A brief history of the cell cycle

Cellular Reproduction is broken down into phases

Cellular components are duplicated

Most are duplicated continuously throughout the cell cycle (RNA, protein, …)

Chromosomes only once S phase

Chromosomes are distributed in M phase

New cell is made: cytokinesis

Phases of the Cell Cycle

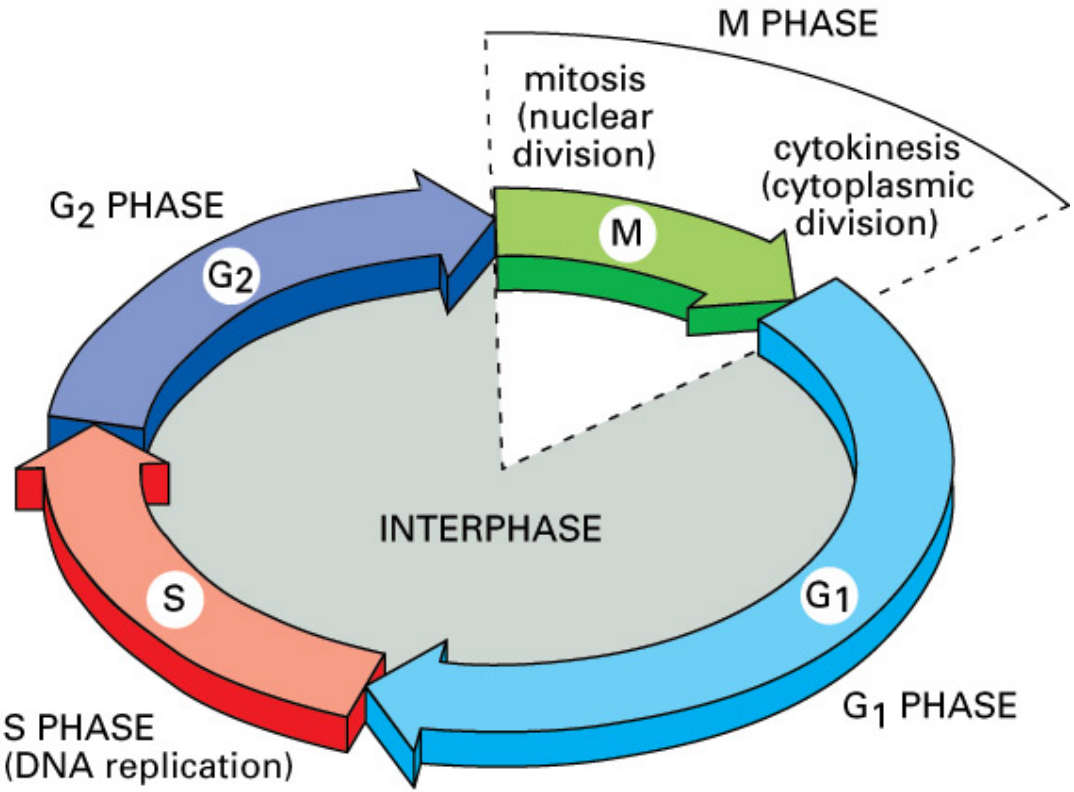


Figure 17–3. Molecular Biology of the Cell, 4th Edition.

Alternatives: fig 1-3, p6 Cell Cycle

How can you construct a cell cycle?

Option 1: Each depend on each other

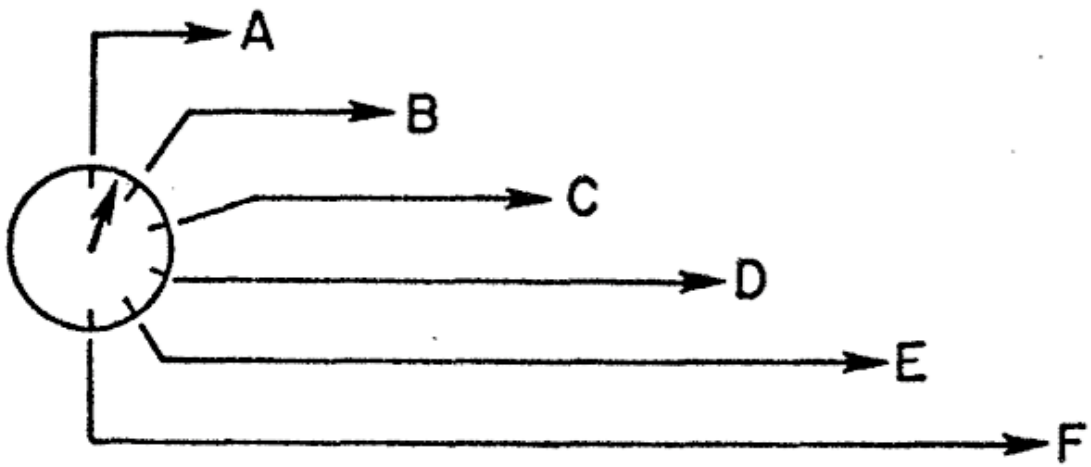
Option 2: Things happen on a timer and the cell keeps track of time

Dependent and Independent models

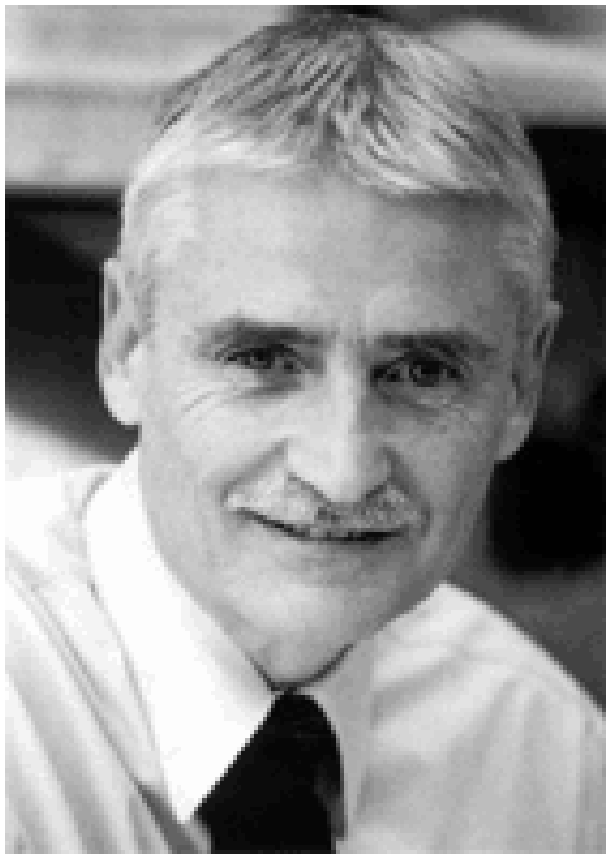
dependent pathway model

A → B → C → D → E → F

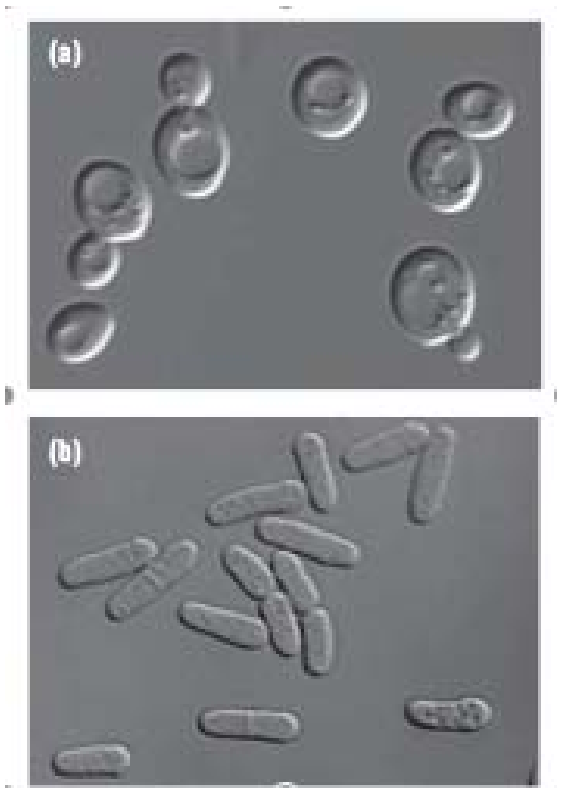
independent pathways model



Lee Hartwell



Hartwell’s yeast



budding yeast Saccharomyces cerevisiae

fission yeast Saccharomyces pombe

Hartwell's yeast

Ovoid cell, 3-5 microns, tough cell wall

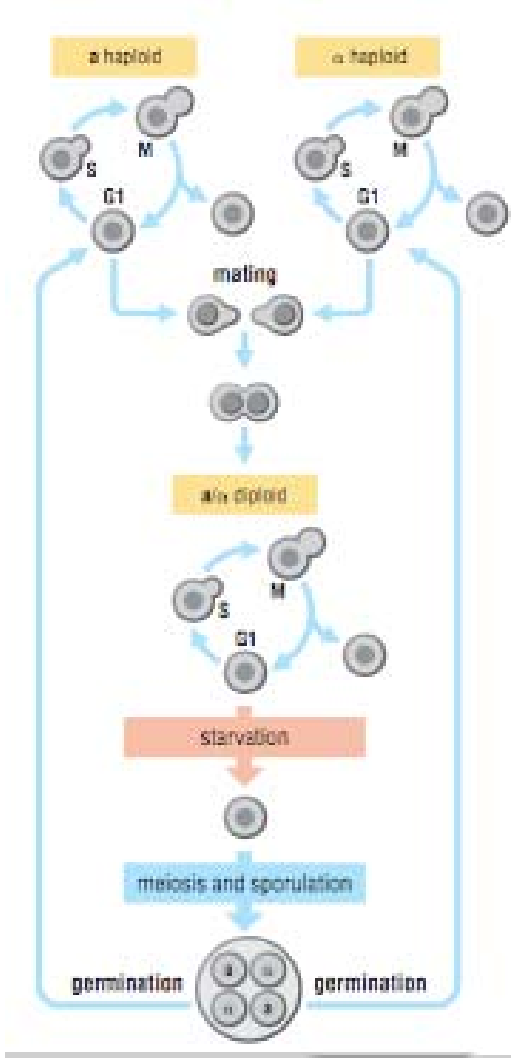
Divides by budding: bud appears at the end of G1 and grows continuously through S and M until size of mother

After mitosis, distribute one set of chromosomes into the bud

Daughter pinches off

Bud size helps define cell cycle position!

Hartwell's budding yeast cell cycle



Harwell's budding yeast: advantages

Both haploids and diploids undergo mitosis

Haploids are useful for genetic screens

Diploids can be grown and used for complementation

Buds define the position within the cell cycle

Temperature sensitive mutants

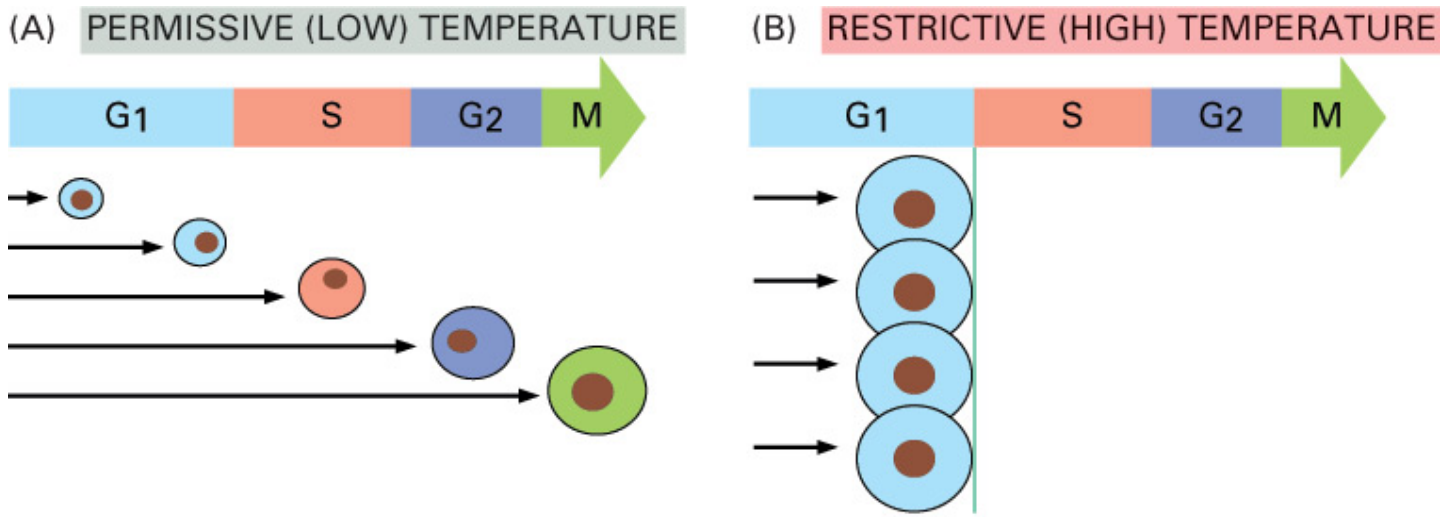


Figure 17–5. Molecular Biology of the Cell, 4th Edition.

