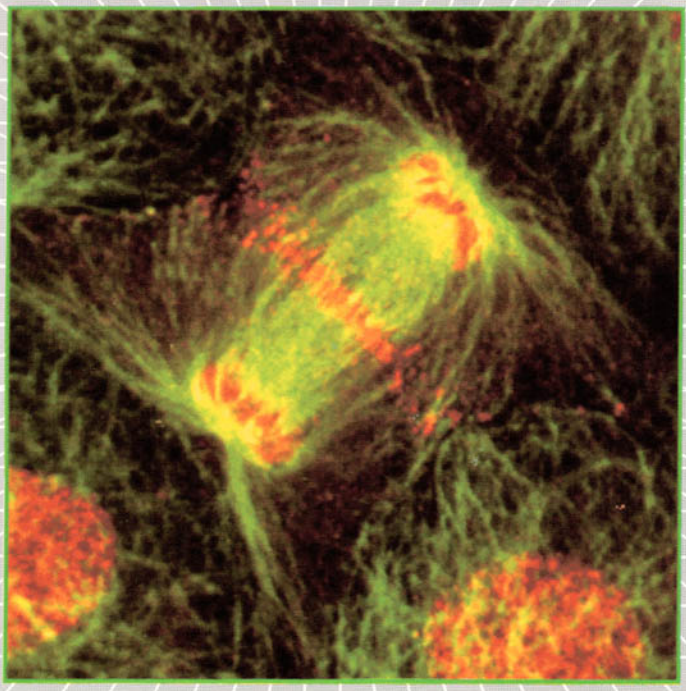
Dynamics of Cell Division



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Dynamics of Cell Division

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Dynamics of Cell Division

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Preface

The major events in the cytology of cell division were described over a century ago by early cell biologists. Only recently have molecular studies begun to have a major impact on our comprehension of the regulation, dynamics, and mechanics of the process. Although recent advances have shed considerable light upon the topic, the new findings raise many questions for the future. In the past decade we have seen a quantum leap in our understanding of the processes that regulate progression of the cell through different stages of the cell cycle. The transitions from one phase of the cycle to the next depend upon the sequential activity of cyclin dependent kinases. To maintain the fidelity of replication and segregation of genetic information, a number of surveillance mechanisms have evolved to ensure that a cell does not progress to the next phase of the cycle before the previous one has been completed. In the context of cell division, it is crucial to ensure that DNA has been completely replicated and any damage repaired before the cell enters mitosis, and there must also be mechanisms that ensure the correct alignment of chromosomes at metaphase, before sister chromatids segregate from one another.

We begin the book with an overview of the regulation of cell division by Yu, Duronio, and Sullivan. Entry into mitosis requires the activation of the major mitotic kinase, $p34^{cdc2}$, which is though to have a wide range of substrates. Breakdown of the nuclear envelope, chromosome condensation, and changes in microtubule dynamics all require the activity of this enzyme, which in many cases acts directly upon substrates that are major components of the cytoskeleton. In the second chapter, Miller and Forbes discuss how nuclear envelope breakdown and reassembly occur as cells progress through mitosis. The function and regulation of spindle pole bodies and centrosomes are reviewed in Chapter 3 by Hagan, Gull, and Glover. These organelles undergo their own cyclical duplication and division cycle that must be coordinated with the nuclear cycle and the overall phase of the cell cycle. The duplication and separation of these bodies is central to the organization of microtubules into bipolar spindles, and thus to ensure correct chromatid segregation in mitosis and meiosis. The process of spindle assembly and chromosome behaviour is the topic examined by Vernos and Karsenti. These authors consider how the dynamics of microtubule assembly and disassembly are coordinated with, and facilitated by, microtubule-associated motor proteins during formation of the mitotic spindle. They emphasize that a bipolar structure can arise through the intrinsic properties of microtubules and motor proteins alone. The following chapter by Sullivan discusses centromeres and kinetochores, and how these vary from yeast to metazoans. Not only the structure of these chromosomal regions is considered, but also the mechanisms by which sister chromatid cohesion is achieved and kinetochore function is regulated during mitosis. The ends of chromosomes present a different set of problems, particularly during

S-phase. Telomeres and the mechanisms by which the ends of chromosomes are replicated are the subjects of the chapter by Hughes and Lundblad. Exciting recent work has led to the identification of the telomerase catalytic subunit in both yeast and human cells. The generation of gametes during meiosis is an area of cell division about which we understand relatively little. The chapter by Karpen and Endow focuses on the special behaviour of the meiotic chromosomes and the dynamics of the meiotic spindle. Two divisions occur during meiosis without an intervening S-phase. The divisions must be integrated into a highly specialized programme of cellular differentiation and must accommodate the exchange of genetic material between maternal and paternal homologues. Perhaps most important within the context of our book, there must be mechanisms to ensure that homologous chromosomes segregate from each other without separation of sister chromatids in the first division, in contrast to the second division in which sisters undergo segregation. In thinking about cell division, we must not forget the partitioning of cytoplasm and organelles between the daughers. This topic is explored by Shima and Warren. Many pressing questions still remain in the area of cytoplasmic inheritance, in particular those that concern the biogenesis and partitioning of membranous organelles like the Golgi apparatus. The final chapter by Goldberg and colleagues concerns cytokinesis, the final event of cell division. The chapter addresses how the site of the cleavage furrow is determined, how initiation of furrow formation is regulated, and the mechanisms of contractile ring assembly and function.

Reviewing the enormous literature for the chapters has been a tremendous task for the authors. We can both speak from personal experience in saying that the insights that have come from writing the chapters that we co-authored were well worth the expended effort. We hope that this is a sentiment felt by the other authors. Our collective efforts should make it easier for others to share the excitement that we all feel about this field. We greatly appreciate the efforts of all the authors. We thank them for their participation in the project, for completing their chapters in a timely fashion, for revising their chapters and bringing them up to date for the book, and for their patience with the editorial process.

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Abbreviations

ADF	actin depolymerizing factor
APC	anaphase-promoting complex
ARS	autonomously replicating sequence
BAPTA	1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid
BFA	brefeldin A
CAK	cyclin-activating complex
CDI	CDK inhibitor
CDK	cyclin-dependent kinase
CENP	centromere protein
CHO	Chinese hamster ovary
CSF	cytostatic factor
DSB	double-strand break
ELC	essential light chain (of myosin)
EMS	ethyl methane sulfonate
ER	endoplasmic reticulum
ERM	ezrin/radixin/moesin
FISH	fluorescent *in situ* hybridization
GAP	GTPase-activating protein
GFP	green fluorescent protein
IGS	intergenic spacer
INCENP	inner centromere protein
IP_3	inositol triphosphate
KLP	kinesin-like protein (or kinesin-related protein)
LAP	lamin-associated protein
LBR	lamin B receptor
LTR	long terminal repeat
MAP	mitogen-activated protein, or microtubule-associated protein
MBC	methylbenzimidazole-2-yl-carbamate (or methyl 2-benzimidazole carbonate)
MCAK	mitotic centromere-associated kinesin
MHC	myosin heavy chain
MLCK	myosin light chain kinase
MMS	methyl methanesulfonate
MPF	maturation- (or mitosis-)promoting factor
MTOC	microtubule organizing centre
NEM	*N*-ethylmaleimide
NSF	NEM-sensitive fusion protein (or NEM-sensitive factor)
NuMA	nuclear mitotic apparatus (associated) protein
ORF	open reading frame

PCB	pericentriolar material
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEST	sequences rich in Pro (P), Glu (E), Ser (S), and Thr (T) which are thought to target the proteins containing them for rapid intracellular degradation
PH	pleckstrin homology
PI	phosphoinositide
PIP_2	phosphatidylinositol-4,5-bisphosphate
RLC	regulatory light chain (of myosin)
RNP	ribonucleoprotein
SC	synaptonemal complex
SMC	stable maintenance of chromosomes proteins—proteins needed for mitotic chromosome condensation
SNARE	soluble NSF-attachment protein receptor
SPB	spindle pole body
TPE	telomere position effect
TERT	telomerase reverse transcriptase

1 Cell cycle checkpoints: safe passage through mitosis

KRISTINA R. YU, ROBERT J. DURONIO, and WILLIAM SULLIVAN

1. Introduction

Mitosis results in the production of two daughter cells containing identical genetic complements. Achieving this requires a carefully orchestrated series of nuclear and cytoplasmic events leading to the accurate replication and segregation of the chromosomes. A powerful combination of molecular genetics, cell biology, and biochemistry have led to the isolation of key structural, enzymatic, and regulatory components governing these events. One of the most gratifying aspects of this research has been the realization that many of these components are highly conserved throughout the phyla. Therefore many of the lessons learned from one system will probably apply to others.

The major transitions in the cell cycle are driven by the successive activation of a family of cyclin-dependent kinases (CDKs) (1–4). The CDKs are structurally related and their activity requires a physical association with cyclin. Regulation of CDK activity occurs through post-translational modifications and through associations with a conserved family of activating cyclins and a family of CDK inhibitors. Entry into mitosis occurs through the activation of a universal mitotic CDK (also known as Cdc2 and p34). Activation occurs in a stepwise fashion: first by its association with cyclin and then through the progressive alteration of the phosphorylation states of key residues. The activated mitotic CDK initiates a diverse array of cytoplasmic and nuclear events driving the cell into mitosis. The mitotic CDK also activates the anaphase-promoting complex (APC), a ubiquitin ligase responsible for the degradation of cyclin and other inhibitors of anaphase (5). This allows the cell to progress through anaphase, forming two daughter cells.

For the most part, these events satisfactorily explain the order and timely progression of events necessary for entry into and exit from mitosis. However, if errors occur or individual steps in the cell cycle are delayed, it is likely that additional regulatory mechanisms are required for the proper progression of the cell cycle. It was through addressing this issue that the concept of cell cycle checkpoints was developed (6). Checkpoints increase the fidelity of the cell cycle by monitoring the accurate completion of specific cellular events. If an event in the cell cycle is not

completed or is improperly completed, checkpoints delay progression of the cell cycle to provide time for repair or completion of the event. This inhibitory phenomenon was initially observed in a classic series of cell fusion experiments. If a cell in S phase was fused with a cell in G_2, the G_2 nucleus delayed entry into mitosis until the second nucleus had completed S phase (7). This suggested that cells which have not completed S phase produce a diffusable inhibitor of mitosis. Mutational analysis in the budding yeast, *Saccharomyces cerevisiae*, led to the first explicit description of the concept of cell cycle checkpoints and the first identification of a checkpoint gene (8, 9). These genetic studies provided the conceptual framework which led to the identification of a number of checkpoints monitoring many events throughout the cell cycle. Checkpoints have been identified in many phyla in both germline and somatic cells and are probably universal components of the cell cycle. In this chapter, we review recent work on how cell cycle checkpoints guide cells into and out of mitosis. In addition, we discuss the role of cell cycle checkpoints in maintaining the fidelity of the synchronous rapid divisions observed during early embryogenisis in many higher eukaryotes. This review is not intended to be comprehensive. Instead we highlight studies that illustrate concepts and issues central to the field.

2. Activation of the mitotic CDK controls entry into mitosis

Entry into mitosis involves a dramatic reorganization of the nucleus and cytoplasm. Centrosomes migrate to opposite poles of the nucleus and establish the microtubule organizing centers and spindle orientation. Microtubule arrays undergo a dramatic reorganization to produce the bipolar mitotic spindle. Also occurring at this time is the breakdown of the nuclear envelope and disassembly of the sheetlike network of lamins lining the inner nuclear membrane. One of the most dramatic mitotic events is condensation of the chromosomes and their alignment along the metaphase plate.

It is clear that phosphorylation plays a key role in the cellular reorganization that accompanies entry into mitosis. As a cell enters mitosis, the total amount of protein-bound phosphate increases. Much of this increase is probably due to the activation of mitotic CDK (1). Activated mitotic CDK initiates many of the nuclear and cytoplasmic rearrangements described above. For example, this complex phosphorylates the nuclear lamins which result in their disassembly at mitosis (10) (see Chapter 2 for a detailed account). In addition, mitotic CDK-directed phosphorylation is responsible for altering microtubule polymerization dynamics and the dramatic reorganization of microtubules into a spindle as the cell enters mitosis (11, 12), as described in Chapter 4. However, most of the *in vivo* substrates remain elusive and little is known about the mechanisms that provide substrate specificity to the activated mitotic CDK (13).

2.1 Activation of mitotic CDK requires an association with cyclin

There is a wealth of information concerning the mechanisms that modulate the activity of mitotic CDK and other CDKs (14). Phosphorylation and dephosphorylation of key residues and specific protein associations are the primary mechanisms of regulating CDK activity (4). As the name implies, activation of CDKs requires a physical association with cyclin (Fig. 1). Cyclins are a large family of proteins originally identified because their abundance oscillates with the cell cycle (15). Each cyclin maintains a cell-cycle-specific pattern of accumulation and rapid proteolysis. Cyclins are generally classified as G_1, S, or M based on the timing of their peak concentrations and association with specific CDKs (1, 16). The orderly progression of key transitions throughout the cell cycle is defined by the phase-specific accumulation of specific cyclin–CDK complexes. Cyclin accumulation is the rate-limiting step in key phase transitions in many cell cycles. For example, in *Xenopus laevis* extracts the accumulation of cyclin B is the rate-limiting step in the activation of mitotic CDK and entry into mitosis (17). In addition, *Drosophila melanogaster* embryos exhibit significant delays in the cortical syncytial cycles when levels of cyclin B are reduced. These delays are more severe when levels of both cyclin A and cyclin B are reduced (18). These studies provide *in vivo* evidence that cyclin A and B levels control the timing of the syncytial cycles in the *Drosophila* embryo. However, cyclin is not the rate-limiting component controlling entry into mitosis in all cell cycles. In *Drosophila* cycle 14 embryos, entry into mitosis is controlled by expression of *string* (19, 20), a

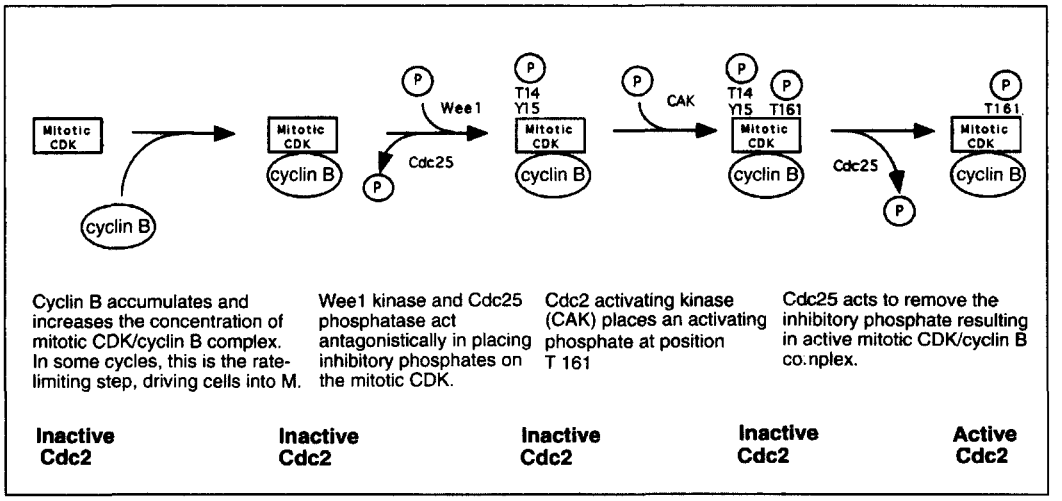


Fig. 1 The G_2–M transition in *S. pombe*. The conserved mitotic CDK ($p34^{cdc2}$) is activated through a physical association with cyclin B followed by series of steps which modify the phosphorylation state of residues Thr14 and Tyr15. The Weel kinase and Cdc25 phosphatase are key enzymes influencing the phosphorylation state of these residues. Mitotic CDK activation also requires phosphorylation on Thr161.

gene that encodes a homolog of *Schizosaccharomyces pombe* cdc25, a phosphatase that targets inhibitory mitotic CDK phosphates.

2.2 The APC mediates cyclin degradation and sister chromosome separation

In addition to regulating events required for entry into mitosis, the mitotic CDK–cyclin B complex activates a pathway leading to the ubiquitin-mediated proteolysis of cyclin B and thus its own inactivation. Proteolysis is dependent on the presence of a conserved 9 amino acid–terminal domain (D-box) that targets cyclin B for ubiquitination and subsequent proteolysis (21–25). The timing of cyclin B destruction is controlled by the late metaphase activation of a multiprotein complex known as the anaphase promoting complex (APC) (Fig. 2) (5). Through an as yet undefined pathway, mitotic CDK–cyclin B activates this complex during late metaphase (26). In addition to targeting cyclin for destruction, the APC also promotes initiation of anaphase by targeting the proteolysis of proteins required for sister chromosome cohesion. Support for this latter activity comes from the observation that undegradable cyclins result in a telophase arrest in which sister chromosomes have completely separated (27, 28). In contrast, disruption of APC activity prevents separation of the sister chromosomes and entry into anaphase (5, 27, 29).

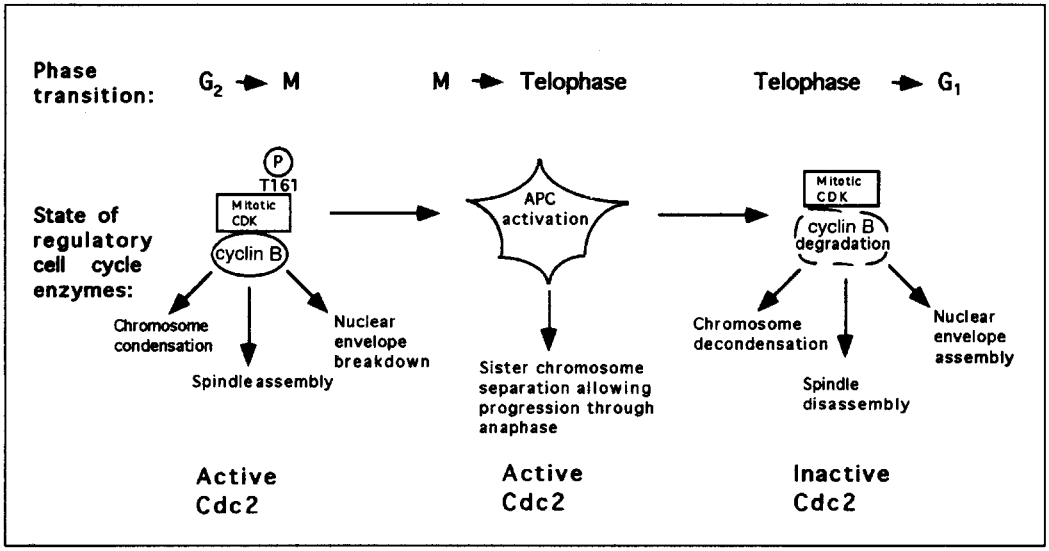


Fig. 2 A model for the role of anaphase promoting complex (APC) in driving exit from metaphase. Active mitotic CDK initiates a number of mitotic events including APC activation. APC is a large ubiquitin-ligase protein complex that inactivates the mitotic CDK by targeting cyclin B proteolysis. APC also acts earlier to promote sister chromosome separation through ubiquitin-dependent proteolysis.

2.3 Proteins that negatively regulate CDK activity

The CDK inhibitors (CDIs) are an emerging class of cell cycle regulatory proteins. In response to external environmental cues and internal signals these proteins associate with CDKs to inhibit their activity. When activated by alpha factor, *S. cerevisiae* Far 1 protein binds to and inhibits the activity of mitotic CDK–Cln2 (a G_1 cyclin). This produces a G_1 arrest (30). Another *S. cerevisiae* protein, Sic 1, binds to and inhibits an S phase cyclin–CDK complex. Sic 1 may be involved in preventing inappropriate rounds of DNA synthesis (31–33). Well known mammalian CDIs include p15, p16, p21, and p27 (34–39). p21 is a target of p53, a gene which is often mutated in human tumors (40). Interestingly p15 and p16 act by preventing the formation of the CDK–cyclin complex (34, 41). The chromosomal region to which both of these genes map in humans is often deleted in individuals with hereditary melanoma (42).

2.4 Post-translational phosphorylation regulates mitotic CDK activity

In addition to cyclin association, CDK activation requires specific phosphorylation and dephosphorylation of key residues. The ATP-binding site of *S. pombe* mitotic CDK contains threonine and tyrosine at residues 14 and 15 respectively. Phosphorylation of one or both of these residues maintains the mitotic CDK–cyclin complex in an inactive state (Fig. 1). In addition, activity of mitotic CDK requires phosphorylation at Thr161 (1, 14). This residue may influence the binding of the substrate to the CDK kinase domain. The enzyme responsible for the phosphorylation of threonine 161 has been historically referred to as cyclin-activating kinase (CAK). CAK activity is associated with the Cdk7–cyclin H complex in higher eukaryotes. However, the *S. cerevisiae* homolog of cdk7–cyclin H does not phosphorylate Thr161 *in vitro* or *in vivo* (43). Recent genetic and biochemical evidence suggests that Cak1/Civ1 is the *S. cerevisiae* kinase responsible for phosphorylating Thr161 *in vivo* (44, 45). It remains to be seen if a Cak1/Civ1 homolog is found in higher eukaryotes and if there are additional classes of physiologically important CAKs.

In *S. pombe*, the conserved phosphatase, Cdc25, and kinases, Wee1 and Mik1, act antagonistically to influence the phosphorylation state of Thr14 and Tyr15 (46, 47). Increasing the dosage of Cdc25 relative to Wee1 results in a shortened G_2 and premature entry into mitosis. Increasing the dosage of Wee1 relative to Cdc25 has the opposite effect: entry into mitosis is delayed. It may be that as a cell normally progresses through G_2, the increase in the Cdc25/Wee1 ratio regulates the timing of entry into mitosis.

3. Cell cycle checkpoints

The initiation of many events in the cell cycle depends on the proper completion of a previous event. For example, in many cells treatment with hydroxyurea, a potent

inhibitor of DNA replication, results in a G_2 delay and failure to progress into mitosis. These studies demonstrate that entry into mitosis depends on completion of DNA synthesis (8, 48). The most extensive description of dependency relationships exists for *S. cerevisiae* and *S. pombe* cell cycles. In these organisms, large numbers of mutations have been isolated that cause an initially asynchronous population of cells to arrest at a specific point in the cell cycle. Analysis of these cell division cycle (*cdc*) mutations enabled the major cytoplasmic and nuclear events of the cell cycle to be placed in one of a series of dependent pathways (49–51).

As described by Hartwell and Weinert (6), these dependency relationships may be either the result of intrinsic substrate–product relationships or may be established by adding external feedback controls enforcing the dependency relationships (Fig. 3). In the former, the product of one step in the pathway serves as a substrate for the next. Therefore if the first step does not occur or is improperly executed, the next step cannot be initiated. Alternatively, surveillance mechanisms extrinsic to the process monitor the accurate completion of each step. If a step fails or is inaccurately completed, the surveillance mechanism prevents the initiation of the next step. In this case, elimination of the surveillance mechanism eliminates the dependency relationship but does not affect the actual process. In contrast, if the dependency relationship is a consequence of a substrate–product relationship, it is unlikely that the dependency can be relieved without disrupting the process itself. Therefore finding conditions (mutations or drugs) that relieve the dependency relationship and allow cell cycle progression provides strong evidence that it is enforced by a feedback mechanism. These negative feedback controls are referred to as cell cycle checkpoints.

In many cells, exposure to low, non-lethal doses of X-irradiation produces an arrest in G_2 and cells do not enter metaphase. This demonstrates that entry into mitosis requires undamaged DNA (52, 53). In the presence of caffeine, entry into mitosis no longer requires intact DNA (54–56). The caffeine-mediated relief of this dependency relationship demonstrates that it is due to a cell cycle checkpoint rather than a substrate–product relationship.

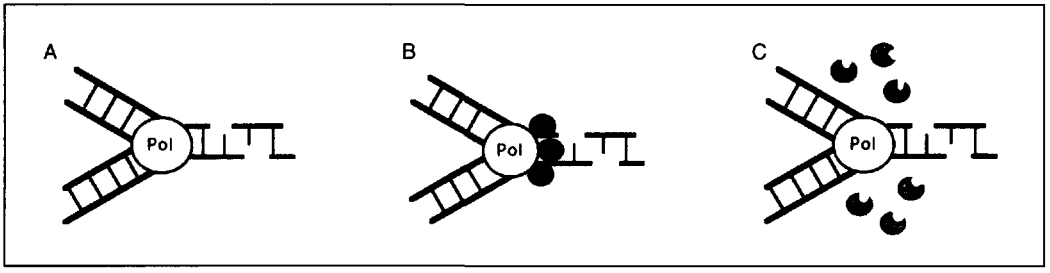


Fig. 3 Normal rates of DNA polymerization rates depend on undamaged DNA. It may be that the lesions physically prevent progression of the DNA polymerase (A, substrate product-mediated delay). Alternatively (B), the lesions may activate feedback controls that slow the polymerization rate (checkpoint-dependent delay). Identifying a condition that relieves the dependency relationship (C) demonstrates that it is the result of a negative feedback mechanism. These feedback mechanisms are called cell cycle checkpoints.

Delays, as well as arrests, in the cell cycle, indicate the presence of a dependency relationship (Fig. 3). For example, studies in mammalian cells demonstrate that DNA damage significantly reduces the rate of DNA replication (57–59). This is a direct consequence of decreasing the frequency of replicon initiation and slowing the rate of strand elongation by DNA polymerase (58). This delay demonstrates that the rate of DNA synthesis depends on undamaged DNA. It is reasonable to assume that this dependency is due to a substrate–product relationship; that is, the slowing of DNA replication is a direct result of the replication machinery navigating lesions in the DNA. However, *S. cerevisiae* mutants have been identified that fail to slow DNA replication in response to methylmethanesulfonate (MMS) induced damage (59). As these mutations relieve the dependency relationship, this indicates that the slowing of S-phase is also enforced by a cell cycle checkpoint.

In mitotic cell cycles, migration of sister centrosomes to opposite nuclear poles precedes spindle formation. In *Drosophila* embryos, mutations that disrupt separation of the centrosomes also disrupt spindle formation (60). This indicates that spindle formation is dependent on proper centrosome separation. The identification of a condition that relieves this dependency relationship is required to demonstrate that this dependency relationship is a consequence of a cell cycle checkpoint rather than a substrate–product relationship. To date such a condition has not been identified. This is not unexpected since it is reasonable to assume that centrosome separation is a necessary first step in the formation of a spindle. However, one cannot conclude that this is the result of a substrate–product relationship. In fact, there are a number of instances in other organisms in which functional spindles form in the absence of centrosomes (61–67). Only by finding a condition that eliminates the dependency relationship, is it possible to conclude that it is the result of a checkpoint rather than a substrate–product relationship.

3.1 Mutational analysis identifies *RAD9* as a DNA damage checkpoint

The first gene shown to be involved in a cell cycle checkpoint was originally recovered because of its sensitivity to X-irradiation (6). In *S. cerevisiae*, as with other cells, X-irradiation produces a delay in G_2. If this delay is the result of a cell cycle checkpoint, two classes of X-irradiation-sensitive mutations are expected: those that are defective in the enzymatic machinery required for repair and those that are defective in the checkpoint-induced delay which provides time for repair to occur (Fig. 4). Among the large collection of X-irradiation-sensitive mutants isolated in *S. cerevisiae*, both classes have been identified (6, 68). For those in the first class, the DNA damage checkpoint is intact and they exhibit a G_2–M delay in response to DNA damage, but repair does not occur. The latter class of mutants have an intact DNA repair system but fail to delay at G_2–M in response to X-irradiation (8, 67). *rad9* was the first of the latter class of mutants to be identified (6). The X-irradiation sensitivity of *rad9* mutants results from progression through mitosis with damaged DNA. This leads to inviable aneuploid daughter cells.

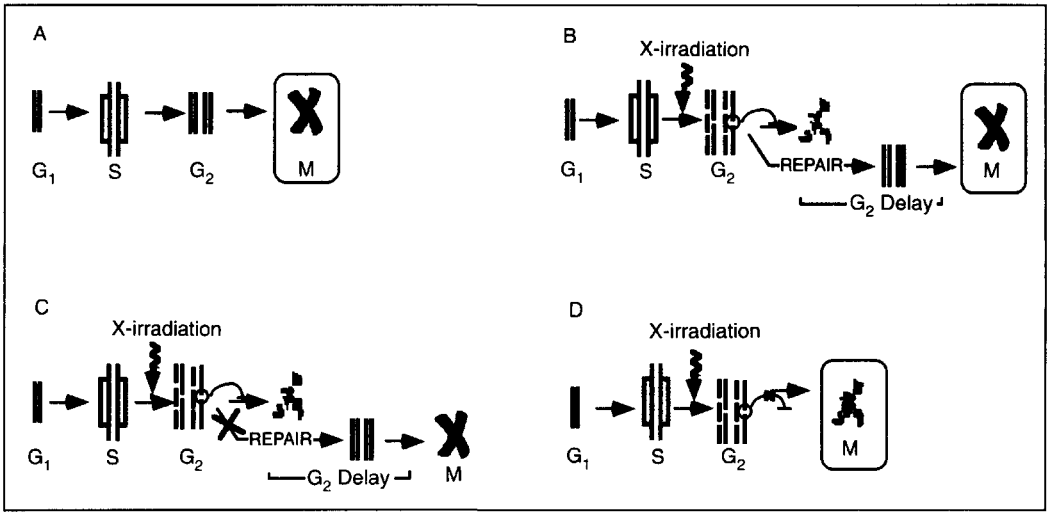


Fig. 4 Identifying DNA damage checkpoint mutations. Checkpoints, by delaying entry into metaphase, provide time for repair (compare unirradiated cells (A) with X-irradiated cells (B)). Consequently, there should be two classes of X-irradiation-sensitive mutations: those that are defective in the repair response (C) and those that are defective in the checkpoint response (D). These classes are distinguished because repair mutations, but not checkpoint mutations, cause a delay in response to X-irradiation. The boxes indicate the outcome for each situation.

This phenotype indicates that the primary function of the *RAD9* gene product is to delay the cell in G_2–M to provide time for repair of damaged DNA before progressing into mitosis. A prediction of this model is that slowing progression through G_2–M should reduce the sensitivity to X-irradiation in *rad9* cells. In *S. cerevisiae*, this experiment is readily performed because spindle formation begins directly after the completion of S phase. Consequently, exposing yeast cells to methylbenzimidazole-2-yl-carbamate (MBC), a drug that depolymerizes microtubules, dramatically slows progression through G_2–M. If *rad9* cells are treated with MBC prior to X-irradiation and are maintained in the drug for an extended period thereafter, the sensitivity to X-irradiation is greatly diminished. This indicates that if time is provided, the damaged DNA is repaired in *rad9* cells (6).

Given that *RAD9* is primarily required for the DNA damage checkpoint, it is not expected to be an essential gene. Null alleles of *rad9* are viable, but they exhibit a 20-fold increase in chromosome loss (6). The high rate of chromosome loss, however, indicates that checkpoints are required during normal growth to maintain cell cycle fidelity. This reflects the fact that cells occasionally require extra time to repair lesions that inevitably occur during S phase and normal progression through the cell cycle. *RAD9* is also involved in DNA damage checkpoints operating during G_1 and S (69, 70). Although the *RAD9* checkpoint operates throughout the cell cycle, it is specific for DNA damage and is not involved in the well-documented DNA synthesis and spindle assembly checkpoints.

3.2 Checkpoints monitor many cellular events and involve signal transduction pathways that link delays in the cell cycle to repair processes

Checkpoints have been identified at all major transitions in the cell cycle. They monitor a diverse array of events including cell size, chromosome condensation, DNA replication, DNA integrity, and spindle assembly (71). Checkpoints monitoring the latter three have been intensively investigated through mutational analysis (72–76). The mechanisms employed by checkpoints to monitor cell cycle events and induce arrest in the cell cycle are not well understood, although it is clear that signal transduction processes are involved (77). Checkpoints require sensors to respond to signals generated from an incomplete or improperly completed event in the cell cycle. Signal transduction processes amplify this signal and activate effectors that mediate cell cycle arrest. The CDKs responsible for driving the major transitions in the cell are a likely target of the checkpoints. As described previously, CDK activity is regulated by diverse mechanisms and the various array of cell cycle checkpoints may act through these mechanisms.

3.3 Ambiguities in the concept of cell cycle checkpoints

Identifying a mutation that eliminates a dependency relationship indicates that the mutated gene is involved in a cell cycle checkpoint. This operational definition has provided a valuable framework for genetically identifying components involved in monitoring cell cycle progression. However, it has also led to some confusion because mutations in the enzymatic machinery driving major cell cycle transitions have been identified that also eliminate cell cycle dependency relationships (15). For example, certain mutations in mitotic CDK eliminate the dependency of M phase on a previously completed S phase (78). In addition, mutations in DNA replication enzymes disrupt the DNA synthesis checkpoint (79). While the behavior of these mutations is in accord with the operational definition of cell cycle checkpoints, they are not in accord with the conceptual definition of a surveillance system that monitors but is extrinsic to the basic events of the cell cycle.

In general, cell cycle checkpoints are thought to monitor the cell cycle but are considered unnecessary for completion of an event or a repair process. The relief of dependence criteria has served to identify many genes involved in these checkpoints. However, for some potential cell cycle checkpoint genes a more stringent criterion has been applied: not only do mutations in these genes relieve a dependency relationship, but they can be rescued by slowing the cell cycle to provide time for repair or completion of an event (8, 78). Rescuing the mutant phenotype solely by providing additional time clearly demonstrates that the gene is not involved in the processes required for repair or completion of an event. Although it is not always practical to apply this criterion, when it is successfully applied it clearly establishes that these genes are involved in cell cycle checkpoints.

4. Lessons from budding yeast: the role of checkpoints in monitoring the completion of S phase and DNA damage

The *cdc* mutations disrupt specific events in the cell cycle and produce a phase-specific cell cycle arrest. These provide a useful set of tools to test both the phase and signal specificity of a given cell cycle checkpoint. For example, *CDC9* encodes a DNA ligase; at restrictive temperatures, *cdc9* mutants produce unligated Okazaki fragments and arrest in late S–G_2. Even in this arrested state, the mutant cells maintain viability for hours. The unligated DNA fragments produced at the restrictive temperature in *cdc9* mutants activate a cell cycle checkpoint that arrests the cell in S–G_2. This idea is confirmed by the observation that *cdc9–rad9* double mutants fail to arrest when placed at a restrictive temperature (80). Unlike the arrested cells, these double mutants rapidly lose viability. This is probably a consequence of progressing through mitosis with damaged DNA. The double mutant demonstrates that the *cdc9* arrest is dependent on the *RAD9* checkpoint. This also indicates that the *RAD9*-dependent checkpoint operates during S–G_2 in response the DNA damage.

Analysis of a series of *cdc–rad9* double mutants strengthens the notion that the *RAD9*–dependent checkpoint responds specifically to DNA damage. Of 12 *cdc* mutants tested, four were dependent on the *RAD9* checkpoint for their arrest. Three of these four encode known DNA replication enzymes (DNA ligase, DNA polymerase I, and DNA polymerase III) (81–85). In addition, the cell cycle arrest induced by hydroxyurea, a potent inhibitor of S phase, is not dependent on *RAD9*. Therefore incomplete DNA replication does not activate the *RAD9*-dependent checkpoint. Functional *RAD9* also is not required for the spindle assembly checkpoint (6). Taken together, these data indicate that *RAD9* functions during late S–G_2 in a DNA damage, but not a DNA replication checkpoint.

Screening the existing collection of radiation-sensitive mutations resulted in the identification of a second cell cycle checkpoint mutation *rad17* (80). *rad17–cdc* double mutants exhibit a pattern of phenotypes identical to that of the *rad9–cdc* double mutants. This indicates that both genes are involved in the same DNA damage checkpoint.

4.1 Synthetic lethal screens provide an efficient means of identifying additional checkpoint mutations

The synthetic lethal phenotype of specific *rad9–cdc* double mutants led to the development of a general strategy for isolating additional checkpoint mutants. The *CDC13* gene encodes a protein that binds to and protects the telomere from degradation and facilitates telomerase loading (86). *cdc13* mutations result in the accumulation of single-stranded telomeric DNA (86). This accumulation activates the arrest induced by a DNA damage checkpoint. As with other *cdc* mutant cells,

these remain viable for hours in this arrested condition. Without a functional checkpoint, these cells fail to arrest and die as a consequence of progressing through mitosis with damaged telomeres.

EMS screens for new checkpoint mutations were performed by isolating mutants that resulted in lethality in the absence of *CDC13* function (87). The screen identified four additional cell cycle checkpoint genes: *MEC1* (mitosis entry checkpoint), *MEC2/RAD53*, *MEC3*, and *RAD24*. None of these mutations delayed G_2 when exposed to X-irradiation and all exhibited an increased sensitivity to X-irradiation. Since the failed G_2 delay was an unselected phenotype, this provided strong evidence that the mutations disrupted genes involved in a cell cycle checkpoint.

Similar screens based on synthetic lethality and drug sensitivity have identified S phase and G_2 checkpoint mutations in *S. pombe* and spindle assembly checkpoints in *S. cerevisiae* (88–91). Although these screens involve different aspects of the cell cycle, they are all based on the common principle that by delaying the cell cycle, checkpoints provide time for repair and increase the tolerance of a cell to both internally and externally induced damage.

4.2 Detecting DNA damage

Although the specific signals responsible for activating DNA damage cell cycle checkpoints have not been identified, properties of these signals have been defined. The DNA damage checkpoint is activated by UV and X-irradiation, but not by drugs that inhibit DNA replication (76). That is, it is activated by signals specific to damaged rather than unreplicated DNA. The checkpoint is extremely sensitive and is activated by a single double-strand break in the *S. cerevisiae* genome (92, 93). The signals generated from DNA damage are capable of eliciting a checkpoint response throughout the cell cycle. For example, the checkpoint genes *RAD9*, *RAD17*, and *RAD24* monitor the state of the DNA during G_1–S and G_2–M (92).

Of the identified checkpoint genes, *RAD9*, *RAD17*, *RAD24*, and *MEC3* appear to be most directly involved in monitoring signals generated from DNA damage (Table 1). These genes are required for the DNA damage but not the DNA replication checkpoint (80, 87). In addition, they influence the processing of damaged DNA. As described above, *cdc13* mutations disrupt the stability of telomeric DNA and exhibit a G_2–M checkpoint-induced arrest. While arrested, these mutations accumulate telomeric single-stranded DNA. If *rad24*, *rad17*, and *mec3* are maintained in a *cdc13* background, the single-stranded telomeric DNA accumulates much more slowly (72). In contrast, *rad9–cdc13* double mutants accumulate single-stranded DNA more rapidly. In accord with these results *RAD24* encodes a protein with some homology to Rfc, a protein that binds gapped DNA, and *RAD17* encodes a putative exonuclease (72, 94). These results suggest that these genes may play a role in processing and repair of damaged DNA in addition to their checkpoint function. Damaged DNA is processed by multiple pathways: modified and crosslinked bases are often repaired by an excision-based repair process while breaks are repaired by a recombination-based process (68, 95). This processing may be required to generate signals

Table 1 Checkpoint genes

Gene	Organism	Checkpoint position	Function	Homologs	References
BUB1	*S. cerevisiae*	G_2/M		*mBUB* (mouse)	155
BUB2	*S. cerevisiae*	G_2/M			155
BUB3	*S. cerevisiae*	G_2/M			155
chk1/rad27	*S. pombe*	G_2	protein kinase	*grp* (*Drosophila*)	90, 135, 186, 193
DUN1	*S. cerevisiae*	S			194
MAD1	*S. cerevisiae*	G_2/M			3
MAD2	*S. cerevisiae*	G_2/M		*hsMAD2* (human) *XMAD2* (Xenopus)	3, 155, 168, 169
MAD3	*S. cerevisiae*	G_2/M			3, 155
MEC1	*S. cerevisiae*	G_1/S, S, S/M, G_2M	protein/lipid kinase	*rad3* (*S. pombe*) *ATM* (human) *MEI41* (*Drosophila*)	3, 155, 168, 169
MEC2	*S. cerevisiae*	G_1/S, S, S/M, G_2/M	protein kinase		87
MEC3	*S. cerevisiae*	G_2/M			87
RAD53	*S. pombe*	G_1/S, S, S/M, G_2/M	protein kinase	*cds1*$^+$ (*S. pombe*)	87, 101–103, 195
MPS1	*S. cerevisiae*	G_2/M			150
Pol2	*S. cerevisiae*	S	DNA polymerase subunit	*cds20*$^+$ (*S. pombe*)	79
Polε	*S. cerevisiae*	S/M	DNA polymerase with 3′→5′ exonuclease		79
RAD9	*S. cerevisiae*	G_1/S, G_2/M			6, 9, 69, 79, 102
RAD17	*S. cerevisiae*	G_1/S, G_2/M	3′→5′ exonuclease	*rad1*$^+$ (*S. pombe*) *rec* (*U. maydis*)	72, 80, 94, 197
RAD24	*S. cerevisiae*	G_1/S, G_2/M	weak homology to RFC	*rad17*$^+$ (*S. pombe*)	72, 87, 197

recognized by DNA damage checkpoints. In addition, activating a given checkpoint may require a specific form of processed DNA.

4.3 Monitoring completion of S phase

In *S. cerevisiae*, DNA polymerase II is a multiprotein complex required for DNA replication. The largest member of this complex is the 256 kDa polymerase encoded by *POL2* (96). Genetic analysis demonstrates that this protein possesses an N-terminal domain required for polymerase activity and a separable C-terminal domain required for the S phase checkpoint (79). Mutations have been identified that disrupt each of these functions separately.

The C-terminal domain of this polymerase is also required for complex formation with other proteins (97). Multicopy suppressor screens of C-terminal domain mutations identified the DpbII protein (98). DpbII is homologous to the product of the fission yeast *RAD4/CUT5* checkpoint gene and associates with DNA polymerase II during replication (99). Null alleles of *DpbII* demonstrate that it is essential. In addition, temperature-sensitive alleles of *DpbII* also demonstrate that the gene is required for an S phase checkpoint.

Rfc5, a component in the small subunit of the DNA replication factor C complex, was identified in a screen designed to identify genes that interact with the S phase checkpoint gene, *MEC2* (100). Replication factor C binds gapped DNA and recruits proliferating cell nuclear antigen (PCNA) (101). This DNA–protein complex recruits polymerases ε and δ to form a functional replication complex. A temperature-sensitive allele of the *RFC* gene, *rfc5-1*, was recovered because it is suppressed by overexpression of the DNA-damage gene, *MEC2*. At the restrictive temperature, *rfc5* mutations do not complete replication. In addition, they fail to arrest or delay in G_2 and progress into mitosis with incompletely replicated DNA. This results in aneuploidy and loss of viability. As with DNA polymerase II and DpbII, the *RFC* gene product is involved in DNA replication and in the checkpoint that monitors completion of DNA replication. These studies demonstrate that DNA polymerase is a key component of the S phase checkpoint (102).

4.4 Signal transduction

The properties of mutations in *S. cerevisiae MEC1* and *RAD53* genes indicate that they act as central components in the signal transduction pathway leading to checkpoint-induced cell cycle arrest (Fig. 5). *mec1* and *rad53* mutations disrupt both DNA damage and DNA replication checkpoints operating throughout the cell cycle (87). Both are required for the expression of DUN1, a protein necessary for the transcriptional response that normally accompanies checkpoint activation (102). *MEC1* is also required for a meiotic cell cycle checkpoint (87). These studies demonstrate that MEC1 and DUN1 process signals derived from multiple cell checkpoints. Consistent with their role in a centralized signal transduction process, both are essential protein kinases. In addition, they function downstream of *POL2* and *RAD9*, genes that are required early in the checkpoint response to monitor signals from improperly replicated DNA (102).

MEC1 and *RAD53* are also required for a checkpoint that operates during S phase in response to DNA damage (103). In *S. cerevisiae*, exposure to low doses of the DNA damaging agent MMS results in a six-fold decrease in the rate of S phase. This DNA damage-induced reduction in the rate of S phase has also been observed in mammalian cells. As described previously, in *mec1* and *rad53* mutants, the rate of S phase is not slowed in response to DNA damage.

MEC1 encodes a phosphatidylinositol-3 kinase and has *Drosophila* and mammalian homologs (104, 105). Mutations in the human homolog, *A-T* (ataxia telangiectasia), behave as autosomal recessives. Homozygotes experience symptoms that include progressive neurodegeneration, permanently dilated blood vessels and an elevated occurrence of cancerous tumors. There is also evidence suggesting that heterozygotes at the *A-T* locus have a slightly elevated risk of breast cancer (106). *A-T* mammalian cell lines lack G_1 and G_2 DNA damage checkpoints (107). In addition, they fail to slow progression of S phase in response to DNA damage. G_2 and S phase DNA damage checkpoints require *MEC1* and it is probably required for the G_1 DNA

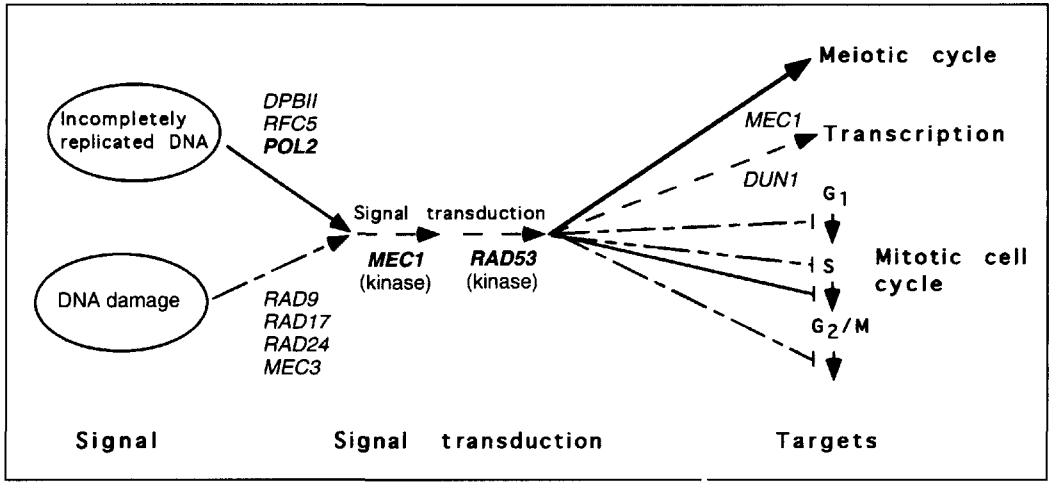


Fig. 5 Outline of the *S. cerevisiae* DNA damage and replication checkpoint signal transduction pathway. The genes indicated in bold (*POL2*, *MEC1*, *RAD53*) are essential. Common pathways are denoted by solid, striped or dashed lines. See Table 1 for references. In *S. cerevisiae*, G_2 and M phase events temporally overlap. In addition to participating in the phase-specific mitotic arrest, some of these genes are also required for meiotic checkpoint function and the transcriptional induction that accompanies checkpoint activation. Mec1 and Rad53 act as central processors in the DNA damage and replication checkpoint response.

damage checkpoint as well. Therefore the Mec1 and A-T proteins are likely to be functional as well as structural homologs.

4.5 p53 is a mammalian checkpoint gene that functions during G_1–S and G_2–M

In addition to *A-T*, the mammalian p53 and p21 genes are also required for the G_1 DNA damage checkpoint (108, 109). Cells lacking p53 are severely compromised in their ability to arrest in G_1 in response to DNA damage, while the G_1 arrest is only mildly compromised in cells lacking p21. This suggests that the mammalian G_1 arrest involves multiple pathways and that p53 may be required for a number of these pathways. This is readily explained by the fact that the p53 gene encodes a transcription factor that may regulate a battery of genes involved in the G_1 checkpoint (110). p53 exhibits rapid turnover, possibly in response to ubiquitin-dependent proteolysis (111). In response to DNA damage, both the activity and stability of the p53 protein are increased by mechanisms that are not well understood (108, 112). In cells lacking A-T, the activation of p53 in response to DNA damage is attenuated, indicating that A-T is an upstream regulator of p53 (113, 114). Like A-T, p53 is frequently mutated in human cancers, indicating that both are essential for maintaining genome stability (115–117). As well as inducing a repair response, p53 is required for DNA-damage induced apoptosis (114, 118, 119).

There is mounting evidence that p53 functions during G_2 and M as well as G_1–S. Mouse embryonic fibroblasts lacking p53 are much more sensitive to low doses of caffeine, a potent inhibitor of the G_2–M DNA damage checkpoint, than genetically matched wild-type controls (120). This indicates that p53 is involved in the G_2–M DNA damage response. Further support is provided from studies demonstrating that immortalized Li Fraumeni fibroblasts lacking p53 exhibit a defective G_2 DNA damage checkpoint response (121). Temperature-sensitive alleles of p53 have also been used to demonstrate that p53 may regulate the cell cycle at G_2–M and as well as G_1–S (122). Driving p53 expression with an inducible promoter can arrest cells at G_2–M (123). p53 undergoes a G_2–M cyclin-dependent phosphorylation that alters its DNA-binding activity (124). Concluding that p53 is involved in a G_2 DNA damage checkpoint must be tempered by the possibility that this may be a secondary effect of increased genetic instability of cells lacking p53. As will be described below, p53 is also required for the spindle assembly checkpoint.

The p21 gene is a target of the p53 transcription factor. The p21 protein is an inhibitor of the Cdk2 and Cdk4 cyclin-dependent kinases required to drive cells into S phase. p21 is activated by and requires p53 (40). Embryonic cells derived from p21 null mice are defective in their ability to arrest in G_1 in response to DNA damage (109). However, unlike p53 knockouts they do not develop tumors, nor are they defective in thymocyte apoptosis or the spindle checkpoint. It is likely that other targets of the p53 transcription factor operate during G_2 and M.

4.6 The mammalian p53 gene is involved in a spindle assembly checkpoint

Studies demonstrating that loss of p53 activity often leads to polyploidy suggests that it may be involved in checkpoints operating during metaphase as well as G_1 (110, 125, 126). To test this idea directly, wild-type and p53-deficient cells were exposed to low doses of the microtubule inhibitors nocodazole and colcemid (127). Due to activation of the spindle assembly checkpoint, wild-type cells delay in metaphase directly after exposure to nocodazole and exhibit a dramatically increased mitotic index. Cells lacking p53 exhibit only a small increase in the mitotic index in response to nocodazole, indicating that the spindle assembly checkpoint is compromised in these cells. Consequently, after 44 h exposure to nocodazole, none of the wild-type cells exhibited an 8*N* DNA content while 44% of the p53 minus cells exhibited an 8*N* content. Cells lacking p53 also undergo unregulated centrosome duplication (128). Thirty per cent of the cells derived from mouse embryonic fibroblasts contained from three to ten centrosomes. Often these additional centrosomes nucleate microtubules that associate with chromosomes and result in their mis-segregation. Whether the abnormal centrosome duplication is a direct consequence of a defective spindle assembly checkpoint in cells lacking p53 remains to be determined.

4.7 Arresting the cell cycle

Since checkpoints delay the cell cycle, core cell cycle regulators such as mitotic CDK are probably the targets of checkpoint-activated signal transduction pathways. Nevertheless, a number of experiments indicate that mitotic CDK is not the target of DNA damage and S phase checkpoints in *S. cerevisiae* (129, 130). Mitotic CDK activity is high in cells arrested due to the activation of an S phase or DNA damage checkpoint. In addition, extensive targeted mutagenesis screens of mitotic CDK in *S. cerevisiae* failed to generate mutations disrupted in the S phase checkpoint.

In many organisms, activation of mitotic CDK during the G_2–M transition depends on dephosphorylation of a conserved tyrosine (Tyr19 in *S. cerevisiae*). However, the S phase and DNA damage checkpoints remain intact when the equivalent residue is mutated. These experiments indicate that in *S. cerevisiae* the checkpoint-induced arrest is not achieved by influencing mitotic CDK activity. This may be a consequence of the fact that in *S. cerevisiae*, the DNA damage checkpoints prevent exit from rather than entry into metaphase. In this organism there is no clear distinction between G_2 and M phases. Spindle assembly and other mitotic events begin in G_2 directly after S phase. This cytological observation explains why inhibitors of microtubule assembly alleviate the X-irradiation sensitivity of DNA damage checkpoints (6). These inhibitors activate the spindle assembly checkpoint and slow progression through G_2–M. This provides time for repair of DNA damage in spite of the absence of a functional DNA damage checkpoint. Consequently, genes that regulate exit from metaphase are the probable targets of the DNA damage checkpoints in *S. cerevisiae*.

Recent studies indicate that the product of the *PDS* gene may mediate the checkpoint-induced mitotic arrest. This gene has been identified as the most downstream element in the DNA damage and spindle assembly checkpoint pathways in budding yeast (131). *pds* mutants were originally discovered because of their sensitivity to microtubule inhibitors and their lack of a spindle assembly checkpoint (132, 133). Gamma irradiation studies demonstrate that Pds1 is also necessary for the G_2–M DNA damage checkpoint. In addition, there is evidence that Pds1 is a target of the APC (131). Pds1 is ubiquitinated and degraded during the onset of anaphase in an APC-dependent manner. Nondegradable forms of Pds prevent exit from mitosis. These studies implicate Pds rather than Cdc2 as a target of cell cycle checkpoints in *S. cerevisiae*.

In *S. pombe* there is strong evidence that the G_2 DNA damage and replication checkpoints arrest the cell cycle by inhibiting the activity of Cdc2. The G_2 arrest mediated by the DNA replication checkpoint requires an intact inhibitory Tyr15 phosphorylation site on Cdc2 (134). Disrupting this site results in the loss of the DNA replication checkpoint. In accord with this result, mutations that disrupt normal Cdc25 control of Cdc2 activity (overexpression of *cdc25* for instance) also eliminate the dependence of mitosis on DNA synthesis (134).

chk1 / rad27 has proven to be a key gene in the pathway linking the activation of the DNA damage checkpoint to cell cycle arrest in *S. pombe* (135). A number of observations are consistent with the hypothesis that Chk1/Rad27 mediates its

checkpoint function by inhibiting Cdc2 activity in response to DNA damage (90, 119). Recent work suggests that Chk1 acting through Wee1 induces a G_2 arrest by maintaining Cdc2 in a tyrosine-inhibited form. Overexpression of Chk1 in undamaged cells produces a G_2 arrest with Cdc2 in a tyrosine-inhibited form. In addition, overexpression of Chk1 has no effect in cells lacking Wee1 kinase activity. Further support that Wee1 is a target of the Chk1 kinase comes from studies demonstrating that Chk1 phosphorylates Wee1 *in vitro* and that Wee1 is hyperphosphorylated in cells delayed in G_2 by exposure to UV irradiation or by overexpression of Chk1 (136). Furthermore, Chk1 activity may be responsible for Cdc25 phosphatase inhibition (137). Taken together, these studies strongly implicate Cdc2 tyrosine phosphorylation in the Chk1 mediated DNA-damage checkpoint.

4.8 Adaptation releases checkpoint-induced arrest

Irreparable DNA damage results in prolonged exposure to checkpoint-activating signals. Eventually, the cells habituate and are released from their checkpoint-induced arrest. This phenomenon, known as adaptation, is observed in many cell cycle checkpoints. Without it, cells suffering irreparable damage would remain arrested and die. Adaptation may have evolved because it allows cell cycle progression in spite of damage. This provides an opportunity, although slight, for the cells to weather the damage and survive. Although little is known about the molecular basis of adaptation, mutations that affect this process have been identified (73).

5. The role of checkpoints in monitoring spindle assembly

The mitotic spindle is an extremely complex and dynamic microtubule-based structure and its proper assembly is essential for the accurate segregation of sister chromatids. Sister centrosome separation marks the initiation of spindle assembly and determines the orientation of the bipolar spindle. The mature spindle consists of three sets of microtubules originating from each centrosome (138, 139). Polar microtubules extend from each centrosome and overlap in the middle of the spindle. These are responsible for spindle stability and separation of spindle poles during anaphase. Kinetochore microtubules extend from the centrosomes to the centromeres of each chromosome. They attach to a defined region of the centromere known as the kinetochore and play a key role in segregating sister chromatids to opposing spindle poles. Astral microtubules radiate from each centrosome into the surrounding cytoplasm and are involved in centrosome separation and spindle orientation within the cell.

Much of spindle assembly relies on the phenomenon that slowly growing and rapidly shrinking populations of microtubules co-exist simultaneously in the cell as described in detail in Chapter 4. In addition, individual microtubules frequently switch from growing to shrinking. This behavior is known as dynamic instability

(140). As a cell progresses into mitosis, the rate at which a microtubule switches from growing to shrinking increases (and the rate at which a microtubule switches from shrinking to growing decreases) (141). This creates an extremely dynamic microtubule population and facilitates the reorganization of the interphase microtubule array into a spindle. Spindle formation is achieved by the fact that growing microtubules are stabilized by associations with a kinetochore or other microtubules. Microtubule-based motor proteins also play a role in spindle formation and separation of the sister chromosomes during anaphase (142, 143).

Accurate segregation of sister chromosomes requires that each chromosome is properly attached to a microtubule. The kinetochore plays a key role in this process. Kinetochores are large protein complexes existing within the region of the chromosome known as the centromere (144). Centromeres and kinetochores are discussed in further detail in Chapter 5. The kinetochore provides a number of functions during metaphase, including microtubule capture, alignment and balancing of the chromosomes on the metaphase plate, and segregation and poleward movement of sister chromosomes during anaphase (145). Given these diverse tasks, it is not surprising that it is a complex organelle. Kinesin-like proteins, cytoplasmic dyneins, other microtubule-associated proteins, phosphatases, and kinases are concentrated at the kinetochore. The motor proteins appear to convert the energy of ATP hydrolysis and microtubule depolymerization into chromosome movement (146). The CENP-E motor protein is a fundamental component of the kinetochore. Injection of polyclonal antibodies against the CENP-E protein disrupts the depolymerization-driven movement of the chromosomes (147). These experiments suggest that CENP-E is the motor protein involved in coupling chromosome movement to microtubule depolymerization.

In most cells, exposure to drugs that disrupt microtubule polymerization prevents exit from metaphase (148, 149). Tubulin mutations that compromise spindle structure also produce a similar mitotic arrest. These studies demonstrate that exit from mitosis depends on a properly assembled spindle and may be the result of a cell cycle checkpoint.

5.1 Genetic identification of the spindle assembly checkpoint

Using a rationale similar to that used for the identification of DNA damage checkpoints, screens for mutants sensitive to microtubule depolymerization drugs were employed to identify genes included in the spindle assembly checkpoint. Under normal conditions a dividing cell does not require a spindle assembly checkpoint because the time required to inactivate mitotic CDK is longer than the time required to assemble a spindle. However if spindle assembly is slowed by exposing the cell to low doses of a microtubule-depolymerizing drug, a checkpoint is required to prevent exit from mitosis before the spindle is properly assembled. Through this approach, two independent screens isolated six non-essential spindle assembly checkpoint mutants: *mad1*, *mad2*, *mad3*, *bub1*, *bub2*, and *bub3* (88, 89). The drug-induced lethality is a consequence of cells progressing into anaphase in the

absence of a completely formed spindle. This produces an increased frequency of nondisjunction, chromosome loss, and inviable aneuploid daughter cells. Even in the absence of microtubule inhibitors, the cells bearing these mutations exhibit an increased rate of chromosome loss. This is expected as normally dividing cells occasionally require extra time for spindle assembly and establishment of the appropriate kinetochore–microtubule associations.

As with other cell cycle mutants, *mad* and *bub* mutants are primarily defective in their ability to delay progression through G_2–M in response to slowed spindle assembly. Therefore, providing an alternative means of slowing progression through G_2–M should eliminate the sensitivity of these mutations to microtubule inhibitors. In *S. cerevisiae*, this is achieved by the addition of hydroxyurea which inhibits DNA replication and activates an S phase checkpoint. As predicted, the sensitivity of the *mad* mutants to microtubule depolymerizing drugs is relieved by exposing the cells to low doses of hydroxyurea (92).

Another spindle assembly checkpoint mutant, *mps1*, was originally recovered as a member of a class of mutants that disrupts spindle pole formation (150). Mps1 also functions as a spindle assembly checkpoint; it fails to cause arrest in metaphase in the presence of the microtubule inhibitor nocodazole.

The identification of mutants that disrupt the spindle assembly checkpoint provides a means of rapidly determining which aspects of spindle assembly are being monitored (73). A mutation or condition that disrupts spindle assembly and results in a checkpoint-dependent metaphase arrest indicates that the process disrupted is monitored by the checkpoint. This approach shows that the checkpoint monitors a variety of aspects of spindle formation including chromosome number, centromeric DNA, centrosome duplication, microtubule polymerization, kinetochores, and microtubule motors (88, 89, 149–155). As found for the DNA damage checkpoint, different aspects of spindle assembly may be monitored. For example, the state of the centrosome and the state of the microtubules may be monitored independently by distinct spindle assembly checkpoints.

5.2 Spindle checkpoints monitor the state of the kinetochore

The conclusion that proper spindle–kinetochore interactions are required for cells to progress into anaphase is supported by live observations in mammalian tissue culture cells. Although there is considerable cell-to-cell variability in the time required for all the kinetochores to become properly attached to spindles, the interval from spindle attachment of the last free kinetochore to the initiation of anaphase is relatively constant (156). This fits with a model in which the spindle assembly checkpoint is activated by negative signals produced by free kinetochores. This idea is supported by analysis of mutations and reagents that compromise kinetochore function (157, 158). Mutations in the *S. cerevisiae* Ctf kinetochore protein activate the spindle assembly checkpoint and cause a delay in metaphase (159). Injection of anti-centromeric antibodies derived from human autoimmune sera disrupt kinetochore assembly and delay progression through mitosis (160, 161). Similar studies were

performed using antibodies directed against CENP-C, a component of the inner kinetochore (162). The injected antibodies severely disrupt and reduce kinetochore size. These cells arrest in metaphase, indicating the presence of a checkpoint monitoring kinetochore integrity.

The kinetochore can be completely eliminated through laser ablation. Surprisingly, this procedure does not elicit an anaphase delay. This clearly demonstrates that the metaphase arrest is a consequence of negative rather than positive signals produced by the kinetochore. A complete removal of the kinetochore renders it undetectable by the spindle assembly checkpoint (163).

5.3 Tension is monitored by the spindle assembly checkpoint

Micromanipulation studies in spermatocytes of praying mantids indicate that the spindle assembly checkpoint monitors tension on the kinetochores (Fig. 6) (164). The spermatocytes of praying mantids manage an XXY sex chromosome constitution by forming a trivalent in which the two X chromosomes pair and segregate from the single Y chromosome. Occasionally the trivalent breaks down because an X chromosome prematurely detaches from the Y, generating an unpaired X. These meiocytes never progress into anaphase. If the free X is placed under tension through micromanipulation, the meiocyte progresses into anaphase. These observations support a model in which a spindle assembly checkpoint monitors the tension at each kinetochore. A single kinotechore not under tension is sufficient to activate the checkpoint and prevent exit from metaphase.

5.4 Molecular changes at the kinetochore in response to tension

These studies indicate that tension alters kinetochore composition and/or conformation and that the spindle assembly checkpoint monitors these alterations. Immunofluorescence analysis using the 3F3 antibody, which recognizes a subset of kinetochore proteins only when they are phosphorylated, has provided insight into the molecular nature of this phenomenon (165). These kinetochore proteins are dephosphorylated on chromosomes that have aligned and properly attached to the spindle. The kinetochore proteins in chromosomes that have not attached to the spindle remain phosphorylated. Microinjection of the 3F3 antibody inhibits kinetochore dephosphorylation and delays entry into anaphase (166). Therefore phosphorylation and dephosphorylation of these kinetochore proteins is correlated with the presence and absence of the signal that activates the spindle assembly checkpoint.

Phosphorylation of kinetochore proteins is directly correlated with tension (167). Experiments in grasshopper spermatocytes demonstrate that detachment of a chromosome from the spindle through micromanipulation results in phosphorylation of kinetochore proteins. Conversely, applying tension to an unattached chromosome through micromanipulation results in dephosphorylation of the kinetochore

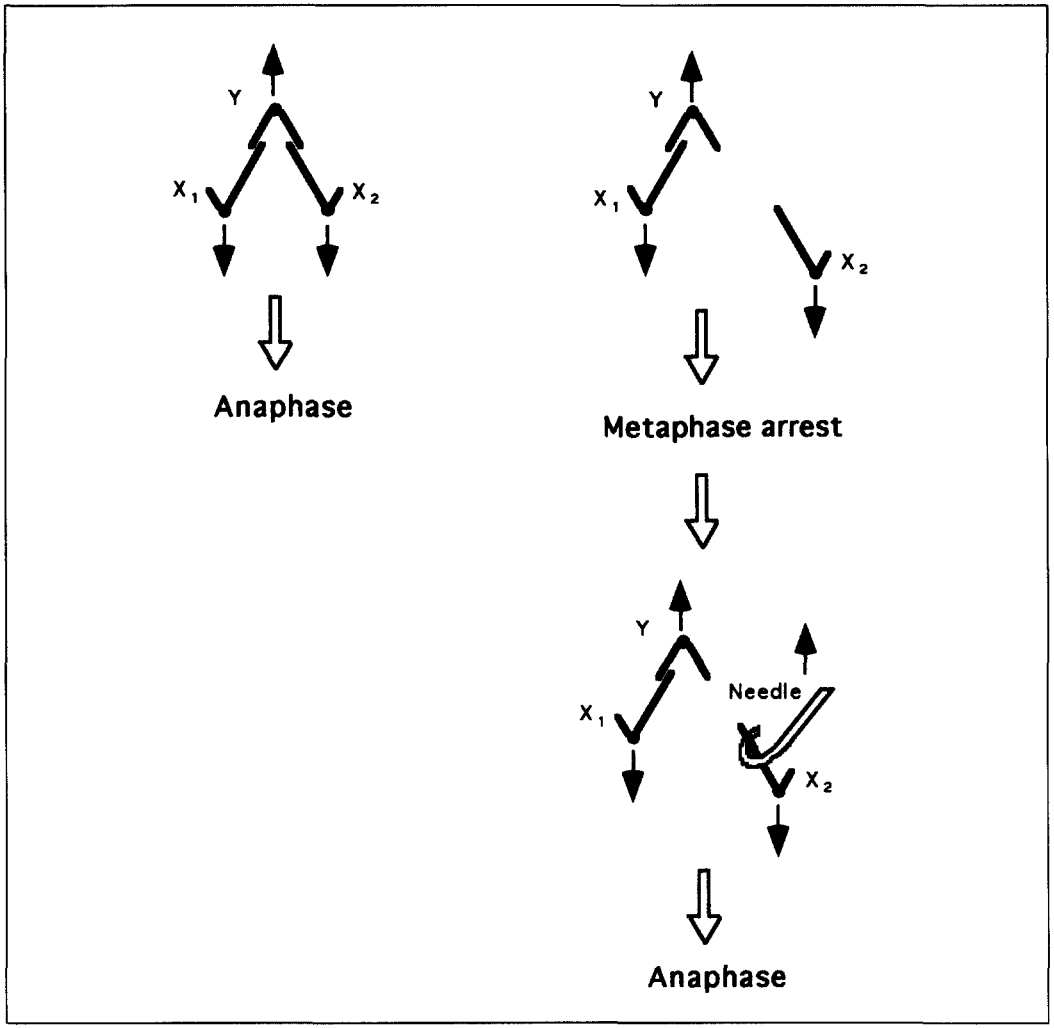


Fig. 6 The spindle assembly checkpoint monitors tension in the spermatocytes of praying mantids. Normally each arm of the Y chromosome pairs with an X chromosome, forming a trivalent in which each kinetochore is under tension. When this occurs, the cells proceed directly into anaphase. Occasionally X–Y pairing is prematurely disrupted, resulting in a free X chromosome no longer under tension. These cells remain arrested in metaphase. If the kinetochore of the free X chromosome is placed under tension through micromanipulation, the cell is release from the metaphase arrest and proceeds into anaphase.

proteins. Tension-sensitive proteins may exist within the kinetochore that regulate the phosphorylation state of these kinetochore proteins (167). The tension-mediated dephosphorylation of these proteins is necessary for release from the spindle assembly checkpoint.

Male grasshoppers contain only a single X chromosome and thus during meiosis the chromosome is unpaired and not under tension. As anaphase is not delayed in

these meiocytes, the unpaired chromosome must not be eliciting the spindle assembly checkpoint. Significantly, the X chromosome kinetochore does not react with the 3F3 antibody, indicating that its kinetochore proteins remain dephosphorylated whether or not they are under tension (167). Consequently, the unpaired X chromosome fails to activate the spindle assembly checkpoint. The observation that these exceptional unpaired X chromosomes also exhibit an exceptional 3F3 kinetochore staining further supports the notion that the checkpoint is monitoring the state of phosphorylation of kinetochore proteins.

Human and *Xenopus* homologs of the MAD2 protein exhibit a localization pattern similar to that of the 3F3 epitope. They localize to the kinetochores as the chromosomes are condensing, but localization is no longer observed once the chromosomes are attached to the spindle (168, 169). Depletion of MAD2 from *Xenopus* embryo extracts inactivates the spindle assembly checkpoint (170). Since *Xenopus* MAD2 is not phosphorylated, the relationship between *Xenopus* MAD2 and the 3F3 phosphoepitope is unclear.

In the *Drosophila* oocyte, tension is employed in a reciprocal manner to that of the grasshopper spermatocyte and is required to prevent premature entry into meiosis (Fig. 7). Analyzing the behavior of specialized compound chromosomes in the *Drosophila* oocyte demonstrates that the tension generated from physical exchange of homologs is required for the programmed metaphase arrest of meiosis I (171). It has been suggested that this distinct response to tension may be a consequence of the fact that progression through female meiosis, but not male meiosis, includes a programmed metaphase arrest (171).

5.5 In some cells, free kinetochores rather than tension activate the spindle assembly checkpoint

Tension is not the primary inducer of the spindle assembly checkpoint in all cells. Ptk cells in which all but one of the kineochores are attached to the metaphase spindle maintain an active spindle assembly checkpoint. Laser ablation of this single unattached kinetochore inactivates the checkpoint and the cells proceed into anaphase even though the sister of the unattached kinetochore is no longer under tension (163). These studies indicate that it is the presence of an unattached kinetochore, rather than the lack of tension, that activates the spindle assembly checkpoint.

5.6 The *MAD* and *BUB* genes are involved in different steps of the spindle assembly checkpoint

To prevent entry into anaphase, a spindle checkpoint requires sensors to detect an improperly formed spindle, a signal transduction pathway, and finally a target that impinges upon the enzymes driving the cell cycle. The *mad* and *bub* spindle checkpoint mutants identify components in these processes and epistasis analysis indicates that they function in a common pathway (155). The finding that Mad1

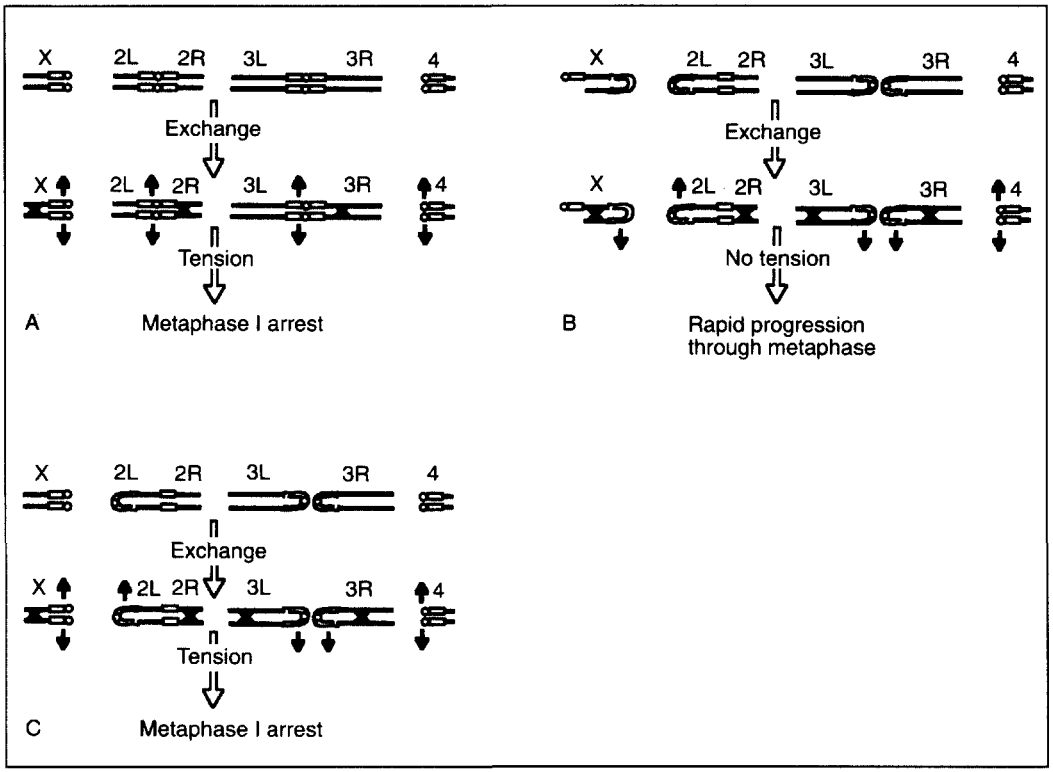


Fig. 7 In the *Drosophila* oocyte, the tension produced by recombination between homologues is required for the programmed meiosis I metaphase arrest (A). Oocytes bearing a set of compound chromosomes in which homologous recombination does not produce kinetochore tension fail to arrest in metaphase (B). Replacing the compound X chromosome with a pair of normal X chromosomes restores the metaphase arrest because recombination now produces tension at the kinetochores (C). The fourth chromosome does not undergo homologous recombination.

is hyperphosphorylated when the spindle assembly checkpoint is induced provides a means of partially ordering the action of spindle checkpoint genes (3). *MSP1*, *MAD2*, *BUB1*, and *BUB3*, but not *MAD3* and *BUB2*, are required for hyperphosphorylation of Mad1 (155). These studies indicate that *MAD2*, *BUB1*, and *BUB3* act upstream of *MAD1* while *BUB2* and *MAD3* act downstream or in parallel to *MAD1*.

MPS1 encodes a protein kinase that may be responsible for the *in vivo* phosphorylation of Mad1. *mps1* mutations disrupt spindle pole body duplication during G_1 and also disrupt the spindle assembly checkpoint (150, 172). Overexpression of *Mps1* results in hyperphosphorylation of Mad1 and a mitotic arrest that is dependent on an intact spindle assembly checkpoint. This indicates that *MPS1* functions in the same MAD and *BUB* spindle-dependent pathway and acts upstream of *MAD1* (73).

5.7 MAP kinase is required for the spindle assembly checkpoint in cell cycle extracts

Mitogen-activated protein (MAP) kinases are a family of proteins that are activated by a wide range of cellular signals and relay them via phosphorylation to other proteins. Recently, a member of this family of signaling proteins has proven to be essential for the spindle assembly checkpoint in *Xenopus* egg extracts (170, 173, 174). Minshull *et al.* found that a *Xenopus* MAP kinase, p44ERK2, is transiently activated in cycling egg extracts. When treated with MAP kinase phosphatase, these extracts in which the spindle assembly checkpoint has been activated, failed to arrest in the presence of the microtubule inhibitor nocadazole (170). Furthermore, extracts that are immunodepleted of MAP kinase fail to arrest in response to microtubule depolymerization, but introduction of recombinant MAP kinase to these extracts restores the spindle assembly checkpoint (174). Immunofluorescence studies localize the MAP kinase to the mitotic spindle during prometaphase and metaphase, suggesting that it is involved in monitoring the state of the mitotic spindle and/or associated proteins (173). Other proposed roles for MAP kinase in the spindle assembly checkpoint include regulation of cyclin destruction machinery, or conversion of cyclins to non-destructable forms (173).

5.8 The APC may be the target of the spindle assembly checkpoint

As described above, a great deal is known about the protein complexes that normally drive cells into anaphase and it is likely that the spindle assembly checkpoint influences their activity to prevent entry into anaphase. Passage through anaphase and entry into G_1 is driven by the activation of a previously described large protein complex known as the APC. APC mediates sister chromatid separation and inactivation of CDC2. Through ubiquitin-mediated proteolysis, activated APC drives sister chromatid separation and cyclin B degradation. Activation of the spindle assembly checkpoint prevents cyclin degradation and sister chromatid separation. Therefore, the spindle assembly checkpoint may arrest the cell in metaphase by inhibiting APC activity. This model is in accord with the observation that mutations disrupting APC activity are epistatic to spindle assembly checkpoint mutations (74).

6. The role of checkpoints in the initial embryonic cell cycles

The eggs of many higher eukaryotes are endowed with large volumes of maternally supplied cytoplasm. Consequently early embryogenesis is characterized by cleavage divisions which divide the cytoplasm into successively smaller units. This allows for rapid, synchronous divisions alternating between M and S phases without

measurable G_1 and G_2 phases. For example, *Xenopus* embryos undergo 12 synchronous cleavage divisions each approximately 30 min in length. The cell cycles lengthen, exhibit gap phases, and become asynchronous after the twelfth division. This dramatic alteration in the regulation of the cell cycle, known as the mid-blastula transition, is activated by an increase in the ratio of nuclei to volume of cytoplasm (Table 2) (175, 176).

In *Drosophila* and other insects not only do these initial divisions occur without cell growth, they are syncytial, that is they occur without cytokinesis (177). The early *Drosophila* embryo undergoes 13 rapid synchronous nuclear divisions with extremely reduced gap phases and cellularizes during interphase of nuclear cycle 14. These divisions occur in waves that initiate near the poles of the embryo. Nuclear cycles 1–10 occur in as little as 8 min, then during nuclear cycles 11–14, the cycles gradually lengthen to 25 min.

The extent to which cell cycle checkpoints maintain the fidelity of these early divisions in *Xenopus* and *Drosophila* is unclear (8, 178). Many checkpoints operate during gap phases which have been effectively eliminated in early embyrogenesis. In addition, checkpoint-induced delays would disrupt the synchrony of these division cycles. This suggests either that the operation of checkpoints is modified or that other

Table 2 Estimates of the ratio of DNA (pg) to cytoplasm (μl) in *Xenopus* embryos. In *Xenopus* embryos, checkpoints are activated around the 700–800 cell stage (cycles 10 and 11) when the DNA:cytoplasm ratio is above 2000.

Cycle	# Nuclei	DNA content (pg)	DNA/total cytoplasm (pg/μl)[1]
1	1	6.4	3.6
2	2	12.8	7.1
3	4	25.6	14.2
4	8	51.2	28.4
5	16	102.4	56.9
6	32	204.8	113.8
7	64	409.6	227.6
8	128	819.2	455.1
9	256	1638.4	910.2
10	512	3276.8	1820.4
11	1024	6553.6	3640.9
12	2048	13107.2	7281.8

[1]Assuming the egg is a sphere of 1.5 mm in diameter, the volume is 1.8 μl and the DNA content is 6.4 pg/cell.

mechanisms are employed to maintain fidelity during these early embryonic divisions.

6.1 Relative timing of mitotic events may be the primary mechanism maintaining fidelity of division in early *Xenopus* embryos

In *Xenopus*, the mitotic cycles do not slow down until the mid-blastula transition during nuclear cycle 12. In addition, during these initial cycles entry into mitosis does not depend on completely replicated DNA and entry into anaphase does depend on a properly assembled spindle (179–181). X-irradiation does not affect the rate of progression through these initial cell cycles. This indicates that cell cycle checkpoints are not active at this time and fidelity must be maintained through alternative mechanisms. It is likely that the relative timing of basic cell cycle events is the primary mechanism by which fidelity is maintained during the initial *Xenopus* divisions (93). During these early cycles, the time required to drive the cells into mitosis is longer than the time required to complete S phase. Therefore, under normal conditions, embryos enter mitosis with fully replicated DNA. Relying on relative timing, however, leaves embryos vulnerable to X-irradiation and other treatments that extend the time required to properly complete events of the cell cycle.

As the replication machinery is titered out with increasing nuclear concentration during the later cycles in *Xenopus* embryos, S phase slows down. Relative timing is no longer an effective mechanism of ensuring that S phase is completed before the cell enters mitosis. Consequently, checkpoints are required during these later cycles.

6.2 Checkpoint control mechanisms are present but not activated in early *Xenopus* embryos

The lack of dependency relationships in the early *Xenopus* embryo could result from checkpoints not being present, or being present but not being activated. Studies using *Xenopus* embryonic cytoplasmic extracts support the latter model (182). Extracts derived from *Xenopus* eggs cycle between S and M phases. In these extracts, the length of S phase, but not M phase, increases with increasing nuclear density. In addition, the dependency of mitosis on properly replicated DNA occurs only when sperm nuclei are added to the extract. These results suggest the presence of a DNA replication checkpoint that requires a critical concentration of unreplicated DNA for activation. Entry into mitosis is prevented by inactivation of the Cdc2–cyclin complex through post-translational modification, presumably phosphorylation (182). As found in somatic cells, caffeine relieves this dependency relationship.

These results are in accordance with *in vivo* studies in which *Xenopus* embryos are injected with the DNA synthesis inhibitor aphidicolin, which delays progression of the cell cycle only after the embryo contains more than 700–800 cells (after nuclear

cycle 10) suggesting that threshold concentrations of nuclei are required for checkpoint activation (Fig. 8) (182). Analogous studies demonstrate that a spindle assembly checkpoint is also present in early *Xenopus* embryos but requires a threshold concentration of nuclei for activation (170).

6.3 The syncytial *Drosophila* nuclear cycles exhibit a number of dependency relationships

Early *Drosophila* development begins with a series of synchronous syncytial nuclear divisions (177). These divisions are rapid and alternate between M and S phases with extremely abbreviated G_1 and G_2 phases. Nuclear cycles 1–8 occur in the interior of the embryo. During nuclear cycles 9 and 10, the majority of the nuclei migrate to the cortex. Once at the cortex, they undergo four more rounds of synchronous divisions before cellularizing during interphase of nuclear cycle 14 (Table 3).

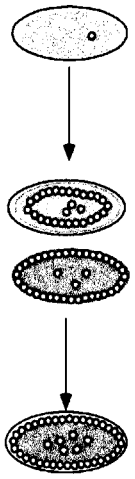
During division cycles 2–7, cell cycle oscillations do not occur in cyclin levels, Cdc2 phosphorylation, or Cdc2 activity (18). It has been proposed that during these early cycles only localized pools of cyclin closely associated with each spindle or nucleus are degraded. Cycles 8–13 exhibit more conventional oscillations in cyclin abundance and Cdc2 activity. During nuclear cycle 14, entry into mitosis is controlled by zygotic transcription of *string*, a homolog of the *S. pombe* gene *cdc25* (20).

These cortical syncytial divisions maintain a surprising number of dependency relationships. Treating syncytial embryos with drugs that disrupt spindle formation produces a metaphase arrest (183). Altered chromosome structure extends metaphase and delays entry into anaphase (178). Exposing embryos to aphidicolin, a DNA synthesis inhibitor, slows the nuclear cycles (184). Centrosome duplication also depends on DNA synthesis (185). X-irradiation delays progression through metaphase (186).

It is possible that the regulation of the *Drosophila* syncytial divisions is similar to that of the early *Xenopus* embryo: checkpoints are present but require a critical concentration of nuclei to become activated. The dependency relationships described above occur during the cortical syncytial divisions when nuclei exist in a monolayer just beneath the plasma membrane. This uniform distribution effectively increases the nucleus:cytoplasm ratio at the cortex and may be sufficient to activate checkpoints enforcing dependency relationships.

However, unpublished observations indicate that even before nuclear migration in the *Drosophila* embryo, disruption of the spindle arrests the nuclei in mitosis (J. Corbin and W. Sullivan, unpublished data). This demonstrates that the *Drosophila* spindle assembly checkpoint operates even at low nucleus:cytoplasm ratios. Similar nucleus:cytoplasm ratios would not activate the spindle assembly checkpoint in *Xenopus* embryos. The ratio of each nucleus and its sphere of tightly associated cytoplasm may be the critical ratio controlling progression through the division cycle in the *Drosophila* embryo. The observation that individual nuclei delay initiation of anaphase during the syncytial *Drosophila* divisions supports the view that regulation of the division cycle occurs at the level of a single nucleus (178). In addition,

Table 3 Estimates of the ratio of DNA (pg) to cytoplasm (μl) in *Drosophila* embryos. After nuclear migration (nuclear cycle 10) in the *Drosophila* embryo, the nuclei reside in a monolayer directly beneath the plasma membrane. This disruption effectively increases the DNA:cytoplasm ratio. Consequently, we calculated the nucleus:cortical cytoplasm ratio (the ratio of nuclei to the ~ 5 μm layer of yolk-free cytoplasm that exists at the periphery of the embryo). Approximately 30 nuclei fail to reach the cortex at nuclear cycle 10. In *Drosophila*, a value of 2000 for the DNA:cytoplasm ratio is not reached unless one considers the DNA:cortical cytoplasm ratio.



Cycle	# Nuclei	DNA content (pg)	DNA/total cytoplasm (pg/μl)[1]	DNA/cortical cytoplasm (pg/μl)[2]
1	1	0.32	0.35×10^2	
2	2	0.64	0.70	
3	4	1.28	1.39	
4	8	2.56	2.78	
5	16	5.12	5.57	
6	32	10.24	11.13	
7	64	20.48	22.26	
8	128	40.96	44.52	
9	256	81.92	89.04	
10	480	153.60	167.00	731.43×10^3
11	960	307.20	333.91	1462.86
12	1920	614.40	667.83	2925.71
13	3840	1228.80	1335.65	5851.43

[1]Assuming the egg is an ellipsoid 450 × 200 μm, the egg volume is 0.92×10^{-2} μl and the diploid DNA content is 0.32 pg.
[2]Assuming the cortical cytoplasm extends 5 μm in from the surface, the cortical volume is 0.021×10^{-2} μl.

chromosome loss during the early divisions results in individual nuclei losing division synchrony with neighboring nuclei (187).

6.4 A DNA replication/DNA damage checkpoint may operate during the late syncytial divisions of *Drosophila*

Interphase increases in length during the late syncytial divisions of *Drosophila* (188). This is thought to be a direct consequence of the increase in time required to complete DNA replication, as maternally supplied replication factors are progressively depleted by an exponentially increasing population of nuclei. As S phase lengthens with each division cycle, feedback controls may be required to prevent entry into mitosis until DNA replication is completed (Fig. 8).

A number of experiments suggest that the *Drosophila* maternally supplied gene product Grp is required for this feedback control. In normal *Drosophila* embryos,

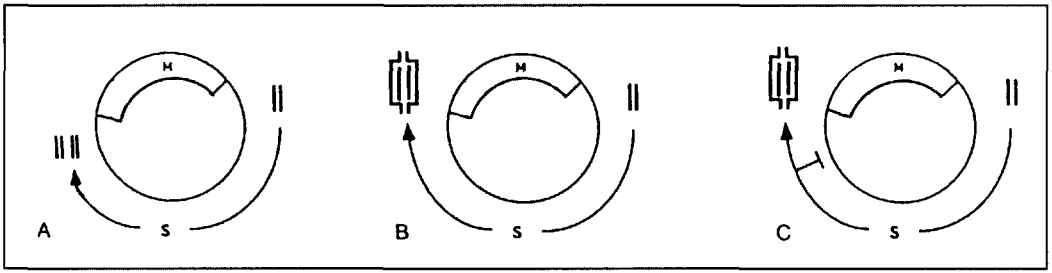


Fig. 8 (A) In the initial embryonic cycles, relative timing ensures that DNA replication is completed before the cell enters mitosis. (B) Later, DNA replication slows down and relative timing no longer solves the completion problem. (C) Eventually, DNA replication checkpoints may be activated to delay entry into mitosis until DNA replication is complete. The *Drosophila grp* gene, a homologue of *S. pombe chk*1, may be required for this checkpoint function during the syncytial cortical divisions.

interphase becomes progressively longer during the late syncytial cycles. This lengthening of interphase does not occur in *grp*-derived embryos (189). Embryos derived from *grp* mutant mothers are specifically disrupted during the late syncytial cycles when there is a dramatic increase in the length of interphase and presumably S phase (190). X-irradiation of normal syncytial embryos results in sister telophase nuclei snapping back and fusing with one another. This is probably a consequence of dicentric bridges formed from damaged chromosomes inappropriately progressing through mitosis. Unirradiated *grp*-derived embryos exhibit a similar snap-back and fusion of sister telophase nuclei during nuclear cycle 12. These fusions are probably a consequence of nuclei in *grp*-derived embryos progressing into mitosis with incompletely replicated DNA. In accordance with this model, *grp*-derived embryos exhibit a five-fold increase in DNA damage relative to normal embryos (186).

Molecular and biochemical analyses also support a model in which *Grp* functions in a developmentally regulated interphase checkpoint during the late syncytial divisions. *Grp* encodes a serine/threonine kinase with extensive homology to the *chk1/rad27* gene of *S. pombe* (186). *chk1/rad27* is a G_2 DNA-damage checkpoint gene, as previously mentioned. A number of studies indicate that *chk1* inhibits Cdc2 activity in response to DNA damage (135–137). Overexpression of Chk1 in undamaged cells produces a G_2 arrest with Cdc2 in the tyrosine-inhibited form. There is evidence that Grp may induce an interphase delay during the late syncytial divisions by modulating the levels of tyrosine phosphorylation on Cdc2. Wild-type embryos accumulate high levels of an inhibited phosphorylated isoform of Cdc2 during interphase of nuclear cycle 14. This does not occur in *grp*-derived embryos (186). Whether this effect is direct or the consequence of a failure to progress through normal developmental transitions that occur at this time remains to be determined. As in *Xenopus* embryos, the Grp checkpoint is probably present and functional throughout the early divisions but it may require a threshold concentration of unreplicated DNA for activation.

6.5 In the syncytial *Drosophila* embryo, checkpoints link delays in the cell cycle to nuclear elimination

The above studies indicate that at least some cell cycle checkpoints function during the rapid synchronous divisions of early embryogenesis in both *Xenopus* and *Drosophila*. This result is paradoxical because the delays induced by cell cycle checkpoints would disrupt the synchrony of these early divisions and thus disrupt early development. This issue was addressed by examining the effects of an abnormally long and rearranged *Drosophila* chromosome on the embryonic syncytial divisions (178). This chromosome dramatically increases the error rate of the syncytial embryonic divisions but has little effect on the error rate of the later larval neuroblast divisions. Live analysis demonstrates that in embryos bearing this long chromosome individual nuclei in the syncytial population of dividing nuclei often delay initiation of anaphase. The telophase products of nuclei delayed in entering anaphase recede into the yolk and are effectively removed from the dividing population of syncytial nuclei. Many studies demonstrate that the products of a broad range of division errors are eliminated in a similar fashion (60, 191, 192). In many cases, removal is preceded by a delay in anaphase. These delays suggest the presence of a checkpoint operating during the cortical syncytial divisions. However, unlike many somatic cell cycle checkpoints which link delays to repair processes, these checkpoints link delays to elimination processes. This results in the increase of the fidelity of the population of dividing nuclei by culling abnormal nuclei. This process is analogous to the checkpoint-induced apoptic response observed in mammalian systems (172).

