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Control of S Phase

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Sustained cell proliferation requires the duplication and segregation between daughter cells of every cell constituent. Because chromosomes are present in only one or two copies per cell, their duplication and segregation are particularly tightly regulated. To ensure that daughter cells inherit at least one copy of each of their genes and to maintain an appropriate balance in their relative numbers, the vast majority of eukaryotic cells replicate each sequence in their genomes once and only once per cell cycle. To ensure that daughter cells inherit sufficient but not excessive cytoplasm for the execution of their genetic programs, i.e., for protein synthesis, the duplication and segregation of chromosomes must occur no more or less frequently than cells duplicate the rest of their constituents. Finally, to ensure that cells maintain their ploidy, which is essential if they are to contribute to the germ line, reduplication must not recur until sister chromatids have been segregated at anaphase.

STARTING DNA REPLICATION IN G_1

Coordinating DNA Replication and Cell Growth

Pulse labeling with radioactive tracers showed that DNA replication occupies a defined window within the interdivision period, which is separated from the previous and preceding M phases by two gap periods, called G_1 and G_2, respectively (Howard and Pelc 1951). Chromosome duplication and segregation are therefore periodic processes. Unlike chromosomes, most cellular constituents like ribosomes and enzymes are present in large numbers and are synthesized fairly continuously during the cell cycle (Mitchison 1970). During sustained cell proliferation, successive rounds of DNA replication and chromosome segregation (known as the chromosome cycle) must occur with the same frequency as cells double the number of their ribosomes and other cell constituents, known as cell growth (see Fig. 1). Coordination between these two processes is

DNA Replication in Eukaryotic Cells

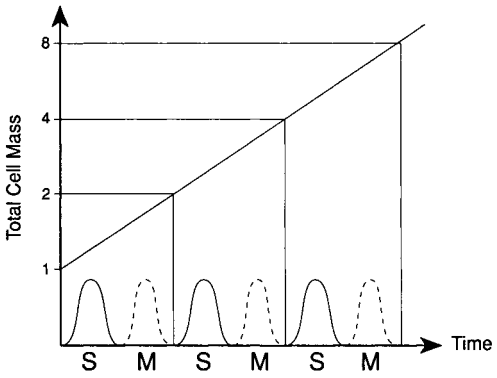

Figure 1 Coordinating the chromosome cycle and cell growth. Total cell mass increases exponentially (for a short while even in the absence of progression through the chromosome cycle), and the signals that trigger S and M phases (shown as oscillations on the abcissa) must occur with the same frequency as cytoplasmic mass doubling if nuclear/cytoplasmic ratios are to be kept within narrow bounds.

needed to maintain the ratio between nucleus and cytoplasm within certain bounds. It may be achieved either by a dependence of the chromosome cycle on the growth cycle or vice versa.

Independence of Cell Growth from the Chromosome Cycle

Dependence of cell growth on the chromosome cycle has been most carefully analyzed in the the fission yeast *Schizosaccharomyces pombe*, where increases in the rates of total protein or RNA synthesis are largely unaffected for several hours after inactivation of the Cdk1 (cdc2) protein kinase, which specifically blocks the chromosome cycle (Creanor and Mitchison 1984). To a first approximation, therefore, DNA replication does not limit the rate of cell growth. A similar conclusion can be drawn from the behavior of chromosome cycle mutants of the budding yeast *Saccharomyces cerevisiae* (Hartwell et al. 1974). A lack of DNA replication does eventually cause cell growth to slow down, after two to three generation times. Presumably, templates for mRNA synthesis eventually become limiting, at least for a few key growth genes.

Mutants in key cell cycle regulators do not exist to test the dependence of growth on the chromosome cycle in mammalian cells, so inhibitors have instead been employed. Some cell lines, such as HeLa, appear to shut down protein synthesis in the presence of DNA synthesis

inhibitors, whereas others, such as CHO, continue cell growth like yeast *cdk1* mutants (Kung et al. 1993). Whether the growth cycle is largely independent of the chromosome cycle in animal or plant cells is therefore an unresolved but important issue that would best be investigated using primary cell lines, or better still, analyzing cells in their natural environment in vivo. Arrest in late G_1 using newly identified cyclin kinase inhibitors would be preferable to replication inhibitors, because the shutdown in protein synthesis seen in HeLa cells could be a specific response to arrest in S phase itself. Anecdotal evidence on the behavior of senescent cells, whose cell cycles are blocked in G_1, suggests that they become very large, indicating that their cell cycle arrest does not interfere with their growth (Lucibello et al. 1993). Many differentiated cells, for example, neurons, must continue growth long after they have ceased proliferating. An intrinsic independence of growth from the chromosome cycle would therefore not only favor microorganisms whose growth rates would be restricted by a tight linkage, but would also facilitate the formation of very differently sized differentiated cells in metazoa.

Dependence of S Phase on Growth

In the absence of any tight dependence of growth on the chromosome cycle, nuclear/cytoplasmic ratios are largely maintained by a dependence of the chromosome cycle on growth. The chromosome cycles of most eukaryotic cells are, with few exceptions, tightly dependent on RNA and protein synthesis. The dependence of DNA replication on cell growth was first noticed by Killander and Zetterberg, who observed that there was much greater variation in the size of fibroblast cells at birth than at the onset of S phase and proposed that DNA replication depends on growth to a critical cell size (Killander and Zetterberg 1965). A particularly clear example of this size control is found in the yeast *S. cerevisiae*, whose asymmetric division by budding gives rise to mother and daughter cells with very different sizes. Mother cells are born equal to or larger than the critical size and have very short G_1 periods, whereas the daughter cells are born well below the critical size and must spend long periods growing before they can enter S phase (Hartwell and Unger 1977). Many eukaryotic cells like amoeba, *Physarum*, and the fission yeast *S. pombe* have very short G_1 periods and appear to break this rule. Size controls over S-phase onset nevertheless exist, even in these organisms. In *S. pombe*, for example, cells are usually born larger than the size needed for S-phase onset due to size controls over entry into mitosis. Abolition of this mitotic control creates cells smaller than the

critical size, which, like fibroblasts or budding yeast daughter cells, must grow considerably before they can undergo DNA replication (Nurse and Thuriaux 1977). Thus, size controls over the onset of S phase can be obscured by controls that restrict passage through other cell cycle transitions. An extreme example is the repeated rounds of replication without any growth during embryonic cleavage divisions, which are possible only because prior growth of the egg ensures that it can undergo many divisions before cells below a critical size are produced.

One consequence of the asymmetric interdependence of cell growth and the chromosome cycle is that sustained increases in cellular proliferation cannot be driven simply by accelerating the chromosome cycle but must instead be driven by the forces that cause cells to grow. This is well established for microorganisms, where accelerating the chromosome cycle merely leads to reductions in cell size (Nurse 1975), but it is not widely appreciated that it might be valid also for metazoan cell proliferation. It is often assumed that deregulation of chromosome cycle activators, for example Cdk1, -2, and -4 cyclin-dependent kinases, should accelerate cell division (Quelle et al. 1993) and thereby contribute to transformation. This can only be true if the chromosome cycle or regulators concerned with it also affect the rate of a cell's growth, which does not occur in microorganisms and remains to be established for mammalian cells.

Growth-related Protein Synthesis Needed for S-phase Onset

How cell growth to a critical size triggers S phase is an important but still largely unsolved problem. Size per se is not the critical variable but rather the nuclear/cytoplasmic ratio, because cell size is directly proportional to ploidy. One corollary of the observation that increases in the rate of protein synthesis are largely independent of the chromosome cycle is that a cell's rate of protein synthesis is largely proportional to cell size in growing G_1 cells. Thus, the total rate of protein synthesis in a cell may be a good measure of its size, and a critical level may be required for S-phase onset. The widespread observations that passage from G_1 to S phase is extremely sensitive to inhibitors of protein synthesis are consistent with this notion (Cross et al. 1989).

In yeast, there are at least two broadly different programs of protein synthesis, one occurring in quiescent stationary-phase cells, which includes synthesis of several heat shock and polyubiquitin proteins (Iida and Yahara 1984), and another occurring in growing cells, which includes synthesis of growth-related proteins like ribosomal proteins and

presumably those needed specifically for the chromosome cycle. These two programs of protein synthesis are distinguished by their different dependence on the initiation factor eIF-4e, which binds to mRNA caps and is encoded by *CDC33* (Brenner et al. 1988). Growth-related but not stationary-phase-related protein synthesis depends on eIF-4e. The G_1 arrest of temperature-sensitive *cdc33* mutations at the restrictive temperature implies, not surprisingly, that S-phase onset depends on proteins synthesized as part of the growth-related program. How cells turn on the eIF-4e dependent and growth-related program of protein synthesis in response to nutrients or growth factors is therefore a very important aspect of S-phase control but is outside the realm of this review. Growth-factor- or nutrient-induced transcription of growth-related genes clearly also has an important part in the stimulation of growth-related protein synthesis, but changes in the translational apparatus that allow these mRNAs to be translated may be equally, if not more, important.

Two Parables about Growth and Division

Certain phenomena concerning the control of S phase cannot be understood without appreciating the distinction between cell growth and the chromosome cycle. A good example is the part played by cAMP in *S. cerevisiae*. Mutants defective in adenyl cyclase or cAMP-dependent protein kinases cannot sustain growth-related protein synthesis, and they arrest in G_1 (Matsumoto et al. 1982), from which it has been concluded that cAMP must be a positive effector of S phase. More recently, it has been found that cAMP is involved in modulating cell size in response to nutrients. Cells growing in poor media are smaller than those growing in rich media, principally because of a reduction in the critical size needed for entry into S phase and budding (Lorincz and Carter 1979). Mutants whose cAMP-dependent protein kinases cannot respond to cAMP cannot alter their size in response to nutrients (Tokiwa et al. 1994). Furthermore, addition of cAMP alone to the medium mimics the effects of transferring cells to a richer medium; that is, it increases the cell size needed for S phase. It is thought to do this by inhibiting the accumulation of G_1 cyclins needed for S phase (Baroni et al. 1994; Tokiwa et al. 1994). cAMP must therefore be considered as an inhibitor of S phase; but how can it be both an inhibitor and a promoter of S phase? This paradox is resolved only if one appreciates that cAMP is regulating two very different types of physiological processes, both of which are needed for S phase. On the one hand, it is involved (directly or indirectly) in signaling activation of growth-related protein synthesis, whereas, on the other

hand, it inhibits the accumulation of mRNAs for G_1 cyclins, whose translation could be part of the growth-related protein synthesis program and which have highly specific roles in triggering S phase. In having these two effects, cAMP helps to increase the rate of protein synthesis in cells transferred to rich media while simultaneously increasing their cell size. The function of this size control is not clear, but it could be beneficial for the rapid execution of the chromosome cycle due to a cell size-related increase in protein synthetic capacity.

A factor that reduces growth-related protein synthesis while accelerating the onset of S phase (i.e., the opposite effect to cAMP) may have an important role in the differentiation of erythrocytes (Dolznig et al. 1995). Undifferentiated precursor cells whose proliferation is supported by mixture of growth factors can be induced to differentiate synchronously into mature nondividing erythrocytes by transfer to medium containing erythropoetin. The rate of protein synthesis remains fairly constant during the first 72 hours but gradually switches from growth-related proteins to proteins specific to erythrocytes like hemoglobin. During much of this period, cell division is temporarily accelerated due to a dramatic reduction in the length of G_1, which is all the more remarkable because the end product is a cell that cannot proliferate at all. Thus, like cAMP, erythropoetin has at least two effects: It promotes the chromosome cycle, presumably by increasing the activity of cyclin-dependent protein kinases (see later), but it also shuts down growth-related protein synthesis. The consequence of accelerating division but not cell growth is the production of erythrocytes that are many times smaller than their precursor cells. A moral to be drawn from these two stories is that changes in cell size are sometimes a better indicator of changes in the regulation of the chromosome cycle than are changes in mass doubling times.

S- and M-phase Promoting Factors

What is synthesized by growing cells that triggers DNA replication and then mitosis? What do oocytes store up that allows fertilized eggs to undergo successive cleavages in the absence of any growth? What do differentiating erythrocytes transiently make more of that reduces their G_1 period? Do cells by mysterious means measure all their constituents, for example, the number of ribosomes or the total rate of growth-related protein synthesis, or do they instead simply measure the quantity of one or a small number of components that are synthesized as part of the general growth program of the cell and whose abundance is proportional to

cell size? Evidence for such a "sampling" mechanism has come from two approaches: the isolation of yeast mutants with an altered cell size and the identification of factors and genes needed for the onset of S phase.

Lessons from Cell Fusion Studies

Due to their large size, many multinucleate cells can be readily coalesced by cutting and grafting. This enabled early cell cycle researchers to address what would happen to the chromosome cycle when cells at different stages were combined into one (Johnson and Rao 1971). Experiments with ciliates, amoebae, and, in particular, the myxomycete plasmodia like *Physarum polycephalum* showed that nuclear synchrony was achieved very rapidly. Nuclei derived from "early" cells were accelerated, whereas those from "late" cells were retarded in their progression toward mitosis. The larger the plasmodium from late cells, the more rapidly were the early nuclei accelerated. These studies suggested that a substance capable of triggering mitosis gradually accumulated during G_2.

Most of the cells on which early coalescence experiments were performed have very short G_1 periods, and it was not possible to address the nature of S-phase inducers. These were eventually addressed in experiments with HeLa cells (Rao and Johnson 1970), where it was found that fusion between G_1 and S-phase cells advanced the entry of G_1 nuclei into S phase, but fusion of G_1 with G_2 cells did not. Significantly, DNA replication of S-phase nuclei was barely, if at all, retarded by the addition of G_1 cytoplasm and nuclei. Fusion between S and G_2 cells failed to induce S phase in G_2 nuclei, but again the addition of G_2 cytoplasm and nuclei did not retard DNA replication within S-phase nuclei. These results indicated that S but neither G_1 nor G_2 cells contained a substance (*S*-phase *p*romoting *f*actor or SPF) capable of inducing DNA replication in G_1 but not in G_2 nuclei. Similar experiments suggested that mitotic HeLa cells, like those from *Physarum* (Rusch et al. 1966), contain a substance (now refered to as MPF) that is capable of inducing premature M phase in nuclei at all other stages of the cell cycle (Johnson and Rao 1970). The picture that emerged from these cell fusion experiments is one in which the abundance of S- and M-phase inducers oscillates during the cell cycle with different phases. The former appear in late G_1 and disappear as cells enter G_2, and the latter appear in late G_2 and disappear at the end of mitosis.

An M-phase-promoting factor was eventually purified from mature *Xenopus* eggs (Masui and Markert 1971), which are arrested in metaphase of meiosis II, using an assay that measured its ability to in-

duce chromosome condensation and nuclear membrane breakdown (Lohka et al. 1988). It proved to be identical to the cdc2 protein kinase, which had by this time been implicated in the control of mitosis in *S. pombe* due to the existence of rare dominant alleles that caused cells to divide at half the normal cell size (Nurse and Thuriaux 1980; Nurse 1990). No convenient biochemical assay was ever developed for S-phase-promoting factors, and the present candidates for these factors were identified by genetic studies of the yeast cell cycle.

S-phase-promoting Factors in *S. cerevisiae*

Genes Required for the Onset of S Phase

S. cerevisiae mutants defective in the chromosome cycle fall into two classes: those with primary defects in nuclear division and those with primary defects in entering S phase (Pringle and Hartwell 1981). However, all mutants with S-phase defects eventually also ceased to undergo nuclear division, whereas most mutants with M-phase defects also ceased eventually to replicate DNA, confirming earlier conclusions, drawn for the use of inhibitors, that S and M phases were interdependent. Why cells must normally complete M phase before they can rereplicate their chromosomes is an important question that we address below.

When *S. cerevisiae* cells reach a critical size, they initiate not only DNA replication but also budding, the first step toward cytokinesis, and spindle pole body duplication, the first step toward building a bipolar mitotic spindle. This point in the yeast cell cycle, which is called Start, is also the point at which haploid cells become refractory to cell cycle inhibition by sexual pheromones and the point at which protein synthesis and nutrients become less critical for completing the cell cycle (Pringle and Hartwell 1981). Mutants with primary defects in entering S phase fell into three groups according to their degrees of pleiotropy. The least pleiotropic class included, initially, mutants in only a single gene, *CDC7*, which encodes a protein kinase that is activated in late G_1 (Hollingsworth and Sclafani 1990). *cdc7* mutants fail to initiate DNA replication. Nevertheless, they bud normally, duplicate and separate their spindle pole bodies, form mitotic spindles, and switch off the budding process, with the result that they arrest as large single-budded cells containing an undivided nucleus with a fully formed mitotic spindle (Pringle and Hartwell 1981). Subsequent studies have shown that alleles of *DBF4* (Chapman and Johnston 1989), *CDC6* (Bueno and Russell 1992), *CDC46*, *CDC47*, *CDC54* (Hennessy et al. 1991), *MCM2*, and *MCM3* (Gibson et al. 1990) arrest with similar phenotypes. *DBF4* encodes a

regulatory subunit of the Cdc7 protein kinase (Jackson et al. 1993), whereas *CDC46*, *MCM3*, and *MCM5* encode members of a family of related proteins with ATPase motifs characteristic of DNA helicases (Tye 1994), homologs of which are needed for the initiation of DNA replication also in fission yeast (Coxon et al. 1992) and in vertebrates (Thommes et al. 1992). The initiation of DNA replication might be the exclusive function of this group of genes.

The next class of mutations mapped to three genes: *CDC4*, *CDC34*, and *CDC53*. These mutants are more pleiotropic than the *CDC7* class, because they fail not only to enter S phase but also to form mitotic spindles (Pringle and Hartwell 1981). However, they duplicate their spindle pole bodies and form buds. Another of their characteristics is that they fail to shut off the budding process after the formation of the first bud, with the result that the mutant cells accumulate with multiple buds (Hartwell et al. 1974). The discovery that *CDC34* encodes an E2 ubiquitin-conjugating enzyme implicated in proteolysis suggests that protein degradation as well as synthesis is needed for S-phase entry and the formation of mitotic spindles in yeast (Goebl et al. 1988).

Cyclin-dependent Kinases

The last, most pleiotropic, class of mutants, represented initially by only a single allele of *CDC28* (Hartwell 1993), failed to undergo any of the four events associated with Start; they failed to enter S phase, to duplicate spindle pole bodies, to form buds, and (in haploids) to shut off sensitivity to cell cycle arrest by mating pheromones (Hartwell et al. 1974; Reid and Hartwell 1977; Pringle and Hartwell 1981). *CDC28* encodes a type of protein kinase (Lorincz and Reed 1984) whose activity is now known to require regulatory subunits called cyclins (Nurse 1990), which had been discovered as proteins whose abundance fluctuated during sea urchin cleavage divisions (Evans et al. 1983). How crucial a role the Cdc28 protein kinase might have in cell cycle control was only fully appreciated upon the subsequent discovery that *cdc2* from *S. pombe*, which had been shown to regulate mitosis, encoded a homologous protein kinase (Beach et al. 1982). Cdc2 from *S. pombe* and Cdc28 from *S. cerevisiae* are the founding members of a large family of similar cyclin-dependent kinases now known as Cdks; they have therefore a second name, Cdk1. Through their association with different cyclin subunits, the two yeast Cdk1 kinases (Forsburg and Nurse 1991a; Nasmyth 1993) have many different cell cycle functions: starting the cell cycle, initiating S phase, orchestrating nuclear division, and ensuring the de-

pendence of S upon M phase, to name but a few. In mammalian cells, the Cdks that participate in these different events differ in both kinase and cyclin subunits (see below). Cyclin-dependent kinases are not exclusively concerned with cell cycle control. For example, distant relatives, cyclin H/Cdk7 from mammals (Fisher and Morgan 1994) and Ccl1/kin28 (Valay et al. 1993) from yeast have been implicated in the phosphorylation of the CTD tail of RNA polymerase II (Kim et al. 1994), whereas the yeast Cdk Pho85 regulates genes involved in phosphate metabolism through its phosphorylation of the Pho4 transcription factor (Kaffman et al. 1994).

Cdks as S-phase-promoting Factors

The first real indication that the onset of S phase might be triggered by cyclin-dependent kinases like Cdk1 (Cdc28) stemmed from the isolation of yeast mutants that started their cell cycles with a smaller than normal cell size. Mutants with a reduced cell size are particularly informative about cell cycle control, because this phenotype can only arise due to hyperactivation of the chromosome cycle (Nurse 1975), whereas mutants with an increased cell size can arise due to defects in any process needed for chromosome duplication or segregation. Dominant alleles of a gene now known as *CLN3* cause yeast to start the cell cycle at half the normal cell size (Sudbery et al. 1980; Cross 1988). *CLN3* encodes a protein distantly related to cyclins A and B, which are subunits of the Cdk1 kinase that promotes M phase in *Xenopus*. The mutant alleles encoded stable variants of Cln3 due to truncation of PEST sequences at its carboxyl terminus (Nash et al. 1988). Drawing on the analogy with mitotic cyclins and the knowledge that Cdk1 is needed for starting the yeast cell cycle, it was proposed that Cln3 might be a partner of Cdk1 and that a Cln3/Cdk1 kinase might trigger early yeast cell cycle events including S phase; i.e., it might correspond to an S-phase-promoting factor.

Deletion of *CLN3* greatly increases the size needed to start the yeast cell cycle, but it is not lethal, suggesting that other cyclins might exist with overlapping functions. Many other genes encoding cyclin partners of Cdk1 have subsequently been isolated, frequently as genes whose overexpression suppresses the temperature-sensitive growth of *cdk1* (*cdc28*) mutants: *CLN1* and *CLN2*, which encode a related pair of cyclins with only distant similarity to mitotic cyclins or to CLN3 (Hadwiger et al. 1989), and *CLB1*, *CLB2*, *CLB3*, *CLB4*, *CLB5*, and *CLB6*, which encode cyclins more homologous to the mitotic B-type cyclins from animals (Ghiara et al. 1991; Surana et al. 1991; Epstein and Cross 1992;

Schwob and Nasmyth 1993). None of these genes had previously been identified by conventional genetic analyses of the cell cycle because none of them are essential genes.

Do Clns and Clbs Correspond to SPFs and MPFs?

Because of the viability of single mutants, much of the phenotypic analysis of cyclin mutants has been performed on strains carrying multiple mutations. The finding that certain combinations of cyclin mutations cause arrest specifically in G_1 or G_2 has usually been taken to mean that the cyclin genes affected had similar functions. For example, inactivation of all three Cln cyclins causes cells to arrest indefinitely as unbudded G_1 cells, whereas all combinations of double mutants are viable, although their cells are much larger than normal (Richardson et al. 1989). The conclusion was that the three Cln cyclins had equivalent functions in promoting budding and S phase. Similar studies indicated, before the discovery of Clb5 and Clb6, that the four B-type cyclins Clb1–Clb4 all participated to a greater or lesser extent in the formation of mitotic spindles but were not needed for the initiation of S phase (Fitch et al. 1992; Richardson et al. 1992).

These two sets of G_1- and G_2-specific cyclins (i.e., Clns and Clbs) are all unstable proteins whose abundance, with the exception of Cln3, fluctuates during the cell cycle. Their periodic accumulation is partly due to transcriptional controls and partly due to fluctuations in their rates of proteolysis. All genes but *CLN3* are transcribed transiently during the cell cycle: *CLN1* and *CLN2* from late G_1 to the middle of S phase (Wittenberg et al. 1990), *CLB3* and *CLB4* from S phase till the end of mitosis (Fitch et al. 1992; Grandin and Reed 1993), and *CLB1* and *CLB2* during G_2 and M phases (Ghiara et al. 1991; Surana et al. 1991). Newly synthesized Cln1, Cln2, and Cln3 proteins have half-lives between 3 and 5 minutes (Deshaies et al. 1995; Yaglom et al. 1995), which have not been reported to vary during the cell cycle. The half-life of Clb2, in contrast, varies dramatically during the cell cycle, switching from 1 minute or less during the interval between the end of mitosis and Start to 60 minutes during the interval between Start and the onset of anaphase (Amon et al. 1994). The periodic accumulation of G_1- and G_2-specific cyclins is consistent with the notion that S phase is triggered by the accumulation or activation of Cln/Cdk1 complexes in late G_1, whereas M phase is triggered by the accumulation or activation of Clb/Cdk1 complexes in G_2. The case for Cln1 and Cln2/Cdk1 kinases being S-phase triggers is particularly strong, not only because these kinases are ac-

tivated shortly before S phase, but also because the onset of S phase is advanced by premature activation of *CLN2* transcription in early G_1 (see below).

Clns Promote S Phase by Activating Clbs

The simple picture of Clns and Clbs corresponding to S- and M-phase-promoting factors, respectively, was dealt a mortal blow by the discovery of two further members of the B-type class of cyclins, Clb5 and Clb6. *CLB5* and *CLB6* mRNAs are of low abundance in early G_1 cells and, like those for *CLN1* and *CLN2*, accumulate to high levels shortly before S phase (Epstein and Cross 1992; Schwob and Nasmyth 1993). Clb5 associates with Cdk1, and the kinase activity of these complexes fluctuates during the cell cycle, being absent in early G_1 and appearing shortly before S phase, only slowly declining during G_2 and M phases, and finally disappearing as cells enter anaphase (Schwob et al. 1994). Like other cyclins, neither Clb5 nor Clb6 is essential, but the onset of S phase is greatly delayed in their absence (Schwob and Nasmyth 1993). S phase is normally coincident with budding, but in *clb5 clb6* double mutants it starts at least 30 minutes later.

New data show that B-type cyclins do not merely support Cln/Cdk1 kinases in triggering S phase but have a central role in this process. There is an important distinction between the S-phase delay of *cln1 cln2* double mutants and that of *clb5 clb6* double mutants. Both budding and S phase are delayed in the former but only S phase in the latter. The lesser pleiotropy of the Clb defect indicated that Clb5 and Clb6 might have a more direct role in the initiation of S phase than Cln1 and Cln2. However, if activation of the Clb5 or Clb6/Cdk1 kinases were "the" S-phase trigger, why is S phase merely delayed in the *clb5 clb6* double mutant? The surprising answer is that the supposedly "mitotic" B-type cyclins Clb1–Clb4 and not the G_1-specific Cln cyclins assume the S-phase-promoting functions of Clb5 and Clb6 in their absence. S phase is delayed yet longer in *clb3 clb4 clb5 clb6* quadruple mutants and never occurs in sextuple mutants lacking all six Clb cyclins, despite the activity of Cln1 and Cln2/Cdk1 kinases (Schwob et al. 1994). Activation of Cln/Cdk1 kinases is therefore not sufficient for initiating S phase; Clb/Cdk1 kinases are essential. In some circumstances, Clns are not even necessary. Cells arrested in G_1 by being deprived of all three Cln cyclins can be triggered to enter S phase highly synchronously by inducing *CLB5* or even *CLB2* expression from the *GAL1-10* promoter (Schwob and Nasmyth 1993; Amon et al. 1994). None of the *CLB* cyclin genes is

efficiently transcribed in the absence of Cln cyclins, and *GAL*-driven expression circumvents this. These data indicate that it is through their activation of Clb cyclins that Cln/Cdk1 kinases promote S phase. An equally important conclusion is that the type of Cdk1 kinase activated in G_1 cells does not determine whether they undergo S phase or M phase (see below).

