FIG. 1. Landmarks of the S. cerevisiae cell division cycle. Abbreviations: PD, plaque duplication; BE, bud emergence; iDS, initiation of DNA synthesis; DS, DNA synthesis; PS, plaque separation; NM, nuclear migration; mND, medial nuclear division; SE, spindle elongation; lND, late nuclear division; CK, cytokinesis; CS, cell separation. Distance between events does not necessarily reflect interval of time between events.
Isolation of temperature sensitive mutants

1500 ts mutants
146 Cdc- phenotype
32 cdc complementation groups
FIG. 1. Landmarks of the S. cerevisiae cell division cycle. Abbreviations: PD, plaque duplication; BE, bud emergence; iDS, initiation of DNA synthesis; DS, DNA synthesis; PS, plaque separation; NM, nuclear migration; mND, medial nuclear division; SE, spindle elongation; lND, late nuclear division; CK, cytokinesis; CS, cell separation. Distance between events does not necessarily reflect interval of time between events.
A cdc mutant – permissive temperature
A *cdc* mutant – restrictive temperature
Execution point: position in cell cycle after which Cdc protein is not needed.

Shift to restrictive temperature; time lapse photography; arrange cells by position in cell cycle at temp shift.
Cdc phenotypes suggest a dependency relationship for landmark events.

Table 1. Summary of mutant phenotypes. Cells were shifted from 23°C to 36°C at the time of cell separation. Abbreviations are as in Fig. 1. A minus sign indicates that an event does not occur, a plus indicates that the event occurs once, and a double plus indicates that the event occurs more than once.

<table>
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<tr>
<th>$cdc^o$</th>
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<th>BE</th>
<th>iDS</th>
<th>DS</th>
<th>NM</th>
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<td>++</td>
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<td>(19)</td>
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* Although mutations in 32 $cdc$ genes have been discovered, only 19 of these genes are included here for consideration in developing a model of the cell cycle. Most of those not included were left out because they progress through several cycles at the restrictive temperature before development is arrested and this prevents an analysis of DNA synthesis during their terminal cycle. The mutant $cdc$ 1 (19) was excluded because macromolecule synthesis, as well as bud emergence, is rapidly arrested in this mutant at the restrictive temperature, and we suspect that this inhibition of growth prevents the occurrence of some events which are not normally dependent upon bud emergence, but which are dependent on growth.
Possible relationships between two gene-mediated steps

1. Dependent

2. Dependent

3. Independent

4. Interdependent
Reciprocal shift experiments
(to determine gene function order)

1. Shift culture to arrest under one condition (Condition A; eg, high temp)
2. Release from that arrest and apply second condition (Condition B; eg, DNA synthesis inhibitor)
3. Observe phenotype. Did cells complete the cell cycle after release from the first arrest condition? If so, it argues that the first condition blocks a step after the step blocked by the second condition.
4. Perform shifts in opposite order and observe phenotype. Is it consistent with order deduced in (3) above?
Schematic representation of dependent pathways of cell cycle events

- SPB Duplication
- SPB Separation
- DNA Synthesis
- Initiation DNA Synthesis
- Late Nuclear Division
- Medial Nuclear Division
- Nuclear Migration
- Cytokinesis
- Cell Separation
- cdc 3, 10, 11, 13
- cdc 5, 14, 15
- cdc 28
- cdc 4
- cdc 7
- cdc 8, 21
- cdc 9, 13, 16, 17, 20, 23
- cdc 24
Cell cycle dependency relationships
Yeast Life Cycle

Mating factor arrests haploid cells in G1, prior to spindle pole body duplication

Sporulation conditions arrest diploids in G1, prior to spindle pole body duplication
The concept of START

a point at which cells commit to initiating and completing a cell cycle

Mating factor causes arrest at START

Sporulation causes arrest at START

Starvation for nutrients causes arrest at START
eg, P, N, C, S

\textit{cdc28} mutants arrest at START

\textit{CDC28} seems to be a pretty interesting gene
Oscillation of a protein named cyclin following fertilization of sea urchin embryos

Figure 2. Correlation of the Level of Cyclin with the Cell Division Cycle

A suspension of eggs was fertilized, and after 6 min, $^{35}$S-methionine was added to a final concentration of 25 μCi/ml. Samples were taken for analysis on gels at 10 min intervals, starting at 16 min after fertilization. Samples were taken 20–30 sec later into 1% glutaraldehyde in calcium-free artificial seawater for later microscopic examination; the cleavage index is shown thus: □---□. The autoradiograph shown as an inset was scanned to yield the data plotted thus: cyclin, •••••; protein B, ▲••••▲.
Pulse-chase experiment demonstrates cyclin is synthesized throughout the cell cycle.