7. Future directions

Checkpoints are central to maintaining the fidelity of the eukaryotic cell cycle. The identification and characterization of a number of checkpoint components have established the outlines of the signal transduction pathways by which they operate. However, much remains unknown about many aspects of these pathways. In no case has the signal responsible for activating a checkpoint been defined at the molecular level. For example, although it is clear that the spindle assembly checkpoint monitors kinetochore tension, the kinetochore proteins involved in the checkpoint response are only just beginning to be identified. Identifying and understanding at the molecular level how these proteins respond to tension is certain to be a focus of future investigation. In addition, many steps in the checkpoint signal transduction pathways remain to be defined and the targets producing cell cycle arrest remain elusive.

In response to irreparable damage, a process known as adaptation occurs; the checkpoint-induced arrest is overridden and the cell inappropriately progresses through the cycle. This process is largely unexplored. Little is known about the genetic basis of adaptation and the factors that control the timing of the adaptive

response. In addition, the molecular basis of release from the checkpoint-induced cell cycle arrest remains to be determined.

Mutational analysis of checkpoints demonstrates that cell cycle checkpoints are essential for maintaining division fidelity in higher eukaryotes. Disruption of mammalian cell cycle checkpoints often leads to tumorigenesis. Checkpoint genes, such as p53, maintain fidelity of large populations of cells by either inducing an arrest and repair response or an apoptotic elimination response. Little is known about the environmental and genetic factors influencing which alternative is chosen. In addition, the relationship between these two responses has not been determined. For example, it is not known whether the cell cycle arrest and apoptotic pathways are independent processes or whether they represent branchpoints of a common pathway.

Given the role of cell cycle checkpoints in maintaining division fidelity in mammals, it will be important to identify those checkpoint genes that are functionally as well as structurally conserved as these are likely to be involved in core aspects of the checkpoint process. Analysis of these conserved checkpoint components in model genetic organisms will provide the most direct insight into their role in mammals.

Higher eukaryotic development is quite resistant to both externally and internally generated damage. It may be that developmental mechanisms formally equivalent to cell cycle checkpoints are responsible for this resilience. In response to an incomplete or improperly executed developmental event, the organism may arrest development and properly complete the event before proceeding to the next developmental stage. These developmental checkpoints may also exhibit a number of other properties similar to cell cycle checkpoints. For example, they may only be required under conditions of unusual stress. Progress in this area will require the identification and characterization of key components of this process. It is likely that the general strategies developed for the genetic analysis of yeast cell cycle checkpoints will serve as an excellent guide in this new area of research.

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2 Mitotic changes in the nuclear envelope

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1. Overview

In higher eukaryotes, the disassembly of the nuclear envelope is one of the most dramatic events to occur at mitosis. The nuclear envelope consists first and foremost of a double membrane system surrounding the chromosomes. Lying under the inner nuclear membrane is a filamentous network composed of the nuclear lamins and associated proteins. At multiple places in the envelope, the inner and outer nuclear membranes fuse at the nuclear pore complexes. Nuclear pores are large, multi-protein structures responsible for the signal-mediated transport of molecules into and out of the nucleus (Fig. 1). The lamina, pores, and the membrane system itself are all disassembled at the beginning of mitosis and reassembled during telophase. This review will focus on the changes that occur within these structures at mitosis.

2. The nuclear lamina and membrane

2.1 Changes to the nuclear lamina at mitosis

The nuclear lamina consists of a highly branched filamentous network of intermediate filament-related proteins, the nuclear lamins (1–8). This network is closely associated with the inner nuclear envelope and extends some distance into the nuclear interior. On the basis of its localization, the lamina is postulated to play a role in the maintenance of nuclear organization.

The lamins of higher eukaryotes can be segregated into two types, A and B, on the basis of their sequence and biochemical properties (9, 10). The B-type lamins are expressed ubiquitously throughout development, and are stably modified by the addition of a C-terminal prenyl group (11, 12). This hydrophobic modification most likely plays a role in the stable association of a proportion of B-type lamins with membrane vesicles after nuclear envelope disassembly. The A-type lamins, in contrast, are restricted in their expression to differentiated tissues (13). Although A-type lamins are also initially modified by the addition of a prenyl group, removal of the C-terminal 18 amino acids of the protein by a nuclear protease clips off this modification (12, 14).

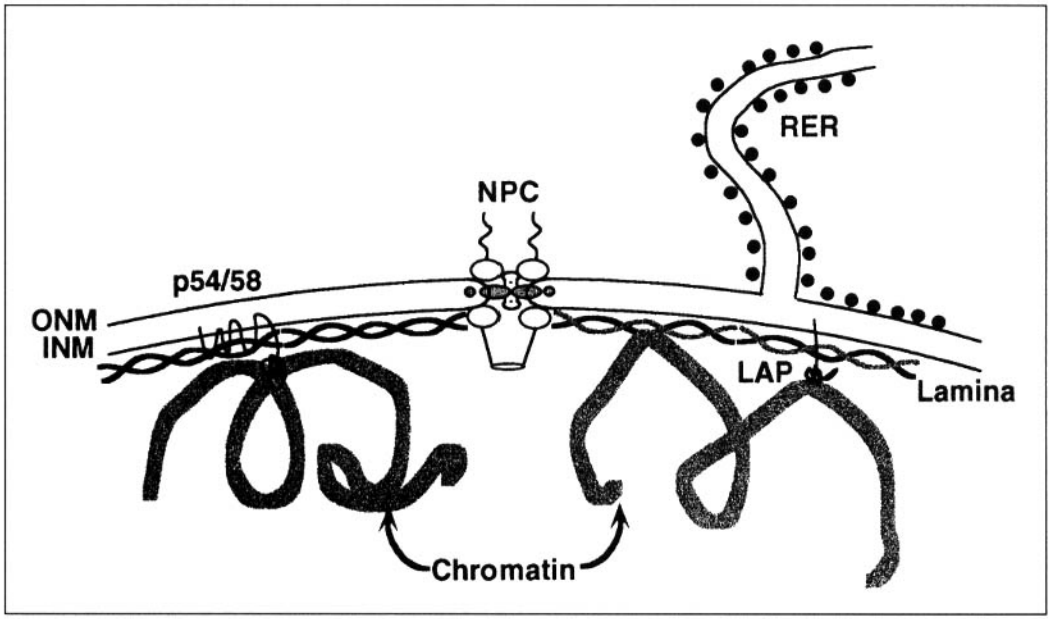


Fig. 1 Diagram of a section though the nuclear envelope. The outer nuclear membrane (ONM) is contiguous with the rough ER (RER). Nuclear pore complexes (NPC) are present at specialized junctions between the outer and inner nuclear membranes. Just beneath the inner nuclear membrane lies the lamina, an intermediate filament-like network assembled from lamin A and B heterodimer. Two integral membrane proteins of the inner nuclear membrane are shown: p54/p58 and a lamin-associated protein, LAP. These two polypeptides make contacts with both the lamina and chromatin.

In addition to a structural role, the lamins also appear to be involved in the maintenance of chromatin structure and DNA replication (15–20). Interestingly, mutation of the gene for lamin D_0 (the B-type lamin in this organism) in *Drosophila* resulted in a severe phenotype affecting survival, locomotion, and fertility (21). The observed cellular phenotype included incomplete nuclear envelopes, nuclear pore clustering, and the appearance of cytoplasmic annulate lamellae, indicating that lamin D_0 is essential for normal nuclear envelope assembly in *Drosophila* (21).

At an early stage in mitosis, the lamina disappears before nuclear membrane breakdown (1, 2, 22). During prophase, both A- and B-type lamins become phosphorylated, and this phosphorylation coincides exactly with the depolymerization of the nuclear lamina (23, 24). Each type of lamin contains multiple copies of the consensus sequence for phosphorylation by the mitotic kinase, cdc2/cyclin B. It is thought that the level of polymerization of the lamins is directly regulated by phosphorylation. Indeed, purified lamins that have been assembled into paracrystals and short filaments can be solubilized by the addition of either mitotic cytosol or purified cdc2/cyclin B kinase (23). In addition, mitotic phosphorylation of the lamins can be blocked by mutation of the serine residues at the cdc2/cyclin B consensus sites

(25). When transfected into fibroblasts, incorporation of the mutant lamin polypeptides into the nuclear lamina blocks normal mitotic disassembly (25). It is therefore believed that the mitotic disassembly of the lamina is directly regulated by the presence of active cdc2/cyclin B kinase.

2.2 Lamin-associated proteins

A number of proteins of the nuclear envelope have been identified as either lamin-binding proteins or lamina-associated proteins. One of the first to be identified was a molecule in avian and human cells called p54/p58 (it has also been termed lamin B receptor, or LBR). Surprisingly, sequence analysis revealed that p54/p58 was highly homologous to a number of sterol biosynthetic enzymes (26–28). Whether or not p54/p58 is specifically a receptor for lamin B remains controversial; however, p54/p58 is localized to the inner nuclear envelope and has been shown to be able to bind both to chromatin and to chromatin-associated proteins (Fig. 1; 29). This suggests that the p54/p58 protein may play a number of different roles within the cell. The p54/p58 protein is phosphorylated at mitosis by a tightly associated kinase, and phosphorylation modifies the ability of p54/p58 to associate with both DNA and lamin B *in vitro* (30, 31).

In addition to p54/p58, a number of integral membrane lamin-associated proteins (LAPs) have been identified by monoclonal antibodies raised against nuclear lamina preparations (32–34). Three of these proteins, LAP1A to LAP1C, are generated by alternative splicing of a single gene (35). The different isoforms differ in their affinity for lamins and chromatin, and are expressed in a tissue-specific manner. A fourth protein, LAP2, is also an integral membrane protein capable of binding chromatin and lamin B (33, 34). The binding of LAP2 to these ligands is abolished when LAP2 is phosphorylated by mitotic cytosol (33). This suggests that the role of LAP2 may be to coordinate binding of nuclear membrane vesicles to the lamina and the chromatin in a cell-cycle dependent manner.

2.3 Regulation of nuclear membrane dynamics by phosphorylation

Cell-free nuclear assembly assays have provided a number of insights into the process of envelope assembly. A simplified form of a *Xenopus* nuclear reconstitution assay revealed that binding of nuclear envelope precursor vesicles to chromatin can occur in the complete absence of cytosol (36, 37). When vesicle-bound chromatin is then incubated in interphase egg cytosol in the presence of the phosphatase inhibitor okadaic acid, the bound vesicles are released; they are not released if a kinase inhibitor is included. This indicated that the binding of nuclear envelope vesicles is mediated by a kinase/phosphatase system. Vesicle release could also be achieved by the direct addition of mitotic cytosol (converted to a mitotic state by the addition of cdc2/cyclin B) (36). Fractionation of the cytosol revealed that the mitotic membrane-

release kinase is distinct from cdc2/cyclin B, but this kinase is activated by cdc2/cyclin B at mitosis (38).

2.4 Disassembly of the lamina and nuclear membrane

Since both the state of assembly of the lamina and the association of the membrane with chromatin are regulated by active cdc2/cyclin B, it would seem likely that vesiculation of the envelope would occur at the same time as lamina breakdown. However, at least experimentally, the two events can be temporally separated using *in vitro* assays. For example, in mitotic *Xenopus* extracts, vesiculation of the nuclear membranes requires a stoichiometric component or components: vesiculation can be completely eliminated by the addition of a large excess of nuclei to the extract (39). Since the outer nuclear membrane is contiguous with and can perform all the biochemical functions of the endoplasmic reticulum (ER), it is possible that the coatamer proteins involved in ER vesicle trafficking are also involved in nuclear envelope breakdown. Excess nuclei would titrate out the soluble pool of coatamer proteins, specifically blocking the mitotic membrane vesiculation. In contrast, in the same *in vitro* system, the mitotic disassembly of the lamina (as assayed by the release of soluble lamin proteins) appeared to require enzymatic components only. Specifically, addition of a large excess of nuclei to a mitotic extract did not block lamin solubilization, and the rate of lamin removal reached a steady state level (39). *In vivo*, during meiosis in both frogs and chickens, the lamins are solubilized and released from nuclei which still contain intact envelopes and nuclear pore complexes (40). This suggests that the disassembly of the nuclear envelope is preceded by the depolymerization of the lamina, a separation supported by the *in vitro* results.

The fate of the nuclear membrane during mitosis is somewhat unclear. One model is that the nuclear envelope is disassembled into membrane vesicles which retain an identity distinct from the rest of the ER. In support of this model, mitotic vesicles competent for nuclear assembly can be isolated from *Xenopus* eggs and can be partially separated from the bulk of vesicles containing ER markers (41). In addition, a number of studies have reported that there are multiple classes of mitotic vesicles derived from the nuclear envelope (42–46). These studies suggest that the nuclear membrane is fragmented into domain-specific vesicles during mitosis.

Much of the evidence for multiple classes of nuclear envelope precursor vesicles is derived from *in vitro* studies using cell extracts (42, 43). Recently, different results were obtained when the dynamics of a LBR–GFP (lamin B receptor–green fluorescent protein) fusion protein were followed in living cells. In this system, the LBR–GFP-labelled nuclear envelope appeared to physically retract into the ER network at mitosis, without obvious vesiculation of either structure (47). Another study used antibodies to both inner nuclear envelope and ER membrane proteins and followed their distribution throughout the cell cycle; this study reached identical conclusions (48). These studies are consistent with a model where specific integral membrane proteins are immobilized to the inner nuclear membrane by interaction with intranuclear ligands such as the lamins or chromatin, now defining a 'nuclear'

membrane domain distinct from the adjacent membrane comprising the bulk of the ER. Reversal of these interactions at mitosis would allow the nuclear membrane proteins and membrane to flow back into the ER.

The discrepancy between the LBR–GFP results and the studies discussed above on vesicularization at mitosis remain to be resolved. One way in which the disparate results could be reconciled is in realizing that the *in vitro* reconstitution extracts are derived from mechanically disrupted cells. The ER network, which in *Xenopus* eggs contains large stored amounts of integral nuclear membrane proteins, would probably become fragmented into many small vesicles (or microsomes) upon cell lysis. A subset of the vesicles, those containing integral nuclear membrane proteins capable of binding to chromatin, might well be separable from vesicles not containing nuclear membrane proteins, creating multiple classes of vesicles upon separation. Similarly, lysis of tissue culture cells at mitosis would also create microsomes. *In vivo*, the reticular network containing ER and nuclear membrane proteins would remain intact. Integral *nuclear* membrane proteins would show an affinity for the chromatin and/or attached lamins in interphase. Thus, during times of nuclear assembly, these integral membrane proteins would draw a portion of the ER membrane to the chromatin, thereby initiating formation of a nuclear envelope. The proteins would lose that affinity at mitosis and the nuclear membranes would detach from the chromatin and lamins and retract into the ER, thereby disassembling the nucleus.

3. Nuclear pore complexes

The nuclear pore complex, composed of approximately 1000 proteins, is assembled in the nuclear envelope and forms a junction between the outer and inner nuclear membranes. The membrane at the border of the pores has been termed the pore membrane (Fig. 2; 49–52). At an early step in mitosis the pore complexes are removed from the envelope. Indeed, a series of electron microscopic studies reveal that the pores are removed from the nuclear envelope before nuclear membrane breakdown, leaving holes in the remaining intact nucleus (53–55). As is the case for the lamins, mitotic phosphorylation of pore proteins is thought to play a role in disassembly of the pore complexes. A number of nucleoporins have been shown to become hyperphosphorylated at mitosis (56, 57). Moreover, two nucleoporins can be directly phosphorylated by purified cdc2/cyclin B kinase *in vitro* (57).

Structurally, the nuclear pore consists of two eight-fold symmetric rings located on either side of the nuclear membranes (Fig. 2). These rings bracket a third ring of spoke elements, which are thought to contain at their hub a central transporter (58, 59). A set of eight short filaments extend from the ring on the cytoplasmic side of the pore, the cytoplasmic filaments. Conversely, a set of long thin filaments terminating in a ring, the nuclear basket, extends 1000 Å from the nuclear side of the pore. At present, it is not known whether these structures are removed from the pore at mitosis in an ordered, sequential fashion, or are simultaneously disassembled.

One intriguing aspect of disassembly is that the nuclear pore does not appear to

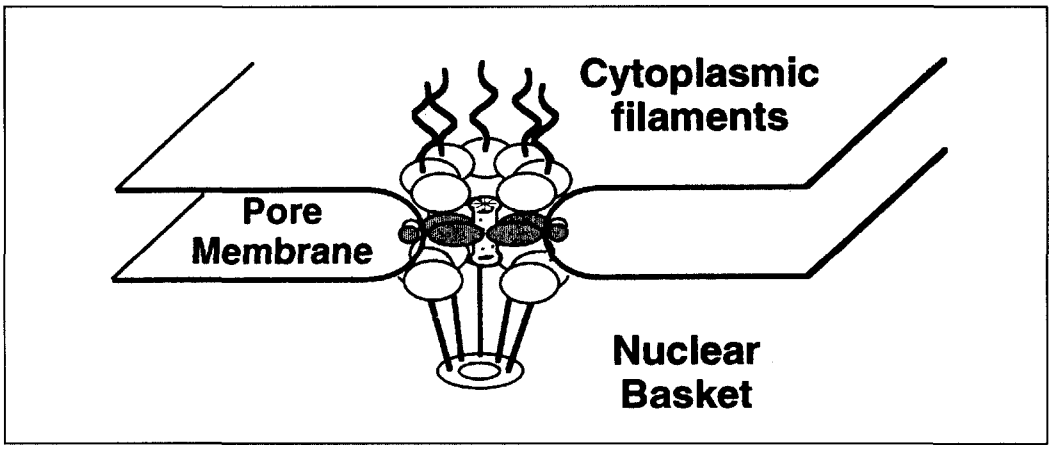


Fig. 2 The nuclear pore complex mediates macromolecular transport across the nuclear envelope. This large (~125 MDa) structure is composed of three eight-fold symmetric rings. Eight short, thick filaments project from the cytoplasmic face of the pore, and are thought to provide docking sites for nuclear import substrates. The nuclear basket is composed of eight thin filaments joined to a small ring approximately 1000 Å inside the nucleoplasm. The pore is anchored in the nuclear envelope by integral membrane pore proteins. These areas where the inner and outer nuclear membranes are joined has been called the pore membrane.

disassemble into completely monomeric polypeptides. A number of nucleoporins have been shown to exist in relatively large heteromeric complexes when unassembled in interphase *Xenopus* eggs ($\leq$1000 kDa), although these complexes are much smaller than a complete nuclear pore (~120,000 kDa). The observed mitotic phosphorylation of individual subunits of these complexes does not result in detectable changes to the size or composition of the complexes (57, 60). These protein complexes appear to represent individual modular portions of the nuclear pore. The mitotic phosphorylation of certain individual pore proteins may well serve to disassemble a nuclear pore into its constituent modules, rather than into individual monomeric pore proteins.

4. Reassembly of the nuclear envelope

The nuclear envelope is completely disassembled during the early phases of mitosis. However, by the end of mitosis, two complete and functional nuclear membranes must be correctly assembled around each of the daughter nuclei. The exact mechanism by which this occurs is still somewhat of a mystery, even more so now that the mechanism of nuclear membrane disassembly is in question. One possibility is that lamins act to guide membrane vesicles containing nuclear envelope proteins to the telophase chromosomes. A fraction of lamin B proteins remains associated with membrane vesicles during mitosis (at least in somatic cells), and lamin B has been found capable of binding to chromatin. Lamin B-binding would thus provide a simple mechanism for the specific recruitment of nuclear envelope precursor vesicles

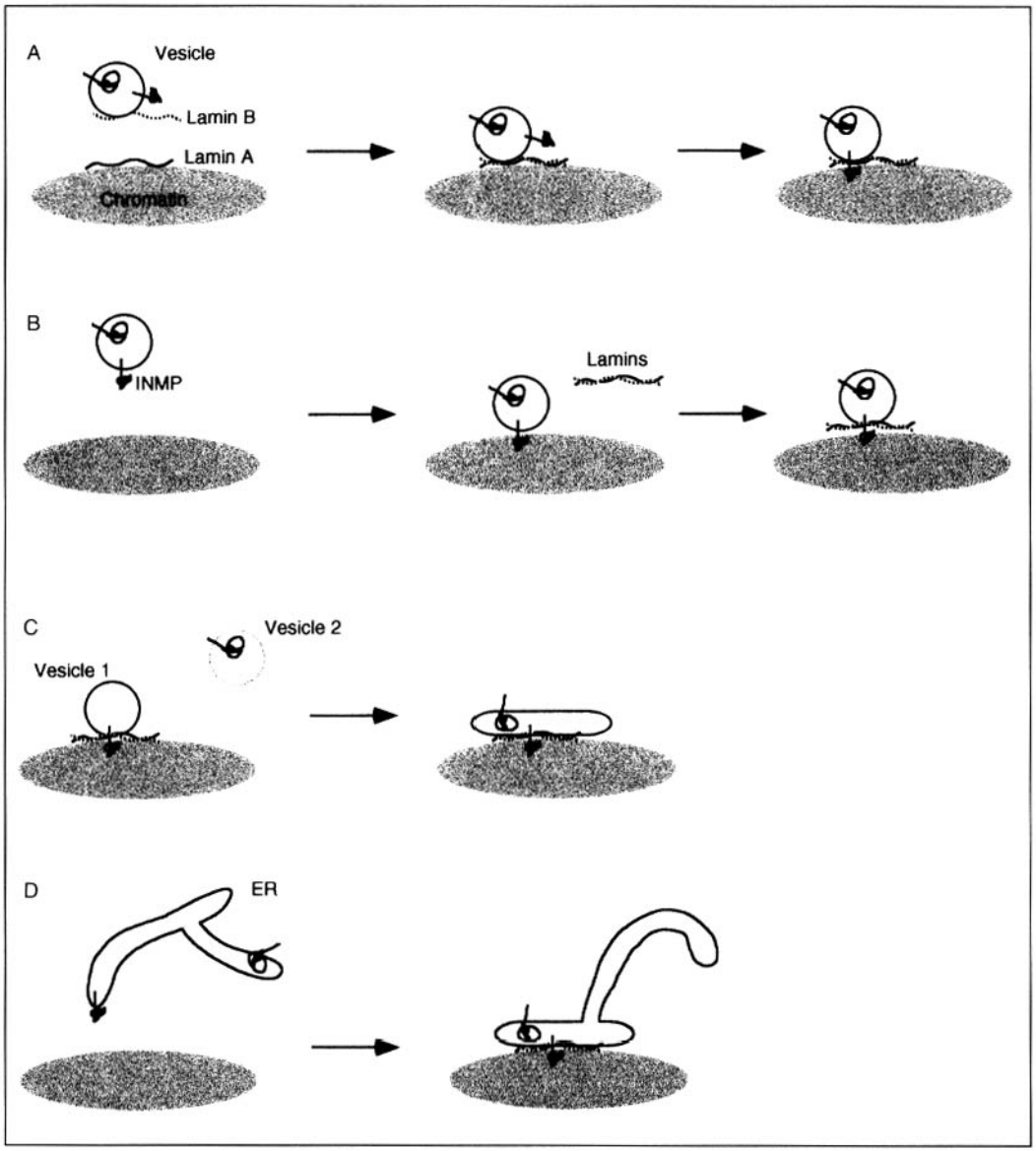
to chromosomes. A-type lamins have also been reported to bind to metaphase chromosomes (61). Chromatin-bound lamin A could provide docking sites for lamin B- or LAP-containing vesicles (Fig. 3A). In support of such a model, it was reported that immunodepletion of A-type lamins from Chinese hamster ovary (CHO) extracts inhibited *in vitro* nuclear formation by almost two-thirds. Depletion of lamin B from the CHO extracts inhibited nuclear formation by roughly one-third, suggesting possible additive contribution of lamin function in nuclear assembly (60). Similar results have been reported in studies using extracts prepared from *Drosophila* embryos and *Xenopus* eggs (62).

There are, however, several reports suggesting that lamin removal from *Xenopus* extracts did not block nuclear formation. Extracts depleted of B-type lamin L_{III} formed small, fragile nuclei that were incapable of DNA replication (15, 18). These data would suggest that lamins do not play an obligatory role in nuclear membrane assembly. Instead, formation of a lamina may be necessary for maintenance of normal nuclear function after assembly of the envelope. One caveat of these studies is that while the antibody used removed all of lamin L_{III}, the major species of lamin in the *Xenopus* eggs, it did not completely remove all lamin subtypes from the extracts. A small percentage of the lamins in the egg extract (estimated at 5–10% of the total) is of the membrane-bound L_{II} subtype, and would not have been removed by the antibodies used in these reports (63, 64). It is therefore possible that the small amount of B-type L_{II} lamins left in the extract are both necessary and sufficient for nuclear membrane vesicle binding to chromatin *in vitro*.

Additional confusion on the role of lamins in the targeting of the vesicles to chromatin comes from immunofluorescence studies. Experiments examining the timing of the association of integral nuclear membrane proteins to chromosomes during nuclear reassembly revealed that two of the proteins of the inner nuclear envelope, p54/58 and LAP2, associate with chromosomes well before detectable lamin B association at telophase (33, 44). It may be possible that p54/58 and LAP2 are recognizing metaphase chromatin-associated lamin A bound to the chromosomes (61). If so, then the two proteins could be responsible for binding nuclear membrane to the chromosomes, and lamin B would later accumulate at the interface of the membrane and chromatin (Fig. 3B). The timing of the association of lamins with chromatin needs to be carefully addressed.

Finally, it is experimentally clear that vesicles can be targeted to chromatin in the absence of cytosolic factors in the *Xenopus* system (37, 41–43). Again, it is possible that the very small amount of membrane-bound lamins (in *Xenopus* eggs the vast majority of B-type lamins are soluble at interphase) are responsible for this. It is

Fig. 3 Models for nuclear envelope assembly. At the end of mitosis, the disassembled nuclear envelope must reform around daughter chromosomes. Multiple models for envelope reformation have been proposed. (A) Targeting by lamins. In this model, chromatin-bound lamin A serves as a docking site for nuclear envelope precursor vesicles carrying membrane-bound lamin B. (B) Targeting by integral nuclear envelope membrane proteins (INMP). Integral membrane proteins such as one of the LAPs may target vesicles to the chromatin, with lamin association as a secondary event. In this and the preceding model, all of the nuclear membrane is supplied by a single, specific vesicular precursor. (C) Multiple vesicular precursors to the nuclear envelope. This model



contains at least two classes of nuclear envelope precursor. One class provides recognition and binds chromatin by either of the above models. The chromatin-targeted vesicles are then recognized by other vesicles containing the rest of the components of the nuclear membrane system. (D) Reformation of the envelope from the ER. In this model, during mitosis, the nuclear membranes are retracted into the ER, and the integral membrane proteins of the inner nuclear envelope mix with those of the ER. To reform the nuclear envelope, recognition of the chromatin by either membrane-bound lamins or by an integral membrane protein such as one of the LAPs occurs and causes the chromatin to become enveloped by membrane.

possible that there are multiple classes of vesicles, each with its own chromosome-targeting protein, involved in nuclear formation. There have been a number of reports of multiple classes of nuclear vesicle precursors (42–44). In perhaps the clearest demonstration of this, it has been shown that the association of the p54/58 membrane proteins with chromatin occurs long before the association of the integral membrane nuclear pore protein gp210 (44). The complete assembly of a functional nuclear envelope *in vitro* may require contributions from multiple vesicular precursors. Some of the vesicles could be targeted to the chromatin by lamins, some by LAPs or other membrane proteins. After initial enclosure of the nuclear envelope, growth of the nucleus could then proceed by vesicle–vesicle interactions (Figure 3C) or, if the model where nuclear membranes are retracted into the ER at mitosis is true, then some integral membrane proteins such as the LAPs may return early, binding the membrane to the chromatin and establishing a nuclear envelope. Later, integral membrane pore proteins could diffuse through the plane of the ER membranes to the nucleus, and be induced to stay there by protein–protein interactions (Fig. 3D). These models are summarized in Fig. 3.

5. Nuclear pore assembly

At some point during envelope assembly, the nuclear pore complexes must assemble, fusing both the inner and outer nuclear membranes at the pore. Exactly how this occurs is still largely unknown. One model was that pore assembly occurs on the surface of chromatin and is simultaneous with vesicle fusion. Assembly of pores between vesicles, followed by vesicle–vesicle fusion, would guarantee the insertion of the pores across the double membrane. Certain electron microscopic evidence supported this possibility (65). However, this appears not to be the case. *In vivo*, new pores assemble into pre-existing, intact nuclear membranes at S phase (reviewed in 49, 52, 66). In addition, morphologically normal nuclear pores can be assembled in membranous organelles called annulate lamellae, both *in vivo* and *in vitro*, in the absence of any chromatin substrate (67, 68).

In vitro, nuclear pores assemble only into a pre-existing double nuclear membrane (69). In the absence of cytosol, the membrane vesicles derived from egg extracts are capable of binding to chromatin, as stated above (37, 70, 71). When ATP and GTP are added, vesicle–vesicle fusion occurs, generating a nuclear intermediate containing patches of flattened double membrane interspersed with chromatin-bound, unfused vesicles (Fig. 4a). Upon the addition of cytosol to the intermediate, nuclear pores can assemble. Interestingly, the pores only assemble in the regions of flattened double membrane, not between vesicles. Thus, pore assembly obligatorily requires a pre-existing double nuclear membrane (69).

Although the pore formation observed above occurred only in pre-existing patches of double nuclear membrane, it was possible that pore formation was initiated on the surface of the chromatin, and the pores then inserted into the membrane in a second step. This chromatin seeding mechanism was ruled out by

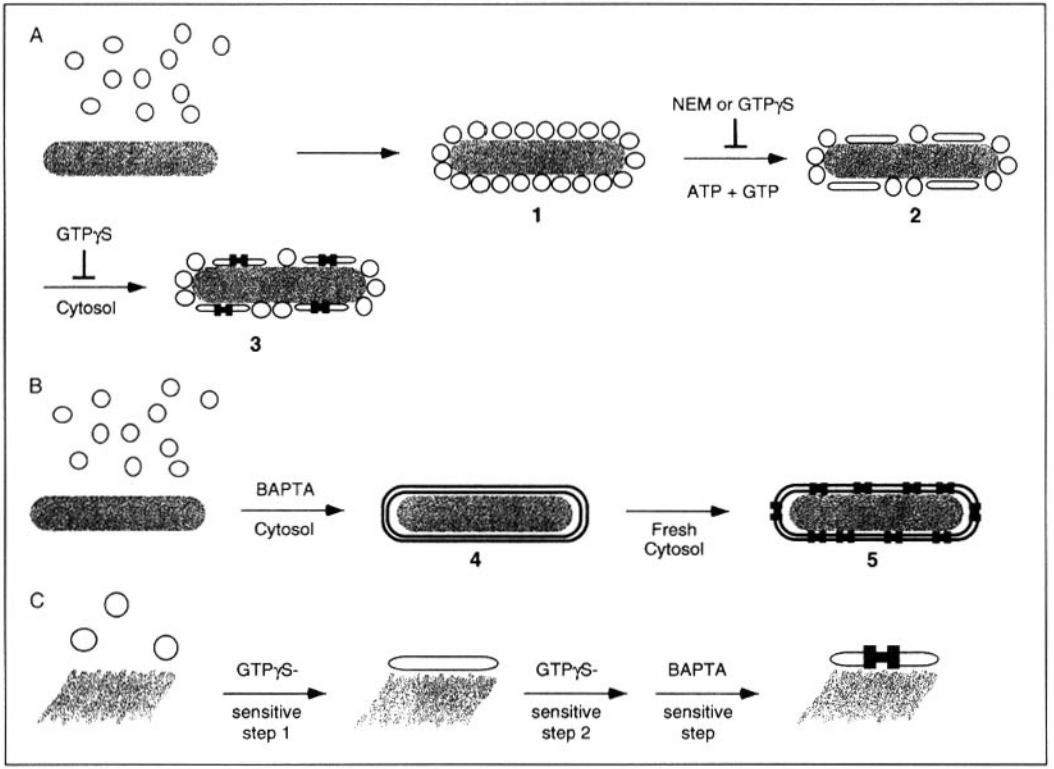


Fig. 4 *Steps in nuclear pore assembly, as revealed by chemical inhibitors.* (A) Two separate membrane fusion events are required for pore assembly. In the absence of cytosol or nucleotides, membrane vesicles can bind to the chromatin (**1**). Addition of ATP and GTP allows a limited amount of vesicle–vesicle fusion along the surface of the chromatin, resulting in the formation of small patches of flattened double membrane (**2**). This fusion event is blocked by either treatment of the vesicles with NEM, or the inclusion of the non-hydrolyzable GTP analog GTP-γS in the reaction. Addition of cytosol to the reaction promotes the assembly of pore complexes into the regions of flattened double membranes (pore membrane fusion) (**3**). The pore assembly initiated by cytosol is blocked by the addition of GTP-γS. (B) Nuclear membrane assembly can occur in the absence of pore assembly. When a complete nuclear reconstitution assay is mixed with the Ca^{2+} chelator BAPTA, the resulting nuclei are surrounded by a complete, double nuclear membrane system devoid of nuclear pore complexes (**4**), demonstrating that BAPTA blocks a step in pore assembly. Replacement of the BAPTA-containing cytosol with fresh cytosol allows assembly of functional nuclear pore complexes even in the presence of GTP-γS (**5**), indicating that the BAPTA-sensitive step in pore assembly is downstream from both GTP-γS-sensitive steps in pore assembly. (C) Summary of the steps in pore assembly revealed by the experiments in (A) and (B).

utilizing the finding that, in the presence of the Ca^{2+} chelator BAPTA, pore-free nuclei containing complete double nuclear membranes surrounding the chromatin could be formed (69). BAPTA had been shown previously to slow vesicle fusion and to block nuclear growth in this system (72, 73). When the pore-free nuclei were incubated in fresh cytosol lacking BAPTA, numerous functional nuclear pores were assembled in the nuclear membranes (Fig. 4B; 69). Thus, pore assembly occurs after double membrane assembly and is presumably initiated by a factor present in the

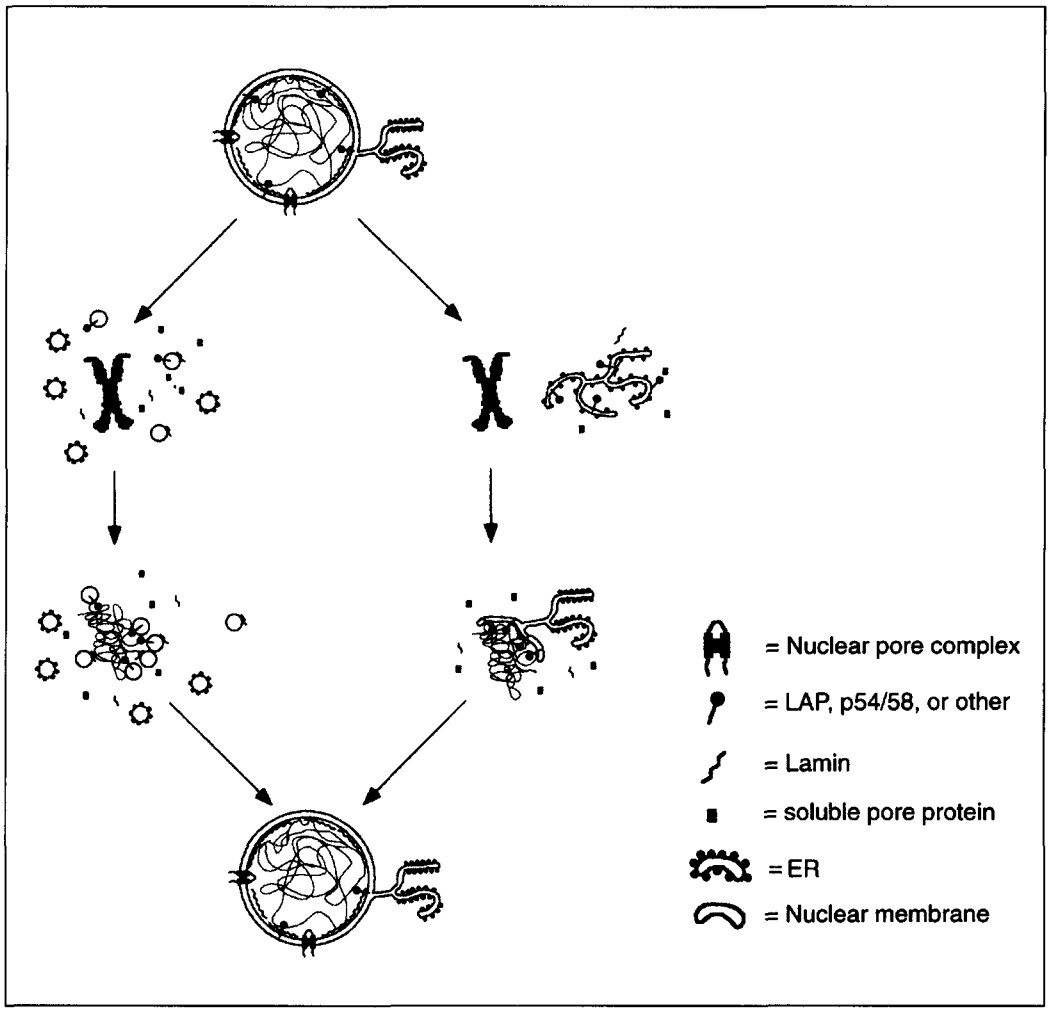


Fig. 5 Model for changes to the nuclear envelope at mitosis. Shown are two possible pathways for nuclear envelope breakdown and reassembly during mitosis. In both models, phosphorylation of nuclear envelope proteins, initiated by cdc2/cyclin B, leads to the disassembly of the nuclear pore complexes, the nuclear lamina, and release of the lamin-associated proteins from the chromatin. In the model on the left, the nuclear envelope is fragmented into specific vesicles, distinct from the putative vesicles formed from intracellular organelles such as the ER at mitosis. During telophase, reduction in the levels of mitotic kinases allows for the rebinding of nuclear vesicular precursors to the chromatin. Bound vesicles fuse and flatten on to the surface of the chromatin, forming the double nuclear membrane system. Nuclear pores reassemble into the nuclear envelope and the rest of the nuclear constituents are re-imported from the cytoplasm. An alternative model is shown as the right-hand pathway. As in the model above, disassembly of the pores and lamina is initiated by cdc2/cyclin B. Instead of fragmentation into specific vesicles, the nuclear envelope is retracted into and mixes with the ER membrane system, which also remains intact. Reduction in cdc2/cyclin B activity allows chromatin recognition by nuclear envelope proteins, and the chromatin once again becomes enveloped by membrane. As in the first model, regions of double nuclear envelope serve as targets for nuclear pore assembly.

soluble cytosol. Intriguingly, sequential blocks in the pore assembly process could be identified through the use of the inhibitors *N*-ethylmaleimide (NEM), GTP-γS, and BAPTA (66, 69; Fig. 4C). These results indicated that the block in pore formation caused by BAPTA was downstream of two GTP-γS-sensitive steps in nuclear pore assembly. Exactly how the nuclear pores are assembled into the nuclear envelope, and what specifies this membrane for pore insertion remain questions for future study.

From the studies reviewed here, a model for nuclear envelope assembly can be proposed (Fig. 5). The initial step probably involves the specific recognition of chromatin by nuclear envelope precursors, either in the form of specific vesicles, or derived from the ER. This recognition may be mediated either by lamin B, or by the LAPs, p54/58, or by some as yet undiscovered protein. It is possible that there are in fact multiple classes of nuclear envelope precursors, each using a different targeting protein. After binding, the membranes fuse and flatten on to the chromatin, creating the double nuclear membrane system. The nuclear membranes then serve as a target for nuclear pore assembly, initiated by a soluble cytosolic protein. At the site of a future nuclear pore, the inner and outer nuclear membranes fuse and the 1000 estimated proteins comprising the structure of the pore assemble. Once the nuclei become completely surrounded by a closed nuclear envelope containing nuclear pores, nuclear import can occur through the newly assembled pores. The soluble lamins are presumably then transported through the pores and subsequently assembled into the nuclear lamina, giving rise to a functional interphase nuclear envelope.

6. Future directions

The lamins are obviously extremely important in the assembly and function of a normal nucleus. What remains to be clarified are the exact role of the lamins in the assembly of the nuclear envelope, and also the interaction of the lamins with other components of the envelope.

The kinase that reverses nuclear membrane binding to chromatin and its targets remain to be identified at the molecular level. Potential targets of this kinase are LAP1A–C and LAP2, as mitotic phosphorylation of these proteins blocks their binding to chromatin. Whether these proteins are the sole molecules responsible for targeting of the nuclear membranes to chromatin remains to be determined. One key question is the identity of the membranous precursors to the nuclear envelope. Are there multiple classes, each with its own targeting mechanism? If so, how are they generated during mitosis? Or does the nuclear envelope retreat into the ER network during mitosis?

Exactly how nuclear pore complexes are assembled into the nuclear envelope remains an intriguing problem. What soluble and integral membrane proteins are required for the fusion of the inner and outer nuclear envelope? Do these proteins, despite the fact that at least some of them reside within the lumen of the ER, resemble

the cytosolic factors such as NSF (*N*-ethylmaleimide sensitive factor) involved in the recognition and fusion of transport vesicles with their target organelles in the secretory pathway? Once the pore membrane is formed, does the pore self-assemble from its constituent soluble components, or does assembly require a scaffold or chaperone? Finally, is disassembly of the pore merely the reverse of the assembly pathway, or are distinct mechanisms employed?

At present, quite a bit is known about the molecular details of mitotic changes to certain individual molecular constituents of the nuclear envelope, such as the lamins. At the ultrastructural level, the morphological changes that occur during nuclear envelope breakdown and reassembly are also well characterized. What is sorely needed are the connections between the two, and these will remain as fundamental areas of enquiry for some time to come.

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3 Poles apart? Spindle pole bodies and centrosomes differ in ultrastructure yet their function and regulation are conserved

IAIN M. HAGAN, KEITH GULL, and DAVID M. GLOVER

1. Introduction

One of the most overpowering images in biology has to be the simplicity and symmetry of the apparatus that forms in order to segregate the eukaryotic genome before cell division, the spindle. Nothing illustrates more graphically the complexity of structure that can be generated by regulating the function of biological polymers. The essential bipolarity of the spindle requires the function of specialized microtubule organizing centres (MTOCs) at its poles, termed centrosomes in animal cells and spindle pole bodies (SPBs) in fungi. These specialized organelles differ markedly in structure from one class of organisms to another. Notably, they are not found in plant cells, and the meiotic spindles of flies, frogs and mammals become organized without centrosomes at their poles. In eggs the chromosomes themselves appear to act as MTOCs and initiate the nucleation of microtubules, which subsequently become focused at the spindle poles through the concerted action of microtubule motor proteins. The role of the chromosomes and nuclear proteins for the organization of spindles has been emphasized by a number of recent studies performed *in vitro* with egg extracts. Magnetic beads coated with plasmid DNA have been shown to facilitate the formation of a bipolar spindle in *Xenopus* egg extracts (ref 1; also discussed in Chapter 4). Waters and Salmon have reviewed this evidence, and suggested that there may be an alternative form of spindle assembly in cells that appear to lack focused centrosomes (2). In such cells microtubules are envisaged to bind to and thus be stabilized by chromosomes, whilst the action of mitotic motors such as chromosomally located chromokinesins (plus-end-directed motors) may work in concert with minus-end-directed motors which focus these microtubules to

produce a bipolar metaphase spindle with centrally located chromosomes (2). Whilst the mechanisms for the formation and maintenance of spindles in oocytes perhaps represent an extreme example of a self-assembly system, similar features are likely to underlie many of the aspects of spindle function in those cases where MTOCs are nucleating the microtubules at the two poles. The overall outcome is a universal organization of the half-spindle with individually dynamic microtubules having a polarity of minus ends at the MTOC and plus ends distal, either at chromosomes, or interacting with microtubules from the other half spindle, organelles or other cytoskeletal components. Given the ability of the microtubules to self-organize in such a dramatic way in both mitotic (1) and interphase cells (3, 4), the two mitotic MTOCs may serve a moderating function and act as a dominant determinant to ensure that only two poles are formed and that these two poles remain stable throughout division.

In the century that has elapsed since the centrosome was first described and named, there has been a focus on morphological documentation of the extensive variety of form amongst diverse spindle types and in various species (5). The field then developed into one of molecular taxonomy in a process of trying to identify the components of the spindle poles (6). We are now at the dawning of a particularly exciting time in which the combined powers of genetics, biochemistry, and cell biology are being harnessed in a convergent and highly productive fashion to provide considerable insight into some key aspects of pole organization and function.

We will attempt to highlight some of the studies on the structure of the polar MTOC and its function most pertinent to understanding its role in genome segregation. Constraints of space lead us to refer to recent more comprehensive reviews for a more complete picture of the MTOC *per se* (6–11). We will concentrate on data obtained in studies of some key model organisms to exemplify the behaviour of the major types of polar MTOC.

2. Ultrastructure of the spindle poles

2.1 The spindle pole bodies of fungi

There is a bewildering array of different MTOC forms in eukaryotic microorganisms (5). However, the genetic malleability of three model systems, *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Aspergillus nidulans,* has resulted in these organisms receiving most attention in recent years. Of these by far the most is known about the MTOC at the pole of the budding yeast (*S. cerevisiae*) spindle, the SPB. Indeed, at the molecular level, the budding yeast SPB is the best characterized MTOC of any system and its continued study is likely to define some of the ground rules for the analysis of higher eukaryotic MTOCs, as much by its differences from other systems, as by its similarities.

The budding yeast SPB is a multi-layered structure that remains embedded in the nuclear envelope throughout the cell cycle (Figs 1 and 2A). A dense central plaque

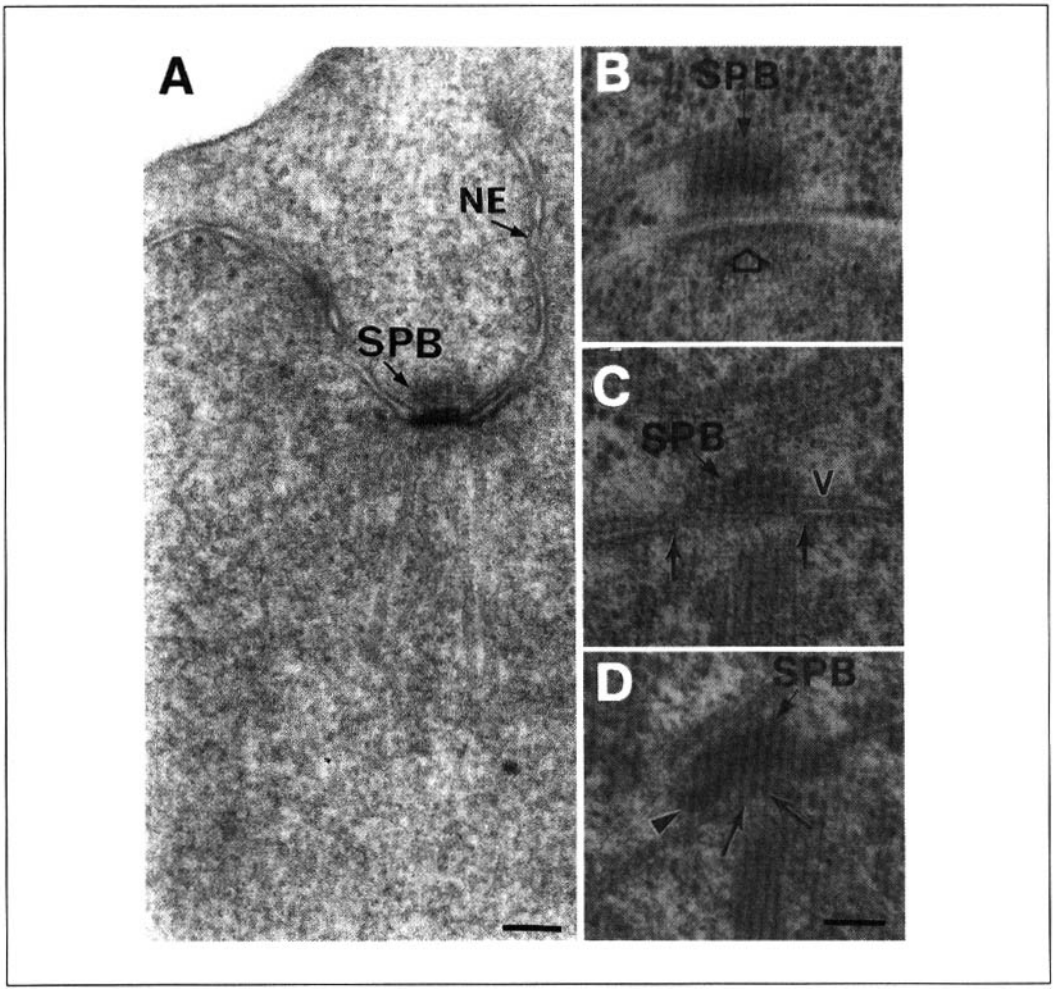

Fig. 1 Electron micrographs illustrating the differences between the *S. cerevisiae* and *S. pombe* spindle pole bodies (SPBs) which are represented schematically in Figs 2 and 3. (A) *S. cerevisiae* SPB residing within the nuclear envelope (NE). (B–D) *S. pombe* SPB at different stages of the cell cycle. During interphase the main body of the SPB sits on the outer face of an apparently contiguous nuclear envelope. The arrowhead indicates a region of dark amorphous γ-tubulin-containing material directly underneath the nuclear envelope under the SPB. Upon commitment to mitosis, the SPB inserts into a newly opened fenestra within the nuclear envelope. During anaphase (B) the nuclear envelope closes and the number of microtubules decreases. The limits of the nuclear membrane are indicated by two arrows in panels C and D. The arrowhead in panel D indicates electron-dense material bound to the nuclear membrane. Scale bars represent 100 nm. Panel A is reproduced from *The Journal of Cell Biology*, 1996, **132**, 887 (ref. 80) by copyright permission of The Rockefeller University Press and (B)–(D) from *Molecular Biology of the Cell*, 1997, **8**, 1461 (ref. 17) with permission of the American Society for Cell Biology.

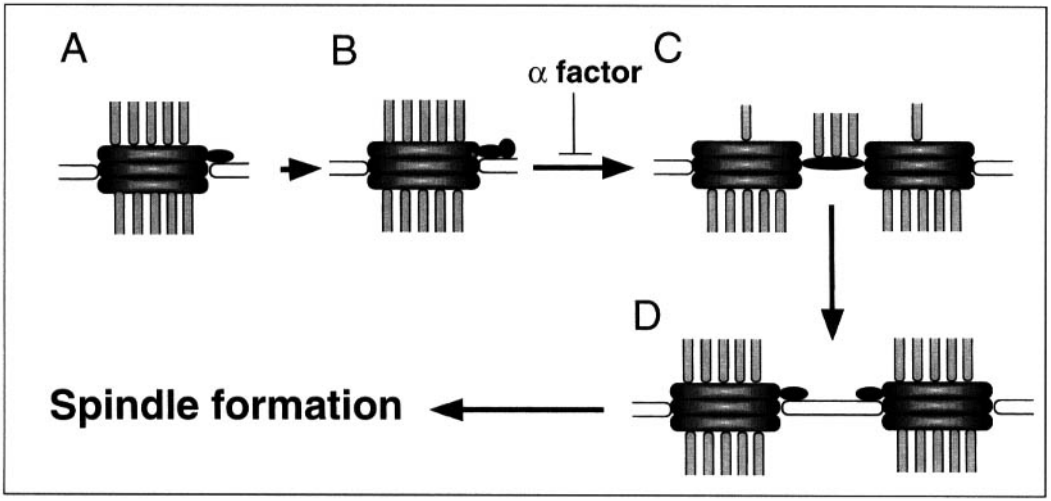

Fig. 2 A cartoon depicting SPB duplication in budding yeast. The SPB resides within the nuclear envelope (depicted by the thin black line curving around either side of the SPB) throughout the cell cycle. The cartoon is drawn with the cytoplasmic face uppermost and the nuclear face below the nuclear envelope. The single half-bridge-bearing SPB (A) gains a satellite structure (B) before a new SPB appears at the end of a larger full-bridge structure (C). Microtubules are nucleated from the cytoplasmic face of the bridge (C) until it is split to produce two separate, half-bridge-bearing SPBs (D).

with a somewhat less dense centre is flanked by two nuclear and three cytoplasmic plaques (12–15). A structure referred to as the half-bridge is associated with the membrane on one side of the SPB.

The synthesis of a new SPB is conservative and initiated from a small amorphous structure at the end of the half-bridge referred to as the satellite (Fig. 2B). The result is two SPBs which are connected by a bridge (Fig. 2C). The development of the satellite into a mature SPB is presumably very rapid as no intermediates have been recorded. The bridge nucleates microtubules from its cytoplasmic face until it breaks towards the end of S phase after which cytoplasmic microtubules are only nucleated from the cytoplasmic plaque and not the half bridge (ref. 12; Fig. 2D). Microtubules extend from the nuclear face of the SPB throughout the cell cycle and differ from those found in most eukaryotes as their ends are sealed by a dome-shaped cap structure (16).

In contrast to the budding yeast SPB, the main part of the SPB in both fission yeast (*S. pombe*) and *A. nidulans* sits on the outside of the nuclear membrane in interphase and inserts into the membrane upon commitment to mitosis (5, 17; Figs 1 and 3). The method of SPB duplication in fission yeast also appears to differ from that in budding yeast as the single interphase SPB splits into two daughter SPBs which then grow from half this final unitary size to create two full-sized SPBs (17). What role the bridge plays in this duplication is unclear, but it is present throughout and the two daughter SPBs end up connected by a bridge structure (Fig. 3C). Upon mitotic commitment the nuclear membrane opens beneath the SPB and the SPB drops into

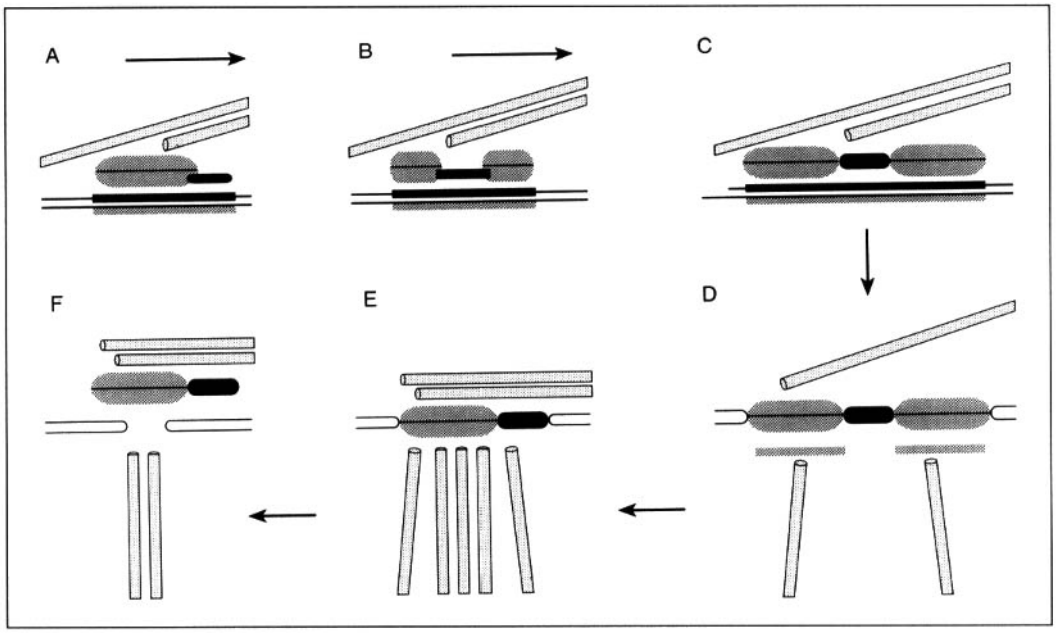

Fig. 3 A cartoon depicting SPB duplication in fission yeast. The double black line in each panel represents the nuclear envelope. The cytoplasm is above this line and the nucleoplasm below it. Arrows indicate temporal progression. (A) A single large, half-bridge-bearing SPB resides on the outside of the nuclear envelope above amorphous material on the nuclear side of the membrane (grey bar). (B) SPB duplication in late G_2 sees a reduction in size of the two SPBs which lie either side of the bridge. These SPBs grow in size (C) until commitment to mitosis when the nuclear envelope breaks down beneath the paired SPBs which slot into the newly formed gap (D). Cytoplasmic microtubules are associated with the cytoplasmic face of the SPB throughout interphase. Nuclear, spindle microtubules are nucleated from the nucleoplasmic face of the SPB upon insertion into the membrane. Cytoplasmic microtubules are nucleated by and run tangentally to the SPB after spindle formation but before the metaphase-anaphase transition (E). After this transition (F) the nuclear envelope starts to close and the number of spindle microtubules decreases. Drawn after ref. 17.

the gap within the membrane and nucleates microtubules. The process of SPB re-exclusion is initiated at the metaphase–anaphase transition and results in a virtual sealing of the membrane in the presence of late spindles (Figs 3F and 1D). During interphase, filamentous structures are seen within the membrane and an electron-dense region is found directly beneath the membrane (Fig. 1B), as is γ-tubulin, strongly suggesting a structural link across the nuclear envelope in interphase. Moreover, centromeres are continuously associated with the interphase SPB region (18), confirming that the functional unit does extend across the apparently contiguous membrane. The SPB–centromere interaction during interphase in fission yeast probably underlies the role of the SPB in directing nuclear positioning (19). As the Ndc10p component of the budding yeast kinetochore is associated with the SPB in interphase (20), SPB–centromere interactions and co-ordinated nuclear positioning may be a common feature of fungal systems.

2.2 Animal cell centrosomes

The centrosomes of animal cells have many functions: (a) they provide the intrinsic dominant control of the number of major sites of microtubule nucleation within the cytoplasm, one in interphase, two in the bipolar mitotic spindle; (b) they modulate the number of microtubules nucleated during the cell cycle (21); and (c) they control the structure of the nucleated microtubules, their polarity and the cellular position of the microtubule array. Thus, by means of the intrinsic properties of polarity and position, both of itself and of the subtended microtubule array, the centrosome is able to influence many of the major events occurring within the eukaryotic cell. These include the directed movement of vesicles, the positioning of membranous organelles, and the processes of mitosis and cytokinesis. These complex, almost holistic properties enabled the importance of the centromere to be recognized as far back as the nineteenth century (21), and yet our understanding of its biochemistry and molecular cell biology is only recently beginning to emerge.

An alternative view of the animal cell centrosome is as a unitary conglomerate of three MTOCs that define the interphase microtubule array, the mitotic spindle, and, in the case of motile cells, the axonemal microtubules (22). Studies of microtubule nucleation/organization sites in eukaryotic microbes provide instructive insight to understanding the animal cell centrosome as in many eukaryotic microbes, particularly protozoa and algae, there is often invariant control over the number and content of microtubule arrays nucleated by MTOCs. Each of these three component MTOCs may either be found at a single site, as in many somatic animal cells, or be dispersed to three different sites, exemplified for instance by the trypanosome cell (Fig. 4) (23). In contrast, the microtubule arrays seen in the amoeboid cell of *Physarum* suggest that the interphase centrosome of these cells exists in a highly organized state, which intriguingly is not so apparent at mitosis (24, 25).

2.2.1 Centrioles

The classical description of the animal cell centrosome with the advent of electron microscopy was of a pair of centrioles surrounded by a rather amorphous cloud of electron-dense pericentriolar material (PCM). Conceptually, we can attribute specific characteristics to each of these two component parts. Firstly, much evidence suggests that microtubule nucleation and anchoring of both cytoplasmic and spindle microtubules is, in the main, a function of the PCM (26). A pair of centrioles normally lies at the heart of the animal cell centrosome (8). We know that the two centrioles within the centrosome are non-identical and that the mature centriole is both ultrastructurally and functionally related to the basal body that acts as the MTOC for the axonemal microtubules of cilia and flagella. Figure 5 represents a schematic view of the centriole pair within a centrosome. The centriole cylinder ($\sim$ 0.2 μm diameter $\times$ 0.4 μm long) is composed of nine triplet microtubules with the outer tubule of the triplet (tubule C) being shorter than tubules A (inner) and B (middle). The interlinked triplets adopt a slightly twisted organization to form the cylinder-like structure or centriole 'pinwheel'. One centriole is defined as mature by its

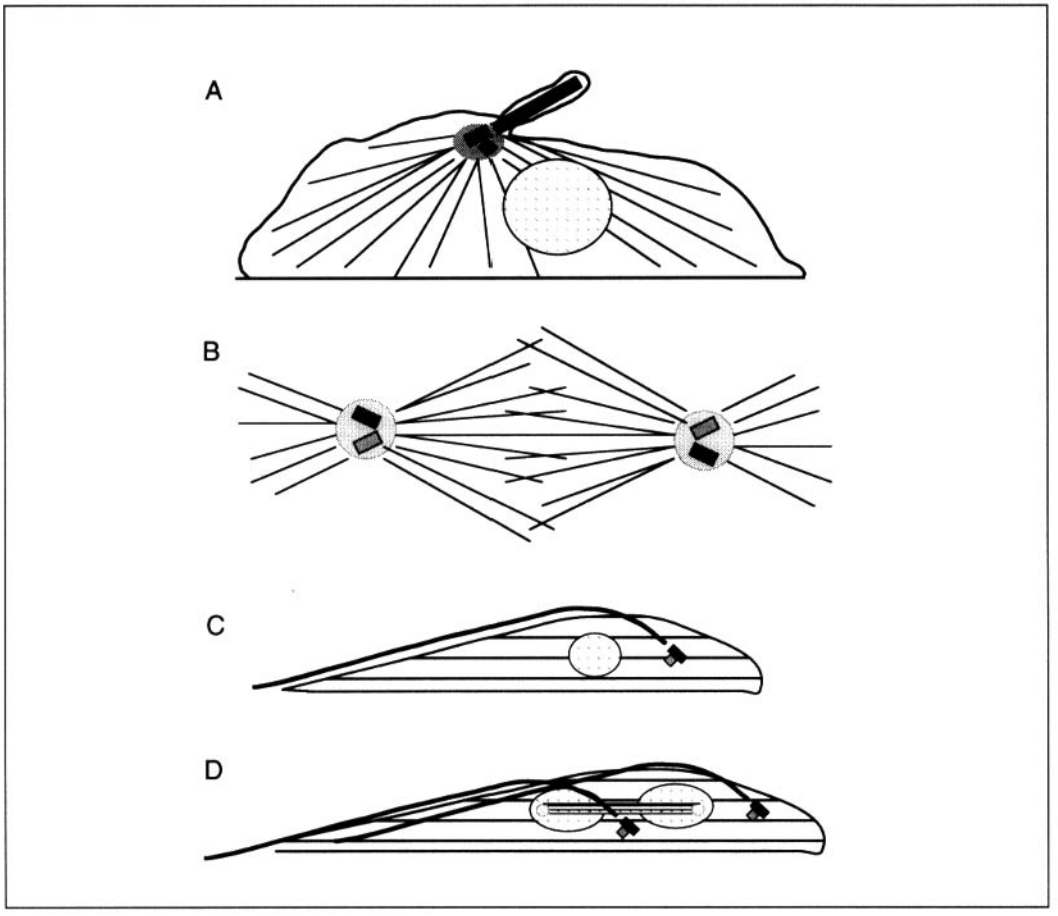

Fig. 4 Examples of grouped and dispersed MTOCs in different systems. The mammalian cell centrosome is a form of grouped MTOC in that it nucleates interphase and mitotic microtubules (A, B) as well as those of the axoneme (the primary cilium in A). The trypanosome cell, in contrast, has dispersed and separate MTOCs for cytoplasmic microtubules and flagellar axonemal microtubules (both present at interphase and mitosis, C and D). In addition it has a separate MTOC for the intranuclear mitotic spindle.

functional capacity to subtend a primary (immotile) cilium. It also bears a set of appendages and satellites that distinguish it from its immature partner. Other structural features define the immature centriole, for instance, its internal cartwheel structure and orthogonal relationship to the mature centriole. The centriole duplication cycle is discussed in relation to centrosome duplication and separation below in Sections 4.2 and 4.3.

2.2.2 Relationship of the centriole to the cilium

The relationship of the centriole to the basal bodies is probably most easily appreciated in microbial systems such as the amoebo-flagellate stages of organisms

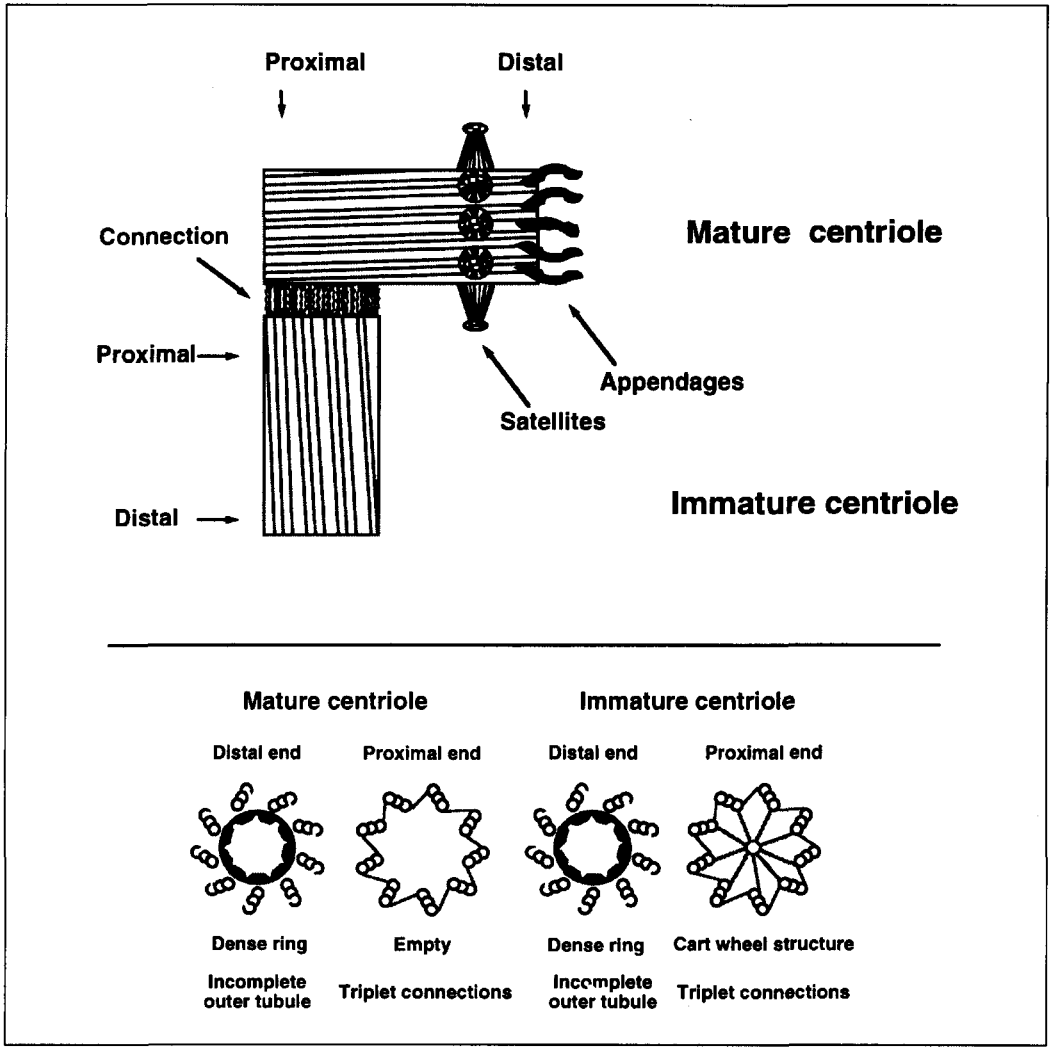

Fig. 5 Schematic view of the centriole pair in animal cells. The two centrioles differ in structure: the mature centriole carries appendages and satellites that the immature centriole lacks. The diagram also illustrates the 'right-angle rule' whereby the long axis of the immature centriole intersects the mature centriole, but the long axis of the mature centriole does not intersect the immature centriole. Also shown is the connection between the two proximal ends of the centrioles. The lower part of the figure shows, in a simplified manner, the internal structures of the centriole as viewed from the outside at each end. The cartwheel structure in the proximal part of the immature centriole is typical of a growing centriole, whilst the distal end of both centrioles contains a dense ring and sometimes an incomplete outer tubule in the microtubule triplet. The proximal ends exhibit discrete connections between A and C tubules. Adapted from ref. 8 with permission of Elsevier Science.

such as *Physarum*. The centrosomal area of the amoebal form possesses two non-identical centrioles, which, when this cell undergoes the reversible transformation to a flagellate, immediately become the basal bodies and act as the MTOC for the two non-identical flagella (25, 27). In animal cells, centriole duplication and maturation within the cell cycle ensures that the early interphase centrosome has one mature and one immature centriole. The potential of the mature centriole to act as an MTOC for an axoneme is also seen during G_1 phase when in many animal cells it forms an immotile, primary cilium. In many microbial systems and in the somatic cells of animals there is a close spatial, temporal, and numerical relationship between existing centrosomal centrioles and the formation of new centrosomal procentrioles. These replication relationships may well be maintained during differentiation of the monoflagellated sperm cells. However, during ciliogenesis in multiciliated epithelial cells, these tight relationships between replication and maturation have to be circumvented. The centriole precursors of the basal bodies of the 200–300 cilia of these differentiated cells assemble close to a mass of electron-dense material, the fibrogranular complex (28).

2.2.3 PCM, microtubule nucleation, and release

In contrast to the very precise description of the centrioles within the centrosome, we have had, until recently, very little information on the substructure of the pericentriolar material. *In vitro* experiments on isolated centrosomes clearly showed that the major microtubule nucleating potential of the centrosome was associated with the PCM (26). However, until recently even high-resolution electron microscopy has revealed little substructure in the PCM (see Section 3.1).

Kimble and Kuriyama (29) suggested that one may expect three levels of organization: (a) a single nucleating element at the minus end of the microtubule, (b) clusters or centres of such nucleating elements, and (c) the organization of many such centres within the pericentriolar material. Their suggestion of different levels of organization does not necessitate that individual microtubules are permanently anchored. In fact many observations suggest that microtubule nucleation, anchoring, attachment, and detachment may all be part of a dynamic process. Vorobjev and Nadezhdina (30) considered the possibility that microtubules may be nucleated and then released from individual sites within a centrosome.

Data from Belmont *et al.* describing the release of individual microtubules from centrosomes within a mitotic microtubule aster in *Xenopus* egg extracts (31) and the existence of a microtubule-severing protein, katanin, in a region just outside the PCM of centrosomes throughout the cell cycle of sea urchin cells (32) suggest that the concept of nucleation and then release may extend to the spindle pole centrosome. Indeed, microtubules are released *en masse* from the *Dictyostelium* interphase centrosome upon commitment to mitosis and from sea urchin poles at telophase (33–36). Such characteristics also provide a logical mechanism to account for the ability of γ-tubulin-nucleated microtubules to depolymerize at the pole after nucleation during metaphase flux (37) (see Section 3.1.2).

3. Components of polar MTOCs and their function

A substantive body of information is now accumulating about structural components of the yeast SPBs. This contrasts with a relative dearth of information about the molecular constitution of the animal cell centrosome. One difficulty is that, although over the years many molecules have been described as being associated with centrosomes, there has often been no means of assessing the functionality of such associations. In a sense, the animal cell centrosome can act as a central railway station served by a vast microtubular railway network. Many proteins arrive at the 'station' as 'passengers' of a variety of 'locomotive' proteins. Nevertheless, positional information in respect of the classification of a protein as a centrosomal component has remained based on a central tenet that it is found in this site by immunofluorescence and immunoelectron microscopy and that this assumption remains even following the depolymerization of microtubules using drugs. Sometimes this positional information can be supported by biochemical or genetic evidence to link its function to its location at this site. However, in classifying any protein as a component of the centrosome one has to rehearse the caveat that this label may not indicate an exclusive location of the total cellular population of such molecules to this centrosomal site. The result is a rapidly lengthening list of proteins with some form of centrosomal location, the catalogue of which has been extensively reviewed (6, 11, 29). In most cases we only have information on position at the centrosome and lack information on likely function.

The first good structural identity of a molecular complex with microtubule-organizing properties in the PCM has been provided by the characterization of the 'gammasome', which probably satisfies the criteria for the first level of organization of the PCM as defined by Kimble and Kuriyama (29), namely the microtubule nucleation element.

3.1 The gammasome

In a few cases, some general principles are emerging about fundamental components of the MTOCs that unify the function of the diverse structures of the yeast SPBs and the animal cell centrosome. Nowhere has this been more apparent than in our rapidly expanding understanding of the function of γ-tubulin. The gene for this protein was first identified through a suppressor mutation in an *A. nidulans* colony on an agar plate that could compensate for the hyperstable microtubules in a *benA33* β-tubulin mutant (38, 39). It was not long before antibodies had been raised against the molecule and used to show that it was located on the spindle poles (40) and that the protein was also present in the centrosomes of higher eukaryotes (41, 42). It was subsequently shown that microinjection of anti-γ-tubulin antibodies blocks microtubule nucleation in mammalian cells (43) and that γ-tubulin is part of a large multiprotein complex, the gammasome (44–46).

It is now established that γ-tubulin is concentrated at MTOCs in many different

organisms and a key role for γ-tubulin in microtubule nucleation has been most clearly illustrated by the disruption to the MTOCs in γ-tubulin mutants of *A. nidulans*, *S. pombe*, *S. cerevisiae*, and *Drosophila melanogaster* (47–51). Given its location at the MTOC, low abundance of the protein and its requirement for microtubule nucleation, Oakley and colleagues (52) proposed that a ring of γ-tubulin within the centrosome could serve as a template for microtubule formation (44, 53, 54).

Genes encoding two γ-tubulin isoforms are known in *Drosophila*. One polypeptide encoded by the 23C gene is expressed in a variety of somatic tissues and developmental stages. Mutations in this gene dramatically disrupt the size and shape of the centrosome and decrease the numbers of microtubules that it nucleates (50). The second polypeptide is only detected in oocytes and embryos and mutations in its gene, located at 37C, disrupt the structure and function of the female meiotic spindle (51). This is a particularly instructive finding since this meiotic spindle in *Drosophila* is anastral, lacks a structurally recognizable centrosome, and shows no obvious localization of centrosomal antigens, including γ-tubulin, by immunofluorescence techniques (see Chapter 7). In the formation of the meiotic spindle, microtubules are initially nucleated by association with chromatin and then bundled and focused into the poles by the action of kinesin-like motor proteins such as Nod and Ncd (55–59). The process was thought not to require specific centrosomal functions. Although the precise role of γ-tubulin in organizing these spindles is not clear, the phenotype of this mutant provides a dramatic illustration that inability to visualize a specific location of a molecule should not be taken as evidence for lack of function in a particular process.

There is now abundant evidence that may support the view of γ-tubulin as a microtubule template during nucleation as γ-tubulin is part of the large 25S gammasome, in embryonic and somatic animal cells. The complex was first isolated from *Xenopus* oocyte extracts and was visualized by electron microscopy as an open ring structure with a diameter of 25–28 nm (44). This particular complex includes six extra proteins including α- and β-tubulin and proteins of 195, 133, 109, and 75 kDa. Interestingly, none of the molecular weights corresponded to the 60 and 190 kDa centrosome-associated proteins, CP60 and CP190, which have been shown to co-purify with γ-tubulin from *Drosophila* embryos (60). Importantly, combined use of immuno-electron microscopy and electron microscope tomography has shown the presence of ring-like structures reminiscent of gammasomes in isolated *Drosophila* centrosomes (45, 61). Convincing arguments for these rings being functional nucleators came from the demonstration that γ-tubulin complexes were close to the minus ends of microtubules nucleated *in vitro* by these isolated centrosomes. Recently, Vogel *et al.* (46) used similar technology to study highly purified centrosomes from *Spisula* oocytes, and again detected ring structures on the centrosomes. Biochemical analyses of the component proteins of the *Spisula* gammasome indicate an unusual stoichiometric ratio of α-tubulin:β-tubulin (being around 1:5 rather than the expected 1:1), suggesting that centrosomes may contain β-tubulin that is complexed with proteins other than α-tubulin—the prime possibility being that β/γ complexes occur at the nucleating site (minus end) of microtubules (46).

3.1.2 Models for microtubule nucleation by the gammasome

Two main models for this centrosomal microtubule nucleation complex have been proposed (44, 52, 62; reviewed in 63) (Fig. 6). Oakley (52) and Zheng *et al.* (44) essentially suggest a model whereby proteins in the nucleation complex provide a molecular scaffold on which γ-tubulin subunits align in a helix. In the simplest form of this model, a tubulin heterodimer would then pair directly with each γ-subunit, providing a template for the first elongation step of microtubule assembly (Fig. 6). In an alternative view, Erickson and Stoffler (62) suggest that γ-tubulin ring complexes, perhaps influenced by other proteins, form curved protofilaments and that these γ-protofilaments interact with both α- and β-tubulin subunits to initiate microtubule nucleation (Fig. 6). This latter mode of action would be more consistent with recent models for microtubule growth in which microtubules polymerize as long sheets which subsequently curl up upon GTP hydrolysis, thus the microtubule is not extending as a uniform tube but by side association into a sheet which then becomes a uniform tube (64). The model of Erickson and Stoffler is also more consistent with the depolymerization of microtubules at the pole during metaphase flux (37). Without invoking severing of every microtubule it is difficult to reconcile such behaviour with the ring nucleation model of Oakley (52) and Zheng *et al.* (44) if γ-tubulin stays on the end of the microtubule throughout division.

3.1.3 A γ-tubulin variant in an organism with closed microtubule ends

The γ-tubulins of *A. nidulans* and *S. pombe* show great similarity to those of higher systems. In fact, human γ-tubulin can substitute for the fission yeast molecule (65). Budding yeast was, for a long time, in the strange position of being one of the most extensively studied systems and yet the one in which γ-tubulin could not be found. This failure challenged the models for γ-tubulin function in microtubule nucleation derived from studies in other systems. The cause of this discrepancy became apparent when the *S. cerevisiae TUB4* sequence was identified through the yeast genome sequencing project; its predicted amino acid sequence exhibits no more than 35–40% homology to other γ-tubulins (66). This contrasts with the usual identities of around 65% between γ-tubulins from different species and explained why the low-stringency hybridization and PCR approaches, which had been successful in identifying virtually all of the other γ-tubulin genes in other systems, had not identified *TUB4*. The divergence of *TUB4* was further highlighted by the inability of human or *Xenopus* γ-tubulin to substitute for its essential function and, conversely, the inability of *TUB4* to substitute for the function of the fission yeast γ-tubulin gene, *gtb1*$^+$ (49, 67).

The analysis of the budding yeast γ-tubulin gene and its interacting genes serves to illustrate the power and incisive nature of genetic approaches in yeast in dissecting biological processes at the molecular level. As anticipated, immunogold labelling with anti-Tub4p antibodies showed that Tub4p is localized to the inner and outer plaques of the SPB (49). Conditional *tub4* mutants exhibit disorganized non-functional spindles with less than the normal complement of microtubules (49, 66,

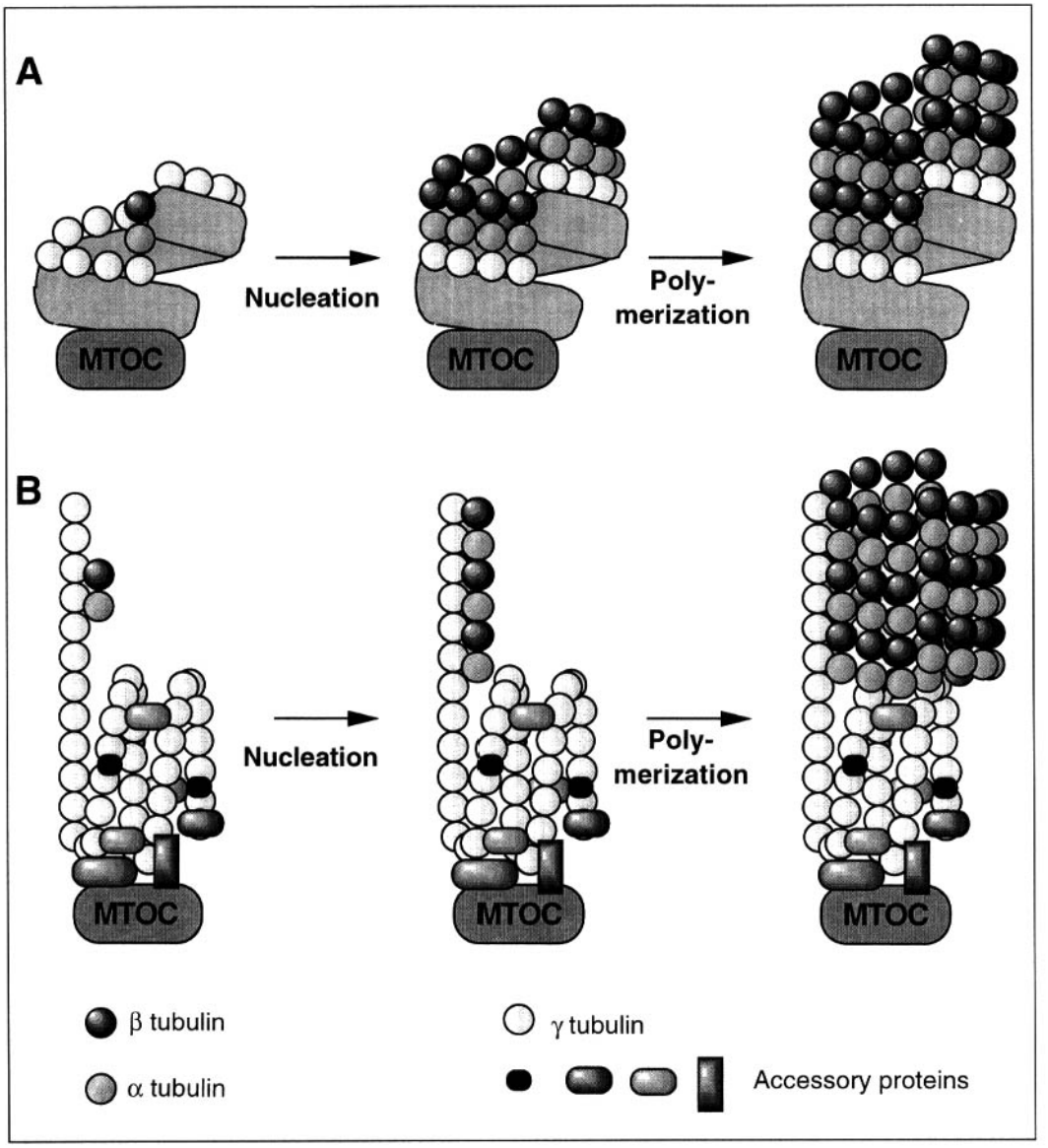

Fig. 6 Illustration of models for the nucleation of microtubules by the γ-tubulin complex at the MTOC (section 3.1.2). (A) In the first model the proteins in the γ-tubulin complex interact with the MTOC and provide a scaffold (grey helix) on which 13 γ-tubulin subunits are aligned. α-Tubulin interacts directly with the γ-tubulin and so determines the polarity of the microtubule. (B) In the second model, γ-tubulin forms a protofilament terminating in a helical structure. The polarity of the γ-tubulin units may be determined by accessory proteins which may also provide the contact sites with proteins of the MTOC. The γ-tubulin protofilament functions as stable seeds upon which additional α and β heterodimer tubulin subunits assemble. The extended microtubule sheet is then closed to a cylinder of α and β heterodimers when it contains 13 protofilaments. Adapted from ref. 63 with permission of the Company of Biologists, Ltd.

67). Spc98p was identified as interacting with Tub4p, as its overexpression rescued a conditional *tub4.1* mutant. *SPC98* encodes a 90 kDa protein identified by Rout and Kilmartin (13) as a structural component of the SPB (see Section 3.2 below). It was shown to be an essential gene and its product localizes to the same parts of the SPB as Tub4p (13, 68). A search for plasmids which, when present in multiple copies, could rescue the conditional *spc98.2* allele, identified a further essential SPB component, Spc97p (69). Extensive genetic and biochemical data convincingly showed that Spc97p, Tub4p, and Spc98p exist in a complex that contains more than one molecule of Tub4p and differs in its sedimentation coefficient from that of the *Xenopus* gammasome in being 6S rather than 24S (63, 69). These differences in γ-tubulin complex composition could provide a molecular basis to account for the closed capping of microtubule ends at the yeast SPB which contrasts with the open structures in other systems (63; see Fig. 6).

3.2 Units of self-assembly in the budding yeast SPB

An emerging theme from the analysis of the budding yeast SPB is of a number of molecules which appear to form discrete, functional, modular groupings that are able to direct self-assembly into specific structural units. As long as some aspect of the process is regulated to define limits, accurate duplication of the organelle occurs in a manner analogous to the assembly of viral coat protein complexes. The cascade of self-assembly of interacting units is initiated and maintained by the biochemical properties of the components themselves. This eccentric, yet attractive, view of SPB assembly may also apply to centriole assembly and the wider process of centrosome duplication in animal cells.

Despite the considerable hurdles placed by the low copy number of SPB components and ignorance of their biochemical characteristics, pioneering work by Rout and Kilmartin identified three spindle components. This was achieved by raising a bank of monoclonal antibodies against highly enriched SPB fractions (13). Using immunogold electron microscopy techniques, they localized molecules identified by these antibodies to different parts of the spindle: a 110 kDa antigen to the inner plaque, a 90 kDa antigen to both the inner and outer plaques (section 3.1.2), and an 80 kDa antigen adjacent to both the SPBs and the kinetochores (Fig. 7).

With the exception of the tubulins, the 110 kDa antigen is the most studied spindle pole component. Its gene, *SPC110*, was identified by three further routes after its initial description by Rout and Kilmartin (13): a screen for insoluble nuclear antigens (70), identification of mutants that compensate for the calmodulin mutation *cmd1.1* (68), and a screen of an expression bank for calmodulin-binding proteins (71). The predicted amino acid sequence of the *SPC110* gene suggests a defined domain structure with a globular N-terminus separated from a calmodulin-binding C-terminal domain by a long coiled-coil region (68, 71–73). In an elegant study, Kilmartin *et al.* demonstrated that while strains lacking the entire gene are inviable, those containing a deletion of virtually the entire coiled-coil domain survive, and the spacing between the site of microtubule nucleation and the main body of the SPB is

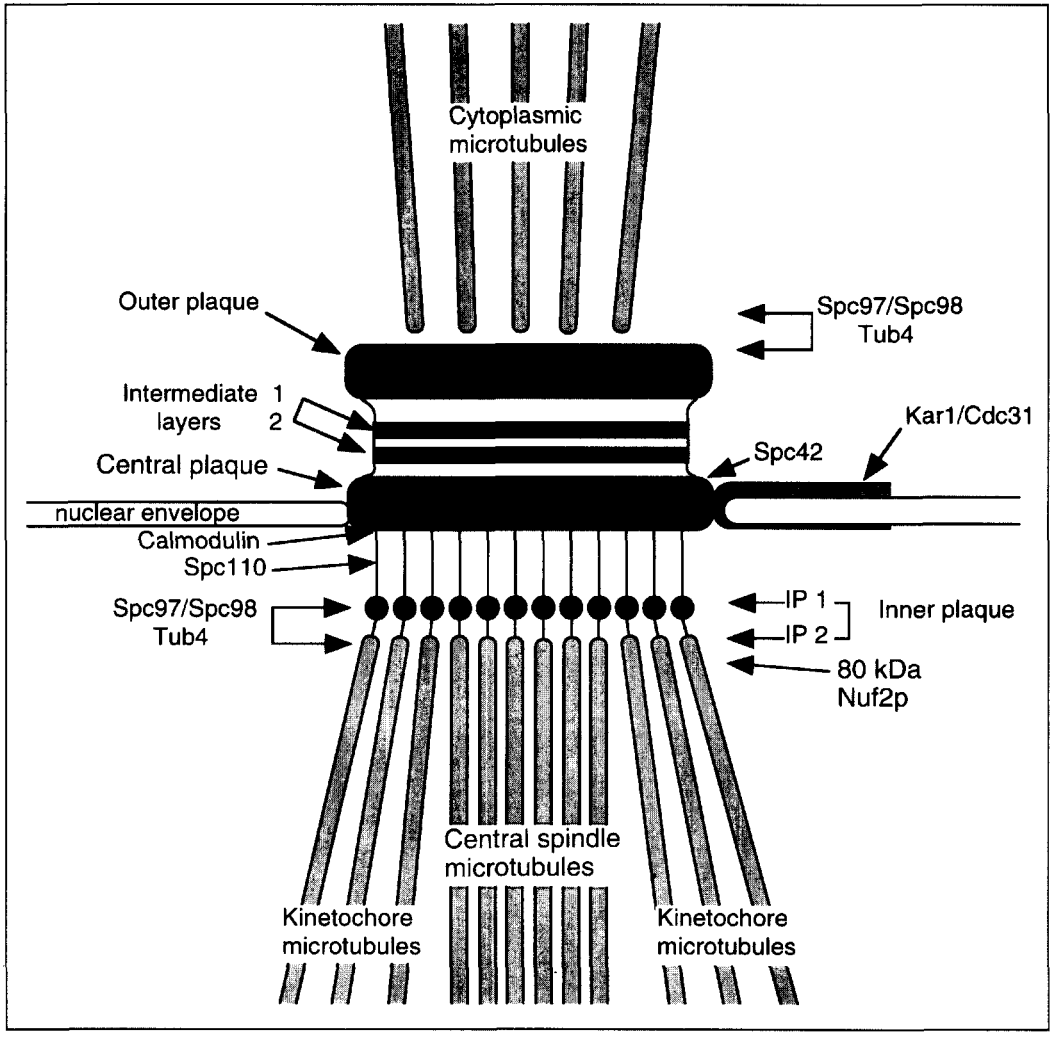

Fig. 7 A cartoon depicting the structure and composition of the budding yeast SPB. The relative location of different components to the different structural features of the SPB is presented. For further details see Section 3.2.

reduced in a manner that correlates with the amount of coil deleted, leading to the proposition that the coiled coil provides a spacing function which must serve some non-essential, but important, purpose (72). It may impart a degree of flexibility to microtubule attachment, thus enhancing the efficiency of microtubule interactions during the initial stages of spindle formation when the adjacent SPBs both have to re-orientate through 90° to face each other (72). It was later shown that the N-terminal domain lies at the inner plaque and the C-terminal domain resides, as does calmodulin, at the central plaque (49, 73, 74).

