Cyclin-Dependent Kinases

J. W. Harper*,† and P. D. Adams‡

Department of Biochemistry and Molecular Biology, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, and Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111

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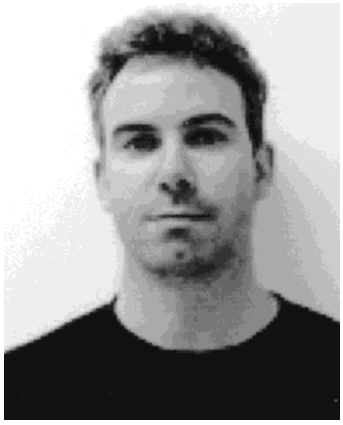
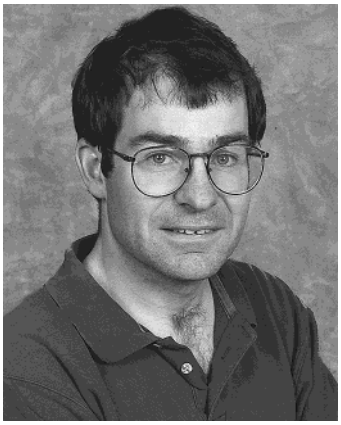


Wade Harper received his Ph.D. degree in Chemistry from the Georgia Institute of Technology in 1984 and then did postdoctoral studies at Harvard Medical School before joining the Department of Biochemistry at Baylor College of Medicine in 1988 as Assistant Professor. He is currently a Professor of Biochemistry and Molecular Biology and a Professor of Molecular Physiology and Biophysics at Baylor College of Medicine. His work focuses on the control of cell division by cyclin-dependent kinases and regulated protein ubiquitination.



Peter Adams obtained his Ph.D. degree from the Imperial Cancer Research Fund in England in 1993. He carried out postdoctoral work at Dana-Farber Cancer Institute in Philadelphia as an Associate Member in 1999. His research focuses on regulation of histone gene expression, DNA replication, and chromatin assembly during the cell cycle.

I. Introduction

Cyclin-dependent kinase holoenzymes are comprised of a catalytic subunit, the Cdk, and a member of a family of regulatory subunits, the cyclins. The hallmark of cyclin-dependent kinases is that they are inactive in their monomeric form and require association with the requisite cyclin partner for activation. This mode of regulation has two major consequences for Cdk function. First, the timing of Cdk activation can be controlled by the timing of expression of a particular cyclin subunit. Second, cyclins can also contribute to the substrate specificity of a particular catalytic subunit by associating tightly with substrates. Thus, the temporal activation of particular Cdk subunits via alterations in the abundance of certain cyclin partners is physically coupled to substrate selection, thereby providing the basis of kinase specificity. These regulatory features, although central to cell cycle control, are by no means

* To whom correspondence should be addressed. Phone: 713-798-6992. Fax: 713-796-9438. E-mail: jharper@bcm.tmc.edu.
† Baylor College of Medicine.
‡ Fox Chase Cancer Center.

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limited to this process. The structural and regulatory framework of Cdks has been used in multiple settings outside the control of cell division, including transcription, DNA repair, and postmitotic processes.

Although the timing of cyclin binding may be sufficient to control the timing of Cdk activation, a number of distinct regulatory pathways are used to provide additional levels of control. These include phosphorylation, tight-binding inhibitors, regulated assembly of holoenzymes, and ubiquitin-mediated proteolysis of cyclins and Cdk inhibitors (reviewed in ref 1). These forms of regulation, some of which are now understood at atomic resolution, are the end points of extrinsic and/or intrinsic signaling pathways intended to coordinate proliferative or antiproliferative decisions. Previous reviews have focused on negative regulation of Cdk activity by tyrosine phosphorylation and by binding of Cdk inhibitors.[1−3] In this review, we outline the biochemical mechanisms underlying Cdk substrate selection and describe how periodic Cdk activation provides order to the cell cycle. Although the discussion is focused on Cdks involved in cell cycle control, many of the principles that dictate substrate choice by cell cycle Cdks will likely be used by all Cdks, including those involved in transcription and postmitotic events.

II. Class Distinction: Cdks in Single and Multicellular Eukaryotes

Budding and fission yeast serve as important genetic systems for understanding Cdk regulation and function. These organisms use primarily one Cdk subunit (Cdc28 in *Saccharomyces cerevisiae* and Cdk1 (aka Cdc2) in *Schizosaccharomyces pombe*) to perform all the major steps of the cell division cycle (Figure 1).[1] To achieve temporal control of cell cycle events, distinct cyclin subunits are synthesized at specific cell cycle phases and these cyclins confer distinct substrate specificities upon the Cdk. In budding yeast, three partially redundant G1 cyclins (Cln1, Cln2, and Cln3) coordinate the processes of budding and spindle pole body duplication and are responsible for events leading to activation of the primary S-phase Cdk, Cdc28 associated with the B-type cyclins Clb5 or Clb6 (reviewed in ref 4). Four other B-type cyclins (Clbs1−4) coordinate late S-phase and mitotic events. In contrast, multicellular eukaryotes contain a somewhat more complex array of Cdk components. In mammalian cells, 5 different Cdk subunits [Cdk1(aka Cdc2), Cdk2, Cdk3, Cdk4, and Cdk6] and 10 distinct cyclin subtypes [A1, A2, A3, B1, B2, D1, D2, D3, E1, E2, and F] contribute directly to the process of cell division. These major cyclin types are found from *C. elegans* to mammals, but in mammals, each cyclin type (with the possible exception of cyclin F) has multiple family members. For some cyclin classes (e.g., D-type cyclins), it is thought that each member functions largely equivalently but is used in tissue-specific ways.[5,6] Moreover, cyclin B1 can compensate for the loss of cyclin B2 in mouse development, implying that cyclin B1 can target Cdk1 to cyclin B2 substrates.[7]

The distribution of different Cdk complexes during the cell cycle in mammals is depicted in Figure 1. D-type cyclins interact preferentially with Cdk4 and Cdk6, and these complexes function primarily during the G1 phase of the cell cycle.[8,9] The synthesis and stability of D-type cyclins are controlled by growth factor levels, and activation of D-type cyclin kinases is rate limiting for progression through G1 in fibroblasts.[9] E-type cyclins form active complexes with Cdk2, and the abundance of these complexes peaks during the G1/S-transition. Indeed, cyclin E and Cdk2 are both required for initiation of DNA replication.[8] Cyclin A can form complexes with both Cdk1 and Cdk2 during S and G2 phases, and these complexes play positive roles in replication and passage through G2. B-type cyclins form complexes with Cdk1 during G2, and these complexes coordinate many of the events required for mitosis. Our understanding of some components of the basic cell cycle machinery is rather limited. For example, the roles of Cdk3[10] and cyclin F[11] are largely undefined.

III. Regulation of Cdk Activity

Much of what we know about Cdk activation comes from the analysis of Cdk2/cyclin A and Cdk4/cyclin D complexes. These two complexes serve as prototypes for the major classes of regulatory pathways that control Cdk activity. The major difference between the two pathways is that Cdk2 is primarily regulated after holoenzyme assembly while D-type cyclin kinases are regulated both at the assembly step and after assembly. Figure 2 summarizes the major pathways known to regulate Cdk activity.

Cdk2 is catalytically inactive in its monomeric form but is activated upon cyclin binding in a two-step mechanism.[1,12,13] There are two obvious reasons why monomeric Cdk2 is inactive. First, the so-called T-loop which contains the site of activating phosphorylation is draped across the substrate binding site and occludes the close approach of peptide substrates.[14] In addition, several catalytic residues are misaligned with respect to their positions in the active enzyme. Upon cyclin A binding, which can occur spontaneously in vitro, the intrinsic activity of Cdk2 is increased by at least 5 orders of magnitude.[15] This activation appears to be due to conformation changes in the Cdk protein induced by cyclin binding that alter the positions of residues Asp145, Glu51, and Lys33. These residues function in orienting the ATP for catalysis.[16] In addition, the T-loop undergoes a large conformational change that displaces it from the substrate-binding cleft. In the second step, Thr160 (Thr161 in Cdk1) is phosphorylated by Cdk-activating kinase (CAK), leading to a further 100-fold activation.[15,17] The major CAK activity in mammalian cells comes from a complex composed of Cdk7, cyclin H, and the ring finger protein MAT1 (reviewed in refs 1, 18, and 19). The ability of CAK to phosphorylate Cdk2 is stimulated by cyclin binding. This likely reflects the fact that the T-loop is repositioned upon cyclin binding, making Thr160 available for recognition by Cdk7/cyclin H.[16] Thus, both cyclin binding and T-loop phosphorylation are required for full activation and biological activity of cyclin A/Cdk2. The major structural consequence of T160 phosphorylation is

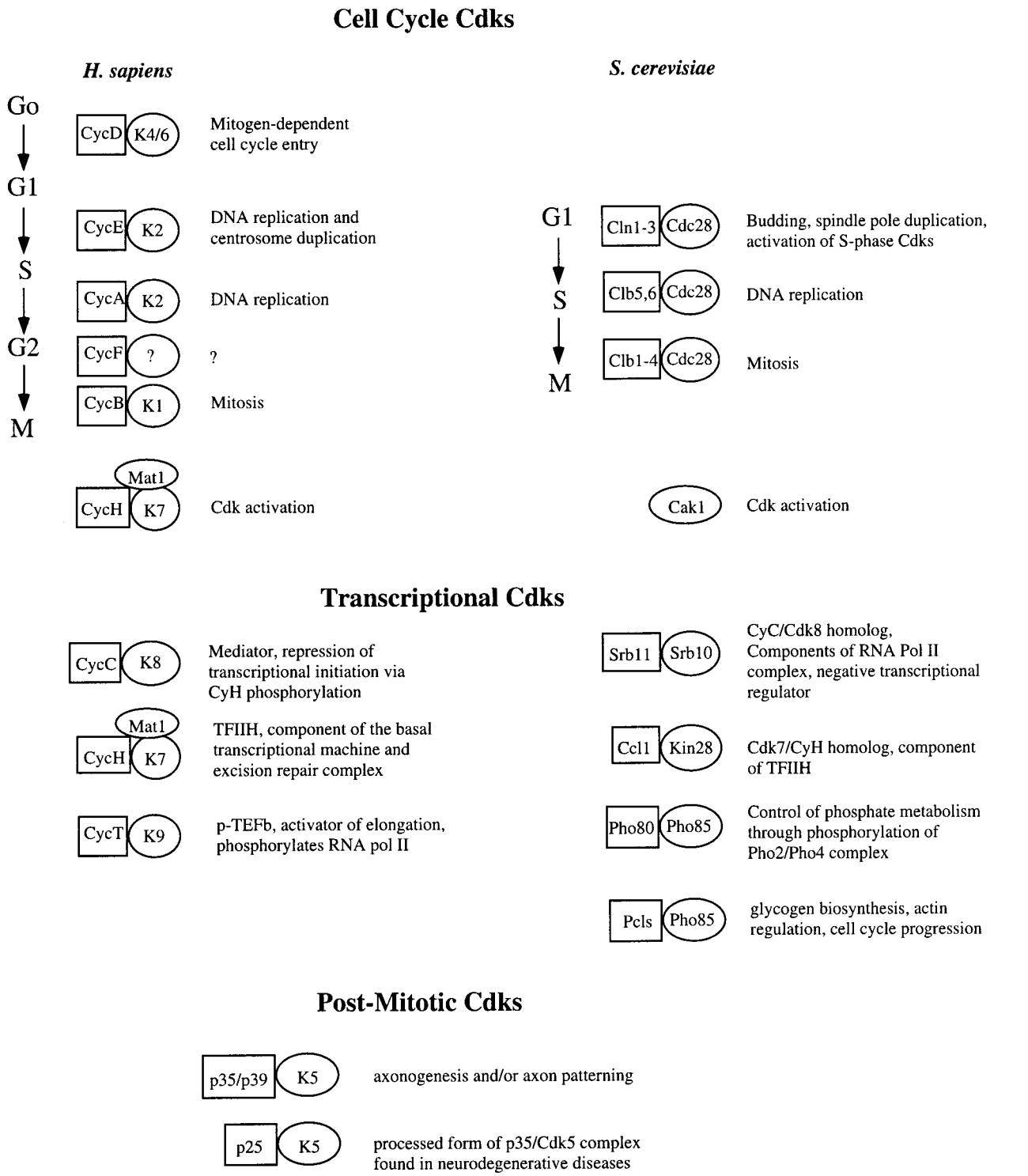

Figure 1. Cyclin-dependent kinases. The major Cdks involved in controlling cell cycle progression and transcription in human and *S. cerevisiae* as well as Cdk5 involved in neural function are shown. Cell cycle kinases are displayed according to their timing of expression during the cell cycle. In budding yeast, S-phase and mitosis overlap.

that the T-loop moves further away from the peptide substrate binding site, a structure that is stabilized by a network of hydrogen bonds between Cdk2 side chains and the phosphate on T160.[20]

Activated Cdk2/cyclin A complexes are subject to regulation by both phosphorylation on inhibitory sites and by binding of Cdk inhibitors of the CIP/KIP class (p21, p27, and p57). CKIs are expressed in a tissue-specific manner and are required for proper cell cycle arrest in response to various differentiation signals during development. These molecules and their regulation have been reviewed extensively.[2−4] The structure of the trimer cyclin A/Cdk2/p27 complex has been solved. p27 makes contacts with both the kinase and cyclin subunits.[21] In particular, an N-terminal region of p27 (residues 30−33) binds to a hybrophobic patch on cyclin A made up from the conserved cyclin box motif while residues 60−65 of p27 interact extensively with the N-terminal lobe of the Cdk2 subunit, expelling ATP.

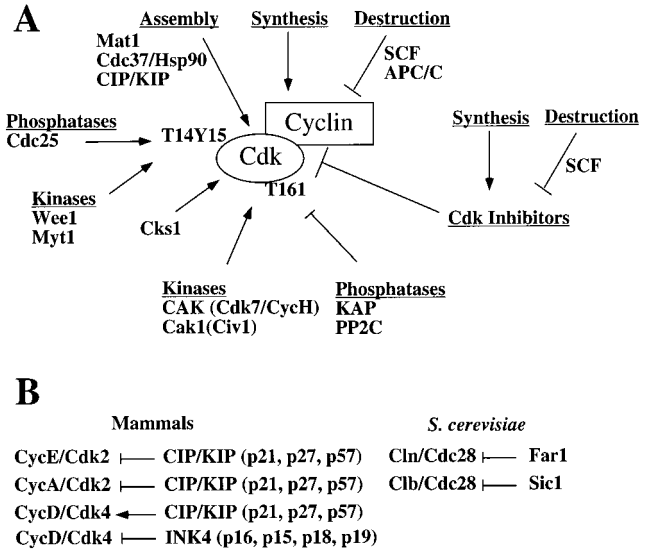

Figure 2. Regulation of cyclin-dependent kinases. (A) Cdks are regulated by several mechanisms, including accumulation (synthesis) of cyclins, assembly of cyclin/Cdk complexes, positive and negative phosphorylation, tight-binding Cdk inhibitors, and ubiquitin-mediated proteolysis of cyclins and Cdk inhibitors by both the SCF (Skp1/Cul1/F-box protein) ubiquitin ligase and the APC/C (anaphase promoting complex/cyclosome). The CIP/KIP family of proteins function as both assembly factors for D-type cyclin kinases and as inhibitors of cyclin E- and A-dependent kinases. Some Cdks interact with Cks1, which has been implicated in multiple aspects of cell cycle progression. CAK activities in budding yeast and metazoans are provied by distinct enzymes (reviewed in ref 19). In metazoans, CAK activity is provided by Cdk7/cyclin H, which also has a role in regulation of transcription. In budding yeast, a distinct monomeric kinase, Cak1 (or Civ1, Cak in vivo), provides this activity. The yeast homologue of Cdk7 (Kin28) has a role in transcription control. (B) Distinct classes of Cdk inhibitors preferentially interact with distinct Cdk complexes. In mammals, CIP/KIP proteins function as inhibitors of Cdk2 and function as both activators and inhibitors (at higher concentrations) of Cdk4 and Cdk6/cyclin D complexes. The INK4 class of inhibitors are specific for Cdk4 and Cdk6. In budding yeast, the Far1 protein inhibits Cln-dependent Cdc28 activity in response to mating pheromone while Sic1 inhibits Clb/Cdc28 complexes.

The mechanism of activation of cyclin D1/Cdk4 complexes is more complex and regulated by the stability of both Cdk4 and cyclin D1 subunits and by their assembly (Figure 3).[22] Newly synthesized Cdk4 appears to be intrinsically unstable and assembles into a high molecular weight complex containing Hsp90 and $p50^{Cdc37}$.[23,24] This chaperone complex is thought to stabilize and/or help fold Cdk4, and this function appears to be required for productive interaction with cyclin D1. This same Hsp90/Cdc37 complex also associates with several other kinases, including Raf-1 and src homologues, which are also intrinsically unstable. Once Cdk4 is properly stabilized, it is thought to be released from the chaperone in what is a poorly understood step. The abundance of cyclin D1 is regulated by at least two mechanisms.[22] First, cyclin D1 transcription is dependent upon the presence of growth factors. Several studies have demonstrated that cyclin D1 transcription is induced in response to ras activation. Second, cyclin D1 levels are controlled by ubiquitin-mediated proteolysis, and this process is also controlled by growth

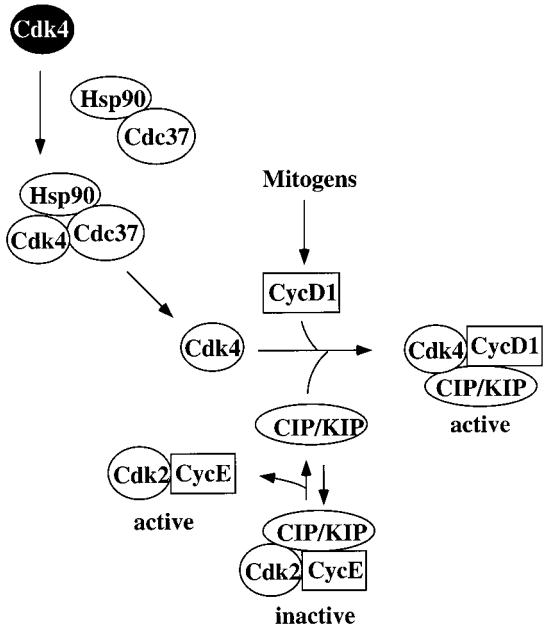

Figure 3. Model for assembly and activation of D-type cyclin kinases. In this model, Cdk4 is unstable in its newly synthesized form as associates transiently with a chaperone complex composed of Cdc37 and Hsp90 in the cytoplasm. Cdk4 is released from this complex in a potentially activatable form and then interacts with D-type cyclins in a process that is dependent upon the availability of CIP/KIP proteins (p21, p27, or p57). Cyclin D synthesis is under mitogen control via the Ras/MAP kinase pathway. CIP/KIP proteins used for assembly may be either newly synthesized proteins or proteins released from a Cdk2/cyclin complex. Thus, accumulation of cyclin D and Cdk4 subunits favors the reequilibration of Cdk2-bound CIP/KIP proteins to the cyclin D complex, thereby resulting in the activation of Cdk2 kinase activity by sequestration of CIP/KIP proteins. CIP/KIP-associated cyclin D/Cdk4 complexes retain enzymatic activity at stoichiometric levels of CIP/KIP proteins, but higher levels of CIP/KIP proteins appear to be inhibitory.

factors.[25] In particular, GSK3β is thought to phosphorylate cyclin D1, thereby promoting its destruction through an as yet unknown E3 ubiquitin ligase pathway. In the presence of growth factor signals, GSK3β activity is diminished, thereby promoting cyclin D1 stabilization.

In stark contrast with Cdk2/cyclin complex formation, free Cdk4 and cyclin D1 associate very poorly in the absence of an assembly function.[26] Recent work has demonstrated that cyclin D1/Cdk4 assembly involves the action of the CIP/KIP family of proteins (reviewed in ref 22). Two major lines of evidence are consistent with this idea. First, when cyclin D1 and Cdk4 are overexpressed in tissue culture cells, complex formation is inefficient. However, concomitant expression of CIP/KIP proteins leads to efficient assembly of holoenzyme complexes containing a CIP/KIP protein.[27] At low CIP/KIP concentrations, assembly is promoted and the resulting complex is catalytically active, while at higher levels, inhibition of kinase activity is observed. Interestingly, assembly of the CIP/KIP-cyclin D/Cdk4 complex is also required for localization of the kinase in the nucleus.[27] Second, mouse embryonic fibroblasts lacking p21 and p27, the major CIP/KIP family members in these cells, poorly assemble D-type cyclin kinase complexes.

However, when p21 or p27 is reintroduced into these cells, assembly of an active cyclin D1/Cdk4 complex is observed.[28] Thus, CIP/KIP proteins are necessary and sufficient to assemble cyclin D1/Cdk4 complexes (Figure 3). The fact that CIP/KIP proteins function as both inhibitors of Cdk2 and activators of the upstream Cdk4 kinase has important implications for the G1/S transition, as described below. Like Cdk2, Cdk4 requires phosphorylation in the T-loop for maximal activity. The precise order of T-loop phosphorylation relative to assembly is not clear, since CIP/KIP proteins are thought to prohibit phosphorylation of the T-loop of Cdk2 by CAK[29] but not Cdk4.[30]

IV. Variations on Cdk Assembly and Activation

Although the Cdk2 and Cdk4 activation pathways are representative of many Cdk complexes, there are some variations on the theme. One variation involves the Cdk-activating kinase CAK. Here, there are two routes to formation of an active enzyme complex.[31,32] In one pathway, Cdk7 and cyclin H assembly is linked to phosphorylation of Thr170 in the T-loop by "CAK-activating kinase". This phosphorylation results in stabilization of the assembled complex and activation of Cdk7. In an alternative pathway, the RING-finger protein MAT1 functions to assemble an otherwise inefficient Cdk7/cyclin H complex. In this setting, Cdk7 is activated in the absence of T-loop phosphorylation. The precise mechanism of activation employed in vivo is not well defined, and it is conceivable that the majority of Cdk7 is both phosphorylated on T170 and associated with MAT1.

Relatively unique among Cdks is the neural-specific p35/Cdk5 complex (reviewed in ref 33). Although Cdk5 is expressed in many cell types, its activators (p35 and p39) are largely restricted to neuronal lineages where they function together with Cdk5 in axonogenesis and/or axon patterning. Initial studies indicated that Cdk5 could be activated by p35 (or a 25 kDa fragment of p35) without phosphorylation on the T-loop.[34] Moreover, Cdk7/cyclin H was unable to phosphorylate Cdk5/p35 complexes. However, this view has recently been challenged by the finding that Cdk5 is phosphorylated in vivo and that neuronal extracts contain a Cdk5-activating kinase that phosphorylates Ser159 (*35*). Thus, like cyclin A/Cdk2 complexes, maximal Cdk5 activity may involve both cyclin binding and T-loop phosphorylation. Although casein kinase I is capable of catalyzing Cdk5 activation in vitro,[35] it is not clear whether this kinase is responsible in vivo.

V. Distinct Modes of Regulation of Cdc28

In budding yeast, a single Cdk orchestrates multiple cell cycle transitions through interaction with several G1 and mitotic cyclins.[4]. While many features of the Cdc28 activation process are similar to those displayed by cyclin A/Cdk2, there are some clear differences. First, Cdc28 requires the activity of the Cdc37 for activation.[36,37] This is in contrast with Cdk2 and Cdc2 in mammalian cells, which do not appear to require Cdc37 for stabilization.[23] Second, budding yeast contains a distinct kinase (Cak1 or Civ1) that is responsible for phosphorylation of Cdc28 on the T-loop (reviewed in refs 18 and 19). In contrast to the mammalian Cdk7/cyclin H/Mat1 enzyme, Cak1 is monomeric and is unrelated to Cdk7.[38−40] A second distinction of Cak1 is that it efficiently phosphorylates monomeric Cdc28, while Cdk7 prefers cyclin-associated Cdk subunits.[41] A further distinction concerns the Cdk binding protein Cks1. Cks1 is the budding yeast homologue of $p13^{suc1+}$ originally identified as a component of the Cdc2 complex in *S. pombe*. This protein is conserved throughout eukaryotes, but its precise function has remained elusive. Cks1 associates with both monomeric and cyclin-associated forms of Cdks: Cdc28 in budding yeast, Cdc2 in *S. pombe*, and Cdk1-Cdk3 in mammals. Temperature-sensitive mutants indicate roles for Cks1 in both the G1/S and G2/M transitions in budding yeast.[42] The G2/M role may reflect association of Cks1 with subunits of the proteasome, which is involved in Clb destruction during this phase of the cell cycle.[43] Recent work suggests that Cks1 is required for activity of Cln/Cdc28 complexes both in vitro and in vivo but is not for activity with Clb/Cdc28 complexes.[44] Cln/Cdc28 complexes prepared in insect cells lack activity unless Cks1 is added, and *cks1* mutants display defects in Cln autophosphorylation in vivo. At present, this requirement for G1 Cdk activity appears to be unique to budding yeast since active Cdk2/cyclin complexes can be prepared in the absence of Cks1 and Cdk4 does not associate with Cks1.

VI. Cell Cycle Regulatory Circuits

The eukaryotic cell cycle minimally consists of alternating DNA replication (S) and chromosome segregation (M) phases. However, generally these two phases are separated by two gap phases, termed G1 and G2. Associated with the S- and M-phases are a host of coordinately regulated biosynthetic processes that are necessary for proper division. For example, histone synthesis occurs simultaneously with DNA replication and the histones are required to incorporate newly replicated DNA into chromatin.

It has been known for many years that progression through the cell cycle is, in large part, driven by the sequential and periodic activation of cyclin/Cdks. Different cyclin/Cdks are active at different points in the cycle. For example, cyclin E/Cdk2 drives the cell from late G1 into the S-phase and cyclin B/Cdc2 promotes progression from G2 into mitosis. Genetic analysis indicates that there is considerable redundancy between the various complexes but also that the different cyclin/Cdk combinations are designed to perform specific functions at their respective phase of the cell cycle. For example, no single cyclin is essential in yeast and most double and some triple and quadruple mutants are also viable. Strikingly, in *S. pombe* the cell cycle can function on a single cyclin gene, Cdc13, and *S. cerevisiae* is viable with only a single Clb-type cyclin if it is expressed at a high level.[45−47] However, the G1-phase Clns cannot activate the S-phase and Clb5 is a more efficient inducer of the S-phase than is Clb2.[4,48] On the other

hand, Clb5 is less efficient than Clb2 at blocking exit from mitosis.[49]

Activity and function of cyclin/Cdks through the cell cycle is inextricably connected with cell-cycle-regulated ubiquitin-dependent proteolysis.[50,51] Covalent attachment of tandem ubiquitin moieties to a protein targets that protein for degradation by the 26S proteasome. Ubiquitin ligases are responsible for covalently linking ubiquitin to proteins through isopeptide linkages with lysine residues. At least two multisubunit ubiquitin ligases, the Skp1/Cdc53/F-box complex (SCF) and the anaphase-promoting complex (APC) are known to play central roles in the cell cycle. Each of these complexes use substrate-specific activator proteins to target it to its substrates. Two APC activators, Cdc20 and Cdh1, are particularly relevent to the following discussion. Cdc20 is required for degradation of substrates during mitosis, and Cdh1 is required at the end of mitosis and in the G1-phase. Proteins containing F-boxes within their primary sequence (so-named because the motif was first identified in cyclin F) act as substrate-specific receptors of the SCF. The F-box protein Grr1 is required for degradation of cyclins, Cln1 and 2, and F-box protein Cdc4 is required for degradation of $p40^{Sic1}$ and Cdc6.

At its simplest, the cell cycle can be viewed as alternating periods of high cyclin/Cdk activity/low APC activity and low cyclin/Cdk activity/high APC activity. Under favorable growth conditions, the cell automatically switches from one state to another. Under less favorable conditions, one of these switches is suppressed and the cell arrests in the G1-phase. This alternation between periods of high kinase activity and high proteolysis provides the biochemical basis for alternating rounds of DNA replication and mitosis (Figure 4).

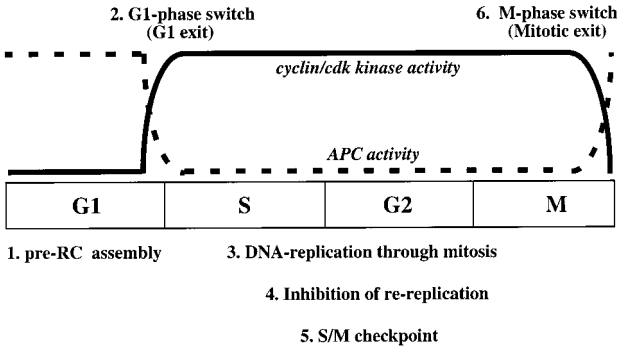

Figure 4. Cyclin/Cdk kinase activity and APC-dependent proteolysis drive the cell cycle. Periods of high cyclin/Cdk and APC activity are indicated. Low kinase activity and high APC activity in the G1-phase are permissive for pre-RC formation (step 1). In late G1 cyclin/Cdk, activity increases and APC activity declines (step 2). High kinase activity from the S-phase through mitosis drives cell cycle events in this period, such as DNA replication and histone synthesis (step 3). High cyclin/Cdk activity during S-phase prevents reformation of pre-RCs after an origin has fired and so prevents re-replication of DNA (step 4). Through a poorly defined mechanism, onset of mitosis is prevented until completion of DNA replication (step 5). At the end of mitosis, APC activity increases and cyclin/Cdk activity falls, and this drop in cyclin/Cdk activity is responsible for exit from mitosis and resets the cell in G1-phase where pre-RCs can form (step 6).

The remainder of this review considers the cell cycle in terms of its most fundamental steps and addresses the role of the cyclin/Cdks at each step. These six steps are illustrated in Figure 4. Particular emphasis will be placed on substrates and modes of regulation that are specific to each cyclin/Cdk complex and important for the function of that complex at that stage in the cell cycle.

A. Step 1: Assembly of the Prereplication Complex (pre-RC) at Origins of DNA Replication

The pre-RC is a complex of proteins, made up of ORCs1−6, Cdc6, Cdt1, and Mcm2−7, that is required for initiation of DNA replication at a replication origin (although a recognizable Cdt1 homologue has not yet been found in *S. cerevisae*). In yeast and mammalian cells, the ORCs are present at the origin constitutively throughout the cell cycle. In contrast, Cdc6, Cdt1, and then Mcm2−7 are loaded sequentially in the G1-phase.[52−54] Formation of the pre-RC requires the low S-, G2-, and M-phase cyclin/Cdk activity that is characteristic of the G1-phase.[55,56] Low activity of these kinases is maintained in the G1-phase by high APC^{Cdh1} activity, which targets the S-, G2-, and M-phase cyclins for degradation,[57] and by expression of Cdk inhibitors ($p40^{Sic1}$ in *S. cerevisiae*, $p25^{rum1}$ in *S. pombe*, $p27^{Kip1}$ in mammalian cells and $p27^{Xic1}$ in *X. laevis*), which inhibit the non-G1 cyclin/Cdks.[58−62] Once an origin has fired in the S-phase, high S-phase kinase activity prevents reformation of a pre-RC. This ensures that each sequence of DNA in the genome is replicated only once per cell cycle, and this is discussed in more detail below (step 4).

B. Step 2: Inactivation of APC-Dependent Proteolysis and Activation of the S-, G2-, and M-Phase Cyclin/Cdk Activity

In the late G1-phase, a key biochemical "switch" occurs (Figure 4). That is, the cell switches from a state of high APC/low S-, G2-, M-phase kinase activity to a state of low APC/high kinase activity. This switch results from the accumulation of G1 cyclin/Cdk kinase activity. In the late G1-phase, this kinase accumulates to such a level that it triggers activation of S-phase kinases and inactivation of the APC. Accumulation of the G1 cyclins and activation of the switch in both yeast and mammalian cells is dependent upon a favorable extracellular environment and coincides with "start" in yeast and the "restriction point" in mammalian cells.[4,63]

In vegetatively growing *S. cerevisiae*, expression of Cln3 is largely cell cycle independent. Cln3/Cdc28 kinase is responsible for activation of the SBF transcription factor, which drives expression of Cln1 and Cln2 in late G1.[64] Accumulation of the Clns is facilitated by their insensitivity to degradation promoted by the APC^{Cdh1}.[65] Moreover, the Cln/Cdc28 kinases are refractory to inhibition by $p40^{Sic1}$.[58] Thus, in growing cells, Cln/Cdc28 kinase activity accumulates as the cells progress through G1. However, the presence of extracellular mating pheromone or nutrient starvation blocks the accumulation of Cln/Cdc28 kinase activity. For example, mating pheromone

activates a signal transduction cascade that upregulates the expression of the Cln/Cdc28 inhibitor, Far1.[66,67] This arrests the cells in the G1-phase in preparation for mating.

The S-phase cyclins, Clb5 and Clb6, are transcribed in the late G1-phase at the same time as Cln1 and 2.[68,69] Interestingly, compared to other Clb-type cyclins such as Clb2, Clb5 appears to be relatively insensitive to the G1-phase APC^{Cdh1}.[70] This presumably facilitates accumulation of this cyclin in the G1-phase.[48] However, in contrast to Cln/Cdc28, Clb5/Cdc28 is sensitive to inhibition by $p40^{Sic1}$ and so its activity is initially kept in check.[58] Phosphorylation of $p40^{Sic1}$ by Cln/Cdc28 targets it for ubiquitination by SCF^{Cdc4} and subsequent degradation.[71−74] Thus, increasing Cln/Cdc28 kinase activity results in decreased $p40^{Sic1}$ and activation of the S-phase cyclin/Cdks, Clb5, and Clb6/Cdc28. In *S. pombe*, the G1 cyclin Puc1 is thought to play an analogous role to the Clns in that it accumulates in G1 and is insensitive to $p25^{rum1}$. Puc1/Cdc2 phosphorylates $p25^{rum1}$ to promote its degradation and relieve the inhibition of the S-phase cyclin/Cdk, Cig2/Cdc2.[75]

Like their G1 counterparts in yeast, the activity of mammalian cyclin D/Cdk4 kinases is linked to the extracellular environment. As detailed above, transcription, stability, and nuclear localization of cyclin D1 all require mitogen-dependent activation of the Ras-pathway. Mitogen deprivation represses transcription, destabilizes the protein, and causes relocalization to the cytoplasm. This causes the cell to enter the quiescent or G_0 state.

In contrast to yeast, where inactivation of only $p40^{Sic1}$ is required for activation of S-phase kinases, in mammalian cells complete activation of the S-phase kinases requires inactivation of at least two proteins, pRB and $p27^{Kip1}$ [22,76] (Figure 5). In the presence of extracellular mitogens, cyclin D/Cdk4 complexes accumulate in the G1-phase, phosphorylate pRB, and activate expression of E2F target genes, such as cyclin E. Initially, cyclin E/Cdk2 is kept inactive by binding to the inhibitor, $p27^{Kip1}$. However, accumulating cyclin D/Cdk4 complexes sequester $p27^{Kip1}$ as part of the assembly process (Figure 3), without themselves being inactivated, and relieve the inhibition of cyclin E/Cdk2. Newly activated cyclin E/Cdk2 contributes to pRB phosphorylation, further activating E2F and initiating a positive feedback loop of pRB phosphorylation and cyclin E production. Eventually, cyclin E/Cdk2 kinase accumulates to such a level that it phosphorylates $p27^{Kip1}$ and targets it for ubiquitination by the SCF and degradation by the proteasome. