not shown). In contrast, Cdc27, a key regulator of the exit from mitosis, and MP44, an unidentified mitotic phosphoprotein with molecular weight of 44 kD [32], both required a longer period of time to become fully phosphorylated. These proteins, along with several others, showed a continuous pattern of shifts requiring 30–40 min to complete. In all of these proteins, the gel shift has been correlated with phosphorylation ([22, 23, 33, 34] Cdc25; [35–37] Wee1; [26, 38] Cdc27; [32] MP44). These results indicate that the timing of phosphorylation during mitosis varies greatly for different proteins.

In Vitro-Translated Cdc27 Is Incorporated into the Endogenous APC in *Xenopus* Extracts

The Cdc27 phosphorylation experiment contained some ambiguity in that we did not know if the in vitro-translated (IVT) Cdc27 was phosphorylated in a free state or whether it was being phosphorylated after being incorporated into the 1500 kD APC complex [38, 39]. We therefore assayed for exchange of in vitro-translated Cdc27 into APC by sucrose gradient sedimentation. The IVT Cdc27 protein was either preincubated for 30 min in an aqueous buffer or in an interphase egg extract. In the experiment involving preincubation in buffer, most of the protein sedimented at 9S, a little faster than might be expected for monomeric Cdc27, and none sedimented at 20S. In contrast, about 20% of the Cdc27 protein that had been premixed with extract sedimented at 20S, the same position as has been reported for endogenous *Xenopus* APC [38] and also the position identified with an antibody to the APC subunit APC2 (data not shown, see Experimental Procedures). These results suggest that some of the IVT Cdc27 is rapidly exchanged into APC in extracts. Though not all of the Cdc27 ex-

Table 1. Summary of the Timing of Shifts of Identified Mitotic Phosphoproteins

Clone	Homolog	Possible Function	GenBank Accession Number	Timing of Phosphorylation
Cdc25[a]	Cdc25C	cell cycle	AAA49671	early
Wee1	Wee1	cell cycle	AAB99952	early
MP45	glycogenin	glycosyltransferase	AF419148	early
Xn NO38	NPM	nucleolar-localized protein	CAA39511	middle
MP42[c]	none	unknown	U95103,U95104	middle
Map kinase interacting kinase		signal transduction	AF419156	middle
Cdc27[a]	Cdc27	cell cycle	P30260	late
MP22	mFrizzled	signal transduction	AF419149	late
MP36	hSet07	unknown	AF419150	late
MP38	hcDNA ZD76B08	unknown	AF419151	late
MP39	hTAR	DNA binding	AF419152	late
MP43[b,c]	none	unknown	U95097	late
MP44[c]	none	unknown	U95098	late
MP67	none	unknown	AF419153	late
HoxD1[c]	xHoxD1	transcription factor	AAA03480.1	late
MP77	Hs PRGP1	proline rich Gla protein	AF419154	late
Groucho	xESG	transcription	AAG01027	late
MP140	ZABC1	putative transcription	AF419155	late

Early, shift begins at the G2/M transition (5–10 min PCA [post cyclin addition], coincident with H1 kinase activation) and is complete by 15 min PCA; middle, shift begins at 5–10 min PCA and is complete by 20 min PCA; late, shift begins 5–15 min PCA and is complete by 25–40 min PCA.

[a] Known mitotic phosphoprotein but not identified in our screens.

[b] Exhibits very early shift coincident with Cdc25 and Wee1, but this shift is not cyclin B dependent. Late shift is cyclin B dependent.

[c] Mitotic phosphoprotein previously identified and published in reference [29].