A wealth of genetic and biochemical evidence demonstrates that correct inter-

action between calmodulin and Spc110p is vital for the correct function of the full-length molecule (68, 72, 73, 75, 76). Deletion of the C-terminal region containing the calmodulin binding site suppresses the lethality of the calmodulin mutant *cmd1.1* (68). Mutations in the calmodulin gene define four internal complementation groups involved in different cellular processes: polarized growth, spindle formation, and two non-cell-cycle events (77). It would thus seem that the role of calmodulin in spindle formation is mediated through its interaction with Spc110p (71, 73, 74, 78, 79).

One interpretation of these findings is that calmodulin overcomes a block that prevents Spc110p incorporation into the SPB. This would be consistent with the phenotype of an *spc110* mutation which is unable to interact with calmodulin at the restrictive temperature (76). In a particular genetic background, cells harbouring this mutation accumulate large, amorphous intranuclear aggregates which are capable of nucleating microtubules (73, 74, 76). Furthermore, the overexpression of either full-length or internally truncated Spc110p resulted in the accumulation of remarkable polygonal nuclear structures containing Spc110p and calmodulin (73) (Fig. 8). Such complexes could be as large as 0.5 μm in diameter within the 2.5 μm diameter nucleus.

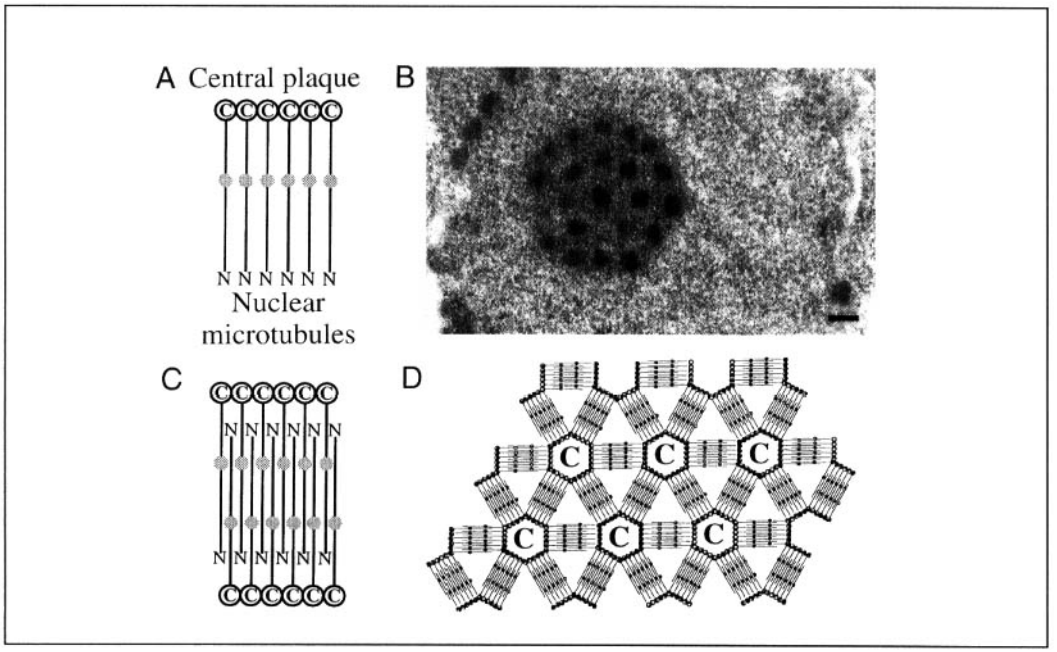

Fig. 8 Self-assembly of excess Spc 110p into polygonal structures within the nucleus. (A) Normal orientation of Spc110p as it extends with its calmodulin binding C-terminus towards the central plaque and its N-terminus at the inner plaque. (B) a electron micrograph of a nuclear Spc110p polymer; (C) and (D) the structure of this polyhedron as deduced from immunoelectron analysis of purified polymers. Scale bar represents 100 nm. Adapted from *The EMBO Journal*, 1996, **15**, 4592 (ref. 73) by permission of Oxford University Press.

The ability of Spc110p and calmodulin to assemble with such order and symmetry demonstrates that the underlying architecture of SPB modules probably lies within the primary amino acid sequence of the molecules themselves. The ability of the mutant aggregates to nucleate microtubules (74) suggests they may be able to recruit the Spc98p/Spc97p/Tub4p microtubule nucleating complex, either directly or through as yet unidentified intermediaries.

SPC42 encodes another example of an SPB component with striking self-assembly properties (15, 80). This protein is a component of the central plaque which is predicted to contain a central coiled-coil domain and is required for correct SPB duplication. *SPC42* overexpression produces a large polymeric disc composed entirely of phosphorylated Spc42p which extends from the side of the central plaque along the cytoplasmic face of the nuclear membrane (Fig. 9). The endoplasmic reticulum appears to associate with this novel structure. Electron microscope tomography of frozen hydrated preparations of the Spc42p raft reveal a remarkably organized crystalline array with hexagonal symmetry (15). This has been interpreted as representing three Spc42p dimers at each density maximum of the hexagon. Furthermore, comparison of a low resolution image of these crystalline structures

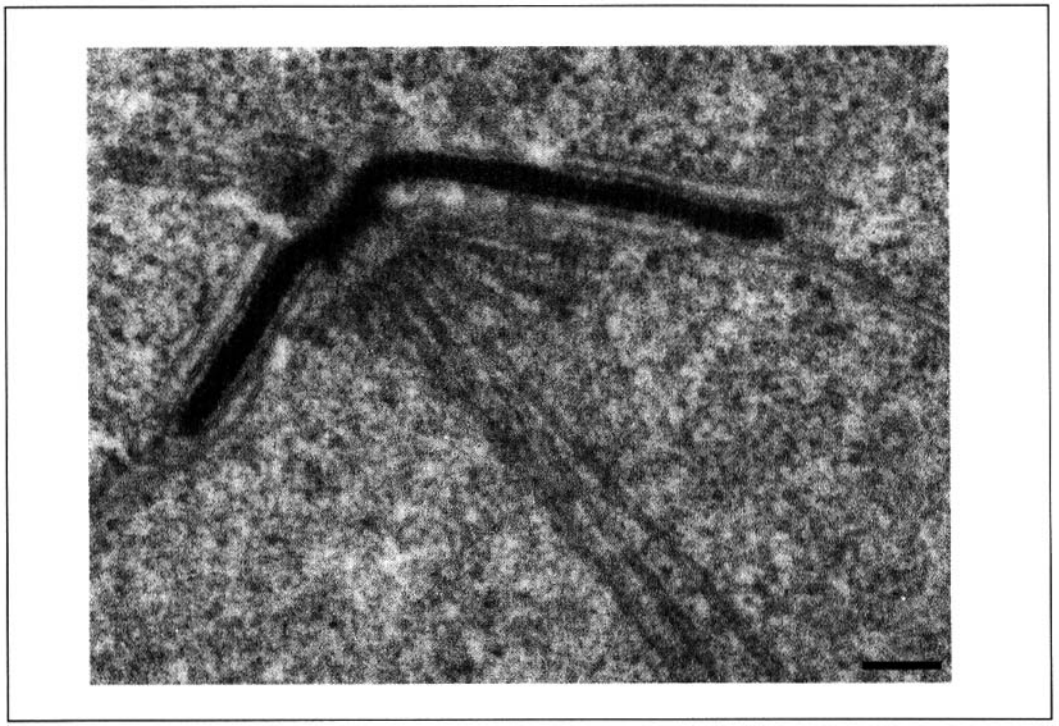

Fig. 9 Overproduction of Spc42p results in an extension of a Spc42 crystalline array from the central plaque along the outer face of the nuclear envelope. Multiple nuclear microtubules extend downwards into the nucleoplasm from the region below the main body of the SPB and not from the region underlying the large Spc42p crystalline array. Reproduced from *The Journal of Cell Biology*, 1996, **132**, 887 (ref. 80) by copyright permission of The Rockefeller University Press. Bar represents 100 nm.

with structural information obtained in the same way from heparin-extracted SPBs suggest that one layer of the SPB exhibits identical geometry to the Spc42p raft, suggesting that the core of the SPB is a highly organized crystal lattice of Spc42p upon which further modules assemble to form the SPB (15).

3.3 Yeast SPB components identified through genetic screening

At the heart of the genetic approach lies the ability to identify the role of proteins based solely upon the consequences of loss of their normal function. We will exemplify the approach by outlining studies that help to understand the participation of Kar1p and Cdc31p in the 'half-bridge' component of the budding yeast SPB.

The *KAR1* gene was identified through mutants that block nuclear fusion during mating (81). *CDC31*, on the other hand, was identified in a screen for cell division cycle mutants (82). Kar1p contains a domain that is essential for karyogamy, another that is required for both viability, SPB localization and association with Cdc31p, and a C-terminal region which is hydrophobic and directs fusion proteins to the nuclear periphery, suggesting that it acts as a nuclear membrane anchoring sequence (14, 83–85).

Strains lacking Kar1p or Cdc31p block cell cycle progression with an enlarged single SPB with an indistinct half-bridge and lacking the satellite structure (86, 87) (Fig. 10). Early studies used fusions of Kar1p with β-galactosidase (84, 85) to show that Kar1p localized to the outer face of the SPB upon overexpression and that Cdc31p co-localized at the outer face. Later immunolocalization of the wild-type molecule at the light and electron microscope level showed that this localization was artefactual and that both Cdc31p and Kar1p are components of the bridge and half-bridge (14, 88). Cdc31p requires Kar1p to localize to the SPB but there is no such reciprocal requirement (14). However, dominant *cdc31* mutations (89) bypass a requirement for Kar1p (85) and direct SPB localization in the absence of Kar1p. This suggests the presence of an additional component in the complex to which these dominant mutant forms of Cdc31p can bind in the absence of Kar1p function. Steps towards the identification of such molecules have been taken by Rose and co-workers (89) who propose that they may be encoded by suppressors of the *kar1* mutant such as the *DSK2* gene. Schiebel and colleagues (14) have also reported preliminary data suggesting that a 30 kDa protein can be cross-linked to Cdc31p. It seems that Kar1p and Cdc31p are required at a discrete stage of the cell cycle for duplication of the SPB rather than growth, since mutations in both genes lead to the absence of a satellite and the timing of the requirement for cdc31p function coincides with satellite emergence (90). Given the nucleation of microtubules by the bridge structure before SPB separation, the Cdc31p/Kar1p complex may interact at some level with the Tub4p complex.

The overall picture that emerges from studies of the SPB in budding yeast is one of functional interdependency between different modular units. This is apparent not

only from the striking oligomerization of Spc110p and Spc42p, but also from the observation that mutation of Spc110p can lead to complete instability and disappearance of the entire SPB under certain conditions (73). The startling images of heparin-extracted SPBs suggest that a crystalline array of Spc42 nucleates a symmetry of form that extends throughout the SPB that is gradually lost as one works one's way to the flexible microtubule attachment sites. This concept further enhances the view of the SPB as the product of a remarkably simple feat of assembly of interdependent substructures (15). Because a diploid SPB is larger than a haploid SPB (12), the identification of mutants where the relationship between nuclear and SPB size is abnormal may provide a key to understanding the quantum nature of MTOC duplication in eukaryotes. The potential of yeast genetics to uncover further aspects of the basic principles of MTOC organization and function is all too apparent.

3.4 Components of the SPB in *S. pombe*

The list of SPB components in fission yeast is far shorter than that amassed for budding yeast. It extends to γ-tubulin, Sad1, Cut12, and calmodulin (48, 91–93). In addition, the kinesin-related molecule Cut7, $p34^{cdc2}$/cyclin, and the protein phosphatase-related molecule Dis1 have all been shown to associate with or lie adjacent to the SPB at some time during the life cycle (18, 94–97). The 58 kDa protein encoded by the $sad1^{+}$ gene is required for the correct formation of the mitotic spindle (91, 98). It has an acidic region near its N-terminus which is followed by a stretch of 19 hydrophobic amino acids that may, as has been suggested for the C-terminal stretch of amino acids in Kar1p, direct nuclear membrane interactions. Loss of *cut12* function results in monopolar spindle formation and surprisingly semi-dominant mutants bypass the requirement for the MPF activator, Cdc25 (92).

3.5 Components of animal cell centrosomes

In the absence of genetics, animal cell centrosomal proteins have often been recognized via antibodies raised to isolated centrosomes or spindles or by the use of autoimmune antisera. Use of such antibodies has facilitated the characterization of centrosomal proteins such as pericentrin (99), CHO2 (100), and the centriolar protein, cenexin (8). The list of proteins that show some form of centrosomal location (given the caveats rehearsed above in Section 3.0) have been extensively reviewed (6, 11, 29), Some components of the vertebrate cell centrosome have been discovered via molecular studies of eukaryotic microbes; specific examples include γ-tubulin (Section 3.1) and centrin. The budding yeast gene *CDC31* described in Section 3.3 encodes centrin (101). This calcium-binding, calmodulin-related protein was first found in the flagellar apparatus of the unicellular alga *Chlamydomonas reinhardtii* where it provides a contractile function which may be directly related to its role in calcium-induced flagellar excision (9, 102). Its role in the centrosome, where it locates to the pericentriolar matrix, satellites, and striated roots of centrioles, is less clear. As

with a number of other such proteins it is apparent that there is a large pool of non-centrosomal centrin within cells (103). The fibrous material connecting the centrioles of isolated centrosomes contracts upon the addition of calcium ions (104) and it may be that centrin or a related molecule (105) plays some role in this process. Calcium spikes have been recorded during mitosis (106) but how these relate to the activity of calcium-binding proteins at the pole is also still not clear. The presence of centrin and calmodulin (see Section 3.2) at spindle poles suggest that at some level MTOC function may be regulated by calcium ions (9).

Pericentrin, which has a widespread distribution (99), was characterized using the 5051 autoimmune serum which was used extensively as a cytological marker for the centrosome. The 218 kDa protein is not abundant in cells: it has been calculated that there are around 500 molecules per centrosome. Micro-injection of anti-pericentrin antibodies into mouse oocytes resulted in serious abnormalities in spindle formation (99). Pericentrin may be a critical component of the pericentriolar matrix important for microtubule nucleation and so may be involved in gammasome recruitment.

Studies of other proteins such as NuMA (nuclear mitotic apparatus protein) (107) show that the form and structure of the centrosome may be influenced not only by nucleating microtubules but also by the action of molecules on those microtubules. NuMA is phosphorylated and released from the interphase nucleus on breakdown of the envelope at mitosis and becomes associated with spindle microtubules (108). Comparison of immunofluorescence given by anti-pericentrin and anti-NuMA antibodies suggests that NuMA does not become a component of the centrosome itself but rather is associated with the emerging cloud of microtubules concentrating at the mitotic spindle poles. Immunodepletion experiments show that NuMA forms a complex with cytoplasmic dynein and dynactin, so tethering microtubules (109). Such studies are illustrative of the likely dynamics at both interphase and mitotic spindle centrosomes, leading again to concepts of some functions being essentially directly mediated by components of the centrosome (nucleation) whilst others (microtubule movements and cross-linking) (54) are mediated by components that come to congregate at the centrosome by means of their activity. The importance of this is to understand that the form, structure and perhaps composition of the centrosome in any particular cell type will be a summation of the balance between these activities at any one particular point in time.

We have already encountered an excellent example to illustrate the importance of this balance in the structure of the centrosome in *Drosophila* γ-tubulin mutants (Section 3.1). In this case centrosomal components adopt extended amorphous morphologies simply because they cannot nucleate microtubules (50). This suggests that the very spherical nature of the centrosome itself is due to the presence of nucleated microtubules. So dependent is the centrosome upon the activity of motor proteins that, when microtubules are depolymerized in mitotic cells and then allowed to regrow, multiple asters form, indicating a dispersal of PCM (110). Taking these arguments to their logical conclusion it may be that the apparent flattening of the PCM sphere during telophase may be due to alteration of the forces acting on the pole by the motor proteins at this stage of mitosis (see Section 4.2.3).

4. Duplication cycles

4.1 SPB duplication and separation in *S. cerevisiae*

The formation of a second SPB is, along with bud emergence, one of the first landmarks of commitment to the cell cycle (Fig. 2). Conditional mutants defective in budding yeast *cdk1*, Cdc28p, arrest as unbudded cells with a single SPB and a satellite-bearing half-bridge (Fig. 10). The ordering of events in the yeast cell cycle is controlled by the successive activation of the Cdc28p protein kinase through its interactions with different regulatory, cyclin subunits encoded by the *CLN* and *CLB* genes (for a detailed review of the budding yeast cell cycle see ref. 111). The precise combination that is active at a particular stage is further determined by protein stability and a specific Cdc28p/Clbp inhibitor protein. *cdc4* mutants arrest marginally after the *cdc28* arrest point, with high Cln1p- and Cln2p-associated Cdc28p kinase activity due to an inability to degrade the Cdc28p Clb5/6p kinase

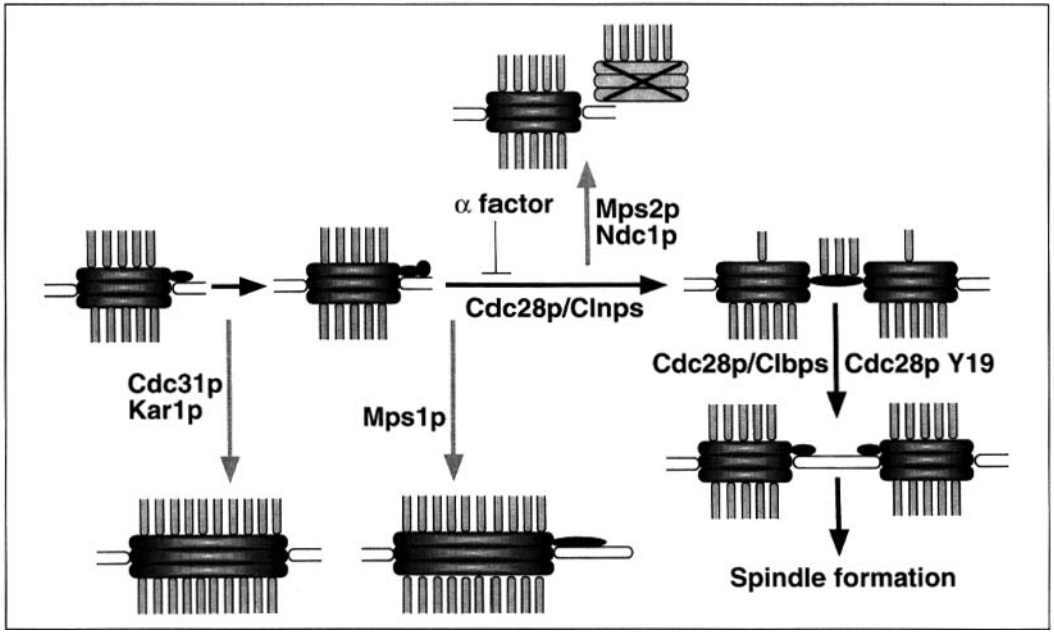

Fig. 10 The genetics of SPB duplication in budding yeast. The SPB resides within the nuclear envelope (depicted by the thin black line curving around either side of the SPB) throughout the cell cycle. The cartoon is drawn with the cytoplasmic face uppermost and the nuclear face below the nuclear envelope. The single half-bridge-bearing SPB gains a satellite structure before a new SPB appears at the end of a larger full bridge structure. Microtubules are nucleated from the cytoplasmic face of the bridge until it is split to produce two separate, half-bridge-bearing SPBs. The protein names indicate arrest points for defects in the respective proteins, whilst the grey arrows show novel morphologies which arise from defects in the indicated gene products. The relationship between the nucleation of microtubules from the bridge and the timing of action of Cdc28p/Clbp complexes, Cdc28p Tyr19 mutants and the *cin8 kip1* and *tub4.1* checkpoint arrests awaits clarification but is drawn here with microtubule nucleation occurring before all of the blocks.

inhibitor, Sic1p. These cells have duplicated SPBs connected by a full bridge structure (12, 111). Thus Cdc28p/Clnp activity is required to initiate SPB duplication. A Cdc28p/Clbp kinase activity is responsible for the breakdown of the bridge that connects newly duplicated SPBs as strains lacking *CLB1* to *CLB4* arrest with duplicated but unseparated SPBs. This artificial situation may be mimicking the inhibition of Cdc28p activity via the reversible phosphorylation of the tyrosine residue at position 19 (Thr19) in the ATP-binding domain of Cdc28p. Either mutation of this residue to glutamate or induction of a kinase capable of phosphorylating Thr19 on wild-type Cdc28p, results in an identical phenotype (112). Thr19 phosphorylation has been implicated in the checkpoint controls that link cell cycle arrest to incorrect bud emergence (113) and chromosome alignment (114, 115). It may also be involved in checkpoint pathways that sense spindle status as a *tub4* allele and *cin8/kip1* double mutants all arrest cell cycle progression with the two SPBs connected by an intact bridge (67, 116, 117). Interestingly, the microtubules emanating from the SPBs in both *clb1–4* deletants and E19 strains are angled towards each other as if attempting interdigitation. In contrast, microtubules in *cdc4* mutants show perpendicular extension from the SPB. This suggests that mitotic microtubule motor activity is active in *clb* deletants, but not at the *cdc4* arrest point (112, 118).

Several genes have now been identified in the budding yeast that are required for SPB duplication (reviewed in ref. 119). Loss of function of such genes results in the cell having only a single functional SPB and so leads to mitotic arrest with a monopolar spindle. The spindle checkpoint genes are responsible for mediating this mitotic arrest, and so, in the presence of a second mutation in such a checkpoint gene, cells undergo a monopolar mitosis to double their ploidy. *MPS1* and *MPS2* were identified in a visual screen for temperature-sensitive mutants which blocked spindle formation with a monopolar spindle (120). The *MPS1* gene encodes a protein kinase that is required both for SPB duplication and the checkpoint pathway that arrests cell cycle progression if there are defects in spindle structure (121–123). As with *kar1* and *cdc31*, the *mps1.1* mutant arrests with a large spindle pole body. However, it differs from these other mutations in being required after, rather than before, cell cycle arrest at START by alpha factor and having a prominent half-bridge as in *cdc28* mutant or alpha factor arrested cells (12, 120).

mps2.1 and *ndc1* mutants exhibit identical phenotypes to each other and direct the formation of a new SPB which is small and malformed and nucleates cytoplasmic but not nuclear microtubules (120, 124, 125). However, whereas in the *mps2.1* mutant the defective SPB fails to insert into the nuclear membrane, in the *ndc1.1* and *ndc1.4* mutants the defective SPB is associated with the nuclear membrane. The similar morphology of the defective SPBs, the fact that the predicted amino acid sequence of the Ndc1 protein contains six or seven potential membrane-spanning domains, and the ability of the molecule to associate with the nuclear periphery upon overexpression suggest that Mps2p and Ndc1p may be both involved in integration of the SPB into the nuclear membrane.

4.2 The centrosome cycle: maturation of the centriole

The intimate connection between the centrosome cycle and the cell cycle in metazoans was perhaps stated most eloquently by Mazia *et al.* (126) as a result of the finding that when mitosis was prolonged in sea urchin zygotes by one of several methods, the two spindle poles split to give four poles that could not double before the next mitosis, resulting in the formation of four monopolar spindles at division. This led to the idea that each centrosome has two determinants, and that in the sea urchin egg these normally split into their two component parts at telophase. Prolonging prometaphase did not affect this time of splitting, and yet duplication of the determinants did not occur until the cell had passed through the metaphase–anaphase transition. Subsequently, Sluder and Rieder's (127) ultrastructural examination of sea urchin eggs having undergone this treatment indicated that those centrosomes capable only of forming monopolar structures have a single centriole, in contrast to those with the ability to duplicate, which have two centrioles.

Duplication of the centrioles is semi-conservative with each daughter cell receiving (via the spindle pole centrosome) a mature and an immature centriole (128) (Fig. 11), the immature centriole being generated in the previous cell cycle and the mature centriole at least one cell cycle before that. The duplication of centrioles usually begins during S phase. The newly formed procentrioles elongate to generate full-length centrioles during S to late G_2 phase. At this stage, a cell will contain two mother and two daughter centrioles, but only one of the mother centrioles is mature. Maturation is accompanied by gaining, at the G_2–M transition, reactivity to an antibody recognizing a 96 kDa protein, cenexin (8). In mammalian cells, the formation of the procentrioles does not appear to depend upon DNA replication and, following a 48 h block to DNA synthesis, the centriole cycle will continue to the following mitosis (129, 130). However, Balczon and co-workers (131) have shown in CHO cells that centrioles and centrosomes have an ability to continue replication that is dependent upon the continued presence of growth factors in the medium.

Thus, during interphase and mitosis there are very precise events of duplication, morphogenesis, and reorientation of the centriolar components at the heart of the centrosome. It is as yet unclear how these events relate to cell cycle related changes in pericentriolar material morphology which also exhibits changes during progression through interphase, undergoing transitions from spherical to plate-like to spherical during various stages of mitosis (summarized in ref. 29). The increased nucleating capacity of the centrosome at mitosis (129, 132) correlates with a decondensation of the pericentriolar material (133). When present, the role of the centrioles within the centrosome is still unclear. However, the capacity of a centrosome as an efficient cellular organizer appears to correlate with the number of centrioles it contains (134). This has led to the idea of a role for centrioles as organizing sites for the aggregation of pericentriolar material in centriole-containing centrosomes (8).

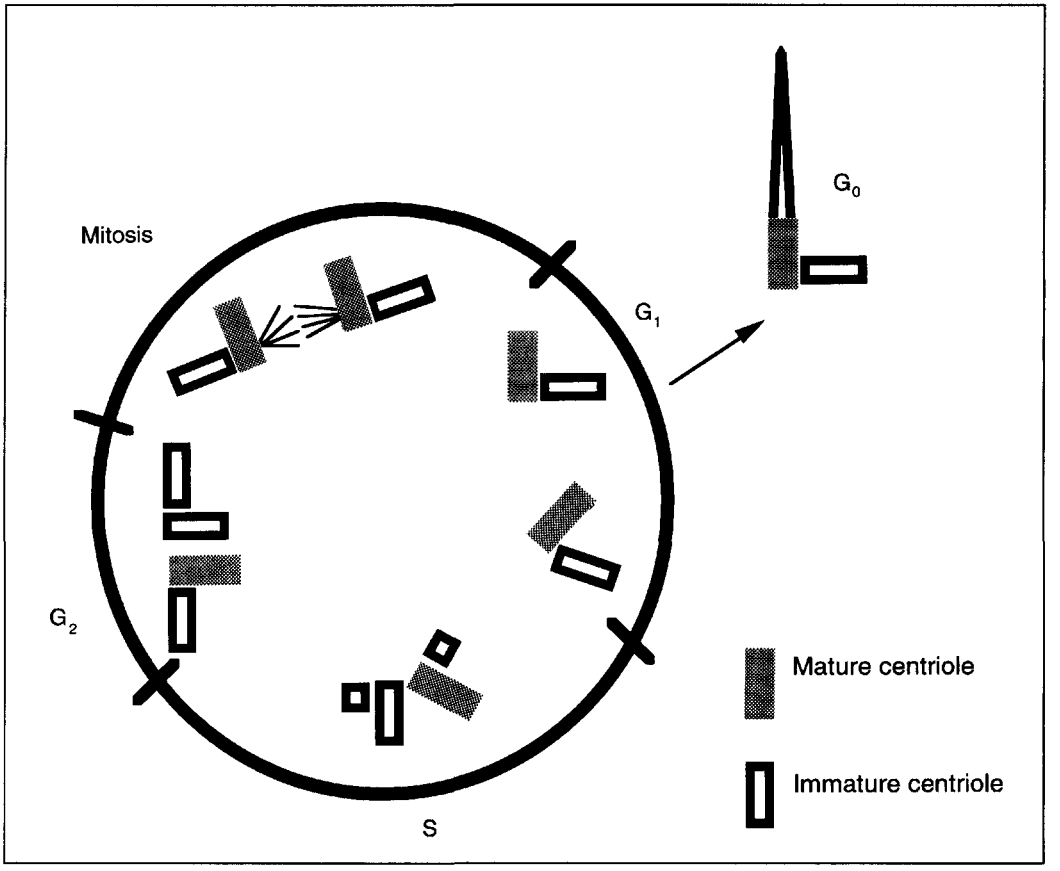

Fig. 11 The modifications to the centriole in the mammalian cell cycle. The G_1 cell has a mature centriole and an immature one (the mature centriole can subtend the primary cilium). Centriole duplication occurs around S phase, but acquisition of cenexin and centriole maturation does not occur until mitosis. G_1, G_2 and S phase represent the different stages of interphase whilst G_0 indicates a quiescent or differentiated state. Reproduced from *The Journal of Cell Biology*, 1995, **130**, 919 by copyright permission of The Rockefeller University Press.

4.3 Co-ordination of the centrosome cycle with the cell cycle

The tendency of centrosomes to attempt to continue their own division cycles following perturbation of the cell cycle was evident from Mazia's early work. The extent to which this can occur is nevertheless impressive. When aphidicolin is used to block DNA synthesis in sea urchin embryos, centrosomes continue to replicate (135). Similar results were reported by Raff and Glover (136) who, working on syncytial *Drosophila* embryos, observed an increase in the ratio of centrosomes to nuclei after aphidicolin injection, although the degree to which centrosomes can continue to replicate in this system has recently been questioned by Debec and co-workers (137) from real-time observations. Additional rounds of centrosome

duplication can occur in embryonic systems in the total absence of DNA. When sea urchin eggs are physically enucleated to leave only a single centrosome in the cell, it undergoes duplication as seen by a geometric increase in the numbers of asters to eight or more per cell (138). Sluder and his colleagues (139) have also observed continued centrosome duplication after the inhibition of protein synthesis in sea urchin zygotes, a phenomenon also reported by Gard and co-workers (140) in *Xenopus* embryos. These experiments indicate an independence of the centrosome cycle from the mitotic cycles that require protein synthesis to regenerate mitotic cyclins. This lack of a requirement for protein synthesis also reflects the fact that most embryos contain a stockpile of maternally derived proteins that include centrosomal components. In cultured cells, there is no maternal stockpile of proteins, and protein synthesis is required in late G_1 phase to permit procentriole synthesis (141).

The above experiments point to an independence of the centrosome cycle from other cell cycle events in embryonic systems. This uncoupling affects not only the centrosome cycle, but also other aspects of mitosis. Raff and Glover's (136) experiments, for example, showed that after blocking DNA synthesis, there were continued cycles of chromosome condensation, nuclear envelope breakdown, and cortical cytoplasmic contractions. This is indicative of the lack of certain of the cell cycle checkpoint controls at this developmental stage to prevent mitotic progression in the presence of incompletely replicated DNA. Such checkpoint controls come into play at later developmental stages. The syncytial *Drosophila* embryo is not entirely devoid of checkpoint controls, however, as DNA damage brought about by X-irradiation will induce a mitotic delay. In the *Drosophila* embryos derived from females carrying the *grapes* mutation, however, X-irradiation leads to a metaphase block (142, 143). The *grapes* gene encodes a homologue of the fission yeast checkpoint kinase gene *chk1*, which functions to prevent yeast cells with damaged DNA from entering mitosis (144) (discussed in detail in Chapter 1). It will be of interest to examine centrosome behaviour with respect to this checkpoint.

Much remains to be discovered about how centrosome behaviour is co-ordinated with the checkpoints that monitor cell cycle progression. In mammalian cells the tumour suppressor protein p53 plays two main roles in preventing cell cycle progression in response to DNA damage (145). First of all, it provides a checkpoint control that reversibly arrests the cell at the G_1–S transition to allow time for DNA repair. Secondly, if the damage is extensive, it may induce the cell death pathway. The suggestion of additional checkpoint roles to influence centrosome behaviour comes from a number of observations including the recent finding that fibroblasts derived from mouse embryos that lack p53 often fail in centrosome assembly and can generate multiple centrosomes in a single cell cycle (146, 147). Moreover, a separate study has shown that p53-deficient fibroblasts are also deficient in the spindle assembly checkpoint control, and fail to arrest cell cycle progression in the presence of microtubule-depolymerizing drugs (148). Perhaps these observations go some way to explaining the spindle abnormalities that were first described to be associated with cancer cells in the last century.

4.4 Centrosome separation

4.4.1 Regulation by protein phosphorylation

polo-like kinases

Whereas the conserved protein kinase $p34^{cdc2}$ brings about the major changes to cellular organization upon entry into mitosis, it has recently become clear that another group of conserved kinases act to regulate specific cytoskeletal events as cells progress through the mitotic cycle. These are the 'polo-like kinases', named after the *polo* gene of *Drosophila* (reviewed in 149, 150). Mutations in *polo* result in a variety of spindle abnormalities which include the formation of disorganized centrosomes and monopolar spindles (151, 152). Strikingly, disruption of the homologous gene of fission yeast, $plo1^+$, also prevents spindle pole body separation, leading to the formation of a monopolar structure (153) (Fig. 12). Furthermore, centrosome separation is prevented by injection of antibodies to the human polo-like kinase into cultured HeLa cells (150). Thus in spite of the huge differences in structure of the metazoan centrosome and the yeast SPB, their separation to initiate the formation of a bipolar spindle would appear to be under the control of a conserved protein kinase. This is not the only role of the polo-like kinases, however, and, at least in the yeasts, there is a requirement for the enzyme for cell division (discussed in Chapter 9). In *S. pombe*, $plo1^+$ is required to initiate both the formation of an actin ring and the deposition of septal material in cytokinesis (153), and in *S. cerevisiae*, the polo-like kinase homolgue, Cdc5, is required for late events of cell division, mutants arresting in a late anaphase-like state with the daughter cell failing to separate. Strikingly, the overexpression of $plo1^+$ in fission yeast will drive the whole process of septation in cells arrested at any stage of the cell cycle. Thus the Plo1 kinase can overcome the normal dependence of septation upon the completion of mitosis. This suggests that it is near the top of the regulatory cascade downstream of $p34^{cdc2}$ that triggers cell division and would be consistent with the localization of both plk1 and $p34^{cdc2}$ to the pole in animal cells (154–157). In animal cells the process that positions the cleavage furrow is dependent upon the position of the spindle mid-body and, in some organisms, upon the sites of the asters (see Chapter 9). Although it is fanciful to imagine that polo-like kinases might play a role in regulating such a process, this has not been demonstrated. Another mystery surrounding the polo-like kinases emerges from the purification of a *Xenopus* polo-like kinase homologue as an enzyme that will phosphorylate and so activate cdc25 (158). It remains to be seen whether this is a means of sustaining the mitotic state rather than a means of initiating entry into mitosis.

4.4.2 Other regulatory phosphorylation events

Whereas the polo-like kinases appear to have a number of functions in addition to regulating centrosome behaviour, another conserved kinase in *Drosophila* encoded by the *aurora* gene seems to be required solely for centrosome separation. Null mutants at this locus develop circular arrays of chromosomes around centrosomes that nucleate monopolar spindles (159). A budding yeast homologue, Ip11, is required for normal chromosome segregation (160). Moreover, mammalian

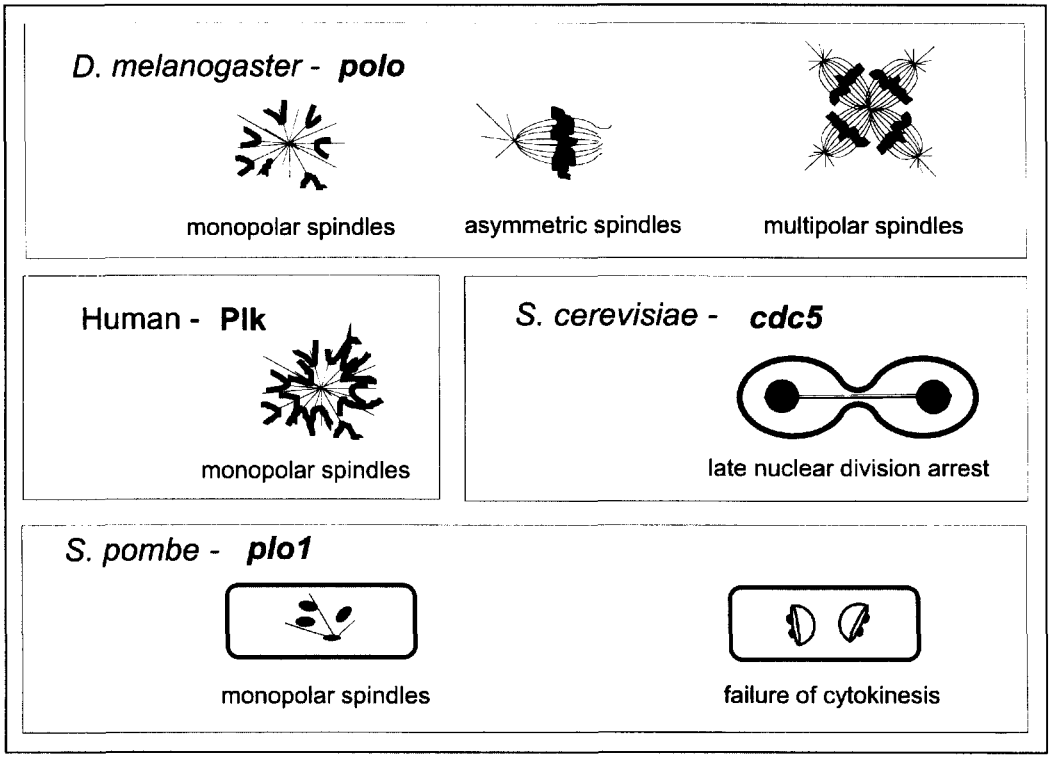

Fig. 12 Mutant phenotypes of polo-like kinases in different organisms. In humans, monopolar spindles are observed when anti-Plk1 antibodies are injected into cultured cells. Reproduced from *The Journal of Cell Biology*, 1996, **135**, 1681 (ref. 149) by copyright permission of The Rockefeller University Press.

homologues of this kinase have now been identified and found to localize to centrosomes (161; P. Donovan, personal communication). The substrates for the kinases of either of these two families remain to be determined. It is conceivable, however, that the enzymes phosphorylate and thereby activate molecules directly involved in the mechanics of centrosome separation.

4.4.3 Phosphorylation and other methods of regulation

It is clear that phosphorylation cascades must play a key role in the regulation of spindle formation and centrosome activity, although the precise pathways remain to be deciphered. In addition to the indirect evidence provided by genetic definition of so many protein kinases as spindle formation molecules, direct evidence for enhanced phosphorylation of the poles upon commitment to mitosis is provided by enhanced reactivity of mitotic poles to MPM2, a monoclonal antibody that recognizes a phospho-epitope in mitotic cells (162). Furthermore, other protein kinases—such as LK6 in *Drosophila*—have been directly localized to the centrosomes (163) and protein phosphatases such as PPX1 have also been localized to the pole of mammalian spindles (164) and so are prime candidates for dephosphorylating these mitotic substrates after they have executed their appropriate function.

In addition to phosphorylation it is likely that other forms of regulation may help to determine pole maturation and activity. A number of observations suggest that heat shock proteins are centrosome-associated, suggesting that protein folding may be another mechanism by which centrosome activity is routinely modulated (165). At what level this could be occurring is not clear but γ-tubulin is known to be complexed with the chaperone-like Tcp proteins, the Cdc37 protein, which is required for activity of the budding yeast SPB maturation kinase, and Mps1p, a member of the Hsp90 family (166). This is one of several areas that merits closer attention. A further area that may well assume increasing significance is protein degradation. BimA, which is a component of the APC complex that targets cyclin for destruction at the metaphase–anaphase transition, has been localized to the SPB in *A. nidulans* (167).

4.4.4 The mechanics of centrosome separation

The principal motile force for centrosome separation is likely to be supplied through a microtubule-based mechanism. Force is mediated upon microtubular structures through molecular motors of the dynein and kinesin-like protein families. Several families of kinesin-like proteins seem to be required for centrosome separation, of which perhaps the best studied is the *bimC* family named after the *A. nidulans* gene. Mutations in this gene and in the related fission yeast gene *cut7*$^+$ both prevent the interdigitation of astral microtubules to form a spindle (98, 168). The Cut7 protein is associated with or adjacent to the SPBs throughout mitosis, but also associates with the mid-zone of the spindle in anaphase (94). Spindle association with a tendency to concentrate near the poles has also been described for the metazoan homologues of these plus-end-directed motors (169, 170), the loss of function of which has been studied using antibodies or through mutation. The immunodepletion of the homologue Eg5 from *Xenopus* egg extracts reduces the extent of spindle formation (169, 171). Similarly, the microinjection of antibodies against human Eg5 into cultured human cells leads to a mitotic arrest with monopolar spindles (170). Mutations in the *Drosophila* gene *Klp61F* encoding a related protein also appear to prevent centrosome separation (172). However, centrosome behaviour can also be affected in a more complicated manner in some mutant alleles of *Klp61F*, resulting in the formation of bipolar monastral spindles that only have organized centrosomes at one pole (173). This ability of spindle poles to organize in the absence of an organized centrosome is not fully understood. It is discussed by Vernos and Karsenti in some detail in Chapter 4, and is thought to relate to the ability of opposing motor activities to be able to focus microtubules nucleated by chromosomes. However, as we discussed in section 3.1, the absence of any obvious pattern of immunolocalization of an antigen cannot be taken as evidence for the lack of function. Experiments by Gaglio and co-workers (174) explore the role of Eg5 in some detail both *in vivo* and *in vitro*. They show that injecting anti-Eg5 antibodies resulted in the extension of the centrosome into a ring-like structure at the MTOC of the monopolar spindles. They also show loss of function of cytoplasmic dynein results in the loss of microtubule-organizing ability. This leads to the hypothesis that the plus-end-directed motor activity of Eg5 antagonizes the minus-end-directed activity of dynein to organize the

pole. It is unlikely that Eg5 homologues are the only kinesin-like proteins that play a role in centrosome separation. Boleti *et al.* (175) have demonstrated a role for another kinesin-like protein, Xklp2, in this process, and it seems likely that the co-operation of several motors is required to maintain the integrity of the spindle structure *per se*, and to form and position it within the cell.

Reider and colleagues (176) have shown that centrosome separation is not dependent upon microtubule interdigitation in newt lung cells, suggesting some other mode of pole separation. A likely candidate could be the actin/myosin system, but its role is less clear. Such a role is implicit from the phenotype of mutations in the *twinstar* gene of *Drosophila* that affect both centrosome migration and cytokinesis. *twinstar* encodes a member of the cofilin family of small actin-severing proteins; in the absence of its function, large actin aggregates accumulate in association with centrosomes (177). The mechanisms whereby these disruptions to the actin cytoskeleton affect centrosome migration are unclear, although a role for the actin cytoskeleton in tethering microtubules to establish spindle orientation has been proposed in both yeasts and nematodes (10, 178).

5. Conclusions and perspectives

The elucidation of the function of the SPB or centrosome through an understanding of its components has been hampered by the biochemical challenges of working with molecules present in low amounts in the cell. However, a reliance upon immunological approaches has been justly rewarded. Such long-term investments may now be superseded by the application of novel approaches such as mass spectroscopy for protein sequencing and peptide identification from gel slices containing proteins of interest. Genetic approaches which are less constrained by problems of quantity as some of the novel biochemical approaches will continue to play a major role in the identification of the molecular components of eukaryotic centrosomes. However a protein is identified, there is considerable anticipation that a number of genome sequencing projects currently underway will enable the rapid identification of homologues in different species. There will be an unprecedented opportunity to move between different experimental organisms to exploit particular advantages of different systems.

Progress will be aided by technical advances in cell biology such as the recent application of fluorescent markers for studying the subcellular behaviour of molecules in real time. This may be achieved either by tagging genes with a sequence that encodes the jellyfish green fluorescent protein (179), or by the micro-injection of labelled components directly into living cells (e.g. 137, 180). These approaches will enable analysis of the centrosome cycle in real time in a number of different organisms and equate these events with other landmark events of division. A powerful complement to the *in vivo* studies will be provided by the parallel development of *in vitro* systems for centrosome duplication and activation assays that will enable functional studies by depletion and addition of specific molecules (181–185). However, even taking the potential of these developments into consideration, our

ability to accomplish the goal of understanding the duplication cycle and function of spindle pole MTOCs will be a test of ingenuity.

Many questions remain unanswered, and no doubt many problems still lie undisturbed. How is the replication cycle of the SPB controlled? What is the signal that initiates its duplication and finalises its completion? What determines the final size of the SPB? What constitutes the duplication mechanism of either the SPB or the centriole? How is the replication and function of the PCM influenced by the centrioles? How is the size of the replicating centrioles and PCM regulated? How many motor proteins are present in the SPB or centrosome, how do they co-operate at different stages of the cycle, and how is their role in centrosome separation and spindle function regulated? What is the relationship between the regulation of centrosome separation and cytokinesis? What regulates the positioning of centrosomes within a cell prior to mitosis? To what extent does the spindle pole act as an organizing centre in terms of regulating cell cycle progression?

We are at a turning point in cell biology. The opportunities to understand this crucial organelle, first described over a century ago, lie within our grasp.

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4 Microtubule dynamics, molecular motors, and chromosome behavior

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1. Introduction

Perhaps because of its aesthetically pleasing and exquisite geometrical shape, the mitotic spindle has fascinated researchers for more than a hundred years. In 1896, Wilson (see ref. 1) had already described accurately the shape of the spindle and the principal phases of mitosis. He described how two centrosomes moved around the nucleus, how fibers grew between the migrating centrosomes and how chromosomes eventually aligned on the 'equatorial plate' of the mitotic spindle. It was already clear that chromosome segregation took place on the fibers of the spindle during anaphase, providing the two daughter cells with equivalent chromosome numbers.

We now know that these fibers are microtubules and that it is their arrangement into two antiparallel arrays that defines the shape and bipolarity of the spindle (Fig. 1). This bipolar arrangement ensures that chromatids are segregated to two opposite poles to form two daughter cells. Positioning of the chromatids on the metaphase plate before their segregation is also very important because this is the mechanism by which each daughter cell inherits only one of the two chromatids from each chromosome during cell division (Fig. 1).

Assembly of the bipolar spindle and the positioning of chromosomes on the metaphase plate require precise control of the length and orientation of microtubules. This involves a tight regulation of their dynamics, both globally in the cytoplasm and locally around the chromosomes, a regulation of microtubule nucleation, and the action of motors, both cytoplasmic and chromosome-bound (2–5). Most of these events are probably regulated by phosphorylation–dephosphorylation reactions under the control of the master mitotic kinase, the cyclin-B-dependent kinase (6). In fact, this enzyme probably activates a network of kinases and phosphatases that determines the shape of the spindle by modulating precisely microtubule dynamics and the activity of motors. This enzymatic network seems to behave as a sort of 'coordinator' of the mitotic dance. In the light of the

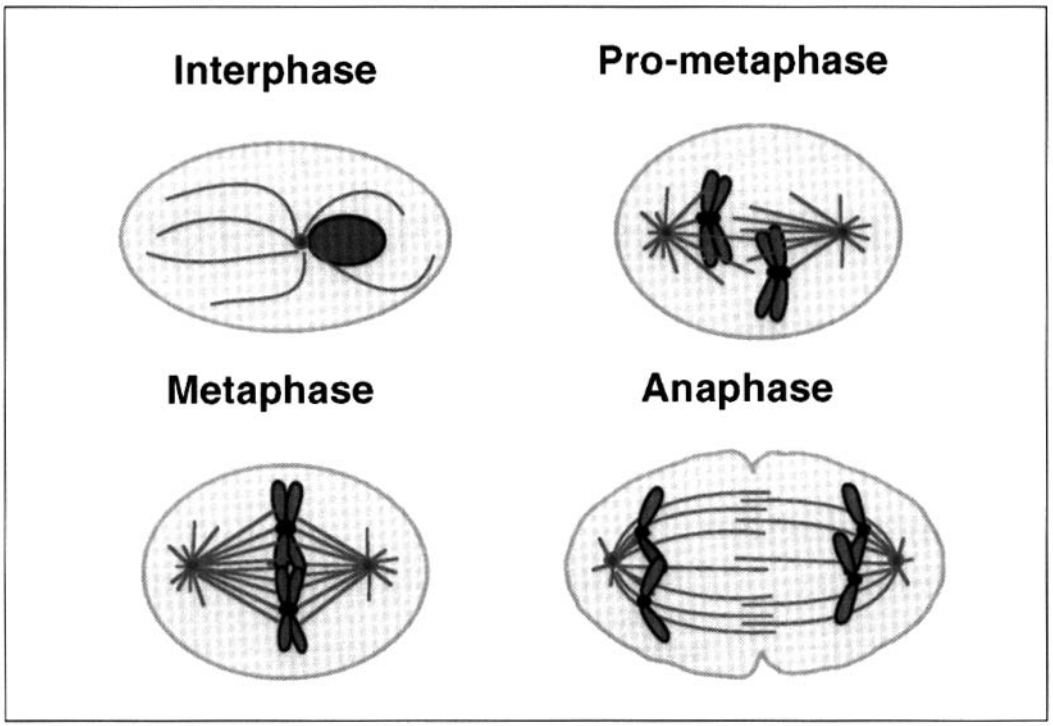

Fig. 1 Schematic representation of microtubule organization during the cell cycle. Centrosomes are represented as grey circles and kinetochores as black circles. In the interphase cell, microtubules irradiate from a centrally located centrosome towards the cell periphery. During mitosis the microtubule interphase network disassembles. Two centrosomes nucleate dynamic microtubules which start to interact with condensed chromosomes. The two antiparallel microtubule arrays overlap near the chromosomes. Metaphase is characterized by the alignment of chromosomes on the equatorial plate. At anaphase sister chromatids migrate to opposite poles and the spindle elongates, increasing the distance between the separating chromatids.

recent work done in this field, we will examine in this chapter how the dynamic properties of microtubules, the activity of motors, and phosphorylation–dephosphorylation reactions generate a functional mitotic spindle that can segregate chromosomes faithfully.

2. Microtubule dynamics during the cell cycle

When cells enter mitosis, there is a dramatic change in microtubule dynamics which is associated with the assembly of the mitotic spindle. The half-life of individual microtubules changes from about 10 min in the interphase network to seconds in non-kinetochore microtubules (7–9). Since the discovery of dynamic instability (10), we know that microtubules exist in dynamic equilibrium with tubulin subunits, growing and shrinking by addition or loss of tubulin dimers from the ends of the microtubules (11). Individual microtubules switch stochastically between phases of

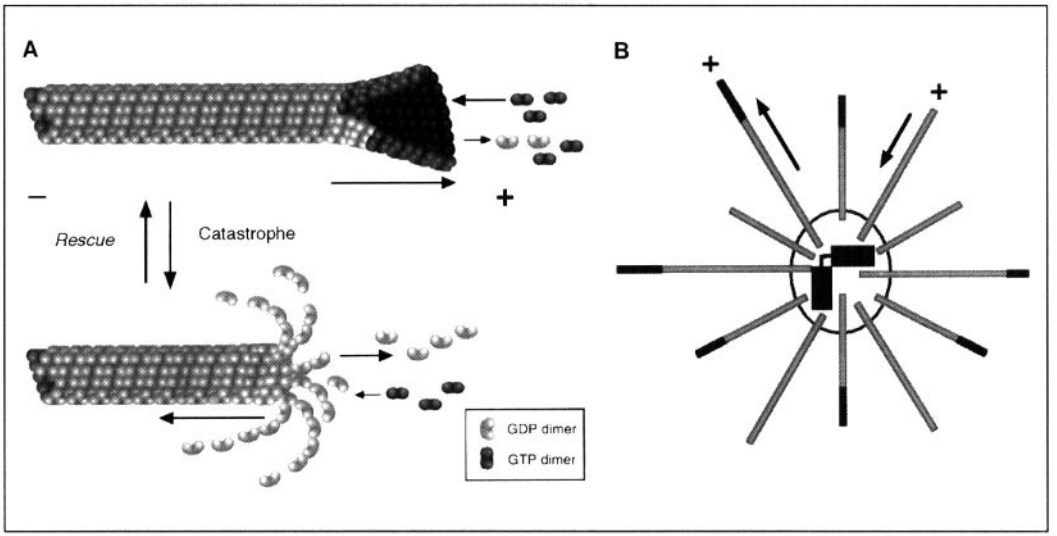

Fig. 2 Microtubule dynamic instability. (A) The upper microtubule is growing. GTP–tubulin subunits are incorporated at its plus end, which has an open sheet conformation. The lower microtubule is depolymerizing. GDP subunits are released. The microtubule plus end has protofilaments that are curling. The switch of microtubules from the growing to the shrinking state is called 'catastrophe', while the transition between the shrinking and the growing states is called 'rescue'. (B) Schematic representation of microtubules nucleated at a centrosome. At a given time some microtubules are growing while others are shrinking. Microtubules can switch between periods of growing and shrinking. The centrosome is represented by a circle containing two black centrioles. Microtubules in the shrinking state are represented by an open box corresponding to the GDP–tubulin lattice. Microtubules in the growing state have a black box at the plus end corresponding to GTP–tubulin.

slow growth and fast shrinkage so that in a microtubule population some will be growing and some shrinking (12). Microtubule dynamics is therefore defined by the rate of growth, the rate of shrinkage, the transition frequencies between growing and shrinking (catastrophe) and shrinking to growing (rescue) (Fig. 2). During the interphase–mitosis transition, microtubule dynamics is increased through an increase in catastrophe rate (13, 14). The molecular analysis of the regulation of microtubule dynamics during entry into mitosis is just beginning. Before describing recent discoveries that shed some light on this interesting problem we will briefly summarize the present knowledge of the nature of microtubule dynamics in pure tubulin solutions.

2.1 Microtubule dynamics *in vitro*

In vitro, the growth of microtubules can be nucleated by the addition of centrosomes to pure tubulin. Under these conditions, the wall of the microtubules is formed of 13 protofilaments (Fig. 3) (15, 16). Microtubules elongate by addition of tubulin subunits to the plus ends of the protofilaments, and polymerization appears to be a diffusion-limited reaction (17). Each tubulin dimer brings two molecules of GTP into the lattice, one of which is rapidly hydrolyzed (18, 19). GTP hydrolysis is not required for

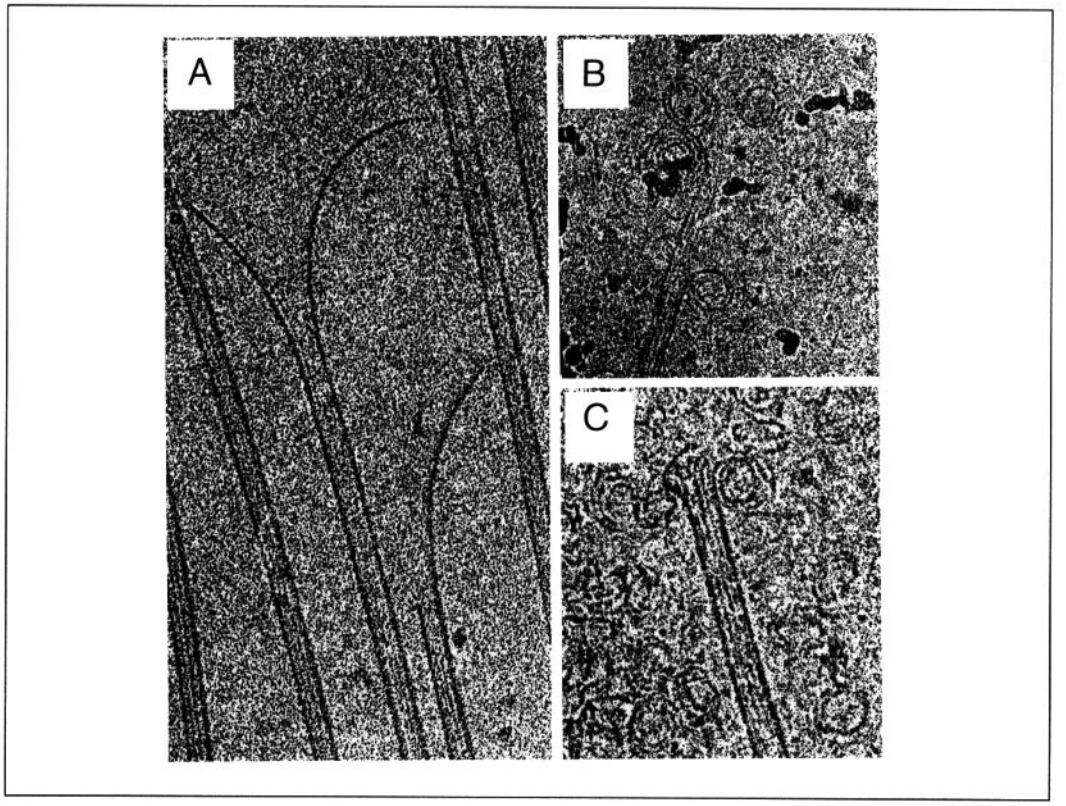

Fig. 3 Cryo-electron microscopy of vitrified microtubules. (A) Growing microtubules have an outwardly curved sheet at their plus end which closes to form the microtubule cylinder (photo courtesy of Denis Chrétien). (B, C) Depolymerizing microtubules disassemble by release of small oligomers that curl from the plus end (photos courtesy of Thomas Müller-Reichert).

assembly but is instead essential to produce unstable microtubules (17, 20). Although it is not yet entirely clear why microtubules undergo catastrophes stochastically, recent experiments indicate that a few GTP-like subunits at the end of a microtubule are sufficient to stabilize it against depolymerization (21). Interestingly, the structure of microtubule ends is different during growing and shrinking phases (Fig. 3). Microtubules grow by the elongation of sheets of slightly curved protofilaments that close into a tube further down the lattice. They shrink by losing bits of protofilaments that curl over as they 'peel' off from the microtubule lattice (Fig. 3) (22–24). A catastrophe occurs therefore when the structure of microtubule ends switches from a sheet to curled protofilaments. It seems that GTP hydrolysis changes the conformation of the tubulin subunit from straight to curled (25–27). So, it may be that when, by chance, all subunits at the tip of a growing microtubule are in a GDP form, lateral interactions between protofilaments become weak because of the curled shape of the subunits and the protofilaments start to peel off. It has been proposed that GTP

hydrolysis is forced by closure of the tube into a cylinder, thereby suggesting that the stochastic occurrence of catastrophes is due to the stochastic closure of sheets into tubes (3, 28). Microtubules start to grow off centrosomes when the tubulin concentration is higher than 7 μM. Catastrophes are observed below 12–15 μM. At 20 μM, roughly the free tubulin concentration in the cell cytoplasm, microtubules grow at about 2 μm/min and catastrophes never occur (29).

2.2 Microtubule dynamics *in vivo*

In vivo, microtubules grow about five times faster than *in vitro* for an equivalent concentration of tubulin (9, 13, 29–31). This stimulation of growth rate is due to microtubule-associated proteins (MAPs) which bind to the wall of the microtubule and it can indeed be mimicked *in vitro* by addition of MAPs to pure tubulin (32–35). Two kinds of MAPs have been identified: thermostable MAPs, like MAP4 (36, 37) and *Xenopus* XMAP230 (32, 38, 39) suppress catastrophes and moderately increase the growth rate whereas another *Xenopus* MAP (XMAP 210) (35, 40) does not suppress catastrophes but increases the growth rate dramatically. In contrast to what happens in pure tubulin solutions, the high growth rate observed *in vivo* is accompanied by a high catastrophe frequency. This suggests that other factors may be involved in microtubule destabilization. Two destabilizing factors, XKCM1 and OP18/stathmin, have been identified recently. XKCM1 is a *Xenopus* kinesin-like protein (KLP) (41) homologous to the human kinetochore protein MCAK (42). OP18/stathmin is a phosphoprotein present at elevated levels in many cancer cells (43–46). Immunodepletion of either protein from mitotic *Xenopus* egg extracts results in an increased microtubule length (41, 47). It was also shown that spindle assembly in XKCM1-depleted extracts is abnormal, with centrally localized chromatin from which long microtubules emanate. Analysis of the effect of immunodepletion of XKCM1 or Op18/stathmin on individual microtubule dynamics showed that these proteins increase the catastrophe rate without affecting the other parameters of dynamic instability. Moreover, immunodepletion of both factors is additive, suggesting that they act independently on microtubule dynamics. It is not yet clear how these factors work at the molecular level. Op18/stathmin may work by binding to the tubulin dimer in solution and affecting its properties for assembly. XKCM1 may 'walk' towards the microtubule plus ends and trigger a catastrophe by forcing the splaying apart of protofilaments that is observed in shrinking microtubules (22, 23, 41). Therefore, *in vivo*, microtubule dynamics is determined and governed by the tubulin concentration present in the cell cytoplasm, the nature and concentration of MAPs which stabilize and increase the growth rate and the activity of factors that destabilize microtubules (Fig. 4).

2.3 Regulation of microtubule dynamics during the cell cycle

How can these parameters be modulated in such a way as to generate a mitotic spindle during mitosis? First, how are the dynamics increased during the interphase–mitosis

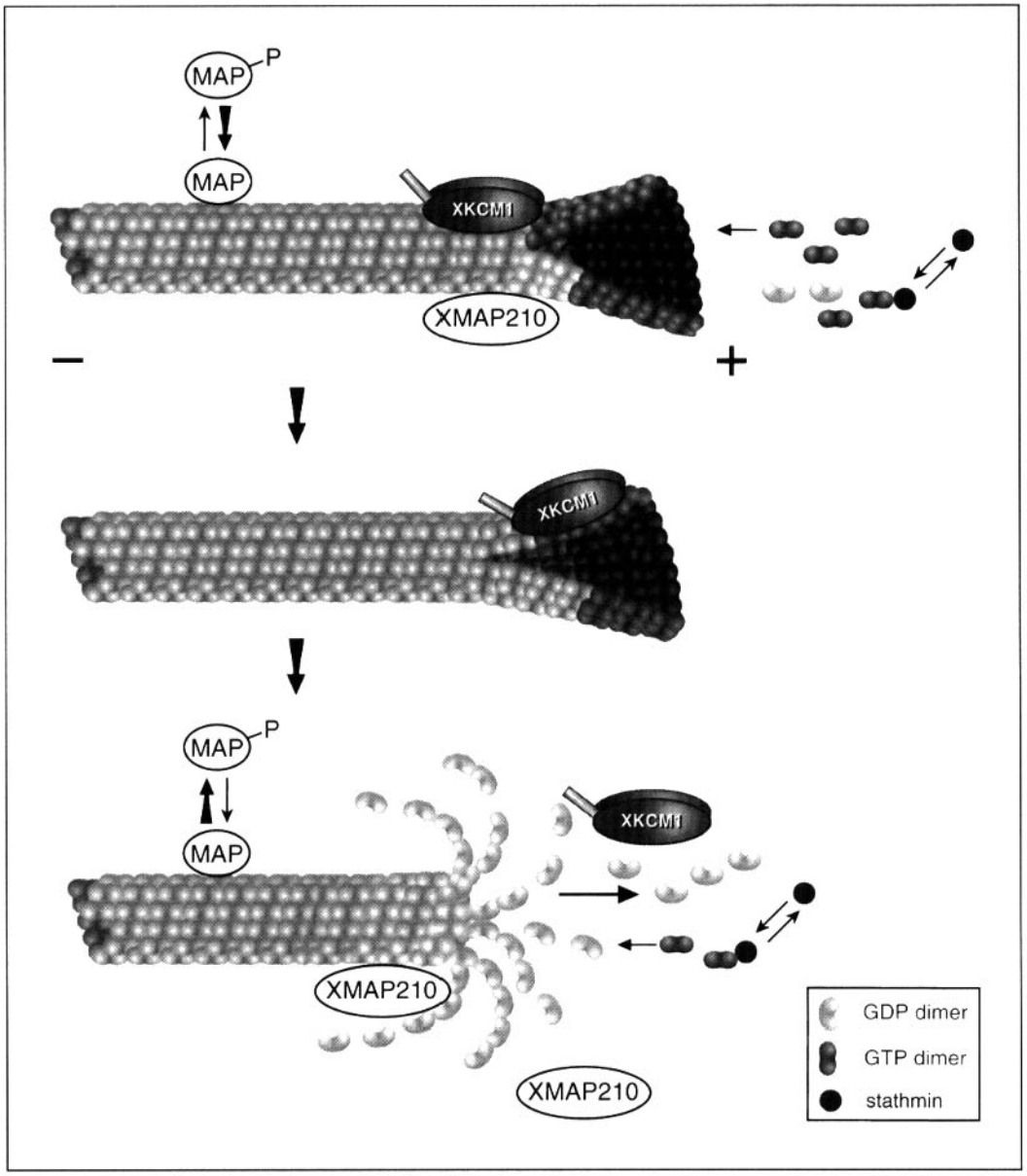

Fig. 4 Speculative view of the role of XKCM1, OP18/stathmin and MAPs in the regulation of microtubule dynamics. The upper microtubule is growing. Dephosphorylated MAPs (like XMAP230) attach to the microtubule wall and stabilize it. Others (like XMAP210) also bind to the microtubule wall and increase its growth rate. OP18/stathmin is a soluble cytoplasmic factor that binds to tubulin subunits. By moving towards the microtubule's plus end, XKCM1 promotes microtubule depolymerization, perhaps by pulling the protofilaments apart. The lower microtubule depolymerizes. MAPs like XMAP230 are phosphorylated and released from the microtubule wall. OP18/stathmin binds to tubulin dimers, lowering the amount of free tubulin available for polymerization. The net result is microtubule depolymerization.

transition? It is now clear that protein phosphorylation dependent on cdc2 kinase activity is responsible for the increased catastrophe rate observed in mitosis (13, 31, 48). Stabilizing factors such as XMAP230 and MAP4 appear to be inactivated by a cdc2-dependent phosphorylation during mitosis (32, 38, 39, 49, 50). On the contrary, XMAP210, which promotes the high growth rate observed *in vivo*, is probably not inactivated during mitosis since if anything, there is an increase in growth rate. Destabilizing factors like OP18/stathmin and XKCM1 may also be regulated during mitosis but we still know very little about this. OP18/stathmin becomes highly phosphorylated during mitosis, suggesting that phosphorylation activates its destabilizing activity (51). Overexpression of the wild-type protein, or the protein mutated in the phosphorylation sites by cell transfection, results in microtubule destabilization in interphase. The mutant protein destabilizes the spindle microtubules, but, unexpectedly the wild-type protein does not, suggesting that mitotic phosphorylation of OP18/stathmin inhibits its activity (51). This does not fit with the observation that depletion of OP18/stathmin from mitotic extracts results in an increase in microtubule length (47) and further studies are necessary to understand the regulation of the activity of this protein (Fig. 5).

Overall, one could propose the following scenario to explain the change in microtubule dynamics that takes place during the interphase to mitotic transition (Fig. 4): MAPs, like XMAP230, stabilize microtubules in interphase by binding along their length and keep the catastrophe frequency at a relatively low level. During mitosis, these MAPs are phosphorylated, their affinity for microtubules is reduced and the catastrophe frequency is increased. MAPs of the type of XMAP210 that generate a high growth rate both in interphase and mitosis, do not participate in the change in microtubule dynamics upon entry in mitosis. How the activity of destabilizing factors like OP18/stathmin and XKCM1 is modulated between interphase and mitosis is still unclear. However, some factor of this type must function during mitosis to explain the high microtubule dynamics observed. This scenario is still highly speculative but it should be possible to test it in the next few years.

The regulation of microtubule dynamics during the interphase to mitotic transition may have at least two functions. First the highly dynamic microtubules nucleated in mitotic cells result in microtubule populations with defined steady-state lengths (31, 52). This certainly plays an important role in the determination of the size of the mitotic spindle. Second it provides a possibility for the differential stabilization of a subset of microtubules in specific cellular domains (11). This can be achieved by capture and stabilization of microtubules at specific sites and/or by local modulation of the activity of MAPs and destabilizing factors. Microtubule dynamics is also coupled to chromosome movements as will be discussed in the next section.

3. Chromosome movements during mitosis

Following nuclear envelope breakdown at prometaphase, chromosomes are released in the cytoplasm and start to interact with astral microtubules (53). The first clear chromosome movements occur when microtubules emanating from the two

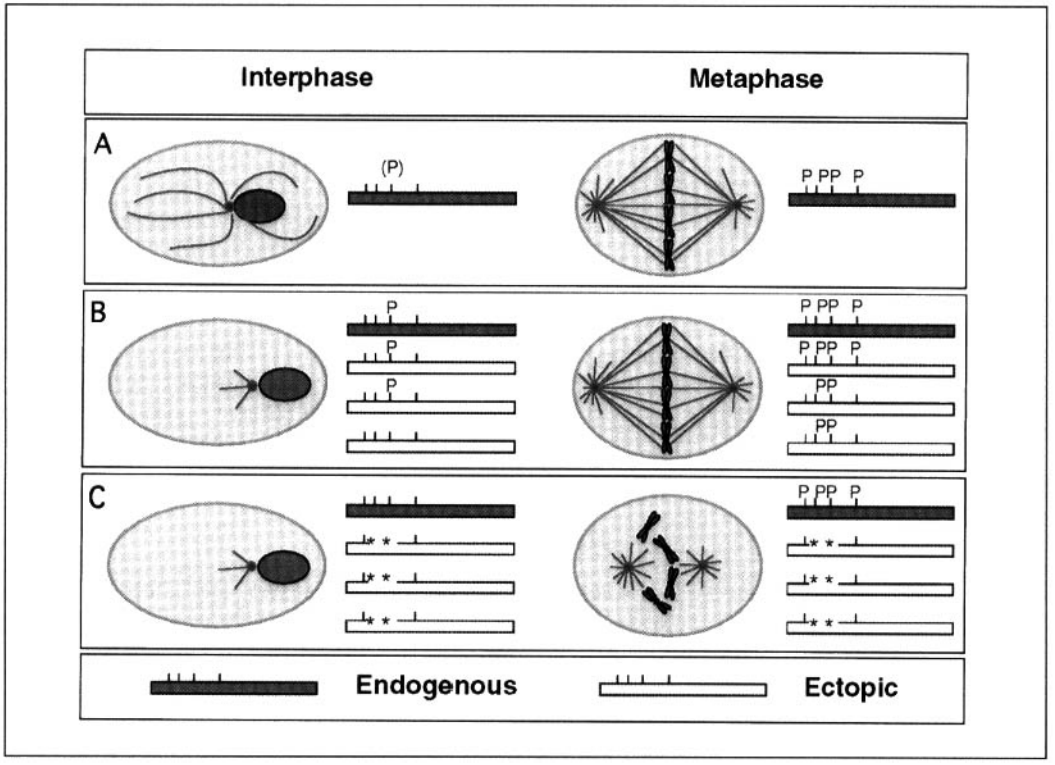

Fig. 5 Overexpression of OP18/stathmin in culture cells. Wild-type OP18/stathmin, or the protein mutated at phosphorylation sites, was overexpressed by cell transfection. The four phosphorylation sites and their phosphorylation state is shown for every case. Mutated phosphorylated sites are represented by a star. (A) In non-transfected cells, OP18/stathmin is not phosphorylated in interphase, and microtubules are long and relatively stable. In mitosis, OP18/stathmin is phosphorylated and dynamic microtubules are organized into a mitotic spindle. (B) Overexpression of wild-type Op18/stathmin induces the shortening of microtubules during interphase but the mitotic spindle can assemble normally during mitosis. (C) Overexpression of OP18/stathmin mutated at two phosphorylation sites induces the shortening of microtubules during interphase. Microtubules remain very short during mitosis and a mitotic spindle cannot assemble. Adapted from ref. 51.

centrosomes hit a kinetochore and become attached to it (54, 55). For centrally located chromosomes, each kinetochore becomes attached to microtubules nucleated at the closest pole, resulting in a rapid bi-orientation. The chromosomes which are close to one pole become mono-oriented because only one kinetochore interacts with and captures the microtubules emanating from that pole. While the chromosome slides along these microtubules towards the pole, more microtubules make contact with the kinetochore resulting in the formation of a kinetochore fiber. Mono-oriented chromosomes capture microtubules emanating from the opposite pole at the free kinetochore and become bioriented. Chromosomes start to oscillate, switching rapidly between movements towards and away from the poles (56). These oscillations culminate in the alignment of chromosomes on the metaphase plate (Fig. 6).

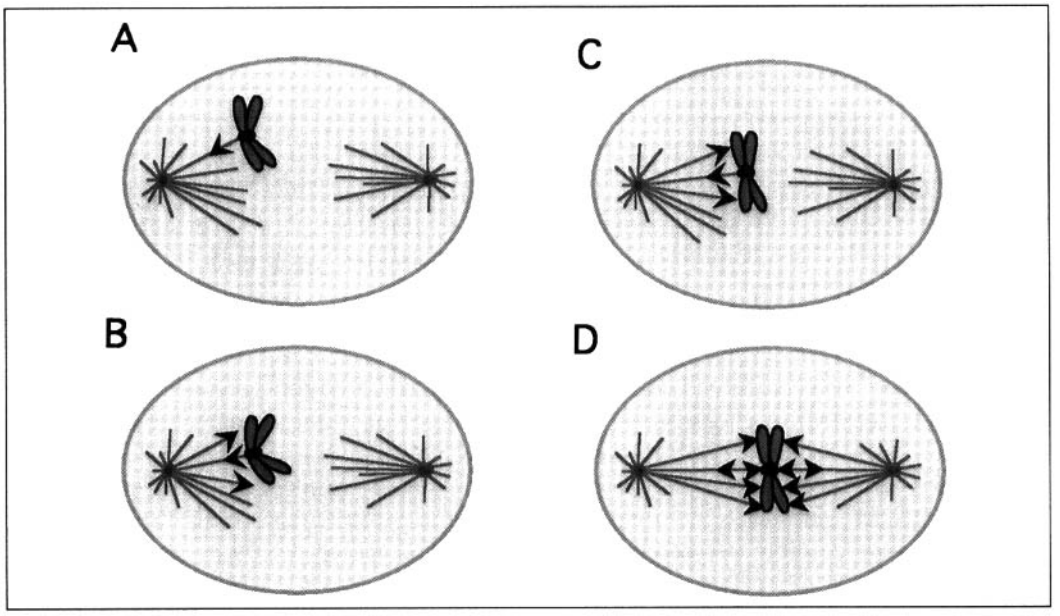

Fig. 6 Chromosome capture and oscillations in the initial phases of mitosis. Only one chromosome is shown for simplicity. (A) A condensed chromosome interacts at the kinetochore with microtubules emanating from the closest pole and moves towards it. (B) Polar microtubules emanating from that pole interact with chromosomes arms and push them away from the pole. (C) The movement back towards the equator favors the interaction of microtubules emanating from the opposite pole with the sister kinetochore. (D) Chromosomes oscillate between the pole and the equator and eventually congress at the equator of the spindle at metaphase. In this diagram, the forces acting on the chromosome are represented by black arrows.

To explain how chromosomes move between the equator and the poles, Ostergren in 1951 proposed a 'traction fiber model' (57). In this model, the force that moves a kinetochore away from its associated pole is generated by a force exerted at its sister kinetochore pulling it towards the opposite pole. Congression occurs because the strength of the pulling force is proportional to the length of the kinetochore fiber. In metaphase both kinetochore fibers have the same length and thus the antagonistic pulling forces are equal. Several observations have challenged this model, in particular the following: a kinetochore can move away from its pole even when its sister kinetochore is unattached or absent (58); severing one of the two kinetochore fibers at metaphase does not significantly shift the chromosome towards the opposite pole (59, 60; see also ref. 61 for more references). These observations indicate that the kinetochore fiber itself is not exerting the force that moves kinetochores. Finally the centromere is stretched poleward during chromosome poleward motion while it is flattened or indented during chromosome movement away from the pole (62). These observations favour the idea that the forces moving kinetochores during prometaphase are produced at the kinetochore itself and not along the kinetochore fiber.

3.1 Microtubule dynamics at kinetochores and chromosome movements

Skibbens and co-workers (63) observed that kinetochores switch autonomously and abruptly between phases of constant movement towards one pole and phases of constant movement away from that pole. They called this property 'directional instability'. Two important factors affect kinetochore directional instability, namely the assembly and disassembly of the kinetochore fibers and the tension produced at the kinetochores.

For vertebrate tissue cells, movement of kinetochores away from the pole is coupled to microtubule assembly while poleward movement is coupled to microtubule disassembly. Laser photobleaching and severing experiments have shown that microtubule assembly and disassembly occurs at the kinetochore (64–68). This raises the question of how microtubule dynamics is regulated at the kinetochore and how kinetochores follow dynamic microtubule ends. The coordination of chromosome movement with microtubule assembly and disassembly at kinetochores may involve kinetochore motors as suggested by the assay of Lombillo *et al.* (69). They showed that beads coated with pure plus-end-directed motors can attach to the plus ends of microtubules emanating from *Tetrahymena* pellicles and remain attached to them when microtubules are induced to depolymerize by tubulin dilution, mimicking a minus-end movement. This assay mimics in some ways the attachment of a kinetochore to a microtubule depolymerizing end and shows that a motor can function in this process. Two kinesin-like proteins, CENP-E and MCAK/XKCM1, have been localized at kinetochores. CENP-E has an N-terminal motor domain and is localized in the corona region of kinetochores (70). MCAK has a central motor domain and is localized throughout the centromere region and between the kinetochore plates of isolated CHO chromosomes (42) (Fig. 7). Antibodies against CENP-E block microtubule disassembly-dependent chromosome motion suggesting that this protein could function *in vivo* by tethering chromosomes to depolymerizing microtubules. In contrast, antibodies against MCAK do not block chromosome motion under the same conditions (71). Thus, CENP-E is the only motor for which there is experimental evidence indicating that it could coordinate chromosome movements with microtubule assembly and disassembly. However, the structural organization of MCAK and its location suggest that it could also participate in the coordination of microtubule dynamics with chromosome movements although there is no functional experiment supporting this idea. Interestingly, however, a homolog of MCAK, called XKCM1, has been shown to increase the catastrophe frequency of microtubules in *Xenopus* egg extracts (41). This supports the idea that motors like MCAK could regulate microtubule dynamics at the kinetochore. The other minus-end-directed kinetochore motor known is dynein. It is localized in the corona region of kinetochores. Given its directionality of movement, it is tempting to suggest that it is involved in the poleward movement of chromosomes although, again, there is no functional experiment to support this idea.

The other parameter that could regulate kinetochore 'directional instability' is the

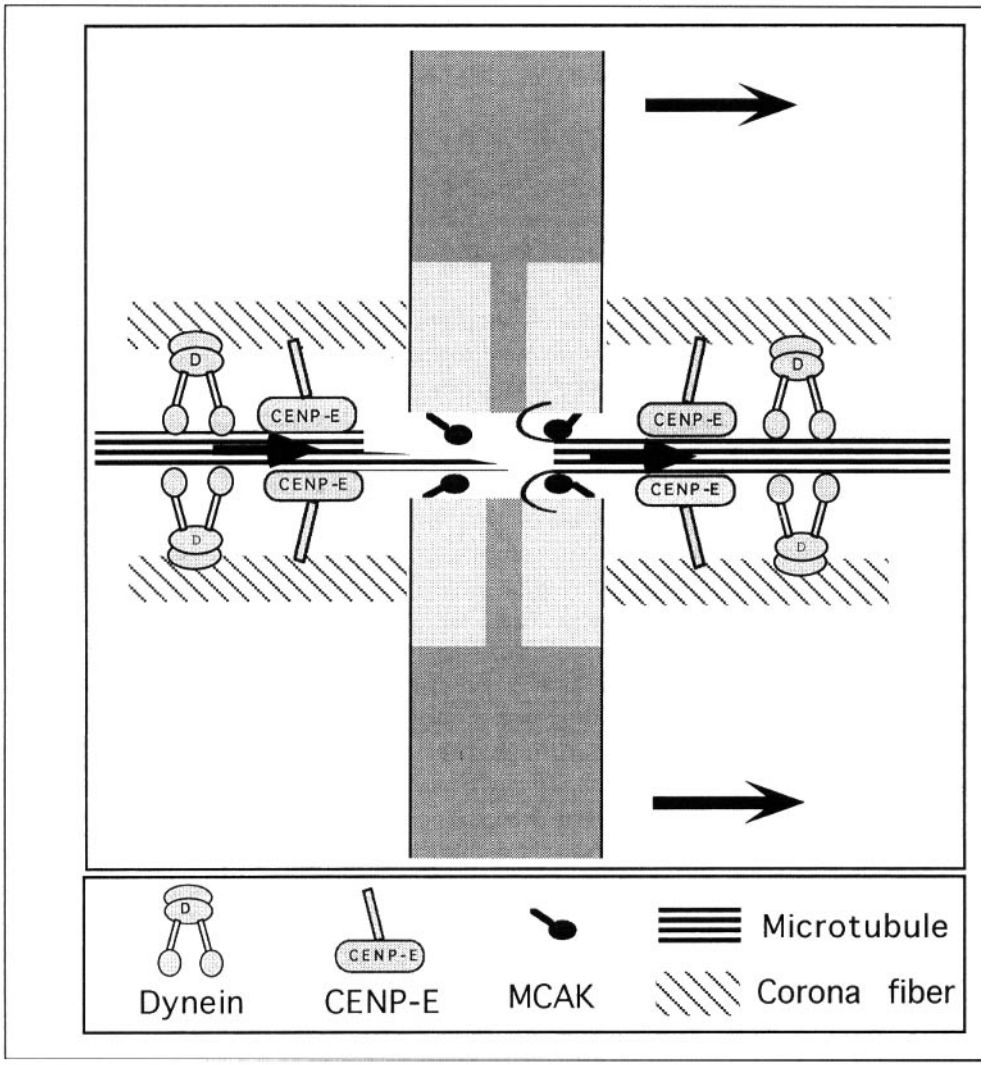

Fig. 7 Localization and potential functions of kinetochore motors. In this drawing the chromosome is moving to the right. On the left, a kinetochore microtubule is growing (with a sheet extension at its end) and on the right a kinetochore microtubule is shrinking (with coiled oligomers at its end). Dynein may move towards the minus end of the depolymerizing microtubule. CENP-E could tether growing and shrinking microtubules to the kinetochore. MCAK/XKCM1 may be localized between the kinetochore plaques as suggested by a recent paper (42) and may be involved in coordinating microtubule dynamics at the kinetochore.

physical tension that occurs at kinetochores. Tension has been proposed to regulate the kinetochore switch by indicating to chromosomes their position on the spindle (72, 73). It has also been proposed that phosphorylation of kinetochore components might control the activity of motor proteins. Since tension has been shown to affect the phosphorylation level of kinetochore proteins directly, it is tempting to speculate

that it could control the activity of kinetochore motors and thus the directionality of movements of kinetochores.

3.2 The role of chromosome arms in chromosome movements

A number of observations indicate that kinetochore motility alone is not sufficient to explain chromosome behavior. The oscillations can indeed cover substantial distances, persist throughout all stages and continue on mono-oriented chromosomes (in which only one kinetochore is attached to one pole) (61). Chromosome fragments lacking kinetochores are actively transported away from the closest pole (at 2 μm/min) indicating that there is another mechanism involved in chromosome movement away from the pole (74). This mechanism seems to be dependent on the dynamic astral microtubules and on the size of the chromosomes. Severing the arms of a mono-oriented chromosome results in the movement of the kinetochore-containing fragment towards the pole (75). Its final position depends on the size of the chromosome surface. There is also a direct correlation between the number of astral microtubules interacting with a mono-oriented chromosome and its kinetochore-to-pole distance (58). Thus, in addition to the forces exerted at kinetochores, moving them towards and away from the poles, there are other forces produced by half-spindle microtubule arrays that push chromosomes away from the pole. These forces have been called 'polar ejection forces'. It has been proposed that these forces are produced by growing microtubules pushing on chromosome arms (61). Although this may be part of the story, recent findings indicate that motors are also almost certainly at play. Two distinct types of KLPs have been localized on chromosome arms: Nod (76) and Xklp1/chromokinesin (77, 78). The phenotype of *nod* mutations in *Drosphila* indicated that it is required for the proper positioning of non-exchange chromosomes between the poles and the equator of the spindle (79–81). This suggested that it could be involved in polar ejection forces, an idea that has been reinforced by the finding that Nod is associated with meiotic chromosome arms. It would still be essential to know the motility properties of Nod, in particular its direction of movement, in order to confirm its function. *Xenopus* Xklp1 and chicken chromokinesin are also associated with chromosome arms (77, 78) (see Fig. 12 for Xklp1 localization). Antisense and *in vitro* spindle assembly experiments have shown that Xklp1 is required for chromosome positioning and spindle bipolarity stabilization. Xklp1 may function by capturing and stabilizing non-kinetochore microtubules on the surface of the chromosome arms. By moving towards microtubule plus ends, it may contribute to the polar ejection forces. In addition, by stabilizing microtubules around the chromosomes it may favor the establishment of interactions between antiparallel microtubules (Fig. 8).

In summary, chromosome oscillations in prometaphase seem to be achieved through a balance of forces produced both at the kinetochores and by astral microtubules. Poleward movements are produced by pulling forces exerted at the kinetochore. The movements away from the poles seem to be produced by a combination of motor activities present at the kinetochores and of ejection forces

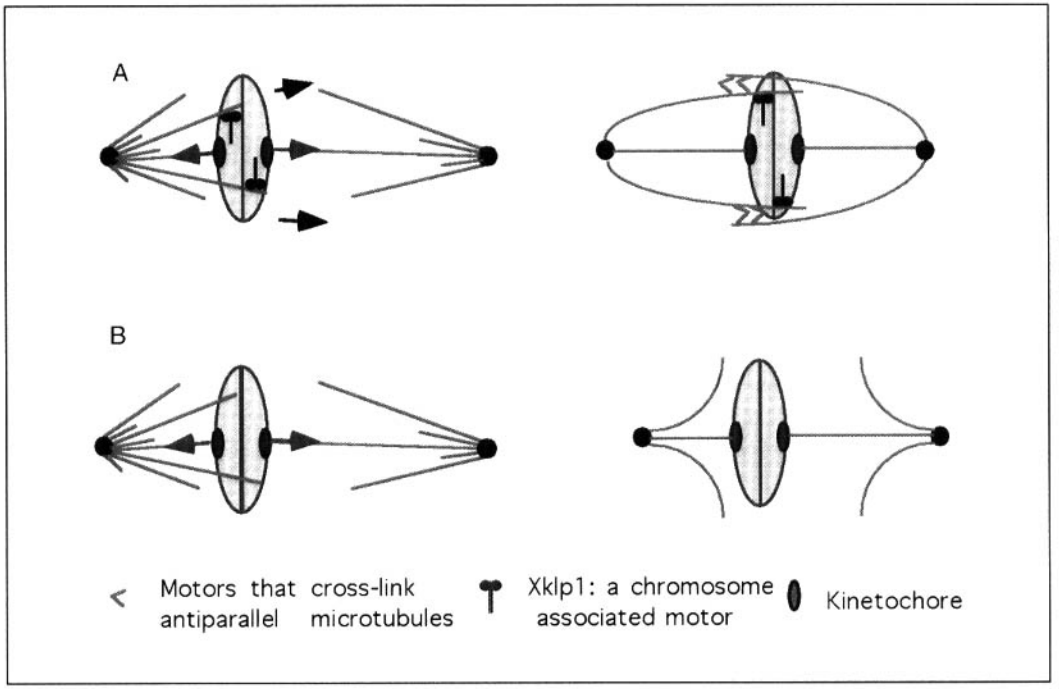

Fig. 8 Xklp1 function in spindle assembly. (A) Xklp1 present on chromosome arms captures astral microtubules. Although its direction of movement has not been determined yet, it probably moves towards microtubule plus-ends (as does the related KIF4). This tends to push chromosomes away from the poles and contributes to the congression of chromosomes on the metaphase plate. By maintaining non-kinetochore microtubules along the surface of chromosomes, Xklp1 favors interactions between antiparallel microtubules, allowing other plus-end-directed motors to cross-link these microtubules and thereby stabilize spindle bipolarity. (B) In the absence of functional Xklp1, only kinetochore microtubules connect poles to chromosomes. Although plus- and minus-end-directed motors are present at kinetochores, the lack of a constant strong force pushing chromosome arms towards the spindle equator results in constant oscillation of chromosomes that never congress on the metaphase plate. The lack of stabilization of microtubules on the chromosome surface results in failure to establish stable antiparalell interactions and eventually in spindle disassembly.

produced by the polar array of microtubules pushing on the chromosome arms (61). However, the recent finding that a symmetric bipolar spindle assembles around chromatin beads lacking kinetochores demonstrates that the positioning of chromosomes on the metaphase plate does not require kinetochore pushing forces (82). The coordination between microtubule dynamics and chromosome movements seems to be achieved at least in part by kinetochore motors, but the activity of motors associated with chromosome arms may also participate in this regulation.

The movement of chromosomes during anaphase A may occur by a change in the balance of forces produced by plus- and minus-end-directed kinetochore motors, by the inhibition of the activity of plus-end motors associated with chromosome arms or by the force produced by depolymerization of kinetochore microtubules. In this context, it is interesting to note that CENP-E is still localized on kinetochores during

anaphase A and thus could tether chromosomes to shortening anaphase kinetochore microtubules (83).

4. Role of kinetochores and chromosome arms in spindle assembly

At the same time as chromosomes interact with microtubules and move to become positioned on the metaphase plate, microtubules are reorganized into the bipolar array that constitutes the mitotic spindle. Since the beginning of the century, chromosomes were thought to be fairly passive in the process of spindle assembly (84). Centrosomes were considered to be the 'division organ of the cell' that determined the assembly and bipolarity of the mitotic spindle. In fact, being based on the simple observation of chromosome and centrosome movements during mitosis in animal cells, this conclusion was justified. However, several observations challenged this conclusion very early on as spindles form in the absence of centrosomes in plants and during meiosis (see Chapter 7 for a detailed discussion of meiotic spindle assembly). More recent experiments pointed out the possibility that chromosomes could induce spindle assembly in the absence of centrosomes and that spindle bipolarity could arise in the absence of the cues provided by centrosomes and kinetochores (85, 86). In other words, it became apparent that chromosomes were not only transported as 'passive corpses' by the fibers of the mitotic spindle, as suggested by Mazia (84), but that they first instructed in some way the assembly of the apparatus that would in the end distribute them to the two daughter cells. What kind of activity associated with chromosomes could drive spindle assembly?

