Cyclin synthesis drives the early embryonic cell cycle

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We have produced extracts of frog eggs that can perform multiple cell cycles *in vitro*. Destruction of the endogenous messenger RNA arrests the extracts in interphase. The addition of exogenous cyclin mRNA is sufficient to produce multiple cell cycles. The newly synthesized cyclin protein accumulates during each interphase and is degraded at the end of each mitosis.

THE early embryonic cell cycle of many organisms is a rapid alternation of interphase and mitotic states. The transition from interphase to mitosis is induced by the appearance of an activity named maturation promoting factor (MPF)[1-3], which is highly conserved[4]. The cytoplasm of meiotic and mitotic cells of a wide range of eukaryotes contains MPF activity, which can be measured by its ability to induce maturation in *Xenopus* oocytes. MPF is now believed to be a protein kinase that initiates a cascade of reactions which lead ultimately to nuclear envelope breakdown, chromosome condensation and the assembly of the mitotic spindle[5]. This view is supported by the recent finding that one of the subunits of MPF is homologous to the gene product of the *cdc2* gene of *Schizosaccharomyces pombe*, whose activity is required for entry into mitosis[6-8]. The gene product of the *cdc2* gene is a protein of relative molecular mass 34,000, referred to as $p34^{cdc2}$, which shares homology with known protein kinases. Immunoprecipitates made from yeast, *Xenopus* and starfish using anti-$p34^{cdc2}$ antisera have protein kinase activity on a number of substrates including histone H1(refs 7-11).

The fluctuation of MPF activity in *Xenopus* oocytes, eggs and embryos is shown schematically in Fig. 1. Fully grown immature oocytes are arrested in prophase of meiosis I with no detectable MPF activity. Secretion of progesterone by the follicle cells that surround the oocyte induces the post-translational activation of an inactive form of MPF and meiosis I (ref. 3). MPF activity then falls before rising again at the onset of meiosis II. The mature oocytes (which pass down the oviduct and emerge as unfertilized eggs) arrest at metaphase of meiosis II by virtue of a calcium-sensitive activity named cytostatic factor (CSF), which stabilizes MPF activity[12]. Fertilization or artificial activation triggers a rise in the intracellular calcium level, the inactivation of CSF, a decline in MPF activity and entry into interphase of the first mitotic cell cycle. At the end of each interphase MPF activity rises transiently, leading to the induction of mitosis; as MPF activity falls the cell enters the next interphase[3].

In a wide variety of organisms protein synthesis is required for the appearance of active MPF in meiosis II and in mitosis[3,13-15]. In the mitotic cell cycles protein synthesis is required during each and every interphase for the induction of the subsequent mitosis. Because early embryos have large stores of the enzymes and structural proteins required for DNA synthesis and mitosis, the requirement for protein synthesis is likely to represent the synthesis of important cell-cycle regulatory molecules. One attractive candidate for a newly synthesized inducer of mitosis is cyclin[15,16]. The cyclins were identified as embryonic proteins that accumulate during interphase and decline precipitously in abundance during mitosis[15]. Cyclin abundance is regulated by controlling the half life of the protein; it is long during interphase and declines during mitosis. A role for cyclin in regulating mitosis was suggested by experiments which show that cyclin mRNA induces maturation in *Xenopus* oocytes[16,17]. Cyclins have now been identified in many organisms, including sea urchins[17], clams[16], starfish[18], *Xenopus*[19], *Drosophila*[20] and *S. pombe*[21-24], and have been divided into two classes, A and B, on the basis of their sizes[15], kinetics of appearance[15] and sequence homology[19].

To study the role of cyclin in the mitotic cell cycle, we turned to extracts of amphibian eggs that would perform the key reactions of the cell cycle[25-31]. We have developed procedures for producing *Xenopus* extracts that undergo multiple cell cycles, extending the work of Lohka and Masui, who produced a single cell cycle *in vitro*[25]. We have used these extracts to show that cyclin is the only newly synthesized protein required to induce mitosis.

Xenopus cyclin *in vivo*

We looked for *Xenopus* cyclins in eggs that had been released from the CSF-mediated metaphase arrest by electrical activation. Shortly after activation the eggs were injected with [^{35}S]methionine, incubated for various times before lysis, and the newly synthesized proteins analysed on SDS-polyacrylamide gels. A closely spaced set of bands, running with apparent relative molecular mass (M_r) of 55,000 (55K), increased in intensity throughout the first cell cycle but then decreased dramatically between 60 and 75 min after activation. This corresponds to the time during the first cell cycle when MPF activity would normally fall at the onset of anaphase. These bands then increased in intensity, only to decline again at the time (85-95 min) when MPF would normally fall (data not shown). The pattern of accumulation and disappearance of the 55K bands suggests that these proteins are cyclins, although they are much less abundant than their counterparts in clams and sea urchins.

In vitro cell cycle

We prepared *Xenopus* egg extracts that undergo multiple cell cycles *in vitro*. Concentrated extracts were prepared from electrically activated eggs that had been washed in a buffer designed to mimic the ionic composition of egg cytoplasm, crushed and fractionated by centrifugation. Demembranated sperm nuclei and an ATP regenerating system were then added to the concen-

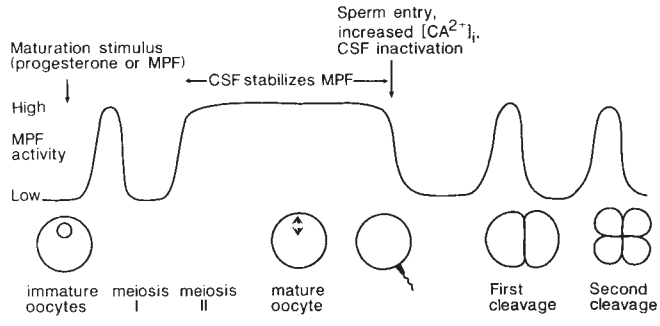
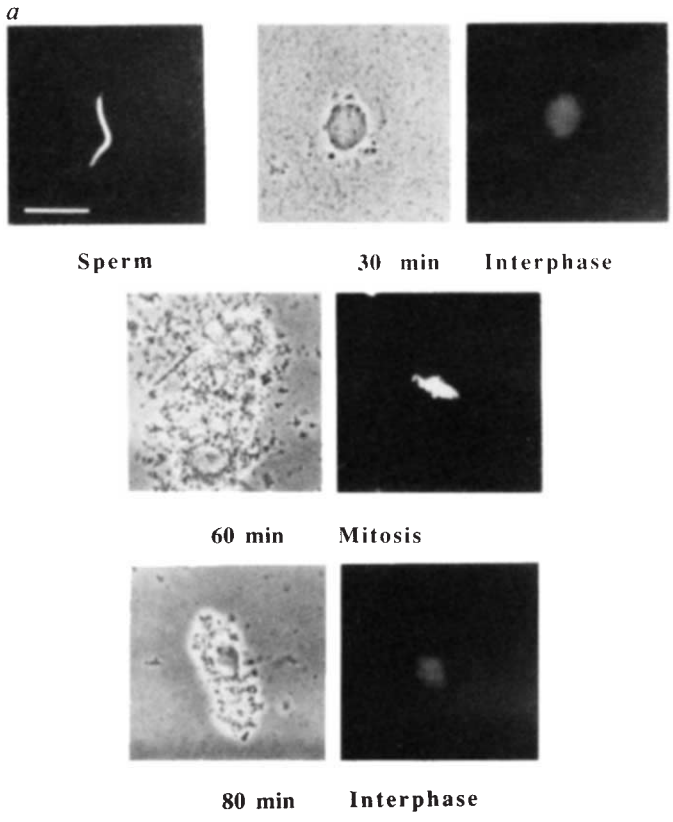


FIG. 1 MPF levels during early *Xenopus* embryonic development. The fluctuation in MPF levels as an immature oocyte passes through meiotic maturation, fertilization and the first two mitotic cell cycles is shown. For further details see the text.

trated cytoplasmic extract. The extracts we have produced have cell-cycle times varying between 35 and 55 min, compared with *in vivo* cycle times of 25–30 min. These extracts, which we refer to as cycling extracts, routinely produce at least three complete cell cycles.

We monitored the progress of the cell cycle in extracts by examining the morphology of the added sperm nuclei in fixed samples stained with a DNA-binding fluorescent dye. We could distinguish five states of the nuclei: interphase, prophase, mitosis and early and late telophase. In the experiment shown in Fig. 2*a*, the sperm nuclei decondensed to form interphase nuclei after 30 min incubation. The nuclear envelope was visible both by phase contrast and immunofluorescence with anti-lamin antibodies (data not shown). At 45 min the nuclei were in prophase,



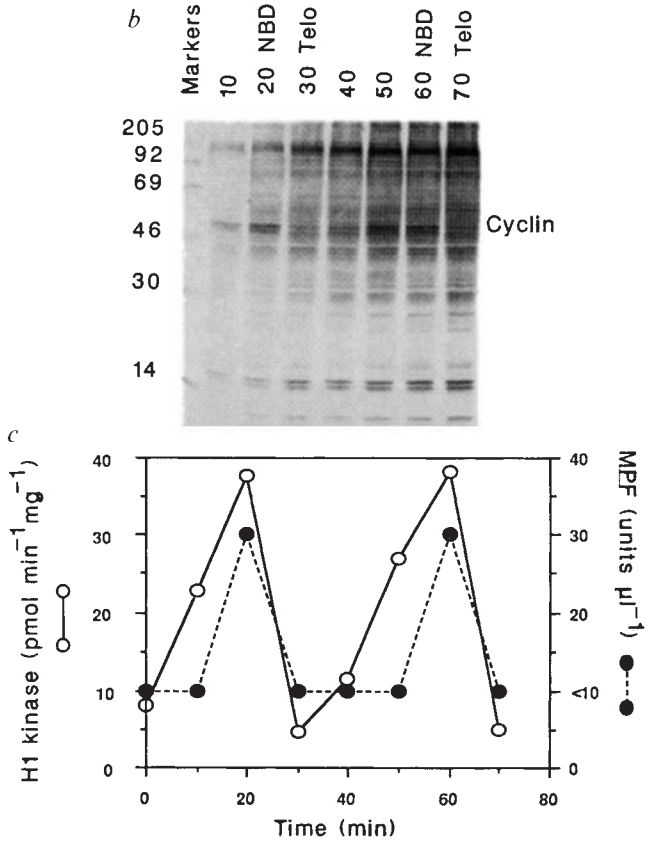


Fig. 2 Behaviour of *in vitro* cell-cycle extracts. *a*, Nuclear morphology at the indicated times of an *in vitro* cell-cycle extract to which sperm nuclei had been added at time zero. Each pair of images consists of a phase contrast image on the left and a fluorescent image of the DNA-binding dye, Hoechst 33342, on the right. Only the fluorescent image of the intact sperm is shown. Scale bar, 20 μm. *b*, Autoradiograph showing the labelled proteins synthesized in an *in vitro* cell-cycle extract to which [^{35}S]methionine had been added at time zero. Samples were taken at the indicated times and either diluted in sample buffer and run on a 12.5% polyacrylamide gel, or fixed for determination of the nuclear morphology. The times of nuclear envelope breakdown (NBD) and telophase (Telo), the positions of the molecular weight markers (in thousands) and the set of cyclin bands are indicated. *c*, The levels of H1 kinase (○—○) and MPF (●·····●) activity during incubation of the extract shown in *b*.