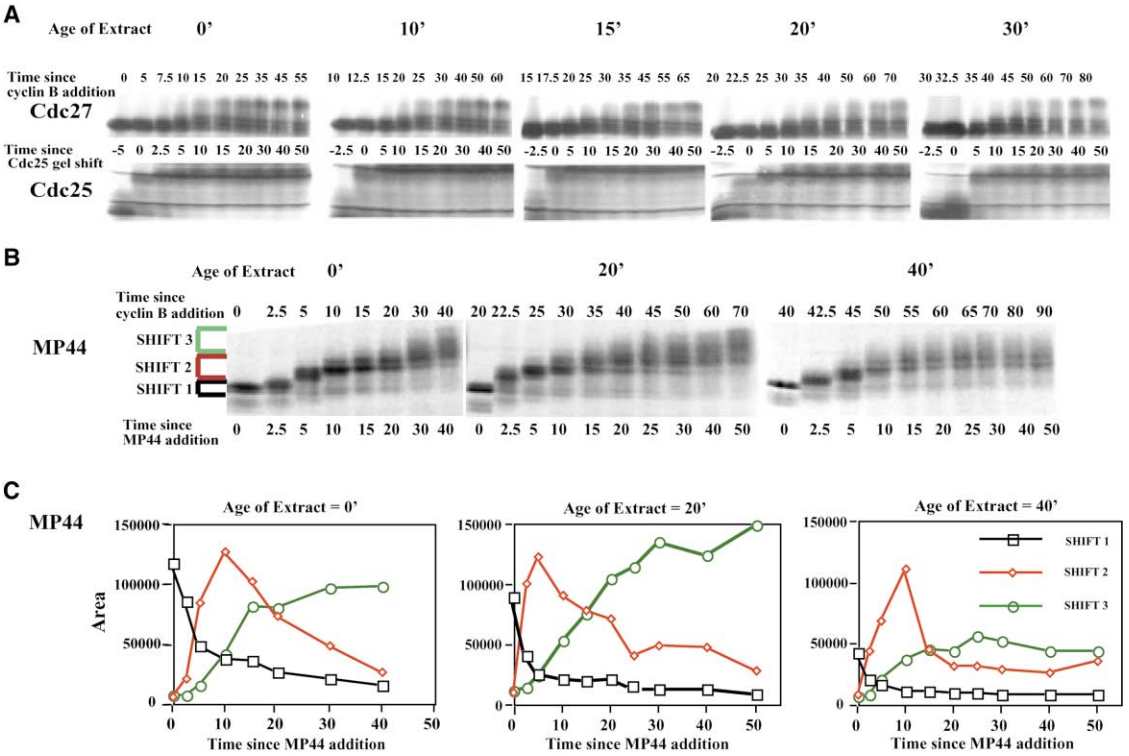

Figure 3. The Lag Time between the Cdc25 Shift and the Cdc27 or MP44 Hypershifts Is a Function of the Substrates and Not the Presence of Regulators in the Extract

Both Cdc27 ([A], top panel) and MP44 (B) are phosphorylated in steps and require more time in mitotic extracts to become fully phosphorylated than Cdc25 ([A], bottom panel), which shifts abruptly at the G2/M transition. (A) In vitro-translated Cdc27 labeled with [^{35}S]methionine was added to *Xenopus* interphase egg extracts at room temperature for 40 min to allow exchange of IVT protein into the APC. The extract plus Cdc27 and IVT ^{35}S-labeled Cdc25 were added simultaneously to extracts at 10, 15, 20, or 30 min post cyclin B addition, and samples were taken for gel analysis. Control reactions contained IVT Cdc27 and Cdc25 added to interphase extracts at the same time as cyclin B (0 min). The top set of labels above the panel, Age of the Extract, refers to the length of time between addition to the extracts of GST-cyclin B and substrate. Time points immediately above the panels indicate the cumulative time since addition of GST-cyclin B to the extracts, and those below the panels indicate the length of time since Cdc25 and Cdc27 were added to the extracts. In the latter case, the 0 min time point is set to the Cdc25 shift as an internal reference point. (B) IVT MP44 protein was added to interphase extracts at the same time as GST-cyclin B or at 20 or 40 min after cyclin B addition. The top set of labels represents the Age of the Extract as described above. The labeling of the time points above the panels is the total time after Gst-cyclin B addition as above, while the time points below the panel indicate the elapsed time from substrate (MP44) addition. (C) Graph of three domains of the MP44 shift. The intensity of the signal for each time point was calculated by integrating of the values determined by densitometry tracings of the gels for each of the shifts (see Experimental Procedures).

changed into endogenous APC, we found that all of the phosphorylated protein ran at 20S; the monomeric fraction showed no gel shift at all (data not shown). Because only Cdc27 incorporated into the APC is phosphorylated, we improved our assay by adding the IVT Cdc27 to an interphase extract for 40 min before using it in all subsequent experiments to allow the protein exchange into APC.

The Phosphorylation Rate of Late-Shifting Proteins Is Unchanged When They Are Added to Extracts at Various Times after Entry into Mitosis

In vitro-translated and labeled Cdc27 that had been preincubated in interphase extract to allow exchange into APC (see Experimental Procedures) was diluted into extracts at either the same time as glutathione-S-transferase-tagged *Xenopus* cyclin B1 (GST-cyclin B) (Figure 3A, 0 min) or at various times after addition of GST-cyclin B (Figure 3A, 10 min…40 min). In every experiment, we determined the time of mitotic entry by two independent methods. First, the activation of Cdc2 kinase was followed in an HI kinase assay [40]. Second, we added an IVT N terminus fragment of Cdc25, which gives the dramatic shift due to phosphorylation but lacks a phosphatase domain and therefore does not affect cell cycle progression. For clarity in exposition, two time lines are shown in Figure 3: the time after GST-cyclin B was added to the interphase extract (shown above the lanes) and the time for which Cdc27 and Cdc25 were incubated (shown below the lanes). For the latter, 0 time is set as the time at which Cdc25 shifted—an internal timing control that allows more accurate comparison between experiments. Thus, in the initial lane of the 0 min extract, GST-cyclin B, Cdc27, and Cdc25 were added simultaneously. The time shown below the lane, −5 min, indicates that it is 5 min before the Cdc25 gel shift. Similarly, in the 20 min extract example, the first lane corresponds to the time when the labeled Cdc27 and Cdc25 were added, which is 20 min after cyclin B was added. Below

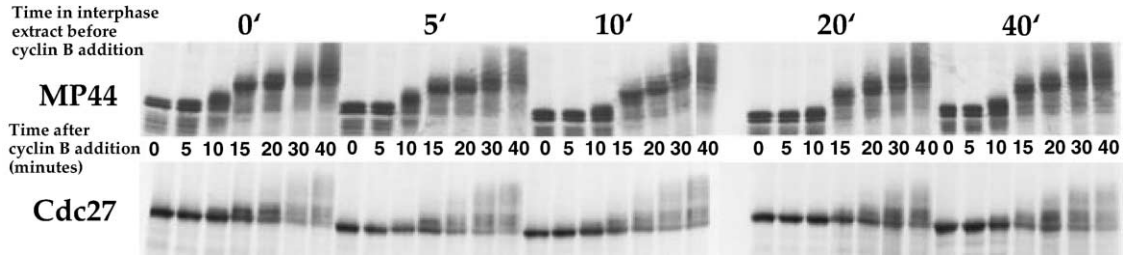

Figure 4. The Timing of the Phosphorylation Shifts of Cdc27 and MP44 Is Unaffected by Incubation in Interphase Extracts before Activation by Cyclin B

Labeled, in vitro-translated substrates were incubated in interphase extracts for 0 min, 5 min, 10 min, 20 min, or 40 min (numbers above the panel) prior to addition of GST-cyclin B to drive the extracts into mitosis. The reactions were incubated at room temperature and samples were withdrawn for gel analysis at the indicated times (numbers between the panels).

the lane, the time is indicated as −2.5 min, because it is 2.5 min before the Cdc25 gel shift. Therefore, the next gel lane to the right, which is the time of the Cdc25 shift, represents 22.5 min after cyclin B was added and 2.5 min after the marker substrates were added.

