Viewpoint: Putting the Cell Cycle in Order

Kim Nasmyth

"All things began in order, so shall they end, and so shall they begin again; according to the ordainer of order and mystical mathematics of the city of heaven." —Sir Thomas Browne, 1658

And so it is for the cell cycle. Most eukaryotic cells never re-duplicate their chromosomes before sister chromatids have been segregated at the previous mitosis, never embark on mitosis before DNA duplication is completed, nor attempt to segregate sister chromatids until all pairs are aligned on the mitotic spindle at metaphase. How cells ensure that chromosome duplication and segregation occur in the correct order and only when the previous events have been successfully completed have been called the "alternation" and "completion" problems (*1*). A series of Articles in this issue of *Science* describe major progress in understanding them.

A bit of history. Early light microscopic studies recognized that cell division was preceded by mitosis (M phase), during which cells condensed their chromosomes, aligned them on a microtubular spindle, and segregated sister chromatids to opposite poles of the cell. Little could be seen during the interval between succeeding mitoses (known as interphase) except that cells grew in volume. Interphase remained a black box until the discovery that DNA carries the information stored in chromosomes. Chromosome duplication was then detected and shown to occur during a narrow window of time during interphase (*2*). This split interphase into three intervals: G_1, the gap between mitosis and the onset of DNA replication; S phase, the period of DNA synthesis; and G_2, the gap between S and M phases.

G_1, S, G_2, and M gradually came to be thought of as major cell cycle states, and identifying the factors that trigger the transitions between these states became one of the major goals of cell cycle research. Their first sighting came from experiments in which cells at different stages of the cell cycle were fused (*3*). This was initially done with unicellular protozoa whose large cells were easily manipulated and with the huge syncitial cells of *Physarum polycephalum*. The results indicated that late G_2 or M phase cells contained an M phase–promoting factor (MPF) capable of accelerating the onset of mitosis in early G_2 cells. These particular experimental organisms have very short G_1 and S periods and it was therefore not possible to investigate other cell cycle transitions. The subsequent discovery that S phase cells might contain an S phase–promoting factor (SPF) capable of accelerating S phase in G_1 nuclei had to await techniques to fuse tissue culture cells (*4*). These cell fusion studies suggested that MPF could induce chromosome condensation in nuclei at all stages of the cell cycle but that SPF could only induce G_1, not G_2, nuclei to enter S phase. Unfortunately, no assay to purify SPF or MPF emerged from this field of inquiry.

The discovery of an activity in meiotic frog eggs capable of inducing M phase in immature G_2 oocytes (*5*) led to a robust assay for factors capable of inducing nuclear disassembly and chromosome condensation, and an MPF was eventually purified (*6*). Genes for S phase– and M phase–promoting proteins had meanwhile been identified by genetic studies in yeast (*7, 8*) and shown to encode a special class of protein kinases. The symbiosis between yeast genetics, frog biochemistry, and mammalian tissue culture led rapidly to the current notion that DNA replication and mitosis are induced by the activation of S phase– and M phase–specific cyclin-dependent protein kinases (CDKs). The MPF purified from frog eggs was an M-phase CDK. The catalytic subunits of these kinases are only active when complexed with unstable regulatory subunits whose fluctuations in abundance during the cell cycle led to their being called cyclins (*9*). In animal cells, S phase is induced by CDK2 complexed with S-phase cyclins (E or A types) and M phase by CDK1 complexed with M-phase cyclins (A and B types), whereas in both budding and fission yeast, S and M phases are induced by CDK1 associated with S phase– and M phase–specific B-type cyclins. Both yeast and mammalian cells also have G_1-specific CDKs that promote synthesis of proteins needed for chromosome duplication and trigger activation of S-phase CDKs (see Articles by R. W. King *et al.*, p. 1652; C. J. Sherr, p. 1672; and B. Stillman, p. 1659).

Successive waves of S- and M-promoting CDKs occur in most eukaryotic cells and they clearly go some way toward explaining "alternation." Furthermore, we now understand many of the regulatory mechanisms that cause S-phase CDKs to accumulate in late G_1- and M-phase CDKs to do so in late G_2. The regulation of CDKs during development has also been explored. These studies, particularly on *Drosophila*, have advanced our understanding of the control of proliferation during early development and pattern formation (see Article by B. A. Edgar and C. F. Lehner, p. 1646). CDKs are not, however, the only conserved factors whose changes in activity promote S and M phase. Activation in late G_1 of the Cdc7 protein kinase, which depends on a regulatory subunit Dbf4 (and which might therefore be called a Dbf4-dependent kinase or DDK), is also necessary for the initiation of DNA replication (see B. Stillman, p. 1659), whereas activation of the Polo/Cdc5 kinase in late G_2 is essential for mitosis (*10*).