Regulation of S-phase-promoting Cyclins

Most growth-related mRNAs are absent in quiescent yeast cells and appear rapidly upon the addition of fresh nutrients. The same is true for *CLN3*. Its mRNAs are absent in stationary-phase cells, are induced within minutes of nutrient addition, but do not thereafter fluctuate during the cell cycle (Nash et al. 1988; Hubler et al. 1993). Cln3 protein levels behave similarly; in particular, their relative abundance does not greatly vary between the birth of daughter cells born below the size critical for Start and the onset of S phase (Tyers et al. 1993). Despite this lack of control, it is clear that the amount of Cln3 protein per cell is an important determinant of when yeast cells start the cell cycle; two- or more fold increases in *CLN3* gene dosage or mutations that stabilize Cln3 protein reduce the cell size at which cells bud and enter S phase (Cross 1988; Nash et al. 1988). Because Cln3 protein has a short half-life and its synthesis is not cell-cycle-regulated, its abundance per cell should reflect the general rate of protein synthesis. It is therefore an ideal candidate for a "sample" protein whose abundance is used by the cell to measure its rate of growth. Cln3 associates with Cdk1 (Tyers et al. 1992), and genetic data indicate that its main function is to activate Cdk1 (Cross and Blake 1993). The specific activity values of Cln3/Cdk1 complexes using histone H1 as substrate are, however, so low that it has not been possible to measure them as wild-type cells progress through G_1. It is not therefore clear whether the Cln3/Cdk1 kinase is in any way regulated.

In contrast to *CLN3*, the mRNAs from *CLN1*, *CLN2*, *CLB5*, and *CLB6* are all tightly cell-cycle-regulated, being low or absent in early G_1, accumulating as cells reach a critical size in late G_1, and declining as cells progress through S and G_2 phases (Koch and Nasmyth 1994). This pattern is part of a larger program of transcriptional control exerted by a pair of related transcription factors called SBF and MBF. The mRNAs of most genes involved in DNA replication and many involved in spindle pole body duplication have a similar pattern of accumulation, but the vast majority of these genes encode stable proteins, like DNA polymerase-α, whose de novo synthesis in the preceding G_1 is not needed for S-phase

entry (Falconi et al. 1993). In contrast, the short half-lives of Cln1 and Cln2 and Clb5 and Clb6 mean that de novo synthesis of these proteins in late G_1 is an important if not essential step in triggering each and every S phase.

Late G_1-specific Transcription Factors

SBF and MBF bind to SCB and MCB elements found in the promoters of late G_1-specific genes. Both factors are composed of two subunits: a common regulatory subunit Swi6 that is complexed with either Swi4 or Mbp1 DNA-binding proteins (Koch and Nasmyth 1994). Swi4 and Mbp1 have a common architecture, both containing related site-specific DNA-binding domains at their amino termini (Primig et al. 1992; Koch et al. 1993), four related ankyrin motifs within their central regions (Breeden and Nasmyth 1987), and common sequences at their carboxyl termini through which they bind to their common partner Swi6 (Andrews and Moore 1992). Swi6, which lacks a DNA-binding domain, nevertheless has a similar structure, and all three proteins are presumably derived from a common ancestor. Although SBF (Swi4/Swi6) and MBF (Mbp1/Swi6) factors bind preferentially to SCB and MCB elements, respectively, polypeptides containing only their DNA-binding domains bind to both types of sequence. G_1 cyclin genes, such as *CLN1*, *CLN2*, and *HCS26*, are mainly regulated by SBF, whereas most genes encoding DNA replication enzymes are regulated by MBF. In some cases, genes regulated by one factor become in its absence regulated by the other (C. Koch, pers. comm.).

The Yeast Cell Cycle Is Started by the SBF/MBF Transcriptional Program

Several observations suggest that the onset of all events that occur at Start is triggered by the activation of transcription due to SBF and MBF. *CLN1* and *CLN2* mRNAs are lacking in small daughter cells and only appear along with other SBF/MBF-regulated mRNAs shortly before S phase when cells attain a critical size (Koch et al. 1993). Similar levels of a *CLN2* mRNA, albeit with a *MET3* leader sequence, can be induced in early G_1 cells from a *MET3-CLN2* promoter gene fusion, and this advances the onset of S phase and budding (L. Dirick et al., in prep.). More convincing, overexpression of *SWI4* from the *GAL* promoter in cells lacking Mbp1 causes mRNAs from most, if not all, genes normally regulated by SBF and MBF to appear in very small cells; that is, as soon as daughter cells are born (M. Neuberg et al., in prep.). The mRNAs are not

overproduced but merely accumulate prematurely to levels found in wild-type cells shortly before S phase. This causes DNA replication and budding in cells whose size is one-third or less than the size needed for these two Start events in wild-type cells. The proliferation of very small cells by virtue of SBF hyperactivation is dependent on the presence of *CLN1* or *CLN2* but is totally independent of *CLN3*, whose activity normally controls the size at which yeast cells start the cell cycle. This suggests that Cln3's main, if not sole, function is to facilitate activation of the SBF/MBF transcriptional program (Tyers et al. 1993) and that the appearance of Cln1 and/or Cln2/Cdk1 kinases due to this program is crucial for the induction of S phase and budding.

Activation of SBF and MBF

How is transcription due to SBF and MBF normally activated only when cells reach a critical size? The transcriptional program fails to be activated or is greatly delayed in *cdk1* (*cdc28*) mutants (Dirick and Nasmyth 1991). It also fails to occur in mutants lacking all three Cln cyclins but is immediately restored by the reactivation of any one of them (Cross and Tinkelenberg 1991). The ability of Cln1 or Cln2/Cdk1 kinases to trigger the transcription of SBF/MBF-regulated genes suggested that the sudden activation of *CLN1* and *CLN2* transcription only when cells reach a certain size could be facilitated by a positive feedback loop by which Cln1 and Cln2 promote their own transcription along with other late G_1-specific genes. This hypothesis predicts that full activation of the transcriptional program should depend on the activities of Cln1 and Cln2, which is not the case. mRNAs known to be regulated by SBF or MBF accumulate with similar kinetics and at similar cell sizes in wild type and in *cln1 cln2* double mutants, but accumulation of the same mRNAs is delayed by several hours in *cln3* mutants (Dirick et al. 1995). These data emphasize that the three Clns do not have equivalent functions. Cln3 is essential for activating SBF/MBF-mediated transcription at normal cell sizes, whereas Cln1 and Cln2 are not.

The mechanisms by which Cln3/Cdk1 activates the SBF and MBF transcription factors only when cells reach a certain size are not understood. Genomic footprinting has shown that functionally important SCBs in the *CLN2* promoter are fully occupied by SBF in cells arrested in G_1 due to a temperature-sensitive *cdk1* mutation. Lowering the temperature of these mutant cells restores Cdk1 kinase and leads to the immediate activation of *CLN2* transcription, suggesting that Cdk1 alters the activity of complexes that are already bound to SCBs (Koch et al. 1996).

Regulation of Clb/Cdk1 Kinases during G_1

Despite their lack of involvement in the activation of gene expression, Cln1 and Cln2 have a crucial role in triggering the onset of both budding and S phase (Hadwiger et al. 1989), which are advanced by premature expression of *CLN2* and which are delayed in *cln1 cln2* double mutants until cells have grown at least three times the normal size (Dirick et al. 1995). Current data are consistent with the notion that Cln1 and Cln2 promote S phase by stimulating activation of Clb/Cdk1 kinases. They do this by facilitating the accumulation of Clb proteins (Amon et al. 1994) and the disappearance of a Cdk1 inhibitory protein p40Sic1 (Schwob et al. 1994).

Rapid proteolysis of Clb2, which is largely shut off during S and G_2 phases, commences during anaphase and persists until activation of Cln1 and Cln2/Cdk1 kinases during the subsequent G_1 period. The extremely short half-life of Clb2 during early G_1 (1 minute or less) ensures that it cannot accumulate in G_1 cells lacking Cln cyclins, even when highly expressed from the *GAL* promoter (Amon et al. 1994). Clb5 protein, with a half-life of 5 minutes (Seufert et al. 1994), is more stable during this period. Cells arrested in G_1, due to deprivation or physiological inactivation of all three Clns, undergo S phase (S. Irniger, pers. comm.) upon inactivation of proteins such as Cdc16, Cdc23, and Cse1, which are required for Clb2 proteolysis (Irniger et al. 1995). This implies that the persistence of rapid Clb proteolysis until the activation of Cln1 and Cln2 could have an important role in preventing premature accumulation of Clb/Cdk1 kinase activity.

Role of a Cdk Inhibitor, p40Sic1

Probably the most important mechanism regulating the onset of S phase in yeast is the late G_1-specific destruction of a Cdk1 inhibitory protein p40Sic1 (Schwob et al. 1994). It had been found that transcription of *CLB5* in small early G_1 cells failed to advance S phase. This was surprising because premature Cln2 expression did advance S phase and yet it was thought that Clb5 had a more direct role than Cln2 in the initiation of DNA replication. The ectopic expression caused Clb5 protein accumulation, but the resulting Clb5/Cdk1 complexes were inactive due to the presence in early G_1 cells of a factor capable of inhibiting the Clb5/Cdk1 kinase. The inhibitory factor is a 40-kD protein encoded by the *SIC1* gene (Nugroho and Mendenhall 1994), which had previously been purified as a potential substrate of Cdk1 and found to be an inhibitor (Mendenhall 1993). p40Sic1 is a potent inhibitor of Clb/Cdk1 but not of Cln1 or Cln2/Cdk1 kinase activities (M. Mendenhall, pers. comm.).

Early G_1 cells contain high levels of p40Sic1 protein, but it disappears shortly before the onset of S phase and does not reappear until cells complete mitosis. This pattern of accumulation is partly due to the periodic accumulation of *SIC1* mRNAs, whose abundance increases transiently from a basal level as cells exit from mitosis (Donovan et al. 1994; Schwob et al. 1994). However, cell-cycle-dependent proteolysis mediated by the Cdc34 ubiquitin-conjugating enzyme is implicated in the sudden disappearance of p40Sic1 in late G_1. The inhibitor accumulates at the end of mitosis to similar levels in wild type and *cdc34* mutants but fails to disappear in the latter when cells ought to be entering S phase (Schwob et al. 1994). This defect is highly specific, because the SBF/MBF transcriptional program, activation of Cln1 and Cln2/Cdk1 kinases, and budding all take place in *cdc34* mutants. The accumulation of p40Sic1 probably inhibits activation of all Clb/Cdk1 kinases in *cdc34* mutants, and this must be responsible for their G_1 arrest because deletion of the *SIC1* gene permits them to undergo DNA replication. The G_1 arrest of *cdc4* and *cdc53* mutants is also due to a failure to destroy p40Sic1.

These data suggest that proteolysis of p40Sic1 mediated by Cdc4, Cdc34, and Cdc53 proteins is an essential step toward the initiation of S phase in *S. cerevisiae*. How then is this process regulated? p40sic1 protein persists in cells arrested in G_1 due to triple *cln* or *cdk1* mutations but not in sextuple *clb1-6* mutants (T. Bohm, pers. comm.), suggesting that Cln but not Clb Cdk1 kinases play a part in its destruction. Unlike SBF/MBF-mediated transcription, the disappearance of p40Sic1 is delayed in *cln1 cln2* double mutants, and this is largely responsible for their delayed S-phase entry (Dirick et al. 1995). Thus, Cln3 is insufficient and Cln1 and Cln2 are necessary to trigger disappearance of p40Sic1 on schedule. p40Sic1 is not an effective inhibitor of the Cln2/Cdk1 kinase either in vivo or in vitro but is an excellent substrate, at least in vitro (M. Mendenhall, pers. comm.). This raises the possibility that phosphorylation of p40Sic1 by Cln1 or Cln2/Cdk1 kinases might be the trigger for its proteolysis via Cdc34. Consistent with this notion, *sic1* mutations that alter potential Cln/Cdk1 phosphorylation sites delay the disappearance of p40Sic1 protein and the onset of S phase (T. Bohm, pers. comm.).

Cdc7

A piece of the S-phase regulatory jigsaw that has not yet been satisfactorily fitted into the above picture is the role and regulation of the Cdc7 kinase. Kinase activity associated with Cdc7 rises in late G_1 along with

that of Clb/Cdk1 kinases (Jackson et al. 1993; Yoon et al. 1993). *cdc7* mutants fail to initiate S phase despite possessing active Clb/Cdk1 kinases. Cdc7 is therefore needed in addition to Clb/Cdk1 kinases. Cdc7 associates with the Dbf4 protein, which is also needed for replication and could be a regulatory subunit with properties (but not sequences) similar to cyclins (Jackson et al. 1993). Dbf4 associates in vivo with proteins that bind to replication origins, suggesting that some of these are substrates for the Cdc7 kinase (Dowell et al. 1994). One scenario is that Clbs activate the replication apparatus via their activation of Cdc7; i.e., Clb/Cdk1 kinases are upstream of Cdc7 on a linear pathway leading to replication (Jackson et al. 1993). It is more likely, however, that Clb/Cdk1 kinases have several roles in activating replication, only one of which might be to promote activation of the Cdc7 kinase. The need for Cdc7 and Dbf4 can be bypassed by *bob1* mutations, suggesting that Cdc7's normal function is to relieve a negative control.