Figure 4. Rates of Synthesis of Three Proteins during the First 2 h of Cleavage

The relative intensities of cyclin, protein C, and the putative histone band were determined from the autoradiograph shown in the right panel of Figure 3 by densitometry.
Plasmid (and other) Libraries

Genomic DNA library
Screening is the key (for any library, actually)
Fractionation of sea urchin maternal mRNA as first step to cloning cyclin cDNA

Fig. 1. Fractionation of sea urchin maternal mRNA on denaturing sucrose gradients. Poly(A)$^+$ RNA was prepared and fractionated on formamide/sucrose gradients as described in Materials and methods. This figure shows the autoradiograph of the translation assay of fractions 6–16. The positions of 18S and 28S ribosomal RNA (indicated above the panel) were determined from a parallel run of poly(A)$^-$ RNA. The positions of cyclin and the small subunit of ribonucleotide reductase are indicated on the right of the figure. Band ‘x’, a predominantly non-adenylated mRNA can be seen faintly in the top right-hand corner; its mobility gives a good indication of how large the other messages are compared to what would be expected.
Identification of cyclin cDNA clone

1. Prepare cDNA library from RNA fractions enriched for cyclin mRNA

2. Hybridize DNA from individual clones to egg RNA

3. Treat with RNase H, which destroys DNA:RNA hybrids, and translate in vitro

39th clone tested showed reduced cyclin translation product
Comparison of sea urchin and clam RNAs and proteins
Egg maturation and early cleavages in Xenopus

**Figure 2.** Schematic view of Xenopus oogenesis, maturation, and early embryogenesis.
MPF – Maturation Promotion Factor

Fig B. Progesterone treatment induces an activity that can transferred to other, immature oocytes and cause maturation

Fig C. MPF activity is also present in mitotically cycling early cleavage embryos. Hence, MPF also stands for mitosis promotion factor

Purified MPF contains 2 proteins, 34 and 45 kD
Sea urchin mRNA induces frog oocyte maturation

Cyclin mRNA is necessary and sufficient for this induction

Table I. Effect of sea urchin RNA on oocyte maturation

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Amount of RNA injected (ng/oocyte)</th>
<th>Number of oocytes injected</th>
<th>% GVBD</th>
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<tr>
<td>(A) A. punctulata total RNA</td>
<td>245</td>
<td>8</td>
<td>100</td>
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<td>100</td>
<td>5</td>
<td>80</td>
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<tr>
<td></td>
<td>25</td>
<td>5</td>
<td>80</td>
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<td>12.5</td>
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<td>80</td>
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<td>6.3</td>
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<td>(D) Anti-sense cyclin mRNA</td>
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<tr>
<td></td>
<td>17.5</td>
<td>11</td>
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Experiment 2
- Progesterone: 7, 57%
- Control (uninjected): 8, 0%
- Total RNA (245 ng): 9, 22%
- Ribonucleotide reductase ablated (245 ng): 12, 25%
- Cyclin ablated RNA (245 ng): 11, 0%
- Pure cyclin mRNA (17.5 ng): 8, 62%
Pure cyclin mRNA induces oocyte maturation

![Graph showing time for 100% GVBD (hours) vs T7 cyclin RNA (ng per oocyte).]

**Fig. 8.** Time course of frog oocyte maturation in response to pure cyclin mRNA. Serial dilutions of synthetic cyclin mRNA made from the pGEM2 cyc4 subclone were injected into groups of five *Xenopus* oocytes in a volume of 50 nL. Oocytes injected with the same volume of water or antisense transcript failed to mature. The time taken for ‘white-spot formation’ was noted and averaged for each group. The time taken for progesterone-induced maturation in a parallel uninjected group of oocytes is indicated by the line and caption ‘progesterone’.
Return to yeast, but a different one, the fission yeast *Schizosaccharomyces pombe*
Fission yeast cdc2 mutants arrest before mitosis

cdc2 is the fission yeast equivalent of budding yeast CDC28
Cloning yeast genes

Genomic DNA library in autonomously replicating plasmid

Clone gene of interest by transforming mutant, selecting for presence of library plasmid, and screening for complementation of mutant defect

Used this strategy to clone fission yeast \textit{cdc2}

Also asked whether there was a budding yeast gene that could complement fission yeast \textit{cdc2} mutant -- How?

\textit{cdc2} is the fission yeast equivalent of budding yeast \textit{CDC28}
MPF contains cdc2

Figure 1. Immunoblotting of MPF with Antibody to p34<sup>cdc2</sup>

(A) Ammonium sulfate fractions of high speed supernatants from unfertilized eggs. High speed supernatants from unfertilized Xenopus eggs were prepared as described previously (Lohka et al., 1988) and subjected to 0%–34% ammonium sulfate precipitation. Aliquots of the precipitated and soluble fractions were resolved on SDS polyacrylamide gels and immunoblotted with PSTAIR antibody as described in Experimental Procedures. An autoradiograph of the blot is shown. The numbers to the right indicate the positions to which molecular weight standards migrated. Lane 1, 0%–34% ammonium sulfate precipitated fraction; lane 2, 0%–34% ammonium sulfate soluble fraction; lane 3, 0%–34% ammonium sulfate precipitated fraction with antibody previously incubated with the 16mer synthetic peptide corresponding to the conserved sequence of p34<sup>cdc2</sup>.

(B) MPF purified through Mono S chromatography. Immunoblotting was performed as described above. Lane 1, total E. coli protein (negative control); lane 2, a peak MPF fraction from a Mono S column; lane 3, total protein from ICRF23 human fibroblasts.

(C) MPF-positive and negative Mono S fractions. Immunoblotting was performed as described in (A). Each lane contains an aliquot of protein from the Mono S fraction series described by Lohka and Maller (1988); the number of each fraction is indicated, together with the presence (+) or absence (−) of full MPF activity, as judged by the ability to induce nuclear envelope breakdown (NEBD) in vitro. “+” indicates induction of 100% NEBD within 60 min; lower levels of activity were detected in fractions to either side of fractions 11 and 12.

Probe fractions purified in various ways with antibodies to cdc2
and purified MPF contains a second protein and also has histone H1 kinase activity.

In fact, it is cdc2 that has the protein kinase activity.

Figure 2. Immunoprecipitation of MPF by Antipeptide Antibody
(A) MPF purified through TSK chromatography. A peak MPF fraction from a TSK 3000 SW column was incubated with $[\gamma-^{32}\text{P}]$ATP, immunoprecipitated with antibody that had (lane 1) or had not (lane 2) been preincubated with the 16mer peptide corresponding to the conserved region of $p34^{\text{cdc2}}$ as described under Experimental Procedures, and subjected to SDS gel electrophoresis. An autoradiograph of the gel is shown.
(B) MPF purified through Mono S chromatography. A peak MPF fraction from a Mono S column was incubated with $[\gamma-^{32}\text{P}]$ATP immunoprecipitated as described in (A) with antibody that had (lane 1) or had not (lane 2) been preincubated with the 16mer peptide.
(C) H1 kinase activity of immunoprecipitated MPF. MPF from a peak fraction of TSK 3000 SW columns was immunoprecipitated, and the immunoprecipitate was used to phosphorylate histone H1 as described in Experimental Procedures. An autoradiograph of the reaction products is shown after SDS gel electrophoresis. The antibody either had (lane 2) or had not (lane 1) been preincubated with the 16mer peptide.
Cyclin, cdc2, and H1 Kinase activity co-purify

Fig. 8. Cyclin cdc2 and H1K co-purification. (A) Elution of H1K (●), cyclin (○) and p34 (△) in DEAE-Sepharose chromatography. The fractions from of the DEAE-Sepharose elution were assayed for H1K activity or run on SDS–PAGE gels before immunoblotting with anti-p34 and anti-cyclin sera. The ¹²⁵I-labelled bands were excised from the immunoblots and counted. (B) Anti-cyclin antibodies immunoprecipitate H1K throughout the purification procedure. H1K prepared from mitotic eggs was purified through various chromatographic steps. Immunoprecipitation with various dilutions of anticyclin antibodies was performed on the peak fraction of the phenyl–Sepharose (●), Reactive yellow 86–agarose (○) and polylysine–agarose (△) columns. The immunoprecipitate was then assayed for H1K activity. No detectable H1K was observed in the pre-immune serum precipitate (P.S.). Results are expressed as the % of maximal H1K precipitated by the antibody (i.e. ~30–50% of the initial H1K in the peak fraction).
Complementation of fission yeast \textit{cdc2} (= budding yeast \textit{cdc28}) by human cDNA
Sequence comparison of budding yeast, fission yeast and human cdc2/Cdc28

General name: cyclin dependent kinase, cdk
Genetics with fission yeast reveals other players
Cdc2 is regulated by phosphorylation

MODEL

Cdc2-P $\xrightarrow{\text{Cdc25}}$ Cdc2

Inactive $\xleftarrow{\text{Wee1}}$ Active
Budding yeast contains multiple cyclins
Mammals have multiple Cdk5 genes, each with a different number of amino acids (aa). The diagram shows a phylogenetic tree with various Cdk proteins, including Cdk1, Cdk2, Cdk3, Cdk4, Cdk5, PCTAI1, PCTAI2, PCTAI3, PITSLRE, CCRK, Cdk9, CkHED, CRK7, Cdk7, Cdk8, and Cdk11. The amino acid counts range from 297 to 1490 aa.
And multiple cyclins
Figure 2 | Genetic interrogation of the roles of cyclin-dependent kinases (CDKs) in the mammalian cell cycle. The genotypes of the various CDK mutant strains used in this analysis are indicated. Wild-type CDKs are indicated in bold font. Ablated CDKs appear in normal font. The arrows indicate the extent to which each of the mouse strains develops with the indicated CDK content. Stop signs indicate the developmental stage at which the strains are no longer viable. The main defects responsible for loss of viability are also indicated. Briefly, mice expressing all interphase CDKs but not CDK1 do not progress beyond the two-cell embryo stage. Mice expressing CDK1 but no interphase CDKs, progress up to mid gestation (embryonic day (E)12.5–E13.5). Mice lacking CDK4 and CDK6 develop until late embryonic development (E16.5–E17.5), whereas those lacking CDK4 and CDK2 die at birth. Finally, mice lacking CDK6 and CDK2 develop to adulthood and have a normal life span. Except for embryos lacking CDK1, none of the mutant embryos or mice display cell cycle defects except in those cell types indicated in the yellow boxes. For specific information regarding the phenotypes of single mutant strains see TABLE 1. P, postnatal day.
Fig. 5 Western blot analysis of *S. pombe* and *H. sapiens* protein. Equivalent protein samples loaded: a, lane 1, human colonic carcinoma cell line HT29; b, lane 1, human colonic carcinoma cell line HT29; lane 2, *S. pombe* wild type; lane 3, *S. pombe, cdc2* overproducer; c, lane 1, *S. pombe* wild type; lane 2, *S. pombe* germinated spores deleted for *cdc2*Sp; lane 3, *S. pombe* deleted for *cdc2*Sp containing pSAB2Hs; d, lane 1, *S. pombe* deleted for *cdc2*Sp containing pSAB2Hs; lane 2, human J6 T cell; lane 3, human HeLa-derived; lane 4, human Daudi B cell; e, lane 1, human J6 T cell; lane 2, human HeLa-derived; lane 3, human Daudi B cell. Blots a, b, c, d were made using affinity purified antibodies against the peptide EGVPSTAIRELLKE and e using serum against the carboxy-terminal 99 amino-acid residues of *cdc2*Sp. The arrowhead marks the position of the 34K protein.

**Methods.** Extraction of proteins and preparation of peptides conjugates for rabbit immunization were as described in ref. 9. Western blot procedures were performed using GeneScreen and GeneScreen Plus membranes according to the manufacturer's (NEN) instructions.
Fig. 1. Summary of post-fertilization cell cycle events in relation to activation of $H_1K$. M-phase-specific histone $H_1K$ assayed in crude cell lysate undergoes a transient activation at the $G_2$–M transition (expressed here in fold stimulation over the unfertilized egg $H_1K$). Cyclin is continuously synthesized but undergoes phosphorylation that begins before nuclear membrane breakdown (NEBD) and proteolysis as the cells exit from metaphase (M). The emetine-sensitive period is that during which protein synthesis is required for $H_1K$ activation and also for the first mitotic cycle to occur.
Fig. 4. Time-course of H\textsubscript{1}K activation in relation to cyclin synthesis, phosphorylation and degradation. Eggs were fertilized and divided into two batches; one was exposed to $[^{35}\text{S}]$methionine at 10–15 min post-fertilization and used to detect cyclin by SDS–PAGE and autoradiography (A). The other batch was used to prepare extracts for assay of H\textsubscript{1}K activity in crude cell lysates (B (●), activity expressed in pmol/10 min/1 μl). The time-course of cleavage in each batch (△, ▲) shows that the two kept in exact synchrony. Quantification of unphosphorylated (□) and phosphorylated (○) cyclin by scanning of the autoradiograph is shown.