The Cdc25 gel shift and H1 kinase activation (data not shown) generally occurred 8–10 min after the addition of cyclin B to an interphase extract. The abrupt shift of Cdc25 clearly marks the transition. When Cdc25 was added to an extract that was already in M phase, the shift was much quicker (complete in 2.5–5 min). Conversely, as shown in Figure 3A, the delay in the initiation and the rate of Cdc27 phosphorylation remained the same relative to the Cdc25 shift (most easily appreciated by looking at the lower time line). Whether the Cdc27 was added to extracts before the G2/M transition point or 10, 15, 20, or 30 min thereafter, the major phosphorylation shift came between 10 and 20 min after the Cdc25 and H1 kinase shifts.

The results with a second protein, MP44 (Figure 4B) support the interpretation that the delay in phosphorylation is independent of the time since the G2/M transition. MP44 was identified in the screen for mitotic phosphoproteins. It has homologs in humans, mice, *Drosophila*, and *C. elegans*, but its function is unknown. We added in vitro-translated MP44 directly to extracts either at the same time as GST-cyclin B (Figure 3B, 0 min) or at 20 or 40 min after GST-cyclin B addition (Figure 3B, 20 min and 40 min). In the 0 min experiment, the initial MP44 shift coincided with the entry into mitosis, but the later hypershift was not complete until 25 min later. When the protein was added at either 20 min or 40 min after GST-cyclin B, the relative timing of the shifts is the same as when it was added at 0 min. The initial MP44 shift occurs rapidly when added to an extract already in mitosis coincident with the Cdc25 shifts; however, in all cases the MP44 hypershift is not complete for another 20–25 min.

To analyze the gel shifts more carefully, we divided the MP44 region of the gel into three domains indicated in Figure 3B. As shown in the graph in Figure 3C, the black line corresponds to the initial shift position of MP44 initiated immediately following the G2/M transition. (It is completed even more rapidly than Cdc25.) The red line corresponds to an intermediate hypershifted position, and the green line represents the most retarded hypershifted position. In all three extracts (0, 20, and 40 min after the addition of GST-cyclin B), the initial (black) shift occurs almost immediately. Its abundance declines quickly, with a half-time of less than 5 min, irrespective of how long the extract has been in mitosis. The intermediate (red) hypershift accumulates to a maximum in 5–10 min after entry in mitosis in all three conditions. Finally, the abundance of the late-shift species (green) increases to a plateau 20–30 min after GST-cyclin B addition. The level of the plateau is decreased in the 40 min extract, which may reflect some changes in the extract following the start of APC-mediated protein degradation. Several other late-shifting proteins identified in the screen also show this pattern of delayed phosphorylation in mitotic extracts relative to Cdc25 (see Table 1). This result suggests that while the phosphorylation of Cdc25 occurs soon after the activation of Cdc2 by cyclin B in the extract, the rate of phosphorylation of Cdc27 and MP44 depends on factors peculiar to these substrates and not on the state of regulators in the extract.

One explanation for the observed delay in the shift of these two proteins could be that time is required for the proteins to interact with cofactors in the extract to be properly folded or exchanged into the endogenous APC as shown for Cdc27. To test this possibility, we added IVT Cdc27 or MP44 to *interphase* extracts for various times before adding GST-cyclin B (Figure 4) then followed the time course of shifts. The timing of the shifts under these conditions was the same as in the 0 min extract. Therefore, the delay in the timing of phosphorylation of Cdc27 or MP44 in mitotic extracts is not due to the time required either for a conformational change in the translated protein or, in the case of Cdc27, for exchange into endogenous APC.

Morphological Changes in Interphase Nuclei Added at Different Times to Mitotic *Xenopus* Extracts

The experiments discussed above describe phosphorylation reactions in mitosis somewhat arbitrarily chosen for ease of measurement. We wanted to know whether these same regulatory properties are found in more complex processes. In particular, we asked whether we could find evidence for regulator-controlled timing or substrate-controlled timing in the morphology of the nucleus and microtubule arrays. To answer this question, we added interphase nuclei, formed from frog sperm [40], to extracts that had been driven into mitosis

for various times and assayed the tempo of structural changes of these nuclei as the extracts proceeded into and through mitosis. When interphase nuclei are added to an extract in which the Cdc2 has been activated by cyclin B, they undergo the same morphological changes as those that occur in mitosis in vivo: chromosome separation, nuclear envelope breakdown, and congression of chromosomes onto the metaphase plate [40]. The extract also faithfully recapitulates events in the cytoplasm of a cell: formation of asters and, subsequently, bipolar spindles; however, they fail to undergo cytokinesis.

To analyze the timing of these structural changes in heterochronic extracts, we performed experiments analogous to those used to study the timing of mitotic phosphorylations. We added interphase nuclei either to interphase or mitotic extracts at 10 min or 30 min after GST-cyclin B addition. Rhodamine-labeled tubulin also was added to follow the formation of asters and bipolar spindles. The extracts were prevented from entering anaphase by the addition of purified MAD2 [41–44] so that morphological changes would not proceed beyond the formation of bipolar spindles and congression of chromosomes to a metaphase plate.

Figure 5A shows that the Cdc25 shift in this experiment is complete by 8 min following GST-cyclin B addition; therefore, this time point has been taken as the start of mitosis (time in mitosis = 0 min). We determined the percentage of nuclei showing nuclear envelope breakdown (Figure 5B). The precision of timing in different extracts is within 5 min. As indicated in Figure 5B, 50% of the nuclei show nuclear envelope breakdown by 5–10 minutes after the Cdc25 shift, irrespective of whether the extracts had been exposed to GST-cyclin B for 0, 10, or 30 min prior to addition of nuclei.

In addition to changes in nuclear morphology, extensive structural changes also occur in the cytoplasm during mitosis. The timing of the appearance of rhodamine-labeled asters and bipolar spindles and chromosome congression onto the metaphase plate was also determined (Table 2). We find that asters first appear coincident with nuclear envelope breakdown at 8–10 min after the nuclei are added to the extract, regardless of how long the extracts have been in mitosis. Further, the timing of the appearance of bipolar spindles and metaphase figures is similar for all three conditions, suggesting that the control of the timing of these cellular changes is also independent of the state of regulators in the cytoplasmic extract.

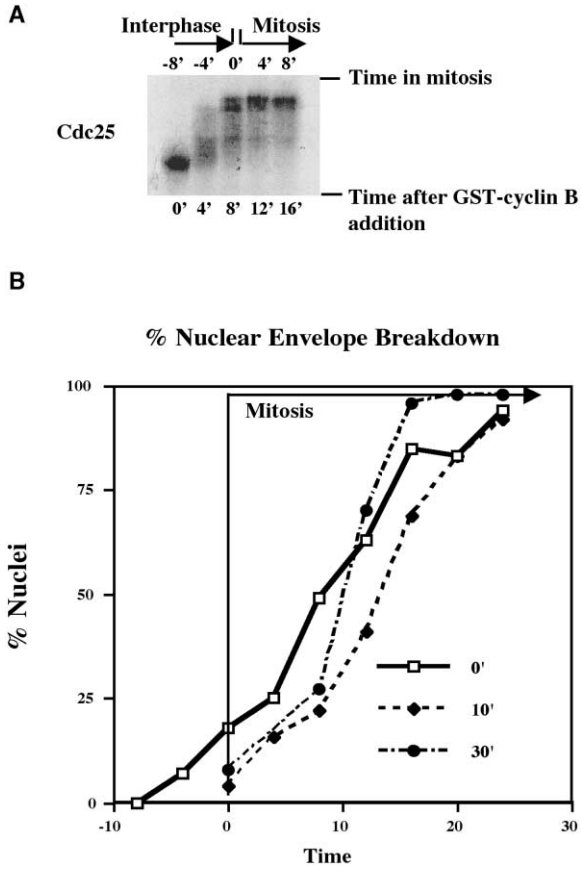

Figure 5. The Timing of Changes in Nuclear Morphology Is Similar Whether Interphase Nuclei Are Added to Interphase Extracts or to Extracts at Different Times during Mitosis

Interphase *Xenopus* nuclei were added to extracts + rhodamine tubulin at the same time as GST-cyclin B or at 10 or 30 min post cyclin addition. At 4 min intervals, 1 μl of extract plus nuclei was removed, fixed, and stained for microscopic examination. Nuclei at each stage were counted for every time point. (A) Cdc25 phosphorylation shift indicating that the G2/M transition in this experiment occurred 8 min after addition of cyclin B. (B) Graph showing the percent of nuclei at the stage of nuclear envelope breakdown for each time point. The solid vertical line represents the relative time at which the nuclei in each of the experiments were first exposed to a mitotic extract (8 min for the 0 min experiment, and 0 min for the 10 min and 30 min experiments). Open squares, 0 min; closed diamonds, 10 min; closed circles, 30 min; time of addition of nuclei to the extract following cyclin B addition.

Discussion

The regulation of the major transitions in the cell cycle, G1/S, G2/M, and metaphase/anaphase, is fairly well understood. However, the regulation of the sequence and timing of events within each stage of the cell cycle has received much less attention. We propose for discussion two extreme models to describe the possible mechanisms for temporal and sequential regulation of events during mitosis (see Figure 1). In the regulator-controlled timing model, successive regulators, such as kinase cascades, are activated (or inactivated) in sequence, and each has a direct effect on its substrate. In contrast, in the substrate-controlled timing model, a single trigger or regulator rapidly initiates a new regulatory state (i.e., mitosis) in which each subsequent reaction is regulated by the peculiar chemistry of the substrates themselves. We have used both biochemical (protein phosphorylation) and morphological (changes in nuclear and cytoplasmic structure) studies to try to distinguish between these two models for the early events in mitosis leading to metaphase. We have used the *Xenopus* cell extract system cognizant that it may not possess all the regulatory features of somatic cells. In fact, no system is likely to possess all the regulatory features found in nature.