4.1 Role of kinetochores in spindle assembly

It was first thought that the only part of the chromosome that played a role in spindle assembly was the kinetochore. Although kinetochores were initially thought of as elements strictly involved in chromosomal movements as described in the previous section, their potential role in spindle assembly was formulated recently (11). It was proposed that kinetochores could capture and stabilize some of the growing and shrinking astral microtubules (Fig. 9). In this way, the kinetochores would favor the establishment of spindle bipolarity by stabilizing two antiparallel arrays of microtubules originating from the two centrosomes. This view was supported by the following experiments. First, in Chinese hamster ovary cells, kinetochores and centrosomes can assemble a bipolar spindle on their own after kinetochores are experimentally detached from the bulk of the chromosome arms (87). Second, in echinoderm egg and newt lung cells, in the absence of chromosomes, centrosomes nucleate two separate asters instead of a bipolar spindle (88, 89); although this experiment was taken as a demonstration of the need of kinetochores to stabilize the spindle, it may mean also that chromosome arms do so. If this were true, one would have to look for additional chromosomal factors affecting spindle assembly. Until

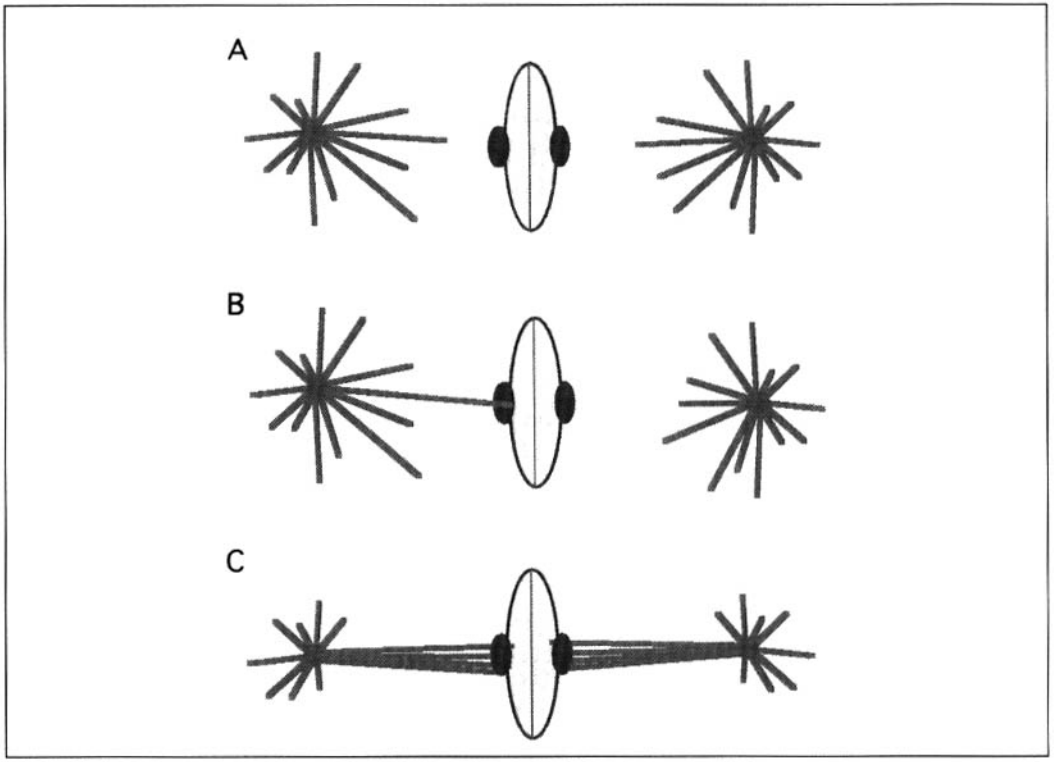

Fig. 9 Search and capture model for spindle assembly. In this model, spindle bipolarity is determined by the presence of two centrosomes and two kinetochores. (A) Dynamic microtubules emanating from the two centrosomes positioned at opposite sides of the chromosome grow and shrink in all directions. (B) Eventually one of them hits a kinetochore and becomes captured. (C) In the same way, its sister kinetochore captures microtubules emanating from the opposite pole. A bipolar spindle is formed.

now, none of the kinetochore proteins has been implicated in the process of spindle assembly.

4.2 Role of chromosome arms in spindle assembly

The ability of non-kinetochore chromatin to promote spindle assembly and organization in the absence of kinetochores was supported by experimental evidence. When phage or bacterial DNA is injected into *Xenopus* eggs, fully functional chromatin assembles by recruitment of stored components. In interphase eggs, this chromatin forms nuclei and in mitotic eggs, it induces a local growth of microtubules that reorganize into bipolar-like arrays (85, 86). A similar result is obtained when magnetic beads coated with fragments of plasmid DNA are used instead of phage DNA (Plate 4) (82). Since neither the phage nor the plasmid DNA have centromere sequences, the chromatin effect is not due to the capture (or stabilization) of microtubules by kinetochores. In this case, the principles ruling spindle assembly

must be different from those proposed by Kirschner and Mitchison in 1986 (11). The mechanism might be one of randomly oriented growth followed by the self-organization of linear polymers into a bipolar array under the influence of mitotic chromatin, perhaps the chromosome arms.

Another approach was used by Zhang and Nicklas (90) to examine the contribution of chromosomes and kinetochores in spindle assembly in meiotic grasshoper spermatocytes. Using glass microneedles they could play with the number and position of chromosomes of different size in the cell. The first conclusion from their experiments is that chromosomes indeed have a direct effect on the microtubule distribution in the spindle and on the total mass of microtubules (the effect of chromosomes on microtubule density had already been observed by Sawin and Mitchison (91) during *in vitro* spindle assembly). This effect is dependent on the size of the chromosome rather than on the number of kinetochores present. Indeed, 'a single large chromosome with two kinetochores has a greater impact than three smaller ones with six kinetochores' (90) (Fig. 10). This result indicates again that chromosome arms and not kinetochores play a direct role in spindle assembly. One apparently contradictory result is that a spindle can be maintained when all the chromosomes are removed from the cell although with a reduced microtubule density. Since the chromosomes were removed from an already formed metaphase spindle this result means that chromosomes are not absolutely required for the maintenance of the spindle but does not question their role during spindle assembly. The stability of these chromosome-less spindles may come from the activity of motors in different areas of the spindle, in particular those that cross-link antiparallel microtubules at the equator. Chromosomes appear to exert a strong effect on the stability of astral microtubules. When the nuclear envelope is ruptured artificially during prophase, a spindle is formed prematurely (92). This strong effect is probably also responsible for the disassembly of the spindle when the only chromosome left is positioned in the cytoplasm away from the spindle, by perturbing the conditions for microtubule assembly in the cytoplasm (Fig. 10). These results suggest that chromosomes act on microtubules from a distance and that this is essential for triggering spindle assembly. This effect may not be necessary for spindle stability, at least for some time.

4.3 Molecular basis of the effect of chromosome arms on spindle assembly

All these observations raise the question of the molecular mechanism by which chromosome arms affect the assembly of a bipolar spindle. They might do so in two ways. First, the chromosome arms may create a micro-environment in the surrounding cytoplasm more favorable to microtubule growth than in the rest of the cytoplasm. Second, the arms may physically capture microtubules.

That mitotic chromatin may favor microtubule assembly in its vicinity was first proposed by Karsenti *et al.* in 1984 (85, 86) at the same time as dynamic instability was discovered. The subsequent discovery that the catastrophe frequency increased

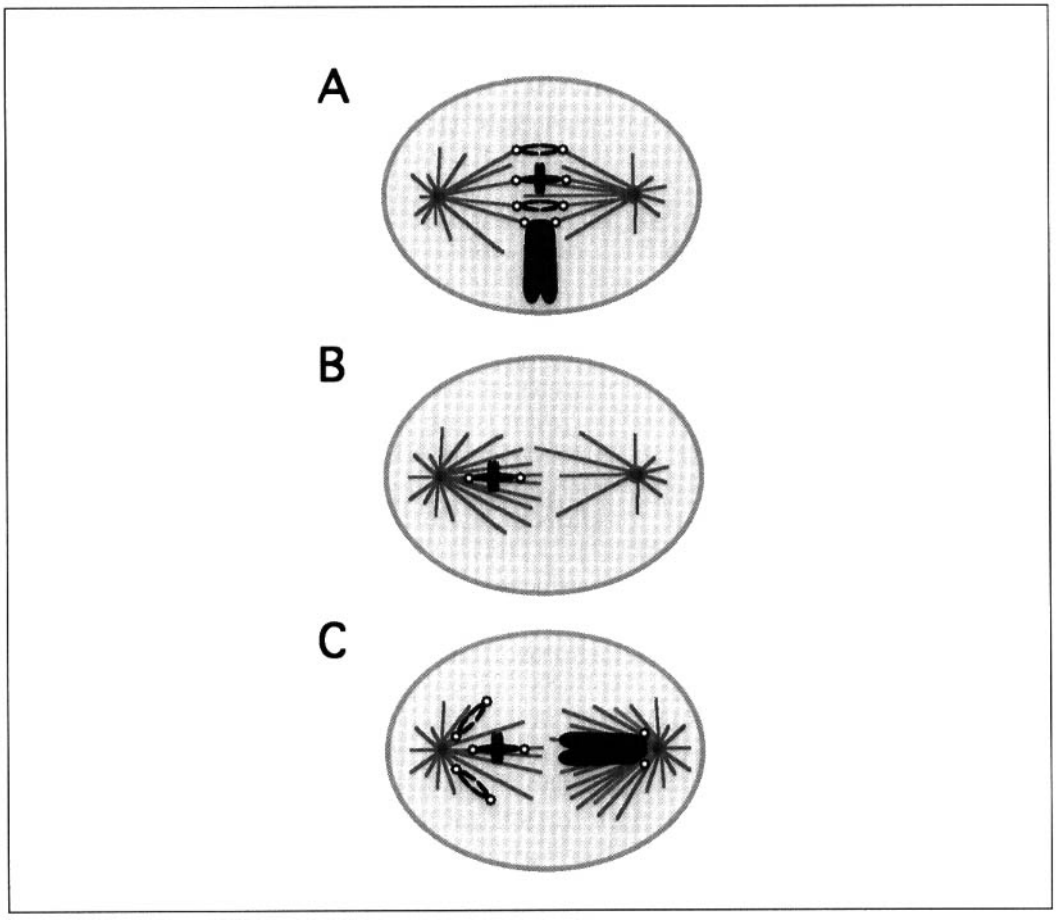

Fig. 10 Role of kinetochore and chromosome arms in spindle assembly. (A) Drawing of a meiotic grasshopper spermatocyte at metaphase. Bioriented chromosomes are aligned at the metaphase plate. Normal cells contain 11 bivalent chromosomes and an X chromosome. (B) All chromosomes except one have been removed experimentally from the cell. Centrosomes have been moved away from the original spindle which then disassembles. The single chromosome left in the cell is placed close to one centrosome. A new spindle is formed that shows four times more microtubules in the half-spindle containing the chromosome. (C) To evaluate the role of kinetochores and chromosome mass in spindle assembly, three small chromosomes containing six kinetochores were positioned at one spindle pole while a single large chromosome containing two kinetochores was positioned at the other pole. In this way one half-spindle contains more kinetochores while the other contains more chromosome mass. The spindle half containing more chromosome mass shows a higher microtubule density than the spindle half containing more kinetochores.

during mitosis by a cdc2 kinase-dependent phosphorylation event led to the suggestion that the local effect of chromatin on microtubule dynamics could be due to a modulation of phosphorylation reactions around the chromosomes, perhaps through the localization of a phosphatase on chromosome arms (93). The idea is as follows: upon entry into mitosis, microtubules become highly dynamic and short because of the activation of the cdc2 kinase. In the same time, the chromosomes

condense and start to dephosphorylate locally substrates of the cdc2 kinase that increase microtubule dynamics when phosphorylated. The result would be a lower catastrophe rate and therefore longer microtubules around the chromosomes. This increase in relative stability of microtubules would then allow their interaction with kinetochores and chromosome arms. Although there is no firm evidence yet for such a mechanism, the fact that microtubules are stabilized around chromosomes without making physical contact with them has been documented recently by Dogterom *et al.* (94). They showed that in low-speed *Xenopus* meiotic extracts, chromatin exerts a short-range stabilization effect on microtubules. This local stabilization is brought about by an effect on three of the four parameters that define microtubule dynamics: the catastrophe frequency is reduced, the rescue frequency is increased and the growth velocity is decreased. This happens fairly close to the chromatin. A longer range effect has also been observed. In this case, it is only the catastrophe frequency that is reduced. Dogterom *et al.* called this effect a 'long range guidance' on microtubules.

Once microtubules have been attracted by the chromosome 'perfume', they must be captured by their arms. Motors of the chromokinesin family appear to be excellent candidates for fulfilling this task. Indeed, inactivation of the function of Xklp1 in *Xenopus* egg extracts results in a lack of stabilization of spindle bipolarity (77). It seems that, in this case, the only part of the chromosomes that can capture microtubules are the kinetochores. Interestingly, this appears to be insufficient to stabilize bipolarity and spindle integrity as chromosomes are lost from the abnormally formed spindles at a high rate (Fig. 8). Positioning of chromosomes on the metaphase plate appears also to result mainly from the action of the plus-end-directed motor Xklp1. Indeed, chromatin beads are always localized in the center of the spindles although kinetochores are absent (82).

In summary, it seems that chromosomes play an important role in directing spindle assembly. They first attract microtubule plus ends, then microtubules are captured and further stabilized by kinetochores and motors present on chromosome arms. Although kinetochores do not appear to be required for spindle assembly, they are probably essential for chromosomal movements in anaphase and chromatid segregation. Therefore, chromosome movements and spindle assembly are tightly interrelated. Chromosomes play an important role in governing both the assembly of the apparatus that moves them around and their own movements.

5. The importance of motor localizations in spindle assembly and function

The localization of motors to specific domains of the spindle is crucial for their function in spindle assembly (Fig. 11) and chromosome segregation. Motors are localized at the poles of the spindle, in the overlap zone, on kinetochores and on chromosome arms (Fig. 12) (2, 5). These localizations are essential since they allow the motors respectively, to organize the poles (dynein), to separate them (Xklp2/BimC family), to sort antiparallel microtubule arrays (CHO1, Cin8, Kip1), to

establish bipolarity (BimC/CHO1/Xklp1), to position chromosomes on the metaphase plate (kinetochore motors, Nod and Xklp1) and to segregate them in anaphase (CENP-E, dynein?) (95, 96). We know that localization is determined in part by specific targeting sequences and by accessory proteins interacting often with the tail of the motors. Moreover, phosphorylation reactions seem to play an important role in the temporal and spatial control of motor localization.

5.1 Targeting by stereospecific interactions

We are just beginning to identify sequences involved in the targeting of mitotic motor proteins to specific cellular domains. Nod has a specific sequence involved in its targeting to DNA (97). Other motors, like Xklp1 and Xklp2, have interesting sequences in their tails that may be responsible for their localization, but there is not yet a definitive proof of their importance in targeting (77, 98). Xklp1 has a zinc finger motif in its tail that may be important for the localization but we still do not know its function. The nuclear localization signals in its stalk are probably responsible for its targeting to the nucleus in interphase. Xklp2 has a leucine zipper in the tail and this has recently been shown to be essential for targeting the protein to the spindle poles (T. Wittmann, H. Boleti, C. Antony, E. Karsenti, and I. Vernos, submitted).

The localization of motors may also be regulated by their association with specific subunits. For instance, KAR3 localization at spindle pole bodies and on spindle microtubules is dependent on its interaction with CIK1 (99).

The structure of the motor is also very important for its localization and function. One can imagine that the motors that are targeted to specific domains of the spindle are dimeric with a recognition signal in the tail. Once bound to their target site, they will move the element containing the site along a microtubule (i.e., Xklp1 will move chromosomes and Xklp2 will move centrosomes or microtubule minus ends). However, multimeric motors may not be targeted to specific sites, but localized by virtue of their mode of interaction with microtubules. For example, motors of the BimC family are tetrameric (100). They could organize microtubules by cross-linking them and moving along two adjacent microtubules towards their plus ends (Fig. 11). This mode of action does not require specific targeting. Dynein also seems to organize spindle poles by cross-linking adjacent microtubules and moving towards their minus ends (82, 101). Interestingly, dynein must be targeted to something that helps it to stay associated with microtubules in order to function in spindle pole organization. This involves the dynactin complex (102). Indeed, the addition of antibodies against the intermediate chain of dynein displaces the dynein heavy chain from spindles (103) and dynamitin disrupts spindle poles (104). It is not yet clear to what dynein is targeted through the intermediate chain and dynactin: microtubules or an unknown complex involved in spindle pole organization.

5.2 Control of targeting by phosphorylation

Some studies suggest that phosphorylation by the cdc2 kinase during mitosis can alter or determine the localization of some KLPs. *Xenopus* Eg5 has a consensus site for

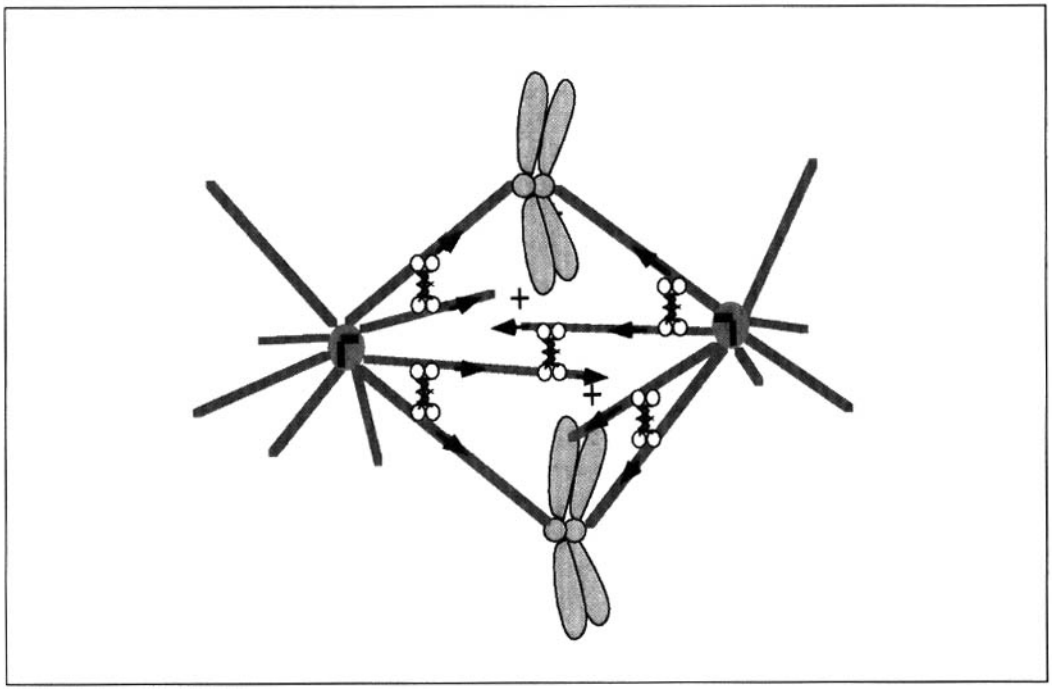

Fig. 11 Possible role of BimC family members in spindle assembly. Kinesin-like proteins from the BimC subfamily move towards microtubule plus ends. They are tetrameric molecules with two motor domains at each end of a stalk region and thus could cross-link microtubules throughout the spindle by moving between adjacent microtubules from the poles to the equator.

phosphorylation by cdc2 in the tail. Mutations at this site inhibit the localization of the protein to the mitotic spindle. Both the N- and C-terminal regions of the protein are required for this localization (105, 106). Since Eg5 belongs to the BimC family and is a tetramer, it is possible that it has to tetramerize to be functional and thus to localize on spindle microtubules. It is not clear yet whether phosphorylation of the tail is directly involved in the targeting or if it affects the conformation of the protein or its level of oligomerization.

Another KLP regulated by phosphorylation, CENP-E, is relocalized from the kinetochores to the spindle midzone during anaphase. CENP-E has a second microtubule binding site in the C-terminal tail domain with a consensus phosphorylation site for cdc2. Phosphorylation of this site inhibits binding of CENP-E to microtubules (107). This result suggests that phosphorylation inhibits CENP-E microtubule binding during mitosis and dephosphorylation allows its relocalization to the spindle midzone during anaphase. Since many mitotic motors have consensus sites for phosphorylation by cdc2, phosphorylation could be a general mechanism for regulating the binding to microtubules and thus the localization and function of motors.

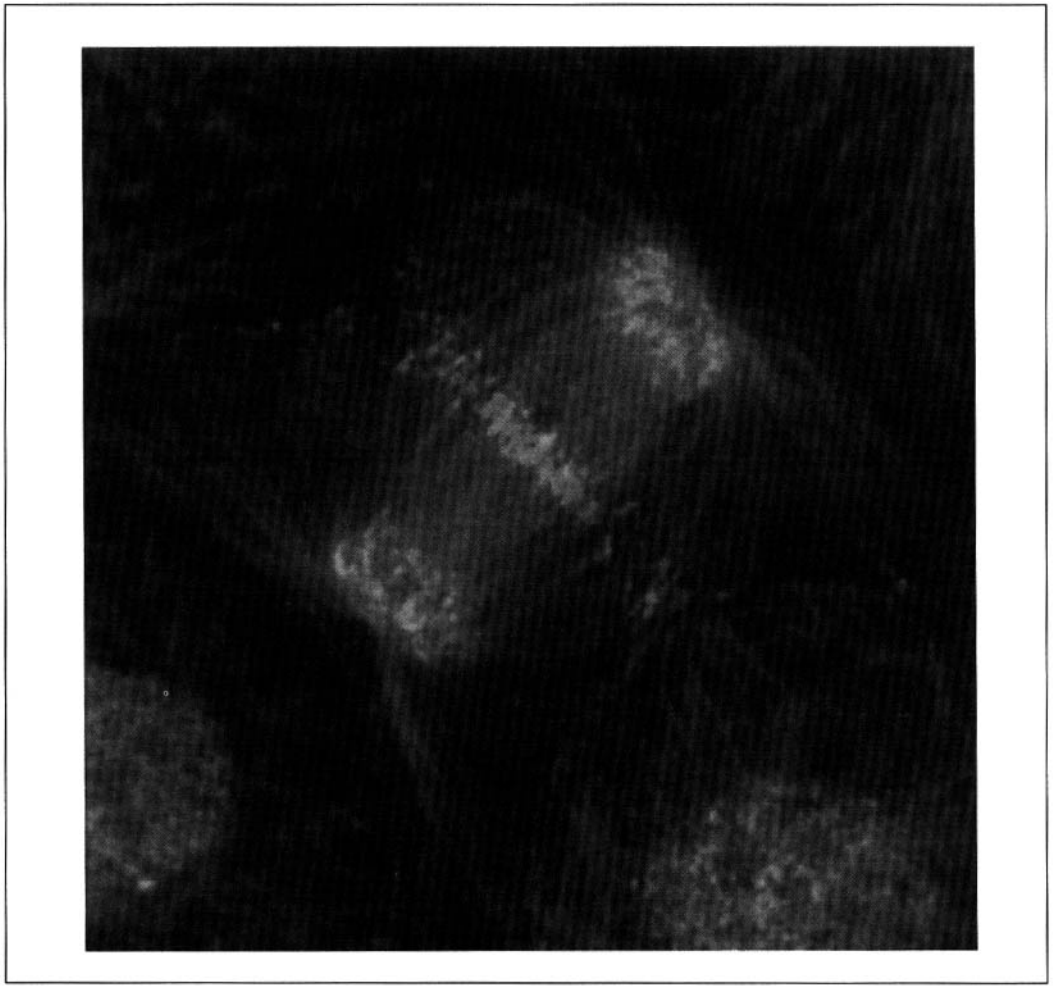

Fig. 12 Xklp1 *localization in an anaphase Xenopus cell. The brighter staining at the poles and the center of the* spindle corresponds to Xklp1 localized on chromosomes and the spindle equator, respectively (see cover).

6. Conclusion

Chromosomes are segregated faithfully on a microtubular apparatus, the mitotic spindle, which assembles once every cell cycle. Chromosome segregation requires chromosomal movements towards two cellular domains that will form the two daughter cells so that each of them inherits the proper genetic information. This is possible because microtubules in the mitotic spindle have a bipolar organization. Although it was first thought that bipolarity was determined by the number of centrosomes and kinetochores in one cell (one pair of each), it became clear recently that bipolarity is the most stable configuration that microtubules take around chromosomes during mitosis. This is due to the local regulation of microtubule

dynamics by chromosomes and the activity of plus- and minus-end-directed motors, in particular motors localized on chromosome arms.

Chromosomes play an important role in spindle assembly. In fact, they govern the assembly of this apparatus in the same time as they are moved by it. Chromosome movements involve a tight coordination between microtubule dynamics at kinetochores and chromosome arms, and motor activities. We still do not understand very well how this coordination is achieved. Phosphorylation reactions may be involved since it has been observed that tension on kinetochore fibers affects the phosphorylation level of kinetochore proteins. In fact, both the assembly of the spindle and chromosome movements are probably coordinated by phosphorylation reactions controlling microtubule dynamics and the activity of motors around chromosomes.

The mitotic spindle appears therefore as a sophisticated self-assembly machine in which structural components and motors are under the control of a regulatory network made of kinases and phosphatases. This network, by controlling the spatial dynamics and motility of microtubules, coordinates the assembly of the spindle. Spatial information comes from the localization of enzymes and motors on the chromosomes which provide the initial asymmetry responsible for the establishment of bipolarity.

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5 A moveable feast: the centromere–kinetochore complex in cell division

KEVIN F. SULLIVAN

1. Introduction

The goal of cell division is to transmit the genome from one generation to the next. This occurs in two essential steps: the genome is first replicated in S phase and then during mitosis the two copies are separated and transported into separate cytoplasmic domains destined to become new cells. This transport function is specified primarily by the centromeres, specialized nucleoprotein domains present in a single copy on each chromosome (1). Centromeres direct assembly of the machinery for microtubule binding and motor activities, known as the kinetochore, at the surface of each sister chromatid, maintain cohesion between sister chromatids, and possess regulatory elements that integrate chromosome motility and spindle function with cell cycle control pathways. During mitosis the kinetochores interact with spindle microtubules first to bind the spindle in prometaphase, then to achieve the crucial bipolar orientation in metaphase and finally to drive poleward movement in anaphase. A parallel system of regulatory elements located within the centromere monitors kinetochore attachment and communicates with the spindle to control anaphase onset. On the other side of the anaphase switch, centromeres very probably function as targets for regulated proteolysis of the 'glue' that holds sister chromatids together.

It is clear now that centromeres, once thought to be a passive 'handle' for microtubule binding, are sophisticated motile devices that integrate multiple forms of propulsion with control circuitry that regulates both local and global features of the mitotic mechanism. Mazia's widely quoted quip is that the chromosomes are like the corpse at a funeral—the reason for the gathering but an inactive participant in the proceedings. Mitosis, then, must be akin to *Finnegans Wake*, where the corpse suddenly wakes to join the party. Other chapters in this volume detail the

mechanisms of microtubule motor functions and sister chromatid cohesion. In this chapter, I will focus on how these functions are specified by the centromere and how they interact within the replicated chromosome and with the regulatory machinery of cell division to ensure transmission of the genome.

2. Centromere structure

What is a centromere? At the genetic level it is a kind of chromosomal singularity, a node of suppressed recombination to which genes are attached left and right forming a linkage group. At the cytological level the centromere is familiar as the primary constriction of a mitotic chromosome, a densely packed heterochromatic domain capped by the trilaminar kinetochore that binds to microtubules in the spindle. Ultimately, the centromere is a chromosomal locus in which DNA assembles into a unique multicomponent nucleoprotein complex that integrates microtubule binding and spindle mechanics with the chromatin fiber (Fig. 1). Most of what we know about the structure of centromeres has come from one of four key systems, each contributing information at a different scale of chromosome structure and lending complementary perspectives on how centromeres are built and how they work. Studies in budding yeast (*Saccharomyces cerevisiae*) have provided the most complete description of a centromere at the molecular level, detailing key protein–DNA interactions required to assemble their compact (125 bp) point centromere. The large (40–100 kb) centromeres of *Schizosaccharomyces pombe* furnish unique insight into the role of chromatin structure and epigenetic features of centromeres. *Drosophila* has

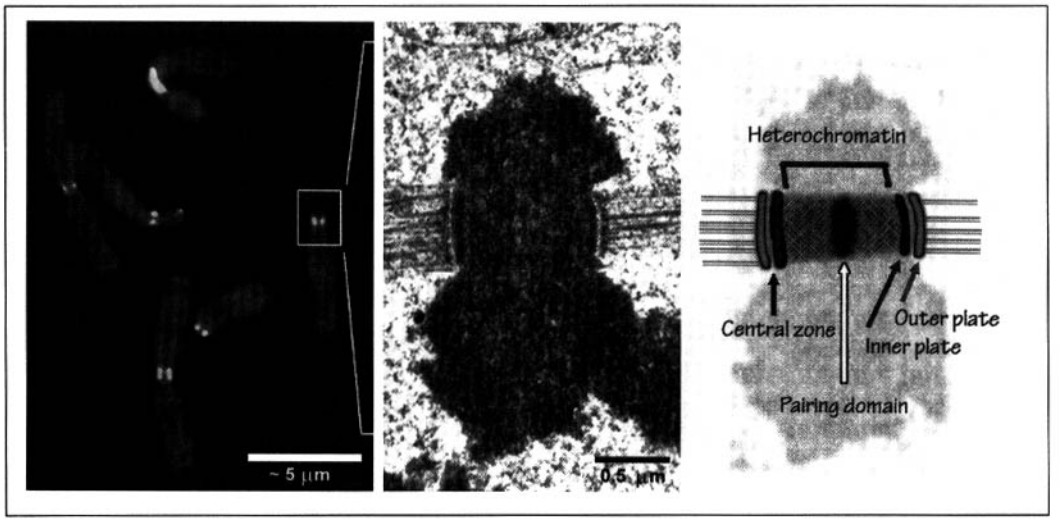

Fig. 1 Organization of the centromere–kinetochore complex. Indian muntjac chromosomes stained with anti-centromere antibodies show the position of centromere/kinetochore proteins at the primary constriction (left). An electron micrograph of the centromere/kinetochore of a mitotic chromosome from the green alga *Oedogonium* (center) reveals the elaboration of the kinetochore as a discrete complex of plates organized at the surface of the centric heterochromatin, as diagrammed on the right. Micrograph courtesy of Dr M. J. Schibler (196), reproduced by permission of Springer-Verlag.

recently provided the first detailed definition of DNA sequence requirements for centromere function in a multicellular organism as well as genetic 'handles' on several important centromere functions. Among vertebrates, human centromeres are the best understood at the level of DNA and associated proteins. While the most daunting in size at 0.5–5 Mb, the identification of a group of centromere proteins as human autoantigans, coupled with structural insight gleaned from adventitiously presented centromere derivatives identified in clinical cytogenetics laboratories, has provided important insight into their functional organization.

2.1 *S. cerevisiae*

Budding yeast has the simplest centromeres known and currently provides the most direct view of the interface between centromeric DNA and the microtubule at the molecular level. The compact *S. cerevisiae* centromere comprises only about 125 bp of DNA, which nucleates the assembly of multisubunit protein complexes which, in turn, carry out the dynamic functions of centromeres in mitosis (for review see ref. 2). Sequence homology between different *S. cerevisiae* centromeres, as well as functional analyses, demonstrate three functional domains of *CEN* DNA: CDEI (8 bp), CDEII, (78–86 bp), and CDEIII (26 bp) (Fig. 2). CDEI and CDEIII both function as binding sites for sequence-specific DNA-binding proteins (3, 4). CDEII, in contrast, is conserved not in DNA sequence but rather in DNA composition and length, suggesting

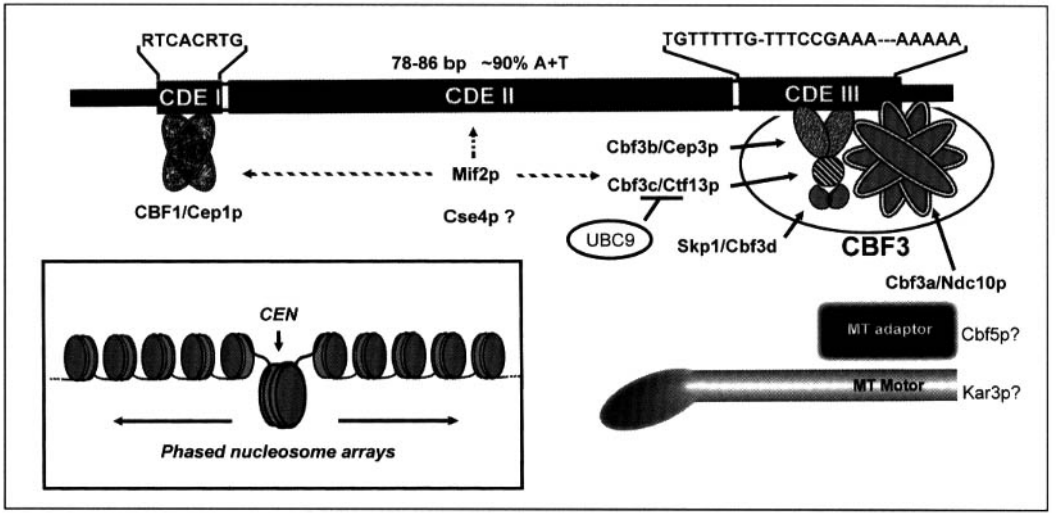

Fig. 2 The budding yeast centromere. The 125 bp consensus determined from each of the 16 centromeres of *S. cerevisiae* is diagrammed with consensus sequences for CDEI and CDEIII shown at the top. Centromere proteins and putative centromere proteins and their interactions are diagrammed below. The stoichiometry and relative positions of CBF3 components are taken from ref. 2. Dashed lines indicate genetic interactions between Mif2p, CBF1 and CBF3 components, and predicted interaction with CDEII. Proteins that are predicted to interact with yeast centromere elements are marked with a '?'. The inset illustrates schematically the highly ordered chromatin surrounding the centromere. Adapted from ref. 2.

that it functions at the level of DNA conformation or folding. The entire centromere is packaged into a nuclease-resistant particle flanked by arrays of highly phased nucleosomes (5). Assembling a functional centromere in *S. cerevisiae* is thus mediated by a hierarchy of interactions: local protein–DNA interactions that fix proteins to specific sites on the DNA, higher order interactions required to fold the centromere locus into a discrete complex, and supramolecular interactions at the level of chromatin that order the chromosomal region around the centromeric nucleoprotein complex.

2.1.1 CDEIII and CBF3: the essential interface

CDEIII is the most critical DNA sequence element for centromere function in the budding yeast, providing the DNA attachment site for the microtubule binding and motility components of the kinetochore. A 26 bp partially palindromic sequence, mutational analysis has shown that single site mutations in CDEIII can abolish *CEN* activity, pointing to direct DNA sequence recognition at CDEIII as a key mechanism for centromere function(6). This was confirmed by affinity purification of a CDEIII DNA-binding activity, yielding a multisubunit protein complex designated CBF3 that bridges CDEIII DNA to microtubules *in vitro* (4, 7, 8). CBF3 contains four distinct polypeptides tightly associated in a complex estimated at 800 kDa (9). Cbf3b/Cep3p, a 68 kDa zinc finger protein, is the best candidate for the DNA-binding subunit of CBF3 (10). It has no DNA-binding activity on its own, but requires assembly of three, possibly four, heterologous subunits for interaction with CDEIII *in vitro* (9). This unusual requirement is in contrast to many protein–DNA interactions during transcription, where sequence-specific proteins can bind DNA on their own and then recruit other factors into a transcription complex (11). One suggestion is that the complex assembly required for CBF3 DNA binding is a fail-safe mechanism to prevent promiscuous kinetochore formation and the catastrophe of dicentric chromosomes (9). The functions of two other proteins of the complex, Cbf3a/Cbf2p/Ndc10p and Cbf3c/Ctf13p, are unknown although Cbf3a possesses a potential GTPase motif and appears to self-assemble to form small oligomers (12).

Purified CBF3 can recruit microtubule-binding and motor activities to immobilized CDEIII DNA sequences *in vitro* (see Section 3.3; 8, 9). A potential component of the microtubule-binding activity was identified as a low affinity CDEIII-binding protein (13). Designated Cbf5p, this protein is essential, binds to microtubules *in vitro* and acts as a high copy suppressor of Cbf2/Ndc10. Surprisingly, Cbf5p is a highly conserved nucleolar protein with prokaryotic homologs (14). While the relationship of Cbf5p to centromere function remains to be clarified, it should be noted that animal centromeres are known to associate with nucleoli at the cytological level and CENP-C, a human centromere protein, also interacts with nucleolar components (15, 16).

A fourth intrinsic subunit of CBF3 was recently identified that establishes an important link between the kinetochore and the cell cycle regulatory apparatus (17). *SKP1* was originally identified in human cells as a potential regulator of cyclin A–CDK2 S-phase kinase (18). It appears to be a versatile adaptor molecule that links

a variety of cell cycle-regulated proteins to the ubiquitin-dependent proteolysis pathway (18). The discovery that Skp1p is an intrinsic kinetochore protein required for assembly of an active CBF3 complex suggests a direct interaction between kinetochore function and cell cycle regulation (9, 17). Whether Skp1p provides an entry point for the proteosome or acts as an adaptor between other molecules at kinetochores remains to be determined. However, the demonstration that Cbf3bp/Cep3p binds to a ubiquitin-conjugating enzyme, UBC9, strongly reinforces the idea that the kinetochore is subject to ubiquitin-mediated proteolysis (19). Both Skp1p and UBC9 are highly conserved across evolution. These proteins illustrate the fundamental relationship between the kinetochore and the machinery of cell cycle regulation that has rapidly expanded into one of the most important concepts in centromere function (see Chapter 1 and Section 5).

2.1.2 CDEI and Cep1p/CBP1

CDEI also functions as a binding site for a sequence-specific DNA-binding protein, Cep1p/CBP1, a classic basic helix–loop–helix protein (3). The role of Cep1p at centromeres is enigmatic; deletion of this element results in a relatively minor decrease in *CEN* activity (20) while it also functions as a transcriptional regulator at non-centromeric sites (21). Cep1p may act by facilitating aspects of chromatin assembly at centromeres as well as promoters, reflecting a general requirement for modulators of chromosome structure for assembly of nucleoprotein complexes on to chromatin-bound DNA (22, 23).

2.1.3 CDEII and the specialized centromeric nucleosome

CDEII plays a different role at the centromere than CDEI or III—its function depends not on DNA sequence, but rather on DNA composition and, it is thought, conformation (24, 25). Centromeres are wrapped in a nuclease-resistant core spanning some 250 bp of DNA which is flanked by highly phased nucleosome arrays (5). This structure is necessary for centromere function, since transcription across a centromere abolishes the protected core and chromosome stability along with it (26). Although no CDEII-specific DNA-binding proteins have been unambiguously identified, a search for genes that can stabilize a minichromosome bearing a mutant CDEII resulted in isolation of a homolog of the core nucleosomal protein histone H3, dubbed CSE4 (27). Cse4p is a chromatin protein required for mitosis and, while not yet experimentally demonstrated to reside at yeast centromeres, it is the yeast homolog of a human kinetochore protein, CENP-A. Coupled with the observations that histones H4 and H2b are required for centromere assembly (28) and that histone H4 interacts genetically with CSE4 to provide an essential mitotic function (29), these data suggest a model in which a modified nucleosome forms the core structure of the yeast kinetochore (see Section 2.4.1).

2.1.4 Putting it all together: the integrated nucleoprotein complex

Available evidence indicates that the known kinetochore components of yeast do not themselves bind directly to microtubules, but rather recruit microtubule-binding

components and, ultimately, motor proteins to the surface of the chromosome (8). Why is this chromosome–spindle interface so complex? Why not simply mount motor proteins with DNA-binding activity—such as Nod (30)—directly on to centromeric DNA? The answer may lie in the mechanical features required of the centromere, which must withstand forces (at least in animal cells) up to 10^4 times those necessary to drag a chromosome through the medium (31) and provide a stable mount for tension-sensitive devices used to monitor chromosome attachment to the spindle. A primary requirement of the nucleoprotein component of the centromere is to provide a fail-safe grip on the DNA fiber.

The product of the *MIF2* gene has the properties of an integrator that acts to fuse the centromeric nucleoprotein complex into a mechanically stable unit (23, 32). Mif2p has convincing homology with mammalian CENP-C, which is co-localized in the inner kinetochore plate with CENP-A (33, 34). Mif2p also contains an AT hook motif found among proteins that bind to A+T-rich DNA, leading to speculation that it may bind the CDEII element of the centromere (32). Genetic analyses also reveal interactions with Cep1p/CBF1 and components of the CBF3 complex (23). In particular, Cep1p/CBF1—which is dispensable for nearly normal chromosome segregation—shows synthetic lethality with temperature-sensitive mutations in *MIF2* and also acts as a dosage-dependent suppressor of such mutations. This has been interpreted to show that Mif2p and Cep1p/CBF1 interact at the centromere and that Cep1p/CBF1 functions to stabilize a higher order protein–DNA complex (23). Mif2p in contact with Cep1p and CBF3 components would also be situated to bind the intervening AT-rich CDEII segment. Thus, like TFIID complexes, which have been proposed to assemble a modified nucleosome-like core particle to recruit or stabilize transcriptional components at promoters (35, 36), the budding yeast kinetochore is configured as a highly ordered three-dimensional nucleoprotein complex that organizes the appropriate binding and enzymatic components onto the chromatin fiber to effect the mechanical functions of mitosis (23).

2.2 *S. pombe*

The centromeres of fission yeast are much larger and more complex than those of budding yeast (Fig. 3; reviewed in ref. 37). They are 40–100 kb in length, almost three orders of magnitude larger than their budding yeast counterparts (38, 39). Unlike in *S. cerevisiae*, centromere function in *S. pombe* is not specified by a unique *cis*-acting DNA sequence. Rather, activation of a functional centromere is the result of interactions between centromere-promoting DNA sequences that fold this chromosomal domain into a unique large-scale configuration. As with budding yeast, the centromere adopts a specific chromatin organization which, while structurally different from the budding yeast model, is an absolute requirement for centromere function. The enigmatic relationship between DNA structure and centromere function in *S. pombe* provides a good model for thinking about centromere structure in multicellular species.

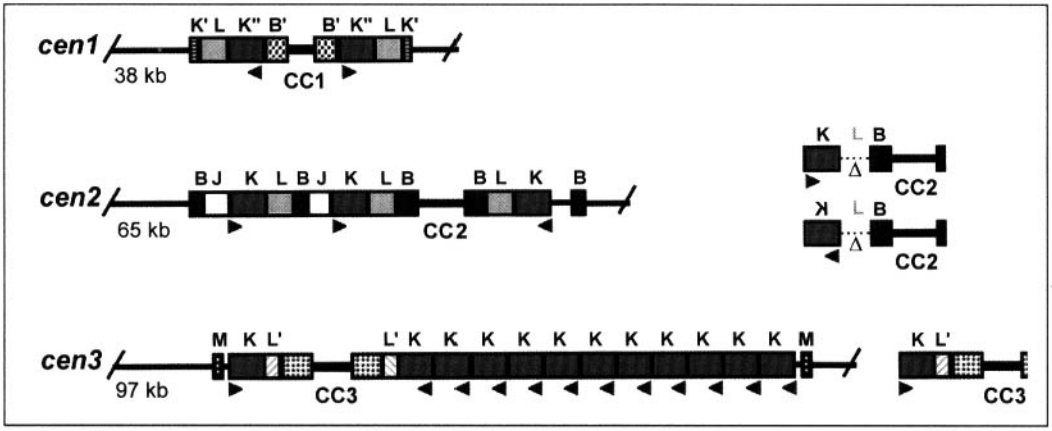

Fig. 3 The fission yeast centromere. The centromeres of each of the three chromosomes of *S. pombe* are organized as complex inverted repeats made up of individual repeat elements (B, B', J, K, K', K", L, L' and M) flanking a central core element present once in each centromere. Arrowheads indicate the orientation of K-type repeats within each repeat unit ensemble. A functional centromere that shows immediate activation of centromere activity requires only a K-type repeat and central core sequences, shown to the right of *cen2*. The large-scale inverted repeat structure of wild-type centromeres is not necessary for centromere function on minichromosomes. Two minimal *cen2* constructs illustrate the orientation-independent nature of the K-type repeat as a centromere enhancer. A comparable construct from *cen3*, shown to the right, displays a requirement for a stochastic activation step revealing a strong epigenetic component required for centromere function in *S. pombe*. Adapted from the data of Clarke and co-workers (45, 47, 56).

2.2.1 DNA structure

Each of the three *S. pombe* centromeres is organized roughly as a large inverted repeat interrupted by a chromosome-specific central core DNA segment 4–7 kb in length (Fig. 3). The inverted repeat motifs have a complex structure, consisting of individual repeat sequence elements (K, L, B, J and M in Fig. 3) which themselves are arrayed into chromosome-specific repeating units (40, 41). The precise arrangement of sequences is highly variable, differing among the three chromosomes and exhibiting polymorphism among laboratory strains (42). The central core regions of centromeres 1 and 3 (cc1 and cc3) share significant homology while cc2 is unique (43). They are packaged into a distinctive type of chromatin characterized by irregular nucleosomal spacing, in contrast to the flanking repeat regions which behave like bulk chromatin (43, 44). Molecular dissection reveals that the inverted repeat organization is not essential for centromere function, nor are most of the individual repeat elements (41, 45). A minimal centromere can be constructed from a central core and one K-type repeat sequence, comprising ~12 kb of DNA (45). However, both the central core domain and the K-type repeats can tolerate internal deletions across their length, indicating functional redundancy within these key sequences (45, 46).

2.2.2 Chromatin structure—distant relations

The unique packaging of central core DNA is strictly correlated with centromere function—the same sequences are packaged into normal chromatin when

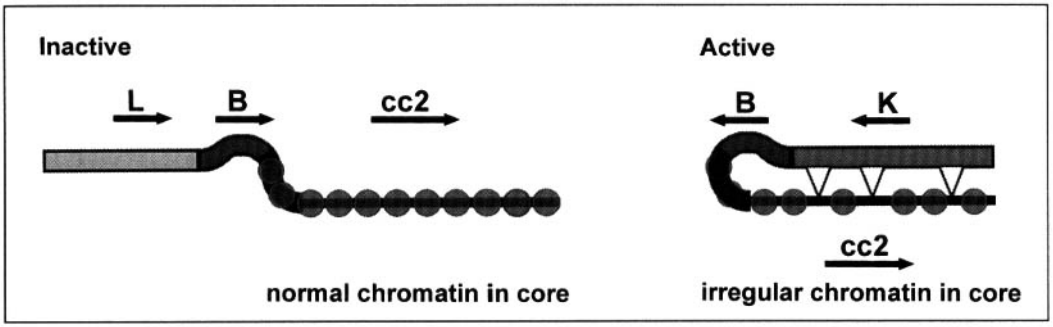

Fig. 4 The *S. pombe* centromere enhancer affects chromatin structure at a distance. The orientation and distance-independent activity of the K-type repeat in association with the central core of *cen2* (cc2) reveals large-scale folding of the chromosomal domain containing the centromere. An inactive centromere derivative shows normal chromatin structure within the central core element (left). In contrast, an active centromere bearing the K-type repeat possesses the disordered chromatin configuration observed for wild-type centromeres (right). The active chromatin configuration is induced without alteration of intervening chromatin and is insensitive to the orientation and distance of the K-type repeat, suggesting a folding or looping of DNA at the centromere. Triangles linking the K-type repeat and the central core illustrate the proposed DNA-binding proteins, perhaps ABP1/CBP1, that link the enhancer to the central core.

propagated in budding yeast or when present on inactive centromere derivatives (44). The central core chromatin signature is found only with a functional K-type repeat, but the orientation and distance between the two DNA elements is unimportant (45, 47). This behaviour is reminiscent of transcriptional enhancers and thus the K-type repeat has been designated a 'centromere enhancer' (45, 46). Central core differentiation occurs without any changes in the chromatin structure of intervening sequences and so the K-type repeat probably acts by looping back over several kilobases to interact directly with the central core (Fig. 4). The picture that emerges is that the centromere of *S. pombe* is organized as a large three-dimensional nucleoprotein complex spanning several kilobases of DNA and that this organization is crucial for presenting microtubule-binding sites on the chromosome surface.

Heterochromatin is a hallmark feature of animal centromeres and this has been found true of *S. pombe* using elegant genetic approaches. Heterochromatin is transcriptionally inactive and genes placed next to it exhibit unstable or variegated gene expression, a phenomenon extensively studied in *Drosophila* as position-effect variegation (for review see ref. 48). Reporter genes placed within the *S. pombe* centromeres also exhibit position-dependent variegation and disruption of chromatin structure associated with the inactive state (49). The variegated phenotype has allowed analysis of genes involved in establishing centromeric chromatin structure, revealing that genes previously identified as necessary for silencing inactive mating loci are also required for normal centromere function (50–52). In particular, Swi6p, a chromodomain protein previously known only for its effects on transcription, is co-localized with centromeres throughout the *S. pombe* cell cycle (51). Identifying the heterochromatic nature of *S. pombe* centromeres has provided an

important entry site for analysis of centromere assembly and its relationship to general chromosomal architecture.

2.2.3 ABP1/CBP1

Despite the degenerate behavior of *S. pombe* centromere DNA sequences, they do appear to contain discrete *cis*-acting sequences that act as binding sites for centromeric proteins (45, 46, 53). Genetic and biochemical experiments aimed at identifying centromere proteins have converged on a DNA-binding protein, ABP1/CBP1p, that bears significant homology to a human centromere DNA-binding protein, CENP-B (53). Deletion or overexpression of ABP1p/CBP1p results in mitotic chromosome instability and it binds with high affinity to multiple sites within cc2. It was independently identified as an autonomously replicating sequence (ARS)-binding protein, raising the possibility that it performs a common role in assembly of functionally distinct nucleoprotein complexes (54). ABP1p/CBP1p joins a family of CENP-B-related proteins that bear homology to transposases, but are predicted to be inactive in transposition (55). One possibility is that these proteins use part of the transposase mechanism to induce unique DNA conformations; indeed, human CENP-B bends its target DNA through an angle of ~70° (K. F. Sullivan, unpublished data).

2.2.4 Centromere activation and epigenetic behavior

Work with *S. pombe* has established that there is a strong epigenetic component of centromere function (56); this was not anticipated from molecular analysis of budding yeast centromeres. The paradigm for centromere function derived from the budding yeast centromere is a transformation assay in which presumptive centromere sequences are placed on a plasmid vector and directly assayed for mitotic stability in the absence of selection (57). A class of *S. pombe* centromere derivatives have been identified that fail to show centromere behavior directly after transformation, but randomly acquire it through a stochastic time-dependent process (56). Centromere activation occurs in the absence of any detectable change in the DNA sequence and DNA from a strain harboring an activated centromere recapitulates the behavior of the parental DNA in the transformation assay, requiring a time-dependent activation event for function. An activated centromere does, however, possess a functional chromatin configuration at the central core. Centromere activation requires the presence of a centromere enhancer, a K-type repeat, and is thought to be a result of the stochastic acquisition of an appropriate chromatin and higher order structure by a sub-optimal centromere (46). Once activated, such centromeres are stable indefinitely, pointing to presence of a self-replicating structure that is associated with, but not strictly templated by, centromere DNA. This is a key concept for trying to understand the complex structure and behaviour of mammalian centromeres.

2.3 Metazoan centromeres

Identification of functional centromere DNA elements in metazoan species remains a

work in progress that has yet to reveal distinct centromere-determining sequences comparable to those found in fungal chromosomes. The emerging theme, derived from experiments primarily in *Drosophila* (58) and humans (59, 60), is that large arrays of repetitive satellite DNA, spanning from ~250 kb to >5 Mb of chromosomal DNA, promote formation of centromeres although the mechanisms involved are not yet understood. Nevertheless, progress is being made rapidly and unravelling the complex issues of centromere structure promises to provide new insight into chromosome structure and function in addition to the practical potential for building artificial mammalian chromosomes.

2.3.1 *Drosophila*

Centromeres of *Drosophila* are cytologically similar to those of vertebrate chromosomes, consisting of large heterochromatin domains with a trilaminar kinetochore (61). An elegant tour de force combining classical X-ray-induced chromosome fragmentation techniques with modern approaches for mapping large DNA segments has recently led to the definition of a 420 kb DNA segment that is necessary and sufficient for complete centromere activity in flies (58, 62). This element, reduced from a 1 Mb heterochromatic region of a *Drosophila* X chromosome-derived minichromosome, consists of simple satellite DNA that is punctuated by 'islands' of complex DNA containing transposon-like sequences (Fig. 5) (62). The relationship of

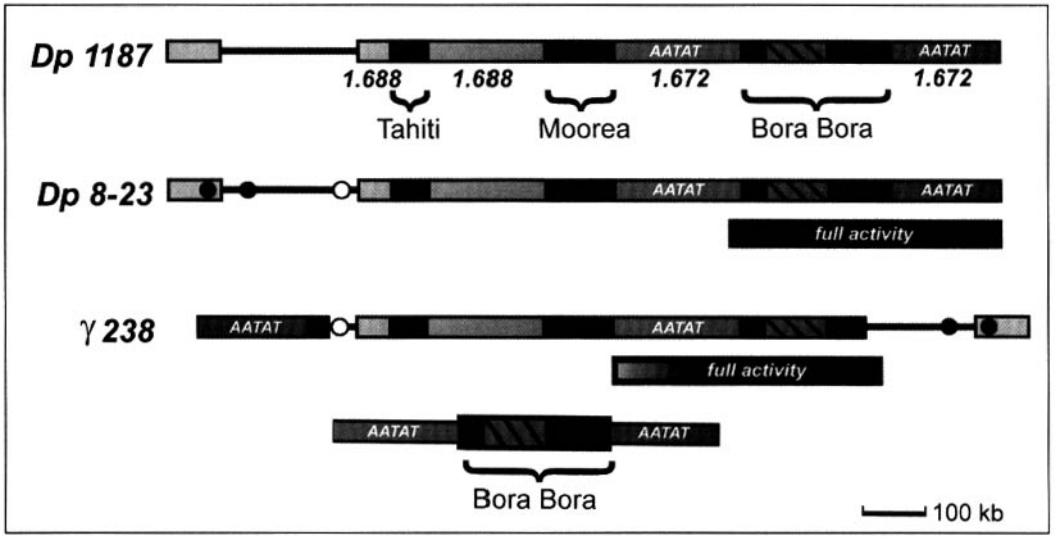

Fig. 5 Definition of a metazoan centromere: *Drosophila*. A 1.3 Mb *Drosophila* minichromosome, Dp1187, was the starting point for dissecting a functional centromere. It consists largely of simple satellite elements designated by their buoyant density in CsCl; the 1.672 satellite sequence, AATAT, is shown. Islands of complex sequence are interspersed within the 1 Mb segment of heterochromatic satellite DNA (Tahiti, Moorea, and Bora Bora). Two derivatives used for mapping experiments and the smallest regions defined as fully functional centromeres are illustrated. The minimal centromere is diagrammed below. It contains the complex Bora Bora sequence flanked by 200 kb of simple satellite, which can reside on either side of Bora Bora. Adapted from the data of Karpen and coworkers (58, 62).

these sequence elements to centromere function is anything but simple, however, as they are neither conserved at the centromeres of other *Drosophila* chromosomes nor are they uniquely localized in the centromere (G. Karpen, personal communication). Further, isolation of an apparently acentric derivative of this chromosome, which lacks the essential 420 kb DNA segment yet has partial centromere activity in male flies, again calls into question the basic idea of DNA sequence-directed centromere and kinetochore formation (58).

Drosophila is uniquely suited for investigating the role of heterochromatin in centromere function, as many genes identified as suppressors [*Su(var)*] or enhancers [*E(var)*] of position-effect variegation have a role in regulating heterochromatin structure or assembly. One of these, HP1 or heterochromatin protein 1, is the archetypal chromodomain protein and has structural homology with the *S. pombe* Swi6 protein (63). Like Swi6, HP1 is concentrated in centric heterochromatin and mutations in the gene cause mitotic abnormalities, although this may be due to a general role in chromosome condensation rather than centromere function per se (64). Nonetheless, investigating the relationships between the (*var*) genes, which affect epigenetic states of genes subject to position-effect variegation, and *Drosophila* centromeres is likely to lead to important insight into epigenetic propagation of the centromere. A more detailed discussion of the structure and meiotic segregation properties of *Drosophila* heterochromatin is provided in Chapter 7.

2.3.2 Humans

Vertebrate centromeres are similar to those of *Drosophila*, consisting of large arrays of tandemly repeated satellite DNA tightly packaged into heterochromatin, but they differ in the details of DNA organization (Fig. 6; 65). The predominant DNA sequence of human centromeres, alpha satellite or alphoid DNA, is a complex satellite sequence consisting of a 171 bp repeating unit that forms tandem arrays of 1500 to >30,000 copies (66, 67). The alpha satellite family has a complex structure, both within chromosomes and across the genome, as individual monomer units differing by as much as 40% in sequence are arranged in higher order repeat units containing from two to >16 monomers which are then tandemly iterated to form large alpha satellite arrays. Individual chromosomes or small chromosome families each have characteristic alpha satellite DNA sequences and higher order repeating units, such that chromosome-specific DNA probes can be generated from cloned alpha satellite sequences (68). The chromosome-specific sequence families are not uniform across the centromeres of most chromosomes, however, but can show internal heterogeneity within arrays (69), be interrupted by non-alpha satellite sequences (70) or coexist with other distinctive alpha satellite sequence families (71). Further, significant sequence variation and array size polymorphism of chromosome-specific alpha satellite arrays within the human population complete the picture of alpha satellite as a bewilderingly diverse and evolutionarily dynamic DNA sequence component (72, 73). The structural organization of complex centromeric satellite DNA in other species follows the broad outlines described for alpha satellite but their sequences, with the exception of the binding site for CENP-B

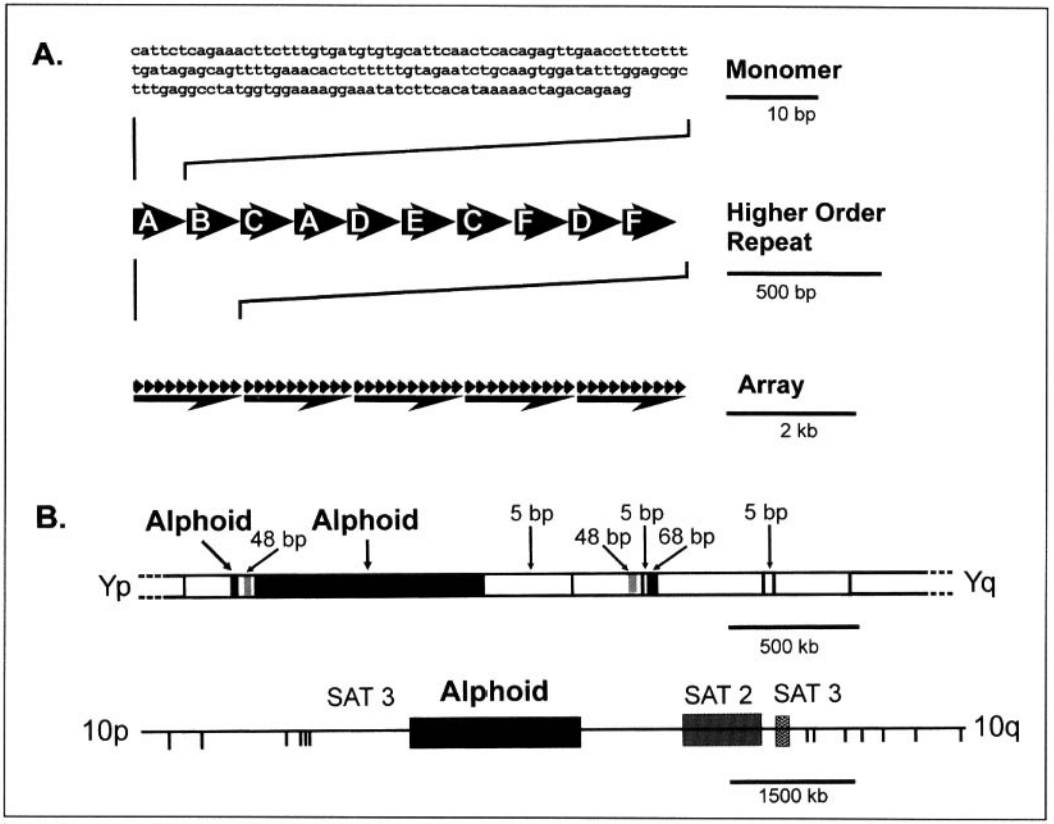

Fig. 6 Human centromere DNA organization. The hierarchical structure of alpha satellite DNA is illustrated in (A). Monomers are arranged in higher-order repeat units containing from two to more than 16 monomer units. Monomer sequence variants are arranged in characteristic order, designated by letters in the higher-order repeat (this example does not show a specific higher-order repeat). Higher-order repeats are then iterated in direct tandem repeats several hundred kilobases to several megabases in length. Two human centromeres that have mapped at high resolution are shown in (B). The Y centromere mapped by Tyler-Smith and coworkers (192–194) is shown at the top. Elements designated by numbers of base pairs correspond to simple satellite elements. A 9.75 Mb region encompassing the centromere of chromosome 10 has been mapped from expressed sequences on both arms using a combination of YAC clones and pulsed field restriction mapping (195).

described below, diverge rapidly from one another, showing detectable homology only among closely related species (74, 75).

As for *S. pombe* and *Drosophila*, available evidence indicates that alpha satellite DNA does not directly specify centromere formation but rather facilitates it in some manner. Examination of natural centromere derivatives isolated from human populations reveals that alpha satellite DNA is neither necessary nor sufficient to specify centromere function. Stable dicentric chromosomes have been identified with an inactive centromere that nonetheless has alpha satellite DNA, forms a constriction in mitosis and binds to CENP-B (34, 76). Conversely, functional centromeres that lack detectable alpha satellite DNA have been identified on several rearranged chromo-

some derivatives (77–79). Nevertheless, alpha satellite DNA has been implicated as a functional element of the Y chromosome centromere by analysis of naturally occurring rearrangements (80) or deletions generated by telomere integration (59). In addition, experimental integration of alpha satellite sequences into chromosomes by transfection produces some features of the centromere, including formation of a constriction and reaction with human anti-centromere antibodies (81, 82). These integration sites did not generate functional centromeres but rather interfered with chromosome movement in anaphase by failing to separate properly. More recently, it has been possible to generate stable microchromosomes in cultured cells by transfection of a mixture containing alpha satellite, telomeres and total genomic DNA, resulting in apparent *de novo* formation of centromeres on the exogenous alpha satellite DNA (60). The available data provide evidence that alpha satellite by itself can specify several features of the centromere and is a functional component of normal centromeres. Activating this centromere-forming potential, however, requires additional components, as discussed below (83).

2.4 Centromere proteins

Despite the complexity of their DNA organization, a good deal is known about the protein components of animal cell centromeres, spurred primarily by the discovery and characterization of human centromere proteins as autoantigens recognized by sera of patients with scleroderma (CREST) (84, 85). The centromere proteins (CENPs) are chromatin-associated structural components of the centromere and play important roles in organizing satellite DNA into a functional state competent to nucleate assembly of a kinetochore in mitosis. The CREST sera have been used as tools to identify novel centromere proteins by biochemical methods (86) and spurred the productive search for additional centromere-related autoantigens (87). Coupled with genetic approaches in *Drosophila* that are beginning to identify centromere-associated proteins based on functional criteria (88, 89) the components required to assemble an animal cell centromere are being uncovered at a rapid pace.

2.4.1 Centromeric chromatin: beginning to make CENPs

CENP-B was the first centromere protein cloned from any species and functions as a sequence-specific alpha satellite DNA-binding protein (90, 91). A highly conserved single copy gene in mammalian cells, CENP-B has a distinctive N-terminal DNA-binding domain that is functional as a monomer, but also associates to form homodimer complexes capable of binding two DNA molecules *in vitro* (92, 93). CENP-B has been proposed to function as a cross-linker for higher order assembly of centromeric DNA because the 17 bp CENP-B box sequence, the DNA-binding site for CENP-B, is iterated with high frequency in alphoid DNA and CENP-B is co-localized with alpha satellite throughout the inner heterochromatic domain of the centromere (93–95). The significance of CENP-B for centromere function is, nevertheless, deeply enigmatic. CENP-B box sequences are conserved in non-human satellite DNAs that

share no other homology with primate alpha satellite (75, 96). However, the CENP-B box cannot be detected in the African green monkey, although it has highly related centromeric alpha satellite sequences as well as CENP-B (97, 98). In addition, inactive centromeres found on stable dicentric chromosomes bind to CENP-B but display no centromere function. Why would a DNA-binding protein be highly conserved (>90% identity among mammals) if its DNA-binding target is dispensable for function? It has been proposed that CENP-B may function to promote recombination within alpha satellite arrays, based on sequence similarity to transposase-like proteins, thus operating at the level of chromosomal evolution without a specific function in mitosis or meiosis (99). Perhaps the identification of ABP1p/CBP1p in fission yeast, with its clear homology to CENP-B, will provide insight into the function of CENP-B-like molecules.

A more direct link to centromere function is seen with the other two founding members of the CENP family, CENPs A and C. CENP-A is a centromere-specific form of the core nucleosomal protein histone H3 and is thought to be the mammalian homolog of yeast Cse4p (100, 101). Biochemical analyses reveal that CENP-A is found in a nucleosome-like particle (102). These data provide evidence for the striking conclusion that the centromere is differentiated from the chromosome arms at the most fundamental level of chromosome organization, that of the nucleosome. CENP-A can target to centromeres in non-human cells, demonstrating that it recognizes a conserved feature of mammalian centromeres (101). Although CENP-A has a unique N-terminal domain unrelated to that of histone H3, this does not specify its distinctive assembly properties; rather, DNA-binding and self-assembly structures within the histone H3 homology domain are necessary for centromeric localization of CENP-A (101, 103). CENP-A is co-localized at the inner kinetochore plate with CENP-C, where it is found only in association with active centromeres (34). Recently, it has been possible to directly isolate human kinetochore DNA for the first time by immunoprecipitation of CENP-A chromatin, demonstrating unambiguously that alpha satellite DNA is kinetochore-associated DNA in human cells (104). Taken together, these results demonstrate that the foundation of the kinetochore, the inner plate, is made from a distinctive form of chromatin.

CENP-C was the first protein identified that tracks with centromere function rather than sequence (76). It is a basic ~110 kDa protein located in the inner kinetochore plate at the level of immunoelectron microscopy, and is essential for normal kinetochore function in human cells (33, 105). Inhibition of CENP-C by antibody microinjection results in reduced kinetochore size and mitotic delay, demonstrating a limiting function for CENP-C in kinetochore replication. This experiment also underscores the relationship between kinetochore assembly and mitotic checkpoint control discussed in Chapter 1 and Section 5. Like CENP-A, CENP-C can assemble at centromeres in a variety of vertebrate species and thus is recognizing some common feature related to kinetochore structure (106). CENP-C has a general DNA-binding activity and the sequences necessary for targeting CENP-C to centromeres are found adjacent to this DNA binding element in the central region of the molecule (106, 107). CENP-C bears sequence similarity with

budding yeast Mif2p, discussed above. Intriguingly, mutations in the Mif2p homology domain disrupt the centromeric targeting of CENP-C (106).

2.4.2 A universal kinetochore nucleoprotein complex?

The inner kinetochore plate is the site where centromeric DNA organizes the kinetochore; exhaustive immunocytochemical analysis at the ultrastructural level has failed to reveal DNA in the outer plate (108). The presence of CENP-A and CENP-C at the inner kinetochore plate in animal centromeres and yeast Cse4p and Mif2p in the core nucleoprotein particle of the budding yeast centromere raises a striking parallel between yeast and human kinetochores. Because of the vast differences in scale and organization, many have thought the two centromeres to be unrecognizably divergent. These results indicate that a similar set of molecules is found at the interface between chromatin and the force-producing apparatus of the kinetochore in both species. Why are these components specifically conserved?

We propose a model in which a complex of CENP-C with a CENP-A nucleosome (or Mif2p with Cse4p) provides the anchor that stably binds the apparatus of the kinetochore directly to the chromatin fiber (Fig. 7). As discussed above, the structure and genetic interactions of Mif2p point toward multiple contacts with kinetochore proteins and with DNA. The pairwise conservation of Mif2p and Cse4p homologs suggests that these two proteins may interact in human kinetochores. CENP-C is proposed to bind asymmetrically on the 'side' of a CENP-A nucleosome, within the gyre of the superhelical DNA path, as proposed for linker histones (109), and also to *CEN* DNA across the DNA path, sandwiching the DNA within a tight protein complex. Linker histones are potent inhibitors of nucleosome sliding on DNA (110, 111). Accordingly, CENP-C/Mif2p could act to provide improved mechanical stability, providing a static linkage to the chromatic fiber. In the yeast centromere, Mif2p is envisioned as integrating a complex of DNA-binding proteins onto the core nucleoprotein particle and these, in turn, function as the spindle interface. The DNA-binding function of CBF3 is necessary to place the complex on a specific DNA locus. It is possible that this recognition function has been replaced by specific protein–protein interactions as centromeres evolved, essentially passing this function up in the hierarchy of nucleoprotein interactions and uncoupling centromere function from DNA sequence per se. This model would account for the lack of DNA sequence conservation among centromeres and the strict correlation between centromere function and the presence of CENP-A/CENP-C (34). It could also explain the persistence of function in kinetochore fragments that has led to the repeated subunit model of the kinetochore, as inner plate chromatin fragments would be predicted to retain kinetochore activity (112, 113). The inner kinetochore plate is proposed to function essentially as a large two-dimensional array of unit kinetochore particles, each one tightly riveted to the DNA strand, displaying key protein–protein interaction domains required to recruit functional components of the kinetochore onto the surface of the chromosome (104).

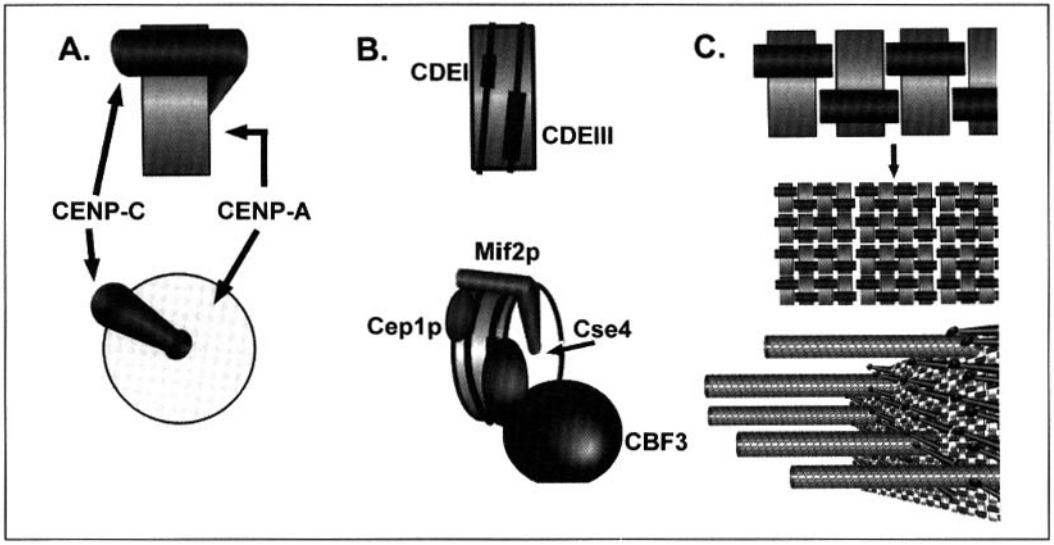

Fig. 7 A model for a conserved centromeric nucleoprotein core. (A) CENP-A and CENP-C are proposed to collaborate in construction of a uniquely stable nucleosome derivative. The model supposes that CENP-C binds to CENP-A nucleosomes in a manner akin to linker histones (168), interacting with CENP-A-specific amino acid residues exposed on the 'side' of the nucleosome within the DNA gyre. A second interaction with the DNA wrapped around the nucleosome serves to pin the DNA in a stable configuration. The third requirement is that the complex has specific protein–protein interaction sites used to recruit other kinetochore proteins. (B) A model that combines the geometry of the nucleosome with the DNA sequence and protein organization of the budding yeast centromere is shown. Wrapping *CEN* DNA around a nucleosome would place CDEI and CDEIII on the same face of the nucleosome, within about 20° of each other (10). Mif2p could contact both CBF1 and CBF3 bound to these elements as well as the adjacent CDEII DNA sequences. A third type of contact with Cse4p completes the model, linking the centromeric nucleoprotein complex into a tightly integrated structure. (C) The inner kinetochore plate is proposed to consist of a tightly packed array of CENP-A/CENP-C nucleosome derivatives. Alpha satellite DNA does in fact exist in phased nucleosomal arrays at the inner kinetochore plate (104). By recruiting microtubule-binding components the inner kinetochore plate creates a field of potential microtubule-binding sites at the chromosome surface, manifested as the outer kinetochore plate.

2.4.3 Kinetochore proteins

Our knowledge of the molecular anatomy of the kinetochore is expanding as numerous proteins, old and new, are being found there (1). Several of these are regulatory proteins and are discussed in Section 5. Others, for example NuMA (114, 115), INCENPs (116) and topoisomerase II (117), are transient residents that redistribute to the kinetochore from other sites and are then shed during or after mitosis, often at anaphase (118). In the case of INCENPs, this appears to be a mechanism for 'measuring' the midplane of the mitotic cell for placement of the cytokinetic furrow (119). A recently discovered autoantigen, CENP-F, is a chromosomal protein that relocates to centromeres in G_2 and is rapidly degraded at the end of mitosis (87, 120). CENP-F is remarkable for its size (367 kDa) and a structure that resembles the SMC family of proteins, with two extended alpha-helical

coiled-coil domains and a putative nucleotide-binding loop. CENP-F is a likely candidate for a scaffolding protein that spans the distance between the chromatin of the inner plate and proteins of the outer kinetochore plates although its ultrastructural distribution remains to be determined. The SMC proteins are involved in a wide variety of chromosomal functions and have been proposed to function as intranuclear motors (121), suggesting a potential mechanical role for CENP-F at kinetochore.

The ZW10 (zeste-white 10) protein of *Drosophila* is a recently identified centromere component, necessary for normal mitotic chromosome distribution, that shows a unique pattern of redistribution during mitosis (89, 122). Entering the nuclear zone after nuclear envelope breakdown, ZW10 concentrates on centromeres in prometaphase. When chromosomes achieve bipolar attachment to the spindle, ZW10 redistributes toward the poles along filaments thought to be spindle microtubules. At anaphase onset it rapidly redistributes to the centromeres of separating chromatids or homologs in meiosis. Homologs of ZW10 are present in a number of species, including worms and mammals, suggesting that it has an important conserved function in mitosis. ZW10 has been proposed to be a component of the tension-sensitive regulatory machinery of the kinetochore, perhaps reporting bipolar attachment to the spindle poles for reasons yet unknown (89).

The burgeoning population of the centromere–kinetochore complex presumably reflects the complexity of events, structural, mechanical and regulatory, that are integrated at the kinetochore to accomplish the foolproof distribution of chromosomes between generations. Beyond identification of these components and demonstration that cells need them to divide, understanding their biochemical activities and the interactions they provide for centromere function will be a significant challenge for cell biologists in the years ahead.

2.5 Chromatin structure and the epigenetic centromere

The properties of centromere DNA from fission yeast, flies and humans fail to support the deterministic paradigm established by *S. cerevisiae* in which *cis*-acting DNA sequences bind to *trans*-acting sequence-specific DNA-binding proteins to nucleate centromere/kinetochore assembly. If centromere formation is not specified at the DNA sequence level, then where? The view that is emerging is that centromere function is specified as an epigenetic feature of the chromosome, dependent not so much on the underlying DNA, but on heritable structures built on to the DNA (34, 49, 56, 58, 83). The most likely candidate is the chromatin structure of the centromere itself, although covalent modification of the DNA, by methylation for example, could play a role in some systems (123).

Epigenetic control of centromere formation is clearly established in two experimental examples provided above. *S. pombe* reveals a requirement for an epigenetic event in activation of latent centromere sequences (46, 56). Establishment of an acentric chromosome derivative in *Drosophila* apparently involves the transfer of centromere function into euchromatic DNA sequences not normally associated with

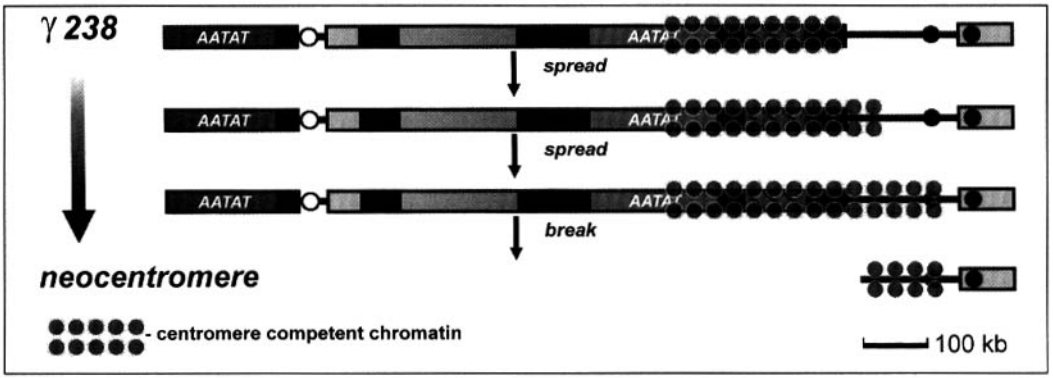

Fig. 8 Neocentromere formation in *Drosophila*. A proposed pathway for generation of a neocentromere in *Drosophila*. Minichromosome derivative γ238 was originally isolated as an inversion derivative of Dp 8-23 which placed centric heterochromatin adjacent to a normally euchromatic DNA segment. A plausible pathway for neocentromere activation involves spreading of the heterochromatic protein organization into adjacent euchromatic DNA. A subsequent deletion event severed the left portion of the chromosome, removing the original centromere and revealing a surprisingly robust centromere that lacks any original centromere DNA sequences. From reference (58).

centromere activity (G. Karpen, personal communication; 58). Formation of this 'neocentromere' was a multistep event involving first the inversion of the centromere, placing it *cis* to the presumptive neocentromere, followed by a deletion that removed the centric heterochromatin entirely (Fig. 8). By analogy with a well documented feature of position-effect variegation (124), one explanation for formation of this neocentromere is that an essential centromeric chromatin structure spread into a normally euchromatic DNA segment while it was juxtaposed to the centromere.

The stability of neocentromeres may reflect a templating phenomenon that propagates chromatin structure through the chromatin assembly pathway or other protein assembly events. Coupled with the variability of DNA sequences capable of supporting centromere formation discussed above, the phenomenon of centromere activation points to a model for the centromere as a self-propagating nucleoprotein complex that templates its own replication rather than relying on *cis*-acting DNA sequences. In this scenario, the centromere may be more similar to the centrioles and spindle pole bodies—whose replication requires a pre-existing structure as a template—than they are to the DNA sequence-directed assembly of transcription factor complexes that have so far provided the major model for understanding how the chromosome integrates DNA sequence with function.

What is the molecular basis for the epigenetic centromere? What is being placed onto the DNA that propagates with high fidelity once a centromere is activated? A *de facto* epigenetic mark on human centromeres is provided by CENP-A and CENP-C as discussed above. But do they actively contribute to specifying a chromosomal locus as a functional centromere? CENP-A assembles onto alpha satellite DNA through a mechanism that requires its differentiated DNA contact surfaces (103). A significant

additional requirement, however, is that CENP-A expression is uncoupled from that of histone H3; CENP-A is expressed in the late S/G_2 phase of the cell cycle, after most bulk histone expression has occurred (103). These observations suggest that replication of the CENP-A chromatin occurs through a mechanism that is distinct, at least in part, from that of bulk chromatin, pointing toward a discrete molecular process that could form the basis for epigenetic propagation of the centromere.

3. Microtubule binding and motor function

Microtubule binding to the kinetochore is the essence of the mitotic mechanism, since it serves simultaneously to attach the chromosomes to the spindle and to generate the key forces that move the chromosomes. The mature kinetochore fiber is bound with microtubule ends buried in the outer plate of the kinetochore and yet microtubule assembly dynamics persist throughout mitosis. The assembly and disassembly of tubulin occurs only at microtubule ends, requiring a kinetochore attachment mechanism dynamic enough to permit subunit exchange. The attachment is highly stable, however, because once chromosomes establish bipolar attachment to spindle fibers they rarely detach. It has become clear that microtubule-dependent motor proteins are important elements of the microtubule–kinetochore interface. What is not as clear is how these proteins contribute to microtubule binding or whether additional microtubule-binding proteins exist at the kinetochore to capture microtubules in a motor-independent fashion. One of the important outstanding questions in mitosis is: what is the source of energy for chromosome movement in anaphase? Is it the ATPase activity of motor proteins or the GTPase activity of tubulin that stores energy in the microtubule lattice, or a combination of the two? The relationship between microtubule dynamics, chromosome movements and motors is discussed in depth in Chapter 4. In this section, I will try to present a centromere's eye view of microtubule binding and motoring.

3.1 A multitude of motors

Barely a decade ago, the idea that the kinetochore was a force-producing 'organelle' on the chromosome was a revolutionary idea (125, 126). The questions currently being asked revolve around which motor is doing what and when: at least three distinct motor-related proteins have been shown to reside at the centromere/kinetochore in animal cells and it is not clear that these represent all the motile activities associated with the centromere (127).

3.1.1 Dynein

A fraction of cytoplasmic dynein can be detected at centromeres by immunocytochemical methods (128, 129). This minus-end-directed motor protein is the most likely candidate for one of the earliest interactions between microtubules and the

kinetochore (130). During prometaphase, the initial attachment of kinetochores to microtubules occurs as a lateral association with the wall of the microtubule and results in rapid poleward motility, the correct directionality for a dynein-based movement. A role for dynein in prometaphase microtubule attachment is bolstered by an experiment in which the 50 kDa subunit of the dynactin complex, a multi-subunit protein complex bound to cytoplasmic dynein, was overexpressed, resulting in the disruption of dynactin complexes within the cell (131). The phenotype of these cells was mitotic arrest in a prometaphase-like state, with condensed chromosomes loosely associated with abnormally structured spindles. While the ultrastructure of microtubule–kinetochore attachment was not examined in this experiment, the phenotype is consistent with a role for dynein in catalyzing the attachment of microtubules to kinetochores. It is not clear whether kinetochore-associated dynein has additional motility functions at later stages of mitosis in animal cells, but in yeast dynein functions to place the spindle in its proper orientation during mitosis rather than in chromosome movement per se (132, 133).

3.1.2 CENP-E

The first candidate for a kinetochore-specific motor protein was CENP-E, a 312 kDa kinesin-related protein that is necessary for proper execution of mitosis (134, 135). CENP-E is located in the fibrous corona/outer kinetochore plate and is one of the earliest known mitosis-specific proteins to associate with kinetochores during prometaphase (X. Yao and D. W. Cleveland, in press). Unlike cytoplasmic dynein, the function of CENP-E is almost certainly restricted to chromosome motility: it does not accumulate until G_2 and is rapidly degraded at the end of mitosis (135, 136). CENP-E distribution is dynamic during mitosis: it is associated with kinetochores until anaphase onset, when it begins to relocate to the spindle midzone (134). A fraction of CENP-E does, however, remain associated with kinetochores throughout anaphase A, where it could play a role in moving chromosomes to the poles (137). The precise function of CENP-E as well as the directionality of its putative motor activity remain to be firmly established. Antibody microinjection experiments result in arrest at or prior to metaphase, indicating that CENP-E could be involved in chromosome congression or necessary for the metaphase–anaphase transition (134,138 One key activity of CENP-E is the ability to bind stably to the track with microtubule ends under conditions of microtubule disassembly, thus possibly acting as a kinetochore–microtubule linker harnessing microtubule lattice energy for movement (139). CENP-E purified from HeLa cells is associated with a ~900 kDa particle that possesses minus-end-directed microtubule motor activity which could be used in chromosome congression, anaphase segregation or both (140). Surprisingly, however, bacterially expressed CENP-E exhibits plus-end-directed motility as determined in standard microtubule gliding assays (K. Wood and D. Cleveland, personal communication). This finding raises the possibility that CENP-E exists as part of a bi-directional motor complex in HeLa cells. The alternative, that CENP-E is itself a bi-directional motor protein, would represent an unprecedented finding in the field of molecular motors. The range of biological properties exhibited

by CENP-E remains to be fully elucidated, but it is clear that this protein forms an important link between the kinetochore and the microtubules of the spindle.

3.1.3 XKCM-1 and MCAK

A distinctive microtubule catastrophe-inducing activity has been ascribed to a recently discovered kinesin-related protein of *Xenopus*, XKCM-1, that is concentrated at centromeres and also localizes to spindle poles and along microtubules (141). Biochemical depletion and add-back experiments demonstrate that XKCM-1 is essential for normal spindle assembly in *Xenopus* extracts: in the absence of XKCM-1, nucleated microtubule arrays grow to enormous lengths (>50 μm), effectively inhibiting normal spindle morphogenesis. Analysis of microtubule assembly in *Xenopus* extracts revealed that the frequency of microtubule catastrophe, the depolymerization phase of microtubule dynamic instability, was reduced four-fold after depletion of XKCM-1. Taken together, these results suggest that XKCM-1 functions at least in part as a regulator of microtubule length and dynamic instability. An attractive model for kinetochore minus end motility can be built from consideration of the combined activities of XKCM-1 and CENP-E (141) (Fig. 9). A plus-end-directed motility of XKCM-1 [proposed on the basis of relatedness to the

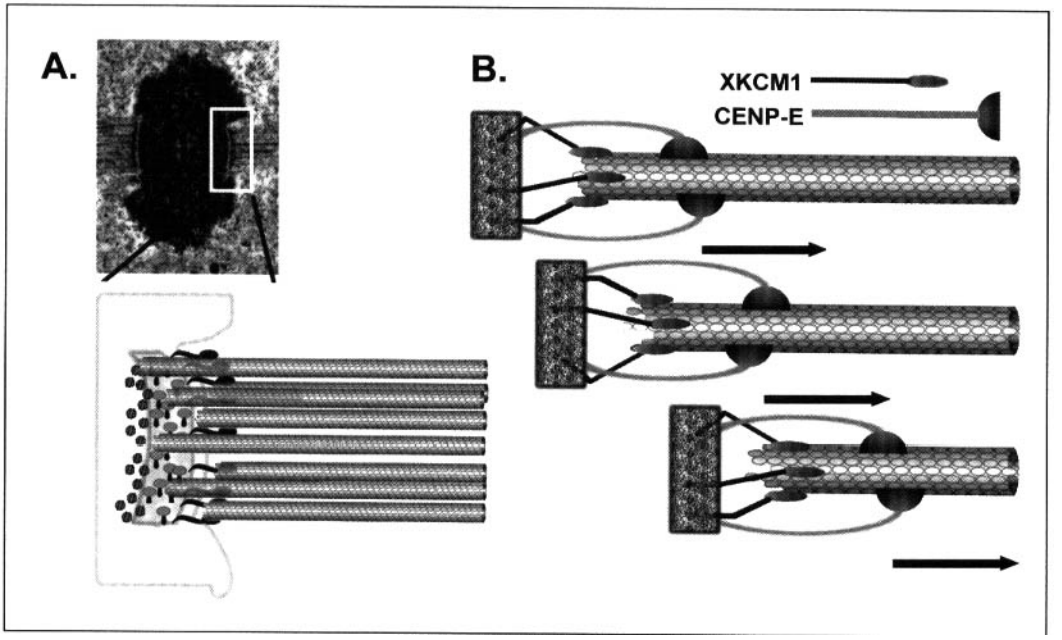

Fig. 9 Functional and molecular organization of the kinetochore. A schematic depiction of the kinetochore is shown in (A), illustrating the collar-like structure of the kinetochore and the proposed segregation of different molecular components at different depths within the kinetochore. A model for induced depolymerization-coupled motility mediated by XKCM-1 in conjunction with CENP-E is shown in (B). XKCM-1 is shown binding the tip of the microtubule, where it induces microtubule catastrophe and conversion to the shrinking phase of dynamic instability. CENP-E maintains a stable attachment to the microtubule, tracking the shrinking plus end and translocating the chromosome toward the microtubule minus end, located at the spindle pole. From reference 141.

known plus-end motor KIF2 (142)] could localize XKCM-1 at kinetochore-bound microtubule plus ends. Induction of catastrophe, presumably through a conformational change induced in the sensitive cap region of the microtubule, would throw the microtubule into the shortening phase of dynamic instability. CENP-E at the kinetochore would maintain association with the depolymerizing end, resulting in an induced poleward motility. These coordinated activities would be not unlike the two feet of a skateboard rider: one (XKCM-1) pushes off against the substrate, in this case by inducing depolymerization, while the other (CENP-E) hangs on to capture the motility so induced. One remarkable aspect of this model is that the presumptive ATPase activities of the two motors need not be directly involved in poleward motility. Rather, they capture the energy of tubulin GTPase activity stored in the microtubule lattice.

XKCM-1 is most closely related to a chromosomal kinesin family protein from mammalian cells: MCAK or mitotic centromere-associated kinesin, originally identified in CHO cells (143). Like CENP-E, MCAK becomes localized to centromeres in mitosis. Unlike CENP-E, however, it localizes to the inner heterochromatic domain of the centromere, between the two sister kinetochores. This would be an appropriate place for a microtubule-depolymerizing activity, if MCAK shares that property with its sister protein from *Xenopus*. However, the conventional wisdom is that microtubules bind and terminate very near the outer plate of the kinetochore, since that is what is observed in most electron micrographs of kinetochores (144). The unexpected localization of MCAK suggests that there may be functional interactions with microtubules that extend deep into the centromere.

3.2 Kinetochores and microtubule dynamics

Once attached to the spindle, movement of chromosomes is tightly coupled to microtubule assembly and disassembly (reviewed in 145, 146). The continual oscillations that chromosomes undergo during congression and metaphase provide *de facto* evidence that tubulin polymerization and depolymerization continue at kinetochore-bound microtubule ends (147). It has long been known that kinetochores affect the dynamic behavior of microtubules to which they are bound (148–150). The sign of this effect—whether kinetochores stabilize or destabilize bound microtubules—is dependent on assay methods as well as on the stage of mitosis: in life they probably do both (149, 151, 152; for review see ref. 145 and Chapter 4). As measured by resistance to depolymerization induced by cold (148, 153) or antimitotic drugs (154), the kinetochore stabilizes microtubules relative to unbound microtubules in the spindle. Net assembly at the kinetochore-bound plus ends of microtubules nevertheless occurs throughout mitosis since fluorescence marking experiments reveal continual poleward flow of tubulin through kinetochore fibers in a process referred to as flux (155) or treadmilling (156). Careful analysis of the dynamic behavior of kinetochore microtubules reveals that it is subject to cell cycle regulation which may promote the stability of the attachment during anaphase (152). In addition, chromosome oscillations require that the 20–50 microtubules comprising

a kinetochore fiber coordinately switch between growing or shortening phases abruptly within 1 s or less (147, 157). These considerations point to the idea that the kinetochore has a sophisticated machinery for manipulating microtubule ends to direct chromosome movement.

3.3 What is the primary kinetochore–microtubule contact?

A critical question that remains regarding the mechanism of microtubule attachment to kinetochores is whether kinetochores have specific non-motor proteins that bind microtubules or whether the binding is mediated solely by motor protein interactions. An important clue comes from analysis of requirements for budding yeast CBF3 binding to microtubules (8). CBF3 preparations bound to CDEIII DNA on beads exhibit motility on microtubules (7). CBF3 was therefore thought to contain intrinsic motor activities, raising for the first time the hypothesis that the primary interface between kinetochores and microtubules is through motor proteins (7). Subsequently it was shown that CBF3 does not contain an integral motor, but recruits an extrinsic motor protein, possibly the kinesin-related Kar3 (158). Through careful dissection of CBF3–DNA complex binding to microtubules *in vitro*, an extrinsic activity that is required for binding to microtubules has been identified (8). CBF3 alone cannot bind microtubules, but it recruits additional components that can be eluted from CBF3 complexes without disrupting DNA-binding activity. However, complexes that were competent to bind to microtubules did not have motile activity. The data are suggestive of a model in which the microtubule binding and motor activities associated with CBF3 are distinct from one another, pointing to a motor-independent mechanism for microtubule binding. Certainly an active motor is not required.

While the structure and components of the microtubule-binding apparatus of the kinetochore remain to be elucidated, the available evidence places some constraints on how it is designed (for a lucid discussion see ref. 145). Microtubules bind end-on to the kinetochore once they are stably attached (144, 159). Because of the requirement to maintain tubulin exchange at the end, most authors draw the kinetochore as a collar-like structure containing multiple microtubule 'receptors' that reach out to touch the microtubule wall near the plus end (Fig. 9; 145, 151). Two features are predicted for the binding complex: (i) that the activation energy for binding is low enough to allow rapid association and dissociation, enabling dynamic attachment, and (ii) that there be a sufficient number of such sites that the probability of complete detachment from the microtubule wall is low; i.e., the aggregate binding energy sums to a high affinity for the microtubule (145). The kinetochore corona fiber complex, a fuzzy surface layer of fine fibrillar material extending ~250 nm away from the kinetochore outer plate (160), is a prime suspect for the multivalent microtubule binding 'collar'. Alternatively or in addition, similar components could reside in the kinetochore outer plate or central region. Indeed, it has been proposed that components with differing functional effects on microtubule binding or stability could be located at different depths within the kinetochore (146, 151). CENP-E and

MCAK are an example of this type of segregation, as discussed above. The distance between the outer plate and the surface of the inner plate of the kinetochore is approximately 50–75 nm at metaphase (144), roughly five to ten tubulin dimers thick, which provides ample opportunity for multiple associations along the wall of the microtubule. In addition to lateral association within the corona or outer region of the kinetochore, it is possible that binding may occur directly on the terminal tubulin molecules at the end of the microtubule. Since there are 13 protofilaments that can be bound, it is possible to conceive of a truly terminal binding complex that could maintain attachment while allowing assembly and disassembly to occur (145). Although a complete understanding of the kinetochore–microtubule interface must await high-resolution ultrastructural analysis, by mapping the biochemical properties of kinetochore-associated proteins such as CENP-E and XKCM-1 onto the kinetochore, a functional understanding of this complex device is beginning to emerge.

4. Chromatid cohesion

The cohesion between sister chromatids, and between homologous chromosomes during meiosis I, provides a mechanical linkage that is essential for accurate chromosome distribution. It ensures that the two sister chromatids will orient toward opposing poles of the spindle (161) and also acts as a physical barrier that prevents progression to anaphase until checkpoint controls have been relieved and the spindle is able to accurately deliver its precious cargo to two new cells. It is clear that the chromatids are joined all along their length throughout the early stages of mitosis, but the evidence also points toward a distinctive form of cohesion at the centromere. Premature separation of centromeres prior to the anaphase transition is a hallmark of Roberts' syndrome and leads to high levels of chromosome mis-segregation, resulting in aneuploidy (162, 163). Conversely, the familiar X shape of mitotic chromosomes after treatment of cells with antimitotic drugs reveals that centromeres can remain paired after the arms of the chromosome have fully separated. Mechanistically, centromere cohesion appears to involve both DNA intercatenation as well as specific 'anaphase glue' proteins, while the proteolytic event(s) necessary for severing the link is one of the key regulatory points of mitosis.

4.1 DNA topoisomerase II

DNA topoisomerases perform essential functions in mitosis, as revealed in yeast where topoisomerase II mutations result in failure of chromatid separation and chromosome breakage (164). Both topoisomerase I and topoisomerase II, a major structural component of the mitotic chromosome scaffold, are necessary for chromosome condensation in prophase (165, 166). Topoisomerase II is also essential for anaphase chromatid separation (167, 168). This was first demonstrated in fission yeast by constructing a strain containing complementary temperature-sensitive topoisomerase II (*top2*) and cold-sensitive β-tubulin (*nda3*) genes (166). Chromo-

somes condense at the permissive temperature for temperature-sensitive top2 in the absence of spindle formation, but fail to separate after a shift to the nonpermissive temperature that activates β-tubulin-dependent spindle formation while inactivating topoisomerase II. Taken together, these results strongly point towards DNA intercatenation as one component of the sister chromatid adhesion mechanism.

Does topoisomerase II have a specific function at the centromere? A recent survey of topoisomerase II distribution in several mammalian species revealed that topoisomerase II is concentrated in the centromere and kinetochore during mitosis, temporally associated with onset of centromeric heterochromatin condensation and persistent until the metaphase–anaphase transition (117). Any functional interpretations of these data amount to 'guilt by localization', but they are clearly suggestive of a role for topoisomerase II in centromere cohesion. Another potential distinction between centromeric and arm cohesion is revealed by analysis of mutations in the *Drosophila barren* (*barr*) gene, which encodes a protein that binds to and modulates the function of topoisomerase II (169). In *barr* mutants, centromeres move apart at the metaphase–anaphase transition while sister chromatids remain attached along their lengths. Thus, topoisomerase II probably plays a direct role in separation of centromeres and this may be regulated through a mechanism that is at least partially distinct from that of the chromosome arms.

4.2 Centromeric cohesion proteins

Three genes have been identified in *Drosophila* that appear to function specifically in chromatid cohesion at centromeres (88, 170, 171). One of these, meiS332, encodes a meiosis-specific centromere protein that presumably links sister chromatids together until anaphase II (88), as discussed in Chapter 7. The other two genes, *pimples* (171) and *three rows* (170), are required for chromatid separation in mitosis. The Pimples protein is unstable and is rapidly degraded at the metaphase–anaphase transition (171). Mutants accumulate polyploid chromosome complexes joined at their centromeres in metaphase after one or more rounds of defective mitosis, a phenotype similar to that of Three rows. Although it has not been shown directly that Pimples is a centromere protein, the apparently normal separation of chromosome arms in *pimples* mutants points to the centromere as its target of action. One possible scenario is that Pimples may promote cohesion directly through protein–protein interactions, acting as a glue to bind the chromatids together. Experimental introduction of *lac* operator arrays into yeast chromosomes, coupled with *lac* repressor expression, results in chromatid cohesion at these sites through self-assembly of *lac* repressor tetramers, demonstrating that protein–protein interactions are sufficient to maintain sister chromatid cohesion during anaphase (172). Another possibility is that Pimples or Three rows act as centromere-specific modulators of topoisomerase II, a complement to the chromosome arm-associated *barr* gene product. These proteins may represent founders of a set of proteins that cooperate to form and regulate the cohesive structure that joins the centromere into an integral structural unit during mitosis.

4.3 Proteolysis and chromatid cohesion

Analysis of anaphase chromatid separation *in vitro* led to the important finding that the onset of anaphase requires a proteolytic event that occurs independently of cyclin degradation (173, 174). Although the actual targets of this ubiquitin-dependent proteolysis have not been identified, association of the ubiquitin-dependent proteolysis pathway with the budding yeast kinetochore discussed above suggests a functional relationship between centromeres and the proteolytic mechanisms of mitosis (9, 17, 19). A second suggestive link with proteolytic pathways is revealed by the discovery of a yeast gene, *SMT3*, that acts as a suppressor of mutations in the *MIF2* gene (P. Meluh, personal communication). *SMT3* encodes a ubiquitin-like protein and although any link between *SMT3* and proteolysis remains purely speculative, the theme of ubiquitin/ubiquitin-like modification of centromere proteins provides support for the hypothesis that proteolysis within centromeres is directly involved in sister chromatid separation. Clearly, proteolytic events mediated by the anaphase-promoting complex (APC) or cyclosome are the key to getting cells out of mitosis (175). Linking APC proteolysis to specific chromosomal and centromeric proteins is an exciting prospect that should be fulfilled in the near future.

5. Regulatory properties of centromeres

A remarkable discovery of the past few years has been that centromeres function as sophisticated signal processing centers, regulating cell cycle progression through checkpoint controls that sense the disposition of the chromosomes and their proper assembly on the spindle (176–178). The noteworthy feature of these systems is that they appear, at least in part, to be mechanosensory pathways that convert tension exerted across the centromere into biological signals that affect global features of mitotic progression as well as local features of individual kinetochores and spindle fibers. Through their regulatory elements, centromeres act as guidance systems that coordinate and report the activities of the chromosomes throughout the critical phase of mitosis leading to the onset of anaphase. At anaphase they switch roles from signal generators to receptors as APC-mediated proteolysis severs the link between sister chromatids, unleashing them for transport to the spindle poles and the next generation. The role of centromeres in cell cycle regulation is detailed in Chapter 1 of this volume so I will make just a few comments here regarding the incorporation of regulatory mechanisms into the structure of the centromere–kinetochore complex.

5.1 Mechanoregulation by kinetochores

Several protein components of the spindle assembly checkpoint and the mechanosensory regulation circuit described in Chapter 1 are structurally associated with the kinetochore. The vertebrate homologs of MAD2 (179, 180) and BUB1 (181) have both been found to reside at centromeres during mitosis. The 3F3 phosphoepitope associated with tension-dependent regulation has been localized to the central region

between the inner and outer kinetochore plates (182, 183). These important results demonstrate that key checkpoint components are in fact structural elements of the centromere and that the spindle assembly/anaphase checkpoint is one of the most highly conserved features of centromere function. An exciting corollary of these findings is that yeast genetics and vertebrate cytology can now directly complement each other in dissecting a critical function of the centromere–kinetochore complex.

The anaphase or spindle assembly checkpoint operates on a global level, but tension also regulates key events at the local level of each kinetochore. Tension is a key factor in stabilizing microtubule binding to the kinetochore, which may be a means of allowing a chromosome to quit and try again if it is having trouble capturing the second pole (184–186). The force-generating mechanisms of the kinetochore also appear to be responsive to tension (147, 187). During prometaphase and metaphase sister kinetochores are engaged in a tug-of-war as each generates force primarily toward one pole (157, 188). As a result chromosomes show nearly continuous oscillations between the poles, stretching centromeric chromatin as kinetochores alternate between driving poleward and remaining passive (147, 187, 189). When kinetochore vectors are correlated with the degree of tension reported by chromatin stretch, there is a correlation between stretching and coordinated kinetochore motion (147, 157). These experiments led to the proposal that the centromere functions as a molecular tensiometer, providing feedback to kinetochore-associated motility components that allow integration of sister kinetochores for coordinated chromosome movement (147, 187).

Taken together, these experiments show that the cell relies on the mechanical connection between sister centromeres for several aspects of spindle assembly, force production and control. Surprisingly, kinetochores (190) and even the chromosomes (191) are dispensable for the construction of bipolar spindles and anaphase-like changes in microtubule dynamics. The spindle apparently consists of several independent programs of morphogenesis and dynamics. Nevertheless, it is clear that integrating these different components, or subroutines, of the mitotic apparatus into an effective program for chromosome distribution requires the mechanical integration afforded by the tension-bearing components of the centromere.

5.2 Kinetochore structure as a process

As the 3F3 antibody documents the changing state of protein phosphorylation in the kinetochore, evidence is mounting that the protein composition of the kinetochore changes as mitosis proceeds. Vertebrate Mad2p and Bub1p are tightly localized to kinetochores during prophase and much of prometaphase, but disappear by metaphase (179–181). In both cases, disassembly of the spindle with antimitotic agents results in sustained accumulation of checkpoint proteins at the centromere, suggesting that the association and dissociation of centromere-associated regulatory proteins represent orchestrated events dependent on spindle function. Coupled with experiments discussed previously, in which proteins like CENP-F, the INCENPs

and ZW10 come and go at the centromere, it becomes clear that the centromere–kinetochore complex is a highly dynamic structure whose composition evolves as mitosis proceeds.

6. Conclusions and perspectives

Our concept of what centromeres are and how they function has evolved a great deal in the past decade. Once viewed as passive attachment sites like the handles on a suitcase, centromeres are now understood to occupy, quite literally, a central position in the structure and regulation of the mitotic spindle. While a great deal has been learned about certain aspects of centromere structure and function, we are now in a position to refine some of the questions necessary for solving the problem of how chromosomes are moved during cell division at a molecular level.

What is the basis for centromere assembly? Biological assembly is understood to proceed through a series of discrete molecular recognition events that are required to establish the network of protein–protein and protein–nucleic acid interactions that knit a structure together. The ultimate paradigm for this is the assembly of T-even phage. With the identification of unique *cis*-acting DNA sequences sufficient for centromere function in budding yeast, it was anticipated that centromere formation in other species would exhibit the same principle: a key protein-DNA binding interaction encoded in the sequence of centromeric DNA that initiates centromere assembly. It seems almost certain that specification of a centromere in higher cells is only remotely related to the underlying DNA sequence and depends much more on the pre-existing nucleoprotein assembly on the chromatin fiber and the functional history of the centromere. Nevertheless, centromere assembly is a highly regulated and specific process; it is expected that a discrete set of molecular interactions occurs to nucleate formation of a functional centromere at a unique site on the chromosome. Identifying these important molecular recognition events, when they occur and how they are regulated, stands as one of the foremost challenges for understanding the complex centromeres of animal cells. Unraveling this assembly process will undoubtedly require a more complete understanding of chromosomal assembly mechanisms—how chromatin is replicated and remodeled for specific functions. In turn, as diverse processes such as transcription and DNA replication are being found to share common features of construction and architecture, we can expect that efforts to understand centromeres will illuminate shared principles of chromosome metabolism (23).

Mitosis and meiosis are, ultimately, mechanical transport processes in which the cell lugs chromosomes back and forth and around on the spindle until everything is in order, whereupon chromosomes disjoin and move to the poles. The forces involved are large and sufficient to stretch the tightly packed heterochromatin of the centromere to twice or more its original dimensions, and yet the link between microtubules and the chromosome is perfectly stable. The conundrum of a dynamic and responsive microtubule linkage that keeps ends free for exchange with soluble tubulin, while at the same time bound tenaciously to the chromosome will be an important one to solve. What is the pathway leading from the chromatin fiber to the

microtubule surface? What accounts for its tensile strength? How does microtubule-generated work feed back to control motility and structure? As experiments are designed to answer these and associated questions it will be important to bear in mind that mitotic inhibition may occur as a result of loss of function or by stimulating checkpoint arrest through disruption of proper assembly. Similarly, it will be difficult to dissect the contributions of microtubule-dependent motor function from those of microtubule assembly dynamics in force production, since microtubule assembly and disassembly are obligately coupled to changes in chromosome position within the spindle. Indeed, microtubule end-dependent functions of proteins like CENP-E and XKCM-1 indicate that it may be important to broadly rethink our concept of cytoskeletal ATPases as 'filament walking' proteins.

The strength and mechanics of the microtubule–chromatin connection form the basis for one of the most remarkable signaling systems yet discovered within the cell: the spindle assembly checkpoint. Mounted within the kinetochore are a group of molecules that interface directly with the mechanical situation of each chromosome during prometaphase and metaphase movements, and translate molecular tension into biological signals that speak directly to the engine driving the cell cycle. The mechanisms that the kinetochore uses to accomplish this translation step from physical strain to biological information will not only be fascinating to unravel but should shed light on basic biomechanical design principles at the molecular level. In addition, the spindle assembly checkpoint pathway may prove to be a fertile site for therapeutic intervention in disease states characterized by aneuploidy, including oncogenesis, fertility and developmental disorders.

Through combined approaches of genetics, molecular biology, biochemistry and cytology, we have reached a sophisticated level of understanding of how centromeres function to produce chromosome movement and have established a clear pathway for future discovery. What was once considered a moribund lump of junk DNA has turned into one of the exciting puzzles of molecular cell biology, a veritable feast of experimental opportunities that promises to turn out new surprises in molecular design and assembly as we elucidate the mechanics of the process by which the cell makes the step from one generation to the next.

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6 Telomeres: structure, synthesis, and cell cycle regulation

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1. Introduction

Genomic integrity depends on a number of different processes affecting chromosome replication and stability. Telomeres are specialized structures found at the ends of linear chromosomes and are functionally defined based on their properties, which differ from those of ends resulting from chromosome breakage. Studies in *Drosophila* and maize from the 1930s and 1940s first demonstrated that broken chromosomes that lack telomeres are unstable and capable of fusing with other newly broken ends. These observations, showing that telomeres are required for proper linear chromosome function, have since been confirmed and extended in a number of experimental systems. Over the last two decades, advances in our molecular knowledge regarding features of telomeric DNA structure, the identification of a novel DNA polymerase responsible for replicating telomeres, and the characterization of numerous other proteins that interact with telomeres have greatly extended our understanding of this essential chromosomal element.

A striking result of these studies is that many features of telomere structure and replication are conserved across the eukaryotic kingdom. With a few notable exceptions, telomeres consist of tandem repeats of simple G-rich sequences, with the G-rich strand oriented 5′ to 3′ towards the end of the chromosome. In addition to this sequence conservation, telomeres exhibit a conserved structural feature, in that the G-rich strand extends as a 3′ overhang beyond the telomeric duplex region. The primary mechanism for replicating chromosomal termini is the enzyme telomerase, which is responsible for elongating the G-rich strand of the telomere; this enzyme is a remarkable RNA-dependent enzyme that employs a novel mode of synthesis to elongate chromosome ends. Recent studies in humans have also suggested that regulation of telomerase activity in different cell types may play roles in both cellular senescence and oncogenesis, thereby directing a focus on the synthesis and regulation of this enzyme in multicellular systems.

In addition to telomerase, the telomere also interacts with both duplex and single-

strand DNA-binding proteins, which are responsible for forming a non-nucleosomal chromatin structure at the end of the chromosome. Among other roles, these proteins are presumed to form a protective cap at chromosomal termini. This protective structure, specific to the telomere, is thought to help distinguish natural chromosome ends from chromosomal breaks, the latter signaling disruptions in cell cycle progression. Other recent reviews (1–5) discuss the above issues as well as aspects of telomere replication and function that are not covered in this chapter.

2. Telomerase

The enzyme telomerase is the primary mechanism by which most eukaryotes ensure complete replication of chromosomal termini, which conventional DNA polymerases are unable to duplicate fully (see Section 3.1). Although telomerase is apparently not absolutely required for cell division, the function of the enzyme is clearly important in continued cell proliferation (6–10). Current studies on the consequences of a telomerase deficiency have concentrated primarily on telomere length decline and the resulting effects on proliferative capacity; the possible involvement of this enzyme in cell cycle checkpoints and/or regulatory interactions with other components of the DNA replication machinery has not yet been addressed in detail (see Section 3).

Initial studies on telomerase were conducted predominantly on enzymes from the ciliated protozoa, in large part due to a unique feature of normal ciliate development. Although single-celled, these organisms have two types of nuclei: a transcriptionally silent diploid micronucleus, responsible for propagating genetic material, and a polygenomic transcriptionally active macronucleus. During the formation of the macronucleus, the micronucleus genome is fragmented into thousands of gene-sized DNA molecules, requiring the *de novo* addition of telomeres to allow maintenance and replication of the newly formed sub-chromosomal molecules (11). As a consequence, extracts prepared during this period of telomere formation are a relatively rich source of telomerase. Telomerase was first identified from the ciliate *Tetrahymena thermophila* as a biochemical activity capable of adding telomeric repeats onto the 3′ terminus of single-stranded telomeric oligomers (12). Biochemical characterization revealed that enzyme activity was sensitive to treatment with RNase, indicating an essential RNA component (13). Subsequent partial purification of the telomerase ribonucleoprotein (RNP) led to the identification of a 159 nucleotide RNA molecule with an internal sequence CAACCCCAA (14), complementary to the $d(TTGGGG)_n$ telomeric sequence synthesized by the *Tetrahymena* enzyme. The presence of this complementary sequence was the basis for the hypothesis that telomerase, unlike other DNA polymerases, carries the necessary information to dictate the sequence of its product; specifically, this model proposes that a short region of an RNA subunit of telomerase serves as a template to dictate the sequence of telomeric repeats that are added onto a chromosomal terminus (Fig. 1; 14). A wealth of experimental manipulations of this RNA subunit, first in *Tetrahymena* and later in a number of other organisms, has proven that the RNA templating model

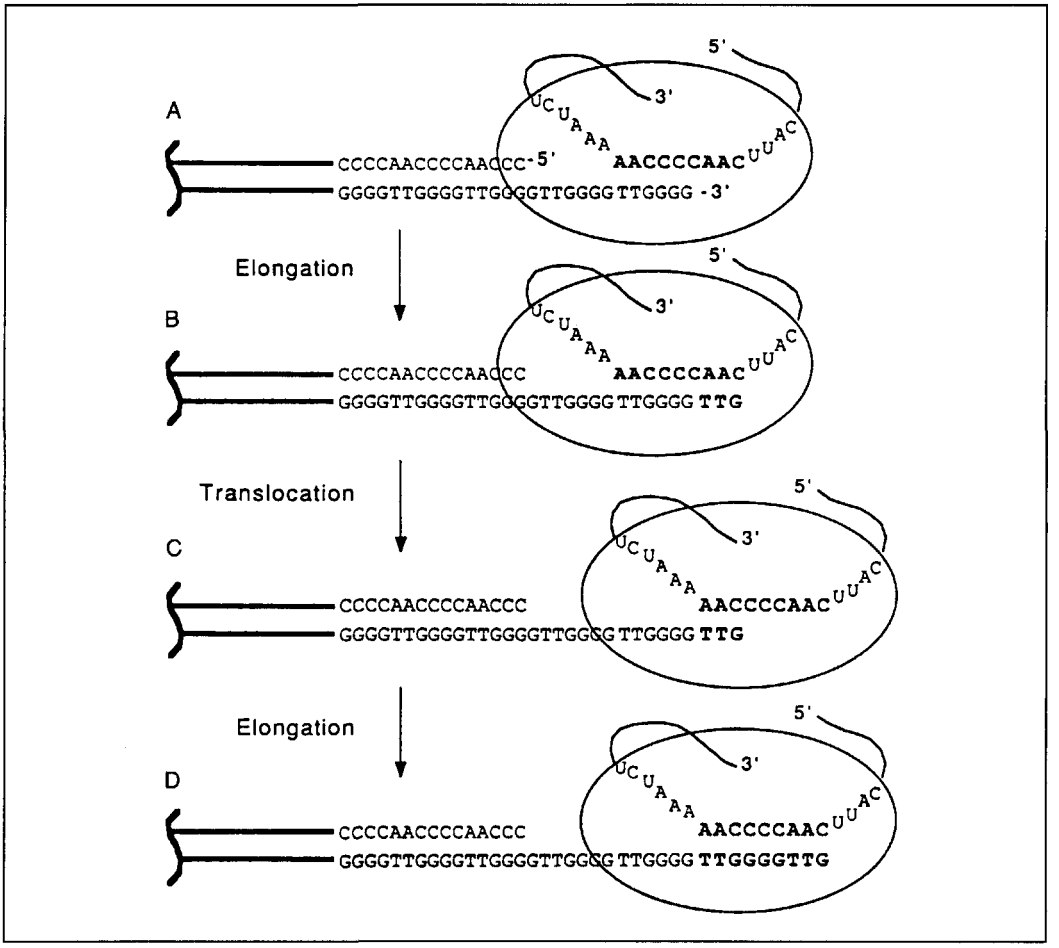

Fig. 1 The RNA subunit of telomerase serves as a template for the telomeric repeat sequence added onto chromosomal termini, reprinted from ref. 14 with permission of the publisher. (A) The telomerase RNA template hybridizes with telomeric 3′ overhangs. (B) Polymerization proceeds to the end of the template. (C) The substrate–enzyme translocates to enable subsequent rounds of elongation, as shown in (D). Reprinted with permission from *Nature*, 1989, **337**, 331 (ref. 14). Copyright 1989 Macmillan Magazines Ltd.

is correct. Therefore, these early studies led to the proposal that this unusual DNA polymerase functions as a specialized reverse transcriptase.

2.1 The RNA subunit of telomerase

Since the initial characterization of this RNA-dependent enzyme from *Tetrahymena*, telomerase identified from every additional source has been shown to be a RNP, and the gene encoding the RNA subunit has now been cloned from over 20 species, including the ciliates, several yeasts, mice and humans. These genes, with one notable exception discussed below, have been primarily cloned using molecular

techniques based on the predicted template region of the RNA (8, 9, 15, 16) or cross-hybridization between related species (17, 18); for two sets of ciliate telomerase RNAs, features such as conserved promoter elements have also contributed to these molecular approaches (19, 20). However, the identification of an RNA displaying a short sequence complementary to telomeric repeats is, in the absence of other data, not a sufficient criterion to prove that the RNA is in fact a telomerase subunit. This problem can be addressed by a functional test to demonstrate that expression *in vivo* of candidate RNA molecules with mutations in the template region results in the corresponding mutant telomeric repeat sequence being introduced into chromosomal termini (6–9). This *in vivo* demonstration is often aided by designing RNA template substitutions such that a restriction site is introduced into the newly synthesized telomeric DNA. Mutation of the template region of candidate RNAs has proven to be a simple and relatively rapid diagnostic test for an RNA component of telomerase, compared to the proof necessary to demonstrate that a potential protein candidate is a component of the enzyme (discussed in Section 2.2).

Strikingly, aside from the template region, there is very little conservation at the primary sequence level among telomerase RNAs from different species. Comparison of the sequence between two budding yeasts (*Saccharomyces cerevisiae* and *Kluveromyces lactis*) shows almost no sequence similarity (7, 8), and even the murine and human RNA subunits show only 65% sequence identity (9, 18). Despite this, it has been possible to construct a conserved secondary structure from telomerase RNAs from multiple, evolutionarily distant, ciliates (17, 19–21). Although some details of the proposed foldings differ somewhat among different ciliate species, a common feature of all of these structural models is the accessibility of the template as an unpaired region of RNA (Fig. 2). Consistent with this secondary prediction, the templating domain can pair with oligonucleotides complementary to this portion of the RNA, as assessed by inhibition of enzyme activity and/or cleavage by RNase H (14–16). The functional role of structural elements other than the template has not yet been determined but, by analogy with other RNPs, at least some of these secondary features will presumably provide specificity for protein binding and hence formation of the telomerase complex. Whether this structural model for the ciliate RNA subunit will extend to telomerase RNAs from other eukaryotes has not yet been determined.

In contrast to the techniques used to isolate most telomerase RNAs, the gene encoding the *S. cerevisiae* telomerase RNA subunit was recovered by a very different approach which may have revealed an additional role at the telomere for this enzyme. This telomerase RNA gene was unexpectedly identified from a genetic screen intended to detect factors that influenced transcription of telomere-located genes (7). Previous studies had demonstrated that reporter genes placed next to the G-rich repeats of the telomere exhibited metastable transcriptional repression (22), a process termed telomere position effect (TPE). Although classic heterochromatin has not been identified in yeast, TPE bears many similarities to the position effects observed in heterochromatin in *Drosophila*, and the phenomenon of TPE can be viewed as a reflection of the non-nucleosomal chromatin structure present at the

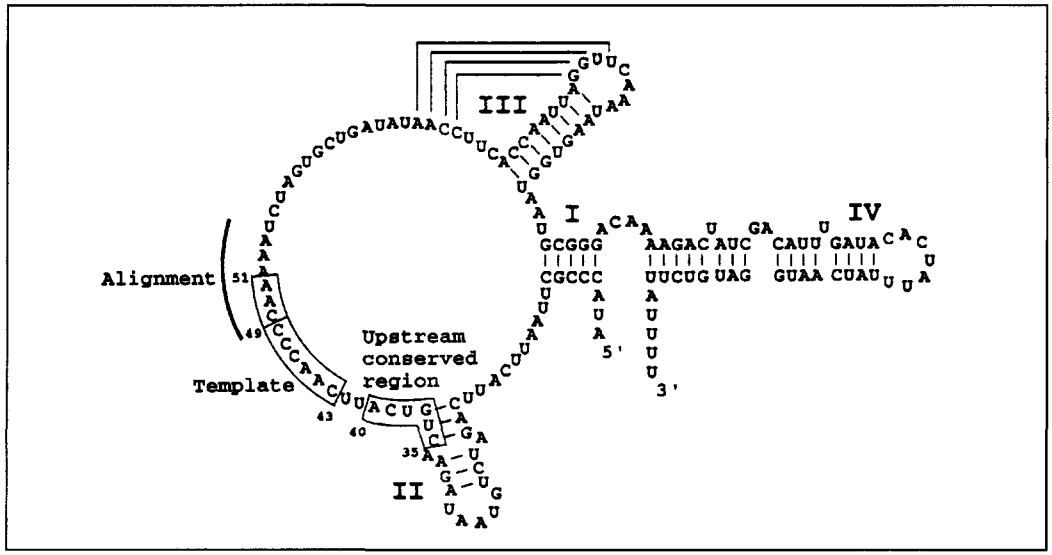

Fig. 2 Secondary structure of the *Tetrahymena* telomerase RNA, reprinted from ref. 63 with permission of the publisher; this secondary structure is conserved in distantly related ciliates, as described in the text. The template and alignment regions of the RNA are indicated; note that this section of the RNA is proposed to be accessible as a single-stranded region. Reprinted with permission from *Genes & Development*, 1995, **9**, 2227 (ref. 63). Copyright 1995 Cold Spring Harbor Laboratory Press.

telomere (discussed in Section 4). In a genetic approach to identify factors that perturb TPE, Singer and Gottschling (7) screened for cDNAs that, when overexpressed, disrupted telomeric silencing. Of the ten genes identified, one gene, called *TLC1*, encoded a 1.3 kb RNA without open reading frames (ORFs) with a 17 nucleotide region previously predicted for the template region of yeast telomerase RNA (23). Introduction of a restriction site into the template site, and the demonstration that this site now appeared in telomeres of strains expressing this RNA, provided proof that *TLC1* encoded the yeast telomerase RNA subunit. The identification of this telomerase component from a screen for alterations in telomeric silencing also argued that telomerase is not only responsible for replicating telomeric DNA but may also directly influence telomeric chromatin structure, possibly as a component of the silencing complex.

The isolation of a telomerase component in a genetically tractable system such as *S. cerevisiae* also provided an *in vivo* examination of the phenotype of a telomerase deficiency. Previous investigation of co-expression of mutant telomerase RNAs in the presence of the wild-type RNA in *Tetrahymena* had implicated telomerase in cell proliferation (6). Complete replacement of the wild-type *TLC1* gene with a *tlc1* deletion resulted in a yeast strain that exhibited telomere shortening, as expected, as well as a decrease in both growth rate and viability (7). The correlation between an absence of telomerase activity and reduced viability in budding yeast provides strong parallels to the proposed mechanism by which higher eukaryotes limit the proliferation of somatic tissues (discussed in Section 5).

2.2 Protein subunits of telomerase

2.2.1 Identification of telomerase protein components in model systems

In comparison to the RNA subunit, characterization of protein subunits of telomerase has until very recently lagged behind that of the RNA subunit, in part due to the difficulty in proving that potential candidates are in fact protein components of the enzyme. Two avenues of investigation have been taken towards solving this problem. The first has been a biochemical approach, via purification of the telomerase complex from a relatively abundant source and characterization of polypeptides that co-purify with activity and the RNA subunit. This has led to the identification of two potential telomerase protein components, 80 and 95 kDa, of the *Tetrahymena* enzyme (24). The larger of the two subunits can be cross-linked to telomeric primers, whereas the 80 kDa protein appears to bind the telomerase RNA. More recently, a similar purification approach has been used to identify two potential protein subunits of 123 and 43 kDa of telomerase from another ciliate (*Euplotes aediculatus*), which are present in apparently equal stoichiometry with the RNA subunit (25). The 123 kDa subunit can be cross-linked to telomeric oligonucleotides and has other properties that suggest it contains a second site involved in recognition of telomeric DNA primers by telomerase (26; discussed in more detail in Section 2.3.2). The disparate sizes of the telomerase protein components from *Euplotes* and *Tetrahymena* could be due to the substantial evolutionary distance between these two species. However, since it is not yet clear how many proteins are a part of the telomerase complex, it is also possible that non-overlapping sets of telomerase protein subunits were recovered from these two purification schemes.

An alternative to the biochemical route is a genetic approach, via the identification of genes which, when mutated, result in phenotypes expected from the elimination of telomerase activity. This has led to the identification of four genes in *S. cerevisiae* that, in addition to *TLC1*, are required for telomerase function. The first of these is the *EST1* gene, long hypothesized to encode a component of telomerase, due to the phenotypes displayed by a strain deleted for *EST1*: *est1*-Δ strains show a progressive telomere shortening as well as a so-called senescence phenotype, manifested as a steady decline in cell viability (27). Since, as described above, deletion of a known component of telomerase (the *TLC1* telomerase RNA gene) resulted in the same phenotypes (7), this provided support for the hypothesis that Est1 could be a component of telomerase, and also indicated that this set of phenotypes could be diagnostic for a telomerase defect. Therefore, using information from the analysis of *est1* strains, an expanded screen for mutants of yeast displaying telomere shortening and cellular senescence was conducted, leading to the identification of three additional *EST* genes (28). Strains mutant for each of these genes resulted in an *est*-like phenotype; furthermore, epistasis analysis demonstrated that all four *EST* genes functioned in the same pathway for telomere replication as defined by the *TLC1* gene, suggesting that these genes encode either components of telomerase or factors which positively regulate telomerase activity.

Convergence between the biochemical and genetic approaches has come with the

demonstration that the p123 subunit of the *Euplotes* enzyme and the *EST2* gene from *S. cerevisiae* are homologs (10). A striking insight into the function of these two proteins resulted from the observation that both Est2p and p123 displayed a set of motifs previously shown to comprise the active site of reverse transcriptases. Introduction of missense mutations in highly conserved residues in these motifs in the Est2 protein abolished telomerase activity *in vitro* and resulted in an *est* phenotype *in vivo* (10, 29), demonstrating that Est2p and p123 are the catalytic protein subunits of the yeast and *Euplotes* telomerase enzymes. This discovery has provided a direct mechanistic basis for the early parallels drawn between the telomerase enzyme and reverse transcriptases, mentioned above. Homologs of the p123 and Est2 proteins have subsequently been identified in the fission yeast *Schizosaccharomyces pombe* and in humans (30, 31), suggesting that a reverse transcriptase-like subunit may be a universal feature of telomerase. Since this is the first example of a reverse transcriptase with a role that is essential for cellular growth, this invokes intriguing speculations about the evolutionary relationship between telomerase and the reverse transcriptase enzymes used by cellular parasites such as transposable elements or retroviruses.

The identification of this catalytic subunit in a number of species raises the question of why telomerase purified from *Tetrahymena* did not have a similar reverse transcriptase subunit. One possibility is that this protein subunit was underrepresented during the purification of the *Tetrahymena* telomerase RNP. Alternatively, the p80- and p95-containing enzyme may use an active site provided by either of these two proteins and therefore not require a p123/Est2-like component. These two possibilities can be distinguished either by the identification of a similar homolog in *Tetrahymena* or via the demonstration that p80, p95 and the RNA subunit are sufficient to reconstitute *Tetrahymena* telomerase activity. A second unexplained puzzle is the absence of easily identifiable homologs of the *Tetrahymena* proteins in the completely sequenced *S. cerevisiae* genome. Since mammalian homologs of p80 have been identified and shown to be associated with telomerase (discussed below), it is unclear why yeast telomerase would lack components that are apparently conserved from ciliates to mammals. One explanation could be that the telomerase holoenzyme consists of a core enzyme with differing sets of accessory proteins in different organisms. Alternatively, yeast proteins that perform the equivalent function to p80 or p95 may not be readily identified by a comparison at the primary amino acid sequence level. If this latter explanation is indeed the case, possible candidates for functionally equivalent yeast proteins could be the remaining Est proteins.

Unlike the situation in the ciliates, it is relatively simple to ask whether extracts prepared from strains mutated for each *EST* gene still retain telomerase activity; a comparable *in vivo* test is not easily performed in the ciliates and thus the *in vivo* consequence of the absence of p80 or p95 activity has not yet been determined. Assaying telomerase activity in extracts prepared from each *est* mutant has shown that, in contrast to Est2p, the other Est proteins are dispensable for yeast telomerase activity *in vitro* (32, 33). Therefore, although each Est protein is essential *in vivo* for

complete telomere replication, only the Est2 protein is required for core enzymatic activity. However, this does not rule out the possibility that one or more of the Est proteins are components of the enzyme and provide other non-catalytic functions. One approach to determining the roles of the other Est proteins in telomere replication has been via biochemical investigation of the individual properties of each protein. Analysis of the Est1 protein has shown that it has the properties of a single-strand telomere binding factor; binding requires a single-stranded DNA substrate with a free 3′ terminus and is specific for G-rich telomeric sequences (34). Est1p also associates with the telomerase RNA *in vivo* (35, 36; T. R. Hughes, unpublished data) and exhibits non-specific RNA-binding activity *in vitro* (34). This has led to the proposal that Est1p functions, via its single-strand DNA-binding activity, as a positive regulator of telomerase by directing the enzyme to the chromosomal terminus. However, the current data do not distinguish between Est1p performing this role as an integral subunit of the enzyme or as a terminus-specific component of telomeric chromatin. Although the RNA interaction would be predicted for a telomerase subunit, it has not yet been determined whether the association of the Est1 protein with the telomerase RNA is stoichiometric, as would be expected of two subunits of the same enzyme complex. If Est1p is instead a component of telomeric chromatin, RNA binding may be a necessary feature of its telomerase loading function. Fractionation of yeast telomerase while monitoring the stoichiometry of the associated proteins will be necessary to differentiate between these two models.

Est1 is not the only single-strand telomere DNA-binding protein identified in yeast. Cloning of the wild-type *EST4* gene revealed that it was identical to the previously identified *CDC13* gene, which has also been shown to encode a single-strand telomere-binding protein (37, 38). Since other features of the Cdc13 protein support the hypothesis that this protein is a component of telomeric chromatin, a more detailed analysis of the role of Cdc13p in telomere function and telomerase regulation is discussed in Section 4.

2.2.2 Mammalian telomerase subunits

Although components of telomerase were first identified in the ciliates and yeast, genes from these lower eukaryotes have now provided the tools for identification of comparable genes in mammals. As a result, two components of the human telomerase core enzyme, the RNA subunit (called hTR; 9) and the reverse transcriptase catalytic protein (referred to as hTERT, for *te*lomerase *r*everse *t*ranscriptase; 30, 31), have been identified. Consistent with studies in the ciliates and yeast, antisense expression directed against the human telomerase RNA leads to telomere shortening and cell crisis in cell culture (9), arguing that telomerase is required for cellular growth in higher eukaryotes as well.

Mammalian homologs of the *Tetrahymena* p80 protein have also recently been identified and shown via immunoprecipitation to be associated with telomerase; however, a stoichiometric association has not yet been demonstrated, leaving open the possibility that this protein may be telomerase-associated but not an integral

component of the enzyme complex (39, 40). Although the mammalian and ciliate p80 homologs show extensive sequence similarity in several regions, the mammalian protein is substantially larger, with N- and C-terminal domains of unknown function.

2.3 Telomerase biochemistry

2.3.1 Telomerase substrate specificity

The preferred substrate for telomerase *in vitro* is a single-stranded oligonucleotide composed of telomeric repeat sequence. However, the sequence of this primer does not need to conform to the telomere repeat of the species from which the enzyme was identified; as was first shown for the *Tetrahymena* enzyme, telomerase shows relaxed substrate specificity *in vitro*, in that the enzyme will efficiently extend telomeric primers from other species (12, 41–44). This loose specificity is also observed *in vivo*, at least in some organisms; in the yeast *S. cerevisiae*, linear molecules with telomeric termini from other species can be converted to functional linear chromosomes, although addition of yeast telomeric repeats is an obligatory step in the conversion to a functional telomere (45–48). In contrast, the sequence requirements for telomere formation in human cells are quite stringent and appear to reflect the DNA-binding specificity of the human telomeric protein TRF1, rather than the primer specificity of telomerase (49, 50).

The relaxed substrate specificity also indicates that the only clear requirement for telomerase primer recognition *in vitro* is the degree of G-richness. Because G-rich primers have a propensity to form secondary structures, most notably G-quartets (51, 52), it was initially proposed that formation of a G-quartet structure might be a prerequisite for telomerase recognition. However, G-quartets are refractory to telomerase elongation *in vitro* (53), leaving open the question of whether these structures have a role in telomere length regulation *in vivo*. Telomerase also has an additional substrate requirement, in that it is unable to extend duplex substrates that have a blunt 3′ terminus, even when such substrates are composed of telomeric repeats; for example, the *Euplotes* enzyme requires a minimum of a four nucleotide 3′ G-rich overhang (25). The inability of telomerase to elongate a blunt substrate *in vitro* has implications for how this enzyme functions *in vivo*, discussed in more detail in Section 3.

2.3.2 Two-site model for telomerase processivity

One consequence of the RNA templating model for elongation of primers by telomerase (Fig. 1) is that the addition of a single round of telomeric repeats alternates with translocation along the template, such that the enzyme either pauses or dissociates prior to translocation. For those enzymes which synthesize regular telomeric repeats, this results in reaction products that display a periodicity when examined after denaturing gel electrophoresis (Fig. 3). This pausing pattern can be used as a diagnostic feature when characterizing telomerases from species with regular telomeric repeats. A further characteristic is that the position of the pause

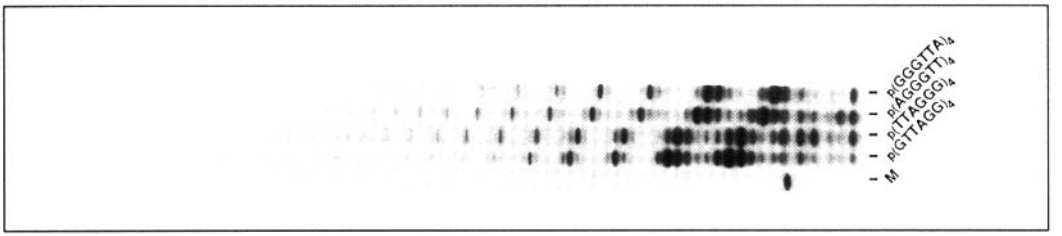

Fig. 3 Radiolabeled human telomerase reaction products run on a sequencing gel, reproduced from ref. 42 with permission of the publisher. The banding pattern reflects pausing or dissociation which occurs when polymerization reaches the end of the telomerase RNA template (step 2 in Fig. 1). Elongation by four different primers is shown, each of which is circularly permuted by one nucleotide; this results in a shift in the pausing pattern. The sequencing gel is shown with the origin at the right. Reproduced with permission from *Cell*, 1989, **59**, 521 (ref. 42). Copyright 1989 Cell Press.

shifts, when comparing elongation products from different telomeric primers which are the same size but circularly permuted relative to each other; the shift in the banding pattern is a consequence of how many nucleotides are added during the first round prior to translocation (Fig. 3). These data indicate that one site for binding telomeric primers is provided by the telomerase RNA template region.

In vitro, ciliate and human telomerases can elongate telomeric primers for hundreds of nucleotides (12, 41, 42, 54, 55). In the case of *Tetrahymena*, these long products have been shown to be the consequence of a processive mode of elongation (54), although under other reaction conditions, the enzyme is highly non-processive (56, 57). A mechanistic basis for telomerase processivity has been provided by two lines of evidence suggesting that in addition to the template site, telomerase is also capable of interacting with primers at a second site, called the 'anchor site' (Fig. 4). This second site is specific for telomeric or G-rich sequences but is distinct from base pairing that can occur between primer and template, and presumably plays a role similar to the posterior legs of an inchworm in preventing dissociation from primers during translocation along the template. The first line of evidence supporting a second site is the demonstration that primer recognition and processivity of the telomerase enzyme is dependent on the presence of G-rich sequence away from the substrate 3′ end. In fact, telomerase will extend primers with non-telomeric or mismatched 3′ termini, provided these substrates contain internal or 5′ telomeric sequence, which can be as far as 36 nucleotides from the 3′ end (13, 58, 59). This would not be predicted if base-pairing to the template region were the sole determinant of substrate specificity.

The second piece of evidence supporting the idea of an anchor site is the observation that the processivity of the *Tetrahymena* telomerase is strongly dependent on substrate length (56, 57). A similar dependence of processivity upon substrate length is seen for human telomerase (42). Short primers (<10 nucleotides) are much less likely to be extended over multiple translocation rounds, and it has been proposed that this may be due to the inability of short substrates to interact with an anchor site, resulting in more rapid primer dissociation (56). However, kinetic analyses of the reaction products of 10- and 12-mers indicate that 5′ sequence

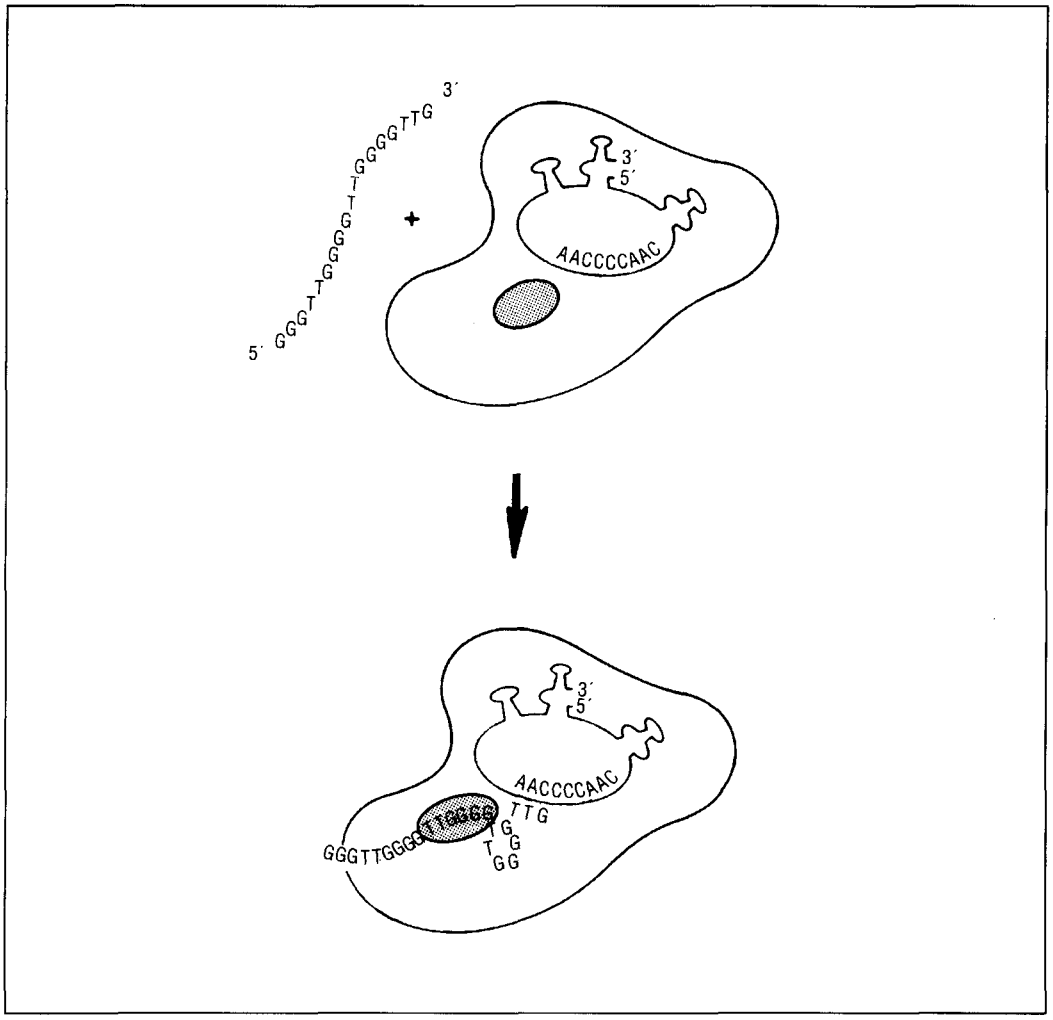

Fig. 4 Schematic representation of the two-site model of telomerase–primer interaction, adapted from ref. 56 with permission of the publisher. The anchor site is shown as a shaded circle, corresponding to a protein subunit of the enzyme. The telomeric primer interacts with both the template of the RNA and the proposed anchor site. The looping out of the primer, once bound, is speculative, but could explain the fact that there is not a precise distance requirement in order for 5′ sequences to impact elongation (58). Adapted with permission from *Genes & Development*, 1993, **7**, 1364 (ref. 56). Copyright 1993 Cold Spring Harbor Laboratory Press.

composition not only affects affinity of the primer for the enzyme, but also has an impact on the reaction rate of the catalytic step (57). There is also an apparent discrepancy in length requirement; processive elongation requires oligomers of >10 bases, yet the presence of G-rich sequence 36 nucleotides away from primer 3′ ends can affect efficiency of elongation (56, 58). This has led to the idea that, rather than the telomerase enzyme sliding along the substrate, it 'loops out' the nascent product; therefore the exact distance between the residues interacting between the anchor site

and the template region may not be as critical as it might otherwise seem (26, 56, 58).

As telomerase RNAs do not contain any sequences outside the template region which can be easily envisioned to promote anchoring to consecutive G residues, the hypothesized anchor site is likely to be a protein/primer interaction. Supporting this is direct physical evidence of primers interacting with telomerase protein subunits. Telomeric DNA substrates can be cross-linked to the large protein subunit of the purified *E. aediculatus* telomerase enzyme, as well as to the telomerase RNA (26). The cross-links between DNA and protein were 20 and 22 residues away from the 3′ end of the telomeric primer. Interestingly, this protein subunit will also cross-link to the duplex portion of partial-duplex structures with 3′ overhangs, analogous to natural telomere structures, suggesting the possibility that *in vivo* telomerase can interact with more than just the terminal single-stranded region of the telomere. The 95 kDa *Tetrahymena* telomerase protein subunit also cross-links to radiolabeled telomeric DNA primers (24).

2.3.3 Telomerase processivity *in vivo*

Additional insights into a telomerase mechanism have come from analysis of the pattern of telomeric repeats produced *in vivo* by telomerase enzymes that synthesize irregular telomeric repeats (either as a consequence of experimental manipulation of the telomerase RNA or due to naturally occurring RNAs). Analysis of cell lines of *Tetrahymena* that express two telomerase RNAs with differing templates results in interspersion of the two telomeric repeat sequences. Since the longest stretch of homogeneous repeats is no longer than eight repeats, this argues that processivity is fairly low *in vivo* (6). The budding yeasts, with telomeres composed of irregular telomeric repeats, provide a natural situation for monitoring enzyme processivity. In *S. cerevisiae*, the phenotype of a strain deleted for the *TLC1* RNA gene is consistent with the idea that there is only one telomerase RNA gene (7). Although sequence analysis of *de novo* telomere formation suggests that telomerase can infrequently use the entire 17 nucleotide template domain (23), the bulk of yeast telomeric DNA is composed of degenerate repeats conforming to the consensus sequence $G_{1\text{–}3}T$. This suggests that the yeast enzyme frequently dissociates while elongating chromosomal termini, leading to degenerate telomeres (7). Consistent with this, *S. cerevisiae* activity is highly non-processive *in vitro* (32, 60). In contrast, comparisons to other budding yeast species indicate that features which influence enzyme processivity *in vivo* can be highly variable. For example, the closely related yeast *Saccharomyces castellii* has much more regular telomeric repeats and an apparently more processive enzyme activity under *in vitro* conditions (32).

2.3.4 Templating and alignment regions of the telomerase RNA

In addition to providing sequence information to be copied on to chromosome ends, the telomerase RNA template base-pairs with and thereby aligns the 3′ ends of telomeric DNA substrates, ensuring that telomeric DNA repeats are added in a regular repeat pattern (Fig. 1). Therefore, a portion of the template region of the telomerase RNA is not copied into DNA and is more properly referred to as the

alignment region (indicated in Fig. 2). Partial reconstitution experiments with the *Tetrahymena* enzyme and *in vitro*-transcribed telomerase RNAs with mutant template regions have defined the templating region as bases 43 to 48, such that mutations in these residues are incorporated into substrates *in vitro* (61). Thus, one boundary of the template defined by this approach is the same as that inferred from analysis of the reaction products of circularly permuted primers, as discussed above in Section 2.3.1. The remainder of the template, residues 49–51, serves as an alignment region; mutations in these residues are not incorporated under standard conditions in this assay, although mutations in bases 50 and 51 result in reduced enzyme activity. The results of experiments involving expression of mutant RNAs *in vivo* are generally consistent with reconstitution experiments, but have indicated that the templating boundary may extend to residue 49 (62).

In addition to providing sequence information, the template may be important in the structure and function of the enzyme active site. Alterations in the template sequence frequently diminish activity *in vivo* (61, 63) and although a reduction in activity could be explained by predicted mismatchings, some template mutations have striking effects on the function of the enzyme (64). Mutations in both the template and alignment domains of the *Tetrahymena* enzyme have been shown to cause premature substrate dissociation, as well as loss of fidelity as manifested by the failure to incorporate nucleotides that are complementary to the templating residues (64). Hence, regions of the template domain of the RNA are crucial to proper enzymatic function.

2.3.5 Cleavage of telomeric primers

In addition to the elongation reaction that partially purified telomerase displays with telomeric primers, the enzyme is also capable of catalyzing primer cleavage, thus removing nucleotides from the 3′ end of certain primer substrates (32, 56, 59). This telomerase-associated nucleolytic activity is not a back-reaction (56); rather, it proceeds by an endonucleolytic mechanism, at least in *Euplotes* (59). In the ciliates, cleavage occurs even in the presence of predicted Watson–Crick base-pairing between the telomerase RNA and the telomeric primer, arguing against a role as a proofreading mechanism (56, 59). Nonetheless, the *Euplotes* enzyme will remove non-telomeric 3′ segments of at least 13 bases in order to access and extend internal telomeric sequence (59), although it is clearly capable of extending non-telomeric 3′ ends. Speculated functions for this nuclease activity include facilitation of elongation or processivity (suggested by comparison to RNA polymerase, which has a similar cleavage activity; 56). Another hypothesis is that the nuclease activity simply ensures that nucleotides beyond the template are not copied on to chromosome ends (59).

3. Coordination of telomere replication with semi-conservative DNA replication

The physical structure at the end of the chromosome is a single-strand extension of

the G-rich strand, providing two critical functions: it serves as a substrate for further elongation by telomerase as well as allowing binding of terminus-specific proteins that protect the end. However, a consideration of the properties of telomerase and the semi-conservative replication machinery demonstrates that our understanding of how this terminal chromosomal structure is replicated and maintained is still incomplete.

3.1 The consequence of leading and lagging strand DNA synthesis for linear chromosomes

The existence of a specialized replication machinery such as telomerase was first predicted due to the inability of conventional DNA polymerases to replicate the lagging strand fully (Fig. 5). As first pointed out by Watson (65) and Olovnikov (66) 25 years ago, if replication of linear chromosomes relies solely on the semi-

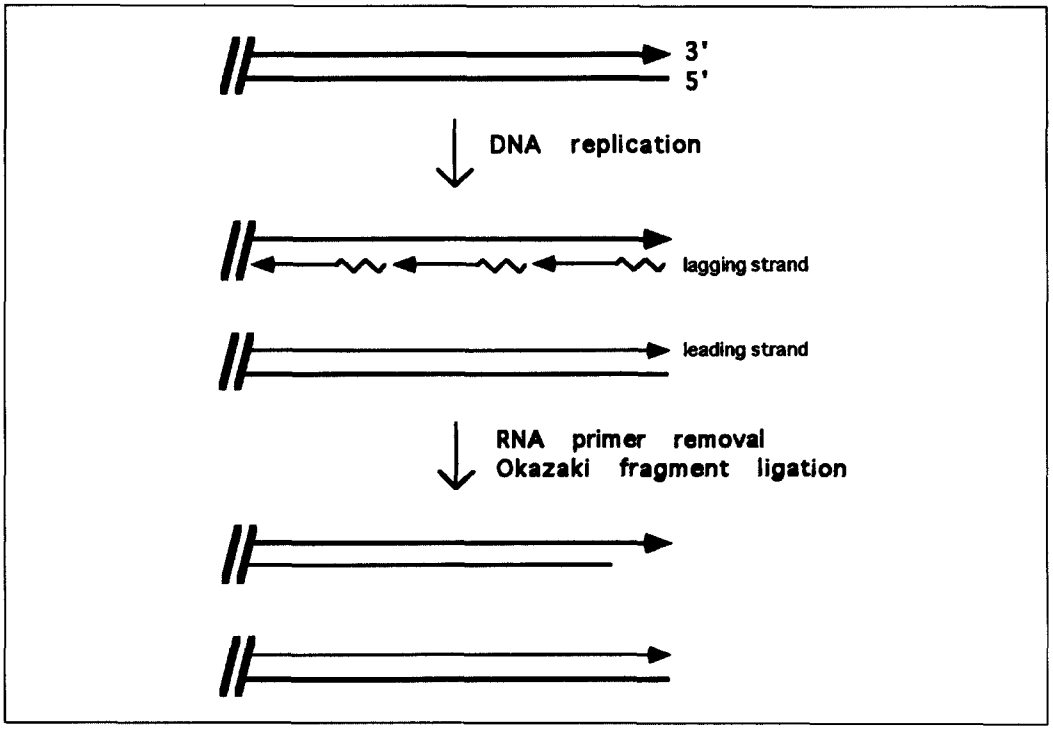

Fig. 5 The end-replication problem that results from the limitations of the semi-conservative replication machinery: because DNA polymerases require primers to initiate synthesis, lagging strand synthesis will be incomplete, leaving a gap on the nascent strand. RNA primers used to initiate synthesis are shown as jagged lines. Although only one round of replication is shown, repeated rounds of cell division in the absence of a mechanism to replicate the terminus fully will result in gradual shortening of chromosomal termini. The parental telomere is shown here with a blunt end for purposes of illustration, although telomeres are likely to have 3' overhangs before replication.

conservative replication machinery, the termini will be incompletely replicated. This problem stems from the fact that DNA polymerases require an RNA primer to initiate strand synthesis. However, once the RNA primer of the terminal Okazaki fragment is used and removed (Fig. 5), if there is no mechanism to replenish the sequence information provided by this primer, repeated rounds of semi-conservative DNA replication would result in gradual degradation of the end of the chromosome. Telomerase provides the necessary enzymatic machinery to prevent this problem, by allowing elongation of the 3′ terminus of the strand that templates lagging strand synthesis. Telomerase could act prior to the onset of DNA replication to elongate this strand, or it could elongate this strand at the completion of lagging strand replication (Fig. 6, step i); there is currently no clear information as to whether telomerase acts before or after lagging strand synthesis.

However, Lingner *et al.* (67) pointed out that the consequence for the leading strand terminus is quite different and presents an alternative problem for generating

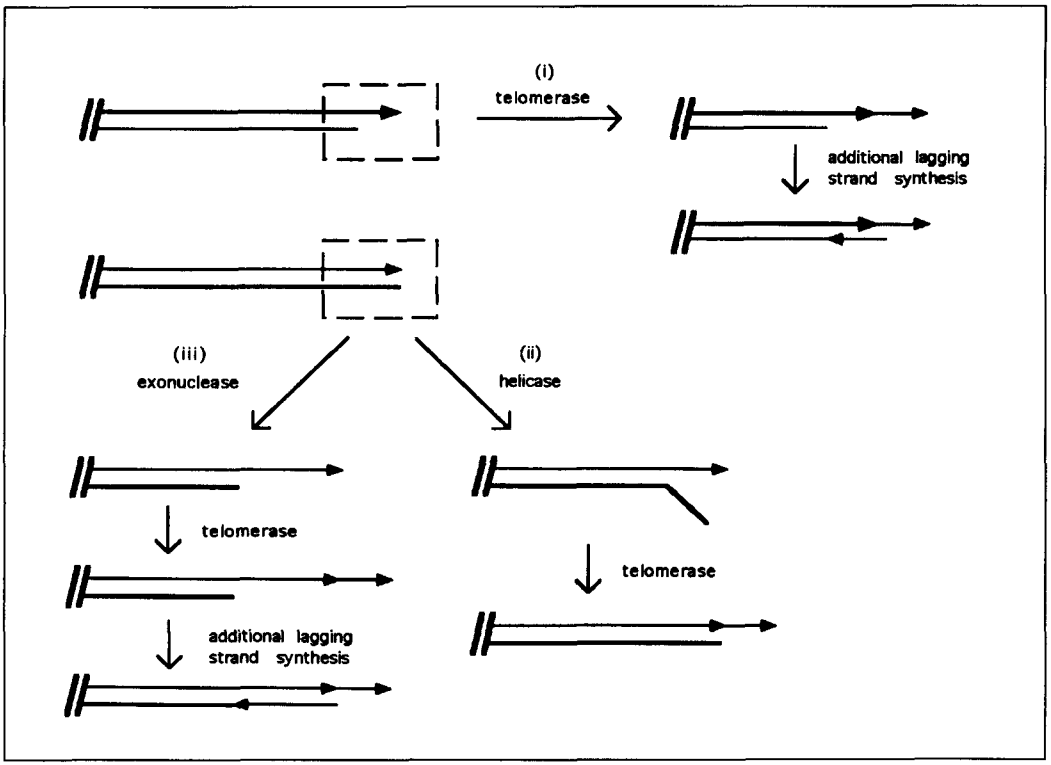

Fig. 6 Possible intermediates in telomere replication. (i) Extension of an overhanging strand by telomerase could be followed by an additional round of lagging strand polymerization in order to avoid sequence loss on daughter molecules. (ii) A helicase activity could unwind a blunt telomeric end, thereby allowing telomerase to extend a terminus and create a G-rich overhang. (iii) Alternatively, blunt termini could be processed by a C-strand-specific exonuclease; action by this nuclease would permit blunt-ended nascent telomeres to be extended by telomerase and filled in with a subsequent round of lagging-strand polymerization.

a fully replicated telomere. Replication of the leading strand is capable of proceeding to the very end of the chromosome (Fig. 5). Although this would, unlike the lagging strand, not result in any net loss of genetic information, it does not generate a G-rich 3′ telomeric overhang. The presence of a G-rich single-strand overhang appears to be a conserved feature of eukaryotic telomeres, and has been observed at chromosomal termini isolated from ciliates, yeast and, more recently, from humans (68–72). The consequence of the absence of this structure should be that proteins specific for the telomeric terminus would be unable to bind, leaving the leading-strand terminus exposed and potentially appearing as a double-strand break. One solution would be for telomerase to regenerate a 3′ extension on these molecules once leading-strand synthesis is complete. However, since telomerase is incapable of elongating a blunt duplex terminus *in vitro* (13, 25), this model invokes the existence of helicases or other activities that would peel apart the blunt leading strand end, providing telomerase access (Fig. 6, step ii). No such telomere-specific blunt-end helicases have yet been identified, although likely candidates might be expected to exhibit a cell-cycle-specific defect (possibly late S phase) when mutated.

A second solution would be to postulate a telomere-specific nuclease that is responsible for processing a blunt terminus to generate the necessary 3′ overhang. This would not only allow binding of terminus-specific telomere proteins, thereby capping the telomere, but would also provide a substrate for telomerase. In this model (Fig. 6, step iii), a 5′ to 3′ exonuclease could act on blunt termini prior to telomerase action, providing an overhanging substrate for telomerase. Nuclease action could occur at a time during the cell cycle separate from telomerase action, although the two activities might be coordinated in order to maintain normal telomere length. For example, telomerase could act prior to DNA replication, a possibility proposed by Lingner *et al.* (67), to hyperextend the telomere; trimming and formation of the overhang would follow the completion of DNA synthesis. Alternatively, as proposed in Fig. 6 (step iii), nuclease action on a blunt duplex terminus after leading strand synthesis is complete would then permit telomerase access and formation of a functional telomere.

Several experimental systems provide support for a nuclease activity that acts at the telomere. Studies of *de novo* telomere formation in the ciliate *Euplotes crassus* have shown that macronuclear-destined molecules initially acquire over-sized telomeres (73, 74). These elongated termini are subsequently pared down to their mature form, consisting of a 28 bp duplex and a 14 nucleotide single-strand extension of the G strand. Trimming to form mature telomeres occurs at a specific time during macronuclear development as the result of an active processing event that is independent of bulk DNA replication (74). In the yeast *S. cerevisiae*, long single-stranded tails are transiently observed at chromosomal termini (70). These single-strand extensions appear only at the end of S phase, and follow activation of a replication origin when assayed on linear plasmids resembling chromosomes (75). Surprisingly, however, the $G_{1-3}T$ tails are generated by a mechanism that does not require telomerase action, as $G_{1-3}T$ tails still appear in a cell-cycle-regulated fashion in *tlc1*-Δ strains of yeast that are defective for telomerase activity (76, 77). This

suggests the existence of an exonuclease that acts on nascent termini, providing a substrate for telomerase via resection of the C strand. Following elongation by telomerase, the lagging strand polymerase would be capable of filling in the C strand. Support for nuclease action at the telomere comes from the observation that, in the absence of the activity of the Cdc13 protein, which presumably binds the single-strand G-rich extension of the telomere (37, 38), there is rapid loss of the C strand (78). Whether a telomere-specific nuclease is responsible (76), or whether nucleases that are required for more general processing of DNA damage are used (79), is unclear.

The above models presume that telomerase activity should be restricted to a specific period of the cell cycle. At least some prior models have assumed that telomerase action occurs primarily at the end of S phase, after the completion of bulk chromosomal DNA replication. This is conceptually tidy, predicting that telomere replication is coordinated with the cell cycle and that complete telomere replication could serve as a means of signaling that S phase is completed. In lower eukaryotes such as yeast, which are immortal and need to maintain telomere length indefinitely, the issue of whether telomerase activity is present only during a specific period of the cell cycle has not yet been addressed, although as described above, other activity(s) that process the terminus are regulated. However, initial studies in mammalian cell lines which are telomerase-plus have shown that enzyme activity is present throughout the cell cycle in actively cycling cells (80, 81). Surprisingly, extensions of the G-rich strand on mammalian telomeres are present in both actively cycling cells as well as normal G_0-arrested cells (72), arguing that at least in some cells, both telomerase and the G-tail processing activity may be constitutive. This would imply that the factors required for converting blunt termini generated as a consequence of leading strand synthesis into functional telomeres may be readily available. However, other factors, including components of the telomeric chromatin, are involved in telomere length regulation in mammalian cells (82) and may limit access of telomerase to the telomere.

3.2 Is telomerase action coordinated with the primary replication machinery?

In addition to the above issues concerning the consequences of semi-conservative replication on telomere structure and replication, little is known about the potential coordination between telomerase activity and bulk DNA replication. These issues are just starting to be addressed, using experimental approaches in ciliates and yeast. Correlative support for the idea that bulk semi-conservative DNA replication is coincident with telomerase action is the demonstration in ciliates that a subfraction of telomerase is concentrated at or near the site of semi-conservative DNA replication (83). In the hypotrichous ciliates, S phase macronuclei are cytologically distinguished by a morphologically distinctive structure called the replication band. This disc-shaped structure contains the macronuclear DNA replication machinery; the band initiates at one end of the macronucleus and migrates towards the other end, with

DNA synthesis occurring in a zone that moves across the band itself (11, 84). Fang and Cech (83) demonstrated that this structure is preferentially enriched for the telomerase RNA as assessed by *in situ* hybridization. The position of the RNA, and presumably telomerase, is also highly dynamic; early in the migration of the replication band, the telomerase RNA lies in the front zone of the band, in a position distinct from where DNA replication is occurring, whereas at later times the position of the RNA is coincident with the bulk of the replication band. This temporal positioning could simply reflect early recruitment of telomerase to the replication process, but also does not rule out the possibility of telomerase action early in DNA replication.

Other observations indicate that the DNA replication machinery can play a role in influencing telomere length, as first evidenced by the demonstration that mutations in DNA polymerase α (*cdc17*) result in elongated telomeres (85). Adams and Holm (86) have explored this initial observation by asking whether alterations in other components required for semi-conservative DNA replication also affect telomere maintenance. Mutations in pol δ (*cdc2*), DNA ligase (*cdc9*), thymidylate synthetase (*cdc21*), thymidylate kinase (*cdc8*) and the large subunit of replication factor C (*cdc44/rfc1*) were examined at permissive and semi-permissive temperatures for effects on telomere length; only *cdc44/rfc1* mutants showed a substantial effect, giving rise to elongated telomeres similar to that previously observed for *cdc17* mutations. In addition, exposure to reagents that disrupt DNA replication, such as hydroxyurea or methyl methanesulfonate, did not alter telomere length. This suggests that general perturbations in DNA replication, or extension of S phase, are not sufficient to disrupt telomere length regulation, arguing that the effects of *cdc17* and *cdc44* on telomere length regulation might be specific. In the case of *cdc17*, telomere elongation was shown to require telomerase, as the introduction of a *tlc1* mutation abolished *cdc17*-dependent telomere lengthening, and this double mutant strain eventually showed the telomere shortening expected of a telomerase-deficient strain (86). One explanation for this phenomenon is that telomere elongation is the result of a more indirect effect of these mutations; for example, as Adams and Holm (86) proposed, these mutations may result in more nicks and gaps at the telomere, thereby reducing the binding of telomere-binding proteins that have been proposed to negatively regulate telomerase action. An alternative possibility is that the effect of *cdc44* and *cdc17* mutations is a direct consequence of disruption of the replication machinery at the telomeres; perhaps telomerase is actually part of a larger complex involving the semi-conservative DNA apparatus. An intriguing observation consistent with such a postulated interaction is the inability to recover *cdc44 tlc1* double mutant strains (86), suggesting synthetic lethality between defects in the DNA polymerase telomerase and replication factor C, previously shown to play a role in mediating exchanges at the replication fork between pol α and pol δ (87). Now that at least one protein component of yeast telomerase has been cloned, it will be possible to explore this possibility in more detail at a molecular level.

Analysis of macronuclear telomere formation in the ciliate *Euplotes crassus* also indicates that synthesis of not only the G-rich strand but also the C-rich strand of the

telomere is highly regulated. The over-sized telomeres formed during the early stages of *de novo* telomere formation are not only longer by about 50 bp, but also display more length heterogeneity than mature telomeres. Strikingly, most of the length heterogeneity is due to variation in the G-strand, whereas the C strand is very tightly controlled with regard to both length and the sequence of the terminal nucleotides, indicating that C-strand synthesis may be a primary factor in telomere length regulation (88). While the G strand is replicated by telomerase, the other strand is presumed to be replicated by the normal semi-conservative replication machinery. These over-sized telomeres, like their mature counterparts, have a single-strand extension of the G-rich strand; removal of the RNA primer used to prime C-strand synthesis could be responsible for generating the observed G-strand overhang. However, the sharply defined length of the C strand implies either precise positioning of the primer, or tightly regulated removal of the RNA primer (and possibly adjacent DNA sequence) to generate a sequence-specific terminal nucleotide. Although there is some evidence for a primase with specificity for telomeric DNA in the ciliate *Oxytricha nova* (89), this primase, like others, does not show strict terminal nucleotide specificity. This has led Vermeesch and Price (88) to propose than an accessory factor bound to the G-strand may regulate second strand synthesis; possible candidates could be either of the two different single-strand telomere-binding proteins identified in *Euplotes* (90, 91).

4. Telomeric chromatin and telomere-binding proteins

Physical analysis of the chromatin structure at the end of the chromosome has provided evidence for two general types of telomeric chromatin. In lower eukaryotes (protozoa and fungi), telomeres have a distinctive non-nucleosomal structure that appears to encompass the entire telomeric repeat DNA tract, often referred to as the telosome (92–95). In *S. cerevisiae*, a distinct nuclease-sensitive interface between telomeric and adjacent nucleosomal chromatin can be observed, allowing release of soluble telomeric chromatin preparations by limited nuclease digestion or directed restriction enzyme digestion at this boundary (95, 96). DNA encompassed by the telosome is also refractory to methylation by the *Escherichia coli dam* methylase, in comparison to other, more accessible, regions of the genome (97), further underscoring the non-nucleosomal character of telomeric chromatin. In contrast, in vertebrates, with telomere length ranging from as short as several kilobases up to as long as >100 kb, the majority of telomeric DNA repeats are packaged into very tightly-spaced nucleosomes (98, 99). The distal portion, however, appears to be present in a non-nucleosomal chromatin structure potentially analogous to that observed at lower eukaryotic telomeres (99). Although it appears that the histones are major constituents of telomeric nucleosomal chromatin, rat telomeric nucleosomes appear to be more condensed than bulk chromatin and have a more compact higher order structure (100). Human telomeres are also attached to the nuclear matrix (101) and, like centric heterochromatin, contain hypoacetylated histone H4 (102). This may reflect a similarity with yeast telomeres, as histone H3

and H4 N-terminal tails are required for telomeric silencing in yeast (103–105). Other factors are also localized to human telomeric chromatin, such as the telomere duplex DNA-binding protein TRF1 (50). The precise distribution of TRF1 is not known, and may potentially be interspersed along the telomeric tract and participate in forming tight nucleosomal spacing. An alternate possibility (and potentially more likely, given its role in telomere length regulation, discussed below) is that it is part of a telosome-like structure at the very terminus, and that the tight telomeric nuclear chromatin structure has another explanation (106).

Characterization of the individual proteins that comprise telomeric chromatin has focused on characterization of two classes of telomere DNA-binding proteins: those that bind duplex telomeric DNA and those that bind the single-strand G-rich extension. A general conclusion of the studies described below is that both types of telomeric proteins play important roles in telomere maintenance and/or cell division and that these functions may be generally applicable in a wide range of organisms.

4.1 Proteins that bind double-stranded telomeric DNA

Double-strand telomeric DNA-binding proteins have been cloned and characterized in budding yeast (Rap1), fission yeast (Taz1) and mammalian cells (TRF1) (50, 82, 107–109). Recent studies on these three proteins have revealed a common role, as negative regulators of telomere length control.

4.1.1 Rap1

The best studied of these telomeric duplex binding proteins is Rap1 (*R*epressor/*a*ctivator *p*rotein) from *S. cerevisiae*. Rap1p is a multifunctional protein which is essential for cell viability. It binds at many other chromosomal locations in addition to telomeres, and depending on the context in which it binds DNA, it can activate transcription or nucleate transcriptional silencing (for review, see ref. 110). Each telomeric tract contains many Rap1 binding sites, and Rap1 is involved in both the formation of telomeric chromatin and the maintenance and replication of telomeres, via multiple protein–protein interactions (108, 111–115). Experiments from the related budding yeast *K. lactis* have led to the proposal that Rap1 negatively regulates telomere length through the formation of a chromatin complex which is inaccessible to telomerase (108). This model stemmed from the observation that certain sequence changes in the telomeric repeat sequence of *K. lactis*, introduced by reprogramming the template region of the telomerase RNA, resulted in explosive elongation of the telomeres (8). The degree of telomere elongation correlated with loss of *K. lactis* Rap1-binding ability *in vitro*, suggesting that alterations in telomeric chromatin via the loss of Rap1 binding allows unregulated access of telomerase to the chromosome terminus. *In vivo* support for this hypothesis is the demonstration of a synergistic effect of combining a telomerase RNA template mutation with a mutant Rap1 protein missing its C-terminus: each mutation had only modest effects on telomere length but in combination produced completely unregulated telomerase activity (108).

The capacity of Rap1 to regulate telomere length was further underscored by recent studies in which the C-terminal domain of Rap1 was tethered to the proximal end of telomeres by fusion to a heterologous DNA-binding domain (116). The length of the terminal telomeric tract decreased in proportion to the number of heterologous binding sites, indicating that the heterologous binding sites of these fusion molecules were counted as part of the telomere. Although the C-terminus of Rap1 interacts with Sir3 and Sir4 (see below), and tethering these molecules restored silencing to a silencing-defective telomere, the tethered Sir proteins were unable to affect telomere length, indicating that the role of Rap1 in regulating telomere length is distinguishable from its role in nucleating the silencing complex. A possible bridge between Rap1 and telomere length control may be the less well characterized proteins Rif1 and Rif2, which were identified on the basis of their interaction with Rap1 in a two-hybrid screen (117, 118). Cells which lack either Rif1 or Rif2 function show a modest increase in telomere length, and the impact on telomere length is additive when both proteins are absent, arguing that they each independently influence the telomere length regulation function of Rap1p.

4.1.2 Taz1 and TRF1

These observations on Rap1 have recently been generalized by analysis of similar telomeric proteins in *S. pombe* and mammalian cells. The *S. pombe* protein Taz1 was identified via a one-hybrid screen for proteins capable of binding to *S. pombe* telomeric sequences linked to a reporter gene (109). A role in telomere length regulation was revealed by the demonstration that a strain deleted for the *taz1* gene results in a massive increase in telomere length, as well as a disruption of telomeric chromatin. A comparable telomeric protein has also been identified in human cells (119). TRF1 was identified initially from HeLa cells as a factor that specifically bound to the human telomeric repeat array TTAGGG *in vitro*, recognizing duplex but not single-stranded telomeric substrates (120). Using indirect immunofluorescence, TRF1 was shown to localize to chromosome ends in human metaphase spreads (50). Direct evidence that TRF1 is a negative regulator of telomere length in mammalian cells came from an examination of the effects of increased dosage of either wild-type or a dominant negative version of TRF1 (deleted for the Myb domain; see below) on mammalian telomere length (82). Over-expression of wild-type TRF1 caused a decrease in telomere length, whereas displacement of endogenous TRF1 protein from its telomeric location via over-expression of the Myb-deleted derivative resulted in telomere elongation. These alterations in telomere length were not the consequence of changes in the level of telomerase activity, leading van Steensel and de Lange (82) to propose that the effects of TRF1 on telomere elongation were the consequence of a competition between TRF1 and telomerase for interaction at the telomere, rather than direct inhibition of enzyme activity. TRF1 is the first example of a mammalian telomeric protein; the parallels between this protein and its counterparts in model organisms such as *S. cerevisiae* and *S. pombe* continues the theme that many features of telomere function and replication are widely conserved.

One additional point of similarity among these three proteins is that all three

appear to use the same conserved protein fold to recognize DNA. The structure of Rap1 complexed with DNA has shown that this protein uses two subdomains that are structurally related to both the Myb DNA-binding motifs found in transcription factors and the homeodomain (121; reviewed in 122). Although Rap1 shows little sequence similarity to Taz1 and TRF1 (nor is a Myb-type domain evident from an examination of the amino acid sequence of Rap1), the primary amino acid sequence of Taz1 and TRF1 reveals that both exhibit strong homology to the Myb DNA binding domain (50, 109). Although these two proteins each only have one such motif, TRF1 is a dimer in solution and therefore it is likely that both Myb domains are used to recognize and bind DNA, thereby extending the analogy to Rap1.

4.2 The yeast Rap1 protein recruits a silencing complex

One role of Rap1 at the telomere is to recruit the Sir2/3/4 complex, which, like Rap1, is apparently a major constituent of telomeric chromatin. Rap1 interacts genetically and physically with Sir3 and Sir4 through its C-terminus (105, 123, 124). The *SIR* genes were originally identified because they are required for the transcriptional silencing of yeast mating-type genes (*SIR* = *S*ilent *i*nformation *r*egulator) (125–127). Therefore, like Rap1, they are not exclusively localized to telomeric regions of the genome. However, these proteins appear to play similar roles at different positions in the genome. For example, telomeres exhibit *SIR*-dependent silencing similar to that at the HM loci; i.e., genes placed near telomeres are transcribed only very infrequently. The Sir complex apparently acts to inhibit transcription directly, through the interaction of Sir3 and Sir4 proteins with the N-terminal tails of histones H3 and H4, which are also required for silencing (103–105). In this regard it is tempting to speculate that TRF1 and Rap1 may play similar roles in modulating the function of histones at telomeres; however, there is currently no evidence for telomeric silencing at mammalian telomeres similar to that observed in yeast, despite efforts by several groups to detect such a phenomenon.

As yet, there is also no evidence that transcriptional silencing at the telomeres is important to any cellular function; in fact, a transcribed telomere can be maintained and propagated (128), and the *SIR* genes are not required for cell viability (127). However, these observations do not rule out a selective advantage. Alternatively, telomeric silencing may simply be a fortuitous consequence of telomeres being placed in transcriptionally inactive chromatin, for the reason that they do not usually house genes. Regardless of its biological significance, telomeric silencing has become a valuable tool for determining whether various conditions, mutations, or genetic manipulations affect the structure of telomeric chromatin. In addition to mutations in *RAP1* and *SIR* genes, a number of other mutations and other perturbations influence TPE (i.e., increase or decrease in the expression of a gene placed near a telomere) (7, 34, 38, 103, 128–133), indicating an impact on telomeric chromatin structure, although in some cases the mechanism remains unknown.

Experiments in which transcription is measured directly in synchronized cells using an induction system reveal that telomere-proximal DNA is accessible, and

presumably telomeric chromatin opened and re-established, at only one point in the cell cycle, G_2/M (132). This is consistent with a breakdown and re-establishment of telomeric chromatin following the end of S phase. It is interesting to note, in light of this finding, that perturbations which increase the length of the yeast cell cycle augment silencing, including telomeric silencing (129). This implies that time required for assembly of the silencing complex may be limiting. A more specific mechanism of regulation of telomeric chromatin is suggested by the recent finding that a deubiquitinating enzyme, UBP3, associates with the Sir complex; deletion of the gene encoding this enzyme augmented silencing without disrupting cell growth (133). Therefore ubiquitination, which controls and coordinates many events related to the cell cycle, may also regulate telomeric chromatin.

4.3 Proteins that bind single-stranded telomeric DNA

4.3.1 Ciliate single-strand telomeric DNA-binding proteins

The most detailed information about telomere end-binding proteins has come from studies in the hypotrichous ciliates, particularly from *Oxytricha nova*. These experiments were initially aided by the large number of telomeres present in the ciliate macronucleus, as well as by the extremely tenacious association of these proteins with telomeric termini, thereby facilitating purification. In *Oxytricha*, the terminus-specific complex consists of two proteins of 56 kDa (α-subunit) and 41 kDa (β-subunit) bound to the 3′ single-strand G-rich extension of the telomere (134, 135). Physical evidence supporting the hypothesis that these proteins form a cap structure to protect the telomere is the resistance of bound telomeric DNA to nuclease digestion and chemical modification (93, 134, 135). *In vitro*, recombinant α- and β-subunits form a complex with telomeric DNA in a cooperative fashion (136). In isolation, the α-subunit can bind single-stranded telomeric DNA whereas the β-subunit does not interact specifically with DNA. However, the β-subunit can promote the formation of G-quartets *in vitro*, although the *in vivo* significance of this activity is unclear (137). *Euplotes* telomeres have a chromatin structure similar to that observed in *Oxytricha* but in contrast, only a single 51 kDa protein with telomeric DNA binding activity has been identified (90), with substantial sequence similarities to the *Oxytricha* α-subunit; there is no evidence for a *Euplotes* counterpart to the β-subunit. An end-binding activity has also been identified in extracts from *Tetrahymena* but has not yet been cloned (138).

From these biochemical data, it is easy to envision the ciliate telomere-binding proteins as forming a cap. It is much less clear how they perform what presumably must be an obligate role in telomere replication. As the half-life of DNA binding of these proteins *in vivo* vastly exceeds the length of the cell cycle (136), it is possible that the proteins do not normally dissociate from chromosome ends during DNA replication, remaining bound to single-stranded DNA. An important observation has shed some light on how the telomere-binding proteins may behave in the context of cell division, which is that while DNA bound by the proteins is inaccessible to nuclease, it is readily extended by telomerase and other template-dependent

polymerases *in vitro* (139). Thus, it may not be necessary for the telomere proteins to dissociate in order for chromosome replication to be completed. However, as telomerase elongates telomeric primers in these experiments to a length well beyond that observed *in vivo* (139), it also raises the question of what is regulating telomerase, if not the telomere-binding proteins. No double-strand-specific telomeric DNA-binding factors analogous to the yeast Rap1 or human TRF1 proteins have been identified in *Euplotes* or *Oxytricha*; the extremely short duplex region of the telomere in the hypotrichous ciliates may in fact eliminate the need for duplex-specific telomere factors. However, there must be a specific length-sensing mechanism in these species, since both *Euplotes* and *Oxytricha* telomeres have precisely determined telomere length (68). In *Euplotes*, one proposed candidate for a length regulator is a second candidate single-strand telomere-binding protein called rTP. rTP was identified based on extensive amino acid identity to the DNA-binding domain of ciliate telomere-binding proteins (91) and presumably also binds telomeric DNA. However, whereas the *Euplotes* telomere-binding protein is distributed throughout the macronucleus, as expected of a protein bound to the bulk of macronuclear telomeres, rTP localization is restricted to the macronuclear replication band, suggesting a specific role for this protein in telomere DNA replication (140). Possible functions include either telomerase recruitment or length regulation.

4.3.2 Single-strand telomeric DNA-binding proteins in budding yeast

With the identification of two candidates for telomeric end-binding proteins in *S. cerevisiae*, *EST1* and *CDC13* (34, 37, 38), it is now possible to test hypotheses regarding the function of these proteins, as budding yeast allows relatively easy *in vivo* analysis. The yeast *CDC13* gene, which is essential for cell viability, encodes a single-strand telomeric DNA-binding protein (37, 38). Genetic analysis of two distinct classes of *cdc13* mutations has argued that this protein serves two functions while bound *in vivo* to the single-strand G-strand terminus. The *cdc13-1*^{ts} temperature-sensitive mutant displays a rapid and extensive loss of the C-strand of telomeric DNA at the restrictive temperature (78), suggesting either a capping function (37) or a role in regulating access of a nuclease required for G-tail formation (38, 76). A second role for Cdc13p, in telomerase regulation, has come from the identification of a novel *cdc13* mutation, called *cdc13-2*^{est}, with a phenotype virtually identical to that of a telomerase-minus strain. Unlike a strain carrying a conditional lethal *cdc13-1*^{ts} mutation, the *cdc13-2*^{est} mutant strain exhibits the senescence and telomere-shortening phenotypes previously observed for a strain deleted for the telomerase RNA (7, 28, 37). This has led to a model proposing that the Cdc13 protein regulates telomerase by mediating, either directly or indirectly, access of telomerase to the chromosomal terminus, with access eliminated by the *cdc13-2*^{est} mutation (37; Fig. 7). Therefore, in contrast to the negative regulation of telomere length exerted by duplex-binding proteins such as Rap1, Cdc13 functions as a positive regulator of telomerase. Whether these positive and negative regulators act competitively to modulate telomerase activity or access, or act at different points during the cell cycle, remains unknown.

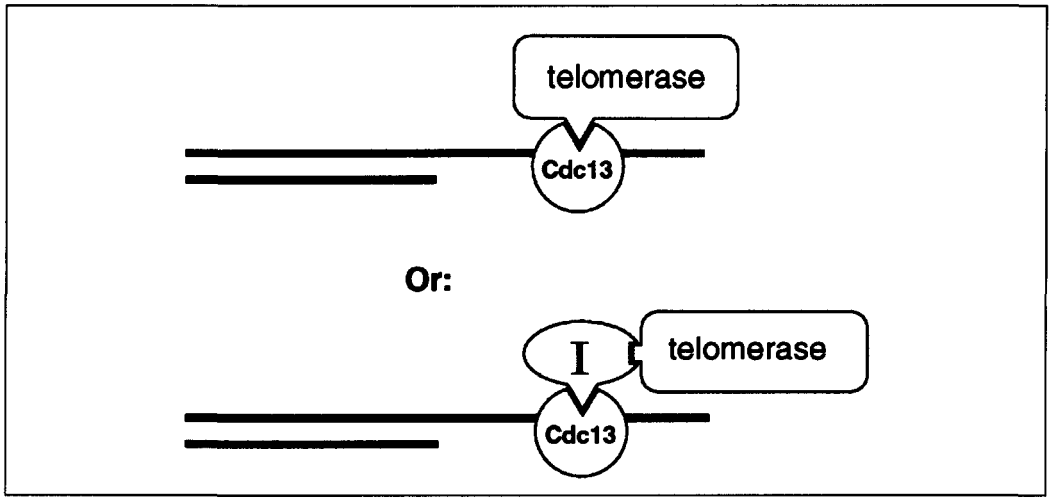

Fig. 7 A model for the function of Cdc13p at yeast telomeres, adapted from ref. 37 with permission of the publisher. The model is based on the fact that Cdc13p binds single-stranded telomeric oligonucleotides *in vitro* (37, 38), and plays a role in both telomere protection (68) and length maintenance via telomerase (37). The novel *est*-like allele of Cdc13 (28, 37) is proposed to define a telomerase-loading function of this protein; this could be mediated through a direct interaction between Cdc13 and telomerase, or via an intermediary protein (indicated as I in the figure). Adapted with permission from *Science*, 1996, **274**, 249 (ref. 37). Copyright 1996 American Association for the Advancement of Science.

Related to the potential role of Cdc13 in telomere function is the observation that conversion of a chromosomal terminus containing a telomere to a double-strand break causes cell cycle arrest in G_2 (141, 142). However, loss of telomerase is not lethal (7, 10) and does not appear to trigger a *RAD9*-dependent checkpoint (C. Nugent and V. Lundblad, unpublished observations), nor does loss of telomerase result in the loss of the G-rich strand extension observed in late S phase (76, 77). This suggests that the critical event at the end of S phase is not telomerase action *per se* but the formation of a protective structure and its association with capping proteins. Consistent with this is the fact that cells arrest in late S/G_2 in the absence of Cdc13 function (143); this specific arrest point also suggests two possibilities. One alternative is that this is the only period of the cell cycle where loss of telomere protection would signal a checkpoint. However, an alternative is that other proteins mask the terminus during other stages of the cell cycle, and the Cdc13 protein only contributes during late S phase/G_2. Asking whether Cdc13p, as well as other factors, are constitutive components of the telosome could start to address these questions.

4.4 Regulation of telomeric proteins

The above data support the idea that telomeric proteins can regulate telomerase, but, so far, little is known about what regulates telomere proteins. Are they bound constitutively to the telomere and/or are there modifications that regulate their activity and hence telomerase access, or even their presence at the telomere? An early

hint of correlations between cell cycle progression and telomere protein modifications has come from analysis of the *Oxytricha* telomere proteins, shown to be phosphorylated *in vivo* (144). Technical limitations unique to the ciliate experimental system prevented investigating whether this post-translational modification was cell-cycle-regulated *in vivo*. However, indirect evidence came from the demonstration that the β-subunit of the *Oxytricha* telomere protein complex exhibited cell-cycle dependent phosphorylation in cycling *Xenopus* egg extracts, with phosphorylation occurring in M-phase and subsequent dephosphorylation during interphase. Furthermore, the β-subunit is phosphorylated *in vitro* by mouse cyclin-dependent kinases (Cdks), consistent with the presence of Cdk consensus recognition sites in this protein. Since phosphorylation does not appear to affect DNA-binding (136, 144), this implies that other activities of these proteins are regulated in a cell-cycle dependent manner. An intriguing possibility is that phosphorylation of the telomere-binding proteins regulates telomerase activity, possibly through the protein's ability to form G-quartets, which are refractory to telomerase elongation (53).

4.5 Other proteins that act at the telomere

An additional factor that may be playing a direct physical role in maintaining telomeric DNA is the heterodimeric Ku protein, which binds DNA ends in a sequence-independent manner. This protein complex functions in mammalian cells in double-strand break repair as well as specialized recombination events such as V(D)J recombination (reviewed in ref. 145). Structurally similar homologs of both Ku subunits have been identified in *S. cerevisiae* and shown to be responsible for the major yeast DNA end-binding activity (146, 147), and genetic analysis of yeast strains deleted for either gene have also demonstrated an *in vivo* role in DNA end joining comparable to that observed in mammalian cells (147). The additional observation that yeast strains deleted for either of these Ku homologs also have a telomere-shortening phenotype suggests the possibility that a target of this end-binding activity *in vivo* is the telomere, possibly via protection of the end from exonuclease action (148, 149). However, an alternative explanation is that the telomere-shortening phenotype is the secondary consequence of this somewhat pleiotropic mutant; a strain defective for Ku protein activity is also inviable at high temperatures and shows phenotypes suggesting a defect in the regulation of DNA replication (146). More investigation will be necessary before a definitive role in telomere function can be attributed to this DNA-binding activity.

5. Telomeres and telomerase regulation in mammals: the telomere hypotheses of cancer and aging

Mammalian telomeres have received a great deal of attention due to a body of correlative evidence associating telomere length and telomerase activity with

tumorigenesis and aging. Since a number of reviews have addressed these issues (1, 4, 150–153), this section will only briefly summarize the basic observations and outstanding questions.

Unicellular immortal organisms such as yeast express telomerase continuously and thereby maintain a constant telomere length and unlimited proliferative ability. However, although telomerase is present in human germ-line cells and telomeres are consequently long, most normal somatic cells in humans are devoid of telomerase activity (154), indicating that there are stringent regulatory mechanisms to permit developmental and tissue-specific repression of this enzyme. Normal somatic cells also display progressive telomere shortening, which correlates with the limited replicative lifespan observed for primary cells in culture (155). This rough inverse correlation between telomere length and proliferative capacity has led to the proposal that gradual telomere shortening over many successive cell divisions is the mechanistic basis or `counting mechanism' that dictates replicative potential (156). Although telomerase is absent and telomere shortening occurs in most non-germ-line cells, the sustained cellular proliferation that is necessary for tumor progression implies that either telomerase or some other mechanism must function to restore and maintain the telomere. Consistent with this expectation, telomerase activity is detectable in tumor-derived cell lines, and ~90% of primary human malignancies are positive for the enzyme (42, 154, 157, 158). This suggests not only that telomerase may be useful as a new marker for malignancy but has also led to the proposal that reactivation of telomerase is crucial for tumor maintenance and progression. An important implication of this model is that telomerase could therefore be a critical target for anti-cancer therapies.

The availability of three genes encoding human telomerase components or telomerase-associated proteins (discussed in Section 2.2.2) has allowed expression studies as a means of determining how telomerase activity is regulated. Consistent with the above model, the human telomerase RNA component, and its murine counterpart, are up-regulated during tumor progression (9, 159, 160). However, the level of the telomerase RNA transcript does not always correlate with the presence or absence of enzyme activity (30, 31, 159, 161, 162). Initial expression studies of the protein catalytic subunit indicates that this component may be more tightly regulated; mRNA levels are low in telomerase-minus cells and tissues and up-regulated during cellular immortalization and in primary human cancers (30, 31). In contrast, Northern analysis indicates that the mammalian p80 homolog may be ubiquitously expressed, including in cell lines which are telomerase-deficient (30, 39, 40). If this protein is an integral component of the enzyme, its widespread expression argues that regulation of telomerase activity is executed at the level of another component, or possibly via modification of the p80 homolog subunit (40).

However, although many reports suggest that telomeres in immortal cells shorten and then stabilize, telomere length in malignant cells and tissues can be highly variable (163, 164). Overall, this suggests that the behavior of telomeres in immortalized cells may be influenced by numerous variables. The ability of telomeres to lengthen in the presence of reverse transcriptase inhibitors which inhibit

telomerase *in vitro* suggests that there may be non-telomerase mechanisms which can lengthen human telomeres (163), discussed in more detail below in Section 6. In addition, several observations suggest that telomerase activity can also be regulated at levels other than expression of the enzyme. Telomerase activity is present in certain human cell lines such as hematopoietic cell lines (165, 166) which nevertheless undergo telomere shortening (167–169). This argues that other factors must also be involved in mediating telomere length control, presumably by regulating telomerase activity *in vivo* or by monitoring access of the enzyme to the chromosomal terminus. Analysis of telomere length in one murine species also does not seem to follow many predictions of the telomere hypothesis. Telomeres in *Mus musculus* are very long and vary considerably in length (170–172), despite that fact that this species has a much shorter lifespan than humans. This extended telomere length could be attributed to detectable levels of telomerase activity present in many mouse tissues (172). In contrast, the closely related species *Mus spretus* has much shorter telomeres, comparable to human telomeres (172). In addition, primary fibroblast cultures from *M. spretus* exhibit telomere shortening and are telomerase-minus, whereas telomerase is present and telomere length stabilizes in spontaneous immortalized clones. Ultimately, the resolution of the role of telomerase in lifespan determination and/or oncogenesis will await an *in vivo* determination of the phenotype of mice in which enzyme activity has been abolished via the deletion of a gene encoding a component of the core enzyme.

All of the above studies on mammalian telomerase regulation have been greatly facilitated by the development of a highly sensitive telomerase assay. Although the conventional biochemical assay used to detect ciliate telomerase activity can also be used to monitor activity in human and mouse cell lines (42, 43), a large number of cells is required, making this technique impractical for screening tissue samples. The development of the TRAP assay (*t*elomeric *r*epeat *a*mplification *p*rotocol; 154), based on a PCR amplification step to detect trace amounts of telomerase extension product, has made it possible to detect telomerase activity even in single cells (173).

6. Alternative pathways for telomere maintenance

Although telomerase is clearly the primary pathway for maintaining and replicating the telomere, a series of reports on budding yeast, *Drosophila* and mammalian cells have argued for the existence of one or more telomerase-independent pathways that can be used to maintain the telomere; the evidence for these pathways is summarized briefly in this section. In *Drosophila* at least, this so-called alternative pathway is the primary means by which chromosomal termini are maintained. These observations raise the possibility that similar mechanisms may be operative at some level in other organisms as well. If such pathways can be activated during mammalian oncogenesis, this has substantial implications for the use of telomerase inhibitors as anti-cancer therapeutics.

In budding yeast, elimination of the telomerase-based pathway for telomere replication results in progressive shortening of telomerase and a resulting reduction

in growth rate and viability (7, 8, 10, 27, 28). However, this does not result in death of all the cells in the culture; late in the outgrowth of these senescing cultures, a small sub-population of cells is able to escape the consequence of the absence of telomerase. These survivors arise as the result of a recombination-mediated bypass pathway for telomerase maintenance, which results in the amplification of both G-rich and (in the case of *S. cerevisiae*) sub-telomeric repeat DNA (28, 174, 175). Therefore, in the absence of telomerase, recombination can be used to continually replenish the telomere with fresh telomeric DNA. In mammalian cells, the establishment of immortal cell lines in culture is also not always correlated with reactivation of telomerase activity; a variety of immortalization techniques can give rise to a substantial percentage of cell lines with no detectable enzyme levels (154, 176). Surprisingly, telomeres do not shorten in such immortalized cell lines; instead, telomeres are extremely long, as much as 30 kb longer than those in telomerase-positive cell lines and tissues. Whether the mechanism used to maintain and elongate telomeres is the same as the recombination-based process described above for yeast has not been determined. The elongated telomeres observed in *M. musculus* may also be a consequence of a similar process, although this would argue that both telomerase-dependent and telomerase-independent pathways are operating simultaneously.

Drosophila termini are maintained by yet another telomerase-independent mechanism, via the continual acquisition of two related non-LTR retrotransposons, TART and HeT-A (177, 178); retrotransposition into termini that are degrading as a consequence of incomplete semi-conservative replication prevents the eventual loss of proximal, essential genes, thereby fulfilling one of the roles that telomeres are thought to play. However, the absence of traditional G-rich repeat sequences and the G-rich overhang structure, necessary for telomere function in other species, leaves open the question of how a protective cap is acquired; no telomere end-binding proteins comparable to those in other species have been identified in *Drosophila*. However, this retrotransposon-based mechanism for dealing with termini may not be unique to *Drosophila*. Two groups have recently shown that double-strand breaks in *S. cerevisiae* can be repaired via a retrotransposon-dependent process, resulting in the acquisition of retroelement-encoded fragments at the healed breaks (179, 180). This observation, combined with the natural occurrence of a particular LTR retrotransposon in or near telomeres (181), suggests potential mechanistic connections to the process of telomere formation in *Drosophila*.

7. Future perspectives

In the 12 years since the discovery of the telomerase enzyme, there has been an ever-increasing supply of surprising discoveries in telomere biology. These include the mechanistic dissection of the enzyme itself, as well as initial steps towards understanding the regulation of telomerase activity *in vivo*. The recent explosion of interest in human telomere length regulation, and its potential implications for cancer biology, promises to propel continued growth in telomere research. The fact that

virtually all eukaryotes have a similar telomeric DNA structure, coupled with evidence that telomere binding proteins and telomerase enzymes play analogous roles in distantly related species, indicates that advances made in model organisms will continue to be applicable to humans.

The identification of the RNA subunit and the protein catalytic component of telomerase in a number of different species now provides the basis for a detailed investigation of the core enzyme. However, what constitutes a complete telomerase holoenzyme has not yet been determined in any organism and will continue to be a goal for future studies. In addition, interactions between telomerase, telomere-binding proteins, and telomeric chromatin are only beginning to be understood, and the coordination of telomere replication with the general DNA replication machinery is almost completely uncharacterized. The presence of G-tails in yeast provides an avenue of approach to addressing the dilemma regarding telomerase and blunt ends; although several models have been proposed, there is presently no clear resolution to this issue. Finally, it will be of great interest to determine the physiological consequences of mutations in telomerase and telomere-associated proteins in mammals, in order to test thoroughly the intriguing connections between telomere length maintenance and the dual processes of aging and oncogenesis.

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7 Meiosis: chromosome behavior and spindle dynamics

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1. General features of meiosis

Meiosis is an alternative pathway of development that leads to the production of haploid cells, usually spores or gametes, for sexual reproduction, rather than the identical or near identical diploid cells produced by mitosis. The recent discovery of analogous proteins that are involved in the regulation of both meiosis and mitosis makes it increasingly apparent that meiosis is closely related to mitosis, and can be regarded as a highly specialized mitotic division. Although meiosis differs from mitosis in several respects, many of these differences are variations of events that occur during mitosis. Some, however, are not. Probably the most radical departure from mitosis occurs during meiosis I and concerns the behavior of the meiotic chromosomes: homologous chromosomes in most organisms undergo pairing (initial alignment of homologs in zygotene), synapsis (intimate association of homologs at pachytene) and recombination (physical exchange) during meiotic prophase, which are not observed in mitotically dividing cells. These events are followed by two successive divisions without an intervening replication, instead of the single division that occurs in mitotic cells, resulting in reduction of the chromosomes to the haploid number (Fig. 1).

Synapsis between homologous chromosomes occurs subsequent to DNA synthesis and is thought to be mediated by specialized proteins that contribute to, or facilitate, recombination between the homologs. Some of these proteins have now been identified in yeast and other fungi (1–3), *Drosophila* (4), vertebrates (5), and higher plants (6). In some organisms, such as males of *Drosophila melanogaster*, in which meiotic recombination does not occur at a detectable frequency (7), synapsis of homologs still takes place but is thought to occur by a different mechanism than for recombination (8, 9). Circumstances also exist in which homologs associate appropriately, but recombination is prevented by chromosomal inversions or translocations (10). This latter type of pairing and synapsis has been studied most extensively during meiosis in *D. melanogaster* females (11, 12), and has recently been

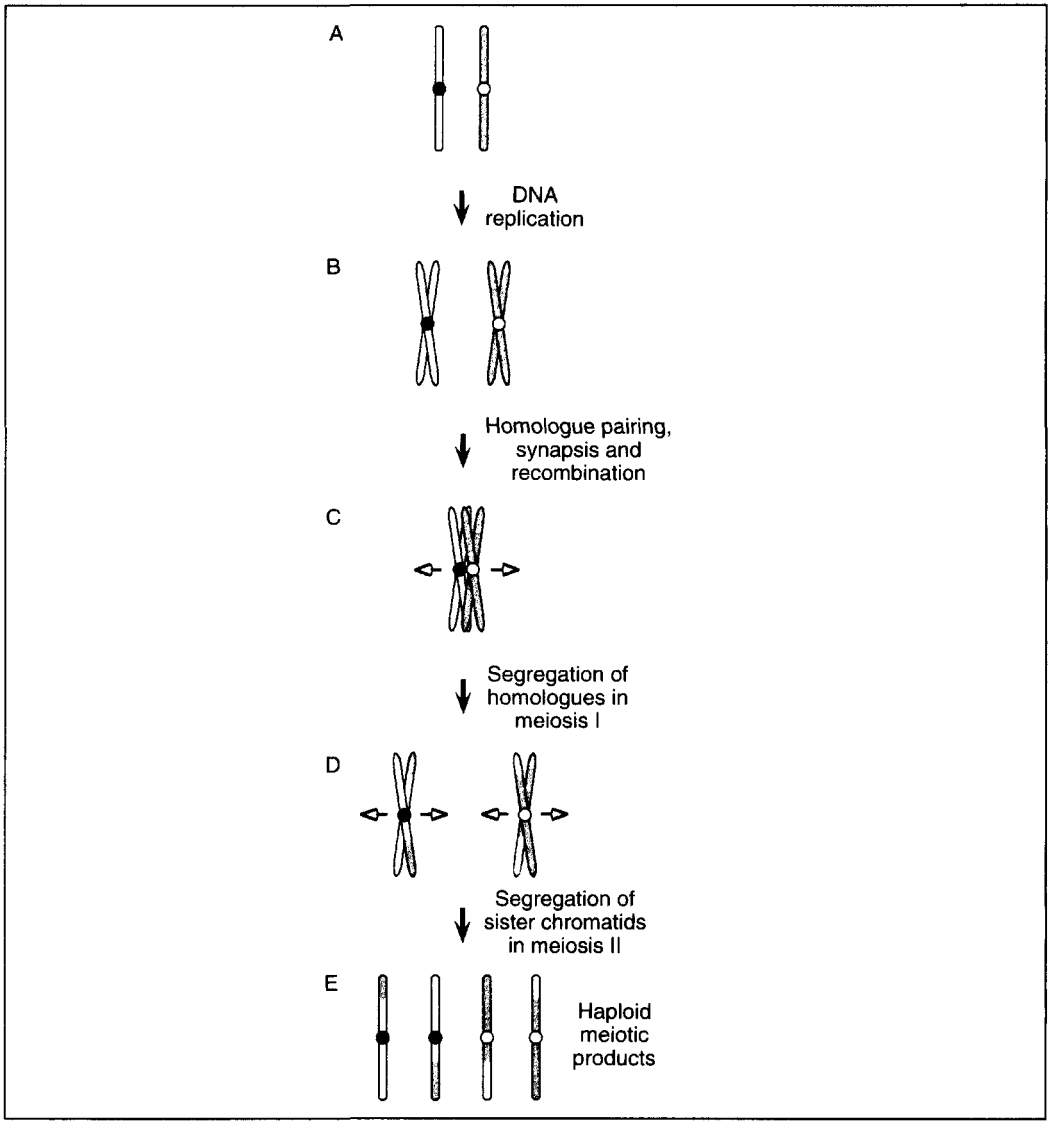


Fig. 1 Replication, pairing, and segregation of chromosomes in meiosis. Two homologs are shown (A) prior to and (B) after replication. Pairing of the replicated chromosomes occurs to form bivalents (C), followed by segregation of the homologs, or half-bivalents during meiosis I. The segregated meiosis I chromosomes (D) each represents the haploid chromosome number. Sister chromatids then segregate from one another in meiosis II to form four haploid meiotic products (E).

referred to as 'achiasmate pairing' (13). Achiasmate pairing of meiotic chromosomes, including pairing in *Drosophila* males, is discussed in greater detail in the first half of this chapter.

Synapsis of homologous chromosomes is followed by the meiotic division(s). In most, but not all organisms, the two meiotic divisions occur with segregation of

homologous chromosomes in meiosis I, followed by segregation of sister chromatids in meiosis II (Fig. 1). Because the number of chromosomes is reduced to the haploid number by the first meiotic division, meiosis I is called the reductional division, and the meiosis II division, in which the number of chromosomes remains the same before and after the division, has been termed the equational division. The meiosis II division has often been compared to mitotic divisions because sister chromatids also segregate from one another in mitosis. The second meiotic division in most organisms is much more similar to a mitotic division than the first meiotic division, although the nuclear events preceding the meiosis II division differ from those of mitotic cells, and this is also true of subsequent events. Moreover, the number of chromosomes that undergoes meiosis II is the haploid number (Fig. 1), rather than the diploid number, as in mitosis.

The relative order in which the reductional and equational divisions occur was the subject of a 'tedious' controversy among early cytologists at the turn of the nineteenth century (14). Although the reductional division precedes the equational division during meiosis in most organisms, exceptions are known to exist. For example, in organisms such as the heteropterous insects, one or more specific chromosome pairs divide equationally first, then reductionally (14). In some species of coccids and lice, reduction to the haploid number of chromosomes occurs in primordial gonial cells prior to the formation of spermatocytes, and spermatocytes undergo only one maturation division, which is equational (15). These and other deviations from the 'normal' sequence of events that occurs in most organisms during meiosis provide evidence for species-specific adaptations of the meiotic pathway of development during evolution. Many of the deviant or variant forms of meiosis that are known in various organisms are reviewed in White (15). These naturally occurring exceptions make it difficult to formulate general statements regarding the events of meiosis.

A further difference between meiosis and mitosis is that haploid spores or gametes are formed following completion of the meiotic divisions, rather than diploid daughter cells. The haploid cell then mates or fuses with another haploid cell, and the resulting diploid cell undergoes division and developmental events that eventually lead to the formation of a complete organism. The processes of sporulation and gametogenesis have been described in many organisms, although the genes required for spore or gamete formation have not yet been completely identified in any organism. Furthermore, although some of the proteins involved in cell mating or sperm entry into the egg during fertilization have been identified, the cellular proteins that mediate pronuclear conjugation and fusion have not been conclusively identified in any organism.

Finally, progression from DNA synthesis through completion of the meiotic divisions can be delayed or interrupted by extended periods of time. These periods of arrest can last as long as months or years in oocytes; thus the cell cycle regulation of meiosis differs dramatically from that of mitosis. Again, some of the cell cycle regulatory proteins that control aspects of the meiotic cell cycle have been identified in some organisms, but a complete pathway, including identification of the proteins

that activate oocytes following periods of arrest, has not been defined for any organism. Regulation of the meiotic cell cycle and the proteins that have been discovered to date are reviewed at the end of this chapter.

The broad area of meiosis cannot easily be covered in a chapter of the length presented here. We therefore focus on several aspects of meiosis that have undergone advances due to recent findings during the past 5 years or so. These include the identification of proteins that underlie chromosome pairing, synapsis, and movement, and facilitate spindle assembly and dynamics. Many of the recent findings have been made in yeast and *Drosophila*, but work in other organisms, including maize and the mouse, is described where appropriate. We apologize to the many scientists whose excellent work elucidating meiotic mechanisms could not be summarized here due to space constraints.

2. Chromosome pairing, synapsis, and movement

The proper disjunction of homologous chromosomes in meiosis I involves two general steps: initiation/maintenance of homolog association (pairing and synapsis), and physical segregation of homologs to opposite poles (Fig. 1). Errors in meiotic chromosome inheritance (loss or nondisjunction) creates aneuploid germ cells, which produce defective or dead zygotes. The importance of meiotic chromosome inheritance to humans is demonstrated by reproduction statistics: 25% of all conceptions suffer from major chromosome abnormalities that cause spontaneous abortions, miscarriages, or birth defects (e.g., Down's syndrome) (16, 17). Despite recent advances in this important field, major gaps exist in our understanding of both chromosome pairing and segregation.

2.1 Homolog synapsis and disjunction

2.1.1 The role of recombination in synapsis and disjunction

Eukaryotic genomes are large, and most contain significant amounts of repeated DNA that is shared among non-homologous chromosomes. How do homologs find each other and maintain pairing with the appropriate partner, given large amounts of 'competitor' DNA? The initiation, maturation, and maintenance of recombination events between homologs play a critical role in ensuring appropriate chromosome synapsis and disjunction. Significant advances have been made in the last decade in our understanding of the biochemical mechanisms responsible for the initiation and maturation of reciprocal exchange and gene conversion events, predominantly from investigations using the yeast *Saccharomyces cerevisiae*. Recent exciting findings regarding meiotic recombination pathways, mechanisms, and proteins will not be covered here; those interested in further reading on these topics should consult recent reviews (5, 3, 18). Here we will focus only on aspects of this important body of work that are relevant to chromosome pairing, synapsis, and disjunction.

The relationship between predominantly euchromatic recombination events and

proper chromosome disjunction was first established by genetic studies in the fruit fly, *Drosophila*, and was later demonstrated to apply to many other organisms (for reviews see refs 19 and 20). Mutations that reduce or abolish homologous recombination cause a significant decrease in the fidelity of homolog disjunction in yeasts, flies, and mammals. In general, exchange is thought to ensure proper disjunction because: (i) homologous recombination events help identify or maintain associations with the appropriate partner, and (ii) chiasmata (the visible connections between recombined homologs) co-orient centromeres to opposite poles at prometaphase. However, exchange alone is not sufficient to ensure proper homolog disjunction. Recent studies in humans, *S. cerevisiae*, and *Drosophila* demonstrate that the location of exchanges along the chromosome arm determines the efficiency of disjunction (21–23). Exchanges in centromere-proximal or telomere-proximal euchromatic regions are associated with frequent disjunction failures, unlike exchanges in the central portion of the arms. Perhaps telomere-proximal events cause bivalents to separate prematurely (before anaphase I), because they interfere with specialized terminal pairing/resolution mechanisms (24, 25; see below), or because distal sister cohesion is insufficient to maintain bivalent stability. Centromere-proximal events may not be resolved before anaphase I, causing the entire bivalent to move to one pole (21–23). Another possibility suggested by these observations is that disjunction is only ensured by the maturation of exchange events into stable chiasmata (23).

The generation of mature exchange events has also been demonstrated to play an important role in meiotic cell cycle control. Female oogenesis arrests at the metaphase–anaphase transition in either meiosis I or II, or premeiotically in G_1 or G_2, depending on the organism. Meiosis is usually completed only after fertilization, which can require that the arrested spindle configuration and homolog associations be maintained for years or decades. In *Drosophila*, mutations that severely suppress meiotic crossing over (e.g., *mei-9* and *mei-218*) cause oocytes to bypass the normal meiosis I arrest; results of these studies also suggest that even a single chiasma is sufficient to arrest the oocyte (26). Elegant studies by Jang *et al.* (27) used compound chromosomes in which both homologs were attached to a single centromere (these chromosomes are usually produced by irradiation mutagenesis; ref. 28) to demonstrate that exchange *per se* is not sufficient to induce meiotic arrest. Instead, chiasmata must join together, or conjoin, chromosomes that contain separate centromeres attached to opposite poles. Therefore, Jang *et al.* (27) suggest that meiotic arrest is induced by the tension produced when poleward forces (through spindle attachments to the kinetochore or chromosome arms) are counterbalanced by the chiasmata connections (see Section 2.3 and Chapter 1 for an in-depth discussion of kinetochore tension and cell cycle regulation).

It is clear that the maturation of exchange events helps to ensure proper disjunction and completion of meiosis, but how do appropriate homologs pair and initiate exchange? What is the nature of homolog pairing and how does it differ from pairing for recombination? Does recombination-based pairing initiate homolog pairing, or does general homolog pairing precede and facilitate recombination-based pairing? In *Drosophila*, genetic mapping studies with translocations suggest that

specific boundaries are required for exchange events to occur in the intervening region (29); there are only four such regions along the entire euchromatic arm of the X chromosome (roughly 25 megabases), and each boundary may correspond to a region of intercalary heterochromatin (see Section 2.2.1). It is still not known whether these sites help to initiate recombination pairing by 'tacking' the homologs together, or act later, in the maintenance or maturation of exchanges.

Studies in *S. cerevisiae* have demonstrated that double-strand breaks (DSBs) can initiate recombination, and that the first recombination steps occur before visible synapsis of the entire chromosome (30–32). Mutants deficient in meiotic recombination also display reduced chromosome synapsis and condensation (33, 34). These observations suggest that homolog synapsis uses the molecular machinery and mechanisms required for recombination. However, the order of the very first events are still unknown—base-pairing could simultaneously initiate exchange and the physical association of homologs, or DSBs (double-strand breaks) may only be formed after local homologous associations are established (32). Kleckner has proposed an attractive model, in which multiple, unstable DNA–DNA contacts ('kissing') between intact duplexes (paranemic interactions) eventually mature into a few stable, appropriate pairings that are competent to base-pair and recombine (reviewed in ref. 18). In one version of this model, overall chromosome synapsis is driven simply by chromosome condensation (the visible compaction of DNA that occurs in prophase of mitosis and meiosis) around the stable recombination contacts (30), which is consistent with the coincident timing of alignment and condensation (31).

The relevance of the yeast observations and models to other organisms is still unknown, and in some cases is under dispute (35). In many plant and animal species, condensed chromosomes are not obviously aligned (36–38), and in maize, chromosome alignment precedes zygotene, the stage at which recombination occurs (39). We have a limited understanding of how appropriate chromosome partners are initially identified for recombination pairing in organisms where chromosomes are not visibly aligned prior to recombination.

2.1.2 The role of telomeres in pairing

Do telomeres play a role in initiating pairing in meiosis? Chromosomes in a variety of organisms display a 'bouquet' structure in prophase of meiosis I, which is characterized by clustering of centromeres and telomeres from different chromosomes (40, 38). In most cases, the bouquets are only observed in early prophase (leptotene/zygotene), which certainly correlates with the timing of early pairing events. Impressive three-dimensional reconstruction studies of meiosis in maize suggest that telomeric regions initiate chromosome pairing by using an active chromosome movement mechanism which may involve the cytoskeleton (39). Early prophase (prezygotene) chromosomes undergo other significant, visible structural rearrangements which may facilitate pairing, including partial separation of sister chromatids and an increase in volume and surface area. In the fission yeast, *Schizosaccharomyces pombe*, telomeres are clustered at the beginning of meiosis and

throughout prophase, but not in vegetative nuclei (41). Similarly, elegant fluorescence *in situ* hybridization (FISH) analyses of human and mouse meiosis demonstrate that centromere and telomere movements and associations are correlated with the beginning stages of chromosome pairing (42). Recent studies of the *Ndj1/Tam1* and *UbcD1* genes in *S. cerevisiae* and *Drosophila*, respectively, suggest that telomeres may interfere with normal meiotic synapsis and segregation if they are not 'processed' appropriately (24, 25, 43); perhaps non-homologous telomeric interactions need to dissociate before proper homologous telomere associations can be generated, by a passive or active (maize-like) mechanism. For example, the *S. cerevisiae* NDJ1/TAM1 protein is present only during meiosis, and is localized to chromosome ends (43). Null mutants display increased homolog nondisjunction, delayed synapsis, and an increase in the number of non-recombinant chromosomes. Recombination *per se* is not impaired in *ndj1/tam1* mutants; only the distribution of exchanges is altered. These results suggest that telomeres may play a positive role in chromosome synapsis and crossover interference. These are exciting findings; however, these analyses do not demonstrate a role for telomeres in initiating pairing, only that telomeres can interfere with proper resolution of bivalents. In addition, ring chromosomes can recombine at normal frequencies in *S. cerevisiae*, suggesting that telomeres may not be essential for pairing in this organism (44). Nevertheless, the possibility that telomeres (or centromeres) provide a common substrate for initiating homolog pairing in unicellular and multicelluar eukaryotes calls for further investigations; specifically, genetic evidence is required to demonstrate that the cytologically-visible associations are required to initiate pairing. It is worth noting here that telomeres play other specialized roles in meiosis. For example, in *S. pombe*, telomeres from all three chromosome pairs associate, then lead the chromosomes in unusual back-and-forth movements during the 'horse-tail' stage, which precedes a normal centromere-mediated homolog segregation in anaphase (45). The significance of these intriguing movements is currently unknown.

2.1.3 The synaptonemal complex

By the end of prophase, most chromosomes are synapsed along their length, and in most (but not all) organisms there is a visible structure called the synaptonemal complex (SC), located in the region between the homologs. SC proteins and ultrastructure have been characterized in a variety of systems (reviewed in refs 3, 18, and 46). The SC often exhibits a ladderlike structure consisting of a central element plus flanking lateral elements. Although early investigations led to the suggestion that the SC was required for pairing, recombination, and synapsis, it is clear from the timing of events in yeast meiosis (31, 32) that the SC is not required to initiate recombination in all organisms. Furthermore, the presence of the SC is not sufficient to ensure recombination. In *S. cerevisiae*, the SC forms in the absence of homologs (in haploids) (47), while in *Drosophila*, the SC forms between non-recombinant homologs in female meiosis (48, 49). The accumulated data suggest that the SC is probably necessary to complete recombination (produce stable chiasmata) and/or control the number of crossovers (regulate crossover interference) (3, 43).

In summary, mature exchange events are intimately involved in ensuring normal homolog pairing and disjunction, but we still do not understand how recombination pairing is initiated in most organisms. The simple hypothesis that base-pairing associated with recombination initiates homolog pairing is probably not correct in most organisms, but currently we have too little information to identify what is responsible for aligning homologs prior to and during recombination. Further analyses are necessary to investigate the exciting suggestion that telomeres may play an important role in initiating pairing, and in resolving bivalents during anaphase I.

2.2 Non-recombinant chromosomes disjoin normally in meiosis I

Although the production of complete chiasmata appears to be sufficient to ensure proper disjunction of homologs, in fact, for an individual chromosome, exchange is not necessary for normal meiosis I pairing and segregation. Non-recombinant (achiasmate) homologs disjoin at high frequency in many different organisms (Fig. 2) (reviewed in ref. 50). Even non-homologous ('heterologous') chromosomes can disjoin from each other when a homologous partner is not present (51, 52), for example compound X and compound 4 chromosomes disjoin regularly in *Drosophila* females as do non-homologs in a haploid yeast strain (47). In this section we will focus on recent analyses that have elucidated some of the *cis* and *trans* regulators of achiasmate disjunction.

2.2.1 Centric heterochromatin determines the efficiency of achiasmate pairing in *Drosophila* females

In meiosis I in *Drosophila* females, 5% of the X chromosome partners and 100% of the 4th chromosomes fail to recombine. Nevertheless, non-disjunction frequencies for the X and 4th chromosomes are less than 0.1% (Fig. 2A) (53, 54). Why are the pairing and segregation of achiasmate chromosomes so efficient? Pioneering studies by Grell dating back to the 1950s described an alternative disjunction system in *Drosophila* females, originally called 'distributive pairing' (reviewed in ref. 54). Partner choice in the distributive system was originally attributed to similarities in chromosome size and shape. More recent cytogenetic analyses suggest that there are two systems to ensure the disjunction of achiasmate chromosomes in females (55). This insightful model is based on observed differences between the disjunction of homologous and non-homologous ('heterologous') chromosomes, when either *cis*-acting chromosome elements or *trans*-acting genes are altered. First, some *trans*-acting mutations affect the disjunction of achiasmate homologs, but not heterologs (*ald, Axs, mei-S51*). Second, partner choice in the 'heterologous' disjunction system is mediated by differences in chromosome size and shape. Heterologous disjunction may not involve physical pairing of partners; instead, chromosomes may disjoin by segregating precociously (Fig. 2A) to the least crowded pole (13), or by moving away from each other along interchromosomal microtubules (56). In contrast, partner

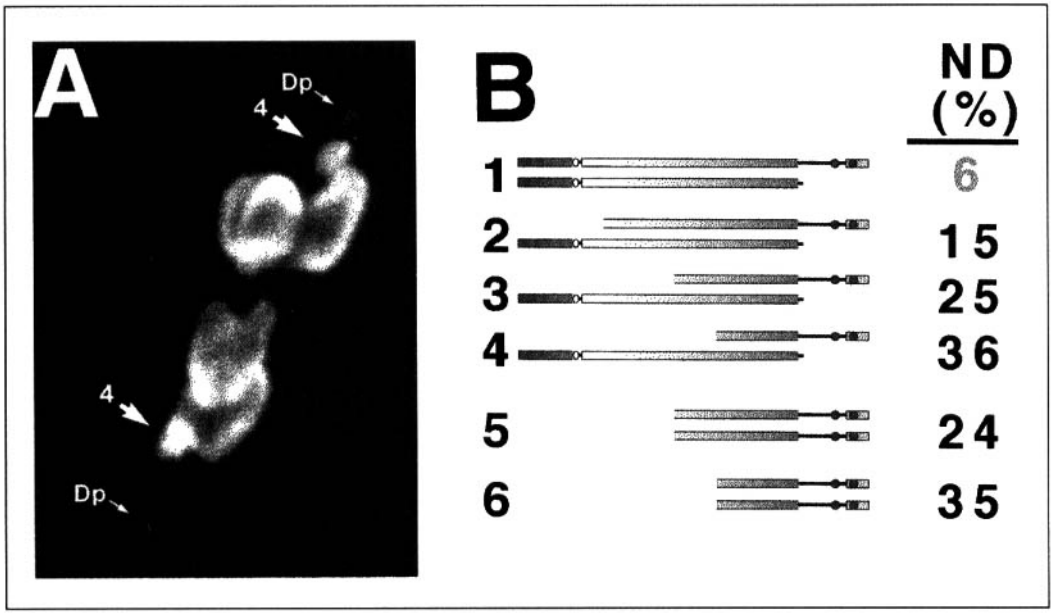


Fig. 2 (A) Chiasmate and achiasmate disjunction in *Drosophila* female meiosis I. An early anaphase preparation is shown. The large chromosomes in the center are positioned near each other and close to the metaphase plate, typical of chiasmate disjunction. The precocious segregation behavior of the small 4th chromosomes is characteristic of achiasmate disjunction (13). Disomic *Dp1187* mini-chromosomes segregate to opposite poles; they display precocious segregation, and are closer to the poles than the larger 4th chromosomes. Photograph by K. Yook, W. Sullivan, and G.H.K. (B) Heterochromatic homology mediates achiasmate meiotic pairing in *Drosophila* females. Molecular genetic dissection of the DNA required for achiasmate pairing was performed using molecularly defined derivatives of the *Dp1187* mini-chromosome. Derivative pairs with 1 Mb of heterochromatic homology efficiently disjoin in female meiosis I (1); less heterochromatic homology results in increased non-disjunction (ND) (2, 3, and 4 differ significantly from 1). Euchromatic homology or similar chromosome size does not result in efficient disjunction (compare 5 with 3 and 6 with 4; see text and ref. 58 for details). Box with gray gradient designates the centric heterochromatin; the gradient indicates orientation. Dark gray box is the sub-telomeric heterochromatin, solid black line is the euchromatin, small open circle is the *yellow*$^+$ gene, filled circles are the two *rosy*$^+$ genes (inserted by P element transformation). Reproduced with permission from *Science*, **273**, 118 (ref. 58). Copyright 1996 American Association for the Advancement of Science.

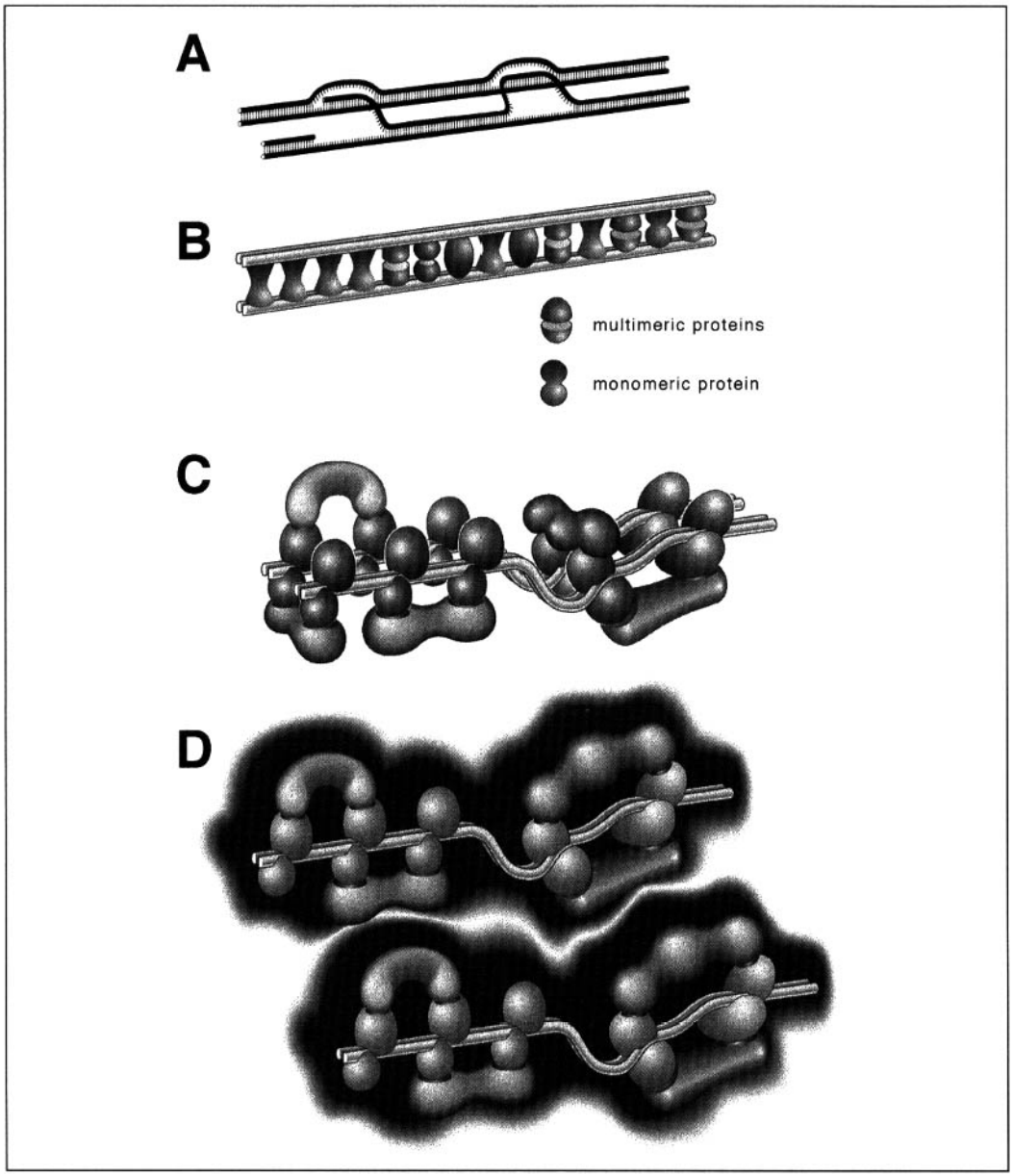
choice in the 'homologous' achiasmate system appeared to depend on heterochromatic homology. However, the rearranged chromosomes used in these early studies could only be characterized cytogenetically (55), so the exact relationship between heterochromatin, pairing, and disjunction could not be determined. A role for heterochromatin in meiotic pairing was first suggested by Gershenson in 1940, based on genetic analyses (57). Carpenter's later electron micrograph investigations of oocytes also suggested that heterochromatic regions are physically paired early in meiosis (in pachytene) (48, 49).

Recent independent molecular genetic and cytogenetic investigations confirm that centric heterochromatin (specifically the heterochromatin located near the centromere, also referred to as 'pericentromeric') mediates homologous achiasmate

disjunction, and suggest possible models and mechanisms. First, molecular genetic evidence for the involvement of heterochromatin in pairing was obtained in studies using *Dp1187*, a 'free duplication' of the X chromosome that exists as an extrachromosomal entity (58). *Dp1187* minichromosomes are completely non-recombinant, yet disjoin frequently via the homologous achiasmate system (Fig. 2). Disjunction of molecularly defined deletion derivatives of *Dp1187* was monitored genetically, which demonstrated that (i) the amount of heterochromatin overlap determined the efficiency of disjunction, (ii) euchromatin did not contribute, and (iii) size similarity was not sufficient to ensure proper disjunction (Fig. 2B). Heterochromatic deletions did not increase non-disjunction by interfering with centromere-mediated segregation during prometaphase or anaphase. For example, many disomic combinations that displayed defective disjunction (e.g., pair 3 in Fig. 2B) did not exhibit chromosome loss. Second, direct localization of chromosome-specific sequences within the oocyte nucleus, by a new method for *in situ* hybridization, demonstrated that heterochromatic regions of homologous achiasmate chromosomes are physically associated throughout prophase of meiosis I (Plate 1) (59). In contrast, heterochromatin from heterologous chromosomes was infrequently paired, as was euchromatin present on homologs. The studies of Dernburg *et al.* (59) and Karpen *et al.* (58) together argue strongly that centric heterochromatin initiates and/or maintains achiasmate pairing between homologs. The minichromosome disjunction studies also suggest that multiple heterochromatic pairing elements exist and that they act additively (58). Thus it is likely that all regions of heterochromatin contribute to pairing, rather than a few, specific sites.

Heterochromatin is an enigmatic component of multicellular eukaryotic genomes; it is sparsely populated with genes, inhibits the function of adjacent euchromatic genes (position effect variegation), replicates late in S phase, and is rich in tandemly repeated 'satellite' sequences (reviewed in refs 60–62). How could heterochromatin mediate pairing? Two general classes of models immediately present themselves: DNA base pairing and meiosis-specific pairing proteins. Recombination-like base pairing between heterochromatic regions (Fig. 3A) is an unlikely mechanism; reciprocal recombination occurs rarely in heterochromatin (63), suggesting that heterochromatin is excluded from base-pair homology searches. Perhaps suppression of recombination in heterochromatin has evolved because there are extensive regions of identity shared by heterochromatin on non-homologous chromosomes (64, 65), which would certainly confuse a base-by-base homology search. Heterochromatic recombination events may also 'poison' meiotic segregation by blocking resolution

Fig. 3 Four models for the role of heterochromatin in meiotic pairing. (A) A DNA homology search mechanism. Black bars represent a single DNA strand, duplexes are from separate homologs. Sister chromatids are not shown. The homology search could involve single- or double-strand breaks (not shown), or single-strand looping with no breaks. (B) Specialized pairing proteins generate homologous pairing. Here, a combinatorial mechanism is shown, which allows only a few proteins to provide specificity, and to circumvent the potential confusion caused by the similarity of heterochromatic DNA sequences on non-homologs. Pairing proteins could bind DNA on one homolog, then dimerize (or multimerize) with similar partners on the other homolog. Alternatively, monomeric proteins with symmetrical binding sites could facilitate pairing by binding the same DNA on both homologs.



Double bars represent sister chromatids. (C) Pairing as an intrinsic property of heterochromatin: cross-homolog multimerization. Multimerization complexes that normally form within the heterochromatin to promote intrinsic heterochromatic functions, such as sister chromatid cohesion, kinetochore formation, or chromatin structure could also form between homologs, providing both specificity and direct pairing contact in meiosis. Double bars represent sister chromatids. (D) Pairing as an intrinsic property of heterochromatin: heterochromatic landscape 'fit'. DNA and protein structures inherent to heterochromatin could produce a chromosome 'landscape' (black background), which ensures partner recognition and alignment by a 'best fit' mechanism. Double bars represent sister chromatids. Reproduced with permission from *Science*, **273**, 118 (ref. 58). Copyright 1996 American Association for the Advancement of Science.

of linked bivalents, as suggested by studies of the distribution of crossovers in non-disjunctional meioses in flies and humans (21, 22). Alternatively, partner recognition and pairing could be carried out by meiosis-specific pairing proteins that interact with identical sequences on each homolog (Fig. 3B). The order of heterochromatic sequences and their corresponding pairing proteins (a combinatorial mechanism) would have to be used to circumvent the problems posed by extensive heterochromatic sequence identities present on non-homologs.

It is possible that heterochromatin accomplishes homologous achiasmate pairing by a less specialized mechanism; perhaps intrinsic structural features of heterochromatin determine pairing specificity and mediate physical associations (Fig. 3C and D) (58). In dipteran somatic mitoses, homologs are somatically paired (66–68); even separating sister chromatids are paired during anaphase (Sullivan, W. and Karpen, G., unpublished). The proteins responsible for heterochromatin 'stickiness' in somatic cells (69, 70) could be co-opted to perform the same function in meiosis. Similarly, the physical pairing of monologs in premeiotic, mitotically dividing germ cells may simply continue into meiosis. In both mechanisms, best-fit pairing could be accomplished by cross-homolog multimeric protein complexes (Fig. 3C), or by recognition of a heterochromatic 'landscape' (Fig. 3D) (58). Similarities exist between the landscape model and Kleckner's 'kissing' model for recombination pairing (18, 71) and these warrant further investigation. Perhaps heterochromatin is responsible for the initial alignment of homologs destined to recombine, in addition to its role in achiasmate disjunction.

Finally, it is interesting to note that the original hypothesis of Grell, suggesting that achiasmate chromosomes pair and segregate according to size (11), may play a role in non-homologous achiasmate segregation in *Drosophila* females (13, 59). However, the minichromosome derivative analyses conclusively demonstrate that size differential does not play a role in the homologous achiasmate system (58). Chromosome size also has no effect on the disjunction of non-homologous or homologous achiasmate chromosomes in the yeast *S. cerevisiae* (72, 73).

2.2.2 Proteins that mediate achiasmate disjunction in *Drosophila* females

Identification of the genes and proteins that affect achiasmate pairing *in trans* would help test the models presented above. For example, the specialized meiotic protein model (Fig. 3B) predicts that proteins essential for achiasmate pairing will only be utilized during meiosis, and only affect achiasmate chromosome disjunction. However, if achiasmate pairing reflects intrinsic features of heterochromatin (Fig. 3C and D), genes that control achiasmate pairing will also be involved in heterochromatin metabolism or structure in other cell types, e.g., position effect variegation (modified expression of a gene due to its position adjacent to heterochromatin) in somatic cells.

Given the dependence of chromosome segregation on spindle function, it is not surprising that mutations in proteins that promote spindle assembly and function, and the attachment of chromosomes to the spindle, affect the disjunction of achiasmate chromosomes (e.g. *nod* and *ncd*; see Section 2.3.2 and 3.2.2). What

proteins mediate physical pairing? Only four known loci are currently candidates for genes involved specifically in pairing or segregation of achiasmate homologs, but none of these genes have yet been demonstrated to play a direct role in homolog pairing, or to interact physically or genetically with heterochromatin. *Axs*, *ald*, and *mwr* specifically reduce the disjunction of homologs without affecting heterologs (74–76); *mei-S51* displays the same phenotype but also reduces euchromatic recombination (77). *ald* also induces high rates of non-disjunction of recombinant chromosomes (74); thus it is possible that *ald* is involved in the disjunction of all chromosomes, with a greater effect on non-recombinant chromosomes due to the absence of chiasmata. *Axs* mutants also display visible spindle defects, but only when non-recombinant chromosomes are present; this suggests that the spindle defects occur in response to aberrant segregation (20). *In situ* hybridization analyses demonstrate that heterochromatic regions are paired in all *ald* and most *Axs* oocytes (59), suggesting that neither gene is involved in the initiation or maintenance of pairing. It is more likely that *ald* and *Axs* play important roles in the release of pairing interactions during metaphase (59), or in the physical segregation of homologs during anaphase. The recently identified *mwr* mutants have normal spindles and rates of recombination, and have no effect on recombinant chromosomes; thus *mwr* is currently the best candidate pairing protein gene (76). Ultimate dissection of the functions of *ald*, *Axs*, and *mwr* requires determination of their protein sequences, biochemical activities, and localizations within cells and chromosomes.

New candidate pairing genes need to be identified and studied. The recent demonstration that heterochromatin mediates achiasmate pairing suggests an overlooked source of potential pairing genes—loci known to affect other types of heterochromatin behavior. Prime examples include the ~130 known dominant suppressors and enhancers of heterochromatin-induced position effect variegation (78). Minichromosome pairing partners with heterochromatic deletions exhibit intermediate levels of non-disjunction (e.g., pair 3 in Fig. 2B, 25% non-disjunction), and thus are excellent 'sensitized' substrates (79) for genetic screens (58).

2.2.3 Chromosome-specific control of achiasmate disjunction in *Drosophila* males

No measurable recombination occurs in meiosis I in *Drosophila* males, yet all homologs segregate with high fidelity (8, 80). Surprisingly, neither of the proposed female achiasmate disjunction systems appears to be utilized in males (reviewed in ref. 4). Instead, male meiotic pairing in *Drosophila* appears to be controlled by chromosome-specific systems (for detailed review, see ref. 81).

X–Y pairing is mediated by the tandemly repeated 18S/28S ribosomal DNA clusters (the nucleolar organizers) present in the heterochromatin of both chromosomes. Light (8) and electron (82) microscope analyses demonstrated that the distal region of the X heterochromatin, the location of the nucleolar organizer, is usually paired with the Y chromosome. Genetic evidence supports this view, since X chromosomes that are deleted for the rDNA cluster (e.g., *Df(1)X-1*) disjoin randomly from the Y chromosome and display high frequencies of meiotic drive (i.e., the

deleted X chromosome is recovered in vast excess of the Y chromosome) and sterility (83). The most convincing evidence comes from the demonstration by McKee and Karpen that a single rDNA transgene inserted into *Df(1)X-1* partially rescues all three meiotic phenotypes, and that increasing the number of X-linked rDNA transgenes to two results in nearly complete rescue (9). X chromosome context appears to be important, since insertions of rDNA in the autosomes did not promote autosome–Y chromosome pairing. McKee has used P-element transformation with deletion constructs of the rDNA to demonstrate that pairing maps to the 240 bp repeats located in the intergenic spacer (IGS) (84, 85). It is surprising that only five or six copies of the 240 bp repeat are sufficient to produce an obvious stimulation in pairing.

How could such a small region of rDNA act to initiate and/or maintain pairing of the much larger X and Y chromosomes (roughly 40 and 45 Mb, respectively)? rDNA is heterochromatic, which on the surface resembles homologous achiasmate pairing in *Drosophila* females. However, heterochromatic pairing sites are much more diffuse and generally distributed in females (see Section 2.2.1), and extensive arrays of rDNA do not promote pairing of X-derived minichromosomes with the normal X chromosome (55). In addition, *trans*-acting mutations that affect achiasmate disjunction in females generally have no effect on male meiosis (4). Most likely X–Y pairing in males uses a different mechanism than achiasmate pairing in females. One attractive model is that rDNA from both the X and Y chromosomes produces nucleoli (86), and that nucleolar fusion mediates pairing by amplifying the information inherent to a small sequence. A single complete rDNA gene can produce a mini-nucleolus at ectopic chromosome locations (87) and mediate X–Y pairing (9). However, IGS-only constructs are not associated with visible mini-nucleoli, yet they are able to promote meiotic pairing (84, 85). Since the 240 bp IGS repeats contain copies of the highly active polymerase I promoter, McKee proposes an alternative model, that transcriptional activity may be linked to pairing ability (81). Transcription factors bound to the promoter may be recruited to act as pairing elements, or transiently single-stranded DNA generated during transcription could make the repeated DNA available for base pairing. One attractive feature of this model is that it accounts for the fact that heterochromatin in general does not participate in pairing in males, because most of the heterochromatin is transcriptionally silent.

Although X–Y pairing is restricted to the rDNA in *Drosophila*, autosomes appear to use multiple euchromatic pairing sites. Studies of chromosome 2 rearrangements demonstrate that heterochromatin is excluded from participation in pairing; most of the euchromatin regions on chromosome 2 individually have a weak pairing capability, except for a large proximal domain (region 39DE) that includes the histone locus (88–90). It is unclear how the histone region displays a strong effect on pairing. The tandemly repeated nature of *Drosophila* histone genes (there are roughly 200 copies at this locus) provides a potential mechanistic link with the rDNA repeats that mediate X–Y pairing in males, and the involvement of repetitive DNA in female achiasmate disjunction. However, tandem satellite repeats are not sufficient for sex chromosome pairing in males (9, 91), and satellite DNA deletions do not affect pairing of any autosome (88). McKee (81) suggests that high rates of histone locus

transcription may help initiate or maintain meiotic pairing, similar to the mechanism proposed for rDNA-mediated sex chromosome pairing. Transgene studies are necessary to prove that the histone loci are directly responsible for pairing activity.

As with achiasmate pairing in females, much could be learned by studying the proteins that mediate pairing in male meiosis. Many mutations have been isolated that cause high rates of non-disjunctional progeny to be recovered from males. However, none of these genes has been demonstrated to play a role specifically in meiosis I pairing; in fact, we know very little about their true biological functions. Baker and Carpenter (92) isolated 20 X-linked mutations that specifically reduced X–Y disjunction—unfortunately, all of the mutant phenotypes disappeared after several generations, an intriguing phenomenon that has been reproduced independently (A. Zitron and R.S. Hawley, personal communication). Similarly, *mei-G17* (93) and *mei-S8* (94) cause non-disjunction of some but not all chromosomes. Non-disjunction of chromosome 2 alone is elevated in *mei-G87* mutants, but this is likely to be caused by defects in maintaining sister chromatid cohesion (see Section 2.3.1), rather than homolog pairing.

The identification of chromosome-specific pairing elements and mutations that affect the disjunction of only a subset of chromosome partners suggests that pairing in male meiosis is accomplished by separate, chromosome-specific mechanisms. However, some mutations affect the disjunction of all *Drosophila* chromosomes, including *mei-O81* (94), *mei-I1*, *mei-I3* (95), and *Dub* (96). *pal* (paternal loss) is generally classified as a male meiotic mutation, but the predominant phenotype is chromosome loss of paternal chromosomes in early embryonic mitoses (97). Thus, the Pal protein probably regulates chromosome transmission in early embryonic divisions and may be regarded as a paternal-effect mutation, rather than a mutation that specifically affects meiosis (K. Owens and B. Wakimoto, personal communication). *Dub* also affects meiosis I disjunction in females, and has a lethal phenotype, suggesting a more general role in meiosis and mitosis (96). Perhaps pairing initiation is mediated by chromosome-specific mechanisms, whereas later steps, such as pairing maintenance or physical separation in anaphase, are regulated by proteins and mechanisms shared by different chromosomes, and even by the sexes. Some of these genes may also play a role in mitosis.

There is currently much confusion about the nature and function of mutations that cause meiotic non-disjunction in males, and we are left with no good candidates for proteins that mediate pairing. The cytological defects caused by the available mutants need to be studied in detail, and the protein products of all these genes need to be identified and localized within meiotic cells. New mutant searches, targeted at genes specifically involved in pairing, are absolutely necessary for this field to progress. Biochemical approaches are also feasible. The identification of a short, specific DNA sequence involved in X–Y pairing (the 240 bp repeats) provides a unique opportunity for biochemical isolation of putative pairing proteins. The tools are available and await exploitation; in the next decade we hope to witness significant progress in our understanding of the mechanisms responsible for chromosome pairing and segregation in male meiosis.

2.3 Chromosome segregation in meiosis

What forces mediate the physical movement of chromosomes in meiotic anaphases? In Section 3, we will discuss spindle assembly and dynamics in detail. Here, we focus on the chromosome side of segregation in meiosis. Specifically, what are the chromosomal elements and proteins that control physical segregation of homologs in meiosis I, and sister chromatid separation in meiosis II?

2.3.1 Centromeres and sister chromatid cohesion

The centromeric DNA is associated with the kinetochore, a tri-laminar plate structure that attaches to microtubules and helps direct chromosome movements along the spindle in mitosis and meiosis (98). Chapter 5 describes the structures and functions of centromeres and kinetochores in detail; here we will only discuss centromere functions that are unique to meiosis.

Replication in both mitosis and meiosis leads to an approximate doubling of kinetochore size and the presence of joined sister chromatids. However, in a normal meiosis I, sister chromatids must stay together during anaphase I, and only separate 'à la mitosis' in anaphase of meiosis II. Kinetochores and sister chromatids use specialized mechanisms to accomplish this unique aspect of the reductional division.

First, there is only one functional kinetochore on each half-bivalent early in prometaphase of meiosis I, and the mitosis-like arrangement of two sister kinetochores, one on each chromatid, is not visible until late prometaphase, after stable microtubule attachments are made and congression of an appropriately oriented bivalent is nearly complete (99). The doubled meiosis I kinetochores are associated with twice the number of microtubules as in the same stage of meiosis II (100). The sister kinetochores can act independently in prometaphase of meiosis I, resulting in erroneous attachment of a half-bivalent to opposite poles. These unfavorable bipolar attachments are apparently resolved by an error correction mechanism that involves rare release and capture events that only cease when appropriate unipolar attachments are achieved (101) (see discussion of tension-sensing mechanisms in Section 2.3.2). Correct attachment of kinetochores to spindle fibers causes a change in the phosphorylation state of an as yet unidentified kinetochore protein, which can be detected using an antibody called 3F3/2 (102–104). The molecular components and mechanisms responsible for the unique regulation of kinetochore structure and function in meiosis I are completely unknown at this time.

Second, sister chromatid cohesion and separation are regulated in an exquisite manner during meiosis [see excellent recent reviews (105–107) for details not discussed here]. The differences between the regulation of meiotic and mitotic sister cohesion are highlighted by the fact that partial deletions of centromeric DNA in *S. pombe*, *S. cerevisiae* and *Drosophila* cause different levels of meiotic and mitotic sister chromatid non-disjunction (108–110; Murphy, T. D., Cook, K. and Karpen, G. H., unpublished). In addition, mutations in genes that specifically affect meiotic sister cohesion have been identified in maize [*desynaptic* (111)], yeasts [*red1* (112) and *rec8* (113)] and *Drosophila* [*ord* and *mei-S332* (114, 115)]. The molecular genetic and cytological analyses of the phenotypes of these mutants have been instrumental in

elucidating the relationship between sister cohesion and proper bivalent function in meiosis. Complex, competing forces must be resolved in a coordinated fashion, or non-disjunction will result. One attractive model is that sister cohesion distal to the sites of exchange is necessary for stabilization of chiasmata and maintenance of the proper alignment of recombinant bivalents during prophase and metaphase of meiosis I (23, 116). However, retention of distal sister cohesion becomes a liability in anaphase of meiosis I, because segregation of recombinant bivalents is hindered by any connection between at least one chromatid on each half-bivalent. This situation cannot be resolved by a complete loss of cohesion, because the sisters in each half-bivalent must remain attached until meiosis II anaphase (111, 112, 117).

Genetic and cytological analyses have led to a simple solution to this conundrum: one hypothesis is that chromatid cohesion in the arms is released first, in anaphase of meiosis I, but sisters do not separate in the pericentromeric region until anaphase of meiosis II (107). Release of arm cohesion distal to sites of exchange would allow recombinant bivalents to separate during anaphase I, while retention of more proximal cohesion in the pericentromeric region would ensure that each half-bivalent acts as a segregating unit. Orr-Weaver's molecular genetic and cytological investigations of the *Drosophila ord* and *mei-S332* genes provide direct support for this hypothesis. Premature sister separation occurs in *mei-S332* mutant males and females, late in anaphase of meiosis I (118). A Mei-S332–green fluorescent protein (GFP) fusion protein localizes to the pericentromeric regions of all *Drosophila* chromosomes in male meiosis I, and the protein is not visible after the start of anaphase of meiosis II (114). The *mei-S332* mutant phenotype and protein localization are consistent with a role in the maintenance of pericentromeric sister cohesion until meiosis II. By comparison, *ord* mutants display precocious sister separation early in meiosis I, when cohesion must be maintained all along the chromosome arms (117). The reduced recombination seen in *ord* mutant females is consistent with a role for this protein in arm cohesion; however, the Ord protein has not been localized within chromosomes (115). Mei-S332 and Ord are novel or pioneer proteins without known homologs in other organisms (114, 115), so further dissection of their functions is necessary to discover whether their roles in sister cohesion are structural or regulatory.

The molecular mechanics of sister chromatid cohesion and separation have been a source of controversy over the last decade. Mutant analyses in *S. cerevisiae* demonstrated that decatenation (removal of the topological intertwining of sister chromatids produced during replication) by topoisomerase II is required to initiate anaphase separation in mitosis and meiosis, which suggests that catenation may in part be responsible for the initiation and/or maintenance of sister cohesion (119, 120). However, intertwining of sisters is not sufficient to maintain cohesion of an *S. cerevisiae* mini-chromosome (121), suggesting an alternative model, in which sister cohesion may be accomplished by 'glue' proteins which must be cleaved or removed to allow appropriate sister separation. In fact, analyses in *S. cerevisiae* demonstrate that the initiation of mitotic anaphase requires ubiquitin-mediated proteolysis (122–124). However, anaphase initiation in mitosis may be mediated by proteolysis

of a regulatory protein (even one involved in catenation), rather than cleavage of a cohesion 'glue' protein. The proteolysis target protein specifically involved in mitotic sister separation is currently unknown, and the role of proteolysis in meiotic sister separation has not been evaluated.

The identification of different proteins that affect arm or pericentromeric cohesion in *Drosophila* provides a possible resolution to the controversy concerning the mechanism of sister cohesion and separation. Bickel and Orr-Weaver (107) suggest that arm cohesion may be mediated by catenation (e.g., through the activity of Ord) and pericentromere cohesion may be accomplished by a 'glue' (e.g., Mei-S332). The tools are available for direct tests of different models, and the next few years should bring advances in our understanding of how meiotic sister cohesion and separation are regulated at the molecular level.

2.3.2 The role of extracentromeric regions in chromosome segregation

Recent investigations suggest a fundamental change in our perception of the forces controlling chromosome segregation in meiosis and mitosis; these processes require more than just spindle microtubule interactions with the kinetochore, and regulation of sister chromatid cohesion and separation. One of the first hints that additional forces must act on chromosomes came from impressive cytological manipulations of live mitoses in newt lung cells by Rieder and Salmon (125). Chromosome fragments lacking a centromere were produced by laser irradiation, and were observed to move away from the pole, while the complementary centromere-containing fragments moved towards the pole. These studies demonstrated the existence of anti-poleward forces on chromosome arms, also known as the 'polar wind'. We will refer to this activity as an 'anti-poleward force'; however, keep in mind that the mechanism could involve interchromosomal interactions, or interactions with the near pole ('true' anti-poleward forces) or even the opposite pole (contra-poleward forces), with respect to the kinetochore microtubules. Recent investigations have identified two classes of kinesin-related microtubule motor proteins known as chromokinesins, which are likely to participate in the generation of anti-poleward forces in meiosis [*Drosophila* Nod (126, 127)] and mitosis [*Xenopus* klp1 (128) and chicken Chrkin (129)]. Chromokinesins are distinguished from other members of the kinesin motor family (see ref. 130) because they are distributed along chromosome arms. Human Kid (131) and *Drosophila* Klp38B (132, 133) kinesin-like proteins are also localized to chromosome arms, yet are phylogenetically distinct from the chromokinesins. Antibody depletion of *Xenopus* klp1 in spindle assembly extracts causes defects in mitotic chromosome positioning and bipolar spindle assembly (128). This has led to the hypothesis that Xklp1 stabilizes a population of microtubules that anchors the bipolar state and positions chromosomes on the metaphase plate.

The *Drosophila* Nod protein is the only chromokinesin currently known to act during meiosis. Loss-of-function mutations in *nod* cause high frequencies of non-disjunction and loss of achiasmate chromosomes (see Sections 2.1 and 2.2) during female meiosis (134). Cytological analyses of *nod* mutant females gave an early hint

that this protein mediated anti-poleward forces; achiasmate chromosomes were positioned on the spindle to lie closer to the poles, in comparison to recombinant bivalents and achiasmate chromosomes in wild-type females (135). Subsequently, a new method was developed to determine which regions of the chromosome interacted genetically with *nod* to promote normal monosome transmission. Normally, deleting extracentromeric regions or reducing the dosage of inheritance genes (as in a mutant/wild-type heterozygote) does not produce defective inheritance phenotypes. However, *Dp1187* mini-chromosome deletion derivatives display significantly increased loss rates in females heterozygous for loss-of-function alleles of *nod* (*nod*/+) (127). The structures of *nod*-sensitive deletions indicated that multiple regions of *Dp1187* interact genetically with *nod* to promote normal chromosome transmission. The surprising result was that extracentromeric regions are required for the function of this putative microtubule motor. Independent biochemical and cytological studies demonstrated conclusively that the genetic/functional *nod* interactions involve direct binding of DNA sequences by Nod protein, and that there is a strong preference *in vitro* for binding an AT-rich satellite DNA (126, 136). The molecular, genetic and cytological data combined to suggest strongly that Nod is a chromokinesin that generates an essential anti-poleward force in female meiosis (126, 127), and may carry out a similar but redundant role in mitosis (137). Whether Nod acts as a true microtubule motor, as a 'brake', or as a regulator of another motor is currently unknown.

How could anti-poleward forces acting at extracentromeric regions be critical for normal meiotic chromosome inheritance? One model proposed for the role of anti-poleward forces in meiotic segregation involves tension-mediated stabilization of kinetochore–spindle interactions (126, 127). Nicklas has performed elegant studies of mitotic divisions in live grasshopper and mantid spermatocytes, and argues that correction of unfavourable bipolar attachments requires infrequent release and capture events that are only stabilized when there is a balanced tension produced by equal kinetochore-mediated forces associated with opposite poles (138). This tension-sensing mechanism is likely to play an important role in a mitotic and meiotic 'spindle assembly checkpoint', which monitors the presence of intact chromosome–spindle attachments and signals to the cell that anaphase can safely begin (139–141) (see Chapter 1). Nicklas' experiments also suggest that tension can act during meiosis to stabilize univalents, and half-bivalents that are conjoined by chiasmata (101, 142, 143). Thus, anti-poleward forces generated on extracentromeric regions during meiosis by putative motor proteins like Nod could act indirectly on centromere function, by maintaining tension on kinetochore microtubules and stabilizing the attachment and reattachment of achiasmate chromosomes to the metaphase spindle (126, 127). One attractive feature of this model is that it can account for the high frequency of non-disjunction and low frequency of chromosome loss observed for *nod* mutant females (127). The general role of extracentromeric regions and chromokinesins in mediating anti-poleward forces and kinetochore tension needs to be evaluated directly for the segregation of exchange chromosomes, in meiosis in other organisms, and in mitosis.

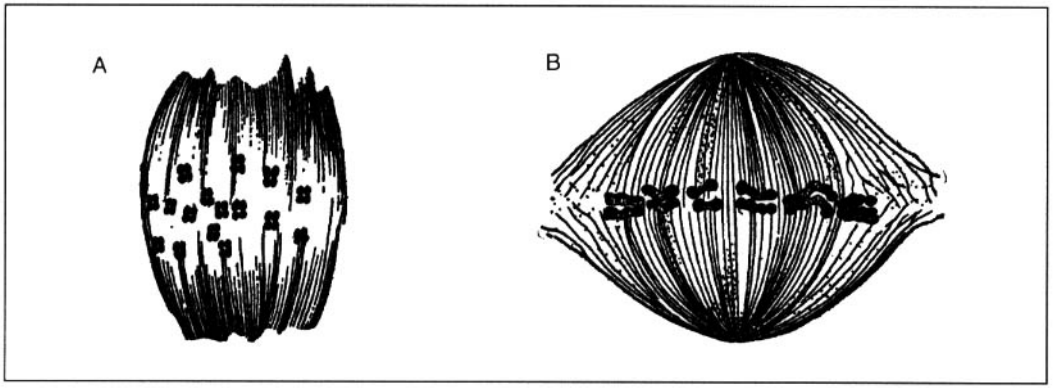


Fig. 4 Anastral oocyte meiotic spindles. (A) Meiosis I spindle with truncated poles in an oocyte of the copepod, *Heterocope*. Chromosomal tetrads are segregating to opposite poles. (B) Meiosis II spindle with rounded poles in an oocyte of the marine gastropod mollusk, *Triton*. Sister chromatids are aligned on the metaphase plate. Reproduced from ref. 14.

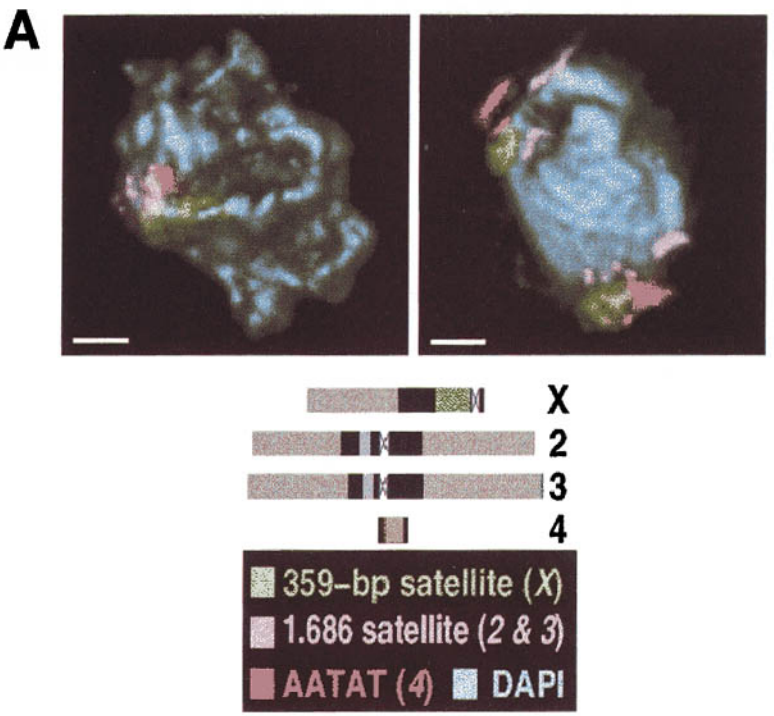
3. Spindle assembly and dynamics

3.1 Meiotic spindle structure

The overall structure of meiotic spindles in many organisms differs strikingly from mitotic spindles in the same organisms. In general, these differences are much greater for oocyte spindles than those of spermatocytes. Spermatocyte spindles of many species are much larger than those of somatic cells in the same organisms, but usually closely resemble mitotic spindles in overall structural features. The large size of the spermatocyte spindles makes them especially favorable for cytological studies, particularly studies involving micromanipulation of spindles and centrosomes.

In contrast, meiotic spindles of oocytes of many species differ dramatically in shape compared to mitotic spindles and, in many oocytes, lack centrosomes, which function as the microtubule organizing centers for spindle assembly in most mitotically dividing animal cells. The absence of centrosomes is correlated with the absence of microtubule asters at the spindle poles, which led early workers to refer to the acentrosomal spindles as 'anastral' spindles (14). Oocyte anastral spindles can resemble mitotic spindles in overall structure, but the absence of centrosomes and asters at the poles makes the spindle poles appear truncated or rounded (Fig. 4). Anastral spindles were observed by biologists in the late nineteenth century in oocytes of copepods, insects, tunicates, and many vertebrates. The absence of asters and centrosomes was at first attributed to an artefact caused by defective fixation, but this was excluded by the presence of asters and centrosomes associated with the fertilizing sperm in the same preparations (14).

The meiotic divisions of oocytes of many species occur with the extrusion of polar bodies, which contain the nuclei that do not contribute further to female pronucleus formation. This is not true of *Drosophila*, however. In *Drosophila* oocytes, the meiosis I



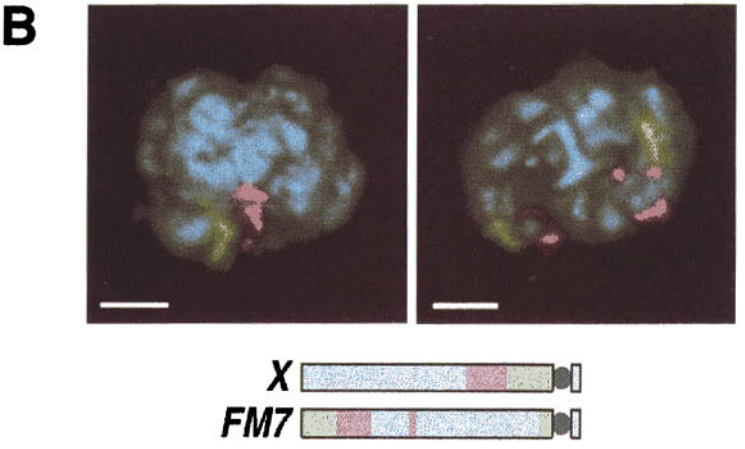


Plate 1 Physical pairing of heterochromatin in *Drosophila* oocytes. (A) FISH and repeated DNA probes were used to reveal the locations of heterochromatic regions in meiosis I. In prophase (left panel), heterochromatic regions from homologs are closely aligned (e.g., the achiasmate 4th chromosomes, orange), and the heterochromatic domains of different chromosomes are only loosely associated. By metaphase (right panel) homologous heterochromatic regions and their centromeres have separated and are oriented toward opposite poles. (B) Achiasmate X chromosome pairs (normal sequence X/FM7, a multiply-inverted balancer chromosome) are clearly juxtaposed in the oocyte in early prophase (left panel, juxtaposed hybridization signals for each X-specific probe), but not in the somatic follicle cell nuclei of the same egg chamber (right panel, distinctly separated hybridization signals for the X and FM7 chromosomes). These studies also demonstrated that the euchromatic histone region is not paired in prophase of meiosis I, and that heterochromatin of heterologous compound chromosomes is not associated with each other (see ref. 59 for examples). Images provided by Abby Dernburg, with permission (59).

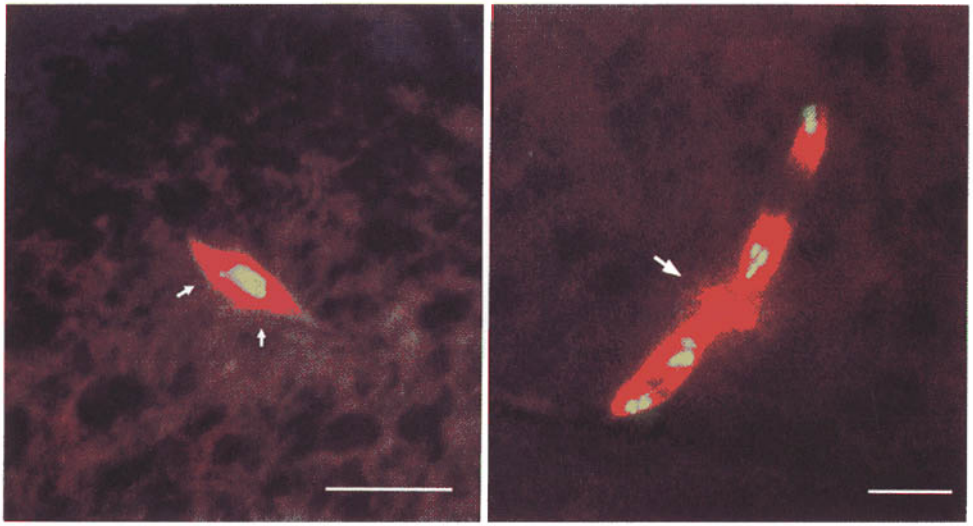


Plate 2 Meiosis I and II spindles of *D. melanogaster* oocytes. Tubulin antibody-stained spindles are shown in red and the DAPI-stained chromosomes in green. (A) Metaphase I-arrested spindle in a mature oocyte. The condensed chromosomes lie in the centre of the tapered spindle. The small chromosomes 4 appear as small protrusions (arrows) at either end of the chromosome mass. (B) Anaphase II spindles in a fertilized egg. The two spindles are aligned in tandem and a ring-like structure (arrow) with radiating microtubules, the central spindle pole body, is present between the two central poles. Bars, 10 μm. Images provided by S.A.E.

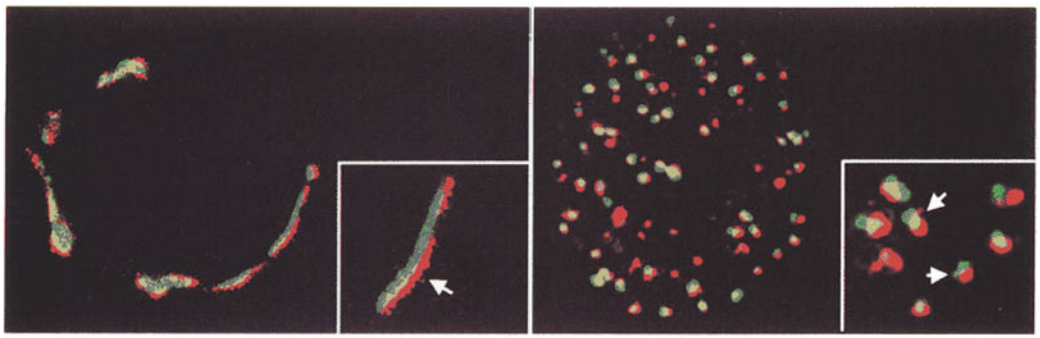


Plate 3 Despite the loss of cisternal morphology (see Fig. 3), resident proteins of the mitotic Golgi cluster maintained a polarized distribution similar to the Golgi stack. Confocal immunofluorescence images demonstrating a polarized distribution of the *cis*-Golgi marker GM130 (red) and a medial/*trans*-localized version of the green fluorescent protein (green), in stacks of the interphase Golgi ribbon (arrow in left panel) and in mitotic clusters (arrows in right panel). Image adapted from the *J. Cell Biol.*, 1997, **137**, 1211–28 by copyright permission of the Rockefeller University Press.

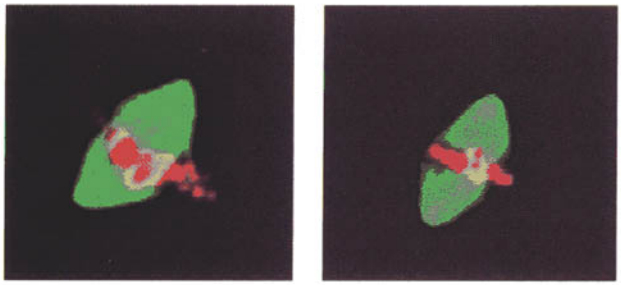


Plate 4 Formation of spindles around DNA-coated beads. Magnetic beads coated with plasmid DNA fragments are incubated in a *Xenopus* egg extract. Chromatin assembles around them and microtubules self-organize into a bipolar spindle. This spindle assembly process takes place in the absence of kinetochores and centrosomes.

and II divisions follow one another in rapid succession and the polar bodies remain in the same cytoplasm as the female pronucleus (144). The meiosis I spindle of *Drosophila* oocytes is anastral, but differs in shape from the anastral spindles shown in Fig. 4, appearing narrow and tapered, rather than barrel-shaped (Plate 2A). The meiosis II spindles of *Drosophila* oocytes are even more unusual in appearance, with the two spindles aligned in tandem with one another and a ring-shaped spindle pole body between the two spindles (145, 146) (Plate 2B). Several aspects of meiotic spindle assembly differ between oocytes of *Drosophila* and vertebrates, such as the mouse, and between meiosis I and II of *Drosophila*, reflecting the differences in anastral spindle structure.

In addition to differences in overall spindle structure, spindle fibers of oocytes of several species that have been examined show differences in length compared to those in mitotic cells. The initial stages of meiosis I spindle assembly in *Drosophila* oocytes (135, 147), and of both meiosis I and II spindle assembly in *Xenopus* oocytes (148), have been reported to involve short microtubules that assemble around the chromosomes, forming a compact mass. The mechanism of attachment of chromosomes to the spindle during meiosis in these organisms differs from that of mitotic cells, where spindle assembly invariably precedes microtubule capture by the kinetochore (149), chromosome attachment to a spindle fiber (150), and congression of chromosomes to the metaphase plate. The mechanism by which the narrow, tapered *Drosophila* meiosis I spindle or the more elongated *Xenopus* meiosis I and II spindles are formed from the initial microtubule:chromatin mass is not clear. The nature of the microtubules in the *Drosophila* and *Xenopus* oocyte meiotic spindles is also not certain—the microtubules appear to be long but have most likely assembled from the initially short microtubules. The polarity of these microtubules, that is, the orientation of the dynamic plus ends and more stable minus ends of the microtubules relative to the spindle poles, and the location of the microtubule ends with respect to the centromeres of the meiotic chromosomes, still need to be defined.

3.2 Assembly of a bipolar meiotic spindle

3.2.1 Chromosomes can organize a meiosis I spindle

Although the assembly of a bipolar meiotic spindle is often assumed to be similar or identical to mitotic spindle assembly, it is apparent from several observations that the two processes can differ considerably from one another. In several organisms, spindle assembly by chromosomes appears to be a specialized feature of meiosis I. This has been demonstrated in *Drosophila* primary spermatocytes by detaching individual chromosomes from the spindle by micromanipulation, monitoring the detached chromosome by video microscopy, then fixing the cells and examining them by serial-sectioning electron microscopy (151). The analysis showed that a small spindle forms around the detached chromosome, on which the half-bivalents separate in anaphase. These ectopic spindles therefore appear to be capable of functioning in chromosome segregation, although the anaphase separation could be

due to loss of sister chromatid cohesion rather than spindle function. The ability of meiosis I chromosomes to assemble a spindle has also been observed in spermatocytes of the crane fly, *Pales ferruginea*, following displacement of the centrosomes from the nucleus in late diakinesis (152). Spindles formed around isolated chromosomes have also been observed in *Drosophila* oocytes. Chromosomes that become detached from the meiosis I or II spindle in mutant oocytes of *Drosophila* due to loss of function of proteins essential for segregation, such as the Ncd or Nod kinesin-related microtubule motor proteins, are often associated with small spindles (135, 147). These spindles, too, are apparently functional, as they can be associated with segregating half-bivalents or sister chromatids (147). The ability of isolated chromosomes to organize a spindle during meiosis is consistent with the idea that the microtubule:chromatin complexes that are observed in the initial stages of meiosis I spindle assembly in *Drosophila* (135, 147) and *Xenopus* (148) oocytes constitute the first step in organization of the meiosis I spindle in these organisms.

The role of chromosomes in spindle assembly in mitotic cells is not so clear (see also discussion of the effect of chromatin/chromosomes on spindle assembly in Chapter 4). The evidence bearing on this is not extensive, but some of the results suggest that mitotic chromosomes are not capable of directing spindle assembly. In one study, the ability of the chromosomes to organize a spindle was tested in a cell entering mitosis (153). Sea urchin eggs were treated with colcemid prior to fertilization to block microtubule assembly and pronuclear conjugation, and were irradiated with ultraviolet light to photo-inactivate the colcemid after fertilization. A bipolar spindle associated with centrosomes formed around the paternal chromosomes, and the spindle then entered anaphase and underwent a normal division. The maternal chromosomes were not associated with a spindle, even though they were present in the same cytoplasm as the paternal spindle, and, in some cases, were positioned near the paternal spindle. These observations have led to the conclusion that chromosomes in mitotically dividing cells require centrosomes for assembly of spindles. Meiotic chromosomes of the sea urchin have not been demonstrated to be capable of spindle assembly, however, and may require specific motors or other proteins that are missing from sea urchin oocytes. Thus, the inability of the sea urchin maternal chromosomes to organize a spindle may reflect a difference in chromatin interactions with microtubules among species, rather than a difference between meiotic and mitotic chromosomes.

The ability of mitotic chromosomes to organize spindles has not yet been properly tested in *in vitro* spindle assembly systems. The spindle assembly extracts that have been developed to date are prepared from *Xenopus* oocytes, which are meiotic, rather than mitotic cells (154, 155). The extracts are made from mature unfertilized oocytes, which are arrested by cytostatic factor (CSF) in metaphase of meiosis II, and treated with calcium to inactivate the CSF and allow the extracts to complete meiosis and enter mitosis (155). The calcium-treated extracts are assumed to be mitotic, but probably more closely resemble meiotic cytoplasm in protein activity and composition. The findings that spindle assembly in these extracts does not require centrosomes or centromeric DNA sequences, and that chromatin is sufficient to direct

spindle assembly, are consistent with previous observations in *Drosophila* oocytes and spermatocytes that chromosomes are capable of organizing spindles. Spindles have been shown to assemble around chromatin or DNA-coated beads in the *in vitro* spindle assembly extracts (155), but have not yet been reported capable of segregating chromosomes. Further functional studies utilizing these *in vitro*-assembled spindles are needed to determine the role of the centromere in regulating spindle fiber:chromosome attachments and chromosome movement during the phases of mitosis. Despite the present limited results, the spindle assembly extracts are anticipated to be of enormous utility in dissecting the roles of various components of the meiotic/mitotic apparatus in spindle assembly.

3.2.2 Nucleation and organization of microtubules for anastral spindle assembly

In addition to apparent differences in the ability of meiotic and mitotic chromosomes to organize spindles, the mechanisms of spindle assembly and spindle pole formation are fundamentally different in meiotic and mitotic cells. As discussed in Section 3.1, meiotic spindles of oocytes of many species lack centrosomes, which are associated with almost all animal mitotic spindles and are thought to provide microtubule nucleating and organizing activity for spindle assembly, as discussed in Chapter 3. This raises the questions of how microtubules are nucleated and organized for spindle assembly and pole formation in anastral spindles. A complicating factor in thinking about anastral spindle assembly is that anastral spindles fall into at least three classes that differ from one another, based on overall structural considerations. These classes can be defined as 'type 1' anastral spindles that contain known centrosomal components, 'type 2' spindles that lack such components, and hybrid anastral:astral spindles. We discuss each of these classes of anastral spindles in turn.

Type 1 anastral spindles

Probably the best characterized example of a type 1 anastral spindle, one that contains known centrosomal components, is the mouse oocyte spindle. A careful study of the pole regions of the anastral mouse oocyte meiosis I and II spindles by electron microscopic analysis of thin-sectioned oocytes (156) showed no centrioles or centrosomes in the spindles. Instead, dispersed foci of electron-dense material were present at the poles of the meiotic spindles, associated with radiating microtubules. The microtubule foci were distributed across the distal ends of the spindle microtubules, forming a broad pole region (Fig. 5). The multiple microtubule foci at the poles have been interpreted as constituting dispersed microtubule organizing centers, in contrast to the single center present at each pole in mitotic spindles with centrosomes. In fact, a centrosomal protein, pericentrin, has been localized to dispersed patches at the broad pole regions of metaphase II-arrested mouse oocytes (Fig. 5) (157). Anti-pericentrin antibodies have also been shown to disrupt spindle organization in injected oocytes (157), providing additional support for the idea that the microtubule foci at the spindle poles represent dispersed microtubule organizing

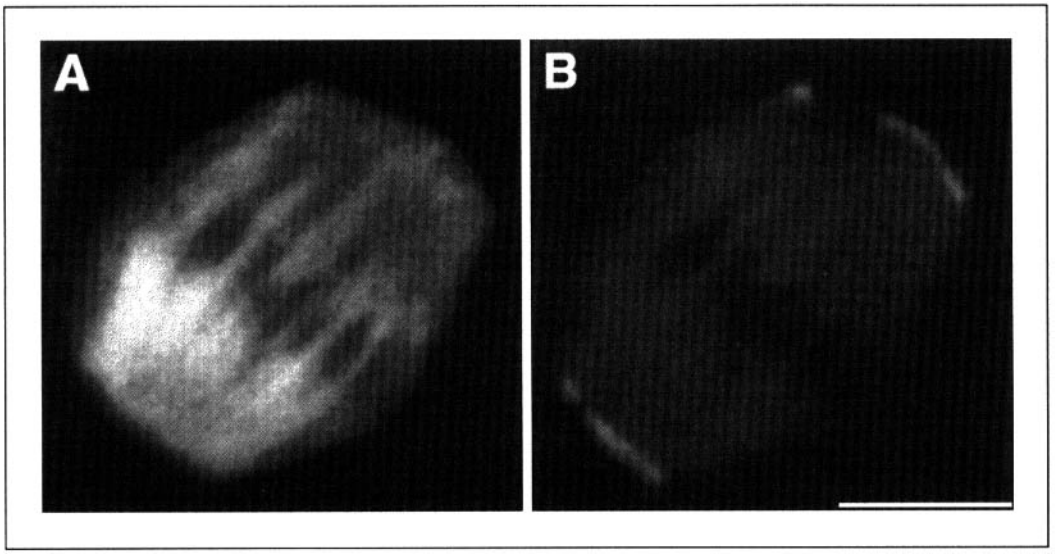


Fig. 5 Anastral meiotic spindle of a mouse oocyte showing tubulin and pericentrin localization. (A) Spindle stained with anti-tubulin antibodies. Note the broad poles of the spindle. (B) The same spindle stained with anti-pericentrin antibodies, showing dispersed localization to several sites at the broad spindle poles. Bar, 40 μm. Reproduced with permission from *Cell*, 1994, **76**, 639 (ref. 157). Copyright 1994 Cell Press.

centers. In addition to the mouse oocyte meiosis I and II spindles, dispersed microtubule foci instead of centrosomes are found associated with the first and second mitotic division spindles of the mouse embryo (158).

Nucleation of microtubules for assembly of type 1 anastral spindles with dispersed organizing centers is likely to occur in a manner similar to that of mitotic spindles with centrosomes. The microtubule nucleating activity associated with centrosomes of mitotic cells probably resides in γ-tubulin, associated with pericentrin. γ-Tubulin is required for assembly of the mitotic apparatus *in vivo* (159) and, in a complex with other proteins, can nucleate microtubules *in vitro* (160). If γ-tubulin is the centrosomal microtubule nucleating activity, it seems very likely that the dispersed foci of pericentrin and γ-tubulin (161) at the poles of type 1 anastral spindles function to nucleate microtubules for spindle assembly, similar to centrosomes of mitotic spindles. The dispersed centers are present *within* the microtubule array that eventually forms the spindle, however, rather than outside it.

Following microtubule growth, spindle assembly and pole formation of type 1 anastral spindles probably occur by a mechanism similar to that of mitotic spindles with centrosomes, except that the forces required for spindle formation are generated from within the spindle, rather than from outside the spindle. These forces are likely to be produced by microtubule motors associated with the spindle microtubules or localized to the dispersed centers. This is almost certainly the case for mitotic spindles, except that the motors are associated with spindle fibers and centrosomes.

Type 2 anastral spindles

In contrast to type 1 anastral spindles, some anastral spindles ('type 2') are thought to lack known centrosomal proteins, such as pericentrin and γ-tubulin. An example of a type 2 spindle is the meiosis I spindle of *Drosophila* oocytes, which is narrow and tapered (Plate 2A), rather than broad at the poles like those of the mouse. This difference in shape implies that the mechanism of pole formation, or the distribution of microtubule organizing centers in the *Drosophila* meiosis I spindle differs from that of the mouse. Centrosomal proteins, including CP190 (DMAP190), CP60 (DMAP60), and γ-tubulin, have been looked for in *Drosophila* oocyte meiosis I spindles but not found (135). How are microtubules nucleated for assembly of these anastral spindles?

One possibility is that microtubule assembly occurs spontaneously, promoted by high local tubulin subunit concentrations. Spontaneous assembly of microtubules would account for the formation of the many short microtubules observed aggregated with the chromosomes of oocytes of some organisms, but would not account for the assembly of these microtubules only near the meiotic chromosomes. An alternative possibility is that binding of tubulin subunits to a substrate such as chromatin might create nucleation centers for microtubule assembly. Microtubules bind non-specifically to negatively charged molecules, such as chromatin, and this is also likely to be true of the dimeric α/β-tubulin subunits. The observation of microtubule:chromatin aggregates in the initial stages of meiotic spindle assembly could be a consequence of charge–charge interactions followed by assembly of microtubules from the tubulin subunits bound to the chromosomes. This hypothesis would account for the many short microtubules associated with bivalent chromosomes of oocytes of some organisms by permitting nucleation to occur at many points over the surface of the chromosomes.

The mechanism by which the initially short microtubules associated with the meiotic chromosomes undergo transition into the anastral meiosis I spindle of *Drosophila* and other oocytes is not known. Microtubule-associated proteins, including motors, may play a role in this process by binding to and stabilizing the short microtubules, permitting their elongation and growth into the apparently long microtubules that comprise the meiotic spindle.

After microtubules have been nucleated, assembly of type 2 anastral spindles is thought to be mediated by minus-end-directed microtubule motors. Current models are based on the ability of the minus-end motor, cytoplasmic dynein, to 'focus' microtubules into polar arrays in *Xenopus* egg extracts (162) and the abnormal spindles observed in *Drosophila* oocytes mutant for the minus-end Ncd motor protein. The *ncd* mutant oocytes show spindle pole defects that include diffuse or multiple poles (Fig. 6) (147, 163, 164), consistent with the idea that the Ncd motor (165, 166) is required for pole formation. Recent observations of spindle assembly in live wild-type and *ncd* mutant oocytes have led to the conclusion that Ncd is required to stabilize spindle poles in metaphase I-arrested oocytes (167), as well as for initial formation of the bipolar meiotic spindles. The minus-end motor, cytoplasmic dynein,

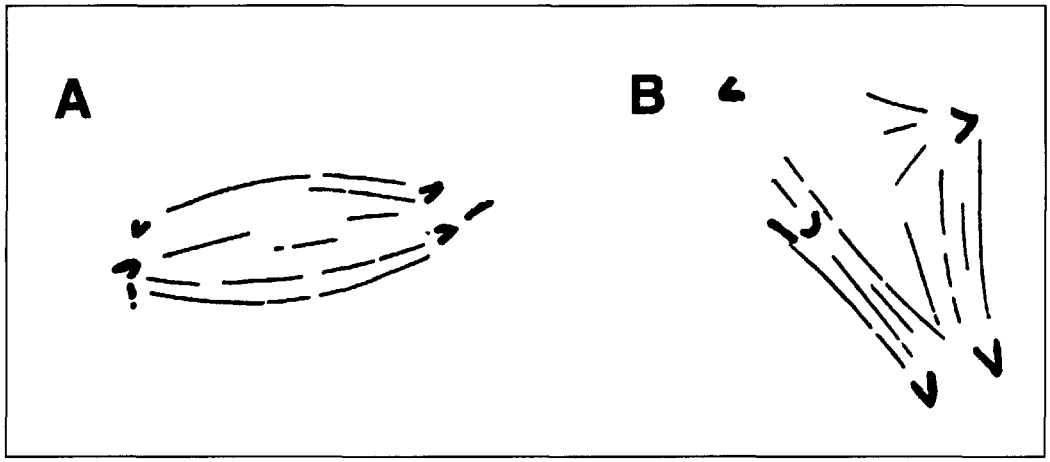


Fig. 6 Wild-type spindle and multiple, abnormal spindles in an oocyte of the *D. simulans* claret (*ca*) mutant. The drawings depict (A) a wild-type spindle in a control oocyte, late anaphase of meiosis I, and (B) *ca* mutant oocyte at late anaphase of meiosis I, showing chromosomes segregating on an abnormal, multipolar spindle that has separated into two components. The *D. simulans ca* mutant (207) corresponds to the *claret non-disjunctional* mutant of *D. melanogaster*, which is a double mutant of *ca* and *ncd* (208, 209). Reproduced from ref. 163.

could also contribute to meiosis I spindle assembly in *Drosophila* oocytes, although evidence for such a role has yet to be reported (168, 169).

One model for spindle assembly in type 2 anastral spindles (170) proposes that movement of a minus-end-directed motor along microtubules of a cross-linked mass is required to focus the microtubule ends into a polar array and keep the minus ends together. Spindle pole formation and maintenance in *Drosophila* oocytes requires both the motor activity and crosslinking activity of Ncd, based on studies of a partial loss-of-function Ncd motor (171). The mutant Ncd motor, which contains a point mutation in the putative microtubule-binding region of the motor domain, showed reduced velocity of microtubule gliding and reduced binding to microtubules *in vitro*. The mutant motor was associated with meiotic spindles of mutant oocytes *in vivo*, but the spindles were abnormal with structurally undefined or multiple poles. These observations provide evidence that the minus-end motility of Ncd, which is expected to drive movement of microtubules and attached chromosomes towards the spindle poles, is critical for wild-type function. The ability of the Ncd motor to cross-link and bundle microtubules is a function of the non-motor 'tail' region of the protein (172), and is thought to contribute to the ability of Ncd to maintain spindle poles and spindle structure during the meiotic divisions. Recent studies have demonstrated a genetic interaction between Ncd and the centromeric DNA of *Drosophila* (79), suggesting that Ncd could mediate spindle assembly by first interacting with the centromeres of the meiotic chromosomes and microtubules, then bundling microtubules associated with the bivalent chromosomes.

Spindle assembly during the meiotic, or spore-forming, divisions of higher plants involves the formation of multipolar spindles as an intermediate step in the assembly

of bipolar anastral spindles (14). The similarity of the plant multipolar and bipolar spindles to those observed in *Drosophila ncd* loss-of-function and wild-type oocytes, respectively, suggests that Ncd-like motors function in meiosis in higher plants. Kinesin-related proteins that resemble Ncd in overall structure have been identified in higher plants (173–176) and one of these has recently been demonstrated to be a minus-end microtubule motor (177). However, evidence for the involvement of these kinesin-related proteins in meiotic spindle assembly in higher plants has not yet been reported.

Hybrid anastral: astral spindles

The third class of anastral spindles comprises spindles that exhibit characteristics both of anastral and astral spindles. Only one member of this class is known so far, the *Drosophila* oocyte meiosis II spindle. The meiosis II spindle of *Drosophila* is unusual in structure in that the two spindles that comprise it are tandemly aligned with one another (Plate 2B), as noted in Section 3.1 above. Recent findings indicate that the meiosis II spindle of *Drosophila* oocytes probably undergoes assembly by a different mechanism than the meiosis I spindle. A ring- or disk-shaped structure, which consists of foci of tubulin with a radiating array of microtubules, is present in the region between the two tandem spindles (145, 146) (Plate 2B, arrow). This structure, referred to here as the central spindle pole body, has been suggested to function in pole organization for meiosis II (145). The presence of a centrosome-specific protein, CP190, in the central spindle pole body (146) provides evidence that known centrosomal components are involved in meiosis II spindle assembly, further supporting the idea that the meiosis II division resembles a mitotic division. Its presence in both unfertilized and fertilized eggs indicates that the CP190 protein is not contributed by the incoming sperm.

The hybrid nature of the meiosis II spindle of *Drosophila* oocytes is suggested by different properties of the central and distal poles. The CP190 protein is associated with the central spindle pole body, but is not present at the distal poles. How are the distal poles formed? The two tandem meiosis II spindles of *Drosophila* oocytes assemble by reorganization of spindle fibers of the meiosis I spindle without disassembly of the meiosis I spindle (178). Thus, the distal spindle poles are likely to be maintained by the forces originally involved in their formation during meiosis I, that is, through the activity of the Ncd microtubule motor and possibly other bundling or focusing activities such as cytoplasmic dynein. Ncd localizes to microtubules of meiosis II as well as meiosis I spindles (179), and *ncd* mutant oocytes display spindle abnormalities in meiosis II and I (147, 164), providing further support for the idea that Ncd is required for pole formation both in meiosis I and II.

The *Drosophila* meiosis II spindle thus forms a new class of hybrid anastral:astral spindles in which the central spindle poles, which form *de novo* for the meiosis II division (178), appear to be organized by an unusual ring- or disk-shaped centrosomal protein-associated structure, the central spindle pole body. The distal spindle poles, apparently retained from the meiosis I division, fall into the second class of anastral spindles. The ring- or disk-shaped central spindle pole body associated

with the central poles of the *Drosophila* meiosis II spindles could be an unusual variant structure of the γ-tubulin + pericentrin centers, with γ-tubulin providing the nucleating activity. It remains to be demonstrated, however, that the central spindle pole body functions in microtubule nucleation and pole organization, and that γ-tubulin is associated with the central and not the distal poles.

3.3 Meiotic spindle dynamics

Following assembly and before undergoing the meiotic divisions, oocyte spindles of some organisms undergo a dramatic change in position, completely reorienting with respect to the axis of the oocyte (Fig. 7). It has been suggested that reorientation of the meiotic spindle is associated with asymmetric or determinative cell divisions (148). The meiotic divisions of most oocytes are asymmetric resulting in two or three polar bodies located at, or near, the cortex of the oocyte and an oocyte nucleus that becomes the female pronucleus. Reorientation of the meiotic spindle perpendicular to the oocyte surface positions the polar nuclei closer to the cortex and the oocyte nucleus more internally. This type of reorientation is likely to be a feature common to oocytes of species that form polar bodies during maturation.

Meiotic spindle dynamics were studied in *Xenopus* using live oocytes injected with fluorescein-conjugated tubulin to label spindle microtubules (148). Time-lapse laser scanning confocal microscope analysis showed that meiosis I and II spindles were positioned parallel to the oocyte surface upon assembly, but reoriented perpendicular to the surface in prometaphase, in alignment with the animal–vegetal axis (Fig. 7). Reorientation of both meiosis I and II spindles was found to be blocked in oocytes treated during maturation with cytochalasin B, an inhibitor of actin assembly (180), indicating that meiotic spindle rotation in *Xenopus* is dependent on cortical actin filaments. In *Drosophila* oocytes, mature meiosis I spindles are positioned parallel to the oocyte cortex. Studies of live oocytes with spindles labeled *in vivo* by expression of the Ncd motor fused to GFP showed that the metaphase-arrested spindles are in a dynamic state, undergoing slight changes in position and small rotational and longitudinal movements (178). Activation of oocytes results in a dramatic expansion and elongation of the meiosis I spindle, accompanied by rapid rotational movements of the spindle around its long axis. Reorientation of the meiosis I spindle into a vertical position relative to the cortex occurs soon after oocyte activation by an acute pivoting movement, accompanied by rapid rotations of the spindle around its long axis (178) (Fig. 7). A similar reorientation of the meiotic spindle has been observed in oocytes of the leech, *Theromyzon rude* (181).

Spindles in mitotically dividing cells can also divide in an orientation different from that of other cells in the same tissue. These mitotic spindle 'reorientations' are associated with asymmetric cell divisions, and may arise by several different mechanisms. Orientation of mitotic spindles perpendicular rather than parallel to the cell surface in *Drosophila* neuroblasts and epithelial cells of the developing central nervous system requires the Inscuteable protein and is dependent on the actin cytoskeleton (182). Expression of Inscuteable in non-neuronal epithelial cells, in

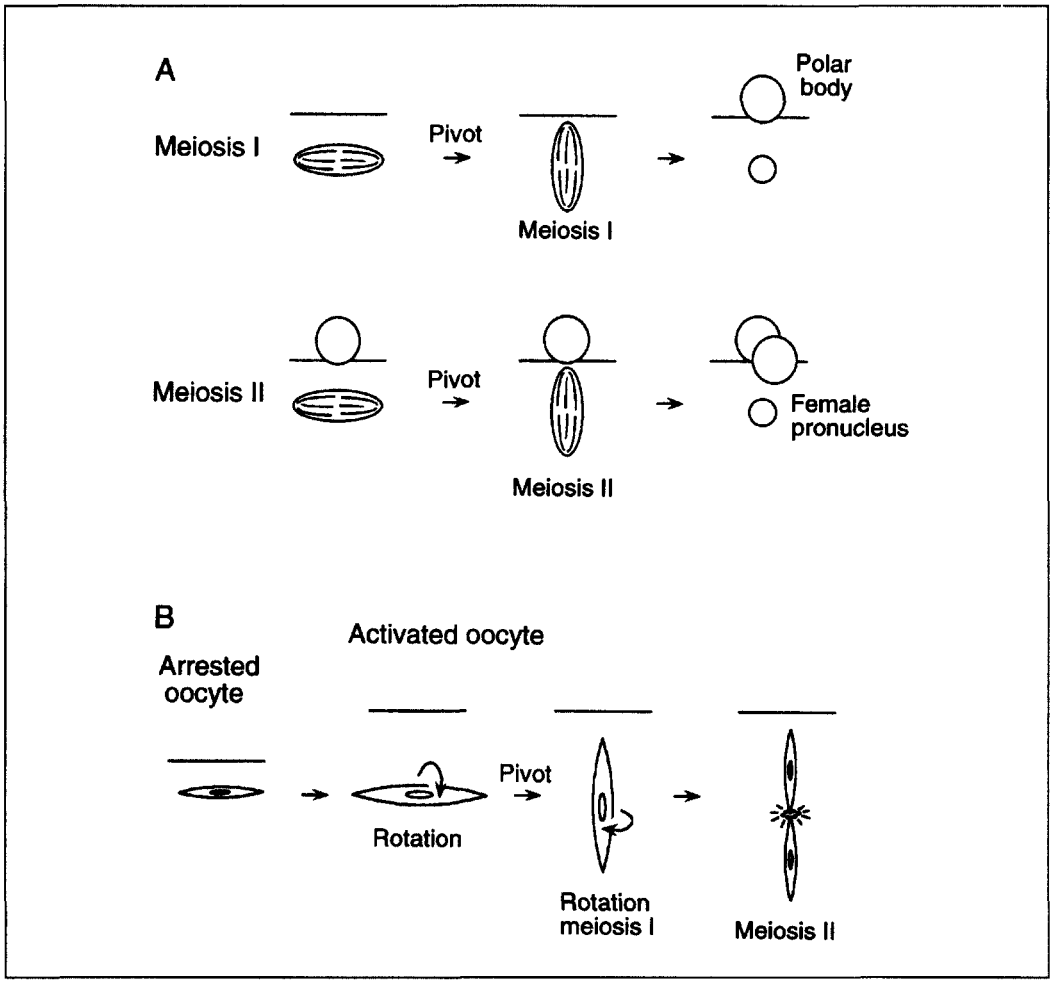


Fig. 7 Spindle reorientation during meiosis in *Xenopus* and *Drosophila* oocytes. (A) Meiotic spindle reorientation in *Xenopus* oocytes. The meiosis I and II spindles are initially parallel to the oocyte cortex (depicted as a line), and during prometaphase rotate into a position perpendicular to the cortex, in alignment with the animal-vegetal axis. Completion of each meiotic division occurs with the spindle perpendicular to the cortex. The nuclei that do not contribute further to the female pronucleus are extruded as polar bodies. See ref. 148 for further details. (B) Meiotic spindle reorientation in *Drosophila* oocytes. The meiosis I spindle is parallel to the oocyte surface upon assembly and in metaphase-arrested oocytes. After activation, the spindle expands, extends in length and undergoes rapid rotations around its long axis. An acute pivoting movement reorients the spindle perpendicular to the cortex, followed by completion of the meiosis I and meiosis II divisions. Adapted from *The Journal of Cell Biology*, 1997, **137**, 1321–36, (ref. 178) with permission of The Rockefeller University Press.

which the spindles are normally oriented parallel to the cell surface, causes the cells to divide abnormally with the spindles perpendicular to the cell surface, indicating that the Inscuteable protein causes the perpendicular orientation of the spindle. The asymmetric nature of the *Drosophila* neuroblast and neuronal epithelial cell divisions and their dependence on the actin cytoskeleton provide parallels to meiotic

spindle reorientation in *Xenopus* and *Drosophila* oocytes. It is currently unknown if Inscuteable or similar proteins function during spindle reorientation in meiosis. Cytoplasmic dynein mutants have been shown to affect spindle orientation in the asymmetric mitotic divisions of the *Drosophila* oocyte (169), implying that the minus-end dynein motor orients the spindle at an early stage of oogenesis.

The meiosis I and II divisions follow any changes in orientation that may occur in the spindle. Meiotic spindles undergo dramatic changes in length and shape during progression through the meiosis I and II divisions. These changes can parallel those observed in mitotic spindles, although the basis of meiotic spindle dynamics has not been carefully examined and the underlying molecular motors and other forces could differ from those of mitotic spindles. Time-lapse microscopy of live wild-type and mutant *Drosophila* oocytes expressing Ncd as a fusion protein with GFP reveals that the Ncd microtubule motor also functions during completion of the meiotic divisions (178). The Ncd motor helps maintain bipolar meiotic spindles, facilitates assembly of the meiosis II spindles, and probably also stabilizes microtubules during completion of the meiotic divisions. The rate of spindle elongation has been determined in *Xenopus* meiotic spindles using time-lapse confocal microscopy (148). The rates of 0.7 μm/min and 1.8 μm/min for meiosis I and II, respectively, are comparable to the average rate of anaphase B spindle elongation of 1.7 μm/min in mitotically dividing cells of the diatom *Stephanopyxis turris* (183). The velocity profiles for elongation of the meiosis I and II spindles did not show clear biphasic modes, indicating that anaphase A- and B-like phases are probably absent during the meiotic divisions of *Xenopus* oocytes.

3.4 Cell cycle regulation of the meiotic divisions

In contrast to mitotic divisions and meiotic divisions in spermatocytes, progression through the meiotic divisions in oocytes is usually interrupted by extended periods of time. The time of arrest with respect to the meiotic cell cycle differs among species. For example, *Rana pipiens* and *Xenopus* oocytes, arrested in prophase I, can be induced by progesterone to re-enter meiosis and undergo the meiosis I division, but are then arrested in metaphase of meiosis II prior to release by fertilization. In contrast, oocytes of *Drosophila* are arrested in metaphase of meiosis I and are released by ovulation to complete the meiotic divisions and by fertilization to enter mitosis. Meiotic development in mammals is arrested at the dictyotene stage of meiotic prophase, which lasts 12 years or longer in humans.

The naturally occurring arrests in the meiotic cell cycle were used more than 25 years ago to demonstrate the existence of a cytoplasmic factor present in mature metaphase II-arrested *R. pipiens* oocytes that, when injected into immature oocytes, could induce maturation and onset of the meiotic divisions (184). The factor, named maturation promoting factor (MPF), has subsequently been recognized as a universal regulator of all meiotic and mitotic cell cycles.

The ability to induce re-entry into the meiotic cell cycle in oocytes was also the basis of functional experiments that identified cyclin as a key cell cycle regulator that

could induce injected *Xenopus* oocytes to undergo maturation and progression through the meiotic cell cycle (185), like MPF. Although cyclin A was used in these initial experiments, it has since been established that cyclin B is the component of MPF that is essential for regulation of meiosis (186) and mitosis (187, 188). Cyclin B associates with a protein kinase, $p34^{cdc2/28}$ to form MPF, which is activated by dephosphorylation of its $p34^{cdc2/28}$ subunit by a phosphatase, cdc25 (reviewed in ref. 189).

The differences in regulation of meiosis and mitosis predict the existence of cell cycle regulatory proteins that are specific to meiosis—such proteins are now beginning to be identified. One of these, a cdc25 homolog that acts in *Drosophila* meiosis and mitosis (190), Twine, was identified by its ability to complement an *S. pombe cdc25* mutant (191, 192) and independently by polymerase chain reaction (PCR) amplification using degenerate primers to conserved regions of the cdc25 phosphatases (193). A loss-of-function *twine* mutant blocks the meiotic divisions in male *Drosophila* and causes severe meiotic defects in females (193, 194). The defect in females has been interpreted as a failure of mature oocytes to arrest in metaphase I (193, 194), followed by continued meiotic divisions (194).

The mutant effect in spermatocytes provides evidence that Twine activity is required for the onset of the meiotic divisions, consistent with a mechanism of function involving activation of an MPF by dephosphorylation, as has been demonstrated for other cdc25 homologs. Twine could act in oocytes to stabilize MPF and maintain metaphase I arrest (27, 193) by an as yet unknown mechanism. A new system for *in vitro* activation of *Drosophila* oocytes has been used recently to demonstrate that new protein synthesis does not contribute to maintaining metaphase arrest (195); thus Twine is more likely to act via dephosphorylation of existing proteins. If so, identification of Twine target proteins would help to establish the biochemical basis of oocyte arrest in *Drosophila* (see Section 2.1 for a discussion of the physical basis for arrest). *twine* and *ncd* loss-of-function mutants display similar disruptions of oocyte meiotic spindles (194), implying that Ncd or proteins that regulate Ncd could be direct or indirect targets of Twine activity.

Oocyte arrest in metaphase II in *R. pipiens* and other amphibia is dependent on a protein named cytostatic factor (CSF), discovered at the same time as MPF using cytoplasmic transfer experiments (184). Cytoplasm transferred from a metaphase II-arrested oocyte into one cell of a two-cell embryo caused the injected cell to arrest in metaphase, but did not affect division of the uninjected cell. CSF is thought to act by inhibiting cyclin proteolysis, causing arrest of the cell cycle (188). Calcium, which is known to activate oocytes of many species, is believed to inactivate CSF, releasing the oocytes into the meiotic cell cycle. A component of CSF is now thought to be the product of the c-*mos* proto-oncogene, which had earlier been demonstrated to be capable of inducing activation of MPF and re-entry of oocytes into the meiotic cell cycle (196). Mos functions in mouse oocytes (197), as well as those of *R. pipiens* and *Xenopus*, to arrest the cell cycle in metaphase II of meiosis (198, 199) through activation of MAP kinase (199).

Accumulating evidence indicates that Mos plays a role in meiotic spindle

assembly. In fixed cells, Mos has been detected associated with the spindle and a Mos 'knockout' mouse shows altered oocyte spindle shape and mispositioning of the spindle (200). The spindles in *mos*-transformed cells that overproduce Mos appear more meiotic-like than mitotic, with no asters and the spindle poles positioned near the cell membrane (201). Finally, Mos has recently been found associated with condensed metaphase I and II chromosomes in live *Xenopus* oocytes (K. Swenson, personal communication). This localization provides an intriguing clue that Mos could be a crucial link between the meiotic chromosomes and their ability to nucleate microtubules: the Mos kinase activity could cause local changes around the chromosomes in phosphorylation of a microtubule-destabilizing/stabilizing protein which could in turn cause microtubule polymerization around the chromosomes, as described in Chapter 4. This would explain the ability of meiotic chromosomes to nucleate and bind microtubules, while providing a key to the regulation of meiotic spindle assembly in oocytes.

Genes have also been identified in *Drosophila* that function during spermatogenesis and, when mutant, cause arrest prior to the first meiotic division (202, 203). The products of these genes [*always early* (*aly*), *cannonball* (*can*), *meiosis I arrest* (*mia*), *spermatocyte arrest* (*sa*), and *pelota* (*pelo*)] have been interpreted to be required for progression through the G_2/M phase of meiosis I. Although these five genes have a similar mutant phenotype of male sterility caused by arrest prior to the meiotic divisions, the genes are thought to represent different control points in the cell cycle (Fig. 8). The first four genes, *aly*, *can*, *mia*, and *sa*, act before the degradation of cyclin A, defining a control point in spermatogenesis that is required both for meiotic division and spermatid differentiation (202). Spermatid differentiation in *pelo* mutants, on the other hand, continues despite the absence of the meiotic divisions, resulting in 4*N* spermatids in 16-cell cysts instead of 64-cell cysts (203). The effects of *pelo* mutants in permitting spermatid differentiation but blocking the meiotic divisions have led to the conclusion that Pelo is required specifically for meiotic divisions. The cytological effects on spermatogenesis of *pelo* mutants are identical to those of *twine* mutants (194, 203), but Pelo is distinct from, and acts downstream from Twine (Fig. 8), which is also required for onset of the meiotic divisions.

The regulation of the meiosis II cell cycle in oogenesis and spermatogenesis is not well understood, although genes have been identified that affect both processes. One of these genes, *roughex* (*rux*), appears to act through cyclin A in premeiotic G_2 to regulate meiosis II during spermatogenesis in *Drosophila* (204). Loss of *rux* function causes entry into an additional division following meiosis II, whereas increased *rux* gene dosage results in failure to undergo meiosis II. The effect of loss of *rux* function can be rescued by lowered *cyclin A* or *twine* gene dosage. These dosage-dependent effects have led to the idea that Rux acts on cyclin A, and the cyclin A–p34$^{cdc2/28}$ kinase then activates a target protein that persists through meiosis and is required for meiosis II (204). Two *Drosophila* genes, *grauzone* (*grau*) and *cortex* (*cort*) that cause oocyte arrest in meiosis II and prevent exit from meiosis have also been identified (205). Further study of these genes in *Drosophila* and the effects of their homologs in other species should provide a more complete picture of the regulation of meiosis II.

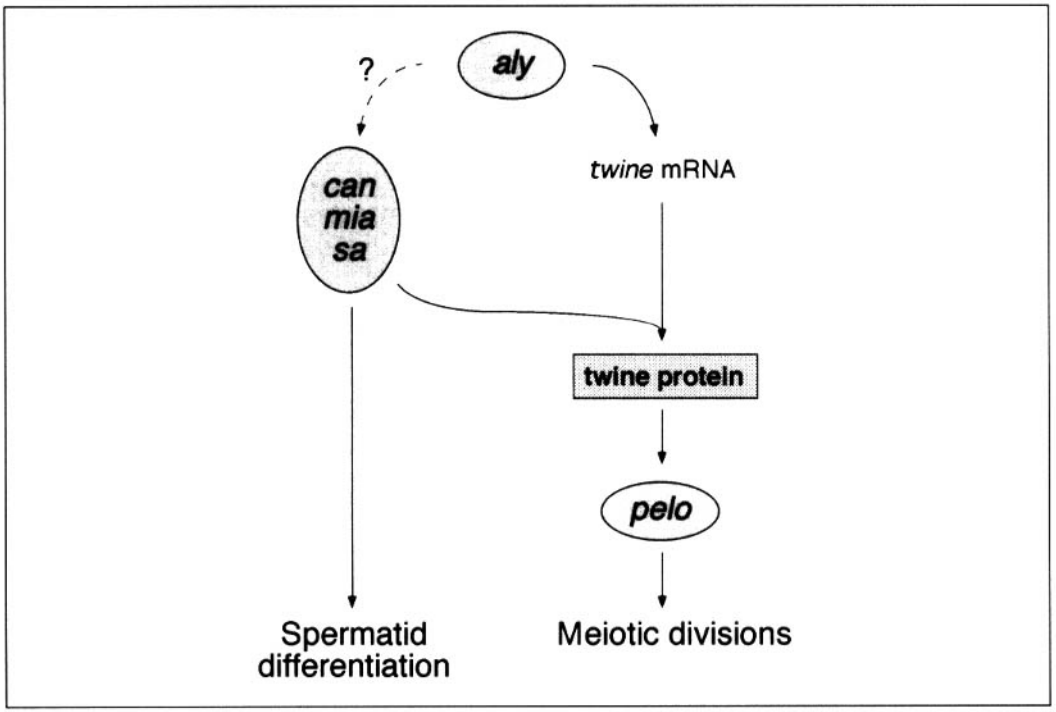


Fig. 8 Proposed pathways for spermatid differentiation and meiosis during spermatogenesis in *Drosophila*. The genes *aly*, *can*, *mia*, and *sa* are required both for spermatid differentiation and the meiotic divisions, and act upstream of *twine* and *pelo*. *twine* and *pelo* are both required for onset of the meiotic divisions, but *pelo* acts downstream of *twine*. Provided by H. White-Cooper and M. Fuller and reproduced from ref. 210 with permission of the Company of Biologists, Ltd.

As is evident from the above discussion, major advances in understanding cell cycle regulation have relied on several of the unusual characteristics of the meiotic cell cycle, which have contributed to the understanding of cell cycle regulation not only in meiotic cells, but also in mitotic cells. Further studies of the regulation of meiosis in organisms other than amphibia and *Drosophila* may well reveal variations in mechanisms that will help in understanding the regulation of differentiated or specialized mitotic cells, or those undergoing aberrant mitoses.

4. Conclusions and future prospects

Significant progress has been made in identifying some of the *cis*-acting DNA elements and *trans*-acting proteins that regulate spindle assembly/dynamics and chromosome pairing/segregation in meiosis. The idea that meiosis is a specialized mitotic division that can contribute to the understanding of cell division in general is becoming more widely accepted, based on the advances in understanding the regulation of all cells that have in many cases come from the study of meiotic cells. Further study of meiosis is likely to contribute to knowledge of other basic aspects of

cell biology, including chromosome–microtubule interactions and dynamics, spindle function, and the impact of chromosome–chromosome interactions during the cell cycle on gene expression. However, we lack a detailed understanding of meiotic chromosome behavior and spindle dynamics, and are especially ignorant about how the exquisite chromosome and spindle behaviors described here are coordinately regulated and integrated. Many questions remain unanswered. For example, how conserved is the role of centric and telomeric heterochromatin in meiotic pairing? Is heterochromatin pairing also a prerequisite for normal recombination pairing in the euchromatin? How is centromere activity regulated so that homologs segregate in meiosis I and sister chromatids in meiosis II, and how are extracentromeric anti-poleward forces integrated with kinetochore-based movements? What are the forces that drive chromosome movement and spindle elongation in meiotic cells? How are these forces regulated to permit oocyte arrest, then reactivation?

Identifying the proteins involved in chromosome pairing, centromere function, and spindle function is critical to future investigations of the meiotic process, and to determine whether the mechanisms and components are conserved among eukaryotes. Significant progress towards answering these questions in the near future will come predominantly from continued studies of the *Drosophila* and yeast model systems. However, insights gained from studies of meiosis in model systems can be applied to mammals, thanks to recent technological advances that allow meiosis to be studied directly in the mouse (206). Investigating the basic biology of chromosome pairing and segregation promises to help us better understand the frequent meiotic chromosome disjunction failures in human females, and ultimately produce useful diagnostic tools and treatments.

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8 Inheritance of the cytoplasm during cell division

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1. Introduction

Within the past decade, a multidisciplinary approach has led to the identification of a large number of biochemical networks involved in the duplication and segregation of nuclear DNA into dividing cells. While the accurate dissemination of genetic information is obviously crucial for an organism's success, cellular mechanisms must also exist to ensure the appropriate inheritance of the cytoplasm, including:

- proteins, such as metabolic enzymes, kinases, phosphatases and transcription factors
- cytoskeletal components and multisubunit protein particles such as proteasomes and nuclear pore complexes
- RNA–protein particles such as mRNA splicing, transport and translation machinery
- membrane-bound organelles such as the endoplasmic reticulum, Golgi apparatus, lysosomes, mitochondria, chloroplasts and peroxisomes

2. Defining inheritance

The inheritance process for nuclear DNA consists of a biogenesis phase (replication) and a partitioning phase (mitosis/meiosis). The inheritance of the cytoplasm also has biogenesis and partitioning phases, in which cytoplasmic components must double in unit mass, then be appropriately divided between nascent daughter cells during cytokinesis, respectively. The need to inherit cytoplasmic constituents such as mitochondria and chloroplasts is obvious since their self-contained DNA is absolutely required for proper organelle and cellular function and therefore must be transmitted to progeny through each cell division (1). Similarly, the endoplasmic reticulum (ER), the major site of synthesis for membrane lipids and proteins, must be derived from pre-existing membranes since there is no apparent mechanism to assembly these cellular membranes *de novo* (2).

The need to inherit other cytoplasmic constituents, such as the proteasome or membrane-bound components derived from the ER (Golgi, lysosomes/vacuoles,

secretory granules) is less obvious. Experimental manipulation resulting in bulk depletion of cytoplasm suggests that cells can recover from inappropriate allotment of cytoplasmic components, but the process is lengthy and would hamper a cell's ability to thrive in its microenvironment since it must divert cellular resources to the task of replenishing critical cytoplasmic components (3, 4). Thus, an organism with a malfunctioning cytoplasmic inheritance process is likely to be at a disadvantage in a competitive population.

Despite its broad-range significance, the field of cytoplasmic inheritance is still in its infancy. One explanation for this is that few unifying concepts or themes exist to relate the mechanisms of inheritance for different cytoplasmic components or among different organisms (5). The reasons for this become obvious when one examines the diverse range of cellular components to be inherited during each cell cycle. For example, biogenesis and partitioning of the single copy, juxtanuclear Golgi apparatus poses a very different problem for the cell than numerous, widely distributed, free ribosomes, or peroxisomes. Moreover, inheritance strategies will probably be different for cells that divide by medial fission (e.g., a typical animal cell or *Schizosaccharomyces pombe*) than for cells that divide through a budding process (e.g., *Saccharomyces cerevisiae*) (6, 7).

The complex nature of the inheritance problem is perhaps best highlighted by a consideration of the secretory pathway, an interconnected, membranous network which extends from the nuclear envelope, through the ER, Golgi apparatus, endosomal/lysosomal system (or vacuolar system in yeast), to the plasma membrane (8). At the onset of mitosis in mammalian cells, the morphological appearance and function of some of these membrane-bound organelles can be drastically altered, supposedly to facilitate the partitioning process. For example, during prophase the nuclear envelope may be disassembled into a collection of vesicles, completely removing the boundaries between the nuclear material and the cytoplasm (9–11) (see Chapter 2 for a discussion of possible mechanisms of nuclear envelope breakdown). However, the membranes of the lysosomes, endosomes and plasma membrane appear to remain intact throughout mitosis, and the membranes of the ER and Golgi apparatus lie somewhere in between, undergoing a variable degree of vesiculation and tubulation that appears to be roughly coordinated in time with nuclear envelope breakdown (9, 10, 12, 13). It is precisely this degree of variability seen in the behaviour of compartments within the secretory pathway which underscores the current difficulty in developing a general framework.

The purpose of this chapter is to discuss recent experimental findings which provide a foundation for our understanding of the cellular mechanisms involved in cytoplasmic inheritance. Rather than catalogue the modes of inheritance used for a variety of cytoplasmic components, we will focus discussion on the Golgi apparatus. The creation and propagation of the intricate Golgi architecture poses a fascinating problem for cell biologists and, for this reason, the Golgi apparatus has become one of the most extensively studied organelles in the area of cytoplasmic inheritance. Where appropriate, we will discuss findings for other cytoplasmic components which aid in reinforcing, reinterpreting or expanding our Golgi-based view of inheritance.

3. Biogenesis

During each cell cycle, the cell doubles in size and divides. Likewise, Golgi membranes must keep pace with the cell cycle by doubling in mass, in preparation for partitioning of Golgi membranes at cytokinesis. The interphase Golgi apparatus in a typical mammalian cell occupies a juxtanuclear, usually pericentriolar, location (14). Each unit comprises a stack of disc-shaped membranes, termed cisternae, bounded on each face by extensive tubular–reticular networks termed the *cis*-Golgi network and the *trans*-Golgi network (15). Tubules join equivalent cisternae in adjacent stacks, forming a ribbon which bifurcates and intersects, yielding a compact, interconnected reticulum (Fig. 1) (16, 17).

The growth of the Golgi apparatus in proliferating cells relies upon both the input and selective retention of material derived from the ER, and the association of Golgi membranes with various peripheral proteins provided by the cytoplasm (18). The ER-derived components arrive at the entry face of the Golgi, and progress sequentially through the cisternal compartments in the form of transport vesicles (19, 20). Whereas cargo en route to the plasma membrane or lysosomal compartments

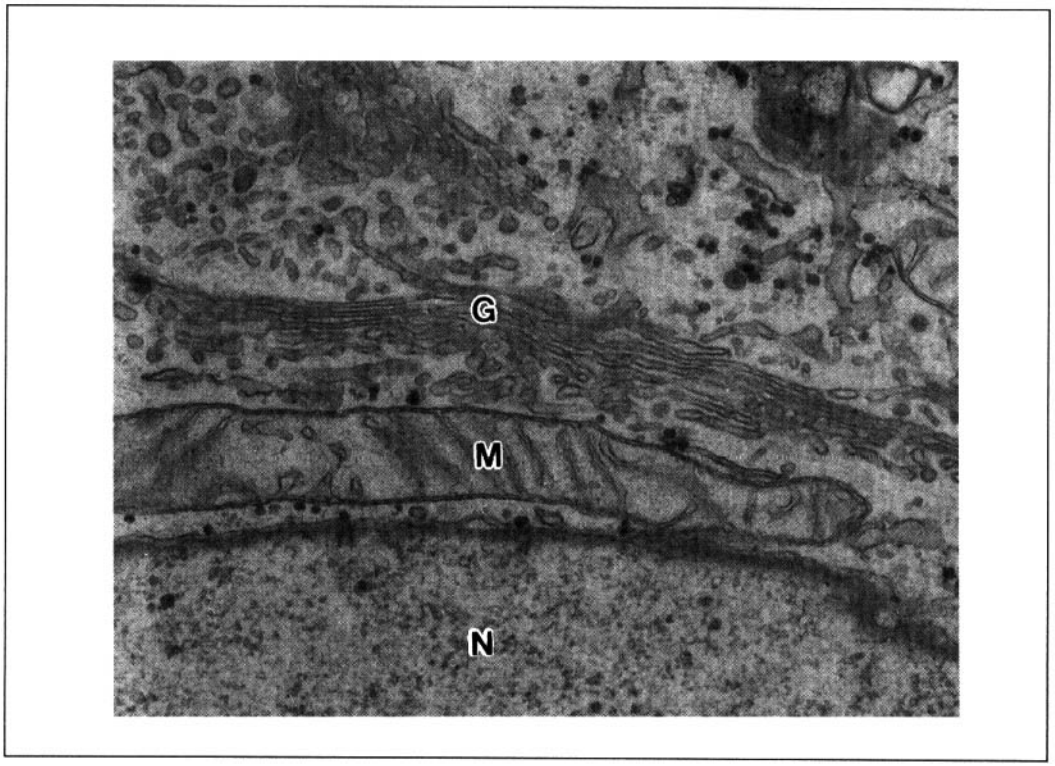

Fig. 1 An ultrastructural view of the interphase Golgi apparatus from a cultured normal rat kidney cell. The typical Golgi ribbon consists of a juxtanuclear collection of interconnected Golgi stacks and extensive tubular reticular networks; G, Golgi apparatus; M, mitochondria; N, nucleus. (Micrograph kindly provided by Eija Jämsä.)

rapidly transits through the Golgi, resident components, such as glycosyltransferases, are maintained within the appropriate Golgi compartment by a combined process of protein retention and retrieval (21–23). Proteins involved in ER-to-Golgi traffic and those ER residents which have escaped from their proper location are also recycled back to their appropriate location by a retrograde transport pathway (24, 25). The net outcome of this complex bidirectional intracellular traffic is a Golgi stack exhibiting both morphological and biochemical polarity (19, 26, 27). Whereas a great deal is known of the origins and transport of membranes between the ER and Golgi apparatus, a major unanswered question is whether the construction of new Golgi stacks requires addition to pre-existing templates, similar to DNA replication, or arises through *de novo* synthesis.

3.1 Clues from disassembly / reassembly of the Golgi apparatus

3.1.1 Evidence for Golgi biogenesis *de novo*

For the Golgi apparatus, we define *de novo* biogenesis as the construction of polarized Golgi stacks by the accretion and self-assembly of vesicles, the smallest membrane units for this and other ER-derived organelles. To achieve this goal, Golgi-bound vesicles would have to have a unique subset of identities which together promote their ordered assembly into an asymmetric organelle (Fig. 2). Evidence that the Golgi can be constructed from ER-derived vesicular units comes from studies of the fungal metabolite, brefeldin A (BFA). BFA treatment of cells results in the rapid, bulk retrograde transfer of Golgi proteins to the ER, and upon drug removal, ER-localized Golgi components are mobilized via transport vesicles from the ER to form a morphologically intact and functional Golgi apparatus (28). Additional evidence for the ability to assemble the Golgi from vesicular building blocks comes from the study of Golgi behaviour during mitosis (5). Starting in prophase, Golgi stacks are progressively transformed into a heterogeneous collection of vesicles, an event believed to facilitate the partitioning process (29). At the onset of cytokinesis, disassembled Golgi membranes rapidly fuse and reorganize into a secretion-competent Golgi stack (30). Lastly, treatment of cells with the sponge metabolite ilimaquinone results in disassembly of the Golgi into a collection of 60 nm vesicles which are capable of fusing to re-establish functional Golgi stacks upon drug removal (31).

3.1.2 Evidence for Golgi templates

Though these observations provide evidence that the Golgi can be rebuilt from vesicle-based starting material, recent findings suggest that organizational templates may have a role in these examples of stack re-assembly. For example, although the Golgi stacks does disassemble into a heterogeneous mixture of vesicles during mitotis, many of these membranes remain grouped into structures termed mitotic clusters (Fig. 3). The mitotic cluster was first identified as a morphological

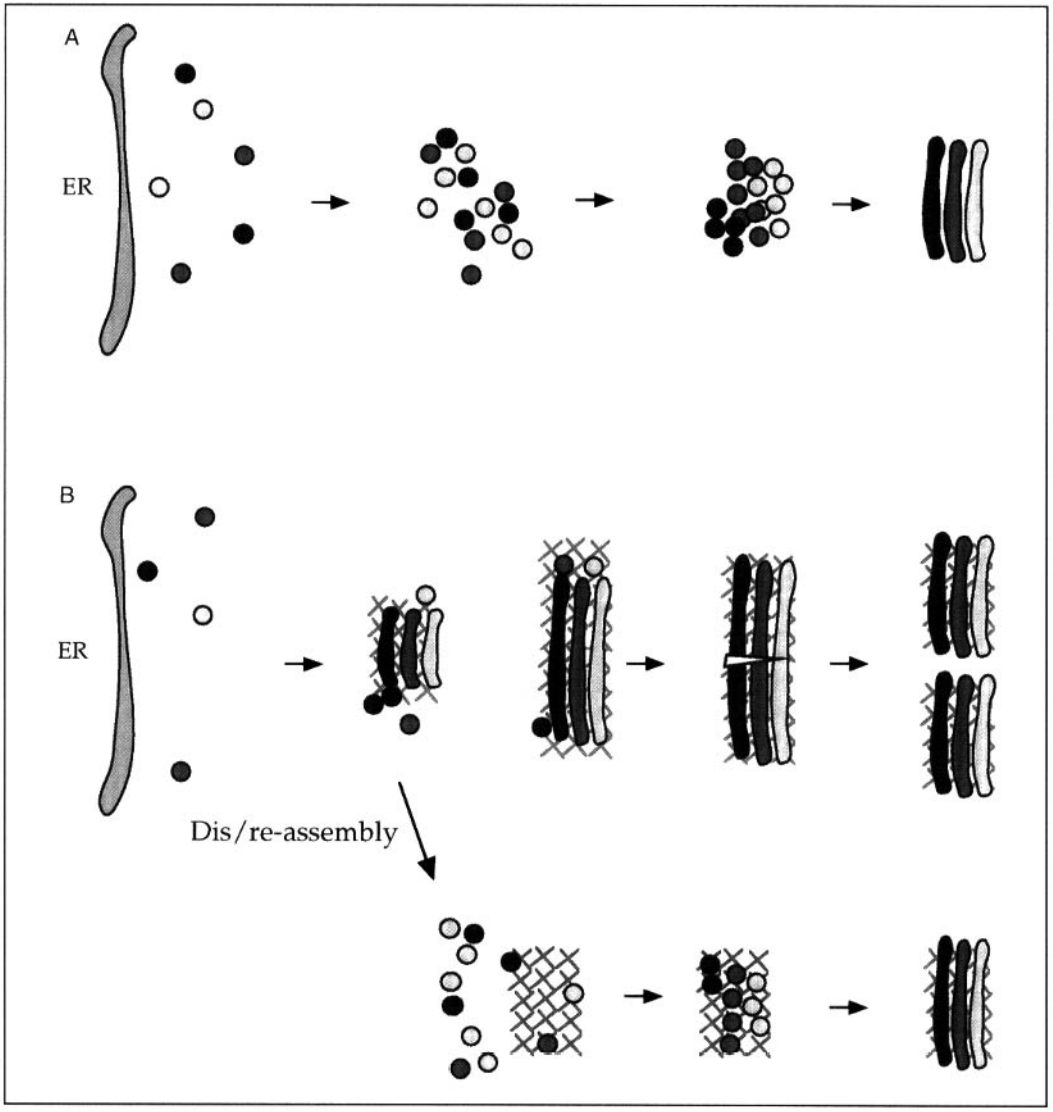

Fig. 2 Models for Golgi biogenesis. Biogenesis *de novo* (A) is the construction of polarized Golgi stacks by the accretion and self-assembly of vesicles derived from the ER. Templated biogenesis (B) is the assembly of Golgi stacks by addition of membranes of a pre-existing template, followed by medial division which results in stack duplication. In one templating scenario, Golgi growth could be accomplished by addition of membrane to pre-existing cisternae, or nascent membranes could be organized upon a polarized Golgi scaffold. The existence of Golgi templates could explain the appearance of Golgi mitotic clusters and the rapid re-assembly of polarized Golgi stacks following mitosis and treatment with BFA or ilimaquinone.

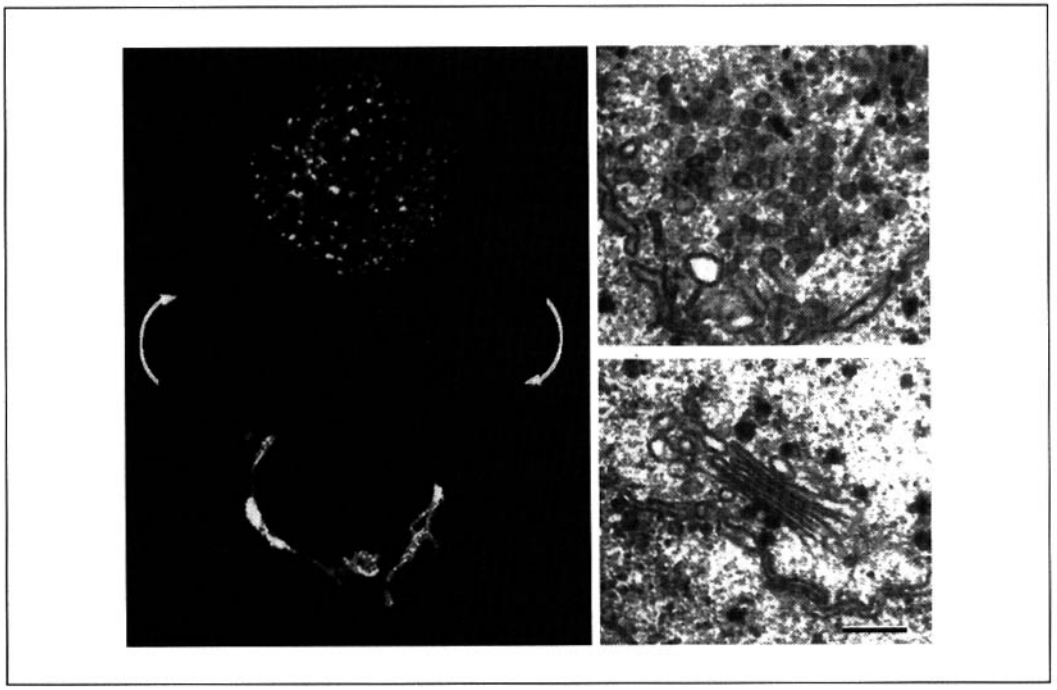

Fig. 3 Views of the Golgi in interphase and mitosis. The left panel shows confocal fluorescence microscopy images of a Golgi-localized version of the green fluorescent protein (see Section 4.3). At the onset of mitosis, the juxtanuclear Golgi ribbon of interphase cells (lower image) is extensively fragmented and dispersed throughout the cell (upper image). The right panel demonstrates the extreme contrast in the ultrastructural appearance of the Golgi during mitosis (upper panel; tubules, tubular networks and vesicles constitute the mitotic cluster) and interphase (lower panel; stacked Golgi cisternae). Note that the clustered tubulo-vesicular elements in the upper panel would correspond to one of the hundreds of mitotic fragments observed in the confocal fluorescence image. Scale bar for electron micrographs: 0.25 μm.

intermediate during mitotic fragmentation of the Golgi in HeLa cells (17), and has since been recognized as a common form of mitotic Golgi in other cultured cell lines and tissues (12, 32, 33). Recent work suggests that certain properties of the mitotic cluster make it a good candidate for a template upon which to re-organize the Golgi stack following mitosis (34). First, the mitotic clusters persist throughout mitosis and are therefore the end-product rather than intermediates of mitotic Golgi fragmentation. The number of clusters within a given metaphase cell population is remarkably constant (130 ± 3), suggesting that the clusters represent a basic structural unit of the Golgi. Moreover, confocal fluorescence microscopy reveals a polarized distribution of Golgi-resident proteins within the mitotic cluster (see Plate 3) despite the complete loss of stacked cisternal Golgi structure. These findings demonstrate that a polarized remnant of the Golgi apparatus persists throughout mitotic disassembly and suggests the existence of an underlying template which is responsible for organizing the biochemical architecture of Golgi membranes (see Fig. 2).

Using ultrastructural and light microscopic techniques, several groups have also

demonstrated the existence of tubulo-vesicular Golgi remnants following BFA-induced disassembly of the Golgi (35–37). Furthermore, it has been shown that TGN residents do not redistribute to the ER (28), and fractionation of membranes following BFA treatment demonstrates that 50% of the resident enzyme galactosyltransferase remains associated with the Golgi peak (38). In other words, not all Golgi material is returned to the ER. Like the mitotic cluster, the BFA remnants of the Golgi could serve as a seed to rebuild the stack following drug removal. Although these membrane remnants are morphologically reminiscent of the cluster (36), their biochemical composition and organization require further examination. Whether a Golgi template remains following ilimaquinone disassembly is not clear, although immunolabelling studies suggest that resident Golgi proteins remain segregated into distinct membrane compartments following ilimaquinone treatment (39).

The ability of the mitotic cluster to maintain biochemically distinct compartments independent of the existence of any obvious cisternal organization raises the possibility that Golgi membranes are organized upon an underlying template, a Golgi matrix, which could potentially exist independent of membrane composition. Consequently, even when Golgi membrane structure is drastically transformed, the blueprint for re-assembly of proper Golgi stacks would remain, and would explain how the complex architecture of the Golgi stack can be restored within minutes following mitosis or drug treatments such as BFA and ilimaquinone (28, 30, 40) (see Fig. 2).

A dense, cytoskeleton-like matrix has been localised to the intracisternal space of Golgi stacks (41) and also within mitotic cluster membranes (32), though the significance of these morphological findings awaits molecular characterization of the putative scaffolding. Good candidates for the scaffold include a collection of cytoskeleton-like proteins that have recently been localized to Golgi membranes, including the autoimmune antigens giantin (42, 43), GM130 (44), Golgin-160 (45), Golgin 245 (46) and p210 (47), and two Golgi-localized variants of the actin/spectrin erythrocyte cytoskeleton: ankyrin-G119 (48) and Golgi β-spectrin (49). The size and structure of these proteins, as well as their localization to specific compartments within the Golgi complex, fit the profile expected of proteins involved in the formation of an organellar scaffold.

3.2 Growth and division of the Golgi stack

A polarized scaffold on to which the animal cell Golgi can be organized is consistent with a templated growth and division mechanism for Golgi biogenesis, though there is still no direct evidence in support of this model. In contrast to the animal Golgi apparatus, which consists of a collection of Golgi stacks arranged into a linked, ribbon-like network, the Golgi of plants and some fungi exist as a collection of tens to thousands of discrete stacks, called dictyosomes, throughout the entire cell cycle (Fig. 4) (50, 51). This morphological feature has greatly facilitated ultrastructural analysis and determination of Golgi number and size during different stages of the cell cycle. For example, in the alga *Micrasterias*, dictyosome cross-sectional diameter

Fig. 4 Ultrastructural appearance of a dictyosome in the fission yeast, *Schizosaccaromyces pombe*. In contrast to the juxtanuclear interphase ribbon of the mammalian Golgi apparatus (see Fig. 1), *S. pombe* dictyosomes exist as discrete stacks and are dispersed throughout the cytoplasm. D, dictyosome; ER, endoplasmic reticulum, often resides adjacent to the plasma membrane, PM; M, mitochondria; N, nucleus. (Micrograph kindly provided by Rose Watson.)

approximately halves following cytokinesis (52, 53), and in cycling cells of the onion root, dictyosome numbers double in the period from prophase to metaphase (54). The simplest interpretation of these observations is that dictyosomes undergo medial fission during mitosis. Numerous ultrastructural images in plant Golgi literature can be interpreted to represent dictyosome fission (53, 55, 56), though it must be stressed that these static images may equally represent fusing dictyosomes. These and other quantitative and qualitative observations of dictyosomes during the cell cycle has led to the general belief that plant Golgi biogenesis occurs through lateral dictyosome growth and medial fission (50). Though tantalizing, definitive identification of an organelle division process for the Golgi awaits a more direct, continuous observation of the birth of two daughter units by organelle fission.

Regulation of Golgi unit number can also be examined in animal cells under conditions where the Golgi ribbon is dispersed to yield individual Golgi units. We have focused our efforts on the mitotic Golgi, which loses its normally juxtanuclear,

ribbon-like appearance to form a well-distributed collection of mitotic Golgi fragments at metaphase (13). Recent study of these fragments suggests that their copy number directly correlates with cell size (34). When cells have been arrested at the G_1/S boundary with the DNA synthesis inhibitor aphidicolin, they continue to grow in size, despite their inability to proceed through the cell cycle (57). After two rounds of aphidicolin arrest and subsequent progression into M phase, cells are approximately twice the size of their unsynchronized counterparts. The number of Golgi mitotic fragments keeps pace with the gain in cell size, increasing in number from about 65 in normal cycling cells to about 130 clusters in cells subjected to the aphidicolin treatment (34). Thus, like the dictyosome, the animal mitotic cluster appears to be a defined structural unit of growth, and therefore could represent a unit of animal Golgi replication. This experimental system supplies a well defined window in which to monitor organelle growth in animal cells and will hopefully provide further clues to the question of whether Golgi biogenesis is based on growth and division, or *de novo* assembly.

3.3 Templated growth: the budding yeast vacuole

Definitive proof for either the *de novo* or templated growth and division pathways for an organelle, like the Golgi, demands a direct visualization and analysis of the biogenesis event. In the absence of such data for the Golgi apparatus, we will now extend our discussion to include elegant work in the budding yeast, *S. cerevisiae*, where investigators have exploited certain fluorescent properties of the vacuole (equivalent to the animal lysosome) to document the mode of biogenesis in living cells (58). The mother cell vacuole normally grows by input of secretory pathway proteins via the Golgi apparatus. At G_1/S, a daughter bud emerges from the mother cell wall and vacuolar material immediately begins to be deposited into the nascent bud; thereafter, a bidirectional exchange of vacuole membranes occurs between the growing bud vacuole and mother cell vacuole until each contains a similarly sized organelle at the time of cytokinesis (59) (Fig. 5).

Vacuolar biogenesis is the best characterized example of templated growth and division, and information about this process has provided an intriguing conceptual framework upon which to organize future investigation in other organelle systems. Rather than build the daughter bud vacuole using vesicles derived directly from the Golgi, the membranes destined for the nascent bud vacuole travel via the mother vacuole. In other words, the mother vacuole grows, then is divided up between the mother and daughter cells. This organelle behaviour suggests a preference for inheriting vacuole-derived membranes, instead of nascent, Golgi-derived vacuolar proteins, perhaps reflecting the requirement to inherit a vacuole template which can only be provided by the mother vacuole. Furthermore, the central role of the mother vacuole in new vacuole biosynthesis underscores the important concept that templates provide a basis for organelle autonomy within the cell; this thought evokes the image of organelles as well-integrated endosymbionts (60).

As the previous example highlights, live-cell microscopy provides an un-

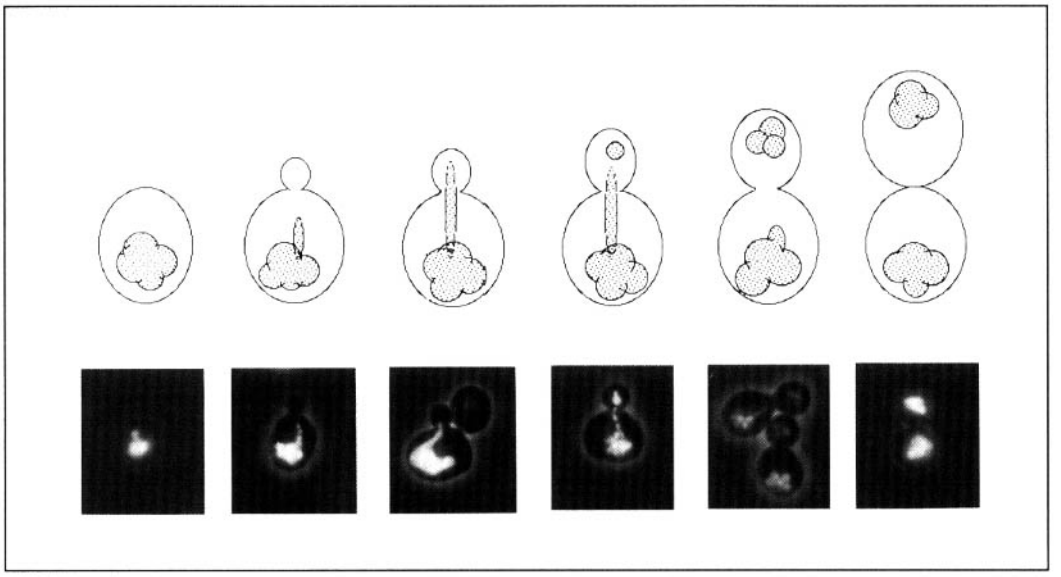

Fig. 5 Vacuole inheritance in *Saccharomyces cerevisiae*. Fluorescence micrographs (lower panels) and schematic illustrations (upper panels) showing the streaming of vacuole-drived vesicles into the growing daughter bud, finally resulting in the accumulation of an equivalent vacuole in the nascent daughter cell following cytokinesis. Kindly provided by William Wickner, Dartmouth Medical School, NH, and adapted from *The Journal of Cell Biology*, 1992, **119**, 1469–79, by permission of The Rockefeller University Press.

interrupted view of the fate of organelles in single cells and is a critical tool for a thorough, definitive analysis of organelle behaviour. The recent advent of the green fluorescent protein (GFP; see Section 4.3) as a vital tag for organelles should greatly facilitate our understanding of these fascinating phenomena (23, 34, 61).

4. Partitioning of the cytoplasm

Two general terms have been used to categorize the modes of partitioning during cell division. The complex segregation of chromosomes at mitosis is the hallmark of an ordered partitioning strategy. As one would expect, it represents the gold standard for highly programmed, and thus highly accurate partitioning. In contrast, subcellular components such as water, ATP, soluble proteins and ribosomes, because of their abundance and extensive distribution throughout the cell, are likely to achieve accurate segregation passively as a consequence of cytokinesis. This is referred to as a stochastic partitioning strategy (Fig. 6) (6, 62).

The error in equal partitioning of an abundant cytoplasmic component (say one with at least 10^5 units/cell) by stochastic means can be estimated, using the binomial theorem, to be less than $\pm 1\%$ (13, 62). Since the accuracy of division furrow placement itself during cytokinesis is estimated to be approximately $\pm 2\%$ (63), the accuracy of partitioning for the abundant class of cytoplasmic components is limited solely by the ability to divide the mother cell into two equal halves.

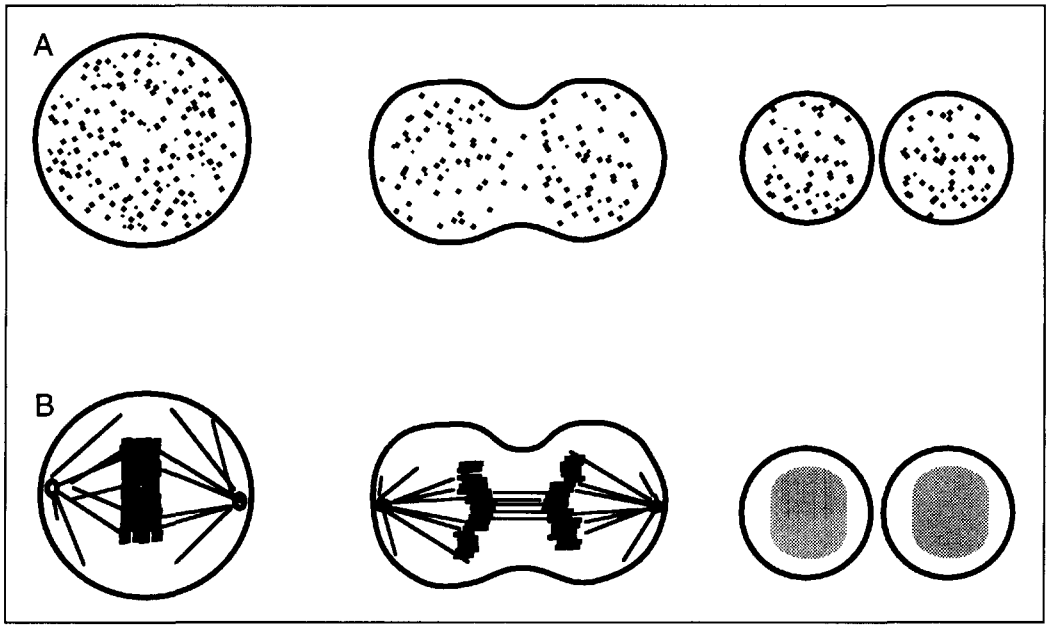

Fig. 6 Schematic representation of the stochastic (A) and ordered (B) modes of partitioning at cell division. Abundant, well-dispersed cytoplasmic constituents may play a passive role during cell division since they are likely to be partitioned equally as a result of cytokinesis. In contrast, a highly ordered, universal mechanism based on the mitotic spindle exists to ensure the accurate partitioning of chromosomes during mitosis.

The organelles do not neatly fit into the categories of stochastic or ordered partitioning. First, morphological analysis of organelle behaviour during the cell cycle suggests that the cellular mechanisms used for partitioning organelles into daughter cells appear to differ from various organelles within a single cell type; moreover, modes of partitioning also differ for a single type of organelle among different cell types and among different organisms. Thus no universal mechanism is apparent.

Secondly, a limited collection of data on the accuracy of partitioning organelles, starting with the work of Wilson on scorpion spermatocyte mitochondria (64) and expanded more recently by the work of Birky and colleagues on algal chloroplasts (1, 62), has led to the puzzling conclusion that these organelles are partitioned with an accuracy that falls somewhere in between that expected for an ordered partitioning scheme and a stochastic one. Birky has described this phenomenon as stochastic partitioning with a tendency to equality (62). The implication is that, for at least some cytoplasmic constituents, there are mechanisms in place to ensure an accuracy better than would be achieved solely through a passive, stochastic partitioning. Whether this property is common to other cytoplasmic components, especially other organelles, is currently unclear. Below, we review recent work on the fate of the mammalian Golgi apparatus during cell division which suggests that, contrary to previous expectations, it also appears to use a mechanism that ensures more accurate

segregation, a mode of partitioning that lies intermediate on the scale between a random and ordered process, and here is termed semi-ordered.

4.1 Ultrastructural view of Golgi partitioning

Ultrastructural observations and stereological analysis laid the foundation for an initial understanding of Golgi behaviour in animal cells during mitosis. During prophase, the interphase Golgi ribbon is initially fragmented into a collection of stacks which are dispersed in the cytoplasm (65). Later, during the transition from late prophase to metaphase, the stacks are transformed into a heterogeneous collection of tubular networks, short tubules and vesicles (17). These observations have given rise to the idea that the pericentriolar, polarized Golgi was converted into numerous, well-distributed membranes to increase the accuracy of a stochastic partitioning process (6). As cells progress to early telophase, the dispersed tubulo-vesicular membranes rapidly coalesce, fuse and rearrange to give rise to intact and functional Golgi stacks before the end of cytokinesis (30). Following cell division, the Golgi stacks congregate in the pericentriolar region to re-form the interconnected reticulum of the interphase Golgi (66, 67).

4.2 Cell-free systems

The morphological transformation of Golgi membranes at mitosis has been mimicked in a cell-free system, which has permitted a more detailed analysis of membrane intermediates and the identification of components involved in the cycle of disassembly and reassembly (68, 69). In this system, 50–65% of Golgi stacks are disassembled by the continuous budding of coated vesicles. This process closely resembles that of transport vesicle formation, and shares a common component, the COP I coat, first identified as a vesicle coat component necessary for Golgi membrane bud formation. The involvement of COP I in disassembly of the Golgi provided the first direct evidence that the formation of mitotic membranes may use the same class of components as vesicle-based transport.

A COP-I-independent pathway has also been implicated in the production of a separate class of mitotic fragments, including tubular networks and larger, heterogeneously sized vesicles and tubules (69). A possible reason for the existence of two disassembly pathways is suggested by the finding that the composition of Golgi residents in the COP I and COP-I-independent membranes differ (70). Golgi residents, including several oligosaccharide processing enzymes, are selectively enriched in the COP-I-independent membrane populations. In contrast, the COP I vesicles are enriched for components involved in transport between the ER and Golgi, such as the putative retrieval receptor for proteins escaped from the ER, the KDEL receptor (71), and a mannose-binding lectin thought to be involved in cargo transport, ERGIC53/p58 (72). Based on these data, it has been suggested that, during mitotic disassembly of the Golgi stack, the COP I pathway acts to remove the cisternal rims, regions of membrane that are specialized for transport, whereas the

COP-I-independent pathway is responsible for the transformation of the remaining cisternae, housing the core of *bona fide* Golgi residents, into a heterogeneous collection of tubular membranes (70).

A working model has been proposed to link the mitotic disassembly of Golgi stacks to the general cessation in exocytic membrane traffic that accompanies the onset of mitosis. The main postulate is that there is an inhibition of membrane fusion during mitosis. If true, this could explain the inhibition of protein secretion, which relies on both the budding of transport vesicles from donor membranes (e.g., ER) and fusion of these vesicles with downstream acceptor membranes (e.g., Golgi). Moreover, the budding of Golgi-derived vesicles in the absence of fusion of new input vesicles would eventually lead to the consumption of Golgi cisternal rims via vesicle budding, explaining the COP-I-dependent morphological changes to the Golgi during mitosis (73).

Recent biochemical evidence supports a role for the putative vesicle docking protein p115 in the inhibition of membrane fusion during mitosis (74). This protein is required for the fusion of transport vesicles with acceptor membranes in a cell-free intra-Golgi transport assay (75), and the yeast counterpart of p115, termed Uso1p, has been demonstrated to be necessary for transport between the ER and Golgi (76). In HeLa cells, p115 is localized to the *cis* face of the Golgi stack during interphase, presumably playing a role in the primary phase of vesicle recognition (34). During mitosis, p115 is released from Golgi membranes, which may explain the inhibition of vesicle fusion, and thus the cessation of membrane traffic and disassembly of the Golgi that are coincident with mitosis (34, 77, 78).

The fusion of mitotic Golgi vesicles and tubules to reform stacks of cisternae has also been mimicked using a cell-free assay (79), which has demonstrated that reassembly can be accomplished using two distinct membrane fusion pathways (80). The first is dependent on the function of the *N*-ethylmaleimide sensitive fusion (NSF) ATPase, a component of the SNARE-dependent membrane fusion machinery implicated in a wide range of vesicle fusion events throughout the cell, including ER to Golgi transport (81), intra-Golgi transport (82) and synaptic transmission (83). The second fusion pathway requires p97, an abundant cytosolic protein and member of the NSF family of ATPases (84). The yeast counterpart of p97, cdc48, was first identified as a cell cycle mutant (85) and has subsequently been implicated in the nuclear membrane fusion required for yeast karyogamy (86).

The relative contributions of these molecules to the reassembly of intact Golgi stacks following disassembly is still unclear, though data from the cell-free assay (80) and from the reassembly of Golgi stacks following ilimaquinone-induced fragmentation (see Section 3.1) (39), suggest that p97 and NSF may be involved in the fusion of distinct Golgi membrane populations.

Fragmentation of the Golgi apparatus is thought to facilitate the partitioning process by increasing the number and distribution of Golgi membranes (5). Since accurate cytokinetic mechanisms exist to divide the mother cell into two equally-sized daughters, any organelle, present in multiple copies, could be inherited by a stochastic process. The identification of vesicles as products of the mitotic

disassembly of the Golgi *in vivo* and *in vitro* fits well with the concept that the Golgi undergoes stochastic partitioning since interpolation of the EM data suggests that up to 10,000 Golgi-derived vesicles could be released into the mitotic cell cytoplasm. With this number of Golgi units, a theoretical accuracy of partitioning of 50 ± 1.5% would be accomplished solely by division of the cell at cytokinesis (29). Together these data suggested that the Golgi-derived vesicle represented the unit of stochastic partitioning.

4.3 Analysis of Golgi partitioning using green fluorescent protein

The process of partitioning has recently been examined using an experimental system which permits the observation of Golgi-resident proteins in living cells. The Golgi of HeLa cells was rendered fluorescent by exploiting the properties of the green fluorescent protein (GFP), a naturally fluorescent protein isolated from the jellyfish *Aequorea victoria* (87). Appending the GFP to the retention signal for the Golgi-resident glycosyltransferase *N*-acetylglucosaminyltransferase localizes fluorescence to the medial/trans compartments of the stack and has permitted us to follow the fate of Golgi membranes in single cells continuously through mitosis (34).

From the analysis of living cells using the GFP-tagged Golgi emerges strong support for an ordered, rather than stochastic partitioning of mitotic Golgi membranes. First, mitotic disassembly does not transform the Golgi into a dispersed collection of free vesicles. Instead, major products of the disassembly process are tubulo-vesicular mitotic clusters, structures which were originally thought to be intermediates, not end-products in the pathway. Definitive evidence for the role of the mitotic cluster as the partitioning unit came from the visualization of single cells which directly demonstrated that clusters are partitioned into daughter cells and, following cytokinesis, assemble to give rise to an interphase Golgi ribbon (Fig. 7). These findings do not preclude a role for free vesicles in the disassembly process, but suggest that the shedding of Golgi-derived vesicles into the cytoplasm does not go to completion. Incorporating findings of the cell-free disassembly assay, we would propose that two distinct populations exist following disassembly. COP-I-coated vesicles consume transport-specialized regions (rims) of the Golgi and may represent a portion of the free vesicle population. What remains following removal of the rims is a core of Golgi residents, the mitotic cluster, whose numbers and relative organization persist throughout mitosis to facilitate reassembly of the interphase Golgi apparatus during cytokinesis.

The existence of mitotic Golgi clusters as a unit of partitioning, and the precise regulation of their number, position and compartmentation (see Section 3.1.2) suggest that an active mechanism exists to coordinate the partitioning of the Golgi at mitosis. Though these characteristics of the cluster do hint at a more ordered partitioning process, the question remains whether these units of partitioning are dispersed throughout the cell and inherited passively through a cytokinetic mechanism, or if there are mechanisms in place actively to control the partitioning of Golgi

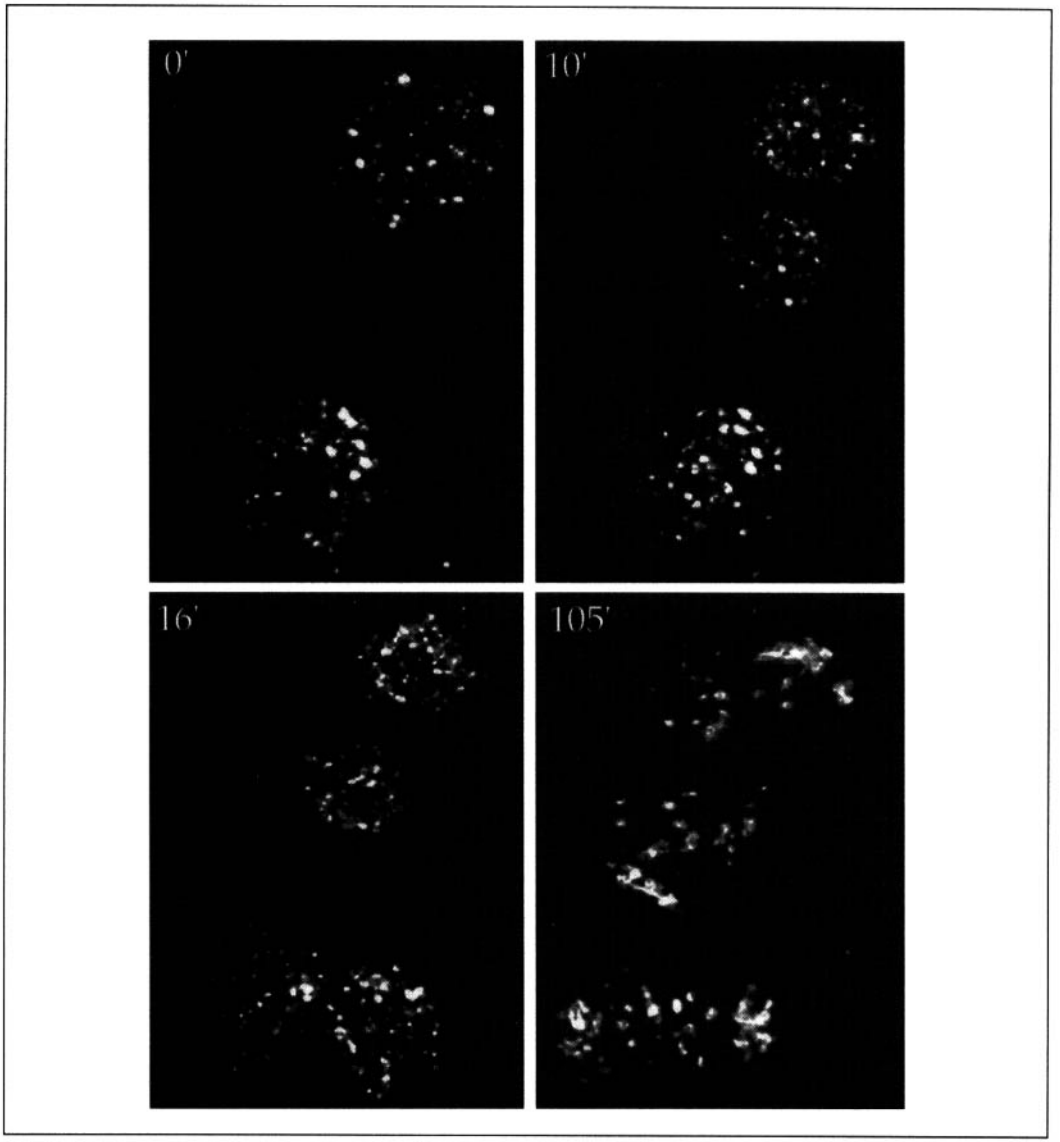

Fig. 7 Partitioning of mitotic Golgi clusters in living cells. GFP-tagged Golgi membranes were monitored in living, mitotic HeLa cells using fluorescence confocal microscopy. Metaphase cells were followed as they progressed into telophase, then G_1 phase of the cell cycle and began to reassemble the interphase Golgi ribbon.

membranes into daughter cells. It is feasible that the accuracy achieved through stochastic partitioning of 130 clusters (instead of 10,000 free vesicles) is still sufficient for cells, perhaps because they are able to synthesize Golgi membrane rapidly to compensate for any deficits. We have attempted to address this issue by determining the accuracy of partitioning of GFP-tagged Golgi. Comparison of partitioned Golgi membranes in late telophase daughter cell pairs suggests, though not perfect, there is

a far greater accuracy of partitioning the Golgi than expected from a solely stochastic process (34). These findings provide strong evidence for the involvement of cellular mechanisms to increase Golgi partitioning accuracy.

4.4 Semi-ordered partitioning

Similar to findings obtained for other cytoplasmic organelles, the Golgi appears to be partitioned with an accuracy characterized by an ordered, but error-prone mechanism, which we have termed semi-ordered partitioning. The implication of such a mechanism is that the cell must be able to count and segregate cytoplasmic components such as organelles. It is difficult to envision how the cell accomplishes this task, given the number and diversity of partitioning units that must be accounted for during mitosis.

A paradigm for a cellular counting mechanism during mitosis exists in the form of the centrosome. In early prophase, the centrosome divides, and each of the resulting pair nucleates a rich astral array of microtubules. As a cell progresses into prometaphase, the centrosomes move to opposite poles of the cell to form a mitotic spindle; then, during anaphase and telophase, each centrosome is partitioned into a daughter cell along with attached microtubules and one pair of daughter chromosomes (88). A reasonable hypothesis to explain the semi-ordered partitioning of organelles during mitosis involves the exploitation of centrosome division and separation to divide organelles between the presumptive daughter cells. Hence, if in addition to the well-known interaction of organelles with interphase microtubules (89–93), organelles (or other cytoplasmic components) are also bound to microtubules during centrosome division and migration, they could be fairly equally distributed on either side of the metaphase plate, and subsequently partitioned into nascent daughter cells during cytokinesis. The ability to visualize organelles and microtubules in living mitotic cells should provide a means to test this and other hypotheses concerning the mechanisms responsible for semi-ordered partitioning.

5. Conclusions and future directions

Recent biochemical, genetic and microscope-based investigations have provided a rudimentary montage of the processes involved in cytoplasmic inheritance. One of the most important concepts to have emerged (albeit slowly) from the study of organelle inheritance is that we need to look beyond the apparent disorder associated with many existing examples of the partitioning and biogenesis process. Though certainly not obvious upon first, or even second observation, at least some organelles appear to rely upon cellular mechanisms that ensure a level of partitioning accuracy beyond those afforded by stochastic means. The nature of these mechanisms, and their general applicability among organelles, is unclear. Moreover, whether mechanisms similar to those employed for organelles could also account for the distribution of a whole range of cytoplasmic components, including proteins, has not yet been addressed.

What we have learned about the mechanisms of inheritance has implications for our understanding of other aspects of cell division, such as asymmetrical cell division during cell fate determination in organisms ranging from yeast to mammals (94–97). Here, deliberate asymmetrical distribution of cytoplasmic components in a mother cell results in the birth of daughter cells with completely different developmental programs. If we assume that cells have developed a means to control the inheritance of certain elements of the cytoplasm during mitosis, it becomes easy to imagine a scenario for adopting these mechanisms to the task of asymmetrical segregation. For instance, unequal distribution of a determinant could be accomplished by inactivating a specific aspect of the partitioning machinery in one half of the cell. Additionally, substantial experimental evidence is in favour of a common mechanism for regulating the asymmetrical segregation of cytoplasmic proteins and the localization of the centrosomes during mitosis (96–98). In sum, a detailed investigation of the inheritance of the cytoplasm during the cell cycle is likely to be central to our eventual understanding of the maintenance of cellular homeostasis and cell growth, and the creation of cellular diversity.

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9 Cytokinesis

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1. Introduction

Cytokinesis is a multi-step process that proceeds linearly with time according to the following general framework. It begins with a signal from the mitotic apparatus that must be transmitted to the cellular cortex at the cleavage site. Receipt of the signal at the cortex sets off a complex set of reactions that transduce the signal into changes in the actin cytoskeleton and lead to formation of an actomyosin-based contractile ring. Mechanical processes in the ring and its interaction with the cortex result in cytokinesis. The distinctions between these steps are probably largely artificial. It is not clear, for example, whether some of the molecules we describe as transducing a cytokinetic signal are in fact part of the signal itself. Even this seemingly innocuous (though admittedly simplified) view of cytokinesis is not free of controversy, and some kinds of cytokinesis almost certainly do not conform to this pattern. Many questions remain unanswered and are addressed in the following sections.

2. How do cells know where to place the cleavage furrow?

2.1 Cleavage site determination in animal cells

2.1.1 Cells regulate the placement of the division plane by controlling the orientation of the mitotic apparatus

The cell division plane must be precisely regulated in order to fulfil two key cellular and organismal imperatives. First, the division plane must be coordinated with nuclear division both temporally and spatially, so that each daughter cell will inherit one nucleus. Second, during development, the choice of the division plane is often essential for establishing the developmental potential of the daughter cells, as well as the shape, size, and position of the cells in the organism. In animal cells, these imperatives can be met because the position of the mitotic apparatus determines the division plane. The mitotic apparatus always correlates with the division plane, and manipulations that move the mitotic apparatus can change the cleavage plane (reviewed in ref. 1). It appears from many such manipulations that virtually any

region of the cell surface that receives signals from the mitotic apparatus at the correct time in the cell cycle can form a cleavage furrow, allowing maximal flexibility in cleavage plane determination.

During development, various cells divide along different division planes; these patterns are often choreographed through rotations or lateral displacements of the mitotic spindle. For instance, asymmetrical cell divisions that occur in different axes contribute to the differentiation in the cell lineage in early *Caenorhabditis elegans* embryos. In certain embryonic cells, microtubules from one of the two centrosomes are captured by a cortical attachment site that includes actin and actin-capping protein (reviewed in ref. 2). This capture causes rotation of the mitotic apparatus when the microtubules are shortened, and thus specifies the axis of the division plane. Genetic studies have identified a set of partioning (*par*) mutations that cause improper rotations of the spindle, and that also produce aberrant symmetrical divisions. Several of these *par* gene products have recently been shown to be proteins that are asymmetrically distributed on the cell cortex (3). This case illustrates an intriguing circularity in cellular logic: the cortex gives instructions to orient the mitotic apparatus, which in turn tells the cortex where to establish the cytokinetic furrow.

2.1.2 Spindle asters are probably the source of the signal in echinoderm eggs

How does the mitotic spindle specify the cleavage furrow? Early experiments involving direct manipulation of large invertebrate eggs specifically implicated the spindle poles as the source of the signal for cleavage furrow formation (4). In Rappaport's 'torus experiment', eggs could be made into a horseshoe shape in which two spindles were present; cleavage occurred not only between the two asters of each spindle, but also between the asters of adjacent spindles (Fig. 1A). Furthermore, by removing various parts of the spindle in sea urchin eggs, Hiramoto (5) showed that asters are essential for cleavage furrow formation while the remainder of the spindle is dispensable. Finally, cleavage furrows are induced even in enucleated eggs that retain centrosomes (6).

2.1.3 In most animal cells, the signal travels from the mitotic apparatus to equatorial regions of the cortex

In the mid-1980s, opposing models were proposed for the site of action of the contractile stimulus. The 'polar relaxation' hypothesis envisioned that the signals from the mitotic apparatus directly influence the polar regions of the cortex at opposite sides of the cell. Receipt of the signal at the polar regions would in some fashion 'relax' the cortex, allowing cortical materials needed for contractile ring formation to flow from both poles toward the equator (7). The opposing 'equatorial stimulation' model posits that the signal is received at the site of furrow formation itself, causing a local increase in contractility. The polar relaxation model still has its adherents, and can be used to model cytokinesis successfully, particularly in large

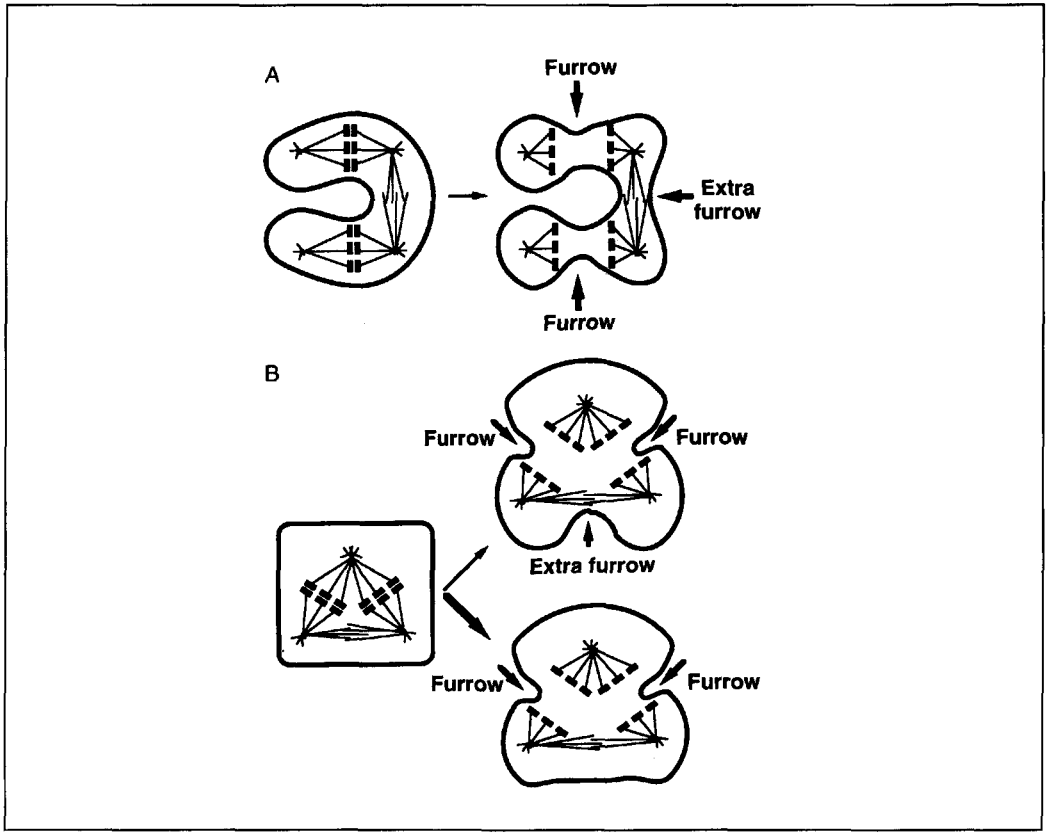

Fig. 1 Cleavage furrows in cells containing two spindles. (A) In Rappaport's classical 'torus' experiment performed on fertilized invertebrate eggs (4), an extra furrow formed between the asters of adjacent spindles in a region devoid of chromosomes. (B) In more recent experiments with mammalian tissue culture cells, an extra furrow usually fails to form at the analogous position. Presence of this additional spindle in a minority of cells might reflect the presence of bundled microtubules between the asters of adjacent spindles. (Based on diagrams in refs 14 and 16.)

embryonic cells (8). However, the equatorial stimulation hypothesis is supported by several persuasive lines of evidence. (a) The cleavage site in certain mechanically-deformed cells is best explained by equatorial stimulation rather than polar relaxation (1). (b) Mechanical interference in the region between the midzone of the anaphase/telophase spindle (the source of the cytokinetic signal in most cell types smaller than amphibian eggs; see below) and the equatorial cortex disrupts cytokinesis. (1, 9). (c) Most convincingly, Burton and Taylor (10) have recently examined the behavior of cells dividing on rubber sheets to locate and measure traction forces generated by dividing cells. They found that as the cell progresses through mitosis, traction forces increase at the cell equator (as predicted by the equatorial stimulation model), but do not decrease at the poles (as would result if

the polar relaxation model were correct). Thus, these findings together suggest that a signal from the mitotic spindle may directly induce contractile events at the equator.

2.1.4 The spindle midzone is the source of the signal in most animal cell types

Although the original echinoderm egg experiments are very elegant and clear, the echinoderm egg may not represent the prototypical cell. Recent studies have shown that for many other cell types, the midzone of the anaphase/telophase spindle is the source of the cytokinesis-inducing signal. The midzone of the spindle, sometimes called the central spindle, is defined as the part of the spindle that lies between the separating chromosome complements (11). Early suggestions for the involvement of the central spindle in cytokinesis were found in the results of micromanipulation experiments that moved or distorted the spindle apparatus in insect neuroblasts or in mammalian tissue culture cells. Cleavage in these cases occurred across the central spindle, with the furrow first appearing in the region of the cortex closest to the spindle midzone (12, 13). More recently, Cao and Wang (9) have shown that creation of a small physical barrier between one side of the spindle midzone and the equatorial cortex of tissue culture cells inhibits contraction specifically in that part of the cortex distal to the barrier. Inhibition of furrowing was exquisitely sensitive to the position of the barrier relative to the spindle, indicating that the source of the signal is highly localized in the spindle midzone. In a second set of experiments from the same research group, cleavage was correlated with the position of midzone microtubule bundles rather than with the spindle poles in tissue culture cells with tripolar mitotic figures [(14); see Fig. 1B]. Similar results have been reported more recently by others (15, 16).

Genetic evidence for the relationship of the spindle midzone and cytokinesis has also been obtained. Mutations in at least two genes that encode proteins that interact with central spindle microtubules block cytokinesis. The *Drosophila* KLP3A kinesin-like protein is a presumptive microtubule motor specifically localized in the spindle midbody (the electron-dense equator of the spindle midzone, sometimes called the stem body matrix). The KLP3A protein is needed for the establishment or stabilization of the central spindle (11). In *KLP3A* mutant spermatocytes, not only is the interdigitation of microtubules in the central anaphase-telophase meiotic spindle disrupted, but cytokinesis in these cells is prevented as well (Fig. 2). Mutations in the *Drosophila* gene *KLP38B*, which encodes another kinesin-like protein, also disrupt cytokinesis, though it is not clear whether the cytokinesis defects in this case similarly reflect aberrations in the central spindle (17). The *Drosophila* gene *l(1)BP1* encodes a protein that also localizes to the spindle midzone; this protein is distantly related to the *Saccharomyces cerevisiae ASE1* gene product (18). Both are microtubule-binding proteins and are necessary for spindle elongation. A high proportion of *l(1)BP1* mutant neuroblasts are highly polyploid, yet the frequency of anaphases is normal. A further indication of a cytokinesis defect is the failure of late anaphase cells to establish a contractile ring (M. Gatti, personal communication).