Summary: Promoting S Phase in S. cerevisiae

There now exists a detailed, but by no means complete, hypothesis for how S-phase-promoting factors are produced in *S. cerevisiae* (see Fig. 2). Let us start with a small quiescent cell that has just been inoculated into fresh medium. The presence of nutrients induces the appearance, largely due to transcriptional controls, of most, if not all, mRNAs needed for cell growth, whose translation is facilitated by the simultaneous activation of translational initiation factors like eIF-4e. Neither of these events is dependent on the forthcoming chromosome cycle, but both are essential for the eventual production of specific S-phase-promoting factors. One of the many proteins whose synthesis is stimulated in this manner is Cln3, a Cdk1 cyclin, whose abundance per cell, due to its short half-life, reflects the overall rate of translation of "growth" mRNAs and thereby the size and growth rate of the cell. Few, if any, other S-phase-promoting factors are made in these small growing cells until they reach a threshold size, at which Cln3/Cdk1 suddenly activates transcription of a large battery of genes, some of which encode unstable replication proteins like Cdc6 (Piatti et al. 1995), whereas others encode further S-phase-promoting cyclins like Cln1 and Cln2 and Clb5 and Clb6. The size at which this transcriptional explosion occurs determines the size at which cells enter S phase and is regulated by external factors like nutrients, which exert their effect via cAMP; an increase in the activity of cAMP-dependent protein kinases in rich medium increases the size needed to trigger the appearance of *CLN1* and *CLN2* mRNAs. With the activation of the late

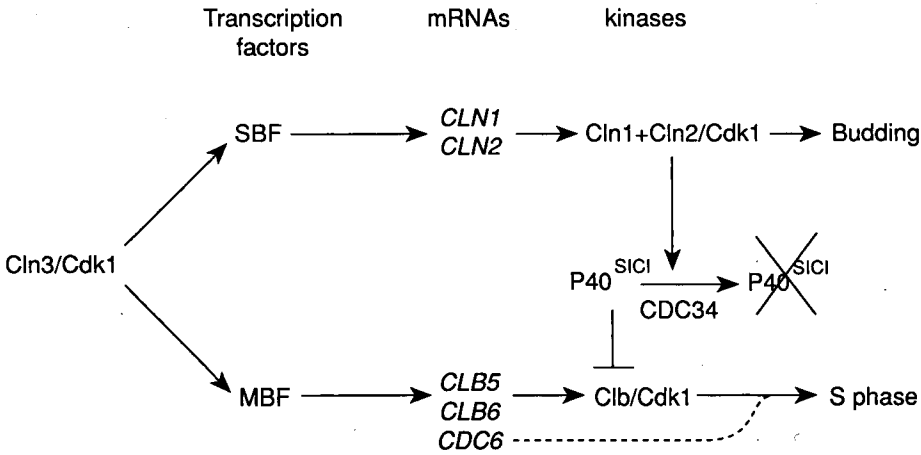

Figure 2 The production of S-phase-promoting factors in *S. cerevisiae*. The process starts with activation of transcription due to SBF and MBF transcription factors when cells reach a critical size. This leads to the synthesis of Cln1/2 and Clb5/6 cyclins and replication proteins like Cdc6 which are needed for the formation of pre-RCs at future replication origins (Cdc6 is also synthesized at the end of mitosis). Clb5 and Clb6/Cdk1 complexes remain inactive until the p40Sic1 inhibitor is destroyed via ubiquitin-mediated proteolysis involving Cdc4, Cdc34, and Cdc53, an event that is thought to be triggered by Cln1 and Cln2/Cdk1 kinases.

G_1-specific transcription program, the "baton," as it were, passes from Cln3 to Cln1 and Cln2. Accumulation of Cln1 or Cln2/Cdk1 kinases, due largely to activation of the SBF transcription factor by Cln3/Cdk1, leads to the next crucial step: activation of Clb/Cdk1 kinases, which are needed to activate the Cdc7 kinase and, presumably, many other replication proteins.

The absence of Clb/Cdk1 kinases in early G_1 cells is due partly to a lack of transcription of *CLB* genes, partly to rapid proteolysis which is started during anaphase but persists during early G_1, and partly to the accumulation at the end of mitosis or in stationary phase of the p40Sic1 protein, which is a potent inhibitor of Clb/Cdk1 kinases. Cln3/Cdk1 induces synthesis of Clbs 5 and 6, whereas Cln1 and Cln2/Cdk1 kinases promote accumulation of active Clb/Cdk1 kinases by drastically reducing proteolysis of cyclins like Clb2 and, more important still, by triggering proteolysis of p40Sic1. How activation of Clb/Cdk1 and Cdc7 kinases triggers the formation of replication forks and why they do this in G_1 but not in G_2 cells will require consideration of the substrates of these kinases.

S-phase-promoting Factors in Other Fungi

A single Cdk kinase related to Cdk1 (known as Cdc2) is needed for the onset of both S and M phases in two other ascomycetes, the fission yeast *S. pombe* (Nurse and Bissett 1981) and the filamentous fungus *Aspergillus nidulans* (Osmani et al. 1994). Less is known, however, about the cyclin partners of Cdk1 (Cdc2) that promote S phase in these two organisms. The *puc1* gene in *S. pombe* encodes a cyclin related in sequence to Cln3 (Forsburg and Nurse 1991b), but there is little or no evidence that *puc1* helps to promote the onset of S phase in vegetative cells (Forsburg and Nurse 1994). This may be less surprising now that we realize that Cln cyclins have only an indirect role in promoting S phase, even in *S. cerevisiae*, and that B-type cyclins are more intimately involved. *S. pombe* contains at least three B-type cyclins encoded by the *cig1* (Bueno et al. 1991), *cig2* (Bueno and Russell 1993; Connolly and Beach 1994; Obara Ishihara and Okayama 1994), and *cdc13* genes (Booher and Beach 1988; Hagan et al. 1988). None is necessary for the G_1-to-S-phase transition in vegetative cells, but *cdc13* is essential for M phase. There are indications that these B-type cyclins are nevertheless important for promoting S phase. For example, *cig2* is important for the terminal rounds of DNA replication that occur as *S. pombe* cells enter stationary phase upon nitrogen starvation (Obara Ishihara and Okayama 1994), and deletion of both *cig1* and *cig2* causes nuclei in vegetatively growing cells to spend longer in G_1 (Connolly and Beach 1994). *S. pombe* might therefore resemble *S. cerevisiae*, in that one of several B-type cyclins is sufficient for triggering S phase.

S phase in *S. pombe* depends on the activation of transcription in late G_1 of key replication genes by a pair of transcription factors Res1/Cdc10 (Tanaka et al. 1992; Caligiuri and Beach 1993) and Res2/Cdc10 (Tanaka et al. 1992; Zhu et al. 1994), which are related to Swi4/Swi6 and Mbp1/Swi6 in *S. cerevisiae*. Cdc10, which resembles Swi6 (Breeden and Nasmyth 1987), is necessary for both factors and is essential for the G_1-to-S-phase transition in *S. pombe* (Nurse et al. 1976). Res1/Cdc10 complexes are more important for S phase in vegetatively growing cells, whereas Res2/Cdc10 complexes are more important for premeiotic DNA replication. Fewer genes involved in DNA replication are regulated by these transcription factors than are regulated by their equivalent factors in *S. cerevisiae*. Nevertheless, transcriptional activation in late G_1 of at least two genes, *cdc18* (a homlog of *CDC6* from *S. cerevisiae* [Kelly et al. 1993]) and *cdt1*, is essential for initiation of the ensuing S phase (Hofmann and Beach 1994). Neither *cig1* nor *cdc13* mRNA are cell-cycle-regulated, but *cig2* mRNAs are periodic (Connolly and Beach 1994), ac-

cumulating to maximal levels in late G_1, and could therefore be regulated by one of the two Res/cdc10 factors. As in *S. cerevisiae*, Cdk1 is needed for late G_1-specific gene activation; in this case, there is evidence that Cdk1 is needed for the DNA-binding activity of Res/cdc10 factors (Reymond et al. 1993). A zinc-finger-containing protein, called Rep2, is needed for the activity of Res2/cdc10 but not Res1/cdc10 complexes in mitotic *S. pombe* cells (Nakashima et al. 1995).

Whether changes in the proteolysis of B-type cyclins in *S. pombe* has any role in regulating their activity during G_1 is not known. There exists, however, a Cdk inhibitory protein, encoded by the *rum1* gene, which delays the onset of S phase under certain circumstances (Moreno and Nurse 1994). In fast-growing cells, daughter cells are born larger than the threshold size needed to start the cell cycle, G_1 is extremely short, and deletion of *rum1* has little or no effect. Inactivation of the Wee1 protein kinase eliminates size control over M phase, and daughter cells are now born below the critical size and must spend one-third of their next cycle growing large enough to enter S phase. This longer G_1 period of *wee1* mutants is largely eliminated by deletion of the *rum1* gene. Rum1 is also implicated in delaying or even preventing activation of B-type cyclin/Cdk1 kinases in nitrogen-starved cells and in cells arrested in G_1 due to *cdc10* mutations. The Rum1 protein is a potent inhibitor of Cdc13/Cdk1 kinase (Correa-Bordes and Nurse 1995), but it has little or no sequence similarity to p40Sic1 from *S. cerevisiae* or Cdk inhibitors from mammalian cells, nor is it known whether or how Rum1 abundance is regulated during the cell cycle. There have been no measurements of specific B-type cyclin/Cdk1 kinase activities during G_1, so it is difficult at this stage to paint even a crude picture of how these kinases are regulated during this stage of the *S. pombe* cell cycle.

Although the details differ considerably, the broad outlines of S-phase control seem conserved between *S. pombe* and *S. cerevisiae*; this includes the synthesis of replication proteins like spCdc18 or scCdc6 due to Cdc10/Swi6-like transcription factors, the role of B-type cyclin Cdk kinases, and the control of these kinases by inhibitory proteins.

S-phase-promoting Factors in Animal Cells

It is not possible to assemble as detailed a picture of the events leading to S phase in animal cells as in yeast, not because fewer components have been identified (the advent of two-hybrid screening has largely solved this problem), but because their functional analysis is still much harder (function cannot be as reliably inactivated by antisense RNA or even

antibody injections as by gene mutation, which although possible in mice, is still laborious and difficult to make conditional) and because studies are performed on many different types of cells whose behavior can be very different. Nevertheless, one can detect several parallels between animal cells and yeast, which are worth pointing out since they may represent the more highly conserved aspects of S-phase control.

The Restriction Point

It has long been known that de novo protein synthesis during G_1 is needed for S phase in tissue-culture cells. However, entry into S phase becomes less sensitive to inhibitors of protein synthesis about 2 hours before DNA replication commences. It is around this point in late G_1, called the restriction point, that many mammalian tissue-culture cells become capable of entering S phase upon withdrawal of growth factors, which are potent effectors of the cell's rate of protein synthesis (Pardee 1989). G_1-specific cyclins and their catalytic subunits may be some of the proteins whose synthesis preceding the restriction point is stimulated by growth factors and is needed for the initiation of DNA replication.

G_1-specific Cdks

Whereas ascomycetes use the same Cdk subunit to regulate S and M phases, animal cells use at least three different kinase subunits. Cdk1 (cdc2) for M phase (Riabowol et al. 1989; Hamaguchi et al. 1992), Cdk2 for S phase (Fang and Newport 1991; Paris et al. 1991), and Cdk4 or Cdk6 for progression from early to late G_1 (Sherr 1994a). As in yeast, multiple cyclins associate with a given Cdk. Cdk4 and Cdk6 associate with D-type cyclins (Matsushime et al. 1992). Cyclin D1 is absent in quiescent cells and is induced by mitogenic growth factors (Matsushime et al. 1991), reaching maximal levels several hours after their addition but long before the onset of S phase. Cyclin D1 has a half-life of around 30 minutes, which, as a percentage of the doubling time, is similar to that of yeast G_1 cyclins. It accumulates in the nucleus during G_1, but its abundance varies little if at all during the cell cycle of exponentially growing cells (Sewing et al. 1993). Antibody injection experiments had suggested that cyclin D1 is essential for progression from G_1 to S phase in several different types of cells (Baldin et al. 1993; Lukas et al. 1994). However, mouse embryos lacking any cyclin D1 gene can undergo all prenatal development (Sicinski et al. 1995). Whether this means that the effects of antibody injection were less specific than anticipated or that

the cell lines used in such studies are more dependent on cyclin D1 than most embryonic cells remains to be determined. It is worth noting that even in yeast few if any cyclin gene deletions are lethal, despite having drastic effects on the kinetics of specific cell-cycle transitions, and the same could be true for mice.

Another means of testing the role of D-type cyclins in promoting S phase has been to analyze the consequences of inducing high levels of synthesis during early G_1. Hyperaccumulation of cyclin D1 caused a modest reduction in G_1 length in cycling cells and a more persuasive reduction during the outgrowth of previously quiescent cells (Quelle et al. 1993; Resnitzky et al. 1994). In a study using regulated cyclin D expression, hyperactivation did not, however, increase the rate of cell proliferation (although one suspects that it reduced cell size), which is consistent with similar findings in yeast and suggests that the G_1-to-S-phase transition is not a rate-determining step for proliferation, at least for the tissue-culture cells used in these studies. Similar experiments, performed with cyclin D transgenes in mice, have little immediate effect on cell proliferation but do eventually give rise to tumors, especially in conjunction with other oncogenes (Bodrug et al. 1994; Lovec et al. 1994).