METHODS. Cycling extracts were prepared as follows. Frogs were induced to ovulate and eggs were collected by squeezing into MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM NaEGTA, 5 mM NaHEPES, pH 7.8)[46] and dejellied with 2% cysteine, pH 7.9, and then electrically activated in 0.2 ×MMR by two 1-s pulses of 12 V a.c. The activated eggs were washed four times with XB (100 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM KHEPES pH 7.7, 50 mM sucrose) and then twice with XB containing 10 μg ml^{-1} each of leupeptin, chymostatin and pepstatin. Finally, the activated eggs were transferred with a minimal volume of XB to a 5 ml polycarbonate centrifuge tube containing 1 ml of XB plus protease inhibitors containing 100 μg ml^{-1} of cytocholasin B. Ten minutes after activation any residual buffer was removed and the eggs were overlaid with 1 ml Versilube F-50 oil (General Electric, $\rho = 1.03$ g ml^{-1}) and spun at 200*g* for 1 min. The buffer that had been displaced by the oil was then removed and at 15 min after activation the eggs were chilled in an ice bath before being spun for 10 min at 15,000*g* at 2 °C. The cytoplasmic layer was collected by side puncture and the following ingredients were added: MgATP to 1 mM, creatine phosphate to 10 mM, EGTA, pH 7.7, to 0.2 mM and leupeptin, chymostatin, pepstatin and cytochalasin B to 10 μg ml^{-1} each. The extract was then spun again at 15,000*g* for 15 min at 2 °C to remove residual yolk and pigment granules. This extract was stored on ice and used within three hours. All the experiments in this paper were performed with fresh extracts and the final volume of added components never exceeded 20% of the volume of cytoplasmic extract. Sperm nuclei were prepared by a slight modification of the method described by Gurdon[47], stored in 30% glycerol at −70 °C and used at a final concentration of 10^5 per ml in the extract. All incubations were performed at 23 °C. Samples were analysed for morphology by spotting 1 μl extract on a microscope slide and then adding 4 μl fixative (MMR containing 50% glycerol (w/v), 10% formalin and 1 μg ml^{-1} Hoechst 33342) before squashing the drop with a coverslip and examining the nuclei by phase contrast and fluorescent microscopy. MPF was assayed by diluting samples twofold into EB (80 mM potassium β-glycerophosphate, 15 mM $MgCl_2$, 20 mM potassium EGTA, adjusted to pH 7.3 after mixing the components) and assaying them for MPF activity in cycloheximide-treated immature oocytes as described[3]. One MPF unit is the amount of MPF activity which when injected in a volume of 50 nl will induce germinal vesicle breakdown in 50% of injected oocytes. H1 kinase activity was assayed by diluting samples fiftyfold into EB and assaying for H1 kinase activity by adding 10 μl to 6 μl containing 1 mg ml^{-1} of calf thymus histones (a gift of J. Minden[48]), and 1 mM ATP, 0.25 μCi μl^{-1} [γ^{32}P] ATP. The phosphorylated H1 kinase was run on 5–15% gradient polyacrylamide gels and the amount of incorporated phosphate quantified by cutting out and counting the gel bands or by densitometry; H1 kinase activity is expressed as pmol incorporated phosphate min^{-1} mg^{-1} of protein (for counted gel bands) or arbitrary units (for samples assayed by densitometry). All the MPF and H1 kinase assays in this paper were performed on samples that had been diluted into EB and then frozen in liquid nitrogen. To analyse the pattern of protein synthesis [^{35}S]methionine was added to a final concentration of 0.4 mCi ml^{-1} and samples were taken, diluted tenfold into sample buffer and run on 12.5% polyacrylamide gels[49]. Detailed protocols for the preparation of cell-cycle extracts are available on request.

which is characterized by the start of chromosome condensation and the further swelling of the nucleus. Nuclear breakdown occurred by 60 min and clusters of condensed chromosomes were seen. Nuclear breakdown and chromosome condensation occurred in all extracts, but only some extracts formed well defined mitotic spindles. At 70 min the chromosomes had started to decondense into the individual mini-nuclei or karyomeres characteristic of early telophase, and by 80 min interphase nuclei were present once more. At 100 min the nuclei were in prophase and a second round of nuclear breakdown followed at 110 min. The synchrony of the nuclei in these extracts was good: at most time points all the nuclei were in the same morphological stage and no time point contained nuclei in more than two stages. *In vitro*, as *in vivo*, protein synthesis was required in each interphase to allow the occurrence of the next round of nuclear breakdown (data not shown).

The pattern of protein synthesis in cycling extracts was monitored by adding [^{35}S]methionine at the start of the reaction and withdrawing samples at intervals during two cell cycles and analysing them on SDS gels. Figure 2*b* shows the behaviour of the cyclin bands *in vitro*, which is essentially identical to that seen *in vivo*. The intensity of the cyclin bands increased throughout the first interphase and then declined dramatically between nuclear breakdown and the onset of telophase. During the second interphase the intensity of these bands again increased, only to fall again after the second round of nuclear breakdown. These bands are indeed the translation products of the *Xenopus* cyclin B genes that have been cloned on the basis of their homology with sea urchin cyclin: treatment with RNase H and anti-sense oligonucleotides derived from the cloned cyclins destroyed the mRNAs that encode these bands (J. Minshull, T. Hunt, A. W. M. and M. W. K., unpublished data). The two primary cyclin translation products undergo post-translational modification to yield as many as five closely spaced bands on gels[19].

To demonstrate that biochemical events occurring *in vivo* also occur in cell-cycle extracts, we monitored DNA replication and the levels of both MPF and H1 kinase activity, which recent evidence suggests is very closely related to MPF[7,9,11]. Figure 2*c* shows that MPF activity appeared at the time of nuclear breakdown and was undetectable by early telophase. Like MPF, H1 kinase activity peaked at the time of nuclear breakdown and was dramatically diminished by telophase, although appreciable activity was detectable in late interphase and prophase, when MPF was undetectable. This apparent discrepancy may simply reflect the high threshold for the MPF assay. We have also shown that DNA replication only occurs during interphase (data not shown), as previously shown in other *Xenopus* extracts[26,28].

Cyclin induces mitosis

To test whether cyclin synthesis in the absence of other protein synthesis can induce mitosis, we added cyclin mRNA to cell-cycle extracts whose endogenous mRNA had been destroyed. We made such extracts, which we call mRNA-dependent extracts, by using pancreatic RNase to destroy the endogenous mRNA in extracts prepared from activated eggs and then inhibiting the RNase with placental RNase inhibitor, RNAsin[32]. Figure 3*a* compares the pattern of protein synthesis in an mRNA-dependent extract, to which no exogenous mRNA had been added, with that in a mock treated extract to which the RNase inhibitor, but no RNase, had been added. In the mock treated extract cyclin accumulated until 30 min, when breakdown of the nuclear envelope occurred. By 40 min the cyclin bands had declined in intensity and telophase had commenced. In the mRNA-dependent extract the rate of protein synthesis was less than 5% of that of the controls. Over a period of 120 min in the RNase-treated extracts, the nuclei swelled but did not break down. Thus RNase treatment can effectively destroy endogenous mRNA and block entry into mitosis. When a strongly translated mRNA such as the viral RNA of tobacco mosaic virus was added to the mRNA dependent extracts, we obtained 50% of the pretreatment level of total protein synthesis but failed to restore the ability of extracts to enter mitosis (data not shown).

We then asked whether the translation of cyclin mRNA was sufficient to allow mRNA-dependent extracts to enter mitosis. Sea urchin cyclin B mRNA was transcribed *in vitro* from a complementary DNA clone (supplied by J. Pines and T. Hunt[17]). This mRNA was added to an mRNA-dependent extract at three concentrations. For each reaction, [^{35}S]methionine was added at the time of RNA addition. The patterns of protein synthesis and nuclear morphology during the course of this experiment are shown in Fig. 3*b* and *c*, respectively. At a cyclin mRNA concentration of 5 μg ml^{-1}, cyclin protein accumulated, reaching a peak at 40 min, at which point the nuclei had broken down. By 50 min the nuclei were in telophase and the cyclin band had dramatically decreased in intensity. The cyclin band then increased in intensity to a second maximum at 120 min when the nuclei had once more broken down (Fig. 3*b*, *c*). Figure 3*d* shows that in mRNA-dependent cell cycle extracts where the cell cycle is driven by the synthesis of sea urchin cyclin, the activities of MPF and H1 kinase increased in prophase, were maintained at high levels during mitosis, and then declined at the end of mitosis. Thus the synthesis of sea urchin cyclin is sufficient to induce both the morphological and biochemical events characteristic of mitosis.

To test whether the rate of cyclin accumulation affected the length of interphase, we added different concentrations of cyclin mRNA to the mRNA-dependent extracts. At 2.5 μg ml^{-1} of cyclin mRNA, the accumulation of cyclin was slightly slower and nuclear envelope breakdown did not occur until 50 min; at 60 min telophase had begun and the cyclin abundance had declined markedly (Fig. 3*b*). At this concentration of mRNA a second round of nuclear envelope breakdown did not occur during the experiment. Finally, at the lowest concentration of mRNA, cyclin accumulated at a slower rate and the nuclei never entered mitosis, nor did the intensity of the labelled cyclin band ever decline (Fig. 3*b*). In experiments that were carried out for longer times, the translation of cyclin mRNA added to mRNA-dependent extracts has induced as many as three rounds of nuclear envelope breakdown. These experiments suggest that cyclin synthesis is sufficient both to induce mitosis and to allow progression from mitosis to the next interphase, and that its rate of accumulation affects the length of interphase.