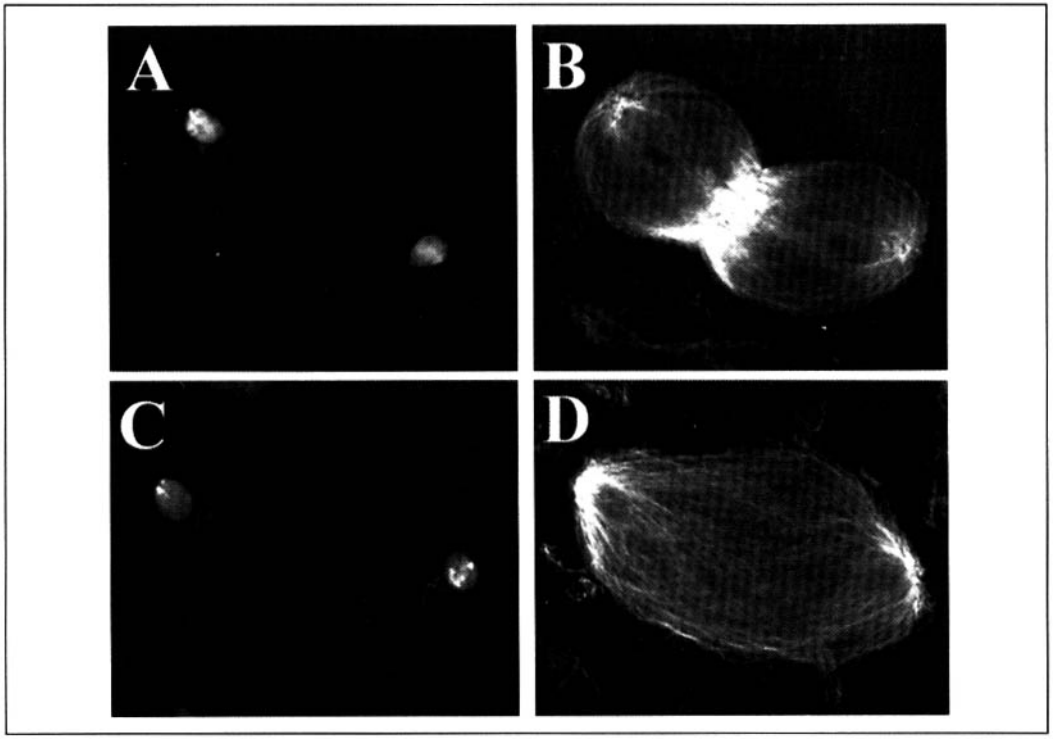

Fig. 2 Possible relationship between the spindle midzone and the contractile ring. (A) A wild-type *Drosophila* spermatocyte in early telophase showing well separated nuclei and (B) a prominent spindle midbody (the interdigitated microtubules between the nuclei). Reflecting the formation of the cleavage furrow, the spindle midbody is pinched inward. (C,D) A *KLP3A* mutant spermatocyte judged to be at a similar cell cycle stage on the basis of the internuclear distance. Note the absence of a prominent spindle midbody and the lack of evidence for cleavage furrow formation. (Pictures courtesy of Dr. Byron Williams, Cornell University.)

2.1.5 The telophase disc hypothesis suggests one way in which the central spindle might supply the signal

Additional circumstantial but nonetheless intriguing support for the involvement of the central spindle in determining the location of the contractile ring is the probable existence of a structure termed the 'telophase disc'. The telophase disc has been defined by immunofluoresence experiments with a human autoimmune serum that recognizes an antigen called TD-60, which completely bisects the anaphase cell at the position of the cleavage furrow, forming a continuous structure that spans the distance between the spindle midzone and the cell cortex (19). Once formed, this disc is quite stable and is not dissociated by drug treatments that destroy spindle microtubules. The presence of such a telophase disc would provide a straightforward mechanism by which the central spindle could transmit cytokinetic signals to the cell surface at the equator (20). Despite the attractiveness of this idea, the concept of the telophase disc is not yet widely accepted because there is no

ultrastructural evidence for its existence, and because neither the TD-60 protein nor other potential components have been characterized molecularly. Nonetheless, recent evidence from two laboratories has considerably strengthened the correlation between TC-60 staining and prefiguration of the site of furrow formation (14, 21).

2.1.6 Are chromosomes the repositories of cytokinetic signalling molecules before formation of the central spindle?

The TD-60 protein is found at the chromosomal centromeres from prophase of the cell cycle to mid-anaphase, when it relocates to the spindle midzone and telophase disc (20). Several other proteins also appear to have a similar cell cycle-dependent intracellular localization, notably the inner centromere proteins (INCENPs; (22). INCENPs appear to be subsequently redistributed in late anaphase and telophase to the cortex, where they may play a structural role in the contractile ring. Importantly, some microtubule motors such as CHO1 and CENP-E behave in a similar fashion. Proteins that move from the chromosomes to the equator of the spindle have been termed 'passenger proteins' (23). The function of any of these proteins in cytokinesis is still not clear. Injection of antibodies against CHO1 (24) disrupts cytokinesis, but this effect could be secondary to defects in the mitotic spindle. More recently, it has been reported that expression of a form of INCENP that remains trapped at the centromere dominantly disrupts the completion of cytokinesis, arguing that its movement to the central spindle is important for its role in cytokinesis (16). One could thus imagine that a plus-end-directed microtubule motor [which is probably not CENP-E, which has been reported to be minus-end-directed (25)] could transport TD-60, INCENPs, or other possible signalling molecules to the midzone. These results have led to the proposition that prior to mid-anaphase, cytokinetic signalling molecules and/or key components of the contractile ring are located at the chromosomal centromeres; only after these molecules have been transported to the central spindle can the midzone act as the source of the signal (16, 20).

A strong argument against this hypothesis in its simplest form is provided by recent experiments of Zhang and Nicklas (26). These investigators removed by micromanipulation all of the chromosomes from grasshopper spermatocytes. Though the operation was performed roughly at prometaphase, the cells were still able to undergo cleavage. These cells retain prominent bundles of microtubules in their equatorial regions, so this finding does not challenge the involvement of the central spindle in the initiation of furrowing. The chromosomes were apparently removed before movements of known passenger proteins to the spindle midzone, although this point is not absolutely certain. TD-60 and other passenger proteins could of course still be involved in cytokinetic signalling if they had the ability to reach the anaphase/telophase central spindle through alternative pathways, such as loading at the spindle poles and moving from there to the midzone via plus-end-directed motors. Clearly, a critical future test of the telophase disc hypothesis will be to determine whether micromanipulated, cleavage-competent cells without chromosomes still form a telophase disc.

2.1.7 The signal probably travels from the mitotic apparatus to the equatorial cortex via microtubules

The identification of the microtubule-rich asters or the central spindle as the source of the signals for furrow formation in animal cells has led to the suggestion that contact between microtubules of the mitotic apparatus and the cell cortex is essential for signalling (1, 27). Microtubule transport of the signal makes considerable sense in echinoderm eggs, where the spindle poles nucleate very large assemblies of astral microtubules. In other cells, where the signal seems to emanate from the central spindle, Zhang (15) has recently observed that bundled interzonal spindle microtubules [most of which do not emanate from the poles (28)] appear to become associated with cortical actin filaments. Disruption of these microtubules by direct manipulation with a needle disrupts cytokinesis. However, it should be cautioned that it is possible that other structures were also affected by mechanical manipulation. Another possibility in at least some cell types is that the signals could be transmitted from the spindle midbody to the cortex along or within the telophase disc, which appears to be able to persist independently of the interzonal microtubules after treatment with microtubule poisons (19).

2.1.8 What is the signal transmitted to the equatorial cortex?

The answer to this fundamental problem is unknown. It is usually assumed that the signal, whatever it is, moves to the equatorial cortex via the action of plus-end-directed microtubule motors. We have already cited evidence that CHO1 and KLP3A, two such motors that are found at the anaphase/telophase central spindle, are required for cytokinesis. Though it is attractive to hypothesize that the role of these proteins is to translocate the putative signal, the possibility that these motors instead function mainly in the establishment and maintenance of the central spindle cannot be ignored. It is also not inconceivable that the microtubules themselves might somehow directly trigger ring formation at the equatorial cortex, without requiring the action of any molecules transported along them.

2.2 Division site determination in *Schizosaccharomyces pombe*

2.2.1 In *S. pombe*, the mitotic apparatus does not determine the position of the division site

Like animal cells, fission yeast cells also divide by medial cleavage using an actin-based ring. Cell division in fission yeast occurs in several discrete steps (29, 30). First, the actin ring forms in the middle of the cell during early mitosis, before nuclear division. This ring, which marks the future site of division, thus forms much earlier than in animal cells. Second, in late mitosis after anaphase, an extracellular cell wall septum forms at the site marked by the actin ring and grows in from the cell surface. During septum formation, the actin ring closes or contracts. It is not known yet whether the ring contains myosin or supplies an active, contractile force in this process. It is also possible that the ring supports or guides the membranes to

guarantee that the membranes are properly closed. Third, the septum itself is digested away, leading to cell separation.

It is still not clear what spatial cues position the actin ring and division plane in fission yeast. In contrast to animal cells, fission yeast ring formation and placement do not require the mitotic spindle. A β-tubulin mutant (*nda2*), which is defective in mitotic spindle assembly (but is probably not as defective in interphase microtubule distribution), exhibits a normal actin ring positioned in the proper location (31). In addition, overexpression of either plo1p or cdc15p (see below) can induce ring formation at the middle of the cell during interphase (32, 33), demonstrating that spatial cues are present even in interphase in the absence of the spindle. The independence of cytokinesis from the spindle in *S. pombe* may reflect key cytological differences between fission yeast and animal cells. For instance, the nuclear membrane in fission yeast does not break down during mitotis, so that the mitotic spindle, which remains inside the nucleus, does not have direct association with the cortex. If the mitotic spindle is dispensable, then what provides the spatial cue at the cell middle?

2.2.2 Interphase microtubules may have a direct role in division site determination in *S. pombe*

Numerous models for division site placement in *S. pombe* have been proposed (34). For instance, the cell middle may be determined by the pattern of cell growth or by a cortical marker left from a previous division. However, these possibilities are not consistent with the fact that many mutants defective in cell shape still exhibit medial placement of the division site (35). Two general classes of hypotheses remain: one where the interphase nucleus (which is also located in the middle) provides the spatial cue; and one in which the interphase microtubule network sends the signal. In support of the first idea, that the nucleus somehow specifies the site of ring assembly, the division plane usually correlates with the position of the interphase nucleus (34). In multinucleate cells, rings often form at the site of each nucleus (36). Mutants with disrupted interphase microtubule organization (*alp* mutants or certain tubulin mutants) exhibit displacement of both the nucleus and the ring (37) We believe, however, that all of these observations can also be considered in light of the second hypothesis: that the signal originates with the interphase microtubule network rather than with the nucleus. In fact, displacement of the actin ring in a mutant with disrupted interphase microtubules can be interpreted in two ways consistent with this line of reasoning: (i) the interphase microtubule cytoskeleton may position the interphase nucleus (or nuclear-associated structure), and the nucleus may send a signal that positions the ring; or (ii) both the interphase nucleus and the ring may be positioned at the cell middle independently by the interphase microtubule cytoskeleton (Fig. 3).

The hypothesis that interphase microtubules may have a direct role in division site determination in *S. pombe* is especially attractive, given its parallels with the role of microtubules in division site determination in animal cells. The interphase micro-

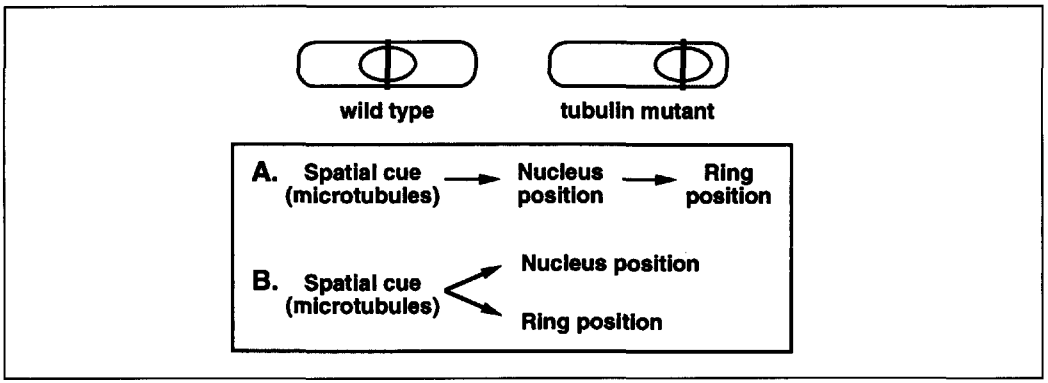

Fig. 3 Two models for positioning the actin ring in *S. pombe*. (A) In the first model, a spatial cue determines the position of the nucleus, which in turn determines the actin ring position. (B) In the second model, a spatial cue independently positions the nucleus and actin ring. The spatial cue for actin ring positioning is thought to originate within the interphase microtubule network.

tubules in fission yeast have certain characteristics similar to the spindle midzone microtubules in animal cells: *S. pombe* interphase microtubules are associated with the cortex, especially near the cell middle, and extend from cell tip to cell tip (38). As described above, mutants that grossly disrupt the interphase microtubule network are defective in positioning the ring. In addition, mutants that have more subtle defects in these microtubules often have multiple rings at the cell middle (F. Verde, personal communication). In analogy to the putative signal in animal cells, we imagine that interphase microtubules may act as tracts for transport of a key cytokinesis protein to the cell middle. These microtubules are required for the placement of the nucleus, and therefore a similar machinery, probably involving motors, may be used to position this putative cytokinesis factor. How interphase microtubules may be used to define the middle is not clear, especially since the polarity of the microtubules is not known. It is possible that the nucleus and spindle pole body somehow organize the microtubules in the proper configuration.

2.2.3 *S. pombe* cdc12p may mark the cleavage site

A candidate protein for a link between the microtubules and ring assembly has been identified in fission yeast. cdc12p is a conserved, large, proline-rich protein component of the cell division ring. It is required for actin ring assembly, and biochemical and genetic interaction evidence suggests that it interacts with the actin-binding protein, profilin (39). As visualized in cells overexpressing cdc12p, cdc12p is located in a discrete spot during interphase that precisely anticipates the position of ring assembly during mitosis (39). Preliminary data suggest that cdc12p may associate with microtubules (F. Chang, unpublished data). These observations support a model in which cdc12p responds to spatial cues from interphase microtubules and/or the nucleus; later, during mitosis, cdc12p specifies the site of actin ring assembly. Further study of the cellular components that position the cdc12p spot will help elucidate the mechanisms of ring placement.

2.2.4 Cells mutant for the *mid1* gene exhibit a specific defect in placement of the ring

Mutations in at least three *S. pombe* genes (*mid1*, *mid2*, and *pos3*) have been identified in which the rings are displaced (31, 40; J. Baehler, personal communication). Thus, these genes are required for proper placement of the division plane. The best characterized is *mid1*, which encodes a novel protein with a nuclear localization signal, PEST sequences (sequences rich in Pro, Glu, Ser, and Thr, which are thought to target the proteins for rapid intracellular degradation) and a pleckstrin homology (PH) protein interaction domain (41). In *mid1* mutants, the microtubules and the position of the nucleus are normal, but the rings are displaced and tilted in a random manner (31, 41). The localization of mid1p is very intriguing: it is located in the nucleus during interphase and is located in early mitosis (possibly even before the arrival of actin) to a ring (41). These properties suggest that mid1p may link the position of the nucleus with the ring. However, the function of the nuclear localization is unknown. Another possibility is that mid1p may have some role in anchoring the ring in the plasma membrane, and the rings are displaced in a *mid1* mutant because they are floating around. The relationships between mid1p, microtubules, and the cdc12p spot remain to be determined.

2.3 Division site determination in *S. cerevisiae*

The budding yeast, *S. cerevisiae*, specifies its division plane in a very different manner from animal cells or fission yeast. In contrast to these other systems, division site determination in *S. cerevisiae* is entirely independent of microtubules and nuclear positioning. Rather, the bud site is determined by two mechanisms, both of which rely on cortical markers placed at a site established by previous divisions or patterns of cell growth. The decision of where to divide is thus made very early in the cell cycle, concurrently with the decision of where to grow, so the molecular mechanisms of cytokinesis and polarized growth in *S. cerevisieae* are quite entangled.

If cell division is not dependent on microtubules, how is cell division coordinated with nuclear division? After the bud site is determined, the actin cytoskeleton orients the mitotic spindle through the spindle pole body during anaphase so that one nucleus enters the bud while the other remains in the mother cell.

The molecular mechanisms of the spatial control of cytokinesis are currently best understood in budding yeast. Analysis of mutants defective in different aspects of bud site selection have lead to the identification of many components of the machinery required to direct the positioning of the bud and has provided a genetic hierarchy for this complex process (for review, see ref. 42).

2.3.1 Haploid and diploid *S. cverevisiae* determine the bud site in different ways

Budding yeast exhibit two patterns of division site placement: haploid cells of mating type **a** or α exhibit an 'axial' pattern, and **a**/α diploid cells exhibit a 'bipolar' pattern.

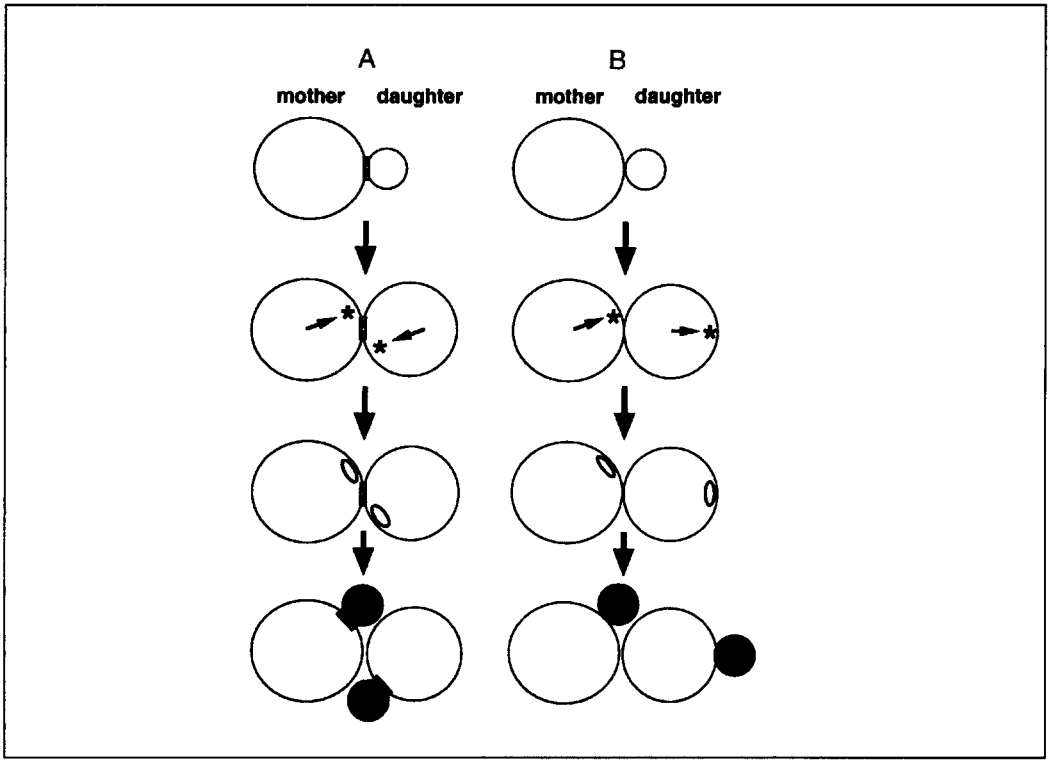

Fig. 4 Localization of division sites in budding yeast. Budding yeast exhibit axial (A) or bipolar (B) budding patterns depending on cell type. **Axial budding** (left): marks (*) are positioned by the septins (striped bar) present at the bud neck between the mother and daughter cells. These marks position the site of ring formation and subsequent new bud formation (black circles) at sites adjacent to the previous division site. **Bipolar budding** (right): marks (*) are positioned at sites of cell growth at the bud tip (distal site) or at the site of division (proximal site). These marks position the site of ring formation and subsequent bud formation (black circles) at sites adjacent to the previous division or at the cell tip.

In the axial pattern, cells always form the next bud adjacent to the site of the previous division. In the bipolar patterns, cells form the next bud at the bud top or at a site of previous division (Fig. 4). What are the spatial cues that direct the bud at these sites? Bud site selection in both cases is independent of microtubules and the mitotic spindle (43, 44). As discussed in the following sections, recent studies have suggested that septins at the bud neck specify the axial pattern, while actin and other proteins may act as positional signals for the bipolar pattern.

2.3.2 In the axial pattern, the septins may provide the original spatial cue

Mutations in genes encoding septin proteins cause a specific defect in axial budding, but not in bipolar budding (45). (See Section 5.3.3 for further information about the biochemistry of septin proteins.) Genetic analysis has identified a number of additional genes required specifically for the axial pattern, including *BUD3*, *BUD4*,

AXL1, and *BUD10* (46, 47). The proteins Bud3p and Bud4p are localized at the bud neck only at the end of the cell cycle during mitosis, and are maintained there until the next bud site has been established (48, 49). The septins may provide a scaffold for positioning Bud3p and Bud4p, since septin mutants disrupt Bud3p and Bud4p localization (48, 49). Thus, the septins position Bud3p and Bud4p, which in turn position the septins for the next cell cycle. Bud10p/AXL2p provides a link to the plasma membrane, since it encodes a glycosylated protein with a transmembrane domain (47, 50). *AXL1* is a key regulator of the budding pattern, since it is only transcribed in haploid cells, and ectopic expression of this gene in diploid cells can change the budding pattern from the bipolar to the axial pattern (51). *AXL1* encodes a protease, but a recent study has shown that the protease function of Axl1p is not required for its role in bud site selection (52). In spite of the complexities of this system, it can be seen that the division site in the axial pattern is dictated by markers located at the previous division site that are positioned on a scaffold of septins.

2.3.3 In the bipolar pattern, actin may be the scaffold for key markers

In diploid cells, buds form either at the previous bud tip (distal site) or at the location of the previous division (proximal site). Actin is located at these two sites during different phases of the cell cycle. Certain actin alleles and mutants in some actin-binding proteins are specifically defective in the bipolar pattern and not in the axial pattern (44). Actin thus may have two roles. First, the actin cytoskeleton may provide the scaffold for positioning specific markers. Second, these markers might in turn organize other actin structures involved in cell polarity and growth. A large genetic screen has identified a number of genes requires for the bipolar pattern (53). Perhaps the most interesting of these genes are *BUD8* and *BUD9*. These are candidates for a proximal marker (Bud8p) and a distal marker (Bud9p), since a *bud8* mutant will only bud at a distal site, and a *bud9* mutant will only bud at a proximal site. How these markers respond to actin or organize actin is still not clear.

2.4 Is a unified view of the control of contractile ring location possible?

Superficially, cells appear to use diverse mechanisms to specify the division plane. However, the basic mechanism underlying cytokinetic signalling may nonetheless be highly conserved. In all systems, there appears to be a signal or marker that is placed at the appropriate location and activates cell division processes. Moreover, as discussed below, the amino acid sequences of many proteins involved in cytokinesis are conserved. These similarities suggest that the actual cytokinetic signal, whatever it is, is in fact the same in many diverse types of cytokinesis. However, the mechanism for placement of this marker is different in different cell types. In both animal cells and *S. pombe*, the signal may be transported via microtubules. In *S. cerevisiae*, the signal often remains at the previous site of division. Genetic screens in a number of organisms that have identified several proteins important in ring assembly or placement may help identify this elusive signal.

3. How do cells know when to begin cleavage?

For a successful cell division, cytokinesis must occur at an appropriate time in the cell cycle; that is, after nuclear division. As with many events in the cell cycle, the cell cycle-dependent kinases (CDKs) and their associated cyclins are thought to play key roles in regulating cytokinesis. A large amount of evidence indicates that in all eukaryotes, cytokinesis usually depends upon the prior initiation of mitosis (reviewed in ref. 30). That is, it appears that cytokinesis will generally only occur if the level of mitosis-promoting factor (MPF) was at one time previously elevated in the cell. Most cytokinetic processes also require the subsequent degradation of MPF. In addition to reading the cell cycle clock, cytokinesis appears to be sensitive to the state of nuclear division, and is thus dependent on checkpoint controls that monitor the mitotic spindle. Finally, in at least fission and budding yeast, cytokinesis occurs in multiple discrete steps, each of which is regulated by different cell cycle signals. The goal is to understand the cell cycle signals that trigger cytokinesis and to identify the cellular targets that respond to these cell cycle signals.

3.1 When is the signal transmitted?

3.1.1 In animal cells, the signal to initiate cytokinesis is probably transmitted after anaphase onset

In those cells where the source of the signal is the spindle midzone, it is probable that the signal is not transmitted to the equatorial cortex until some time after anaphase onset. This is because the interdigitated microtubules of the central spindle are not organized until after anaphase has begun. Strong support for this assumption was recently obtained by Cao and Wang (9). Perforations introduced between the spindle and the equatorial cortex block cleavage if made during metaphase or the first minute of anaphase, but have no such effect later in anaphase. It is important to note that, in animal cells, the possibility that the signal is made (but not transmitted to the cortex) before the onset of anaphase cannot be excluded.

It remains unclear whether the signal from the mitotic apparatus is continuously required throughout cytokinesis. Most classical studies in animal cells indicated that the mitotic apparatus can be removed at some point during anaphase without subsequently interfering with cytokinesis (reviewed in ref. 1). The results of the perforation experiments described above (9) also imply that, once the signal has been transferred to the cortex, no further signalling from the mitotic apparatus is required. In contrast, a continuous need for signalling from midzone microtubules is suggested by the recent finding that furrowing can be reversed even after it has been initiated when all microtubules in the vicinity of the equatorial cortex are eliminated by nocodazole treatment (14).

3.1.2 In yeasts, the initial steps of cytokinesis occur early in the cell cycle

In both fission and budding yeast, the site for division is established well before division itself commences. In *S. cerevisiae*, rings of actin, septins, and cell wall

materials, which specify the bud neck and site of division at the end of mitosis, are deposited at the future division site at START of the cell cycle in late G_1. These events thus are thought to be regulated by Cdc28 and G_1 cyclins at START (54). In *S. pombe*, the actin ring is established in very early mitosis and is dependent on cdc2/cyclin B (MPF) activity (29, 55). Thus, assembly of the actin ring is triggered directly or indirectly by this mitotic kinase. The formation of the septin ring and also the initiation of septation (cell wall formation) occur in late mitosis after anaphase and are thus likely to be triggered by a different set of regulatory signals (34). Finally, cell–cell separation through digestion of the cell wall septum appears to require yet another regulatory step that is poorly understood.

3.2 Cytokinesis and cell cycle controls

3.2.1 Cytokinesis is likely to require inactivation of MPF, but not sister chromatid separation, at the time of anaphase onset

Near the end of mitosis, but before cytokinesis, MPF is degraded and sister chromatids separate; both events are dependent on the 20S anaphase-promoting complex (APC) that appears to degrade by a ubiquitin-based mechanism both cyclin B and the proteins thought to hold the metaphase chromosomes together (56; see also below). As discussed in this section, it appears that MPF degradation, but not sister chromatid separation, is a critical step for the progress of cytokinesis. It should, however, be remembered that some of the earlier steps of cleavage site determination in fission and budding yeasts are almost certainly completed before MPF breakdown.

In the normal cell, the dependence of cytokinesis on degradation of MPF makes considerable sense, as it ensures that cytokinesis will generally not occur until the chromosome complements have begun to separate. The evidence for this idea is based on the effects of manipulations that prevent MPF degradation. Expression of indestructible cyclin B inhibits anaphase and septum formation in *S. pombe* (57), and causes a telophase arrest in *S. cerevisiae* (58). *Xenopus* embryos and tissue culture cells injected with an undegradable form of cyclin B fail to cleave (59, 60). Cytokinesis does not occur in tissue culture cells injected with anti-APC antibodies (61), or in budding yeast with mutations in APC components (62); in both cases, cyclin B degradation does not occur. It should be cautioned that, in all of these cases, the blockage of MPF degradation could affect cytokinesis only indirectly.

Although prior entry into mitosis is required for cytokinesis, successful completion of all aspects of mitotic nuclear division is not. Sister chromatid separation, the hallmark of anaphase onset, is not a strict prerequisite for cytokinesis. Even if all of the cell's chromosomes are removed before the onset of anaphase, cytokinesis can still occur in animal cells (26). Furthermore, in *S. pombe* carrying mutations in the *cut* series of genes (63, 64), and in *S. cerevisiae* mutant for the gene *PDS1* (65, 66), cytokinesis occurs in the absence of sister chromatid separation. It is curious that additional checkpoint controls to ensure the dependence of cytokinesis on prior sister chromatid separation appear not to have evolved.

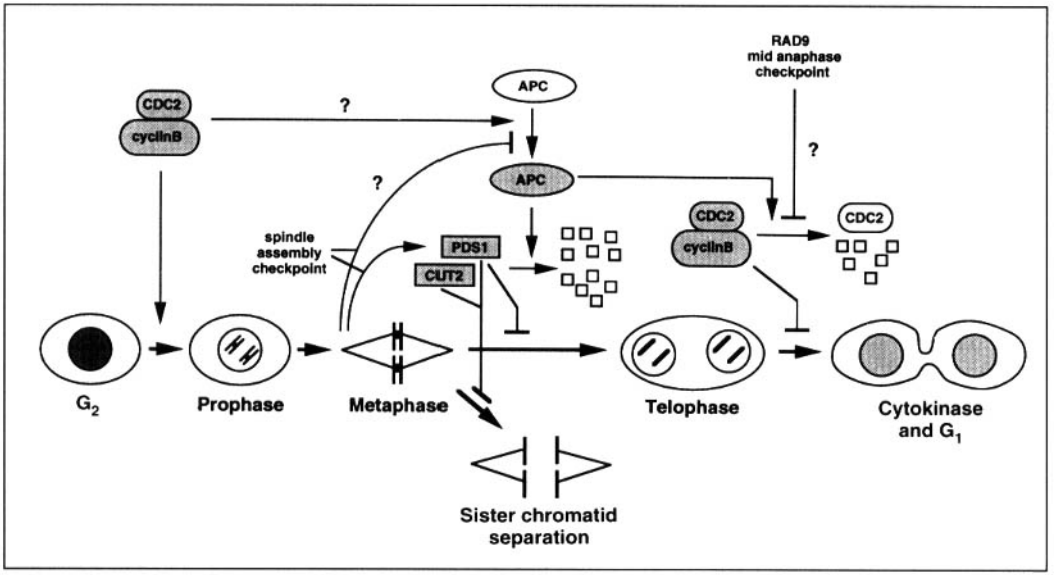

Fig. 5 Cytokinesis and the cell cycle. Active (shaded) forms of MPF (i.e. CDC2 + cyclin B) are thought to activate the anaphase promoting complex (APC), which in turn degrades the cyclin B component of MPF. Degradation of MPF is required for cytokinesis. The APC also degrades *S. pombe* Cut2p and *S. cerevisiae* Pds1p, allowing sister chromatid separation. Degradation of Pds1p is also required in an unknown way for cytokinesis, perhaps because of the participation of this protein in a spindle assembly checkpoint. This figure may be complicated, but is nonetheless oversimplified. For example, mutations inactivating the Cut2p and Pds1p proteins in fact prevent entry into anaphase, presumably because they are required for some previous step not shown here. (Redrawn, with major modification, from ref. 56.)

The results discussed in this section are consistent with current models (56, 67) for the control of events at and subsequent to anaphase onset (Fig. 5). These models suggest that the APC must be activated by MPF, explaining why cells must normally have entered mitosis at some earlier time in order for cytokinesis to take place. Moreover, these models predict that there are at least two classes of substrates for the APC: cyclins such as cyclin B (which must be degraded for cytokinesis to occur), and non-cyclin molecules that specifically inhibit sister chromatid separation until they are degraded by the APC. These predictions appear to be correct: cyclin B degradation does involve APC activity (56, 62), and two APC substrates in this latter class—the products of the *S. cerevisiae* gene *PDS1*, and the *S. pombe* gene *cut2*—have been identified (63, 68). The existence of APC substrates needed specifically for sister chromatid separation but not for other processes would explain the independence of events such as cytokinesis from sister chromatid separation. As anticipated by the model, *S. pombe* cells expressing an undegradable form of cut2p fail to separate their sister chromatids but do undergo cytokinesis (63). However, undegradable forms of Pds1p block both processes (68). This latter result, which does not fit so easily into the hypothesis, may potentially be explained by the participation of Pds1p in cell cycle checkpoints (see below).

3.2.2 Kinases of the polo family may trigger cytokinesis

The polo serine/threonine kinase family has been implicated in multiple events in the regulation of mitotic events (69) and recent evidence suggests that it may directly activate cytokinesis (see also Chapter 3). In *S. pombe*, depletion experiments show that loss of *plo1* function causes defects in mitotic spindle formation and also abolishes the entire septation formation pathway, including deposition of septal material and formation of the actin ring (32). Remarkably, overexpression of *plo1* drives cells to form a septum (and presumably an actin ring first), even in interphase cells with low cdc2 activity (32). Thus, overexpression of *plo1* overcomes the normal requirement for $p34^{cdc2}$ activation in the control of septation, suggesting that *plo1* normally acts downstream of MPF for cytokinesis. Budding yeasts carrying mutations in the polo family gene *CDC5* are also defective in cytokinesis (69).

The importance of the *Drosophila* and mammalian members of the polo family for cytokinesis has not been established. For example, mutants of the *Drosophila polo* gene accumulate polyploid cells, but this may be an indirect consequence of earlier spindle aberrations that are also caused by the mutations. Cytokinesis defects were not observed when anti-human Plk1 (polo-like kinase 1) antibodies were injected into human cells already in metaphase (70). Nonetheless, two observations point to a cytokinetic role for the *Drosophila* and mammalian proteins. First, the kinase activity of *polo* appears to be highest during late anaphase and early telophase in syncytial *Drosophila* embryos (71), though this is not the case in mammalian cells (71, 72). Second, mammalian polo-like kinase shifts location from the centrosome, where it is found from interphase through metaphase, to the midzone of the central spindle during anaphase and telophase (71, 72). This pattern is reminiscent of the 'passenger proteins' described in Section 2.1.6; in fact, it has been reported that mouse polo-like kinase interacts *in vivo* with, and phosphorylates *in vitro*, the kinesin-like motor protein CHO1 (72).

3.2.3 Other genes may act with *plo1* to regulate cytokinesis and septation in *S. pombe*

The Plo1p kinase is not the only protein whose overexpression can drive some or all aspects of cytokinesis in fission yeast, although it differs from all others in also being involved in regulating spindle pole body separation. Overexpression of the *spg1* gene of *S. pombe* also leads to septation at any stage of the cell cycle (192), but the relationship between *spg1* and *plo1* remains to be explored. The *spg1* gene encodes a small GTPase of the Ras superfamily that seems to form a complex with, and is postulated to activate, another protein kinase encoded by $cdc7^+$ (192). The Cdc7p kinase is very similar to the product of the *S. cerevisiae CDC15* gene, which is also needed for cytokinesis. Overproduction of Cdc7p results in multiple septum formation, although this does not happen in G_2-arrested cells (74). The *cdc11* and *cdc14* genes are also required for septation. As with *cdc7* mutants, cells mutant for these genes form an actin ring, but do not septate and the ring disappears (73). Mutations in the *byr4* (75) and *cdc16* (30) genes have the reciprocal phenotype, loss of

function leading to multi-septation. Thus the wild-type gene products act as negative regulators of the septation pathway. Some homology of the Cdc16p sequence to a family of GTPase-activating proteins (GAPs) has led to the suggestion that Cdc16p may be the GAP that regulates Spg1p (192). Of the set of *S. pombe* genes required specifically for the formation of the actin ring, *cdc15* appears to be near the head of the hierarchy (33). Cdc15p contains a Src homology domain 3 (SH3) and its over-expression will induce ring formation during interphase. However, only actin ring formation and not septation is induced. Once again, the regulatory interactions of Cdc15p with the septum-forming pathway and with Plo1p kinase remain to be resolved.

3.2.4 Cytokinesis is sensitive to the spindle assembly checkpoint

Normally, cells treated with drugs that interfere with microtubule function arrest before anaphase, due to action of a spindle assembly checkpoint that appears to be driven by signals from unattached kinetochores (reviewed in refs 67 and 76). As discussed in Chapter 1, a series of genes in *S. cerevisiae* has been implicated in this spindle assembly checkpoint; these include the genes *MPS1*, *BUB1–3*, *MAD1–3*, and *PDS1*. Wild-type cells with a checkpoint activated by drug treatment retain high levels of MPF activity and, probably for this reason, do not attempt cytokinesis. The exact relationship between the checkpoint genes, MPF, and APC, and cytokinesis is currently unclear and may be quite complicated and interesting. As an example, mutations in the *S. pombe cdc16* gene, which is a homolog of *BUB2*, can cause a multi-septation defect (30). In addition, the budding yeast gene *PDS1* appears to be a key effector of both the spindle assembly checkpoint and sister chromatid separation. Absence of *PDS1* function permits cytokinesis, while expression of a non-degradable form of the product of this gene prevents cytokinesis (65, 66, 68). Both *cdc16* and *PDS1* point to cellular mechanisms that can coordinate the mitotic spindle and cell cycle state with cytokinesis.

In *S. cerevisiae*, cytokinesis is also sensitive to a later, mid-anaphase checkpoint that monitors chromosome integrity even after sister chromatid separation. This checkpoint is dependent on the *RAD9* gene, which stops cell cycle progression if damaged DNA is present. It appears that this mid-anaphase checkpoint operates by preventing the degradation of a pool of MPF that remains after anaphase onset (77).

4. What events link cytokinetic initiation signals with elaboration of the contractile ring?

Two types of intracellular cell signalling pathways—one dependent upon calcium ion flux and the other involving Rho family G proteins—have been implicated in the control of cytokinesis. We regard these pathways as signal transduction mechanisms, though the relationship between these pathways, the cytokinetic initiation signal, and events at the contractile ring remains unclear. Transmission of the cytokinetic signal will affect the activity of proteins at the cleavage furrow. For the most part, the

nature of these target proteins is unknown, but we discuss some possibilities based on current knowledge of other cell surface events.

4.1 Calcium in cytokinetic signal transduction

4.1.1 Transient increases of calcium ion concentration near the cleavage furrow help regulate cytokinesis

A role for calcium in the control of cytokinesis is suggested by several observations. The injection of calcium or treatments with the Ca^{2+} ionophore A23187 can stimulate cytokinesis, while the injection of EGTA or the calcium buffer BAPTA (both of which sequester Ca^{2+}) inhibit cytokinesis (reviewed in ref. 78). Using the photoprotein aequorin to visualize calcium ions, Fluck *et al.* (79) found evidence for a micromolar calcium wave that advances slowly just in front of the cleavage furrow. Similar results in zebrafish embryos were obtained more recently by Chang and Meng (80). Finally, expression of calmodulin antisense RNA in *Dictyostelium* inhibits the completion of cytokinesis (81).

One possible source of the calcium ions in the transient is the endoplasmic reticulum, potentially via calcium channels regulated by inositol triphosphate (IP_3). Support for this hypothesis includes the observations that the calcium signal is blocked by an antagonist of IP_3 receptors (80); and that IP_3 and calcium appear to oscillate in tandem (82). If the increase in calcium is indeed directed by IP_3, then there are many possible ways by which the cytokinetic signal could cause local increases in IP_3, including effects on the kinases needed to make phosphatidylinositol-4, 5-bisphosphate (PIP_2), on the phospholipases that cleave PIP_2 into diacylglycerol and IP_3, and on the enzymes that hydrolyze IP_3 or convert it into other compounds. In accordance with this idea, mutants in *PIK1*, a yeast gene encoding phosphatidylinositol-4-kinase (which is in the PIP_2 synthetic pathway) are defective in cytokinesis (83).

4.1.2 What are the possible targets for the increased levels of Ca^2+ that would stimulate the cortical events associated with cytokinesis?

Several proteins involved in cytokinesis may be activated by Ca^{2+} or Ca^{2+}/calmodulin. For example, myosin light chain kinase (MLCK), which catalyzes an activating phosphorylation of myosin light chain (see below), is one of many Ca^{2+}/calmodulin-dependent kinases. In addition, Ca^{2+} can directly affect the activity of actin-binding proteins such as gelsolin (84). Other direct and indirect targets of calcium signalling undoubtedly remain to be found.

4.2 The role of Rho-family G proteins in cytokinesis

4.2.1 Rho-family G proteins initiate signalling cascades that regulate contractile ring formation

The small GTP-binding proteins of the Rho/Rac/Cdc42 G-protein superfamily have emerged as key regulators linking cellular signalling cascades to control of the actin

cytoskeleton in many cellular processes including cytokinesis. In budding yeast, Rho proteins have been implicated in a regulatory hierarchy that determines the organization and placement of the cytoskeleton for budding and cell division. In mammalian cells, Rho, Rac, and Cdc42 can each be activated by different extracellular signals that promote formation of a distinct actin-dependent structure in a given cell type. In fibroblasts, for example, Cdc42 induces filopodia, Rac induces lamellipodia and membrane ruffling, and Rho leads to the formation of stress fibers and focal adhesions (85–87). G proteins cycle between (active) GTP- and (inactive) GDP-bound states, and their activity can likewise be modulated by an array of factors such as GAPs, guanine nucleotide exchange factors (GEFs), and GDP dissociation inhibitors (GDIs). Post-translational modifications of Rho proteins with lipids that target these G proteins to the membrane are also essential for function (88, 89).

Given the known involvement of Rho-family G proteins in controlling the actin cytoskeleton, it is not surprising that evidence for the importance of Rho-family proteins in the regulation of cytokinesis in a variety of systems is now accumulating. The lines of evidence are as follows.

(a) Several Rho-family proteins have been shown to be important for polarization of the cytoskeleton and budding in *S. cerevisiae* (see below).

(b) A genetic screen for cytokinesis defective mutants in *Dictyostelium* has identified a new member of the Rho gene family, *racE*. This gene is required specifically for cytokinesis and no other cell motility event (90).

(c) There is evidence that Rho is translocated to sites of active actin reorganization, including the cleavage furrow (e.g., Myc-tagged RhoA is localized to the cleavage furrow in dividing Swiss 3T3 cells) (91).

(d) Treatments that inactivate Rho (for example, the addition of *Clostridium botulinum* C3 exoenzyme, which specifically ADP-ribosylates Rho, or addition of *rho* GDI inhibitory GDP/GTP exchange factor, which traps Rho in an inactive GDP-bound form) disrupt furrowing and result in loss of contractile ring F-actin in dividing *Xenopus* and sand dollar eggs before or during furrowing (89, 92).

(e) In cleaving *Xenopus* eggs, cytokinesis is blocked by constitutively active or dominant negative forms both of Cdc42 and Rho (93).

Taken together, these results suggest that Rho activity is required both for initiation and maintenance of furrowing, that Rho acts to sustain the integrity of the F-actin-based contractile ring apparatus during cell cleavage, and that cycling of Rho and Cdc42 proteins between active and inactive forms is critical. It should be noted in this context that the role of particular Rho-type G proteins in cytokinesis may not be universal. In *S. pombe*, mutants in genes encoding homologs of Cdc42 and Rho are defective in aspects of actin organization, polarized growth, and septum wall formation, but do not have defects in actin ring formation.

There are several complications in assessing the precise role of Rho-family proteins in cytokinesis. Rho-type G proteins can cause many kinds of changes in the

actin cytoskeleton at the cell surface, so some of the effects of the disruption or inappropriate activation of Rho functions may be the indirect result of cortical disturbances at sites distinct from the contractile ring. Individual Rho-family proteins may initiate more than one type of signalling cascade inside the cell that affect not only the actin cytoskeleton, but may also influence transcriptional regulation, control of cell cycle progression, and even vesicular traffic (see ref. 94). Different downstream responses can apparently be achieved by direct interaction of a single Rho-family protein with separate effector molecules (95). Some effectors seem to be specific for a particular Rho family member, while others are relatively promiscuous (96, 97). Finally, cross-talk between pathways initiated by different Rho family proteins coordinates changes in the actin cytoskeleton [e.g., (98)]. For example, activation of Cdc42 subsequently results in the appearance of Rac- and Rho-dependent structures as well (87). The complexity introduced by the function of Rho-family proteins as major centers for the integration of different types of information in the cell that determine a variety of cellular responses will make it difficult to untangle precisely how these proteins are involved in any one process, such as cytokinesis.

4.2.2 Rho-family G proteins are likely to have several targets in cytokinetic signal transduction

Even though the specific pathways through which Rho-family proteins act have not yet been completely defined, it is clear that their effects on the actin cytoskeleton are multifaceted. Rho-family proteins appear to be directly involved in regulating actin dynamics and the nature of actin cross-linking, as well as the assembly and maintenance of attachments (presumably including those at the contractile ring) between the actin cytoskeleton and membrane structures (reviewed in ref. 99). The number of potential targets for Rho-family proteins is rapidly expanding (96). Several of these targets can be linked either directly or indirectly to effects on the actin cytoskeleton (Fig. 6), but in most cases, the relevance of these targets to cytokinesis has not been unambiguously demonstrated to date.

Other Rho proteins

Results from genetic studies in *S. cerevisiae* indicate that several Ras- and Rho-related G proteins and their associated modulatory factors must be connected in a hierarchical cascade to control bud site selection and to organize actin and septins at the new bud site (see ref. 98 and references therein). The Ras-related Bud1/Rsr1 GTPase, as well as Bud5p (its GEF) and Bud2p (its GAP), are at the top of the hierarchy. Mutations in the genes encoding these proteins cause a randomization of bud site placement but do not affect polarization or cytokinesis. This bud site machinery is thought to activate the Rho-like Cdc42p in a specific region of the cell. Bud1p/Rsr1p may activate Cdc42p activity by binding to Cdc24p, a GEF for Cdc42p (100, 101). Cdc42p in turn polarizes growth and induces both actin and septin ring assembly at the new bud site. One probable downstream target of Cdc42p is Rho1p, a yeast homolog of mammalian RhoA, which is also required for polarized growth and co-localizes with clusters of cortical actin patches at the bud neck and tip (88). The

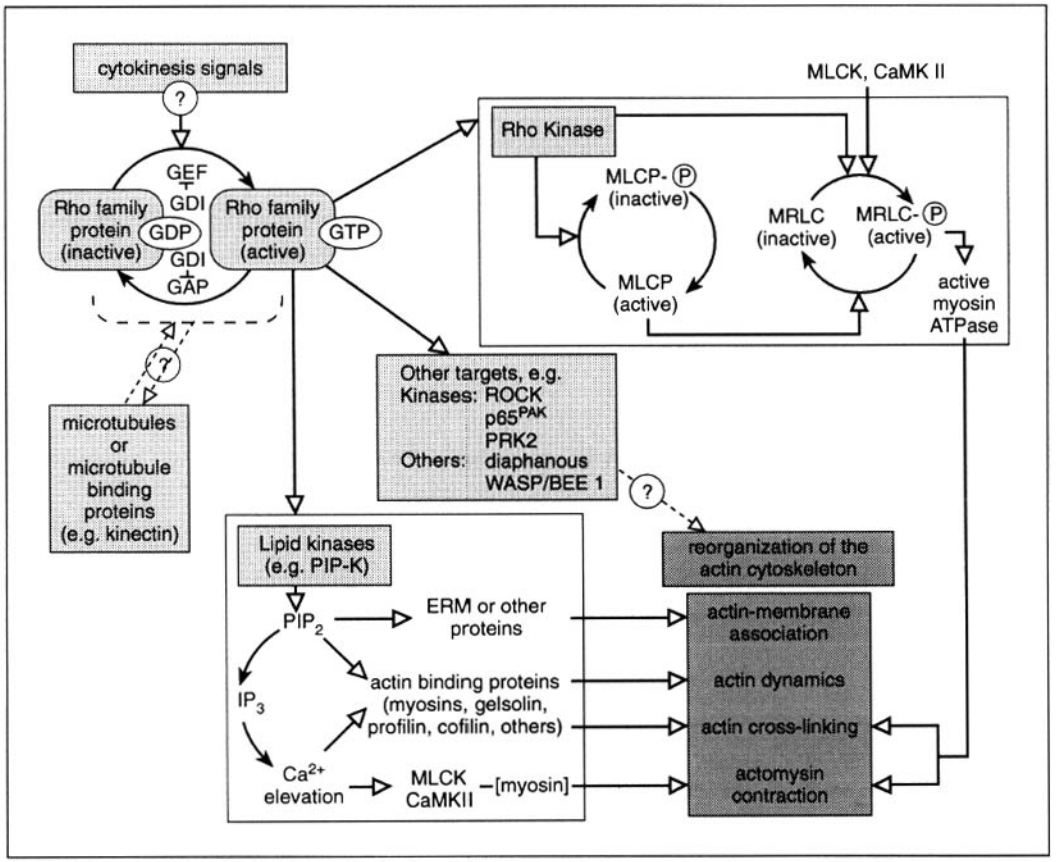

Fig. 6 Possible mechanisms by which Rho-family proteins might control the assembly and function of actin in the contractile ring. Molecules transducing the cytokinetic signal activate particular Rho-family members by unknown means. Activated Rho proteins then regulate actin in the contractile ring indirectly, through one or more of several possible targets (see text for details). This diagram is meant only as a framework for conceptualizing the influence of Rho proteins on cytokinesis. Although many of the interactions depicted here are known to be relevant to other actin-dependent processes, and although many of these molecules are clearly important for cytokinesis, the pathways mediating the organization of actin in the contractile ring have not in fact been defined.

localization of Rho1p appears to be under the control of *CDC42*, since it is disrupted in *cdc42* mutants. Similar hierarchies may operate in cytokinesis in other organisms as well.

Serine-threonine kinases

Recent work has identified a Rho-associated serine-threonine kinase (Rho-K / ROKα) activity downstream of Rho whose kinase domain is closely related to that of myotonic dystrophy kinase (see ref. 102 and references therein; Fig. 6). Activation of Rho-K by Rho–GTP has been found to induce elevated myosin motor activity via a bipartite mechanism: Rho-K phosphorylates the myosin regulatory light chain (RLC or MRLC) itself, and phosphorylates myosin light chain phosphatase (MLCP), thus

inactivating the phosphatase that would remove the RLC phosphorylation (103, 104; reviewed in refs 94 and 96). The target on RLC for both MLCP and Rho-K is serine 19, the same site at which myosin light chain kinase (MLCK) acts. As discussed below, phosphorylation of this serine is required for the activation of myosin ATPase and for the formation of bipolar myosin filaments that are competent to interact with actin. A close relative of Rho-K, p160 Rho-associated coiled-coil-containing protein kinase (ROCK), has been placed in pathways leading to Rac-dependent lamellopodial formation as well as Rho-dependent focal adhesion formation (95, 105).

Another serine/threonine kinase target of Rho-family G proteins is $p65^{PAK}$ (106). Although much work on $p65^{PAK}$ has emphasized its role in Rac- and Cdc42-dependent transcriptional regulatory cascades (95, 107, 108), studies in yeast, *Drosophila* (109) and human cells (110) indicate a role for these proteins in actin-dependent events, including cytokinesis (Fig. 6). Of particular interest in this context, *S. cerevisiae* simultaneously mutant for *CLA4* and *STE20*, both of which encode homologs of $p65^{PAK}$, are defective in cytokinesis and cannot maintain septin rings at the bud neck (111).

Most recently, a p140 kinase (PRK2) has been reported that interacts with Rac and Rho and appears to mediate their effects on the actin cytoskeleton (112).

Lipid kinases

Control of lipid metabolism may be a major route by which Rho proteins regulate actin assembly on the membrane (Fig. 6). Rho has been reported to stimulate phosphoinositide (PI) turnover and to regulate the activities of several phospholipid kinases and phosphatases (113). In particular, Rho has been shown to stimulate phosphatidylinositol-4-phosphate 5-kinase (PIP kinase), whose activity results in the formation of PIP_2 (114) and can lead to increased actin polymerization (115). PIP_2 is a precursor of IP_3 which, as mentioned above, causes release of Ca^{2+} from intracellular stores upon binding to its receptor on the ER.

PIP_2 is also known to affect actin polymerization by regulating the activity of a variety of actin-binding proteins. PIP_2 can inhibit actin binding of actin-capping protein, gelsolin, profilin, and cofilin (84). These combined effects could lead to increased actin polymerization by uncapping existing filaments and by releasing additional monomers from sequestration (116). In support of this line of reasoning, activation of Rac can cause uncapping of actin filaments through PI synthesis (117).

The attachment of actin to the membrane can also be regulated in a Rho-dependent manner, and this may occur at least in part through the influence of PIP_2 on proteins of the ezrin/radixin/moesin (ERM) family (118). ERM proteins are thought to act as bifunctional linkers between actin and transmembrane glycoproteins such as CD44 or CD43. The binding of ERM proteins to transmembrane partners is enhanced significantly by PIP_2 and is correspondingly regulated by Rho both *in vivo* and *in vitro* (113). ERM proteins as well as CD43 have been reported to localize to the cleavage furrow (119, 120), and Myc-tagged Rho co-localizes with ERM proteins, including in the cleavage furrow (91). However, as discussed below, the importance of ERM proteins to the contractile ring is currently controversial.

The Bni1p/cdc12p/Diaphanous protein family

The budding yeast protein Bni1p is a member of a newly identified family of large proline-rich proteins implicated in cytokinesis and other actin-mediated processes such as cell fusion and cell polarity. Members of the family necessary for cytokinesis include *S. pombe* cdc12p (39), *Drosophila* Diaphanous (121), and *Aspergillus* sepA (122) proteins. Whereas these other members are absolutely required for cytokinesis and viability, *S. cerevisiae bni1* mutants are viable but have defects in the morphology of the bud neck and partial defects in cytokinesis (123, 124). In addition, Bni1p has other functions such as actin organization in projection formation during mating, and the localization of a determinant Ash1p on the daughter bud. Both two-hybrid and biochemical evidence suggests that Bni1p interacts with the GTP-bound form of Rho1p (123). A synthetic lethal interaction between *bni1* and *rho1* mutations further indicates that these proteins may interact *in vivo*. Bni1p has also been shown to bind to other Rho proteins including Cdc42, as well as to the actin binding proteins profilin and Bud6p (124; see also below). In addition, it interacts genetically with a septin (53). Thus, Bni1p is a candidate for linking Rho proteins to actin organization during cytokinesis (Fig. 6). It remains to be tested whether members of the Bni1p/cdc12p/Diaphanous family other than Bni1p also respond to Rho proteins; it will also be of interest to determine whether interactions of this protein family with actin-binding proteins are important for cytokinesis.

Other proteins

Additional targets of Rho-family proteins in cytokinesis undoubtedly remain to be found, possibly including components of the actin cytoskeleton. One potential example is the human Wiskott–Aldrich syndrome protein, a component of the actin cytoskeleton of unknown biochemical function that has been linked to changes in actin polymerization in response to Cdc42Hs (125, 126). A gene encoding a related protein has just been discovered in *S. cerevisiae* gene (*BEE1*); disruption of this gene causes defects in budding and cytokinesis that appear to reflect the absence of cortical actin patches in the bud (127).

Rho-family proteins may also be able to interact with microtubule-based cytoskeletal elements. RhoA, Rac1, and Cdc42 have all been found to bind in the yeast two-hybrid system to kinectin, a protein that anchors vesicle membranes to the plus-end-directed microtubule motor, kinesin (128). Rac1, but not RhoA or Cdc42Hs, was recently reported to bind directly to tubulin (129). The *in vivo* significance of these activities is not yet understood. However, it is tempting to speculate that a Rho protein could 'hitch a ride' with kinesin to the spindle midzone through its interaction with kinectin, landing it in the right place at the right time to carry out its functions in cytokinesis.

5. How is the contractile ring assembled and how does it function?

The contractile ring can be viewed as a large cortical complex consisting of actin, myosin, septins, and other proteins that organize these cytoskeletal elements. It is

usually assumed that the contractile ring is anchored to the cell surface by membrane-associated proteins. The contractile ring is likely to share several similarities with other cortical-actin structures in the cell such as focal adhesions and their associated stress fibers. An important unsolved question is whether there are proteins that are specific for cytokinesis that do not have roles in other cellular processes. Although some components of the contractile ring have been identified, little is known about their functions *in vivo*, the protein–protein interactions involved in building the ring, and the order in which they assemble.

5.1 Actin at the cleavage furrow

The importance of actin to cytokinesis has long been recognized, and is based on several observations. (a) In almost all cell types, actin is concentrated in a ring at the site of division. (b) Treatment with anti-actin drugs such as cytochalasin blocks cytokinesis (see ref. 21 for a discussion of what were until recently regarded as exceptions to this rule). (c) Actin (*act1*) mutants of budding yeast form large multinucleate cells with cytokinesis and polarity defects (130). (d) Mutations in genes encoding a number of actin-associated proteins in several organisms disrupt cytokinesis (see below).

5.1.1 Pre-existing actin filaments move along the cell cortex to the site of actin ring formation

In a series of experiments following the distribution of rhodamine-labelled phalloidin or rhodamine-labelled actin microinjected into dividing cells, Cao and Wang (131) observed that actin filaments that existed prior to the onset of cytokinesis became concentrated in the contractile ring, while actin filaments assembled *de novo* during cytokinesis were distributed throughout the cortex. In later experiments employing similar techniques (132), these same authors found that pre-existing actin filaments become associated with the cortex in mid-metaphase, and then appear to flow in the plane of the cortex to concentrate at the contractile ring. These findings indicate that the contractile ring includes pre-existing actin filaments which migrate around the cortex toward the cleavage furrow. These results are of potential importance in interpreting the cytokinesis-defective phenotype associated with several kinds of actin-binding proteins (see below). Such phenotypes may not be the direct result of the absence of action of these proteins at the contractile ring, but may rather be an indirect consequence of an inability of actin filaments in the mutant to form or to move around the cortex toward the equator.

5.1.2 Very little is known about what anchors actin to the plasma membrane at the site of the contractile ring

Some cortical proteins that mediate attachments of actin filaments to the membrane in other situations, such as ERM proteins, spectrin, and α-actinin, are present and perhaps concentrated at the cleavage furrow (reviewed in refs 78 and 133). It has

also been suggested that septin proteins may form a scaffold to direct organization of the contractile ring. However, as discussed below, there are some reasons to question whether these molecules do participate in the anchoring of actin to the ring. Other proteins that appear to arrive at the site of ring formation before actin, such as mid1p in *S. pombe* (41) and (probably) INCENPs in mammalian cells (16), must also be considered as potential candidates for a role in mediating the connection between the actin ring and the underlying cell surface.

5.1.3 Recent observations challenge the purse-string model for actin filament organization and function at the contractile ring

The 'purse-string' model postulates that cytokinesis is caused by the contraction of a circumferential ring composed of actin and myosin II filaments (Fig. 7). Interaction of oppositely polarized actin filaments, anchored to the equatorial cortex at different positions, with bipolar myosin II filaments allows for the generation of tension, much as in a muscle cell. Because the actin filaments are in some fashion anchored in the cell membrane, contraction of the ring acts like tightening of a purse string, leading to constriction of the cell, which is pinched in two (reviewed in ref. 78).

Although the contractile ring contains equatorially-arranged bundles of actin, as predicted by the purse-string model, it also includes actin filaments that are in several other orientations (133, 134). Some filaments associate end-on with the membrane and point into the cytoplasm, while others lie flat along the cortex, but are parallel to the spindle axis (Fig. 7). These unusual filament orientations suggest additional ways in which cytokinesis could be achieved. For example, traction forces applied to the membrane-associated end-on bundles would pull the membrane

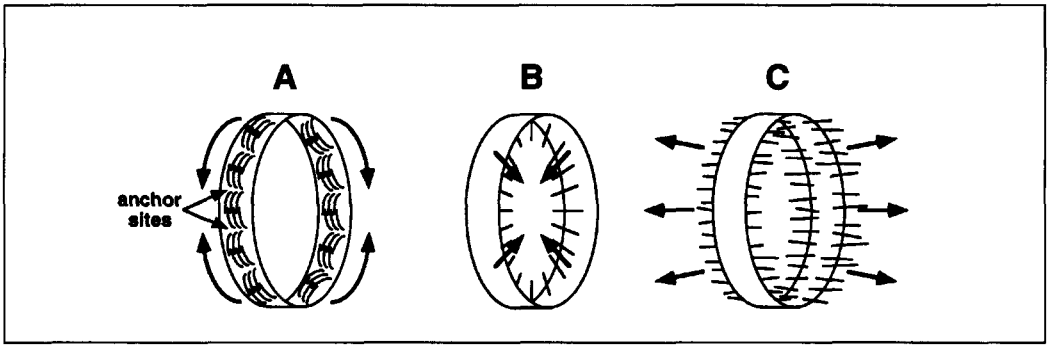

Fig. 7 Observed orientation of actin filaments at the contractile ring. (A) Circumferential bundles of actin filaments around the contractile ring, as anticipated by the purse-string model. Bipolar myosin II filaments (not shown) allow actin filaments anchored by their barbed ends at different cortical sites to interact; the myosin thus applies tension to the cell membrane and constricts the cell at the contractile ring. (B) Some actin filaments have been observed at the contractile ring that point into the cell interior toward the spindle midbody. Forces exerted by these filaments would pull the cell membrane inward, though it is not clear what these filaments might pull against. (C) Some actin filaments in the ring are seen to extend along the spindle axis. Forces from these filaments would help split the cell. All of these types of actin filaments might play a role in cytokinesis. (Adapted from refs 78 and 133.)

toward the spindle midbody. Other investigators have proposed that the 'telophase disc' would allow such inwardly-directed contraction (19). Yet another possibility is 'isotropic contraction', in which sliding of a random network of actin filaments at the cortex would simply make the equatorial region shrink (133).

5.1.4 Genetic evidence suggests that several actin-binding proteins regulate cytokinesis by effects on actin polymerization or bundling

Mutations in genes encoding several actin-associated proteins in various organisms cause defects in cytokinesis. They illustrate that several kinds of perturbations in the actin cytoskeleton can disrupt cytokinesis. Many of the observed effects could well be indirect, particularly in cases where the protein in question does not clearly localize to the contractile ring. Consequently, it is possible that defects in cytokinesis arise through an inability to disassemble pre-existing cytoskeletal structures.

Cofilin

Proteins in the cofilin/actin depolymerizing factor (ADF) family have actin severing and depolymerizing activity, and can bind to actin filaments and monomers (see ref. 159 for review). Recent studies point to the importance of these proteins in accelerating the rate of actin filament turnover in living cells (160, 161). Cofilin mutants in yeast exhibit cytokinesis and polarity defects associated with a stabilization of the actin cytoskeleton (162). Injection into cleaving *Xenopus* eggs either of antibodies against a *Xenopus* cofilin (XAC) or of a constitutively active form of the protein causes regression of the cleavage furrow (163). *Drosophila* carrying hypomorphic mutations in the *twinstar* gene (a cofilin/ADF homolog) show frequent failures in cytokinesis during mitosis in larval neuroblasts and during meiosis in spermatocytes (164). In *twinstar* mutant spermatocytes, actin accumulates at the correct time and place to assemble a contractile ring, but instead forms large aggregates that are morphologically abnormal and fail to disassemble at the time of cleavage (see also below). Cofilin has been found to localize in the vicinity of the contractile ring or cleavage furrow in *Xenopus* embryos (163) and tissue culture cells (165). This localization appears to depend on its ability to interact with actin, as a inactivatable cofilin derivative fails to accumulate in actin-rich regions of the cell (166). Taken together, these observations suggest that cofilin may play a role in the correct organization of F-actin in the contractile ring as well as in F-actin disassembly at cleavage, and that cycling between active and inactive forms is essential for its proper function. *In vitro*, cofilins are influenced by pH, the presence of other phospholipids, other actin-binding proteins, and by an inactivating phosphorylation, providing several ways in which cofilin activity in cytokinesis could be regulated (reviewed in ref. 159).

Profilin

Profilin is a small actin-binding protein that can affect actin filament assembly in numerous ways. Profilin can sequester actin monomers, inhibiting F-actin polymerization, but it can also promote F-actin assembly by lowering the critical

concentration of actin or by catalyzing nucleotide exchange on actin (167–169). This dichotomy reflects different effects on actin filaments with barbed ends that are either capped or uncapped (168). Profilin is also known to bind strongly to PIP_2, suggesting that this actin-binding protein may interact with signal transduction pathways (169).

Mutations in profilin genes are associated with cytokinesis defects in *S. pombe*, *S. cerevisiae*, *Dictyostelium*, and *Drosophila* (170–173). In fly profilin mutants, actin rings do not form at the presumptive site of cleavage (M. Gatti, personal communication), suggesting that profilin plays an active role in F-actin polymerization at the actin ring. However, because abnormal actin aggregates accumulate in these cells, it is also possible that profilin normally acts by sequestering monomers, thereby preventing inappropriate polymerization. Consistent with the first of these possibilities, profilin is concentrated near the cleavage site, though in a less diffuse pattern than F-actin itself, at least in *S. pombe*, *Tetrahymena*, and *Drosophila*.

There is increasing evidence that profilin interacts with the Bni1p/cdc12p/Diaphanous family of cytokinesis-essential proteins described in Section 4.2.2 above. These proteins bind directly to profilin via a conserved proline-rich region (the FH1 domain; e.g. see ref. 39). Mutations in *cdc12* family genes and in profilin genes in several organisms show genetic interactions consistent with the idea that such binding may be physiologically relevant (39). Since *cdc12* is required specifically for ring formation, the cdc12 family of proteins may help localize or somehow alter the activity of profilin to induce actin polymerization at the site of actin ring assembly.

Tropomyosin

Tropomyosin is a coiled-coil protein that forms rods binding along the length of, and presumably stabilizing, actin filaments. The *S. pombe cdc8* gene product (a tropomyosin homolog) is required for actin ring formation and is located at the actin ring (174). Tropomyosin may be required for the formation of long actin cables present in the contractile ring.

Coronin and cortellexins

Mutations in certain *Dictyostelium* cytoskeletal proteins have been reported to disrupt cytokinesis. Coronin is an actin-binding protein with WD repeats and with some similarity to the β-subunits of trimeric G proteins. During separation of wild-type daughter cells, coronin is depleted from the cleavage furrow and is instead strongly enriched at the leading edges of the distal portions of the daughter cells (175). This protein might thus help generate traction forces to separate daughter cells independent of contractile ring function.

Cortexillins I and II are related *Dictyostelium* proteins in the α-actinin/spectrin superfamily that can cross-link and bundle actin filaments. Elimination of both isoforms by gene disruption gives rise to multinucleate cells due to cytokinesis defects (176). Although these proteins accumulate in cortical regions, specific localization in the vicinity of the cleavage furrow was not reported.

5.2 The role of myosin in cytokinesis

Cytoplasmic myosin II, the cytoplasmic (non-muscle) form of the classical long-tailed myosin, has long been assumed to be the molecular motor of cytokinesis. The tight regulation of the motile activity and multimer assembly of myosin resulting from the phosphorylation of key amino acids makes myosin activity an obvious candidate for a major control point during cytokinesis.

A molecule of cytoplasmic myosin II is a hexamer composed of two each of three pairs of polypeptides: the 200 kDa heavy chain (MHC), the 18 kDa essential light chain (ELC) and the 20 kDa regulatory light chain (RLC) Fig. 8; see ref. 135). The N-terminal half of the heavy chain forms the globular motor (or 'head') domain which contains the actin-binding site and actin-activated ATPase. The C-terminal portions of the two heavy chains form an α-helical coiled-coil rod which can oligomerize with other myosin molecules to form short bipolar filaments similar to the myosin filaments of muscle tissue. Between the head and rod domains is the 'neck' region, where the ELC and RLC bind. The state of phosphorylation of the RLC has a major influence on the motor activity of the myosin molecule (see below).

5.2.1 Is myosin II required for normal cytokinesis?

Like actin, cytoplasmic myosin II accumulates in the contractile ring of many organisms (reviewed in ref. 78). Generally, cytokinesis is eliminated or at least impaired in all systems where myosin activity is perturbed, either by mutation or by antibody injection. However, one recent set of findings in *Dictyostelium* raises interesting questions about the universality of this conclusion.

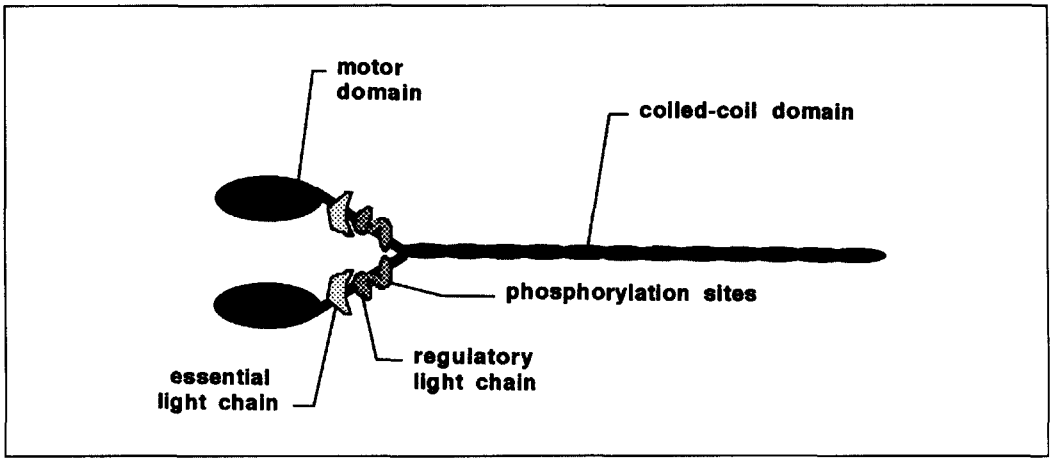

Fig. 8 The structure of myosin II. Myosin II is a heteromultimeric protein consisting of two heavy chains, two essential light chains, and two regulatory light chains. The heavy chains each contain an N-terminal motor domain, followed by a 'neck' region to which the light chains bind. The extended coiled-coil rod is formed by dimerization of two heavy chains. The regulatory light chain is the site of phosphorylations that activate or inhibit activity of myosin II.

Perturbing myosin function

The first experiments demonstrating that cytokinesis depended on myosin were those of Mabuchi (136) and Kiehart (137) who found that injecting anti-myosin antibodies into echinoderm blastomeres blocks cytokinesis without affecting the mitotic cycle. The importance of myosin II was confirmed by the phenotypes of mutants in *Dictyostelium* (138, 139) and in *S. cerevisiae* (140), in which the expression of the MHC of myosin II was eliminated. In *S. cerevisiae*, mutations in the gene encoding the myosin II MHC have imperfect cell division due to abnormal cell wall organization at the bud neck and grow as chains of cells. In *Dictyostelium*, amoebae with a deletion of the MHC gene are unable to cytokinese in suspension cultures, and as a consequence accumulate as large cells with multiple nuclei. The cells are otherwise surprisingly healthy, and are still capable of phagocytosis and certain other aspects of actin-mediated cell motility. Mutations eliminating either the ELC (141, 142) or the RLC (143) of *Dictyostelium* also result in a failure of cytokinesis in suspension, thus establishing that the myosin heavy chain alone is incapable of functioning in cytokinesis.

In *Drosophila*, strong mutations in the *spaghetti-squash* (*sqh*) gene (which encodes the RLC of myosin II) that eliminate most, but not all, light chain expression, profoundly disrupt cytokinesis, leading to the accumulation of many hundreds of chromosomes in the dividing cells of *Drosophila* larvae (144). Ironically, null mutations in *sqh* (145; P. Jordan and R. Karess, unpublished), like null mutations in the gene encoding *Drosophila* MHC, known as *zipper* (146), do not present massive cytokinesis failure in the larvae, but rather die during or soon after embryogenesis from other causes. Cytokinesis defects are not seen in the earliest stages of embryogenesis in these animals because wild-type myosin stockpiled in the egg by the mother masks the deleterious effects of the myosin mutation.

An exception, or a revolution?

Until recently, there was no reason to question the centrality of myosin to cytokinesis. All available data were consistent with the model that myosin is the motive force for construction of the contractile ring. Recently, however, the story has become more equivocal, following the observations by Neujahr *et al.* (147) that under certain conditions, myosin II null cells of *Dictyostelium* can divide surprisingly well.

It had previously been noticed that myosin II null amoebae, when attached to a solid surface, could at times successfully 'divide' by a mechanism called traction-mediated cytofission, in which cells pinch off a nucleus-containing lobe of cytoplasm (148). This process occurs in interphase cells and is thus not linked to the mitotic cycle. Neujahr *et al.* (147) re-examined the behavior of MHC null amoebae when attached to a substratum and discovered a second myosin-independent cleavage mechanism, one which is linked to presence of the mitotic spindle and which has a remarkable visual likeness to *bona fide* cytokinesis. The process is slower (fewer than 10% completing division in under three minutes, compared with more than 80% for wild-type), not fully reliable (10% of the cells fail to divide), and wholly dependent on attachment to a substratum. In addition, F-actin at the cleavage furrow was also

significantly reduced in the mutant cells. Nevertheless, these findings reveal the presence of an alternative, myosin-independent mechanism of cleavage, and raise a serious question about the true role of myosin II, at least in *Dictyostelium*.

Is myosin then largely dispensable to cytokinesis, playing only a supporting role? Or is *Dictyostelium* an exception? One possibility is that the activity of another myosin motor may compensate for the lack of myosin II in these cells. However, with the exception of myosin I associated with the late central spindle of certain cells (149), no other myosin has been seen to accumulate in the contractile ring. Nor have mutants in any other myosin gene revealed any effect on cleavage. Another possibility is that myosin-less cleavage in *Dictyostelium* may be based exclusively on the regulation of stability and cross-linking in the cortical actin network by the mitotic apparatus. In any event, it is premature to conclude from this single report that myosin II is in fact not required for cytokinesis. Its importance remains unchallenged in *Drosophila* and echinoderms. Further studies will be required to determine the precise contribution of myosin to the contraction of the cleavage furrow in different systems.

5.2.2 Myosin RLC phosphorylation provides a potential mechanism for regulating cytokinesis

Myosin can be phosphorylated at a number of sites on the RLC (Fig. 9), and these phosphorylations can profoundly affect the activity and assembly properties of the molecule. Indeed, the state of myosin phosphorylation at any given moment and location within a cell is apparently determined by an elaborate web of signalling pathways altering both kinase and phosphatase activities in response to both internal and external stimuli (reviewed in ref. 150). The description of myosin phosphorylation that follows is for vertebrate non-muscle myosin II except where noted.

Activating phosphorylation

Myosin activity *in vitro* can be measured either on the basis of its actin-activated ATPase, or by following its ability to move actin filaments in an *in vitro* motility

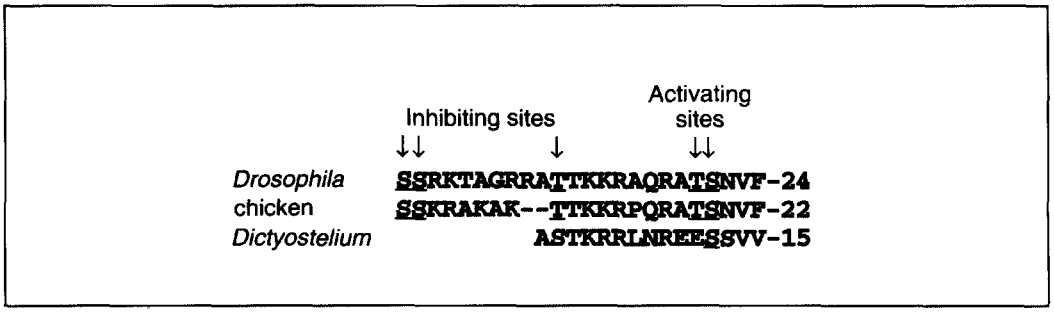

Fig. 9 The N-terminal sequence of non-muscle RLCs. Sequences of the RLCs from *Drosophila*, chicken, and *Dictyostelium* are shown; the sites of inhibiting and activating phosphorylations that influence myosin activity are indicated.

assay. Both of these activities are extremely low in totally unphosphorylated myosin II. In addition, the tail of the unphosphorylated myosin molecule is bent over the head, forming the so-called 10S conformation, which is incapable of oligomerizing into bipolar filaments (135, 151, 152).

Phosphorylation at Ser19 of the RLC is absolutely required for significant myosin II activity. Phosphorylation of this activating serine greatly enhances the actin-activated ATPase and the motor activity of the myosin head, and in addition triggers an unfolding of the myosin tail, thus favoring the polymerization of myosin into bipolar filaments. *In vivo*, phosphorylation of this serine is closely correlated with a number of profound cell shape changes, including smooth muscle contraction, platelet activation, and cytokinesis (see below).

The principle enzyme responsible for this phosphorylation is MLCK, and its only known natural substrate is the RLC Ser19 and, to a lesser extent, the adjacent Thr18. MLCK requires Ca^{2+}/calmodulin for activation, and its activity is modified by phosphorylation by protein kinase A and probably other kinases as well. Also capable of phosphorylating Ser19 is Ca^{2+}/calmodium-dependent protein kinase II, and a Rho-dependent kinase (104), although these latter two activities have not yet been proven to be relevant *in vivo*. Reversing the activating phosphorylations is myosin light chain phosphatase, an enzyme whose own activity is modulated by phosphorylation by the same Rho-dependent kinase mentioned above (103).

Inhibiting phosphorylations

Adding to this tangled regulatory web are the phosphorylations of RLC that inhibit myosin activity. *In vitro*, three sites of inhibitory phosphorylation have been identified: Ser1, Ser2, and Thr9. Phosphorylation at any of these sites has two effects: If the light chain has not already been phosphorylated at the activating site, the inhibitory phosphorylations render the light chain a poorer substrate for activation by MLCK. If, by contrast the light chain is already phosphorylated at the activating site, the addition of these phosphates reduces the level of myosin ATPase, apparently by lowering its affinity for actin (151).

A number of kinases are able to phosphorylate these inhibitory sties *in vitro*, but their physiological relevance is not clear. Protein kinase C, however, does appear to be a major player *in vivo*. At least one other kinase targets these sites as well, as we will see below.

RLC phosphorylations and the cell cycle

Xenopus myosin is phosphorylated on the RLC in a mitosis-specific manner: during interphase, myosin is phosphorylated at the activating sites; but during metaphase, it is primarily phosphorylated at the three RLC inhibitory sites (153, 154). Satterwhite *et al.* (153) proposed that these mitosis-specific inhibitory phosphorylations coordinate cytokinesis with the mitotic cycle. In prometaphase and metaphase, when the inhibitory kinase was active, myosin would be shut off. The inhibitory phosphorylations might promote disassembly of pre-existing myosin filaments, allowing their subsequent reorganization subsequent to anaphase onset. At the

metaphase–anaphase transition, the inhibitory kinase activity disappears and, as phosphatases quickly remove the inhibitory phosphorylations, MLCK targets the activating serines, and myosin assembles into the contractile ring. Indeed, there is approximately a 20-fold increase in Ser19 activating phosphorylation during cytokinesis in mammalian cells (154).

Although Satterwhite *et al.* (153) originally identified $p34^{cdc2}$ kinase as the activity responsible for the inhibitory phosphorylations, more recent studies by others tentatively concluded that a mitosis-specific kinase activity other than p34 is involved (154); the identity of this kinase remains unknown.

5.2.3 Activating phosphorylation of Ser19 of the RLC is critical for assembly of the contractile ring in animal cells, but is less important in *Dictyostelium*

That the activating light chain phosphorylation is likely to be involved in regulating the assembly or contraction of the contractile ring was widely anticipated, given that smooth muscle contraction is triggered by MLCK phosphorylation of smooth muscle RLC. We discuss here experiments designed to test this possibility.

RLC phosphorylation in Dictyostelium

Mutant forms of the *Dictyostelium* RLC in which the targets of MLCK were replaced by unphosphorylatable alanines are capable of almost (but not quite) totally rescuing the cytokinesis defects of *Dictyostelium* RLC null mutant cells (155). Moreover, *Dictyostelium* deficient for a major form of MLCK have recently been demonstrated to have a lowered efficiency of cytokinesis in suspension culture (156). Thus, RLC phosphorylation apparently plays a real but nonetheless surprisingly minor role in cytokinesis in this organism. This conclusion might be explained by the fact that *Dictyostelium* myosin II is apparently less tightly regulated by activating phosphorylation. Due to differences in basal activity levels, the phosphorylated form of the *Dictyostelium* myosin is only five to six times more active than the unphosphorylated form, while for vertebrate myosin the activation is on the order of 50- to 100-fold.

RLC phosphorylation in Drosophila

The *Drosophila* RLC, the product of the *sqh* gene, is structurally closer to its vertebrate counterparts than the *Dictyostelium* RLC is, particularly around the sites of both the inhibiting and activating phosphorylations (144). The Karess laboratory has tested a battery of altered RLCs, lacking either the inhibiting or activating phosphorylation sites, for their ability to complement a *sqh* null mutation (157; R. Karess, unpublished).

Drosophila RLC lacking the principal activating serine is partially defective in cytokinesis, causing polyploidy in 5–10% of mitotic larval cells. If the adjacent threonine is also removed, then the light chain is entirely inactive, and the animal dies just after embryogenesis with the phenotype of the *sqh* null mutation. Thus, *Drosophila* myosin II behaves as predicted by the vertebrate biochemistry: activity *in*

vivo requires a phosphorylatable amino acid at the activating site. Interestingly, an RLC in which the activating serine and threonine were replaced by glutamates (which should mimic the phosphorylation) was able partially to rescue the null phenotype (157).

When the three sites of inhibiting phosphorylation on *Drosophila* RLC are simultaneously replaced by alanines, no deleterious effects on cytokinesis are observed. Thus, at least in *Drosophila*, it would appear that the coordination of mitosis and cytokinesis does not require mitosis-specific phosphorylation at the inhibitory sites of myosin RLC, though such phosphorylation may still help fine-tune the timing of cytokinesis. The applicability of this finding to vertebrates is presently uncertain because it is not yet known whether *Drosophila* RLC is cyclically phosphorylated *in vivo* as is the case for *Xenopus* (see above).

In vivo *imaging of myosin*

In a recent 'high-tech' approach, a fluorescently labelled myosin and a fluorescent biosensor of RLC phosphorylation at Ser19 were injected into cells, which were then monitored by multimode light microscopy and computer analysis (158). It was found that, as the cell enters anaphase, myosin throughout the cell is rapidly phosphorylated at the activating serine (see also ref. 154), but as the cell continues from anaphase to telophase, only the region of the incipient cleavage furrow maintains the highest levels of phosphorylation. These results provide strong circumstantial evidence that Ser19-phosphorylated myosin II does at least accumulate in the equatorial plane and the cleavage furrow of cultured cells.

A synthesis?

At least in metazoan cells, the primary means of coordinating myosin activity in cytokinesis with the onset of anaphase is by regulated phosphorylation of the activating serine of RLC. RLC phosphorylation at the activating site is highly dynamic, and correlates both spatially and temporally with cleavage. A major challenge for the future will be to identify the factors regulating the kinases and phosphatases responsible for this phosphorylation, as these should be closer to the source of the signal emanating from the spindle that sets cytokinesis in motion. In contrast, the inhibitory phosphorylations do not appear to be as important (at least in *Drosophila*) in controlling myosin activity during cytokinesis.

As described above, there is reason to believe that the importance of myosin itself to the regulation of cytokinesis may not be universal. It will be particularly important to determine to what extent *Dictyostelium* differs from other organisms in its use of myosin during cleavage.

5.3 The role of septins in cytokinesis

The genomes of probably all eukaryotic organisms contain multiple genes encoding various members of a family of related proteins called septins, which play a general role in cell surface phenomena (including cytokinesis) involving interactions between the plasma membrane and the cortical cytoskeleton (reviewed in refs 183

and 184). Septin proteins contain, in addition to some family-specific motifs, P-loop consensus sequences indicative of nucleotide-binding functions, and most (though not all) contain a C-terminal coiled-coil domain. Individual septins may not have conserved unique functions because a particular septin in one organism is generally not clearly orthologous to one particular septin in a different organism.

Although some septins in *S. cerevisiae* and *S. pombe* appear to be specific for processes in sporulation and play no obvious role in cytokinesis, the function of most other septins in cytokinesis has been unambiguously demonstrated (reviewed in refs 183 and 184). The original members of the septin family of proteins were identified in *S. cerevisiae* as the product of the cell division cycle genes *CDC3*, *CDC10*, *CDC11*, and *CDC12*, mutants of which yield identical phenotypes with large, multinucleated, multiply budded cells indicating defects in cytokinesis, as well as phenotypes indicating defects in other processes such as bud site selection and bud morphogenesis. Knockout mutations in four of six known *S. pombe* septin genes cause delays (but not complete blocks) in septum formation, leading to the formation of chains of cells. Mutations in the *Drosophila* septin gene *peanut* (*pnut*) also cause the accumulation of multinucleate cells consistent with cytokinesis defects (185).

5.3.1 Septins are concentrated near the contractile ring

In cytokinesis, septins almost certainly act at a highly restricted location between the dividing cells, very near the position of the contractile ring. Immunofluorescence studies using anti-septin antibodies show that septins are concentrated at the cleavage furrows and residual intercellular bridges of dividing *Drosophila* tissue culture and post-cellularization embryonic cells, and at the tips of the pseudocleavage and cellularization furrows in earlier *Drosophila* embryonic stages (185, 186). In dividing *S. cerevisiae* cells, these proteins localize to the neck connecting the mother cell and bud, and are likely to be the major structural components of the 10 nm wide 'neck filaments' that form a ring encircling the neck just beneath the plasma membrane (reviewed in refs 183, 184, and 186).

The role of septins in cytokinesis remains uncertain. The septins could help to position the contractile ring by modifying the plasma membrane or attaching the ring to the membrane, or they could help assemble or regulate the contraction of the contractile ring. However, actin and septin ring assembly appear to be independent of each other in *S. cerevisiae* (187) and, in *S. pombe*, the actin ring forms earlier than the septin ring (184). Septins are therefore unlikely to be required for actin ring assembly or placement. In addition, a direct role for septins in contraction appears unlikely given that the septin ring retains its original diameter during cytokinesis (187).

5.3.2 Septins associate to form complexes

In *S. cerevisiae*, the *CDC3*, *CDC10*, *CDC11*, and *CDC12* gene products co-localize to the mother–bud neck (187); in *Drosophila*, the Pnut, Sep1, and Sep2 septins are similarly co-localized in all tissues analyzed to date (186). Significantly, mutations in any of the four original budding yeast septin genes cause the disappearance of all four septin proteins from the mother–bud neck (as well as loss of the neck filaments),

while Sep1 fails to localize properly in *Drosophila* deficient for Pnut. The four *S. cerevisiae* genes show many genetic interactions, such as synthetic lethality of double mutants and suppression of mutations in one septin gene by overexpression of another (183, 184, 186). Recent biochemical studies provide convincing evidence for the formation of a septin complex: using immunoaffinity purification, a complex containing the septins Pnut, Sep1, and Sep2 has been purified from *Drosophila* embryos (188). The complex appears to contain two of each polypeptide, suggesting that the complex is a heterotrimer of homodimers. Consistent with the P loops that characterize septin proteins, the complex is also associated with six molecules of guanine nucleotide. Intriguingly, the purified septin complex polymerizes into filaments; at higher concentrations these filaments can associate along their lengths in cable-like aggregates. Septins thus appear to define a new class of cytoskeletal filaments that are critical in a currently unknown manner for cytokinesis and other processes at the cell surface.

5.4 Other cytoskeletal proteins at the cleavage furrow

A number of other cytoskeletal proteins localize in the vicinity of the cleavage furrow, but their importance to cytokinesis is unknown. These fall into several categories based on their biochemical functions: actin filament cross-linking or bundling proteins such as α-actinin (177), filamin (178), and the newly-discovered anillin (179); actin-membrane linkers such as talin (180) and ERM proteins (e.g., radixin (119); reviewed in ref 118); those that regulate F-actin assembly or organization in other ways, such as acidic calponin, which inhibits the ATPase activity of phosphorylated myosin (181); and transmembrane glycoproteins such as CD43 (120). It is important to emphasize that for most of these proteins, there is no ultrastructural evidence that they are actual components of the contractile ring. In fact, it is probable that at least some are located in microvilli within the cleavage furrow rather than in the ring itself. In addition, in the few cases tested, mutations in these genes have not led to cytokinesis defects (182). This does not exclude the possibility of important roles in cytokinesis, as the function of these proteins may be redundant.

5.5 Processes related to cytokinesis

The study of cortical events that share similarities with normal cytokinesis should help provide mechanistic comparisons and contrasts. We have already stressed, particularly in Section 4.2 on Rho protein signalling, that precedents of relevance to cytokinesis have come from studies on other membrane/cortical structures such as filopodia, lamellipodia, stress fibers, and focal adhesions (see refs 96 and 99 for further review). Here, we draw attention to several more specialized and perhaps less generally appreciated processes that are related to cytokinesis.

In *Drosophila*, two such phenomena are the formation of metaphase cleavage furrows that transiently separate adjacent spindles of the pre-cellular (syncytial)

blastoderm divisions, and the process of cellularization that eventually sequesters nuclei near the embryonic cortex into individual cells. A relationship to cytokinesis is suggested by the localization of actin, myosin, septins, and anillin to the metaphase furrow and to the cellularization front. However, there are interesting proteins that have been shown to be required specifically for these processes. The products of the genes *nullo, bottleneck,* and *Serendipity-alpha* are expressed only at the time of cellularization, consistent with the finding that mutations in these genes only affect cellularization. The proteins encoded by the genes *sponge, nuclear fallout,* and *scrambled* are required for cleavage furrows, and the latter two for cellularization as well. Homozygotes for the available mutant alleles of these genes survive to adulthood, though homozygous mutant females lay eggs that arrest development during the syncytial blastoderm stages or during cellularization. These processes have recently been discussed in two excellent recent reviews (182, 189).

An interesting variation on cytokinesis is the incomplete cytokinesis that occurs in germline divisions in both *Drosophila* males and females. These divisions are marked by structures known as ring canals that connect the incompletely separated daughter cells and allow the directional transport of cytoplasm between cells, which is critical to oogenesis and spermatogenesis. The egg-chamber ring canals contain novel proteins such as the products of the genes *kelch* and *hu-li tai shao* (*hts*) in addition to actin, proteins containing phosphotyrosine epitopes, and anillin. The spermatocyte ring canals contain septins, epitopes that react with a phosphotyrosine antibody, and anillin, but not actin. The recent review by Robinson and Cooley (190) provides more information on these intriguing stable intercellular bridges.

Finally, during sporulation in yeasts, new membrane and cell wall components are deposited around each nucleus to form the spore. The requirement of specific septins in sporulation suggests that this process may also have important, overlooked analogies to cellularization and cytokinesis (191).

6. What do we still need to learn about cytokinesis?

The last few years have provided insights into the mysteries of cytokinesis. Our understanding of several important aspects of cytokinesis has clearly improved. In particular, there has been increased appreciation for the role of the spindle midzone in determining the site of cleavage furrow formation in most animal cells, a greater understanding of the relationship between cytokinesis and controls on the cell cycle, and a more solid basis for the role of signal transduction, especially through Rho-type G proteins, in cytokinesis. More proteins of potential importance to the formation or stabilization of actin rings or other aspects of cytokinesis have been identified, and the collections of cytokinesis-defective mutations that are being established in several experimental organisms promise to be of great future service.

In spite of these signs of progress, basic questions about cytokinesis remain unresolved. What is the nature of the signal from the mitotic apparatus that establishes the plane of cleavage? How is this signal regulated by the cell cycle machinery? How does this signal activate the signal transduction pathways that

appear to operate during cytokinesis? What is the precise role of myosin and its regulation in cytokinesis? What is the order in which components of the contractile ring become recruited to this structure? How are these components organized with respect to each other? We hope that new investigators with fresh approaches will be attracted to the study of cytokinesis: a subject of fundamental importance that is in many ways still in its infancy.

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