Cdk2 is found mainly associated with cyclins E and A in mammalian cells. Both proteins oscillate in phase with the chromosome cycle, with cyclin E accumulating to maximal levels in late G_1 (Dulic et al. 1992; Koff et al. 1992), possibly coincident with the restriction point, and cyclin A somewhat later during S and G_2 phases (Pines and Hunter 1990; Tsai et al. 1991). Antibody injection experiments suggest that Cdk2 and cyclin A (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Tsai et al. 1993) are essential for S phase in mammalian tissue-culture cells. Cyclin A, however, has been found unnecessary for DNA replication in extracts from *Xenopus* eggs and for at least certain divisions during *Drosophila* embryogenesis (Lehner and O'Farrell 1990; Knoblich and Lehner 1993). Cyclin E, in contrast, seems essential for replication in *Drosophila* and *Xenopus*. Maternal cyclin E transcripts disappear suddenly as *Drosophila* cells complete the last rapid mitotic division during embryogenesis (cycle 16) and start to accumulate for the first time in G_1, but the mRNAs reappear due to zygotic gene expression shortly before S phase in the minority of cells that continue cell division at this point; they also reappear during late embryogenesis in tissues that undergo endoduplication cycles. More convincing still, cyclin E mutants cease all DNA replication after cycle 16, and ectopic expression of cyclin E from a heat shock promoter induces S phase in cells that would otherwise have

arrested in G_1; however, this only occurs if expression is induced soon after their exit from the previous mitosis (Knoblich et al. 1994). *Drosophila* cyclin E protein associates with a Cdk that resembles the mammalian Cdk2. In mammalian tissue culture, the artificial induction of high levels of cyclin E is insufficient to cause quiescent cells to enter S phase, but it does cause a modest reduction in the length of their G_1 period and thereby enables cells to divide at a smaller cell size (Ohtsubo and Roberts 1993; Resnitzky et al. 1994). It does not, however, alter their rate of proliferation. Thus, the accumulation of cyclin E in late G_1 may have an important role in the onset of S phase in both arthropod and vertebrate cells. Neither mammalian nor *Drosophila* cells seem to require Cdk1 (cdc2) or B-type cyclins for S phase.

Cdk Inhibitors

As in yeast, the activities of D and E type cyclin/Cdk complexes are not just regulated by the pattern of cyclin accumulation. There is a family of small proteins (p15, p16, and p18) composed of ankyrin motifs analogous to those first found in Swi6 (Breeden and Nasmyth 1987), which, it is thought, inactivate Cdk4 and Cdk6/cyclin D complexes by binding to the Cdk subunits and displacing cyclin D (Serrano et al. 1993; Guan et al. 1994; Hannon and Beach 1994). Expression of p15 is induced in keratinocytes by antimitogenic factors like transforming growth factor β (TGF-β). p16INK4, on the other hand, increases in abundance following the stimulation of fibroblasts with serum (Tam et al. 1994); its accumulation to maximal levels during S phase might play a role in inhibiting Cdk4 and Cdk6 kinases as cells enter G_2. Whether any of these inhibitors has a role in preventing premature activation of cyclin D/Cdk kinases during early G_1 is unclear. Transient overproduction of p16INK4 causes cells to arrest in G_1, and mutations in the INK4/MST1 gene for p16 are found in most tumor cell lines and in some primary tumors (Hunter and Pines 1994), suggesting that p16 could have an important role in arresting the proliferation of potential tumor cells.

Other cyclin kinase inhibitors, p21CIP1/WAF1/SDI1 (El-Deiry et al. 1993; Harper et al. 1993) and p27KIP1 (Polyak et al. 1994), bind to and inhibit a wide variety of Cdk kinases including cyclin D/Cdk4, cyclin E/Cdk2, and cyclin A/Cdk2. p27 levels decline modestly as quiescent macrophage cells are stimulated to enter S phase by growth factors (Kato et al. 1994). A proposal that this decline is important for entry into S phase remains to be tested rigorously. p27 abundance declines rapidly in T cells in response to interleukin 2 (IL-2), which could be an important

event in the induction of S phase by this factor (Firpo et al. 1994). p21 levels, in contrast, rise upon growth factor stimulation, which might have a role in preventing activation of S-phase-promoting Cdks before cells have made other preparations for S phase. It is still unclear, however, whether inhibitors like p21 and p27 exert a strong effect on the kinetics of S-phase entry in cycling cells. Both certainly have the potential to block cell cycle progression. A good example is the increase in abundance of p21 in response to DNA damage, which is due to activation of p21WAF1 transcription by p53 and has a role in preventing the entry of irradiated cells into S phase (Deng et al. 1995). Other modes of p21 induction, for example, its induction by growth factors, do not involve p53 (Parker et al. 1995), and it is unknown whether p21 levels are sufficient to delay activation of S-phase-promoting Cdks in cells lacking DNA damage and, if so, how cells surmount this barrier.

Phosphorylation

The activities of all Cdks are dependent on phosphorylation of a highly conserved residue (Thr-161 [Solomon et al. 1992]), but the kinase responsible, which is another cyclin-dependent kinase composed of Cdk7(MO15) and cyclin H (Fesquet et al. 1993; Fisher and Morgan 1994; Solomon et al. 1993), and which seems identical to the kinase that phosphorylates the CTD of RNA polymerase II (Roy et al. 1994), is not apparently cell-cycle-regulated (Tassan et al. 1994). Phosphorylation at other conserved sites on Cdk1 (Tyr-15), which inhibits its kinase activity during G_2 in yeast and in animal cells, is reversed by the Cdc25 (*S. pombe*) family of phosphatases. Vertebrates possess at least three members of Cdc25 phosphatases; Cdc25A undergoes phosphorylation and activation in late G_1 and could have a role in the activation of Cdk2 kinases shortly before S phase (Hoffmann et al. 1994).

Late G_1-specific Transcription

As found in yeast, transcripts from many genes implicated in DNA replication or its control oscillate in abundance during the cell cycle, reaching maximal levels in late G_1; examples include mRNAs for thymidine kinase (Dou et al. 1992), dihydrofolate reductase (Bjorklund et al. 1990), cdc2 (Dalton 1992), DNA polymerase-α (Pearson et al. 1991), and cyclin E (Reed et al. 1992). A family of transcription factors collectively called E2F are implicated in this transcriptional program (La Thangue 1994). Most of these factors are heterodimers containing an

E2F and a DP1 subunit. There are several subtypes of each of these subunits, creating many different potential heterodimers, whose regulatory properties almost certainly differ. E2F binds to sequences that resemble the SCBs and MCBs bound by SBF and MBF in yeast, but E2F and DP1 have no sequence similarities with Swi4 or Swi6.

Analyzing patterns of mRNA accumulation in cells lacking particular genes will ultimately be necessary to establish which E2F factors are responsible for regulating which late-G_1-specific genes. Mutation of an E2F gene in *Drosophila* abolishes both S phase and developmentally regulated pulses of DNA replication enzyme mRNAs (Duronio and O'Farrell 1994; Duronio et al. 1995). Analyzing similar mutants in the mouse will undoubtedly be complicated by potential overlaps in the function of different E2F and DP-1 genes, as has been found for SBF and MBF in yeast. Another complication is that genes thought to be activated or controlled by E2F-binding sites do not have a common mode of regulation. The c-*myc* gene, for example, whose promoter contains an E2F site implicated in its transcriptional regulation, is regulated in a very different manner from many other supposedly E2F-regulated genes.

Activation of E2F in Late G_1

At least three different regulatory proteins related to the product of the retinoblastoma gene (Rb itself, p107, and p130) are found associated with different E2Fs during G_1 (Hinds and Weinberg 1994). Rb associates with E2F-1, E2F-2, and E2F-3 and can inhibit their ability to activate transcription in vitro (Dynlacht et al. 1994). Rb is phosphorylated at many residues throughout the cell cycle, but two or three specific residues become phosphorylated only in late G_1 and remain so until the end of mitosis (Sherr 1994b). This hyperphosphorylation prevents Rb from binding to E2F. It is therefore proposed that Rb hyperphosphorylation caused by cyclin D- or cyclin E-associated Cdks that become active in late G_1 enables accumulation of uncomplexed E2F, which is free to activate transcription. Although attractive as a regulatory mechanism, this theory has never been rigorously tested; for example, by characterizing the properties of Rb proteins in which residues whose phosphorylation is cell-cycle-regulated (and not others) have been mutated. The current hypothesis would predict that expression of modest levels of proteins that lack the ability to be hyperphosphorylated should be sufficient to prevent the activation of genes regulated by E2F.

There are two other potential mechanisms for the activation of E2F-regulated genes by G_1-specific Cdks. Phosphorylation of E2F-1's serine

residues 332 and 337 hinders its interaction with Rb but facilitates its interaction with the adenovirus E4 protein. Both residues are largely unphosphorylated during early G_1, become phosphorylated during late G_1, and can be phosphorylated by cyclin D/Cdk4 kinase in vitro (Fagan et al. 1994). Cell-cycle-specific phosphorylation of E2F-1 by cyclin D/Cdks could therefore also contribute to the accumulation of "free" E2F in late G_1. Finally, the abundance of E2F mRNAs increases dramatically in late G_1. It has therefore been proposed that activation of Cdks in late G_1 stimulates synthesis of E2F, which could contribute to its accumulation in a form unbound to the Rb protein (Johnson et al. 1994). The effectiveness of this mechanism might depend on whether phosphorylation of Rb or E2F can actually liberate E2F from Rb. If not, then de novo synthesis of E2F in late G_1 could have a vital role and not just a helping hand in the activation of E2F-regulated genes.

The role of Rb as a key cell-cycle regulator has been questioned recently by the discovery that embryonic stem cells homozygous for Rb deletions can contribute to most tissues in the mouse; that is, cell-autonomous Rb function is not essential for the proliferation and differentiation of most types of mammalian cells (Sherr 1994b). It will be important to establish to what extent the normality of Rb-negative cells in vivo is due to the assumption of Rb's regulatory functions by other Rb-related proteins, to E2F remaining adequately regulated in the absence of any such protein, to regulation of E2F-mediated gene expression being less important than hitherto envisaged, or to deregulated entry into S phase being not particularly damaging to embryonic development because cell proliferation can be equally well if not better regulated by growth control or cell death (apoptosis). There are modest increases in the rates of proliferation within certain neuronal tissues in *rb* mutants but these seem to be compensated by increased apoptosis (Morgenbesser et al. 1994).

Comparisons to Yeast: Are Cyclin D and Rb on the Same Pathway?

The similarities between yeast and animal cells are sufficient to encourage a comparison of their factors that promote S phase. Several facts suggest that cyclin D/Cdk4 kinases perform functions that are analogous to Cln3/Cdk1. First, the patterns of synthesis of cyclin D and Cln3 are similar; neither is tightly periodic and both appear to be stimulated primarily by "growth"-promoting signals rather than chromosome cycle signals. Second, both proteins are very different from other cyclins in-

volved later in the cycle, like the E, A, and B types, and differ greatly in their substrate specificity in that neither Cln3/Cdk1 (Tyers et al. 1992) nor cyclin D/Cdk4 readily phosphorylates histone H1 (Kato et al. 1993). Third, although the sequences of Cln3 and cyclin Ds do not greatly resemble each other, there is some evidence that they can perform equivalent tasks when expressed in yeast (Hatakeyama et al. 1994).

One of the more striking similarities between cyclin D and Cln3 is that the main, if not sole, task of both cyclins may be to activate late G_1-specific transcription. The dependence of S phase on Cln3 in yeast (i.e., its occurrence at a normal cell size) is abolished in cells that express late G_1-specific mRNAs constitutively due to hyperactivation of SBF. Likewise, entry into S phase of mammalian cell lines in which E2F cannot be regulated by Rb (due to mutations in the Rb gene) is sensitive neither to the injection of cyclin-D-specific antibodies (Lukas et al. 1994) nor to the overexpression of the cyclin-D-specific inhibitor p16INK4 (Serrano et al. 1995). Thus, both Cln3 and cyclin D appear to be "upstream" effectors of late G_1-specific transcription whose function becomes redundant when the "downstream" transcription factors are activated by other means.

There are indications that the abundance of cyclin D might be regulated by Rb. Cyclin D levels are very low in cell lines lacking Rb (Bates et al. 1994), suggesting that Rb, to which it is capable of binding in vitro, might play some role in stabilizing cyclin D protein. That cells can proliferate with very low levels of cyclin D when Rb is missing is consistent with its function being bypassed in these cells. Whether this is exclusively due to the resulting hyperactivation of E2F or to the deregulation of other S-phase-promoting events in *rb* mutants remains to be established.

What Are the Animal Equivalents of Clb5 and Clb6?