We have also tested the ability of the *Xenopus* cyclins to induce mitosis. Transcripts of either *Xenopus* B1 or B2 cyclin clones (provided by J. Minshull and T. Hunt) drove the *in vitro* cell cycle (data not shown). Both cyclin proteins increased in abundance during interphase, reached peak levels at nuclear envelope breakdown and had declined by the onset of telophase.

Discussion

We have developed an *in vitro* cell-cycle extract that faithfully reproduces the key features of the cell cycle in early *Xenopus* embryos and performs multiple cell cycles. The endogenous mRNA in such an extract could be destroyed, arresting the cell cycle in interphase and producing an mRNA-dependent extract. In an mRNA-dependent extract, the translation of sea urchin cyclin B or either of two *Xenopus* cyclin B mRNAs was sufficient to drive multiple cell cycles. These results imply that cyclin synthesis can satisfy the protein synthesis requirement both for the activation of MPF and the entry into mitosis, as well as for the degradation of cyclin, inactivation of MPF and progress to the next interphase. The amount of cyclin protein present at mitosis in mRNA-dependent extracts programmed with cyclin mRNA is not substantially different from that translated from the endogenous cyclin mRNA in mock treated extract. This demonstrates that cyclin made in the absence of synthesis of other protein can induce mitosis at about the same level as when the protein is made in untreated extracts.

One limitation to the conclusion that cyclin synthesis is

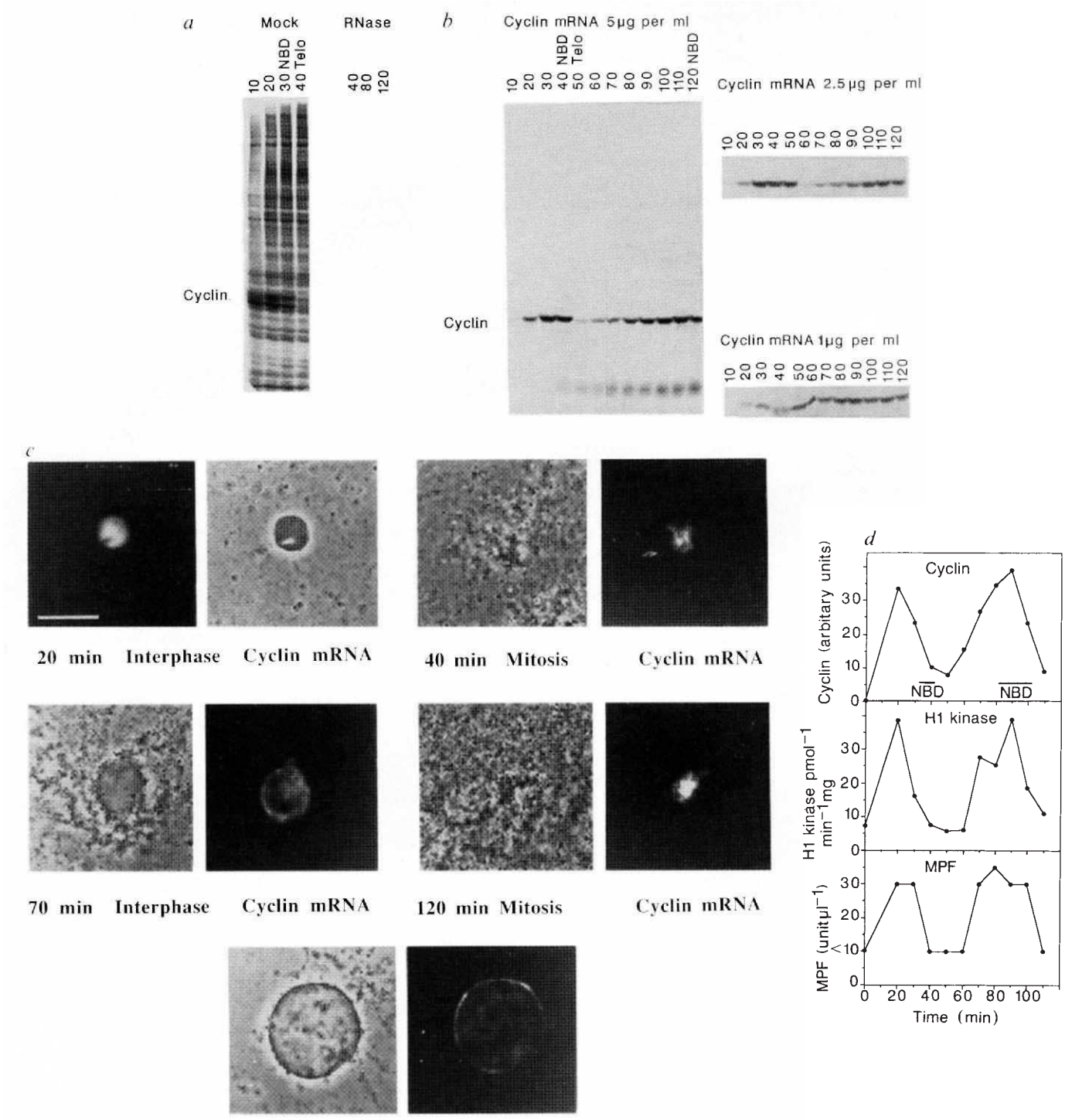


FIG. 3 Cyclin synthesis is sufficient to drive the embryonic cell cycle. *a*, The pattern of protein synthesis in mock treated and RNase-treated extracts. Cell cycle extracts prepared from activated eggs were treated with RNasin (gift of David Drechsel) alone (Mock) or with RNase followed by RNasin (RNase). [^{35}S]methionine was added to the extracts, which were then incubated and samples were removed at the indicated times and run on 10% polyacrylamide gels. The times of nuclear envelope breakdown (NBD) and telophase (Telo) and the position of cyclin are indicated. *b*, The pattern of protein synthesis in mRNA-dependent extracts with different doses of added sea urchin cyclin mRNA. [^{35}S]Methionine and cyclin mRNA were added 10 min after the addition of RNasin and samples were taken for analysis on 10% polyacrylamide gels and for nuclear morphology at the indicated times. The times of nuclear envelope breakdown and telophase are indicated. *c*, Nuclear morphology in the mRNA-dependent extracts with and without added sea urchin cyclin mRNA whose pattern of protein synthesis is shown in *a* and *b*. Each pair of images consists of a phase-contrast image and a fluorescent image of the DNA-binding dye Hoechst 33342, as described in Fig. 2. Scale bar, 20 μm. *d*, The fluctuations in cyclin abundance and MPF and H1 kinase activity in an mRNA-dependent extract to which 5 μg ml^{-1} of sea urchin cyclin mRNA had been added. The times of nuclear breakdown are indicated (NBD).

METHODS. Cycling extracts were prepared and incubated with a final concentration of 0.25 μg ml^{-1} of boiled pancreatic RNase at 10 °C for 20 min before adding 0.05 volume of a solution of placental RNase inhibitor (RNasin, OD_{280} = 0.21) and incubating for a further 10 min at 10 °C and then adding calf liver transfer RNA to 50 μg ml^{-1}, [^{35}S]methionine to 0.4 mCi ml^{-1} and sea urchin cyclin B mRNA in 100 mM KCl and 1 mM $MgCl_2$. The mRNA was transcribed by T7 polymerase from the sea urchin cyclin B cDNA clone as described[17] and then treated with RNase-free DNase to destroy the template DNA. Cyclin abundance was measured by densitometry.

sufficient to drive the cell cycle is that some protein synthesis occurs between activation and the RNase treatment that produces mRNA-dependent extracts. The ability of cyclin synthesis to induce multiple rounds of nuclear envelope breakdown demonstrates that cyclin synthesis is sufficient to induce mitosis in a cell cycle where no other protein synthesis has occurred. Although these experiments show that cyclin synthesis is sufficient to drive later cell cycles, it does not prove that other proteins synthesized in the first cell cycle are not required for the first cell cycle. In the accompanying paper[33] we demonstrate that cyclin protein made in a cell-free translation system can induce entry into mitosis when it is added to an extract where cycloheximide had been used to inhibit protein synthesis before the start of the first mitotic cell cycle. This experiment also shows that the translation of residual endogenous mRNA in the extracts, or of mRNA newly transcribed from the added sperm nuclei, does not supply proteins that are necessary for the induction of mitosis.

The length of the cell cycle increased as the amount of cyclin mRNA was reduced, suggesting that cyclin accumulation has to occur to some critical level to induce entry into mitosis. Thus at least a fraction of the length of interphase in embryonic cell cycles represents the time required to accumulate cyclin to this critical level. This time can be estimated by measuring the time in interphase at which the addition of inhibitors of protein synthesis no longer blocks the occurrence of the next mitosis[34,35]. This time varies in different embryonic cell cycles, *in vivo*, but is always less than half the length of interphase, suggesting that there are slow steps between the accumulation of cyclin and the activation of MPF and occurrence of mitosis.

The lack of other newly synthesized proteins in the mRNA-dependent extracts strongly suggests that when the cyclin band disappears at the end of mitosis, it does so as a result of proteolysis, rather than as a result of some form of post-translational modification which alters its gel mobility so that it is obscured by other bands. Finally, the stability of the newly synthesized cyclin at the lowest dose of cyclin mRNA shows that the rapid degradation of cyclin is not induced unless it is present in doses sufficient to induce the extract to enter mitosis.

Recent mRNA ablation experiments have shown that cyclin synthesis is necessary for entry into mitosis. In these experiments it was necessary to cleave the mRNAs for both *Xenopus* cyclin B1 and B2 to prevent entry into mitosis[19], confirming the functional redundancy we have seen for these two proteins. The demonstration that cyclin synthesis is both necessary and sufficient for entry into mitosis strongly suggests that cyclin is the only newly synthesized protein required for the induction of mitosis in *Xenopus* embryos.