The timing and rate of phosphorylation differ for differ-

Table 2. Summary of the Timing of Morphological Changes in Interphase Nuclei Added to Mitotic Extracts

	Time Nuclei Were Added after Gst-cyclin B (min)		
Nucleus State	0	10	30
A	0–16	0–8	0–4
B	4–16	0–12	0–10
C	10	8	8
D	10	8	8
E	16	12	16
F	24	20	20

A, time period for >40% nuclei as interphase; B, time period for >20% nuclei with condensed chromosomes; C, time from first appearance of nuclear envelope breakdown; D, time from first appearance of asters; E, time from first appearance of bipolar spindles; F, time from first appearance of metaphase figures.

ent proteins during mitosis. Cdc25 and Wee1 are known to be phosphorylated at the G2/M transition [12, 34, 35,] and represent the earliest shifts we found. Although in our screens for mitotic phosphoproteins we found a few proteins that shifted at nearly the same time as Cdc25 and Wee1 (see Table 1), we did not find any proteins that shifted earlier during the lag phase. In addition, the shifts for Cdc25 and Wee1 are coincident with the activation of histone H1 kinase activity and may be the crucial signal for entry into mitosis.

Exit from mitosis and progression into anaphase requires activation of the APC, a multisubunit complex that targets specific proteins for degradation via the ubiquitin pathway. We have looked in detail at the timing of phosphorylation of one of the subunits of the APC, Cdc27, and found that it is specifically phosphorylated in steps over a prolonged period of time beginning a few minutes after the Cdc25 shift is complete and extending later into mitosis. Several previous studies have shown that phosphorylation of the APC in mitosis is required for high levels of activation [28, 44, 45]. Furthermore, Cdc20 binding to APC depends on the phosphorylation of at least some of its subunits [27, 46, 47]. It seems clear that the temporal regulation of APC activation must be tightly controlled to prevent the cell from exiting mitosis prematurely. In somatic cell cycles, the timing of anaphase is regulated by feedback from the spindle assembly checkpoint [4]. In yeast, this checkpoint is not essential for viability under laboratory conditions, and the intrinsic timing mechanism such as the one we describe for Cdc27, may be sufficient. In mammalian cells, however, MAD2 knockouts are not viable, indicating that MAD2 plays a more essential role in these cells [48]. As expected, such an important event as the onset of anaphase is under multiple layers of regulation built on top of the intrinsic timing mechanisms described here.

Cdc27 is not the only protein we have studied with this pattern of prolonged phosphorylation lasting throughout mitosis. MP44, a protein with unknown function, also behaves this way. Significantly, neither Cdc27 nor MP44 shift more rapidly when they are introduced into a late mitotic environment. When Wee1 and Cdc25 are added to extracts already in mitosis, they respond with a very rapid and immediate shift, irrespective of how long the extracts have been in mitosis. Cdc27 and MP44, however, still demonstrate characteristic delayed and prolonged patterns of phosphorylation shifts. If the phosphorylation of Cdc27 and MP44 were controlled by regulators that are activated in a temporal pattern after the G2/M transition, then adding these substrates to late mitotic extracts should produce an accelerated pattern of shifts. As this is not the case, we suggest that phosphorylation is regulated by timing properties intrinsic to these proteins and not by changing conditions in the mitotic extract. These intrinsic mechanisms could include the relative rates of phosphorylation and dephosphorylation at specific sites, the slow build up of multiply phosphorylated species (including positive or negative cooperativity), and slow conformational chaperone association and dissociation reactions.

During mitosis, the cell undergoes dramatic changes both in nuclear and cytoplasmic morphology, and the sequence and timing of these events also must be tightly regulated. Chromosome condensation, nuclear envelope breakdown, spindle formation, and progression of chromosomes onto the metaphase plate must occur before the onset of anaphase and sister chromatid separation. In experiments similar to those described for measuring the rates of phosphorylation of various proteins in mitosis, we compared the timing of these morphological events by adding interphase nuclei to extracts at different times either before or after Cdc2/cyclin B activation. The most obvious conclusion is that nuclei added to extracts that had been in the mitotic state for 30 min still underwent the typical sequence of nuclear and cytoplasmic events. Furthermore, there was no significant acceleration in timing when nuclei were introduced directly into late mitotic extracts. If these events are regulated solely by the activation and inactivation of factors in the cytoplasm, then we might expect that the timing and even the sequence of the structural changes during mitosis would be affected by adding interphase nuclei to an extract 10 or 30 min after cyclin B addition. The fact that the timing and sequence of these events are unaffected under these conditions suggests that, as with Cdc27 and MP44, substrate-controlled timing plays an important role in the ordering of events in prophase, prometaphase, and metaphase.

These experiments give a broad indication that the control of the detailed timing of events in early mitosis depends on the individual responses of the substrates to a common set of regulators. Although there were slight indications that changes might be occurring in these extracts, reflected in small differences in phosphorylation patterns, we found little or no evidence for a succession of regulatory states. Yet, we are not prepared to rule out regulatory control. These experiments were done in a system where the nuclear volume is infinitesimal compared to the cytoplasmic volume. Although this is the physiological state of early embryos, it is not the situation for somatic cells, and there is evidence that nuclear localization of cyclin B and Wee1 can affect timing [49–52]. In somatic cells, Cdc2/cyclin A appears to be activated and inactivated before Cdc2/cyclin B [51], and, even in cycling frog extracts, Minshull et al. [53] showed a succession of accumulation of different cyclin isotypes. This also suggests that a cascade of regulator control could play a role in mitosis. However,

demonstrating a regulatory cascade imposed on top of the substrate control, which we have identified here, will require stringent functional tests. While it is suggestive that several genes show cell cyclic periodic accumulation during the cell division cycle (416 of 6200 genes in yeast) [54, 55], each must be tested rigorously to determine whether the timing of expression has important functional consequences. Furthermore, transcriptional or translational regulatory mechanisms are unlikely to be fast enough to coordinate the events within mitosis. The results provided here might be seen as the core regulatory processes of mitotic timing in much the same way as the frog egg cell cycle oscillations represents the core machinery in all mitotic cycles. We would expect that other forms of regulator control might overlay the core features of substrate control that we have demonstrated. As yet, our experiments have provided little information on how substrates are engineered to respond differently to a common set of signals. There are likely to be many different mechanisms reflecting the biochemical diversity of different substrates. Further study of the kinetics of phosphorylation will help to complete the structural understanding of the mitotic process.

Experimental Procedures

Materials

All chemicals were supplied by Sigma, unless otherwise stated. Anti-APC2 antibodies have been previously described [27]. Anti-Cdc27 monoclonal antibodies were purchased from Transduction Laboratories. Rhodamine tubulin was purchased from Cytoskeleton and used as recommended by the manufacturer. MAD2 was prepared from a construct provided by Guowei Fang as described [41]. *Xenopus* GST-cyclin B1 was expressed in *E. coli* and purified by glutathione affinity chromatography as previously described [53]. A Cdc25 mutant was generated containing the N-terminal regulatory domain but lacking the C-terminal phosphatase. This protein is phosphorylated and shows strong gel shifts but is not active in the extract [56]. This construct was shuttled into pCS2+ by Xba1/Not1 restriction digestion and ligation for expression in IVT reactions.

Preparation and Analysis of Mitotic Phosphoproteins

The small pool screen of cDNA libraries was performed as described [32]. IVT proteins were prepared by using the TNT Coupled Reticulocyte Lysate System from Promega and ^{35}S methonine from New England Nuclear, as previously described [32]. Interphase *Xenopus* egg extracts were prepared as described previously [40]. Mitotic extracts were prepared from interphase extracts by addition of 66 nM nondegradable *Xenopus* GST-cyclin B. Progression of the extract from interphase into mitosis was followed either by analysis of Cdc25 phosphorylation using gel shift as a marker or by measuring the activity of H1 kinase in the extract as previously described [40].

The timing of phosphorylation was followed by gel motility retardation of IVT, ^{35}S-labeled proteins. Cdc25, Wee1, and MP44 IVT proteins were diluted 1:9 in either interphase or mitotic extracts and incubated at 25°C. Cdc27 IVT was added to interphase extracts at a dilution of 1:2 at room temperature for 30 min to allow exchange of the IVT protein into APC in the extract. The mixture was then diluted 1:5 into either interphase or mitotic extracts. The phosphorylation reactions were terminated by addition of 7 μl of sample buffer (10% glycerol, 50 mM Tris [pH 6.8], 2% SDS, 0.1% Bromophenol blue) to 1 μl of the reaction. The labeled proteins were resolved on 5%–15% gradient SDS-PAGE gels and visualized by autoradiography. Quantation of the ^{35}S signal for the three different regions of the MP44 shift was obtained by scanning the gels and integrating the Plot Density function on a BioRad Molecular Imager FX Phosphorimager.

Measurement of Cdc27 Incorporation into APC

The level of incorporation of IVT Cdc27 protein into APC in the extract was determined by comparing the sedimentation coefficient of IVT Cdc27 plus buffer (80 μl IVT reaction: 80 μl extract buffer [100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.7), 50 mM sucrose]) to Cdc27 incubated in extracts (80 μl IVT reaction: 80 μl extract) by sucrose gradient centrifugation using a 10%–30% gradient for 17 hr at 37,000 rpm in a Beckman SW40 rotor. IVT reactions were incubated either with extract buffer or *Xenopus* interphase extracts at room temperature for 30 min before loading. Fractions (0.6 ml) were collected, TCA precipitated (180 μl sucrose fraction, 20 μl 2.25 M TCA, 4°C for 30 min, centrifuged for 10 min at 14,000 rpm in Eppendorf centrifuge, and the pellet solubilized in SDS-PAGE sample buffer, the pH of the precipitate was adjusted with 1–2 μl of 1 M Tris [pH 8.8]), and analyzed on 5%–15% SDS-PAGE gels. Sedimentation coefficients were determined by comparison to standards fractionated in parallel (BSA, 4.41 S; adolase, 7.355 S; catalase, 11.25 S; thyroglobulin, 19.55 S).

The sedimentation coefficients for the endogenous APC2 and Cdc27 in the *Xenopus* extracts were determined by immunoblots [57] of the sucrose gradient fractions. For Western blots, 15 μl of the fractions was run on 5%–15% polyacrylamide gradient gels, and the proteins were transferred onto Schleicher and Schuell Protran nitrocellulose (catalog number 10402480) using a BioRad TransBlot SD Transfer Cell. The immobilized proteins were detected with either anti-APC2 polyclonal or Cdc27 monoclonal antibodies and were visualized by autoradiography using ECL (catalog number RPN2106) from Amersham Pharmacia Biotech.

Sperm Nucleus Preparation

The preparation of sperm nuclei was carried out using a modification of the Murray method [40]. Male frogs were injected with 25 U PMSG 3 days prior to the procedure and then with 50 U HCG 2 days later. The testes were removed and rinsed once in ice-cold 1× MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2m M $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES [pH 7.8]) and then twice in ice-cold XN (50 mM HEPES KOH [pH 7.0], 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine·4 HCl, 0.15 mM spermine·4 HCl). The testes were placed in 5 ml of ice-cold XN and macerated with clean forceps until no clumps remained. The suspension was filtered through eight layers of cheesecloth, and the cheesecloth was then rinsed with an additional 5 ml of cold XN. The sperm were centrifuged for 5 min at 4.5K using a Sorvall HB-6 rotor. The resulting pellet was washed three times with fresh cold XN buffer and resuspended in 500 μl XN. The sperm were demembranated by addition of 100 μl 2 mg/ml lysolecithin. The progress of demembranation was followed at 1 min intervals by staining 1 μl of the sperm mix with 4 μl of 1 μg/ml Hoechst 33342 and observing the sperm under a microscope until all the sperm heads stained (about 10 min). The reaction was quenched by addition of 9.5 ml XN containing 3% BSA (Sigma, fraction V). The sperm were centrifuged and washed three times with cold XN. Following the final rinse, the sperm were resuspended in XN plus 50% glycerol and small aliquots were stored at −80°C.

Interphase nuclei were prepared from *Xenopus* sperm; interphase and mitotic nuclei were fixed and stained as previously described [40].

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References

1. Flemming, W. (1882). Zellsubstanz, Kern und Zelltheilung (Leipzig: FCW Vogel).
2. Wilson, E.B. (1928). The Cell in Development and Heredity, Third edition (New York: Macmillan).
3. King, R.W., Jackson, P.K., and Kirschner, M.W. (1994). Mitosis in transition. Cell *79*, 563–571.
4. Hixon, M.L., and Gualberto, A. (2000). The control of mitosis. Front. Biosci. *5*, d50–57.
5. Murray, A.W., and Kirschner, M.W. (1989). Dominoes and clocks: the union of two views of the cell cycle. Science *246*, 614–621.
6. Hartwell, L.H. (1978). Cell division from a genetic perspective. J. Cell Biol. *77*, 627–637.
7. Lee, M., and Nurse, P. (1988). Cell cycle control genes in fission yeast and mammalian cells. Trends Genet. *4*, 287–290.
8. Murray, A.W., and Kirschner, M.W. (1989). Cyclin synthesis drives the early embryonic cell cycle. Nature *339*, 275–280.
9. Luca, F.C., and Ruderman, J.V. (1989). Control of programmed cyclin destruction in a cell-free system. J. Cell Biol. *109*, 1895–1909.
10. Lohka, M.J., Kyes, J.L., and Maller, J.L. (1987). Metaphase protein phosphorylation in *Xenopus laevis* eggs. Mol. Cell. Biol. *7*, 760–768.
11. Kumagai, A., and Dunphy, W.G. (1991). The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. Cell *64*, 903–914.
12. Solomon, M.J., Lee, T., and Kirschner, M.W. (1992). Role of phosphorylation in $p34^{cdc2}$ activation: identification of an activating kinase. Mol. Biol. Cell *3*, 13–27.
13. Maike-Lye, R., Newport, J., and Kirschner, M.W. (1993). Maturation-promoting factor induces nuclear envelope breakdown in cyclohexamide-arrested embryos of *Xenopus laevis*. J. Cell Biol. *97*, 81–91.
14. Ward, G.E., and Kirschner, M.W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. Cell *61*, 561–577.
15. Peter, M., Nakagawa, J., Doree, M., Labbe, J.C., and Nigg, E.A. (1990). *In vitro* disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. Cell *61*, 591–602.
16. Lowe, M., Gonatas, N.K., and Warren, G. (2000). The mitotic phosphorylation cycle of the cis-Golgi matrix protein GM130. J. Cell Biol. *149*, 341–356.
17. Karsenti, E., Bravo, R., and Kirschner, M.W. (1987). Phosphorylation changes associated with the early cell cycle in *Xenopus* eggs. Dev. Biol. *119*, 442–453.
18. Gard, D.L., and Kirschner, M.W. (1987). Microtubule assembly in cytoplasmic extracts of *Xenopus* oocytes and eggs. J. Cell Biol. *105*, 2191–2201.
19. Belmont, L.D., Hyman, A.A., Sawin, K.E., and Mitchison, T.J. (1990). Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. Cell *10*, 579–589.
20. Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L., and Tobey, R.A. (1978). Histone phosphorylation and chromatin structure during mitosis in chinese hamster cells. Eur. J. Biochem. *84*, 1–15.
21. Hans, F., and Dimitrov, S. (2001). Histone H3 phosphorylation and cell division. Oncogene *20*, 3021–3027.
22. Izumi, T., Walker, D.H., and Maller, J.L. (1992). Periodic changes in phosphorylation of the *Xenopus* cdc25 phosphatase regulate its activity. Mol. Biol. Cell *3*, 927–939.
23. Kumagai, A., and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in *Xenopus* extracts. Cell *70*, 139–151.
24. Izuumi, T., and Maller, J.L. (1993). Elimination of cdc2 phosphorylation sites in the cdc25 phosphatase blocks initiation of M phase. Mol. Cell. Biol. *4*, 1337–1350.
25. Kuang, J., Ashorn, C.L., Gonzalez, K.M., and Penkala, J.E. (1994). Cdc25 is one of the MPM-2 antigens involved in the activation of maturation-promoting factor. Mol. Biol. Cell *5*, 135–145.
26. Kramer, E.R., Scheuringer, N., and Peters, J.-M. (2000). Mitotic regulation of the APC activator proteins CDC20 and CDH1. Mol. Biol. Cell *11*, 1555–1569.
27. Fang, G., Yu, H., and Kirschner, M.W. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell *2*, 163–171.
28. Kotani, S., Tanaka, H., Yasuda, H., and Todokoro, K. (1999). Regulation of APC activity by phosphorylation and regulatory factors. J. Cell Biol. *146*, 791–800.
29. Alexandru, G., Uhlmann, F., Mechtler, K., Poupart, M., and Nasmyth, K. (2001). Phosphorylation of the cohesin subunit scc1 by polo/cdc5 kinase regulates sister chromatid separation in yeast. Cell *105*, 459–472.
30. Rao, P.N., and Johnson, R.T. (1970). Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. Nature *225*, 159–164.
31. Johnson, R.T., and Rao, P.N. (1970). Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. Nature *226*, 717–722.
32. Stukenberg, P.T., Lustig, K., McGarry, T.J., King, R.W., Kuang, J., and Kirschner, M.W. (1997). Systematic identification of mitotic phosphoproteins. Curr. Biol. *7*, 338–348.
33. Kumagai, A., and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. Science *273*, 1377–1380.
34. Kumagai, A., and Dunphy, W.G. (1997). Regulation of Xenopus Cdc25 protein. Methods Enzymol. *283*, 564–571.
35. Tang, Z., Coleman, T.R., and Dunphy, W.G. (1993). Two distinct mechanisms for negative regulation of the Wee1 protein kinase. EMBO J. *12*, 3427–3436.
36. Parker, L.L., Walter, S.A., Young, P.G., and Piwnica-Worms, H. (1993). Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase. Nature *363*, 736–738.
37. Wu, L., and Russell, P. (1993). Nim 1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. Nature *363*, 738–741.
38. King, R.W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279–288.
39. Kotani, S., Tugendreich, S., Fujii, M., Jorgensen, P.-M., Wantanabe, N., Hoog, C., Hieter, P., and Todokoro, K. (1998). PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression. Mol. Cell *1*, 371–380.
40. Murray, A.W. (1991). Cell cycle extracts. In Methods in Cell Biology, B.K. Kay and H.B. Peng, eds. (New York: Academic Press), pp. 581–605.
41. Fang, G., Yu, H., and Kirschner, M.W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev. *12*, 1871–1883.
42. He, X., Patterson, T.E., and Sazer, S. (1997). The S. pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *94*, 7965–7970.
43. Gorbsky, G.J., Chen, R.H., and Murray, A.W. (1998). Microinjection of antibody to Mad2 protein into mammalian cell in mitosis induces premature anaphase. J. Cell Biol. *141*, 1193–1205.
44. Lahav-Baratz, S., Sudakin, V., Ruderman, J.V., and Hershko, A. (1995). Reversible phosphorylation controls the activity of cyclosome-associated cyclin-ubiquitin ligase. Proc. Natl. Acad. Sci. USA *92*, 9303–9307.
45. Peters, J.-M., King, R.W., Hoog, C., and Kirschner, M.W. (1996). Identification of BIME as a subunit of the anaphase-promoting complex. Science *274*, 1199–1201.
46. Kramer, E.R., Gieffers, C., Holz, G., Hengstschlager, M., and Peters, J.-M. (1998). Activation of the human anaphase-promoting complex by proteins of the CDC20/Fizzy family. Curr. Biol. *8*, 1207–1210.
47. Shteinberg, M., Protopopov, Y., Listovsky, T., Brandies, M., and Hershko, A. (1999). Phosphorylation of the cyclosome is required for its stimulation by Fizzy/cdc20. Biochem. Biophys. Res. Commun. *260*, 193–198.
48. Michel, L.S., Liberal, V., Chatterjee, A., Kirchwegger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P.K., Murty, V.V., and Benezra, R. (2001). MAD2 haplo-insufficiency causes premature ana-

phase and chromosome instability in mammalian cells. Nature *409*, 355–359.

49. Takizawa, C.G., and Morgan, D.O. (2000). Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. Curr. Opin. Cell Biol. *12*, 658–665.
50. Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr. Biol. *9*, 680–689.
51. Jackman, M.R., and Pines, J.N. (1997). Cyclins and the G2/M transition. Cancer Surv. *29*, 47–73.
52. Wu, L., Shiozaki, K., Aligue, R., and Russell, P. (1996). Spatial organization of the Nim1-Wee1-Cdc2 mitotic control network in Schizosaccharomyces pombe. Mol. Biol. Cell *7*, 1749–1758.
53. Minshull, J., Golsteyn, R., Hill, C.S., and Hunt, T. (1990). The A- and B-type cyclin associated cdc2 kinases in Xenopus turn on and off at different times in the cell cycle. EMBO J. *9*, 2865–2875.
54. Cho, R.J., Campbell, M.J., Winzeler, E.A., Steinmetz, L., Conway, A., Wodicka, L., Wolfsberg, T.G., Gabrielian, A.E., Landsman, D., Lockhart, D.J., Davis, R.W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell *2*, 65–73.
55. Simon, I., Barnett, J., Hannett, N., Harbison, C.T., Rinaldi, N.J., Volkert, T.L., Wyrick, J.J., Zeitlinger, J., Gifford, D.K., Jaakkola, T.S., et al. (2001). Serial regulation of transcriptional regulators in the yeast cell cycle. Cell *106*, 697–708.
56. Stukenberg, P.T., and Kirschner, M.W. (2001). Pin 1 acts catalytically to promote a conformational change in cdc25. Mol. Cell *7*, 1071–1083.
57. Harlow, E. (1998). Antibodies: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).