Cyclin E/Cdk2 kinases could perform functions similar to those of Cln1 and Cln2/Cdk1 kinases or those of Clb5 and Clb6/Cdk1 kinases. In contrast to Cln3 and cyclin D, accumulation of all these cyclins is tightly regulated by passage through the chromosome cycle. Certain observations suggest that an analogy between cyclin E and the "early" Clb cyclins Clb5 and Clb6 might be more appropriate. The functions of cyclin E seem highly conserved between vertebrates and arthropods, which is analogous to the conserved role of B-type cyclins (but not Cln cyclins) in regulating S phase in *S. pombe* and *S. cerevisiae*. Furthermore, the sequences of cyclins E resemble B-type cyclins much more

than than they do Cln cyclins. The apparent activation of cyclin E/Cdk2 kinases 1 or 2 hours before the onset of S phase (Koff et al. 1992) is, on the other hand, more analogous to the behavior of Cln1 and Cln2, and is at first glance inconsistent with the notion that activation of cyclin E/Cdk2 is the "S phase trigger." If cyclin E/Cdk2 performs functions similar to those of Clb5/Cdk1, then cyclin A/Cdk2 could perform functions similar to those of Clb3 and Clb4/Cdk1 kinases. Cyclins A and Clb3 and Clb4 all accumulate later than cyclins E and Clb5 and Clb6, possibly too late to initiate S phase in wild-type cells, but not too late to facilitate the firing of "late" origins of replication.

Prospects for Genetic Analysis of S Phase in Animals

Analysis of the cell cycle in mammalian cells has until recently been dominated by experiments involving "gains of function"; hyperactivation of cyclins accelerate the cycle, overexpression of Rb suppresses division, cotransfection of transcription factor X can activate reporter gene Y, etc. There are two reasons for this. First, the prime motivation for many who study the mammalian cell cycle has been to understand oncogenesis, which until recently was thought to be largely due to gain-of-function mutations. Second, it is still much easier to study the effects caused by gains of function in mammalian cells than it is to study effects due to loss of function. Antisense has a discredited track record in establishing phenotype, whereas antibody injection is technically demanding and difficult to interpret unambiguously. Gain-of-function approaches only tell us what "can" function, which may be important for oncogenesis, but generally distracts us from understanding how normal cells actually function. The more recent appreciation that many, if not most, mutations that cause cancer are "loss-of-function" mutations and the development of methods to delete genes in mouse ES cells have been a very important step in the right direction. The latter has provided a reliable means of generating either animals or cell lines that are homozygous for mutations in key cell-cycle regulators.

It should not be forgotten, however, that the "reverse genetics" approach pioneered in yeast and now available in the mouse has its limitations, and there will continue to be much scope for the conventional genetic approach of isolating mutants following screening. This is possible in yeast, flies, and (hopefully) in the zebra fish, but is unlikely ever to be practical for mice. In an era when genetic analysis increasingly holds sway in experimental biology, it is seldom appreciated how difficult it is to determine gene function merely by analyzing phenotype. Gene knock-

outs, whether they are performed in mice or yeast, appear either to have little effect or to cause lethality. In the first case, it is difficult to ascertain what might have gone wrong without previous intuitions about the gene's function, whereas in the second case, it is difficult to distinguish primary from secondary phenotypes. Conventional mutant screens, when successful, are highly selective on both counts: Mutants are sought with specific defects in an area of biology in which the investigator is an expert and pleiotropic mutants are usually avoided. Reverse genetics will be most valuable when we have some idea where to look for defects.

STOPPING DNA REPLICATION IN G_2

Could Cell-cycle State Be Determined Solely by Cdks?

How do cells ensure that no DNA sequence is replicated more than once during S phase and that mitosis precedes the next round of replication? There is ample evidence in yeast and animal cells that the timing of S and M phases is determined by the pattern of activation of G_1- and G_2-specific Cdks. Might not just the timing but also whether a cell undergoes S or M phase be determined by the state of Cdk-cyclin complexes? Let us create an imaginary cell that possesses only two types of Cdks, an S-phase- and an M-phase-promoting form (SPF and MPF), which oscillate in abundance with identical frequencies but out of phase with each other. Let us further specify the shape of each wave such that the S-phase-promoting Cdk is inactive during the first quarter of the cycle, active during the second, but inactive again during the third and fourth quarters and such that the M-phase-promoting Cdk is inactive throughout first and second quarters, partially active during the third, and fully active during the fourth. Let us further assume that whether or not the cell undergoes chromosome duplication (S phase) or segregation (M phase) is determined entirely by the state of the two Cdks: If SPF is active and MPF inactive, then cells replicate DNA, whereas if MPF is fully active and SPF is inactive, then cells undergo mitosis. Our imaginary cells will undergo alternate S and M phases with intervening gaps equivalent to G_1 and G_2. This hypothesis, that cell-cycle state is defined solely by the state of S- and M-phase-specific Cdks (Nurse 1994), predicts that activation of G_1-specific Cdks in the absence of G_2-specific forms should trigger S phase, whereas activation of G_2-specific Cdks in the absence of G_1-specific forms should trigger M phase, irrespective of a cell's position in the cycle at the time. In other words, it should be possible, by artificial manipulation of SPF and MPF activities, to cause cells to jump between states that are not normally adjacent; that is, it should be

possible to override the cell's history. Two observations in *S. pombe* are consistent with this view (Hayles et al. 1994). First, *S. pombe* mutants that lack the "mitotic" cdc13/Cdk1 kinase fail to enter M phase and instead undergo multiple rounds of DNA replication. Thus, preventing activation of an MPF allows cells to reenter the S-phase state without proceeding through the M-phase state. Second, *cdc10* mutants, which normally arrest in G_1 at their restrictive temperature due to a failure to activate late-G_1-specific transcription, can be induced instead to enter mitosis merely by overproducing the two components of the mitotic Cdk: cdc13 and Cdk1. Thus, premature activation of MPF can induce cells lacking SPF and MPF to enter M phase without any prior S phase.

If, as indicated by these experiments, the "Cdk state" model is correct, then understanding how the cell cycle runs in the correct direction (i.e., how cells ensure that chromosome duplication precedes their segregation) should be simply a question of understanding the dynamics or logic of SPF and MPF oscillations. Much progress has been made in understanding how different cyclin-dependent kinases regulate each other in *S. cerevisiae*. Consider the S-phase-promoting Cln1 and Cln2/Cdk1 kinases and the M-phase-promoting Clb1-Clb4/Cdk1 kinases. Cln1 and Cln2 promote activation of Clbs by turning off their proteolysis and by triggering destruction of p40Sic1. Clbs then repress transcription of *CLN1* and *CLN2*, promote mitosis and finally their own destruction, leading back to a state in which *CLN1* and *CLN2* genes can be activated by Cln3 in the absence of any interference by Clbs. But are such regulatory networks really solely responsible for imposing the procession of S and M phases?

The State of Nuclei Determines the Response to a Given Cdk

A major problem with the Cdk state hypothesis is that it provides no explanation for how cells avoid the repetitive firing of origins during S phase. S-phase-promoting Cdks must presumably be active throughout S phase to fire both early and late origins of replication. How do cells prevent the refiring of early origins as they fire old ones? It can be debated whether this remarkable property is relevant to the problem of how S and M phases alternate (Roberts 1993), but there is no denying that it would be nice to have a theory that could explain both.

Doubts as to whether cell-cycle state could solely be determined by the state of S- and M-phase-promoting factors were raised long before the discovery of Cdks even, when it was found that G_1 but not G_2 nuclei can be triggered into S phase by fusion of G_1 or G_2 cells with S-phase cells (Johnson and Rao 1971). However, this could still be explained by

the "Cdk state" hypothesis if G_2 nuclei continued to harbor G_2-specific Cdks even after fusion to S-phase cells. The Cdk state hypothesis cannot, however, explain the recent discoveries that Clb/Cdk1 and not Cln/Cdk1 kinases are essential for S phase in *S. cerevisiae* (Schwob et al. 1994), that supposedly mitotic Clb/Cdk1 kinases can trigger S phase in G_1 cells (Amon et al. 1994), or that mutants deprived of the mitotic Clb1–Clb4/Cdk1 kinases possess high levels of the S-phase-promoting Clb5/Cdk1 kinase but arrest permanently in G_2 (Amon et al. 1993). These data show that the distinction between S- and M-phase-promoting Cdks is not a clear one. Mitotic Clb/Cdk1 kinases can also promote S phase! Cell-cycle state cannot therefore be determined solely by the presence or absence of specific S- or M-phase-promoting Cdks. It must also be influenced by a cell's history; that is, whether it has recently responded to these Cdks by undergoing S phase. Put in molecular terms, whether or not a cell responds to an S-phase-promoting Cdk by undergoing DNA replication must be determined by the state of Cdk substrates at the time of this Cdk's activation. G_1 nuclei, but not G_2 nuclei, presumably contain substrates whose phosphorylation by S-phase-promoting Cdks triggers DNA replication.

A Two-step Mechanism for S-phase Onset

Licensing Factor

What the crucial difference between a G_1 and a G_2 nucleus might be has until very recently been largely investigated using extracts from activated *Xenopus* eggs (Lohka and Masui 1984) in which exogenous DNA can undergo multiple rounds of DNA replication and where the distinction between G_1 and G_2 nuclei can be measured in vitro (see Laskey and Madine, this volume). *Xenopus* eggs are arrested in metaphase of meiosis II and contain high levels of MPFs like cyclin B/Cdk1. Fertilization, or artificial "activation," triggers destruction of cyclin B, and the eggs exit from mitosis and enter interphase. Extracts made from these "activated" eggs support replication of sperm chromatin following its assembly into nuclei (Blow and Sleeman 1990); the extracts presumably contain SPFs like cyclin E/Cdk2. Shortly after DNA replication, cyclin B/Cdk1 activity accumulates, which causes nuclear envelope breakdown and chromosome condensation, but the kinase later disappears due to cyclin B destruction, which leads to nuclear reformation and, in some cases, a second round of DNA replication (Hutchinson et al. 1987; Blow and Laskey 1988). The extracts thus undergo, at least partly, the natural sequence of events that occurs during cleavage divisions, in that cyclin B levels os-

cillate and DNA replication occurs only once per cell cycle (Murray and Kirschner 1989). The system's resemblance to the in vivo situation is striking in that no DNA molecules replicate more than once per cell cycle (Blow and Laskey 1986).

Addition of cycloheximide to the extracts prevents the appearance of cyclin B/Cdk1 kinase activity but presumably does not block activation of S-phase-promoting Cdks, because a single round of replication still occurs within the freshly assembled nuclei. Addition of an MPF activity to cycloheximide-treated extracts causes nuclear envelope breakdown and allows at least partial rereplication. The cycloheximide-treated extracts also cause G_1 but not G_2 nuclei from HeLa cells to enter S phase. The cycloheximide extracts therefore partly mimic the cytoplasm of S-phase HeLa cells, in that they can trigger S phase in G_1 but not in G_2 nuclei (Leno et al. 1992).

To explain these observations, it has been suggested (Blow and Laskey 1988) that initiation of DNA replication might be a two-step process. The first step would be the binding to origins of a factor needed for the initiation of DNA replication whose entry into the nucleus is restricted to M phase, when nuclear envelopes are disassembled. This factor would license origins to fire upon the subsequent arrival of SPF in the next cell cycle. To explain the lack of rereplication in the absence of MPF, it was envisaged that initiation of DNA replication destroys all licensing factor within the nucleus and that the nuclear membrane must be disassembled before licensing factor residing in the cytoplasm can regain access to origins. In an experiment that is widely regarded as an important confirmation of this hypothesis, it was found that permeabilization of the nuclear envelope permits rereplication of G_2 nuclei in the absence of MPF activation (Blow and Laskey 1988; Coverley et al. 1993). Purification of the putative licensing factor has been thwarted until now due to the lack of a good assay (but see Laskey and Madine, this volume).

Preparations for S Phase as Cells Exit from Mitosis

The *CDC46*, *MCM2*, and *MCM3* gene products in yeast have certain properties reminiscent of the putative licensing factor (Hennessy and Botstein 1991; Hennessy et al. 1991; Tye 1994). They are necessary for the initiation of DNA replication in yeast, and their access to chromatin is under cell cycle control. Cdc46 and Mcm3 proteins accumulate in the cytoplasm during G_2 and only enter nuclei as cells undergo anaphase. Vertebrate homologs of these proteins are not like their yeast counterparts excluded from nuclei during G_2, but their association with chromatin may nevertheless be under cell cycle control (Kimura et al.

1994). The P1 protein, a homolog of Mcm3 that binds to DNA polymerase-α, is distributed in the cytoplasm during metaphase and associates with chromatin only as chromosomes decondense during telophase. The behavior of Cdc46/Mcm proteins and that of the putative licensing factor differ drastically in one important respect. The Cdc46/Mcm proteins enter nuclei or associate with chromatin as cells exit from M phase (Hennessy et al. 1990), as cyclin B/Cdk1 kinase is destroyed (Surana et al. 1993), and not during M phase, when cyclin B/Cdk1 kinase is high, as originally proposed for licensing factor. This difference could be vital because, as we shall see, the cyclin B/Cdk1 kinase might be an important regulator of DNA rereplication (Hayles et al. 1994; Dahmann et al. 1995). Another example of preparations for S phase occurring at the end of M phase concerns the 70-kD subunit of RP-A, a three-subunit single-stranded DNA-binding protein needed for prepriming, whose association with subnuclear foci proposed to be prereplication centers (pre-RCs) as chromosomes decondense following exit from M phase (Adachi and Laemmli 1994) can be inhibited by cyclin B/Cdk1 kinase.