Each mutation has a single defect

Parent strain and made haploid temperature sensitive mutants

Mutagenesis with nitrosoguanidine

Make diploids by crossing each haploid to nontemperature-sensitive strain

Lesions segregate 2:2

Indicates defect in a single nuclear gene

Budding yeast cell cycle

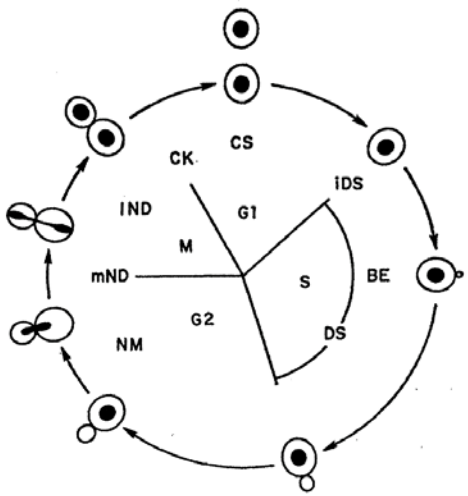
Cell separation

Cytokinesis

Late nuclear division

Medial nuclear division

Nuclear migration



Initiation of DNA synthesis

Bud emergence

DNA synthesis

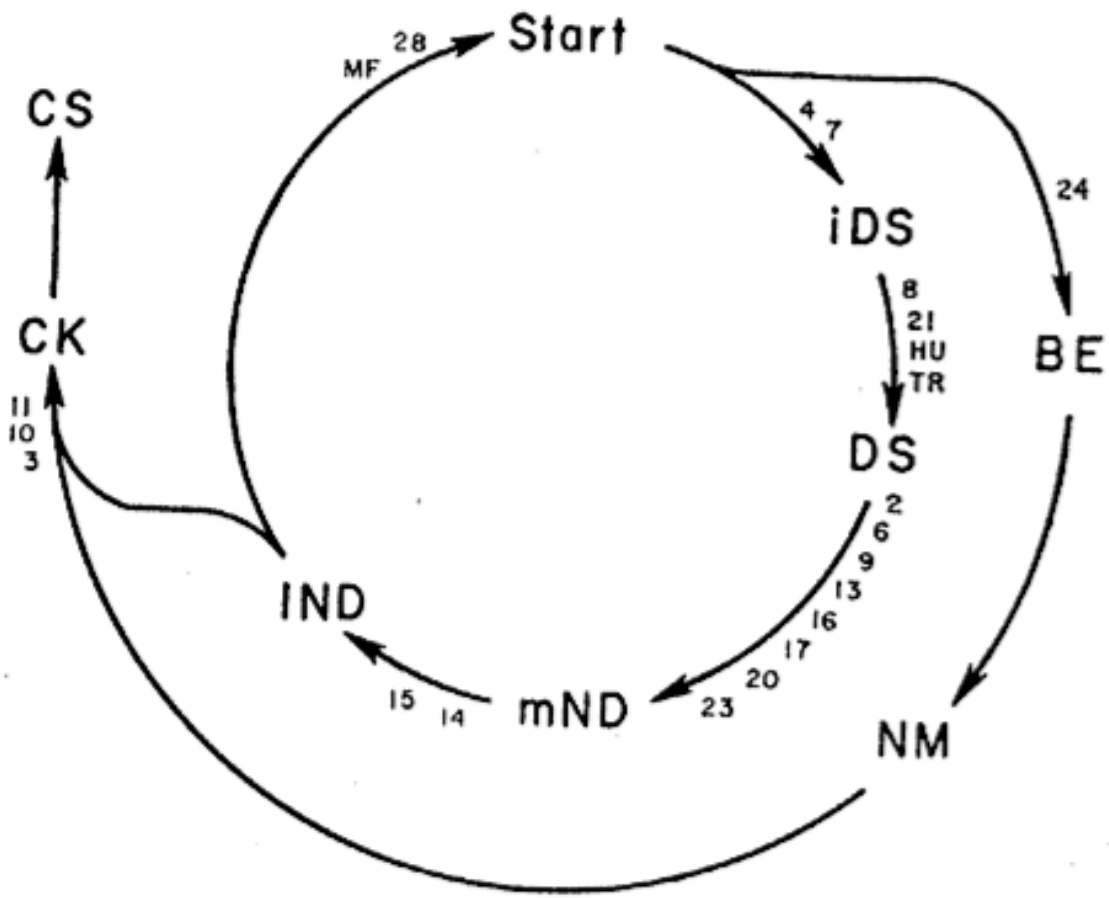
Cdc mutants: Implications for cycle?

*cdc**	Initial defect	Events completed at restrictive temperature							
		BE	iDS	DS	NM	mND	lND	CK	CS
		Bud emergence	Initiation of DNA synthesis	DNA synthesis	Nuclear migration	Medial nuclear division	Late nuclear division	Cytokinesis	Cell separation
28	Start	–	–	–	?	–	–	–	–
24	BE	–	++	++	?	++	++	–	–
4	iDS	++	–	–	+	–	–	–	–
7	iDS	+	–	–	+	–	–	–	–
8	DS	+	+	–	+	–	–	–	–
21	DS	+	+	–	+	–	–	–	–
2	mND	+	+	+	+	–	–	–	–
6	mND	+	+	+	+	–	–	–	–
9	mND	+	+	+	+	–	–	–	–
13	mND	+	+	+	+	–	–	–	–
16	mND	+	+	+	+	–	–	–	–
17	mND	+	+	+	+	–	–	–	–
20	mND	+	+	+	+	–	–	–	–
23	mND	+	+	+	+	–	–	–	–
14	lND	+	+	+	+	+	–	–	–
15	lND	+	+	+	+	+	–	–	–
3	CK	++	++	++	++	++	++	–	–
10	CK	++	++	++	++	++	++	–	–
11	CK	++	++	++	++	++	++	–	–
	CS	++	++	++	++	++	++	++	–

Model of cell division cycle

- Dependent model:
  - Cell separation
  - Cytokinesis
  - Late nuclear division
  - Medial nuclear division
  - DNA synthesis
  - Initiation of DNA synthesis
- Mutant with an initial defect in one of these processes fails to complete any of the events that occur later

Lee Hartwell's cell cycle model



Lee Hartwell's cell cycle model

Common early step for both pathways:

Cdc28 required for both bud emergence and initiation of DNA synthesis even though they are separate

Mating factor produced by cells of mating type alpha blocks bud emergence and initiation of DNA synthesis in cells of mating type alpha

What is the role of cdc28?

Lee Hartwell's cell cycle model

- Alpha factor/cdc28 step precedes cdc4/cdc7 (DNA synthesis) and cdc24 (bud emergence)
- Alpha factor/cdc28 mediate an early event necessary prerequisites for both dependent pathways
- "START"

What happens when cells get through START?

Lee Hartwell's cell cycle model

Completion of START:

Insensitivity to alpha factor in haploids of mating type alpha

Or

Insensitivity to temperature in a cdc28 mutant

What happens if nutrients are limiting? Is it different for different nutrients?

Lee Hartwell's cell cycle model

- START is the beginning of the cell cycle
- Stationary phase populations from limiting a nutrient (glucose, ammonia, sulfate, phosphate) almost exclusively cells arrested at "START"
- Stationary phase of mating type alpha don't undergo bud emergence after inoculation into fresh medium with alpha factor

Lee Hartwell's cell cycle model

START: grow cultures in a chemostat with limiting glucose

Correlation between the generation time and fraction of unbudded cells

Unbudded cells delay the start of new cycles until some requirement for growth or for the accumulation of energy reserves is met, and the time needed for this depends on glucose availability

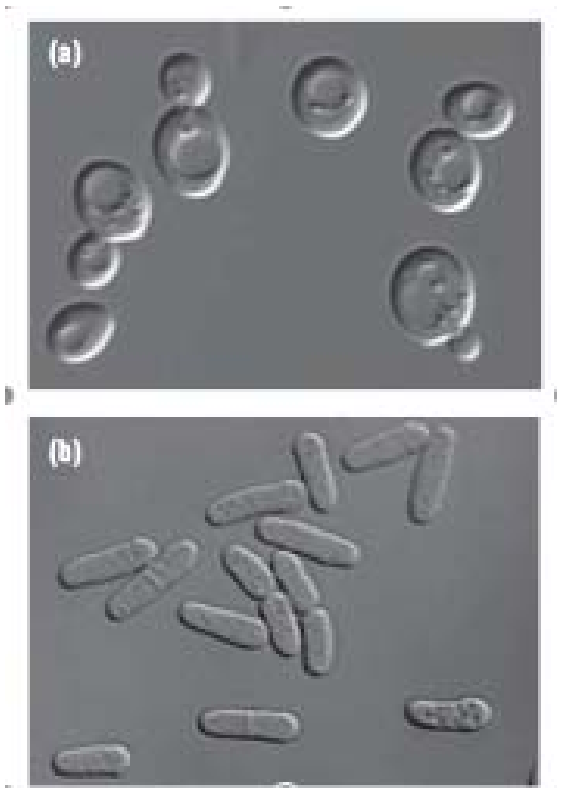
Lee Hartwell's cell cycle model

Passing START is a point of commitment to division

If a cell is beyond start, it proceeds through the cell cycle to cell separation at a normal rate, then both daughter cells become arrested at start

Sir Paul Nurse

Budding and fission yeast



budding yeast Saccharomyces cerevisiae

fission yeast Saccharomyces pombe

Temperature sensitive cdc mutants

>40 cdc mutants in S.cerevisiae

Four commitment to start:

Cdc28, 36, 37, 39

>25 mutants in S.pombe

Cdc2, 10

Necessary techniques

High frequency transformation of S. pombe

Construction of a gene bank in a yeast-bacterial shuttle vector

Get S. pombe cdc2 on a plasmid

Experimental strategy

takes pombe plasmids and complement cdc2 mutation

Morphology of mutants

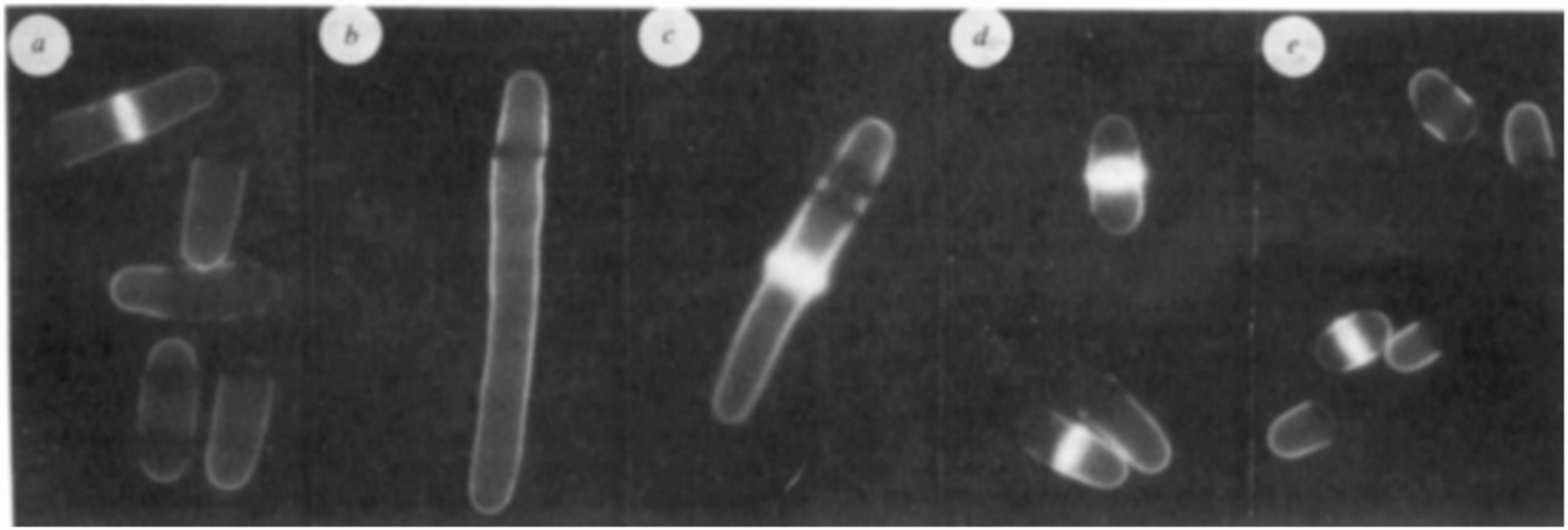
Wild type

cdc 2.33
leu 1.32

cdc 2.33
leu 1.32
pcdc2.3(Sp)

cdc 2.1w

cdc 2.33
leu 1.32
pcdc28



Cdc-2

- Clones that continue to divide at 35° C were isolated and plasmids recovered in E. coli
- One plasmid complemented two cdc2 mutations when retransformed
- Cells were elongated indicating incomplete suppression

Got a plasmid with a sequence that complements two different cdc2 mutations

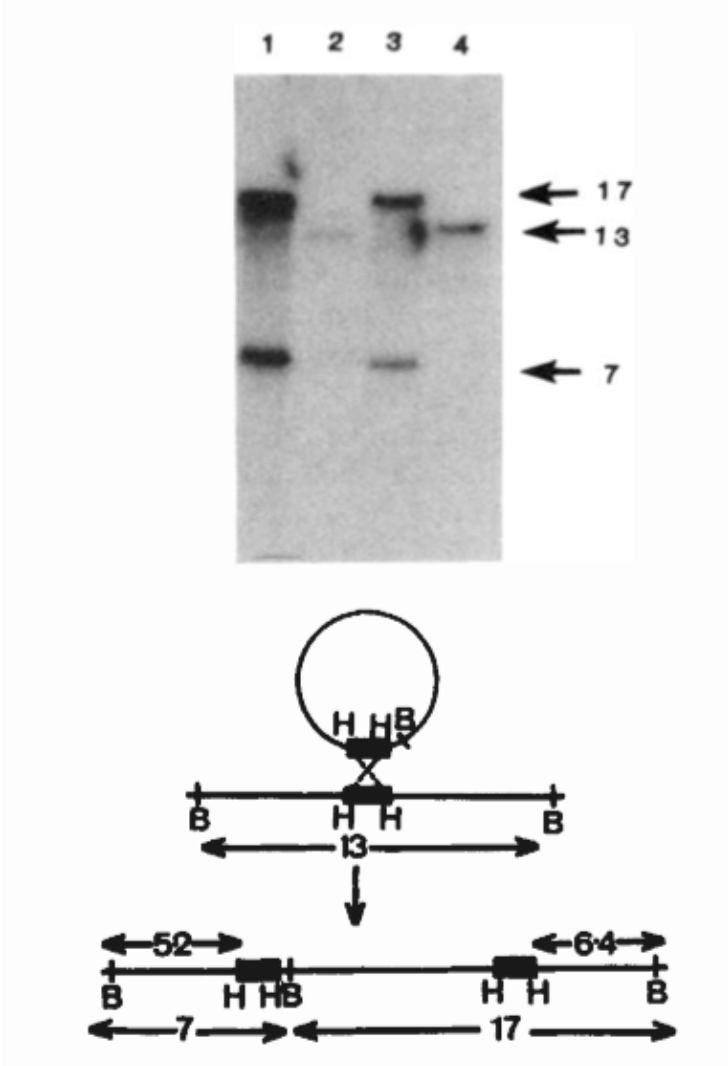
Then wanted to stably integrate cdc2 into S. pombe confirm by Southern blotting and close to his locus

Cdc2 from Pombe

Portion of the sequence recloned and put into S. pombe, transformants presumed to have arisen by plasmid integration into cdc2

Leu1+ marker closely linked to his 3 based on recombination

Pcdc2.32 integrated at the homologous chromosomal site



Put an S cerevisiae gene bank into S pombe

Found a clone with same appearance as complemented S. pombe

S cerevisiae has sequences that complement pombe cdc2

Morphology of mutants

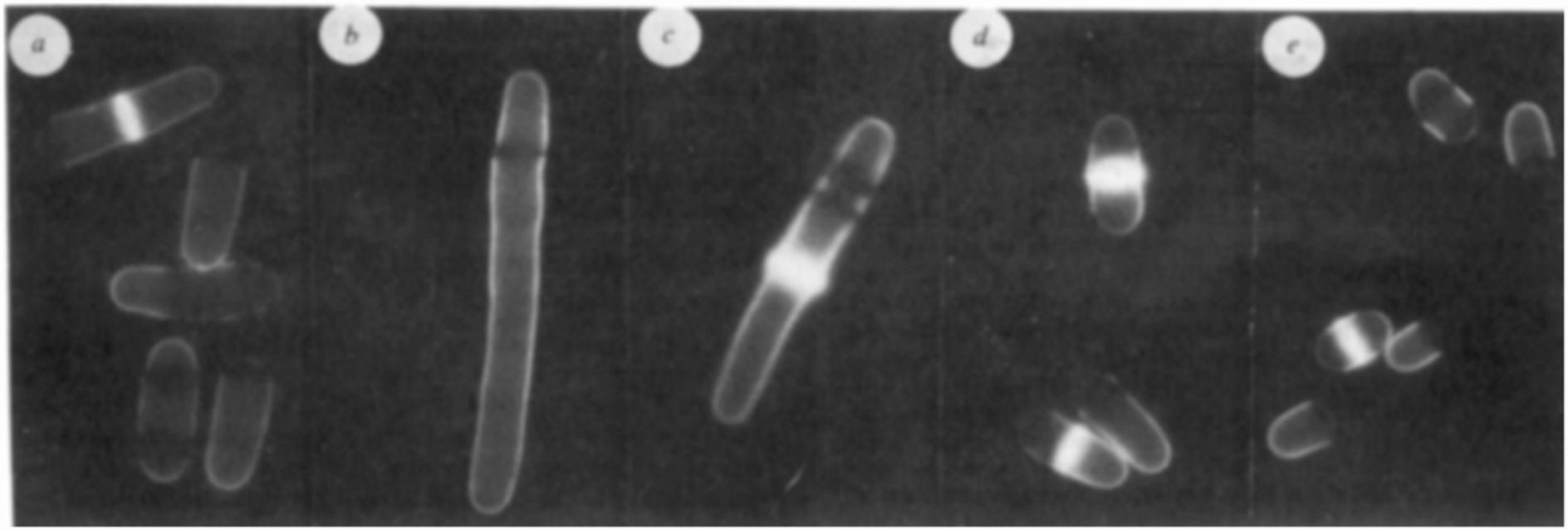
Wild type

cdc 2.33
leu 1.32

cdc 2.33
leu 1.32
pcdc2.3(Sp)

cdc 2.1w

cdc 2.33
leu 1.32
pcdc28

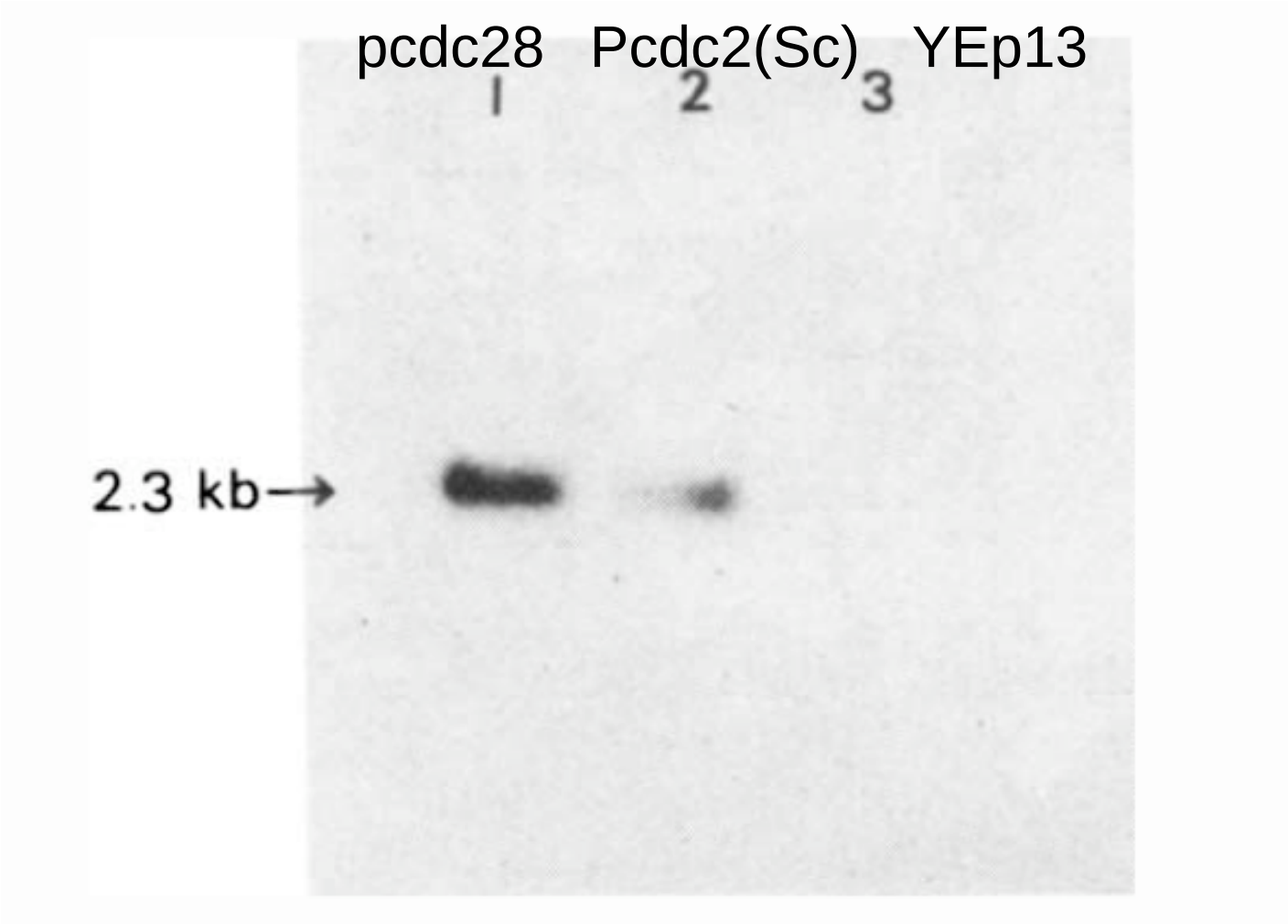


Already have plasmids with cdc28, cdc36 cdc37, cdc39

Probe insert with these plasmids

Hybridization with cdc28 probe

Cross hybridization between cdc2 and cdc28



Cdc28 and cdc2 contain common sequences

Cdc28, 36, 37 and 29 were run digested with HindII, run on Southern and probed with 32P-pcdc2(Sc)

Pcdc2(Sc) and pcdc28 contain common sequences

Confirmation: put pcdc2(Sc) into cdc28 mutant S cerevisiae and show it complements