Checkpoints: new concept or shibboleth? Crucial insight into the completion problem can be traced to studies on bacteria. In *Escherichia coli*, DNA damage or inhibition of DNA replication generates a signal that activates the RecA protein to proteolyse the LexA repressor. This allows induction of a large battery of SOS genes, some of which participate in DNA repair whereas others block cell division (*11*). The key point is that cell cycle arrest caused by damage or incompletion of earlier cell cycle events can be caused not by damage or incompletion per se, but instead by specific surveillance mechanisms that detect mistakes and induce inhibitors of key cell cycle transitions. Similar phenomena were also found in mammalian cells, whose cell cycle arrest in response to DNA damage depends on the ATM gene (*12*), and in fission yeast, whose delay of mitosis until cells reach a critical cell size depends on the Wee1 protein kinase (*13*).

The significance of this way of thinking was not fully appreciated until the discovery that a similar set of surveillance mechanisms in the budding yeast *Saccharomyces cerevisiae* are responsible for the arrest of many DNA replication mutants and irradiated cells in a G_2-like state (*14*) and for delays in the onset of anaphase in cells whose chromosomes have not properly aligned on mitotic spindles (*15, 16*). The discovery that human tumor suppressing genes, such as p53 or ATM, are required for blocking entry into S phase when DNA is damaged or for blocking re-replication when mitotic spindles are damaged suggests that defective cell cycle surveillance mech-

The author is at the Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria.

anisms also contribute to the highly abnormal karyotypes of human tumor cells (see Article by S. Elledge, p. 1664). There has been rapid progress in identifying components of these various surveillance mechanisms but there are few instances where we understand how they inhibit key cell cycle transitions.

The surveillance mechanisms that check completion have been likened to roadblocks where travelers are scrutinized and thus are often called checkpoints (*17*). This term is widely used but there is much confusion as to what exactly it means. Checkpoints are currently the shibboleth of the cell cycle. The confusion stems partly from the inherent ambiguity of the word to check. Does it refer to blocking the cycle or monitoring completion? It also arises because we are already used to the notion of points in the cell cycle. Checkpoints have been thought of as points in the cell cycle at which processes being monitored are completed (*18*) or as points in the cell cycle at which transitions are blocked (*1*). Furthermore, though checkpoints started life as cell cycle surveillance mechanisms, they were later re-defined as any control that ensures the order of cell cycle events (*17*). This would include the functions of most cell cycle regulatory proteins, including CDKs, replication initiation factors, and factors that promote anaphase, which leaves us with not very much to check. Besides, those who actually study the controls that ensure the "alternation" of S and M phases as opposed to their "completion" do not usually refer to them as checkpoints. The checkpoint jargon is probably here to stay but it should remain synonymous with "surveillance mechanisms that block cell cycle transitions" and not lay claim to the soul of the cell cycle.

Cracks in the system? Identification of S- and M-phase CDKs and understanding their regulation has made it possible to alter the timing and indeed the order of CDK activation. Such experiments reveal that M-phase CDKs can assume the function of S-phase CDKs and trigger chromosome duplication in G_1 cells (*19*, *20*)! As a consequence, it has become clear that CDKs capable of promoting S phase in G_1 cells are present from the beginning of S phase until the end of mitosis and yet origins of DNA replication fire once and only once during this period. This echoes the finding from cell fusion studies that S-phase cells trigger G_1 but not G_2 nuclei to enter S phase (*4*). The implication is that the order of S- and M-CDK waves cannot alone be responsible for ordering S and M phases. The state of their substrates must be equally if not more important in determining whether a cell duplicates or segregates its chromosomes. Much recent attention has therefore been focused on analyzing changes that occur at origins of DNA replication during the cell cycle (see B. Stillman, p. 1659).

A well-prepared chromosome is the key to its duplication. Recent work in yeast and frogs suggests that initiation triggered by activation of S-phase CDKs and DDKs depends on the prior assembly at origins of a pre-replication complex (pre-RC) composed of three sets of proteins: ORC, Cdc6p, and Mcms. Throughout most of the cell cycle, origins are marked by the site-specific binding of a group of proteins called the Origin Recognition Complex (ORC). Origins are occupied by ORC alone during G_2 and, in yeast, even during M phase; but they acquire a second set of proteins either as cells exit from mitosis or later during G_1. One of these is an unstable protein called Cdc6p, which is synthesized during G_1. Cdc6p's arrival at origins catalyzes the assembly onto neighboring DNA sequences of a second set of complexes composed of Mcm proteins. How pre-RCs catalyze replication initiation is not known.