Chromatin Structure of Yeast Origins during the Cell Cycle

Yet more persuasive evidence of preparations for replication occurring at the end of mitosis have come from analyses of chromatin structure at DNA replication origins during the cell cycle (Diffley et al. 1994). So far, discrete replication origins have been well defined only in *S. cerevisiae*, where a 200-bp DNA sequence (ARS) containing a core consensus sequence called A and flanking sequences B1, B2, and B3 is sufficient for origin function on episomes and within chromosomes (Marahrens and Stillman 1992). A 250-kD protein complex called the origin recognition complex (ORC) binds to sequences A and B1 (Bell and Stillman 1992; Diffley and Cocker 1992), whereas a separate protein, Abf1, binds to the B3 element (Diffley and Stillman 1989). The state of these origins during the cell cycle has been analyzed by genomic footprinting. Both ORC and Abf1 appear to be bound throughout the cell cycle, but there are important changes in the neighboring chromatin (Diffley et al. 1994). From S phase until late anaphase, the pattern of cleavage by DNase I in vivo resembles that induced by ORC binding in vitro, which causes an adjacent DNA sequence to become hypersensitive. As cells exit from mitosis, this DNase I hypersensitive site disappears and does not reappear until cells enter S phase in the next cycle. The data indicate that a second factor binds next to ORC during G_1 but not at other stages of the cell cycle.

CDC6 *Is Necessary for Pre-RCs*

A key question is whether the cell-cycle-regulated factor that binds to yeast origins is required for DNA replication or for preventing its premature onset. If the former were true, then disappearance of DNase I hypersensitivity at the end of mitosis should depend on proteins needed for origin firing. A good candidate for such a protein is that encoded by *CDC6*, which is required for the initiation of DNA replication in *S. cerevisiae* (Bueno and Russell 1992), as is a homologous gene, *cdc18*, in *S. pombe* (Kelly et al. 1993). Minichromosomes whose replication is dependent on a single ARS sequence are much more frequently lost in *cdc6* mutants than in wild-type cells, but this defect is suppressed when the minichromosome carries multiple ARS sequences, implying that *cdc6* mutants are not defective in DNA replication per se but rather in the function of origins (Hogan and Koshland 1992). The Cdc6 protein is unstable and is synthesized in a burst at the end of mitosis (as well as in late G_1) simultaneous with the change in chromatin structure that occurs at origins (Piatti et al. 1995). More important, the disappearance of ORC-induced DNase I hypersensitivity at origins does not take place when cells exit from mitosis in the absence of Cdc6 synthesis (Cocker et al. 1996). Furthermore, the "protected" state of origins characteristic of cells arrested in G_1 (by pheromone) is temperature sensitive in *ts cdc6* mutants. Cdc6 is therefore necessary for the formation and maintenance of a chromatin configuration at origins that precedes DNA replication and is presumably essential for its initiation. The observation that Cdc6 and ORC can form complexes in vitro (Liang et al. 1995) is consistent with this view.

S-phase onset in yeast might therefore involve two temporarily distinct events at replication origins. The first would be the formation of pre-RCs, whose appearance during telophase depends on *CDC6* and coincides with the destruction of Clb/Cdk1 kinases (MPFs). The second event would be the activation of S-phase-promoting Clb/Cdk1 kinases (Schwob et al. 1994) in late G_1 (SPFs), which would trigger pre-RCs to initiate DNA replication. There is a pleasing congruence between this scheme and the arrival on chromatin at the end of mitosis of RP-A subnuclear foci and of Cdc46/Mcm proteins, which might also be components of yeast prereplicative complexes.

Control of pre-RC Formation

If the formation of pre-RCs at future origins is a prerequisite for initiating DNA replication, then control of pre-RC formation could be as vital

an aspect of S-phase control as the production of S-phase-promoting factors (Cdks). In *S. cerevisiae*, activation of cyclin B/Cdk1 kinases during G_1 causes the onset of S phase, but the very same kinases are active during G_2 and yet cells do not reenter S phase. This can now be explained by the absence in G_2 cells of pre-RCs that are, as it were, the key substrates for S-phase-promoting Cdks. What then excludes the formation of pre-RCs during S, G_2, and M phases?

In yeast, the adoption by origins of a prereplicative state indicative of pre-RCs occurs with very similar kinetics at the end of mitosis as the destruction of cyclin B/Cdk1 kinases (Surana et al. 1993; Diffley et al. 1994). This suggests that cyclin B/Cdk1 kinases could have a role in preventing the de novo assembly of pre-RCs. Direct evidence that destruction of Cdks in G_2 or M phase is sufficient to trigger pre-RCs and thereby creates the potential to rereplicate upon activation of S-phase-promoting Cdks has been obtained using an inducible *SIC1* gene in *S. cerevisiae* (Dahmann et al. 1995). The microtubule-disrupting drug nocodazole prevents nuclear division in *S. cerevisiae* and causes cells to arrest with duplicated DNA, origins in a postreplicative state, and high levels of all six Clb kinases. Induction of high levels of the Clb/Cdk1 inhibitor p40Sic1 from a *GAL-SIC1* gene fusion inhibits completely both Clb2 and Clb5/Cdk1 kinases (and presumably all other Clb kinases as well), causes cells to rebud without nuclear division, causes origins to adopt a prereplicative chromatin structure, but does not induce rereplication. However, reactivation of Clb/Cdk1 kinases by subsequently shutting off p40Sic1 synthesis causes all cells to rereplicate their DNA.

Mutants That Rereplicate

Quite independent evidence implicating cyclin B/Cdk1 kinases in blocking rereplication in G_2 cells stems from the characterization of yeast mutants that undergo successive S phases in the absence of an intervening mitosis. Such mutants have been isolated on the basis of their ability to diploidize and thereby sporulate. Budding yeast mutant cells with a temperature-sensitive allele of the *ESP1* gene replicate normally at the restrictive temperature, but they subsequently fail to undergo anaphase due to an unspecified mitotic spindle defect. Despite their mitotic failure, the mutant cells proceed as usual with destruction of Clb/Cdk1 kinase, and they subsequently embark on a new cell cycle, during which they rereplicate their chromosomes (McGrew et al. 1992; Surana et al. 1993). The behavior of *esp1* mutants shows that Clb destruction is accompanied by rereplication even when mitosis has not occurred. Thus, mitosis per se is not necessary.

Many more diploidizing mutants have subsequently been analyzed in *S. pombe*. The vast majority fall into the *esp1* class, in that they attempt to enter mitosis, fail, and proceed with the next cycle. In rare mutants, however, cells rereplicate DNA without attempting mitosis (although nitrogen starvation is also required). Upon return to the permissive temperature, some of these "G_2" cells rereplicate their DNA before entering mitosis. Mutations in two genes give rise to this phenotype. Remarkably, they encode *cdc2* (*cdk1*) and *cdc13*, the two components of the cyclin B/Cdk needed for mitosis in fission yeast (Broek et al. 1991; Hayles et al. 1994). The behavior of cells completely lacking the *cdc13* gene product is even more striking. Upon inoculation into fresh medium, wild-type spores germinate, replicate their DNA, spend the next 90 minutes in G_2, and then undergo mitosis. The second round of DNA replication follows almost immediately after completion of the first mitosis. Spores lacking *cdc13* resemble wild-type cells up to the point at which the Cdc13/Cdc2 kinase should have become active. Due to its absence, the mutant cells never enter mitosis; G_2 is prolonged, but not indefinitely, because 5 hours after the first S phase, cells rereplicate their DNA and this process recurs thereafter, roughly every generation time. As a consequence, some cells eventually accumulate up to 32 copies of their genome. Therefore, it seems that Cdc13 is necessary to prevent *S. pombe* cells from entering a G_1-like state after they have spent some time in G_2. Why might this be so? One possibility is that loss of Cdc13/Cdk1 kinase encourages the formation of pre-RCs.

Cyclin B/Cdk1 kinases have also been implicated in preventing DNA replication between meiosis I and II. During maturation of *Xenopus* oocytes, cyclin B/Cdk1 is inactivated after meiosis I but, due to the action of c-*mos*, is promptly reactivated prior to meiosis II. Ablation of c-*mos* product causes maturing oocytes instead to reform nuclei and replicate DNA after the first meiotic division (Furuno et al. 1994). The same effect is observed with dominant-negative alleles of Cdk1. Thus, cyclin B/Cdk1 kinases inhibit DNA replication between meioses I and II as well as between S and M phases during vegetative cell cycles. Whether they do so by inhibiting pre-RC formation is yet to be determined.

Preventing Rereplication during S and G_2 Phases: Do S-phase-promoting Cdks Have a Dual Function?

We now have a coherent explanation for the behavior of origins during mitosis and G_1. Assembly of pre-RCs requires destruction of mitotic Cdks and the synthesis of pre-RC components, either at the end of

mitosis or in late G_1. Pre-RCs are then transformed into replication forks by activation of S-phase-promoting Cdks. Thus, the lack of DNA replication during G_1 and M phases has very different causes. G_1 cells that have formed pre-RCs have the potential to enter S phase but do not do so for lack of S-phase-promoting Cdks, whereas M-phase cells could not enter S phase even if they were to express S-phase-promoting Cdks (and some types of cell indeed do) because assembly of pre-RCs is inhibited by mitotic cyclin B/Cdk1 kinases. This model cannot yet account for the behavior of S and G_2 cells. During DNA replication, S-phase-promoting Cdks are active whereas mitotic cyclin B/Cdk1 kinases are inactive, and cells should therefore be able both to form pre-RCs and to turn them into replication forks. What then prevents origins from firing more than once? A similar problem could afflict G_2 cells that have not yet activated cyclin B/Cdk1 kinases but contain Cdks like cyclin A/Cdk2 in animals or Clb1–Clb4/Cdk1 in yeast, which are capable of promoting S phase.

The phenotype of *S. cerevisiae* mutants lacking G_2-specific B-type cyclins Clb1–Clb4 may provide an important clue to this mystery. Strains lacking Clb1, Clb3, and Clb4 and kept alive with a temperature-sensitive allele of *CLB2* (conditional *clb1–clb4* mutants) can replicate normally but fail to build a mitotic spindle at the restrictive temperature. In this regard, their phenotype resembles that of *S. pombe cdc13* deletion mutants. Their phenotype differs greatly in another regard, for they arrest in G_2 indefinitely without rereplicating. Clb5/Cdk1 kinase remains active during this arrest. Is it possible that this kinase also inhibits assembly of pre-RCs and thereby prevents rereplication? Mutations in at least two genes (*SIM*1 and *SIM*2) allow *clb1-4* mutants to rereplicate (C. Dahmann et al., in prep.) Remarkably, the mutants are all defective in maintaining high levels of Clb5/Cdk1 kinase and rebud at the same time as they rereplicate, which is a sign of Clb kinase decline in vivo. Moreover, they fail to rereplicate if *CLB5* gene dosage is increased. The behavior of these mutants shows that Clb5/Cdk1 kinase acts to suppress replication in *clb1-4* mutants, which is all the more remarkable because Clb5/Cdk1 normally promotes S phase.

The apparent contradiction that Clb5/Cdk1 both promotes and inhibits S phase could be explained if the kinase had different replication functions in G_1 and G_2: promoting it during G_1 but blocking it during G_2. Might the very same kinase that promotes transformation of pre-RCs into replication forks also inhibit de novo assembly of pre-RCs (see Fig. 3)? Indeed, it seems likely that all of the six Clb/Cdk1 kinases that are capable of promoting S phase in *S. cerevisiae* also inhibit formation of pre-RCs. Clb5 and Clb6 might be primarily responsible for inhibiting

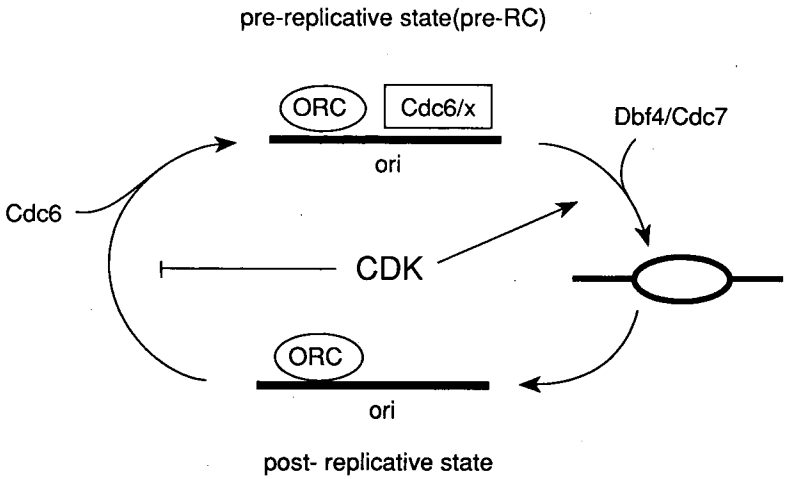

Figure 3 A dual function for Cdks: promoting initiation of DNA replication and inhibiting pre-RC assembly. Initiation is a two-step process, the first step of which is the formation of pre-RCs at future origins. This process depends on the synthesis of the unstable replication protein, Cdc6. It usually occurs at the end of mitosis when there is a burst of Cdc6 synthesis but can also occur in late G_1, when there is a second burst of Cdc6 synthesis, at least in daughter cells. The second step is activation in late G_1 of cyclin B/Cdk1 kinases along with the Cdc7 kinase. It seems that activity of the very same set of cyclin B/Cdk1 kinases prevents de novo assembly of pre-RCs. Each round of initiation therefore depends on a cycle of cyclin activation/destruction (see Fig. 4).

pre-RC assembly during S phase, Clb3 and Clb4 during early G_2, and Clb1 and Clb2 during late G_2 and during M phase, when the respective kinases are most active. If this model were correct, the different behavior of *S. pombe cdc13* mutants, which undergo multiple rounds of replication, and *S. cerevisiae clb1-4* quadruple mutants, which arrest indefinitely in G_2, might be simply one of degree. *cdc13* mutants also do not re-replicate for many hours after their first S phase, during which time other B-type cyclins, e.g., Cig1 and Cig2, might prevent the assembly of pre-RCs. The Cig1 and Cig2 B-type cyclins might either be somewhat less effective than Clb5 and Clb6 and eventually be overcome by the forces driving the assembly of pre-RCs or, for reasons not yet understood, they may simply not remain active during G_2 for as long as Clb5 and Clb6.