Morphology of mutants

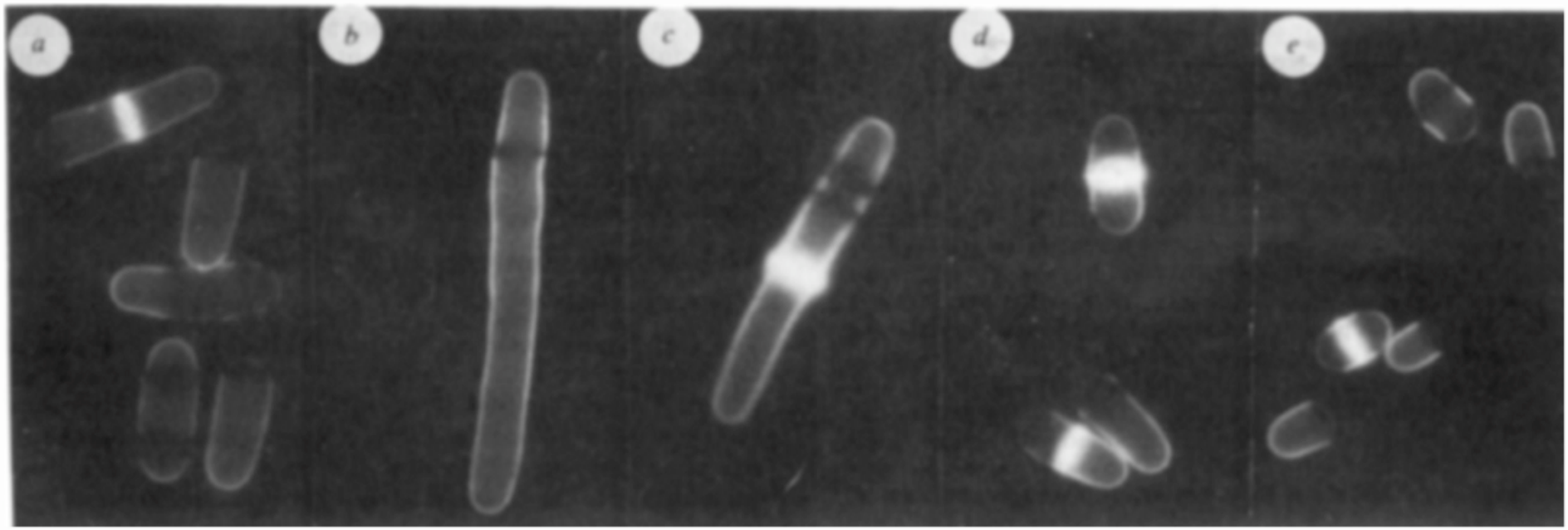
Wild type

cdc 2.33
leu 1.32

cdc 2.33
leu 1.32
pcdc2.3(Sp)

cdc 2.1w

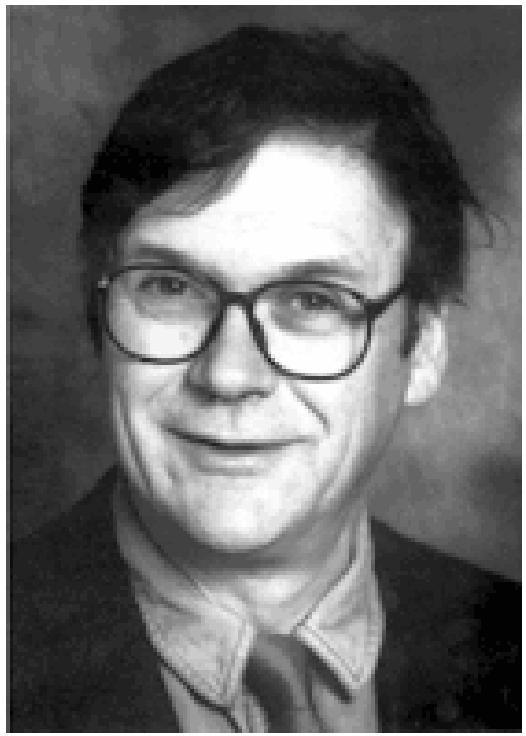
cdc 2.33
leu 1.32
pcdc28



Complementation between strains

- Cdc28 from Sc put into the same pombe, made small cells (like wee) from overexpression of cdc2
- But cdc2 from pombe couldn’t complement cdc28 mutations in S cerevisiae

Tim Hunt



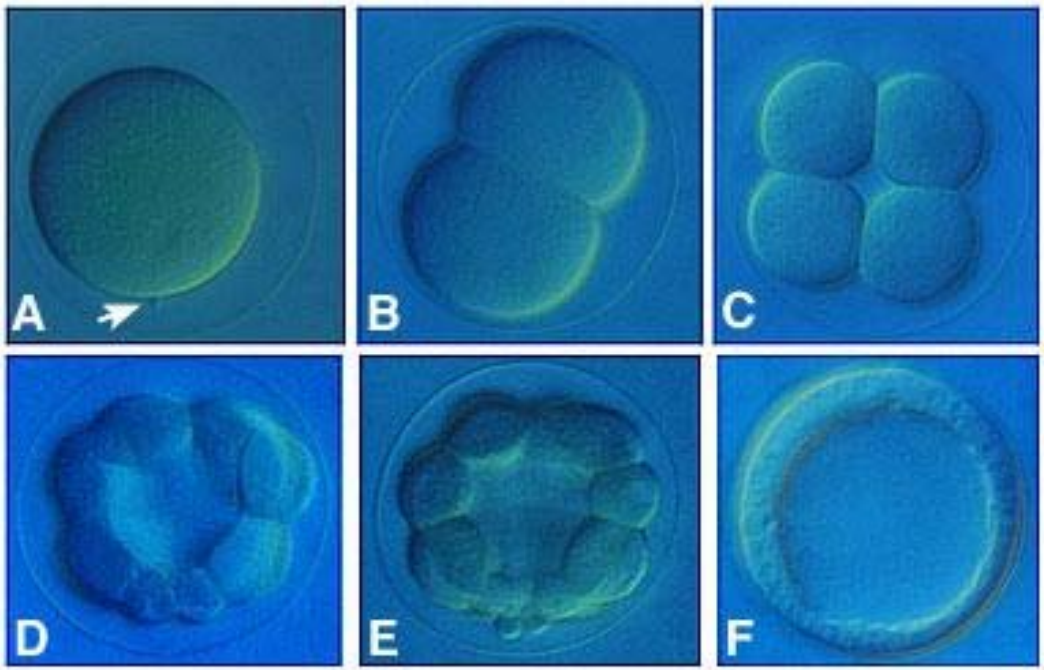
Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division

Evans, Rosenthal, Youngblom, Distel, Hunt

Cell

1983

Sea urchin embryo



Fertlization of eggs or oocytes

Fertilization of eggs or meiotic maturation of oocytes in many organisms leads to increase in rate of protein synthesis programmed by maternal mRNA

Inhibit protein synthesis in fertilized sea urchin eggs: blocks development

Permit normal fertilization, pronuclear fusion and DNA replication, prevent nuclear envelope breakdown, chromosome condensation and mitotic spindle

Delay cycloheximide

If 30 mins later, nuclear envelope breaks down normally, chromosomes condense, spindles form and cells divide, but don’t separate normally

A protein synthesized by one or more maternal RNAs is required for cell division

Activation of sea urchin eggs

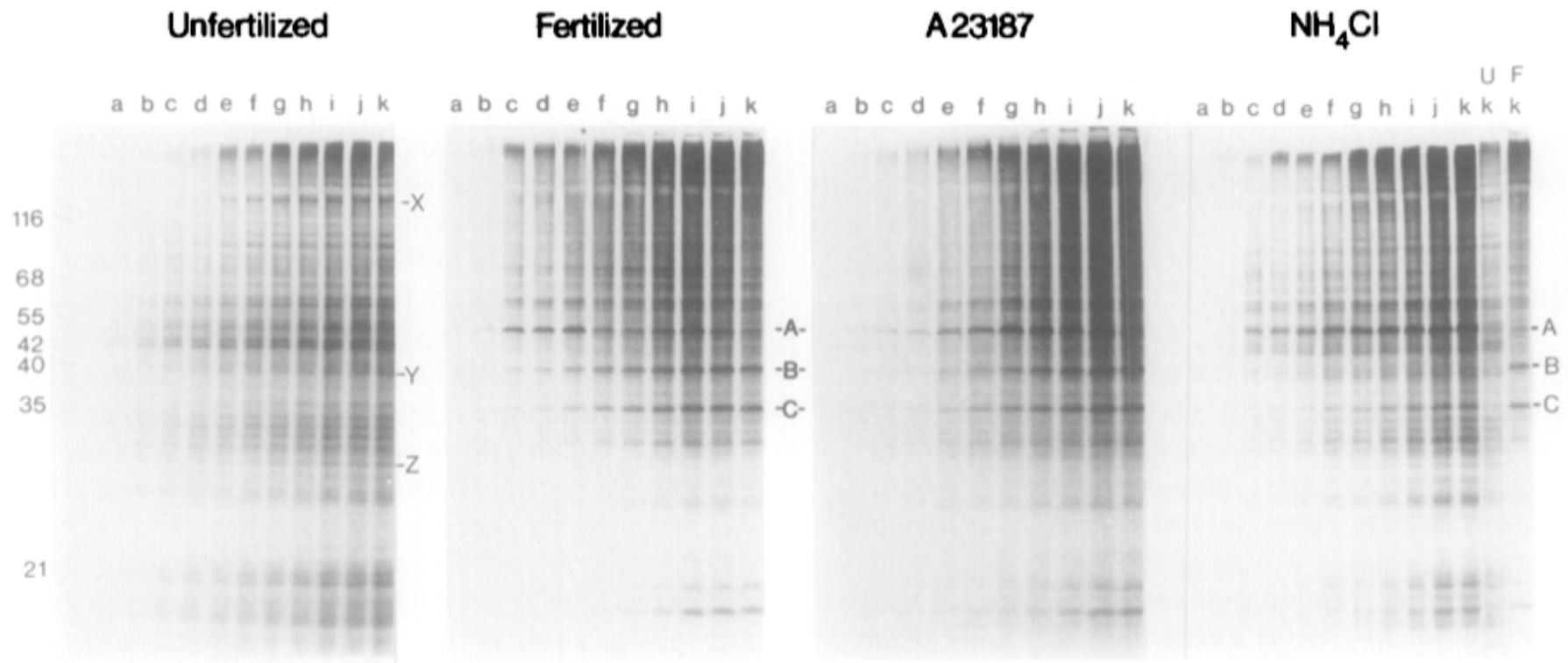
Fertilization or

10 microM A23187 and 10 mM NH4Cl activate DNA and protein synthesis, only 1/2 value

Eggs don’t divide unless further treatment

If then give hypertonic seawater or D2O, some form functional asters and divide

35S-Met labeling of eggs



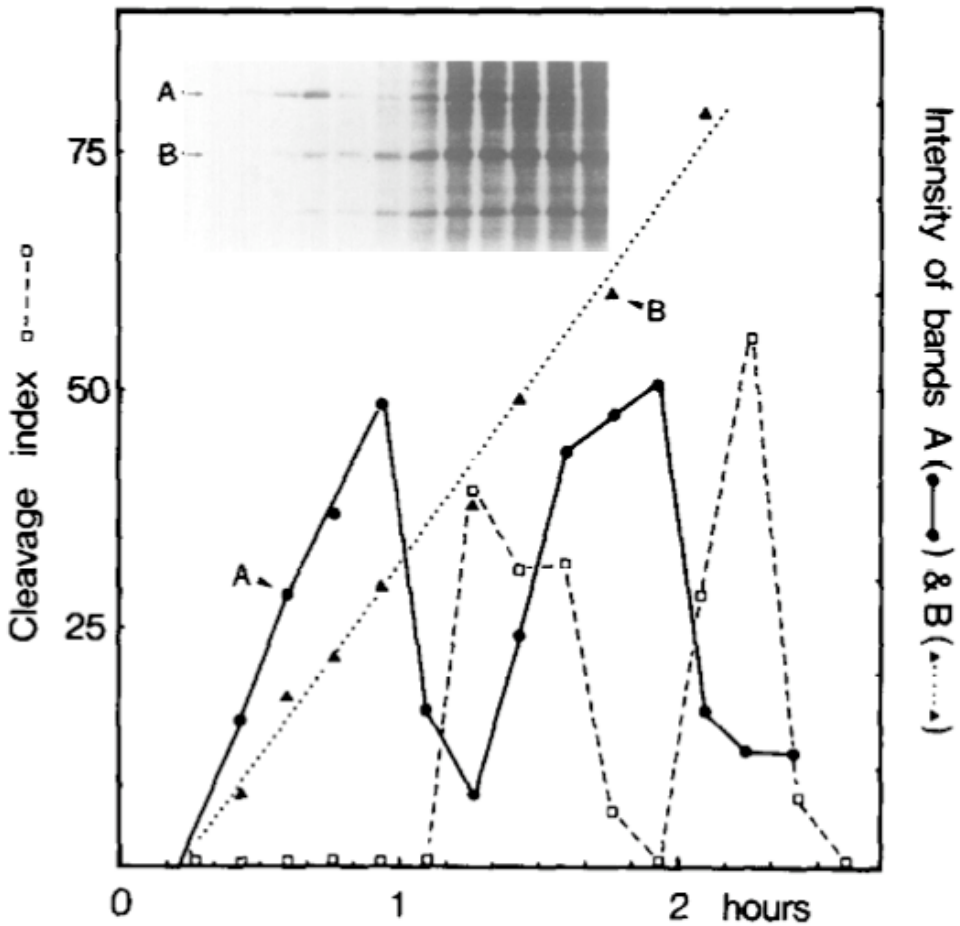
Protein A Cyclin

Most strongly labeled protein at early times after fertilization but by 85 mins (lane g) it disappears, then stronger again in h and I, declines again in lane k

Induced but doesn’t oscillate with A238187 or NH_4Cl

NH_4Cl protein B not turned on

Cyclin correlates with the cell cycle



Cyclin and Cell Cycle

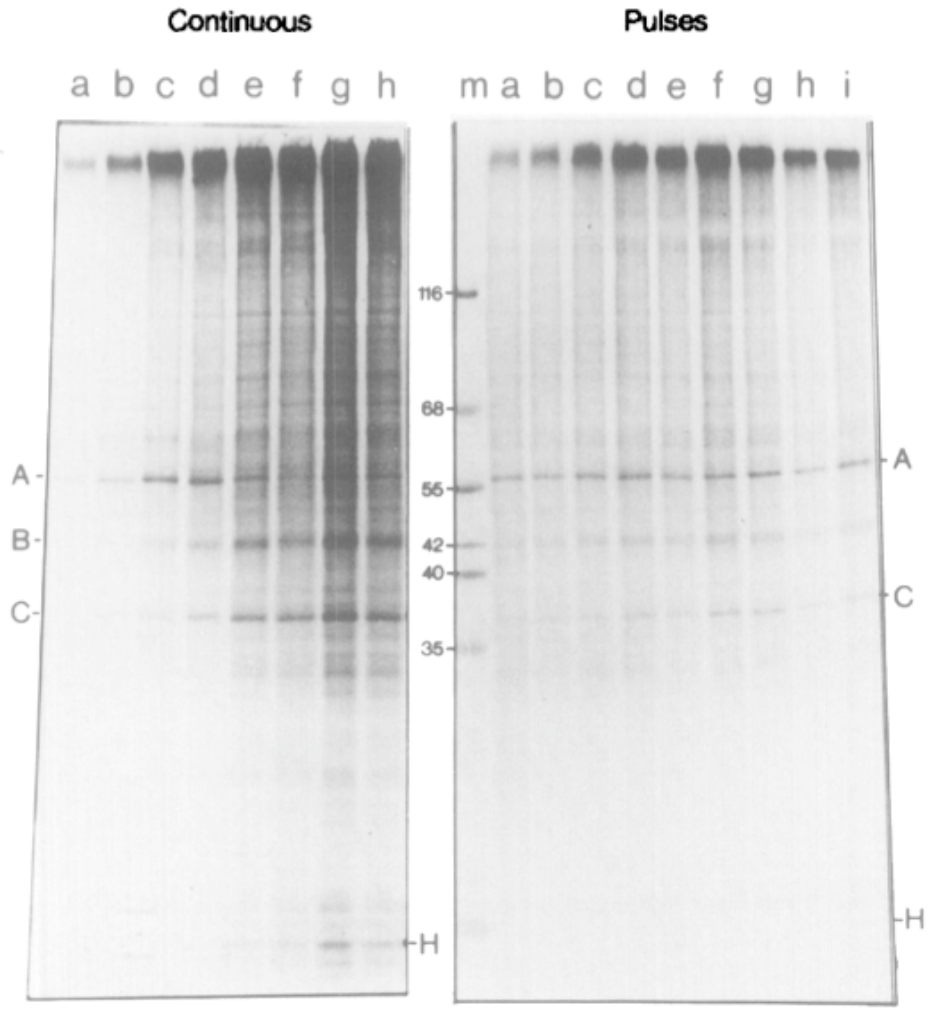
35SMet was added and protein was monitored at time points, and some eggs were fixed in 1% glutaraldehyde for alter examination

Dashed line: cleavage index

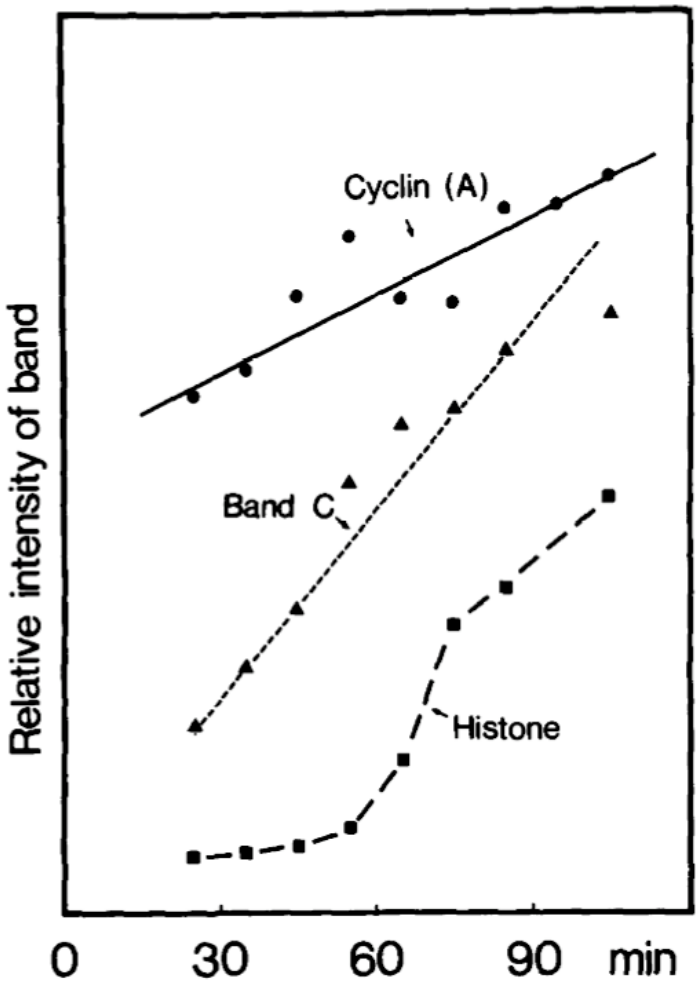
Other, relative intensities of cyclin and protein B

Cyclin A levels fall at onset of cleavage, rise and fall again during 2nd cell cycle

Cyclin is synthesized continuously at a steady pace



Cyclin is synthesized continuously at a steady pace



Continuous and Pulse-Labeled Embryos

C: increase in labeling with time

H: (histone) synthesis rises rapidly at 2-cell stage

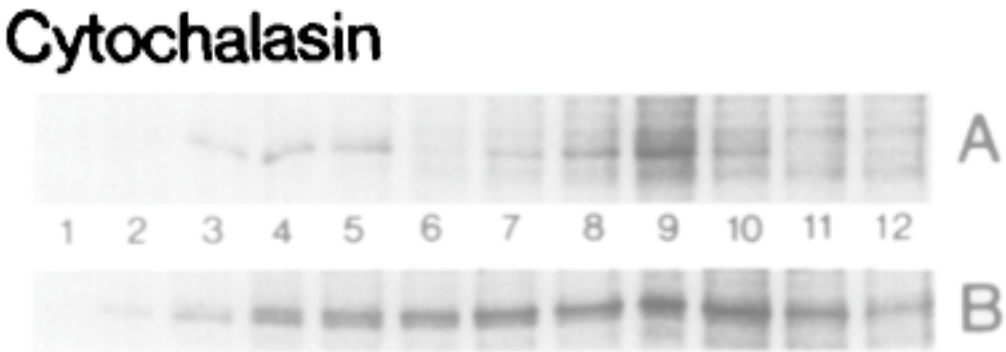
A: rate of synthesis rises rapidly after fertilization, only a relatively small rise after

Continuous and Pulse-Labeled Embryos

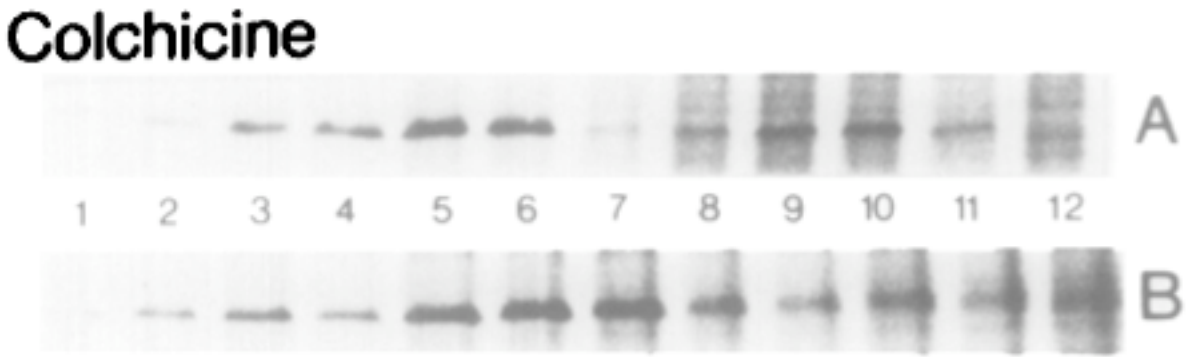
Variations in intensity of cyclin due to destruction by periodic proteolysis, not to periodic synthesis

Newly synthesized cyclin may need to participate in a maturation or assembly process before destruction