S-phase CDKs not only trigger initiation from origins that have formed pre-RCs but also prevent the de novo assembly of pre-RCs. M-phase CDKs also inhibit this process, which means that pre-RCs cannot be formed from the point of S-phase CDK activation in late G_1 until the degradation of M-phase cyclins at anaphase. Initiation of DNA replication therefore depends on a period of low CDK activity during which synthesis of Cdc6p can drive the assembly of pre-RCs, followed by a period of high CDK (and DDK) activity, which permits origin firing. The two opposing effects of CDKs prevent conditions from ever arising that permit both the assembly of pre-RCs and firing of origins that have formed them. The consequence is that origins cannot fire more than once during an S- and M-phase CDK cycle. The disassembly of pre-RCs during S phase might be caused by initiation itself or passage of a neighboring replication fork. This picture provides a satisfying explanation for many aspects of initiation but it should still be regarded as a working hypothesis because many important details have yet to be established (see B. Stillman, p. 1659).

Severing the bond that holds sisters together. Having duplicated its chromosomes, the cell's next task is to generate a bipolar mitotic spindle, to attach sister kinetochores to microtubules that associate with opposite poles of this spindle, and—by means of tension exerted on sister kinetochores—to align each pair of sister chromatids on the metaphase plate. It is key to note that chromosome alignment during metaphase depends not only on splitting forces exerted by microtubules on kinetochores but also on an opposing cohesive force exerted by tethers that hold sister chromatids together. This Newtonian action and reaction is the essence of mitosis, for it is the means by which cells determine which DNA molecules within a cell are sisters. Chromosome alignment is preceded by the partial resolution of sister chromatids from each other, by chromatid condensation, and by disassembly of the nuclear membrane. All these events depend on activation of M-phase CDKs, whose regulation by cyclin synthesis and phosphorylation is quite well understood. Surveillance mechanisms that detect incomplete DNA replication block activation of M-phase CDKs in some organisms (see S. J. Elledge, p. 1664).

The subsequent separation of sister chromatids to opposite poles, known as anaphase, marks the point of no return in the mitotic cycle. Two sorts of processes are involved: loss of cohesion between sister chromatids, which leads to movement of sisters to opposite poles and changes in the dynamics of spindle growth and disassembly that enable separation of spindle poles . The segregation of sister chromatids is accompanied by, but is not dependent on, inactivation of M-phase CDK through cyclin proteolysis (see R. W. King *et al.*, p. 1652).

We still have little idea how sister chromatids are held together, let alone how their cohesion is loosened at the metaphase to anaphase transition. Nevertheless, recent research has identified a very large multisubunit complex called the anaphase-promoting complex (APC) or cyclosome that is essential for both chromosome splitting and destruction of M-phase cyclins. It promotes ubiquitination of proteins that contain specific sequences called destruction boxes and thereby targets them for proteolysis by the 26*S* proteosome (see R. W. King *et al.*, p. 1652). Many different proteins are degraded via APC-mediated ubiquitination during anaphase, including M-phase cyclins, proteins like Pds1p and Cut2p whose destruction is essential for sister separation, proteins like Ase1p that associate with anaphase spindles, and even the M phase–promoting protein kinase Polo/Cdc5 (M. Sharayama, personal communication).

It is currently thought that anaphase is triggered by activation of the APC and that degradation of proteins like Pds1p and Cut2p might have an important role in allowing separation of sister chromatids. The APC remains active for much of the subsequent G_1 period and is not turned off until accumulation of G_1-CDKs, which ensures that M-phase cyclins and presumably many other APC substrates needed for mitosis do not accumulate prematurely (see R. W. King *et al.*, p. 1652). Once a cell has severed the bond holding sister chromatids

together, it has no means of ensuring that sister kinetochores attach to opposite poles of the mitotic spindle. It is therefore imperative that anaphase not be initiated before all pairs of sister chromatids have aligned on the mitotic spindle. Two sorts of mechanism ensure this. The APC is only activated after cells have first activated M-phase CDKs and kept them active for long enough to form mitotic spindles and to align chromosomes on them. How activation of M-phase CDKs leads to APC activation is one of the least well understood steps in the eukaryotic cell cycle, even though this connection is fundamental to mitosis. In addition, most eukaryotic cells have surveillance mechanisms that detect sister kinetochores which have not been properly aligned on the mitotic spindle and block APC function (see S. J. Elledge, p. 1664).