The ability of cyclin synthesis to induce mitosis suggests an attractive model for the early embryonic cell cycle (Fig. 4*a*). In this model, cyclin activates the protein kinase activity of $p34^{cdc2}$ that constitutes MPF activity. An opposing activity inactivates this $p34^{cdc2}$ kinase activity. The level of MPF activity is thus controlled by the ratio of the activities of cyclin and the inactivator. Early in interphase cyclin levels are low and the activity of the inactivator is dominant, keeping MPF in its inactive form. Cyclin accumulates during interphase until its activity exceeds that of the inactivator. At this point, MPF activation begins and ultimately triggers a cascade of reactions which lead to the morphological and biochemical events of mitosis. We postulate that MPF induces the degradation of cyclin. Once cyclin has been destroyed, the inactivator is once more dominant, MPF is inactivated and the cell cycle progresses into the next interphase. In this model both interphase and mitosis are unstable states. The instability of interphase is caused by the accumulation of cyclin, whereas that of mitosis is due to the ability of MPF to induce degradation of cyclin. This simple model of the early *Xenopus* cell cycle predicts that cyclin degradation is required to exit from mitosis. The accompanying paper[33] verifies this prediction and examines the mechanism by which cyclin activates MPF.

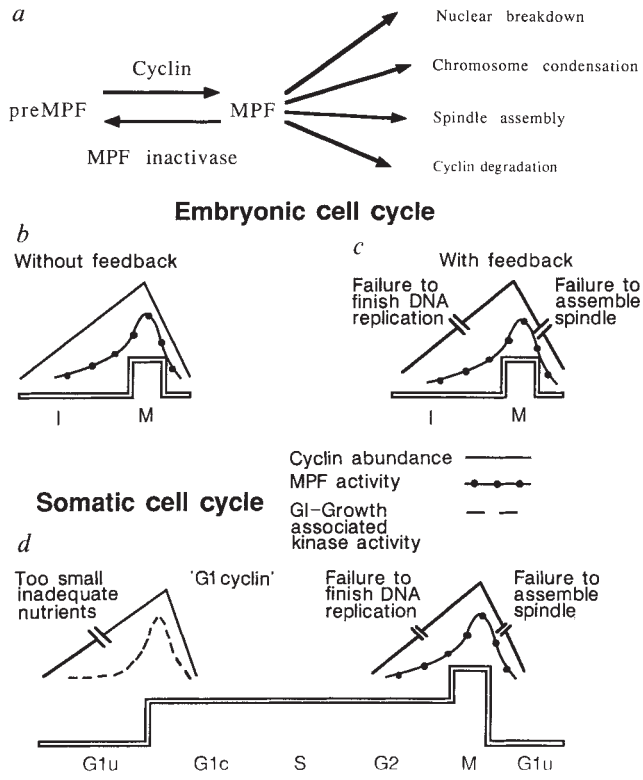


FIG. 4 A model for the cell cycle. *a*, An overview of the proposed reactions controlling passage through the cell cycle. *b*, A diagram of alternation of interphase (I) and mitosis (M), and the levels of cyclin (——) and MPF activity (●—●) in an early embryonic cell cycle without feedback controls. *c*, A diagram of an embryonic cell cycle with feedback controls (II). *d*, A schematic view of a somatic cell cycle showing the role of a Gl cyclin in activating a Gl-specific $p34^{cdc2}$ kinase activity (— —) to drive the cells from a state where they are not committed to the mitotic cell cycle (Glu), to a state where they are committed to enter DNA synthesis and ultimately progress to mitosis (Glc). See text for further details.

In this model the accumulation of cyclin initiates a pathway that leads to the post-translational activation of $p34^{cdc2}$ to a form that has MPF activity. During interphase in early *Xenopus* embryos, none of the events in this pathway, except the concentration of cyclin, seems to be regulated, making cyclin the trigger of mitosis. In the more tightly controlled cell cycle of somatic cells, other steps in the pathway that leads to MPF activation, such as cyclin or $p34^{cdc2}$ phosphorylation, may be subject to regulation. One example of this may be the post-cellularization divisions of *Drosophila* embryos. In these cell cycles, cyclin synthesis is required, but it is the accumulation of the gene product of the *string* gene that appears to trigger mitosis[36]. The *string* gene is the homologue of the *S. pombe cdc25* gene, which acts as a dose-dependent activator of the mitosis-inducing function of *cdc2* (ref. 37).

The cyclin-based oscillator in frog extracts lacks feedback controls and will continue to cycle even when processes such as DNA synthesis or spindle assembly are inhibited by aphidicolin and nocodazole (data not shown; see Fig. 4*b*). The lack of feedback controls in the extract is in good agreement with the previous demonstrations that the oscillations in MPF activity were unaffected by inhibiting DNA synthesis or microtubule polymerization[3,38,39]. In all somatic cells and in the embryos of many organisms the cell cycle manifests feedback controls that prevent the initiation of one step in the cell cycle until the previous step has been successfully completed[40]. The cyclin-based oscillator may be converted to such a cell cycle by imposing controls on the accumulation and degradation of

cyclin (Fig. 4*c*). One example is the action of CSF, which appears to stabilize MPF activity by blocking the degradation of cyclin[35]. In this case, inactivation of CSF and progress into the mitotic cell cycle is made dependent upon an increase in cytoplasmic calcium concentration induced by fertilization. It is easy to imagine that CSF or a related molecule could act in somatic cells to prevent the degradation of cyclin until the spindle has been successfully assembled. In this case the signal that inactivates CSF would be generated by a pathway that monitors some parameter of spindle assembly, such as stable attachment of the kinetochores to microtubules. In sea urchin and clam embryos, microtubule depolymerization greatly increases the length of mitosis and stabilizes cyclin[15]. The completion of DNA synthesis could be used to regulate either the accumulation of cyclin, or some post-translational step in the activation of MPF. The latter possibility is suggested by the ability of aphidicolin to block sea urchin embryos in interphase, even though cyclin accumulates to very high levels (T. Hunt, personal communication).

In early embryos, regulation of the cell cycle appears to be limited to the control of the entry into and the exit from mitosis. Somatic cells possess an additional control point located in G1, where cells can either become committed to passage through the cell cycle or remain in a resting state. This commitment point has been named 'Start' in studies on yeasts and the restriction point in mammalian tissue culture cells[40,41]. Three lines of evidence fuel the speculation that passage through Start requires the accumulation of a molecule related to cyclin that induces a particular spectrum of $p34^{cdc2}$ kinase activity (Fig. 4*d*). First, experiments on tissue culture cells suggest that the accumulation of an unstable protein to some critical level is required to pass the restriction point[42]. Second, the *cdc2* gene of *S. pombe* is required at Start as well as for the induction of mitosis[43]. The Start function of *cdc2* is independent of the genes that regulate its mitotic function, *cdc25*, *weel* and *cdc13*, the *S. pombe* B-type cyclin, suggesting both that the substrate specificity and kinase activity of $p34^{cdc2}$ at Start and the induction of mitosis are different, and that a second group of genes that has not yet been identified exists to modulate the activity of $p34^{cdc2}$ at Start[8]. Third, homologues of both cyclin and $p34^{cdc2}$ have been identified among mutants which affect the ability of mating pheromones to arrest the cell cycle of *S. cerevisiae* at Start[44,45] (W. Courchesne and J. Thorner, personal communication). These lines of evidence strongly suggest that the interaction between members of the cyclin and $p34^{cdc2}$ families will play a key part in the regulation of Start as well as in the induction of mitosis. □

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The role of cyclin synthesis and degradation in the control of maturation promoting factor activity

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We show that cyclin plays a pivotal role in the control of mitosis. A proteolysis-resistant mutant of cyclin prevents the inactivation of maturation promoting factor and the exit from mitosis both *in vivo* and *in vitro*. We have used a fractionated extract to study the activation of MPF by added cyclin protein.

THE activation of a protein complex called maturation promoting factor (MPF) induces cells to enter mitosis and meiosis. In the accompanying paper[1] we show that cyclin is the only newly synthesized protein required to induce MPF activity and the entry into mitosis in the cell cycle of early *Xenopus* embryos. This finding implies that in early embryonic cell cycles, none of the other components of MPF needs to be synthesized in each cell cycle, and that all steps in MPF activation and inactivation other than cyclin accumulation are post-translational events. Here we investigate the role of cyclin synthesis and degradation

in the control of MPF activity.

Two lines of evidence suggest that cyclin degradation may be required for the inactivation of MPF and exit from mitosis and meiosis:(1) Extending the duration of the mitotic state with pharmacological agents increases the stability of cyclin, and (2) high concentrations of protease inhibitors arrest starfish oocytes in meiosis I (ref. 2) and stabilize cyclin (T. Hunt, personal communication).

The recent purification of MPF has led to a more detailed understanding of its molecular nature. MPF appears to be identical to the growth-associated histone H1 kinase and purified preparations of both these activities contain $p34^{cdc2}$, the homologue of the product of the *cdc2* gene of *Schizosaccharomyces pombe*, whose activity is required for entry into mitosis[3-5].

The biochemistry of MPF activation has been studied in immature oocytes. In these cells MPF activation and meiosis I can be induced in the absence of protein synthesis, implying that such oocytes contain an inactive form of MPF, which has been termed pre MPF[6,7]. In *Xenopus* oocytes, the post-translational conversion of preMPF into MPF requires ATP and small quantities of MPF. The role of MPF in this reaction appears to be to antagonize an inhibitory factor named INH, which inhibits the conversion of preMPF into MPF[7].

To examine the role of cyclin proteolysis in the exit from mitosis and meiosis we prepared cell-cycle extracts that preserve the cytostatic factor (CSF)-mediated metaphase arrest of unfertilized eggs. Cyclin is stable in CSF-arrested extracts but is rapidly degraded when these extracts are induced to re-enter the cell cycle by the inactivation of CSF. A mutant cyclin that retains the ability to induce mitosis but is resistant to proteolysis arrests the cell cycle in metaphase even when CSF activity is

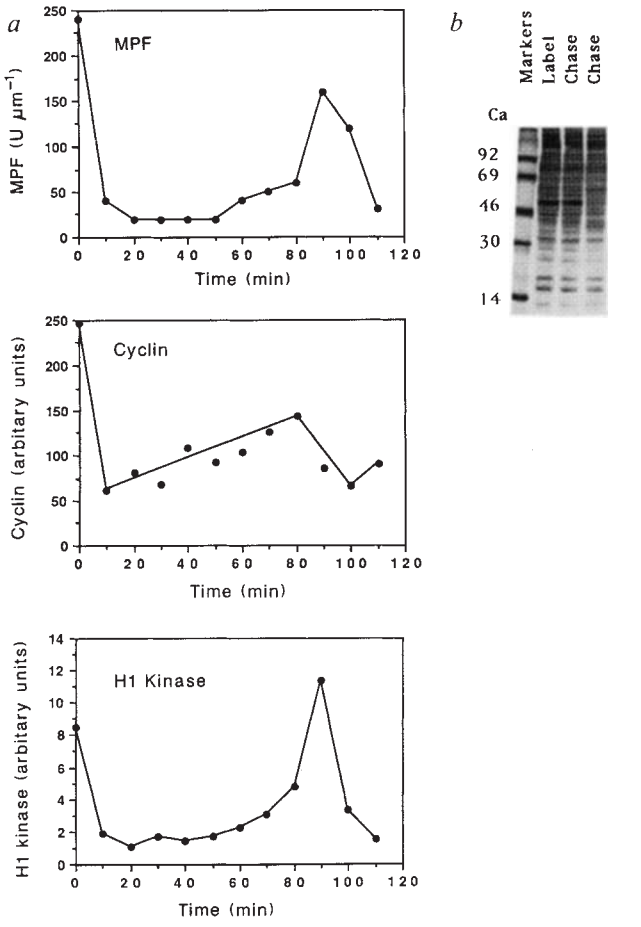

Fig. 1 Behaviour of CSF-arrested extracts. *a*, The abundance of cyclin and the activities of MPF and H1 kinase in a CSF-arrested extract after incubation with [^{35}S]methionine for 30 min before the addition of calcium to 0.2 mM at time zero. *b*, The stability of cyclin in CSF-arrested extracts. 100 μg ml^{-1} of cycloheximide was added to a CSF-arrested extract which had been incubated with [^{35}S]methionine for 60 min (Label) and one half of this extract had $CaCl_2$ added to a final concentration of 0.4 mM. Both calcium-treated and control halves of the extract were subjected to a 40-minute chase (Chase).

METHODS. CSF-arrested extracts were prepared as follows. *Xenopus* eggs were squeezed into MMR (ref. 1), dejellied with cysteine and washed four times with XB (ref. 1) and then twice with XB containing the following additions: 5 mM KEGTA, pH 7.7, 1 mM $MgCl_2$, 10 μg ml^{-1} each of leupeptin, pepstatin and chymostatin. The eggs were then transferred to a 5 ml centrifuge tube containing 1 ml XB with the additions described above plus 100 μg ml^{-1} cytocholasin B. Versilube F-50 oil was used to remove excess buffer for extracts made from activated eggs[1] and the packed eggs were then spun for 10 min at 15,000*g* at 15 °C. The cytoplasmic fraction was made 10 mM in creatine phosphate and 10 μg ml^{-1} each in leupeptin, chymostatin and pepstatin and spun for 15 min at 15,000*g* at 2 °C, then sperm nuclei were added to a final concentration of 10^5 per ml. MPF and H1 kinase activities and cyclin abundance were measured as described[1].

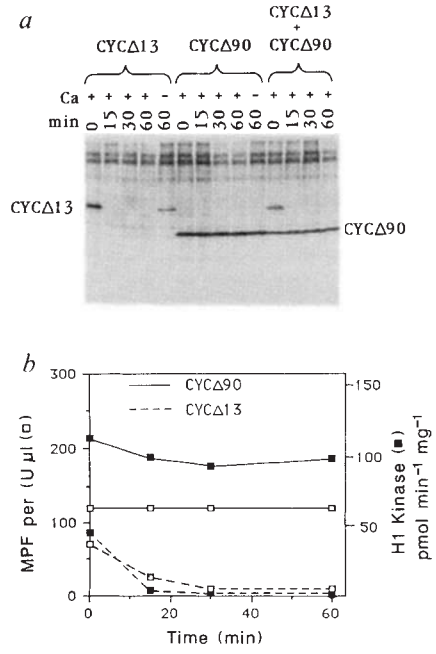

FIG. 2 A truncated cyclin is resistant to degradation. *a* An autoradiograph of a 12.5% polyacrylamide gel of [^{35}S]methionine-labelled CSF-arrested extracts containing the mRNAs encoding CYCΔ13, CYCΔ90 or both mRNAs together, at the indicated times after the addition of calcium. *b* The levels of MPF and H1 kinase activity during calcium-induced release from CSF arrest in extracts containing CYCΔ13 or CYCΔ90 whose pattern of labelled proteins is shown in *a*. The mRNAs were added to a CSF-arrested extract, containing [^{35}S]methionine at 0.4 mCi ml^{-1}, at a final concentration of about 10 μg ml^{-1}. The extract was incubated at 23 °C for 40 min before addition of cycloheximide to 100 μg ml^{-1}. The extract was then split into two portions, $CaCl_2$ was added to one portion to a concentration of 0.4 mM, and samples were taken at the indicated times and diluted in sample buffer for analysis on gels, fixed to visualize their nuclear morphology and processed for MPF, cyclin, and H1 kinase assays as described.

METHODS. The clones CYCΔ13 and CYCΔ90 were constructed as follows. A 1.3-kb *Nco*I–*Hin*dIII fragment from the sea urchin B cyclin complementary DNA containing the coding sequence of the sea urchin cyclin B gene, minus the first 13 amino acids, was ligated to the vector pSPBP4 (ref. 19; a gift from William Hansen) that had been cleaved with *Nco*I and *Xba*I, after filling in the *Hin*dIII and *Xba*I ends with T4 DNA polymerase. The resulting plasmid, A400p27, has the cyclin gene out of frame with the ATG provided by pSPBP4. CYCΔ13 was made by cleaving A400p27 with *Nco*I, filling in the sticky ends with T4 DNA polymerase and ligating with T4 DNA ligase to place the cyclin gene in frame with the ATG. CYCΔ90 was made by cleaving A400p27 with *Bgl*II and *Nco*I, filling in the sticky ends with T4 DNA polymerase and ligating with T4 DNA ligase. Capped CYCΔ13 and CYCΔ90 mRNAs were made by transcribing CYCΔ13 or CYCΔ90 DNA with SP6 RNA polymerase, including GpppG or m^{7}GpppG in the transcription reaction[20].

absent. Finally, we have started to define the biochemical role of cyclin protein by preparing fractionated extracts where the addition of exogenous cyclin induces the activation of MPF.

CSF-arrested extracts

We have used CSF arrested extracts to investigate the fate of cyclin during a physiological metaphase arrest and during the exit from meiosis. Lohka and Masui showed that extracts made from unfertilized *Xenopus* eggs in the presence of EGTA retain the CSF-mediated arrest in meiotic metaphase, directly convert added sperm nuclei into condensed chromosomes on meiotic spindles and can be induced to progress to interphase by the addition of calcium[8]. Thus calcium can release the cell cycle from CSF arrest *in vitro* as well as *in vivo*. We prepared similar, but more concentrated, extracts where the addition of as little as 0.2 mM calcium induced the inactivation of CSF, spindle dissolution, chromosome decondensation and the formation of interphase nuclei within 30 min.

Figure 1*a* shows cyclin levels, MPF and H1 kinase activity in CSF-arrested extracts and in CSF-arrested extracts induced to re-enter the cell cycle by calcium treatment. Before the addition of calcium the levels of cyclin, MPF and H1 kinase activity were all high. Within 10 min of calcium addition, cyclin levels had dramatically decreased, as had those of MPF and H1 kinase activity. During interphase the levels of cyclin increased continuously but H1 kinase and MPF activity appeared suddenly at 90 min corresponding to the time of nuclear envelope breakdown, showing that H1 kinase activity is proportional to MPF activity but not to cyclin abundance. By 100 min the nuclei were in telophase and MPF and H1 kinase activity had decreased. A control portion of the same extract, without added calcium, maintained high levels of cyclin, MPF and H1 kinase activity for more than 80 min (data not shown), suggesting that cyclin degradation is induced by the destruction of CSF.

We examined the stability of cyclin during CSF arrest by performing a pulse-chase experiment. We added [^{35}S] methionine to a CSF-arrested extract for 20 min and then added cycloheximide to prevent further protein synthesis. Figure 1*b* shows that the labelled cyclin was completely stable during a further 40 min incubation. In contrast, when calcium was added immediately after the cycloheximide, the cyclin bands disappeared although the other labelled proteins were completely stable, suggesting that one of the functions of CSF is to prevent the cyclin degradation that normally occurs when MPF is active. CSF-arrested extracts that were induced to enter interphase, after protein synthesis had been inhibited by cycloheximide, never entered mitosis, confirming the protein synthesis requirement for the induction of mitosis.

Proteolysis-resistant cyclin

If the domain of cyclin that is responsible for the activation of MPF activity is separable from the one that directs cyclin proteolysis we could ask directly whether cyclin proteolysis is required for the exit from mitosis. We constructed two plasmids CYCΔ13 and CYCΔ90 that contain deletions of the N-terminal 13 and 90 amino acids of the sea urchin cyclin B, respectively, and which can be transcribed by SP6 RNA polymerase to yield highly translatable messenger RNA. We refer to the proteins encoded by these plasmids as the CYCΔ13 and CYCΔ90 cyclins. We have used the CYCΔ13 cyclin as a control in the following experiments because the CYCΔ13 mRNA is much more efficiently translated than full length sea urchin cyclin mRNA used in the preceding paper[1]. The biological properties of the full-length and CYCΔ13 proteins appear to be identical.

We examined the proteolysis of these truncated cyclins in CSF arrested extracts and during the exit from metaphase after the calcium-induced inactivation of CSF. The synthetic cyclin mRNAs were translated for 40 min in a CSF-arrested *Xenopus* cell-cycle extract containing [^{35}S] methionine before cycloheximide was added to prevent further protein synthesis. Cal-

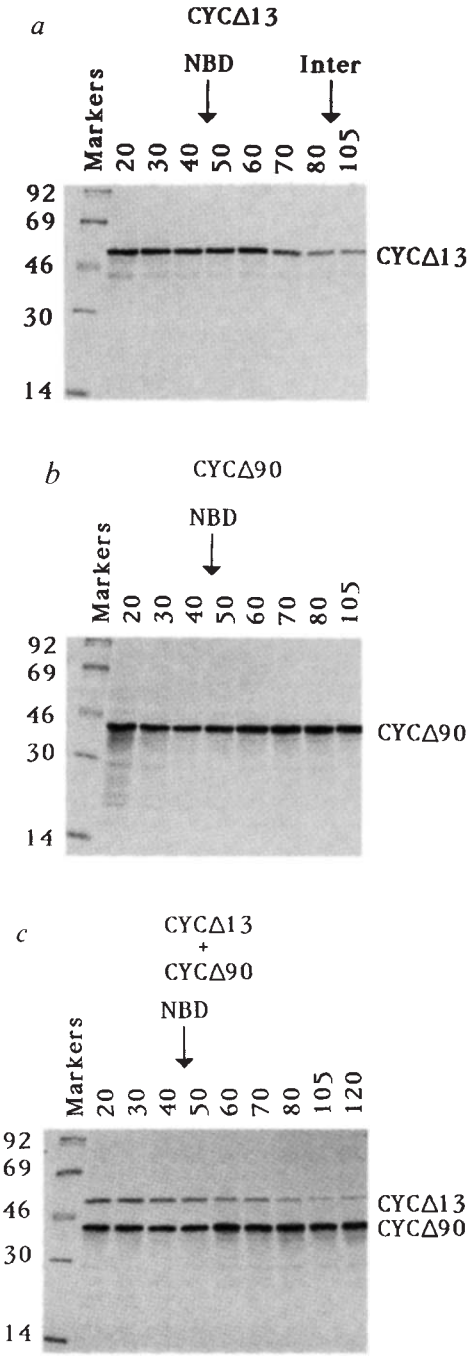

FIG. 3 Induction of mitosis by cyclin made in reticulocyte lysate. *a*, Autoradiograph of a 12.5% polyacrylamide gel showing samples taken at the indicated times after the addition of 10 μl reticulocyte lysate containing approximately 50 nM [^{35}S]methionine-labelled CYCΔ13 cyclin to 20 μl a CSF-arrested extract that had been induced to enter interphase after the addition of cycloheximide. The times of nuclear breakdown (NBD) the reappearance of interphase nuclei (Inter), the position of the CYCΔ13 cyclin and the molecular weight markers (in thousands) are indicated. *b*, Addition of reticulocyte lysate containing ~50 nM CYCΔ90 cyclin; other details as in *a*. *c*, Addition of reticulocyte lysate containing ~15 nM CYCΔ13 and ~35 nM CYCΔ90; other details as in *a*.

METHODS. Sperm nuclei were added to a CSF-arrested extract which was then made 20 μg ml^{-1} in cycloheximide before the addition of $CaCl_2$ to 0.4 mM to induce progression into interphase. Twenty minutes after the addition of calcium, 0.5 volumes of reticulocyte lysate containing translated cyclin protein was added. CYCΔ13 or CYCΔ90 mRNA was translated in message-dependent rabbit reticulocyte lysate[21] and the amount of synthesized protein was calculated from the amount of incorporated methionine, the cold methionine pool size in the reticulocyte lysate and the number of methionines in CYCΔ13 or CYCΔ90.

cium was then added to the extract to induce the inactivation of CSF, destruction of endogenous cyclin and entry into interphase. After calcium addition, we monitored the pattern of labelled proteins, nuclear morphology, MPF and H1 kinase activity. Extracts containing the CYCΔ13 cyclin entered interphase rapidly as judged by the formation of interphase nuclei (data not shown) and the loss of MPF and H1 kinase activity (Fig. 2*b*). The CYCΔ13 cyclin, but not other labelled non-cyclin proteins, was degraded (Fig. 2*a*). In a control extract to which calcium was not added the levels of the CYCΔ13 cyclin, MPF and H1 kinase decreased less than twofold during a 60-min incubation in the presence of cycloheximide. Thus deletion of the first 13 amino acids of cyclin does not alter its susceptibility to the cyclin-specific proteolysis that is induced in the mitotic state or the ability of CSF to protect it from such degradation. In contrast, in extracts containing the CYCΔ90 cyclin, the addition of calcium did not induce the degradation of CYCΔ90, the exit from metaphase, or decrease the activity of MPF and H1 kinase (Fig. 2*a*,*b*). These results are consistent with the hypothesis that CYCΔ90 is resistant to proteolysis and that cyclin degradation is required for exit from CSF arrest. As a further test we added calcium to a CSF arrested extract containing both CYCΔ13 and CYCΔ90 cyclins (Fig. 2*a*). This extract remained in metaphase and the CYCΔ13 cyclin was degraded but the CYCΔ90 cyclin was not, demonstrating that the CYCΔ90 cyclin does not inhibit the inactivation of CSF, or block the proteolysis of intact cyclin molecules in some other way.

We then tested the ability of the deleted cyclins to induce mitosis by adding protein translated in reticulocyte lysate to a CSF-arrested extract that had been induced to enter interphase after the addition of cycloheximide. The addition of reticulocyte lysate containing the translation products of an irrelevant mRNA, such as tobacco mosaic virus (TMV) RNA did not induce mitosis and the translation products were stable in the extract. Figure 3*a* shows that the addition of reticulocyte lysate containing the CYCΔ13 cyclin induced nuclear envelope breakdown after 40–50 min. The cyclin was then degraded and the extract returned to interphase. (Smaller doses of the CYCΔ13 cyclin failed to induce mitosis and the added cyclin was stable for at least two hours (data not shown).) In contrast, the addition of the CYCΔ90 cyclin induced nuclear envelope breakdown, but the cyclin was stable and the extract remained in mitosis for the duration of the experiment (Fig. 3*b*). When CYCΔ13 and CYCΔ90 cyclins were added together the extract entered mitosis and induced the degradation of the CYCΔ13 cyclin, but failed to return to interphase (Fig. 3*c*). These experiments demonstrate that the deletion of the N-terminal 90 amino acids of the sea urchin cyclin does not affect its ability to induce mitosis but renders it resistant to proteolysis and strongly suggest that cyclin degradation is required for the exit from mitosis as well as meiosis.

The presence of the CYCΔ90 cyclin can also arrest the cell cycle *in vivo*. We injected unfertilized eggs with mRNA and allowed the mRNA to translate for two hours before activating the eggs by injecting calcium and recording their behaviour by time-lapse video microscopy. Eggs that received CYCΔ13 RNA, CYCΔ90 RNA, TMV RNA or no RNA showed the normal cortical activation reaction in which the pigment contracts towards the animal pole and the egg rounds up after activation (Fig. 4*b*). In the TMV RNA and control eggs, after the initial contraction the pigment relaxed, the cortex became less stiff and the egg spread out under the force of gravity. Approximately

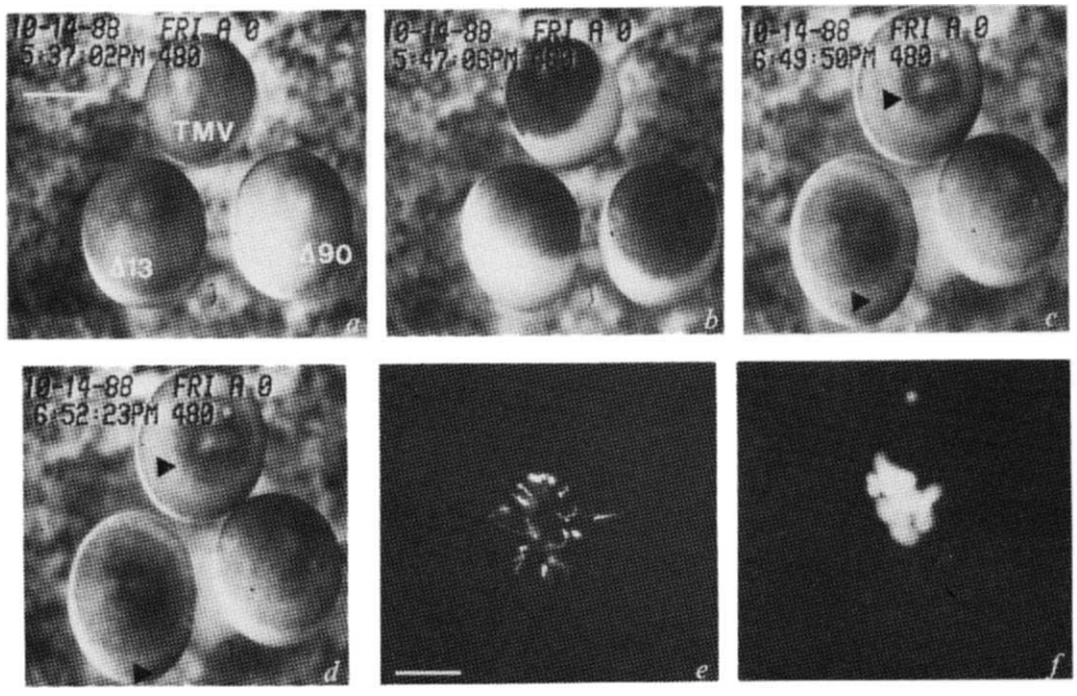

FIG. 4 CYCΔ90 cyclin arrests the embryonic cell cycle *in vivo*. *a*–*d*, Photographs of a time-lapse video recording of eggs that had been injected, in non-activating medium (100 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 25 mM KPIPES, pH 6.5) with 50 nl of a 1 mg ml^{-1} solution of CYCΔ13 (Δ13), CYCΔ90 (Δ90) or tobacco mosaic virus (TMV) RNA two hours before they were activated by injection of 20 nl MMR. The four images show the eggs: *a*, before activation (the pigmentation of all three eggs is identical, but the egg injected with CYCΔ90 mRNA was more strongly illuminated at this time); *b*, 5 min after activation; *c* and *d*, at two points during the passage of the first surface contraction wave across the eggs injected with CYCΔ13 or TMV RNA. The surface contraction waves (►) are visible as rings of lighter pigmentation that originate at the animal pole (the centre of the visible hemisphere of the egg in this figure) and that move across the surface of the egg. There is no visible surface contraction wave in the egg injected with CYCΔ90 mRNA. (Scale bar, 1 mm). *e*, The single set of mitotic chromosomes from a fertilized egg that had been injected with 50 nl of a 1 mg ml^{-1} solution of CYCΔ90 mRNA. The egg was squashed and fixed in the presence of Hoechst 33342 (ref. 1) at 150 min after fertilization. (Scale bar, 20 μm). *f*, One of fourteen telophase nuclei in a fertilized egg that had been injected with 50 nl of a 1 mg ml^{-1} solution of TMV RNA at 20 min after fertilization. The egg was squashed and fixed at 155 min after fertilization. Same magnification as in *e*.