Blocking cell division affects cyclin disappearance



Don’t divide



Don’t divide

Rapid disappearance of cyclin depends on normal cleavage

Identification of Maturation Promoting Factor

Lohka et al PNAS 1988

Oocyte growth and egg cleavage in Xenopus

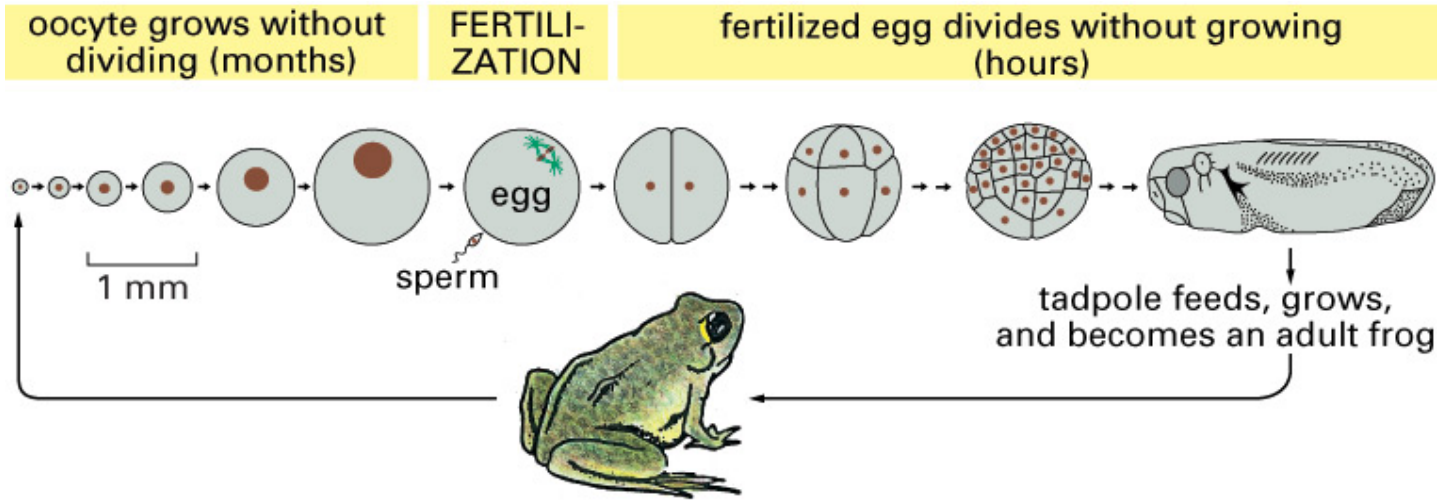
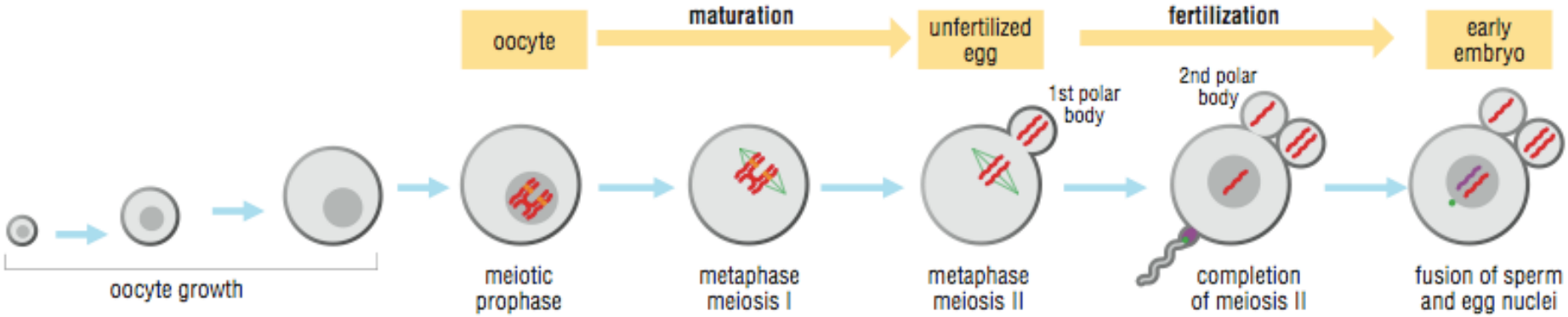


Figure 17–8. Molecular Biology of the Cell, 4th Edition.

Xenopus oocyte model



Mature Xenopus egg ready for fertilization

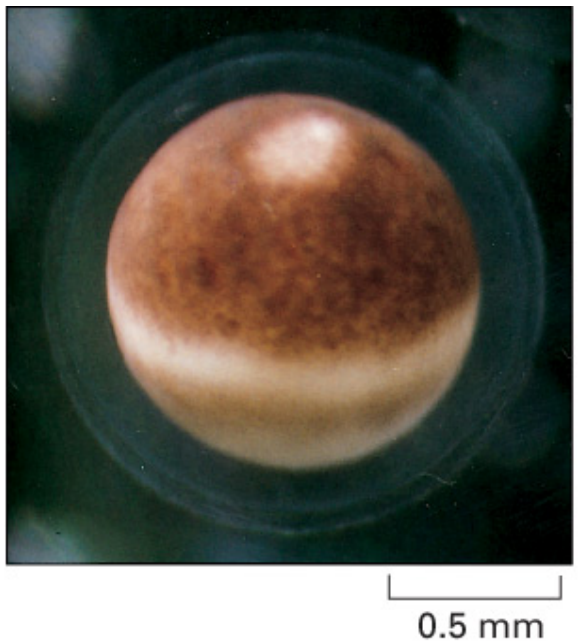


Figure 17–7. Molecular Biology of the Cell, 4th Edition.

Xenopus oocyte model

Diploid oocyte enters the meiotic program and completes meiotic S phase

Arrests in meiotic prophase for several months, grows to 1 mm

In response to hormonal cues from the pituitary gland, the follicle cells surrounding the oocyte secrete progesterone, interacts with the oocyte to initiate oocyte maturation, meiosis I

Xenopus oocyte model

Cell naturally arrested in G2

Amphibian oocyte

In response to hormones, enters M phase and matures into a metaphase-arrested unfertilized egg

Upon fertilization, eggs complete meiosis II and enter S phase

So G2->M at maturation, then M->G1/S at fertilization

Xenopus oocyte model

When cytoplasm from maturing oocytes or eggs is injected into immature oocytes, recipients undergo oocyte maturation, even in the presence of protein synthesis inhibitor

Active component: MPF

Cell-free Xenopus extract system

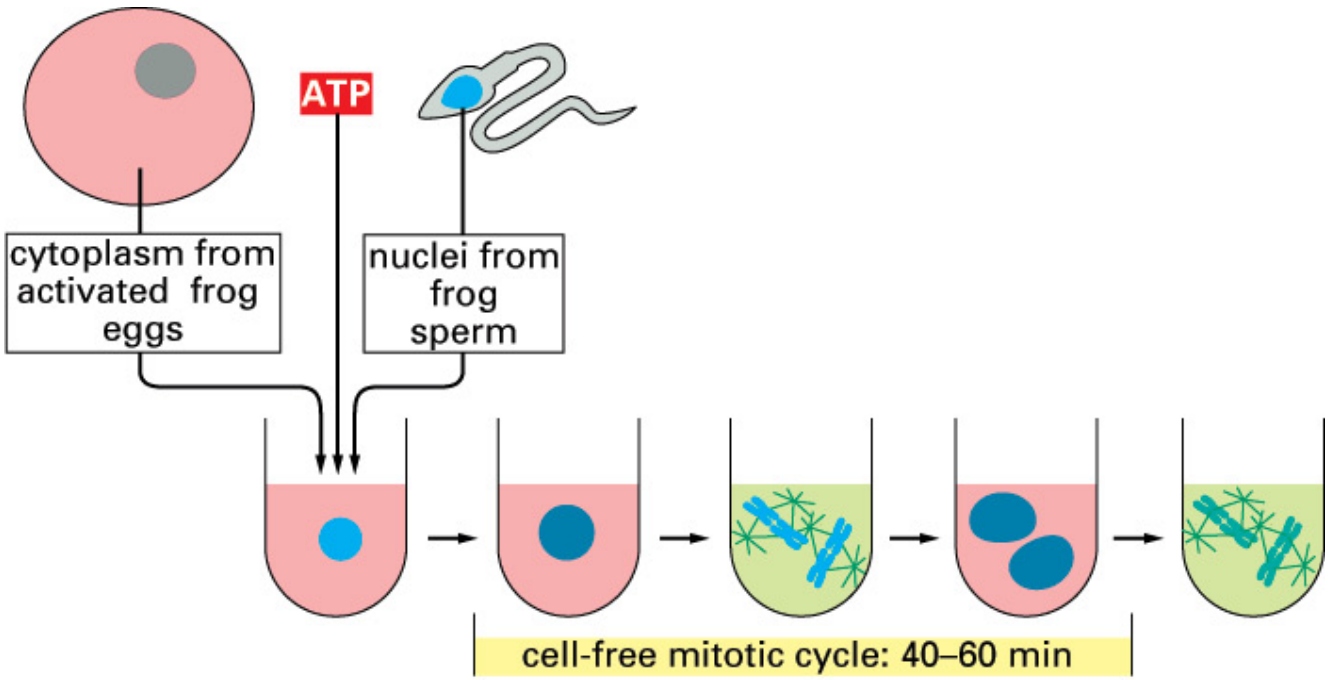


Figure 17–9. Molecular Biology of the Cell, 4th Edition.

Xenopus cell-free model

Xenopus female lays several 1000 unfertilized eggs placed in a dish

“Mock fertilization” with electrical stimulation, calcium

Activated eggs complete meiosis II and begin mitotic cell cycle (no sperm)

Xenopus cell-free model

Centrifuge frog eggs to break apart, stratify, collect egg cytoplasm

Add sperm nuclei stripped of membranes to cytoplasm and the sperm decondense and are packaged

Replication of sperm DNA

Extracts proceed through mitosis and segregate sperm, several rounds of S and M

Xenopus model cell-free model

Cell free system from amphibian eggs

Nuclei induced to undergo early mitotic events by addition of crude or partially purified MPF

Lohka et al Experimental methods

Assay of MPF Activity in a cell-free system

Mix Xenopus extracts and sperm and add to sample

Visualize how many of the pronuclei enter M phase by microscopy

Dilution is indication of potency of sample for MPF

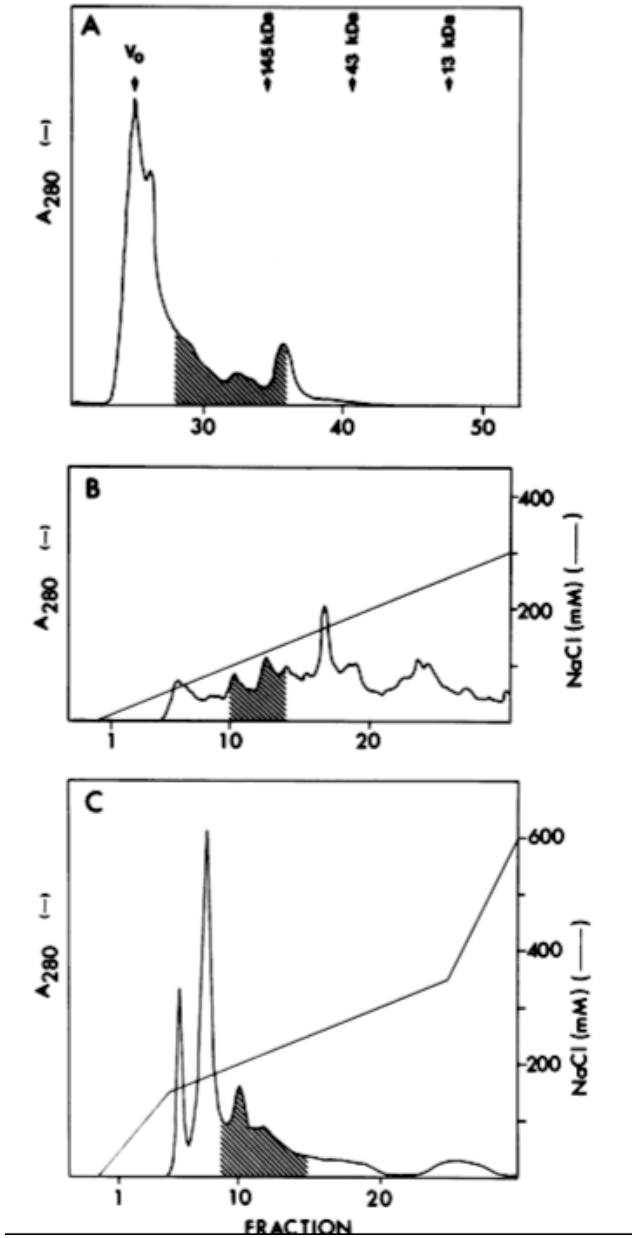
Lohka et al Experimental methods

Assay of MPF activity by microinjection of oocytes

“Microinject” sample (What’s this?)

Fraction with GVBD determined (Germinal vesicle breakdown)

Lohka et al Results

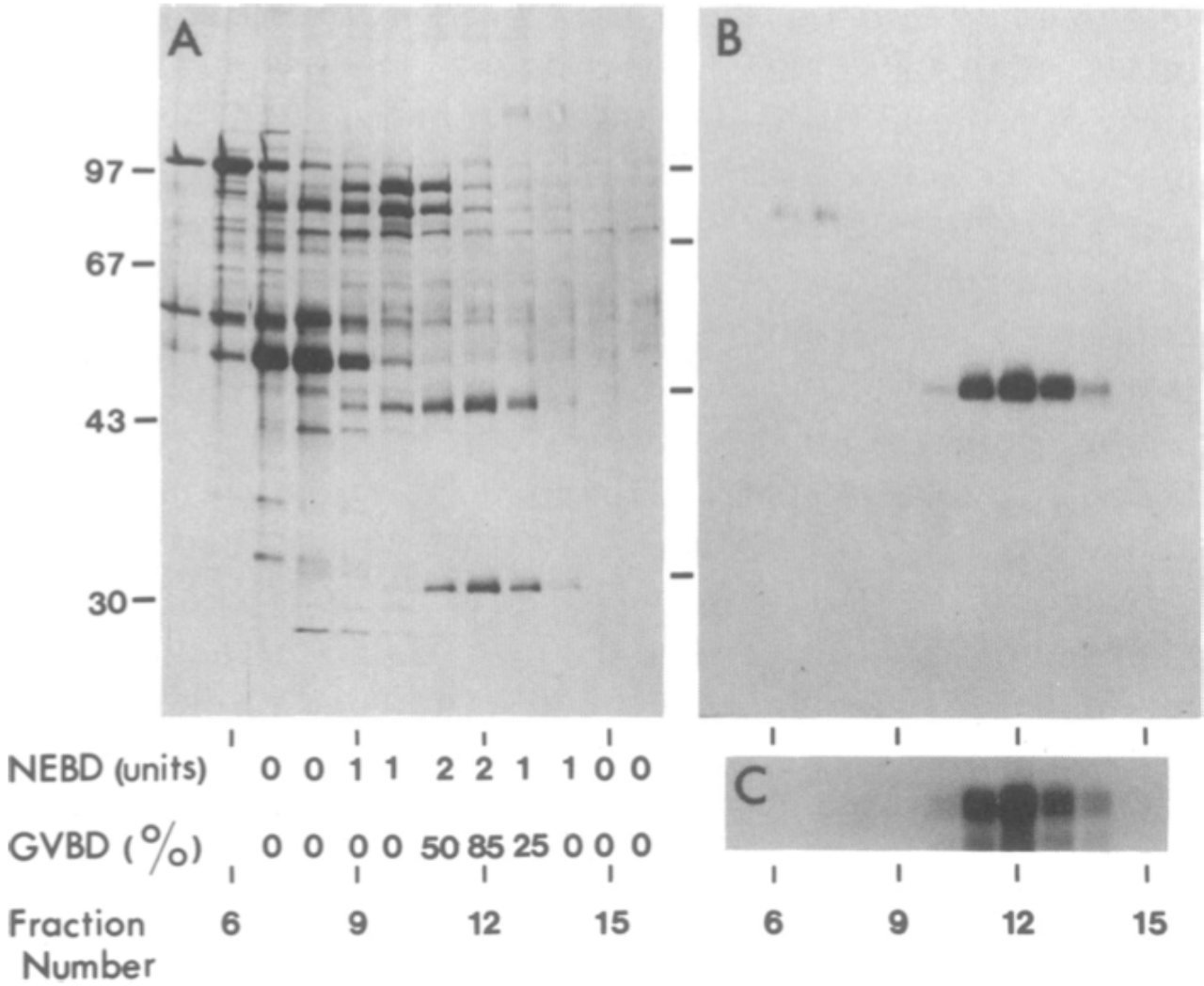


Lohka et al Results

Table 1. Summary of MPF purification from *Xenopus* eggs

Step	Total protein, mg	Total activity, units × 10^{-3}	Specific activity, units/mg	Recovery, %	Purification, fold
Supernatant (250,000 × *g*)	2100	11.0	5.2	100	—
Ammonium sulfate	700	9.0	13	82	2.5
DEAE-Sephacel	—	—	—	—	—
Heparin-Sepharose	31	1.7	55	15	11
Green A Matrex gel	9.9	1.3	130	12	25
TSK 3000SW	1.7	0.48	280	4.4	54
Mono Q	0.07	0.20	2,900	1.8	560
Mono S*	0.0065	0.12	18,000	1.1	3500

Lohka et al Results



The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis

Cell

1988

Dunphy, Brizuela, Beach, Newport

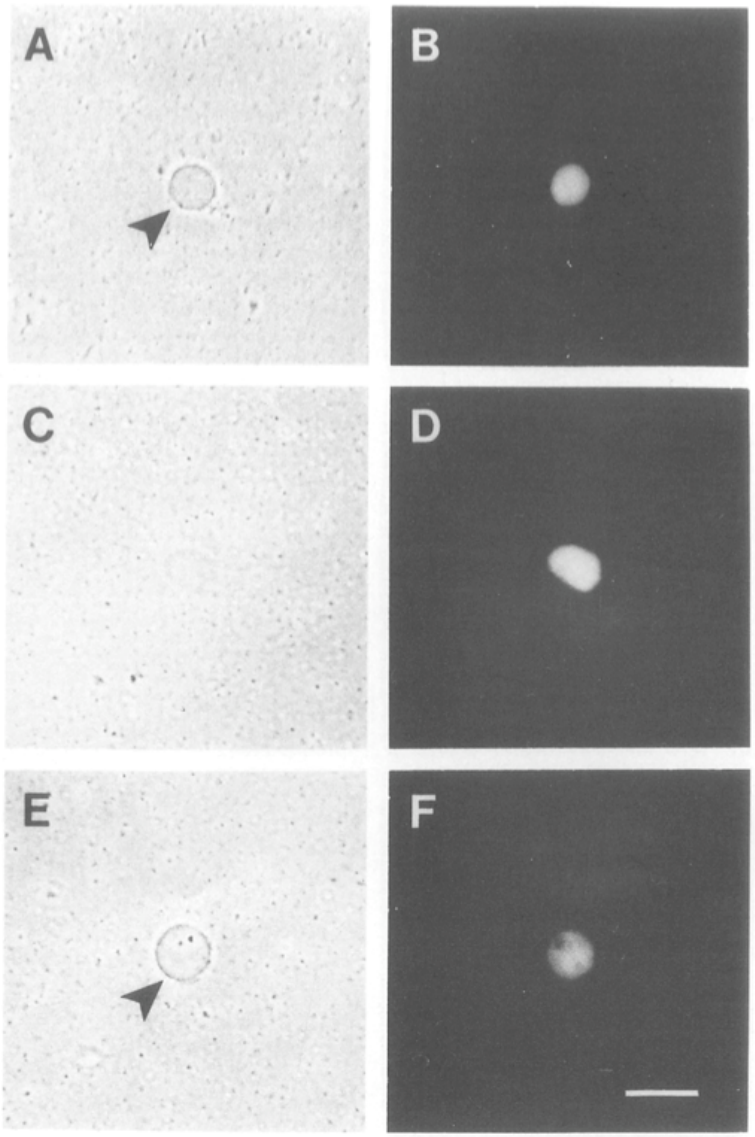
How can the frog and the yeast meet?

Cell-free assays for mitotic induction in yeast? No, so no biochemistry on cdc gene products and no genetics in frogs

Thinking outside the box:

Fission yeast cdc gene product can function in vitro to regulate Xenopus extracts

Fission yeast p13 antagonizes MPF-induced nuclear disassembly in a cell free system



Heterologous system

Known cdc yeast proteins in the Xenopus cell-free mitotic assay

p13 inhibits MPF

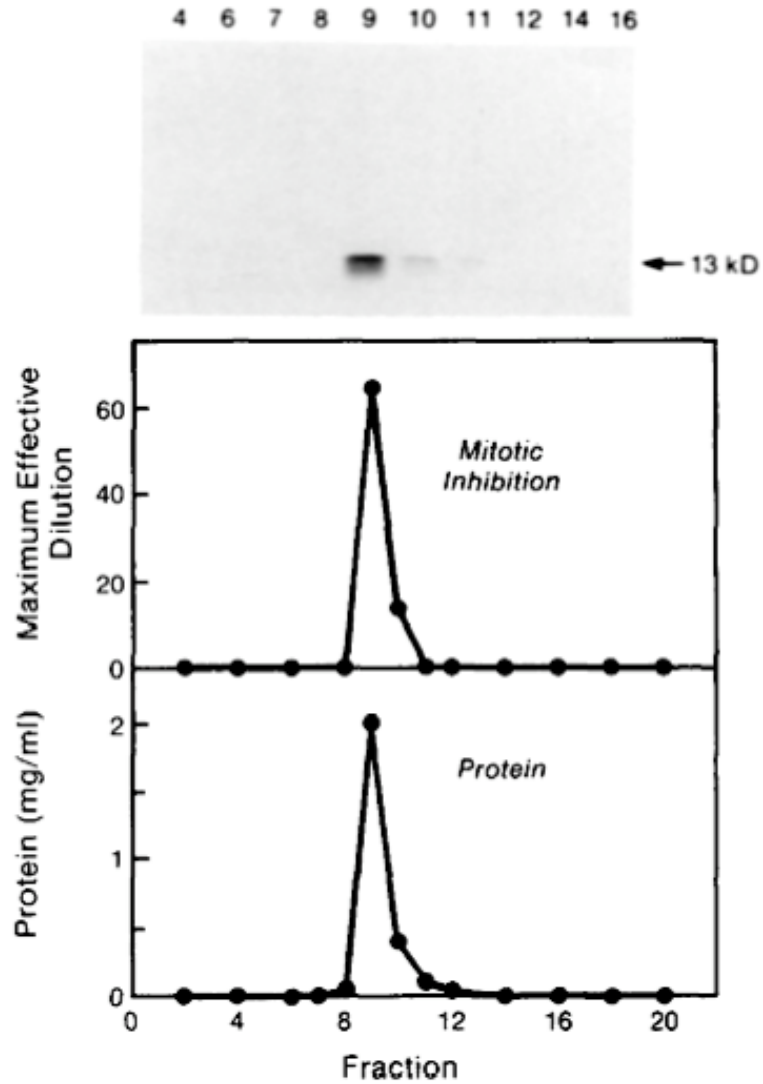
Expresss p13 in E coli, soluble, purify it

With p13, MPF-induced loss of the nuclear envelope in neutralized

Rechromatograph highly purified p13

Inhibitory activity co-chromatographs with p13 and no other proteins are there

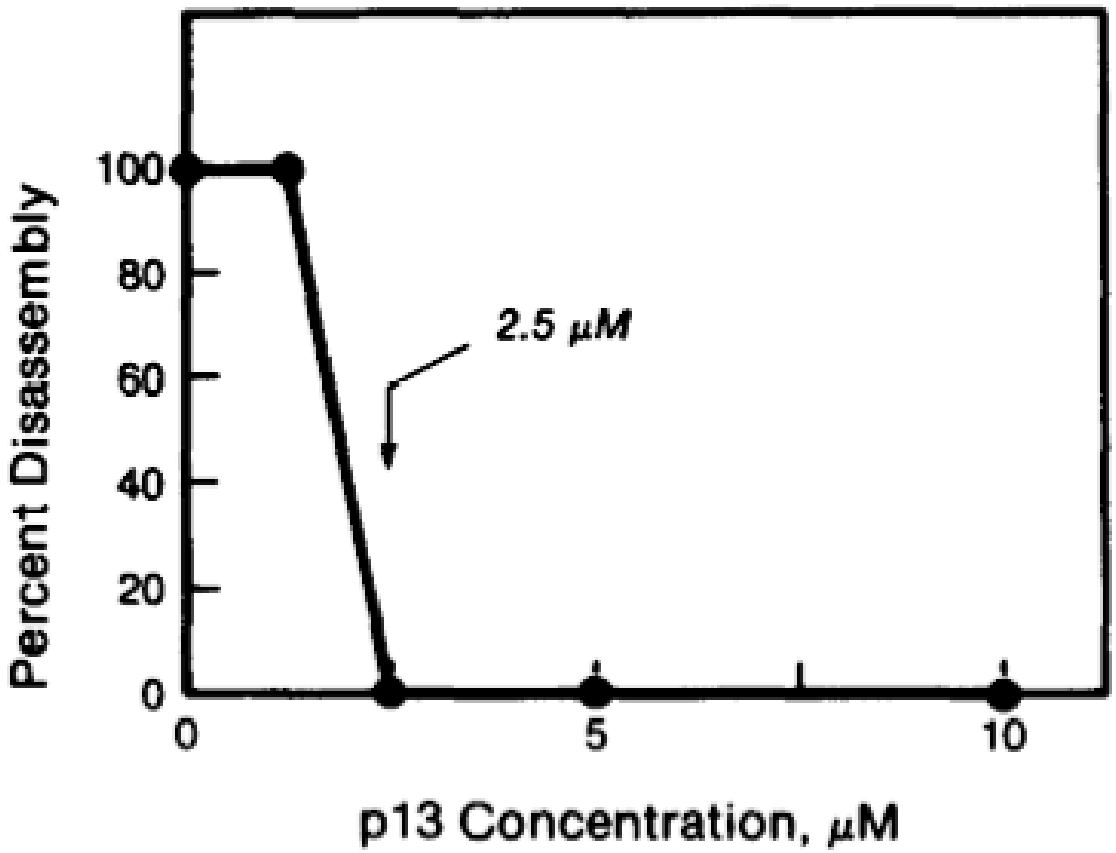
Mitotic inhibitory activity fractionates with homogenous p13



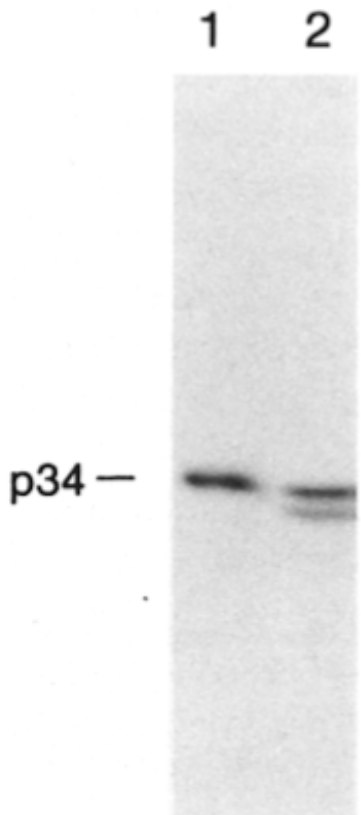
Proof p13 is active

- Requires intact p13, trypsin digestion inhibits its activity
- p13 is heat stable and treatment at 100° C for 10’ had no effect
- Effective at low concentrations 2.5 micromolar
- Inhibited oocyte maturation when injected
- P13 isn’t a general inhibitor: no effect on in vitro nuclear assembly around sperm chromatin
- P13 inhibition is reversible, add back extra MPF
- Very steep threshold: total inhibition at 2.5 micromolar and no effect at 2-fold lower

Dose response of mitotic inhibition by p13



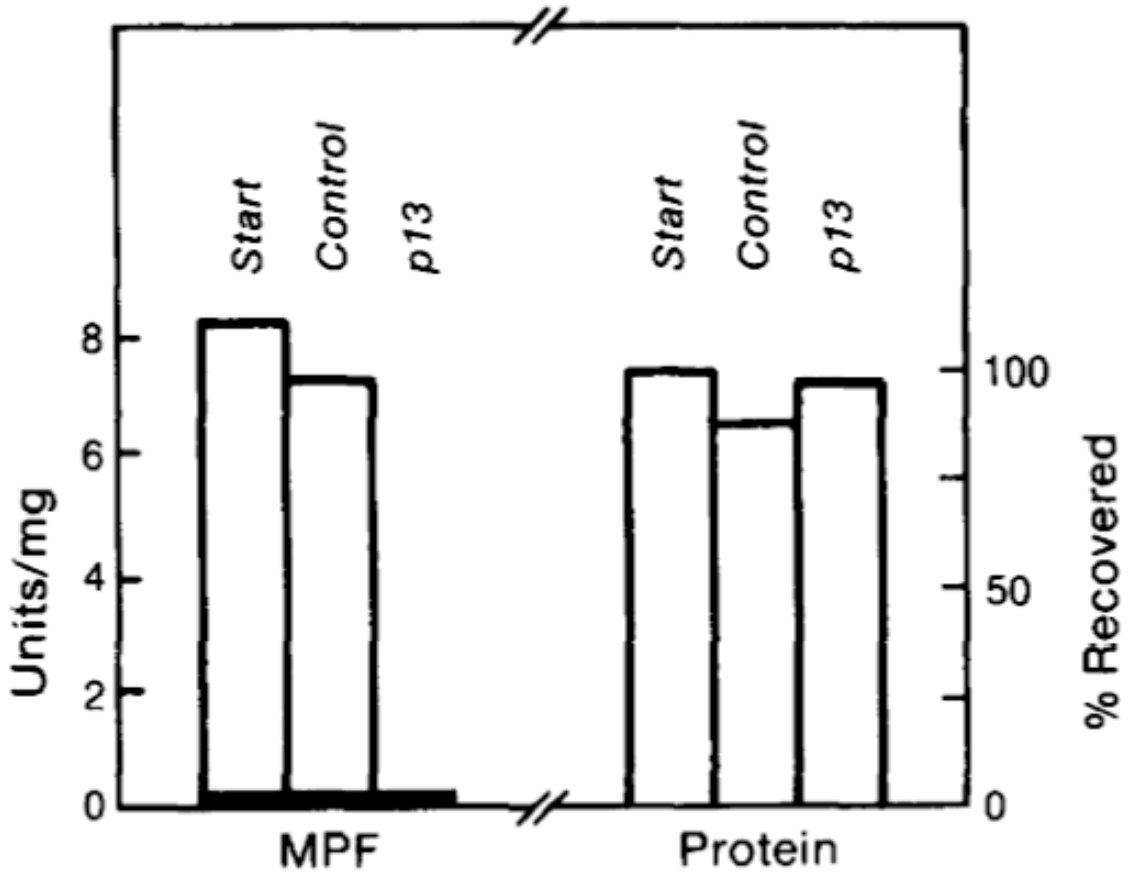
Xenopus eggs contain yeast cdc2 homologs



Xenopus eggs contain yeast cdc2 homologs

p13 interacts physically with cdc2
cdc2 kinase plays a direct role in mitotic induction in yeast
Xenopus egg contain two proteins that react with antibodies to cdc2: a 33 kDa and a 34 kDa protein
cdc2 is conserved from yeast to human

Affinity chromatography on p13-agarose depletes MPF



Affinity chromatography on p13-agarose depletes MPF

Binding of MPF to p13 was rapid and depended on the concentration of p13

Flowthrough from p13 did not inhibit MPF

No p13 leached off

Haven’t been able to elute MPF in an active form from the column (binding to p13 so strong)

p13 chromtaography enriched Xenopus cdc2 and a 42 kDa protein

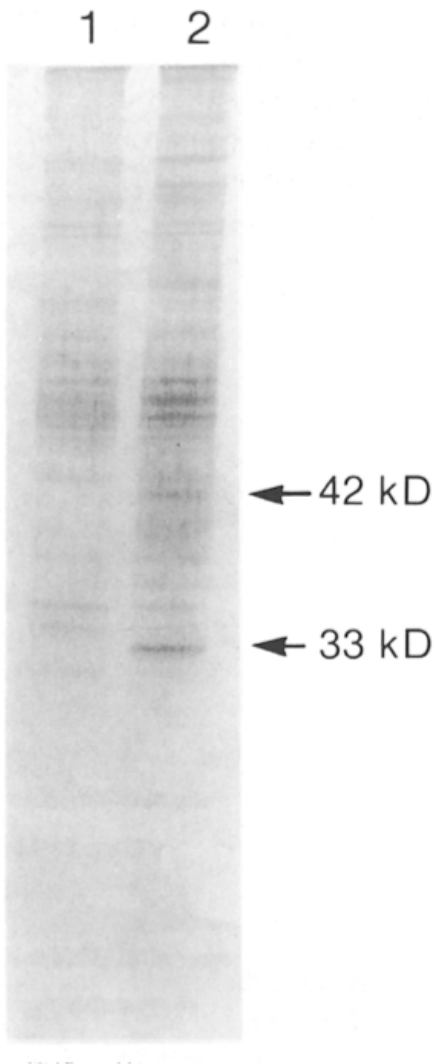
Lohka: 2 polypeptides 32 and 45, likely cdc2 is 32 kD species

P13 affinity chromatography: found a 33 kDa and a 42 kDa protein retained on the column

42 kDa did not react with anti-cdc2 abs

Related to 45 kD Lohka protein?

p13 chromtaography enriched Xenopus cdc2 and a 42 kDa protein



Dunphy

Link between 2 areas of research:

Genetics of division control in unicellular eukaryotes

Large number of mutants but little biochemistry

and

And cellular biochemistry of the cell cycle in early animal embryos

Can’t isolate enough of the proteins to do anything

What is your cell cycle model?
What evidence is there for your model?
What experiments would you do to test your model?

Cyclins and CDKs Drive the Cell Cycle

Cyclins interact with and activate cyclin-dependent kinases

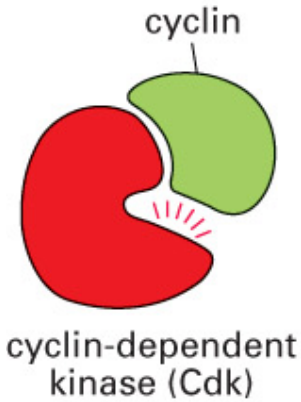


Figure 17–15. Molecular Biology of the Cell, 4th Edition.

Cyclins and CDKs Drive the Cell Cycle

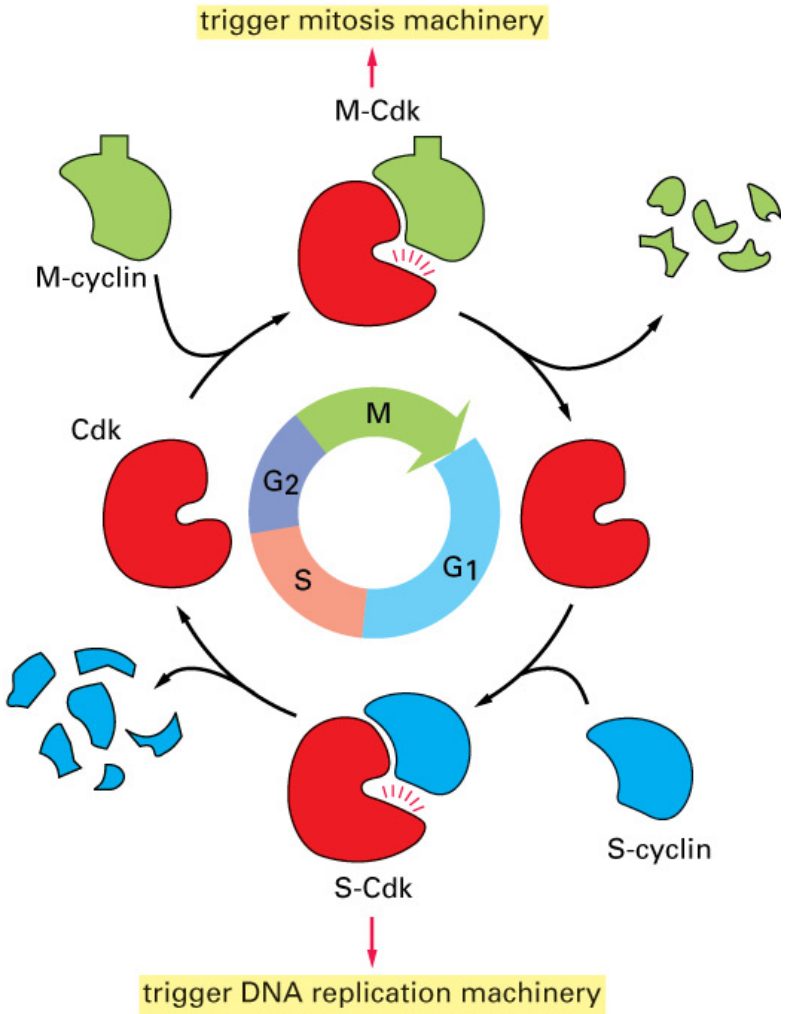


Figure 17–16. Molecular Biology of the Cell, 4th Edition.