Pre-RCs Can Form Either at the End of M Phase or in Late G_1

At first sight, a mechanism whereby S-phase initiation depends on events that occurred at the end of the previous mitosis seems somewhat

precarious. It is hard to imagine how cells ensure that prereplication complexes formed at the end of mitosis could be relied on to survive an extended arrest in G_1, as occurs during stationary phase in yeast. The same problem would afflict quiescent mammalian cells, which can spend months, if not years, arrested in G_1 before they are stimulated to reenter S phase. In fact, the chromatin structure thought to represent pre-RCs at yeast origins can form not only at the end of mitosis, as occurs in cycling cells, but also during G_1 phase when inoculation into fresh medium stimulates stationary-phase yeast cells to reenter the cell cycle (Diffley et al. 1994). This observation is not at odds with the notion that destruction of cyclin B/Cdk1 kinases is necessary for the formation of pre-RCs, according to which pre-RC assembly should be possible throughout G_1, for as long as the inhibitory cyclin B/Cdk1 kinases remain dormant. Thus, DNA replication could be seeded predominantly from pre-RCs formed at the end of mitosis in cells with short G_1 periods, as in yeast mother cells and embryonic cells undergoing cleavage divisions, whereas it could be seeded mainly by pre-RCs formed during G_1 in cells with long G_1 periods.

Democracy of the Genome

The notion that assembly of new pre-RCs might be prevented by the very same set of Cdks that promote their transition into replication forks is an interesting idea, because it explains how cells ensure that there is no stage during the cell cycle in which pre-RCs can both be assembled and be transformed into replication forks (see Fig. 4). S-phase-promoting Cdks with dual functions would preclude any possibility of origins firing more than once during the cell cycle. Furthermore, if we assume that all Cdk1 (or Cdk2) forms can hinder pre-RC formation, we could explain why G_2 nuclei cannot enter S phase even when they are placed in a cytoplasm containing S-phase-promoting Cdks (Johnson and Rao 1971). We presume, by analogy with *S. cerevisiae*, that from S phase until the end of mitosis, animal cells also continuously maintain an environment that is hostile to the formation of pre-RCs. S-phase-promoting Cdks like cyclin E/Cdk2 or cyclin A/Cdk2 might prevent their formation during S phase, whereas cyclin A/Cdk1 and cyclin B/Cdk1 assume this function during G_2 and M phase.

Primordial Cell Cycles

Although multiple Cdks may participate in the exclusion of pre-RC assembly during the S, G_2, and M phases of most present-day eukaryotic

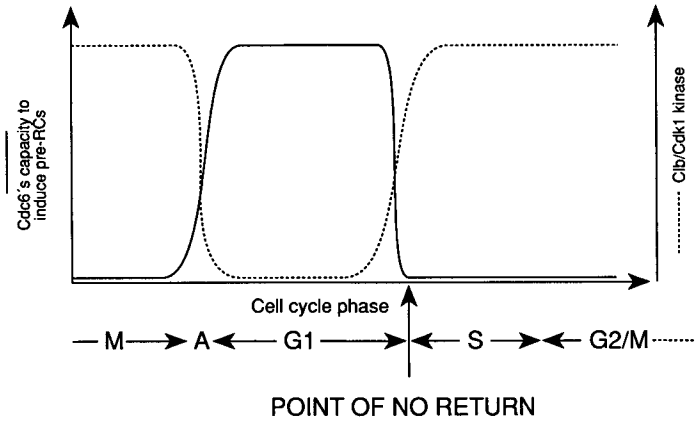

Figure 4 The pre-RC/Cdk cycle in yeast. Cyclin B/Cdk1 kinases prevent the formation of pre-RCs during S, G_2, and M phases. Pre-RCs therefore only form upon proteolysis of cyclin B molecules during anaphase. Reactivation of cyclin B/Cdk1 kinases during the subsequent G_1 period triggers initiation at origins that had formed pre-RCs during G_1, when cyclin B/Cdk1 kinases were inactive.

cells, and specialized Cdks may be employed to transform pre-RCs into replication forks, it is worth considering that discrete rounds of replication could theoretically be driven by the alternate activation and inactivation of a single "dual function" Cdk. In *S. cerevisiae*, where mitosis does not entail extensive chromosome condensation, entry into mitosis and DNA replication can co-exist. It is therefore conceivable that in the primordial eukaryotic cell, activation of a single Cdk might have triggered simultaneously transformation of pre-RCs into replication forks (i.e., S phase) and attachment of chromosomes to mitotic spindles (i.e., M phase). Subsequent inactivation of this same Cdk could have signaled both the assembly of pre-RCs and exit from mitosis. It is therefore possible to envisage how the oscillation of a single Cdk could have formed the heart of our distant ancestors' cell cycle. To function with high fidelity, such a system might have required a surveillance mechanism that ensured that destruction of Cdks was never initiated before DNA replication was complete. This might then have been the cell cycle's oldest checkpoint control.

Dependence of S on Anaphase

The hypothesis that mitotic Cdks prevent pre-RC assembly also explains why sister chromatid separation always precedes a second round of DNA

replication. S-phase-promoting Cdks decline during G_2, G_2-specific Cdks like cyclin A/Cdk1 decline during metaphase, but the destruction of the last remaining Cdks capable of inhibiting the assembly of pre-RCs does not occur until mitosis-specific Cdks like cyclin B/Cdk1 are inactivated due to cyclin B proteolysis at anaphase. The latter depends on activation of a specialized multisubunit ubiquitin ligase, which is necessary also for the separation of sister chromatids and is therefore called the anaphase-promoting complex (APC) (see Fig. 5). The APC is thought to catalyze proteolysis not only of B-type cyclins but also of proteins that inhibit sister chromatid separation (Holloway et al. 1993; Irniger et al. 1995; King et al. 1995). Thus, cells cannot begin to assemble pre-RCs (a pre-condition for chromosome duplication) without having also triggered anaphase.

A Three-parameter Model for the Cell Cycle

We started this section with the notion that cell-cycle state might be determined by the activity of two key parameters: the level of S- and M-phase-promoting Cdks. This idea turns out to be untenable. At least three parameters are required to define the major cell-cycle states of most existing eukaryotic cells: the levels of S- and M-phase-promoting Cdks (SPF and MPF) and the presence or absence of pre-RCs. Early G_1 cells lack SPF and MPF but usually (although not always) contain pre-RCs; cells embarking on S phase contain SPF and pre-RCs; cells in S phase contain SPF and are in the process of converting their pre-RCs into replication forks; G_2 cells have finished this process and may or may not contain SPF but have not fully activated MPF; M-phase cells have fully activated MPF, and anaphase cells are in the process of destroying their MPF and other proteins whose destruction is needed for chromosome segregation and are about to form pre-RCs. Only when we take pre-RCs into account can we explain why G_1 and G_2 nuclei respond differently to activation of S-phase-promoting Cdks. The three-parameter model of the cell cycle also allows us to explain the very different effects of activating mitotic Cdks in G_1-arrested *S. pombe* and *S. cerevisiae* cells; it induced replication in *S. cerevisiae* cells deprived of Clns but failed to do so in *S. pombe cdc10* mutants (Amon et al. 1994; Hayles et al. 1994). *cdc10* is needed for the synthesis of Cdc18, which is homologous to Cdc6 from *S. cerevisiae* and might therefore be necessary for the formation of pre-RCs. The presence of pre-RCs in *cln*-arrested *S. cerevisiae* cells and their absence in *S. pombe cdc10* mutants would explain their different responses.

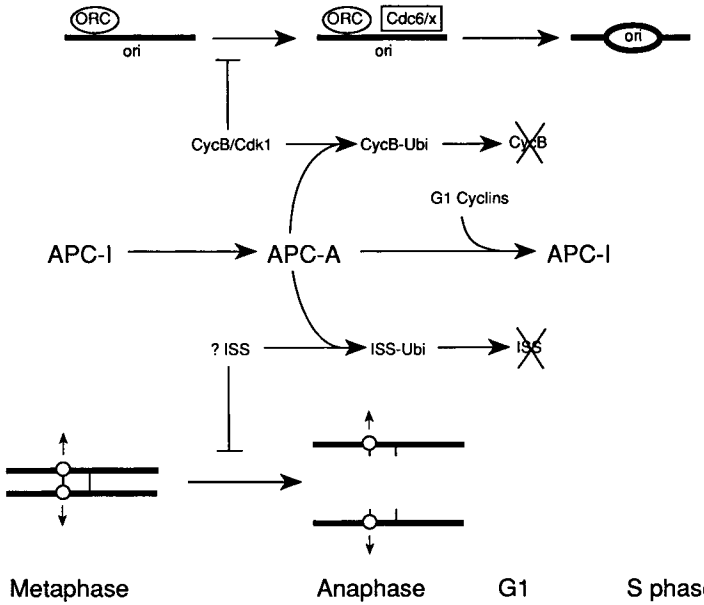

Figure 5 The APC could link S phase to anaphase. The APC is a 20S particle that contains Cdc16, Cdc23, and Cdc27 proteins (among others) and catalyzes the ligation of multiple ubiquitin molecules to cyclin B proteins containing a destruction box, targeting them for destruction by the 26S proteosome. The ubiquitin ligase reaction also requires ATP, ubiquitin, an activating enzyme (E1), and a conjugating enzyme (E2). APC components are needed not only for cyclin B ubiquitination and proteolysis, but also for the onset of anaphase. It is therefore thought that the transition from metaphase to anaphase depends on the destruction of inhibitors of sister chromatid separation (ISS). The APC is thought to be inactive from late G_1 until the onset of anaphase. Its activation then triggers anaphase and the destruction of cyclin B proteins. The latter leads to the formation of pre-RCs and thereby to a new round of DNA replication when cyclin B/Cdk1 kinases are reactivated during the subsequent G_1 period. Activation of Cln1 or Cln2/Cdk1 kinases turns the APC off and allows the accumulation of mitotic cyclin B proteins.

The inclusion of pre-RCs as a major cell-cycle parameter helps to impart a rigid directionality to our cell-cycle model. The presence of these structures is partly determined by the cell's history, but this directionality comes at a price. The timing of cell-cycle events becomes more crucial. For example, activation of S-phase-promoting Cdks before pre-RCs have been assembled, as would be possible in the outgrowth of cells that had long lost pre-RCs inherited from their previous telophase, would com-

pletely finesse the replication machinery and lead to an unreplicated state from which a cell could no longer extract itself. Much of the regulation of pre-RC components and S-phase-promoting Cdks, which is more conventionally thought to be contributing to the control of cellular proliferation, may have the more important role of helping to avoid the disastrous consequences of activating S-phase-promoting Cdks before pre-RCs have had time to form.

Why Do Most Quiescent Cells Arrest in G_1?

A final insight that stems from these ideas concerns why so many metazoan cells choose to enter a quiescent state during G_1 and not during S or G_2 phases. Cells at these latter stages must maintain active Cdks to exclude the assembly of pre-RCs. A temporary inactivation of these Cdks due to unspecified physiological disasters could lead to the assembly of pre-RCs and thereby to an unscheduled round of DNA replication if S-phase-promoting Cdks were activated before mitosis could take place. It is interesting in this regard that rereplication of Cdk1 alleles in *S. pombe* was first observed only upon extreme heat shock and nitrogen starvation (Broek et al. 1991); that is, extreme environmental conditions can contribute to the destruction of Cdks. In contrast, destruction of preassembled pre-RCs in G_1 cells should not be disastrous, as long as Cdks capable of inhibiting pre-RC assembly were not yet activated and as long as cells contained pre-RC components or were capable of entering a program that caused their synthesis. The logic of replication control may therefore dictate that the early G_1 state is one from which a cell can more readily recover its true cell-cycle state following an experience that erases its memory of cell-cycle position. It is the one stage of the cell cycle where history does not matter!

Closing the Cycle

The first section of this review dealt with the events needed to drive a G_1 cell into S phase. We treated the resting G_1 cell as a blank slate and considered how cells produce the factors that trigger it to replicate. In the second half, we considered how cells prevent replication during G_2 and M phases. We can now appreciate how events at the end of one cell cycle lead to the start of the next one; that is, we can begin to appreciate its cyclical nature. There is sufficient knowledge about this process in *S. cerevisiae* to piece together the following picture. During G_2 and M phases, Clb/Cdk1 kinases prevent transcription of S-phase-promoting

kinases Cln1 and Cln2/Cdk1 by preventing the binding of SBF to SCBs in their promoters. These kinases also prevent the assembly of pre-RCs (how is not understood). Thus, destruction of Clb/Cdk1 kinases during anaphase through the activation of Clb proteolysis and the accumulation of p40Sic1 removes the key barrier to transcriptional activation of *CLN1* and *CLN2* by Cln3/Cdk1 and the formation of pre-RCs at origins (which can form at this stage or later in G_1). In the absence of Clb/Cdk1 kinases, growth of daughter cells to a threshold size facilitates transcription of *CLN1*, *CLN2*, and *CDC6*, which produces active Cln/Cdk1 kinases and yet more pre-RCs. Cln1 and Cln2/Cdk1 kinases then trigger the reactivation of Clb/Cdk1 kinases by promoting proteolysis of p40Sic1 and by turning off proteolysis of Clb cyclins, whose activity now promotes the transition of pre-RCs into replication forks.

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