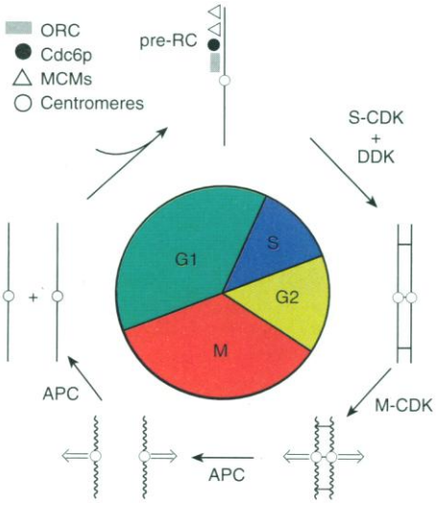

Fig 1. Major transitions during the chromosome cycle of eukaryotic cells. During G_1 there exists a period of low CDK activity due to the presence of cyclin kinase inhibitory proteins (CKIs), the activity of the APC, and the lack of transcription of cyclin genes. Synthesis of Cdc6 protein during this period promotes the binding of Mcm proteins to chromatin and the cell assembles a pre-replication complex at future origins of replication. Activation of S-phase CDKs triggers the firing of origins that had previously formed pre-RCs, while at the same time it blocks any further assembly of pre-RCs. Replication produces pairs of sister chromatids that are attached to each other; they can be aligned on the metaphase plate once activation of M-phase CDKs promotes the formation of a mitotic spindle. M-phase CDKs also promote activity of the APC, which leads to loss of sister chromatid cohesion and to the destruction of M-phase cyclins. The APC remains active during the subsequent G_1 period and is turned off by the accumulation of G_1-CDKs.

Linking duplication and segregation. In addition to promoting anaphase by degrading inhibitors of sister chromatid separation, the APC degrades M-phase cyclins and proteins that stabilize anaphase spindles. It thereby mediates the disassembly of the mitotic spindle once anaphase has been completed. Proteolysis of M-phase cyclins also relieves the block to pre-RC assembly exerted by S-phase and M-phase CDKs, and this may be a key aspect of the mechanism by which eukaryotic cells ensure that reduplication of chromosomes does not precede separation of sister chromatids produced at the previous S phase. By using the APC to regulate degradation of both anaphase inhibitors and M phase cyclins, the cell prevents preparations for S phase before anaphase is initiated.

The restriction of origin firing to once per cycle stems from initiation of DNA replication being a two step process, in which the second step, activation of CDKs, also inhibits the first step, formation of pre-RCs. A similar principle governs mitosis, which must also be a singular event. Mitosis involves two fundamental steps—alignment of sister chromatids on bipolar spindles and splitting of sister chromatids. The enzyme responsible for the second step, the APC, also destroys the M-phase cyclins needed for the first step. Such phenomena are ubiquitous in engineering and are analogous to the cocking and firing cycle of a gun or the cycle of piston movement in a reciprocating steam engine.

The old order changeth. The new insights described in this issue of *Science* give us cause to reflect on our current thinking about G_1, S, G_2, and M phases. These started life as an operationally convenient means of dividing the interval between succeeding divisions. They were as it were the cell cycle's four "seasons." However, they soon acquired the status of major cell cycle states (in the mathematical sense of the word) and the factors that promoted transitions between these states became the holy grail of cell cycle research. This notion has had its flaws (in budding yeast, S and M phases overlap under certain conditions!), but the successful identification of S- and M-phase promoting factors shows that it served a useful purpose. Nevertheless, its period of utility may be drawing to a close. Chromosomes do indeed undergo a series of key transitions in their state during the cell cycle, but these transitions do not correspond exactly to our old friends G_1-S and G_2-M (Fig. 1). The chromosomes of G_1 cells that have formed pre-RCs are clearly in a fundamentally different state from those that have not. Likewise, "G_1" cells that have not activated S-phase CDKs are clearly in a fundamentally different state from those that have done so, but have not yet initiated S phase because DDKs have not yet become active. One of the most irreversible transitions during the cell cycle is the onset of proteolysis mediated by the APC, but this occurs within M phase! Just as the sun's inclination and not season is the primary cause of our weather, so it is the formation of pre-RCs and not whether a cell is in G_1 or G_2 that determines whether cells enter S phase upon activation of CDKs. The time is ripe to return the four cell cycle phases to their rightful status, as cell cycle seasons and to cease thinking of them as states. Seasons are not variables that determine weather but merely intervals during which there occur changes in the state of the weather and in the state of its determining variables, such as the sun's inclination. This applies also to concepts such as Start (*21*) or the restriction point (see C. J. Sherr, p. 1672), which helped in the past to guide the cell cycle field through a phenomenological minefield, but are probably best considered as periods of the cell cycle rather than defined processes. Such an emphasis on mechanism is a sign of the field's growing maturity.

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