50 min after activation these eggs rounded up, as the level of MPF rose. Shortly afterwards a surface contraction wave (SCW) propagated across the surface of the egg (Fig. 4*c*, *d*). Strong circumstantial evidence supports the notion that the SCW is induced by the decline in MPF activity that follows each mitosis[6,9,10]. At 25° C the first SCW occurs about 60 min after activation and subsequent ones occur at 30-min intervals. In eggs that had been injected with CYCΔ13 mRNA (Fig. 4*c*,*d*) the egg also undergoes normal SCWs, but the interval between the later SCWs was shorter than that in uninjected or TMV injected controls, suggesting that the translation of cyclin at high levels may shorten the length of the cell cycle. Although eggs injected with CYCΔ90 mRNA showed a normal cortical reaction upon activation (Fig. 4*b*), the cortex never lost its stiffness, the eggs never spread out and no SCWs were seen (Fig. 4*c*,*d*)). Thus the presence of the deleted cyclin *in vivo* prevents the escape from CSF mediated meiotic arrest. The occurrence of the normal cortical contraction at activation and the elevation of the eggs' vitelline membrane in eggs arrested in metaphase with the deleted cyclin demonstrates that these events are dependent on the increased levels of intracellular calcium induced by activation rather than by the decline in MPF that this calcium spike normally induces.

To show that the deleted cyclin can arrest cells in mitosis as well as in meiosis we injected fertilized eggs with CYCΔ90 or TMV RNA during the first cell cycle and observed them for two and a half hours. The eggs injected with CYCΔ90 mRNA failed to cleave during this period. To visualize the nuclei we squashed eggs and observed their DNA using a fluorescent DNA-binding dye. Figure 4*e* shows the single set of condensed chromosomes found in an embryo that had been injected with CYCΔ90 mRNA, whereas Fig. 4*f* shows one of 14 telophase nuclei found in a single control embryo that had been injected with TMV RNA. We conclude that the presence of the deleted cyclin encoded by CYCΔ90 can arrest the embryonic cell cycle in mitosis *in vivo* as well as *in vitro*, demonstrating that cyclin degradation is required for the exit from both meiosis and mitosis.

Cyclin and MPF activation

We have started to investigate the biochemistry of cyclin. The demonstration that cyclin synthesis is required to drive the embryonic cell cycle into mitosis and that cyclin degradation is required for the exit from mitosis implies that cyclin has a role in the activation and maintenance of MPF activity. Possible mechanisms by which cyclin could activate $p34^{cdc2}$ to yield MPF activity include: binding to $p34^{cdc2}$ to form the active MPF complex; stimulating the post-translational activation of $p34^{cdc2}$; or acting to inhibit an inactivator of the MPF activity of $p34^{cdc2}$.

In the ooctye pre-MPF can be activated to MPF post-translationally, by the removal of an inhibitor fraction called INH, and incubation of the partially purified fraction with ATP or ATPγS, (a more potent activator of MPF than ATP[7], presumably because the thiophosphate group that it introduces into proteins is resistant to the action of phosphatases[11]). We first asked whether removal of INH also leads to the post-translational activation of MPF from CSF-arrested extracts that were activated and returned to an interphase state, that is, whether protein synthesis, or more specifically cyclin synthesis, is required to activate MPF when INH is removed. We prepared an extract deficient in cyclin by activating CSF-arrested extracts in the presence of cycloheximide; as a control, the same CSF-arrested extract was activated in the absence of cycloheximide. Activation with calcium destroys the endogenous cyclin. Samples were taken during the ensuing interphase from both extracts. At various times samples of the extracts were fractionated by precipitation with 33% ammonium sulphate which precipitates MPF and pre MPF and separates them from INH, which precipitates in the 45–55% ammonium sulphate fraction[7,12] (M. S. and T. Lee unpublished data). The 0–33% fractions were incubated with ATPγS and assayed for MPF activity by injection into cycloheximide-arrested oocytes.

The results of this experiment are shown in Fig. 5*a*. In the extract where protein synthesis was allowed to proceed, the ability of ATPγS to convert preMPF into active MPF gradually appeared during interphase. But, none of the fractions prepared from the cycloheximide treated extract could be activated by ATPγS to yield active MPF, demonstrating that protein synthesis is required to produce an activity required for the activation of MPF. Because the only newly synthesized protein required for the appearance of MPF activity in unfractionated extracts is cyclin, this result strongly imples that cyclin is directly required for generating MPF, rather than inhibiting INH.

We then tested directly the ability of exogenous cyclin protein to stimulate the formation of MPF when added to a fraction deficient in cyclin. We prepared a cyclin-deficient extract by activating a CSF-arrested extract in the presence of cycloheximide, as in Fig. 5*a*. From this extract we prepared a 0–33%

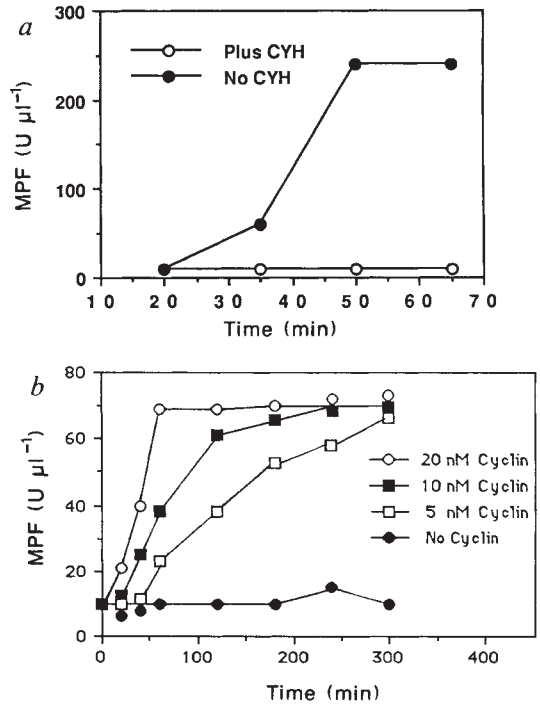

FIG. 5 Cyclin activates MPF *in vitro*. *a*, The time course of the appearance of MPF activity in a CSF-arrested extract that had been induced to progress into interphase in the presence (○—○) or (●—●) absence of cycloheximide. *b*, Kinetics of the induction of MPF activity after addition of cyclin protein. METHODS. *a*, One aliquot of a CSF-arrested extract was made up to 60 μg ml^{-1} in cycloheximide and the other was left untreated. Both portions were induced to progress into interphase by adding $CaCl_2$ to 0.3 mM. At the indicated times 60 μl samples were taken, chilled, diluted with 200 μl EB (composition in ref. 1) containing 5 mM dithiothreitol, 0.25 mM PMSF and 10 μg ml^{-1} each of leupeptin, pepstatin and chymostatin and spun for 20 min at 100,000 r.p.m. in a Beckman Airfuge. The supernatant was precipitated by addition of ammonium sulphate to 33% saturation. The ammonium sulphate pellet was resuspended in 15 μl EB plus protease inhibitors and dialysed for 1 h against two changes of EB plus 5 mM dithiothreitol. (Large preparations of this fraction were made by scaling up this procedure using CSF-arrested extracts to which cycloheximide had been added. After dialysis the fraction was frozen in liquid nitrogen and stored in small aliquots at −70 °C.) The dialysed material was made 9 mM in ATP-γS and incubated for 75 min at 20 °C and then assayed for MPF activity by microinjection into cycloheximide-treated immature *Xenopus* oocytes[1]. Incubation of the fraction without ATP-γS did not activate MPF (data not shown). *b*, Each reaction contained 0.5 volumes of the activated CSF fraction described in *a*, 0.1 volumes 9 mM ATP-γS and 0.4 volumes of reticulocyte lysate containing different amounts of CYCΔ90 cyclin that had been previously synthesized *in vitro* to yield the indicated final cyclin concentrations. After generating the cyclin protein in the reticulocyte lysate, no protein synthesis occurred in any further reactions.

ammonium sulphate fraction and added reticulocyte lysate containing previously synthesized cyclin protein (CYCΔ90). The mixture was incubated with ATPγS and assayed at various times for MPF activity. In the complete reaction, MPF activity and H1 kinase activity were generated showing that added cyclin and ATPγS can activate MPF. The omission of the *Xenopus* egg fraction or ATPγS, or the substitution of reticulocyte lysate containing TMV translation products completely blocked the ability to generate active MPF. The activation of MPF is not a unique property of the proteolysis-resistant cyclin; the addition of the CYCΔ13 cyclin also induced MPF activation (data not shown).

The addition of different amounts of the CYCΔ90 cyclin to the reaction affected the rate at which MPF activity was generated but not its final level (Fig. 5*b*). This suggests that if cyclin is a component of activated MPF the amount of one of the other components found in the 33% ammonium sulphate fraction must be limiting, even at the lowest doses of added cyclin. Alternatively, cyclin may be acting to stimulate the activation of MPF but may not be a tightly bound component of the active MPF complex.

Discussion

We have attempted to verify the prediction, made in the accompanying paper, that cyclin degradation is required for the exit from mitosis. A truncated form of the sea urchin cyclin B, CYCΔ90, is resistant to proteolysis and its presence arrests the cell cycle in mitosis. The simplest interpretation of this finding is that the deletion of the N-terminal 90 amino acids of cyclin removes sequences required for the degradation of cyclin without affecting other properties of the molecule. Alternatively, the N-terminus of cyclin might play some active role in the inactivation of MPF, such as guiding MPF to a site where it is inactivated, and cyclin degradation could be a secondary event, which occurred as a consequence of the inactivation of MPF. If this were the case, the addition of a large excess of the CYCΔ13 cyclin (which behaves as a full-length cyclin) ought to overcome the ability of the CYCΔ90 cyclin to arrest extracts in metaphase. We have found that the ability of the CYCΔ90 cyclin to arrest extracts in mitosis cannot be overcome by a tenfold excess of CYCΔ13, suggesting that CYCΔ90 arrests extracts in mitosis because it is resistant to proteolysis and not because it lacks some function required directly for MPF inactivation. A possibility which is more difficult to exclude is that the ability of cyclin to maintain MPF activity is abrogated by some modification of cyclin, that CYCΔ90 is not a substrate for this modification and that cyclin degradation is a consequence of the inactivation of MPF. Even if it were possible to arrest extracts or cells in mitosis by overproducing a wild-type cyclin, it would be impossible to demonstrate that the arrest was a consequence of saturating cyclin proteolysis, rather than some inhibitory cyclin modification.

At present we understand very little about cyclin proteolysis. Although it can be inhibited by a number of protease inhibitors both in starfish (T. Hunt, personal communication) and *Xenopus* (our unpublished data), the high inhibitor concentrations that are required also inhibit protein synthesis and nuclear decondensation, suggesting that the inhibition of cyclin degradation in these experiments may be non-specific. Even in experiments where cyclin is the only labelled protein, we have failed to see intermediates in degradation, suggesting that once degradation has begun it proceeds rapidly to completion. The stability of CYCΔ90 suggests that in some way the N-terminal region is required for cyclin proteolysis. But without an extensive series of mutants in this region it is impossible to draw any more specific inference. At least one site required for cyclin phosphorylation has been localized to about amino-acid 70 in the sea urchin cyclin sequence (S. Mackie and T. Hunt, personal communication) and it has been shown that inhibitors of protein phosphorylation can block the degradation of cyclin in *Xenopus* egg extracts (M. Felix and E. Karsenti, personal communication). We speculate that the phosphorylation of this sequence is required for cyclin degradation.

We have shown that exogenous cyclin protein can activate MPF in a fractionated cell-cycle extract and presented evidence that, in this reaction, the exogenous cyclin affects the rate but not the final level of MPF activity. These experiments were performed on a fraction that was prepared from CSF-arrested extracts that had been treated with cycloheximide before activation by added calcium. We have shown that under these conditions recently synthesized frog cyclin is largely destroyed. In the absence of antisera to all of the frog cyclins we cannot demonstrate that all of the cyclin in the eggs is destroyed and must therefore admit that there may be some *Xenopus* cyclin in this fraction. Nevertheless, it is clear that the addition of exogenous cyclin is required for the generation of MPF activity and that the final level of MPF is independent of the concentration of cyclin. The simplest interpretation is that either $p34^{cdc2}$ or some other component is limiting or that each cyclin molecule can activate more than one $p34^{cdc2}$ molecule.

Evidence that cyclin need not be a stably bound component of MPF comes from studies of purified H1 kinase, which has high levels of MPF activity, but is reported to contain only $p34^{cdc2}$ (ref. 13). We wish to emphasize that the existence of forms of MPF that lack cyclin may be properties of *in vitro* systems, where inactivating components have been removed. In the natural system, cyclin may be required to stabilize MPF activity from inactivation. We suggest that much of the variability of the polypeptide components of active MPF or H1 kinase preparations may reflect the complex physiological regulation of these activities, and, at least in the case of MPF, the extremely complex system in which it is assayed. For instance, the particular substrate specificity of the protein kinase that is assayed as MPF may be manifested by $p34^{cdc2}$ molecules carrying a particular constellation of post-translational modifications, even when $p34^{cdc2}$ is not associated with other proteins. But, the attainment or maintenance of this phosphorylation state may require the association of other proteins, including, but not necessarily limited to, the cyclins. If the stability of these associations and particular modification states of $p34^{cdc2}$ varied between different organisms and different experimental conditions, then it would be possible to produce a wide variety of protein complexes that had MPF activity.

Evidence that cyclin interacts directly with $p34^{cdc2}$ comes from the genetic demonstration of allele-specific interactions between *cdc2* and *cdc13*, the cyclin homologue in *S. pombe* (refs 14–17), and precipitation of cyclin with anti-$p34^{cdc2}$ antibodies and vice versa[18]. Perhaps cyclin may act in different ways both upstream and downstream of $p34^{cdc2}$. Cyclin accumulation may be required directly or indirectly to activate $p34^{cdc2}$, but may then itself be phosphorylated by the activated $p34^{cdc2}$ and act directly or indirectly in the pathways by which $p34^{cdc2}$ kinase activity produces the mitotic state. Recent evidence in *S. pombe*, supports this idea. Deletion of the *S. pombe* cyclin homologue, *cdc13*, prevents the entry into mitosis and the activation of the $p34^{cdc2}$ kinase activity, but the missense mutation *cdc13-117* arrests cells in an aberrant mitosis where $p34^{cdc2}$ kinase activity is hyperactivated, the chromosomes are hypercondensed, but no mitotic microtubule structures are present (P. Nurse, personal communication).

It is clear that cyclin has an important role in the activation and maintenance of the particular set of post-translational modifications of $p34^{cdc2}$ that constitute MPF activity. However, the molecular details of how cyclin participates in the post-translational activation of $p34^{cdc2}$, the nature of other proteins involved in the activation and inactivation reactions, the physiological substrates of MPF and the mechanism of cyclin degradation remain largely unclear. The next few years seem certain to bring a considerable advance in our understanding of how the entry into and the exit from mitosis are controlled. □

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A new class of extragalactic radio sources with one-sided structure?

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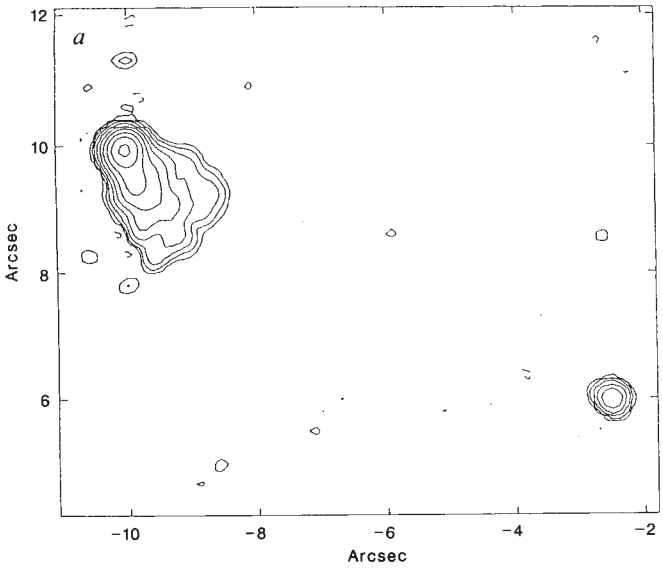
EXTRAGALACTIC radio sources with extended emission on only one side of the active galaxy usually have very dominant radio cores[1–5], large misalignments between the structure inferred from Very Long Baseline Interferometry and that on the arcsecond scale[1,6,7], and often evidence of 'blazar' characteristics and superluminal motion in the nucleus[8]. These properties are usually attributed to relativistic motion of nuclear jets in sources inclined at small angles to the line of sight. From a large survey of possible one-sided sources made with both MERLIN and the Very Large Array, we have been able to identify a class of one-sided sources that are dominated by their extended emission while the lower limits on their degree of asymmetry, inferred from our MERLIN observations, are amongst the highest known. These weak-cored one-sided sources appear to be inconsistent with the 'unified scheme'[9–15], which attempts to explain core-dominated sources as being the relativistically beamed counterparts of the lobe-dominated ones. We discuss possible explanations for this class of sources and suggest tests to distinguish between the different alternatives.

The vast majority of high-luminosity ($\geq 2\times10^{25}$ W Hz^{-1} sr^{-1} at 178 MHz extragalactic radio sources, especially when selected at a low frequency ($\leq$1 GHz), have reasonably symmetric outer lobes on opposite sides of the nucleus; the median value of the distribution of the peak brightness ratio is ~3. However, a small but significant fraction of these sources seem to have radio emission on only one side of the nucleus. These one-sided sources, also referred to as being of the D2 type[16] or C type[1], have very prominent radio cores, and many of their observed properties are consistent with the predictions of 'unified schemes'. The one-sidedness of some of the core-dominated sources must then be due largely to relativistic beaming of the extended emission so that the approaching component is Doppler-boosted and the receding one is diminished[10,11]. The brightness ratio is given by $\{(1+\beta_e \cos\phi)/(1-\beta_e \cos\phi)\}^{2+\alpha}$, where $v_e = \beta_e c$ is the bulk velocity of the extended emission, ϕ is the angle of inclination of the source axis to the line of sight and α is the radio spectral index, defined by the relationship between flux S and frequency ν: $S \propto \nu^{-\alpha}$. Examples of core-dominated, one-sided sources are NRAO140 (0333+321) (refs 17, 18), 3C273 (1226+023) (ref. 19) and 3C454.3 (2251+158) (refs 4, 17), which are also well-known superluminal sources[8].

In order to make a systematic study of one-sided radio sources, we have been making maps with high angular resolution and good dynamic range of all known candidates with both MER-



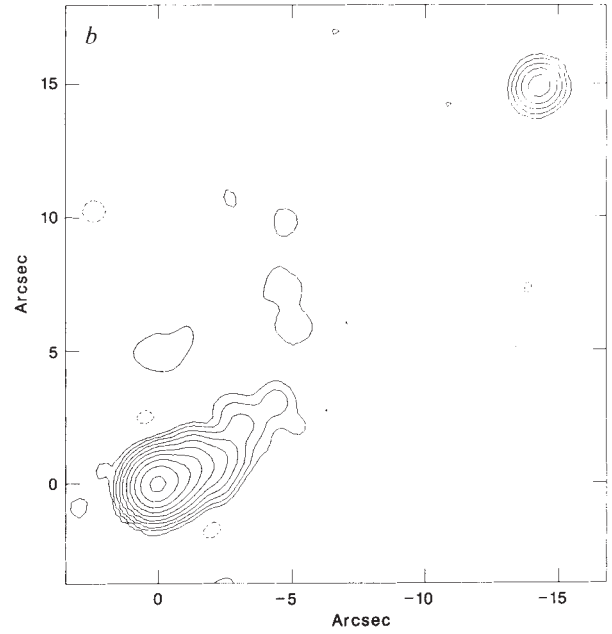


FIG. 1 *a*, MERLIN map of 0404+177 at a wavelength of 18 cm. Peak brightness = 121 mJy per beam. Contours are 0.4 × (−1, 1, 2, 4, 8, 16, 32, 64, 128, 256) mJy per beam. Restoring beam = 0.3 × 0.3 arcsec. *b*, MERLIN map of 1729+501 at a wavelength of 73 cm. Peak brightness = 1,236 mJy per beam. Contours are 2 × (−1, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512) mJy per beam. Restoring beam = 1 × 1 arcsec.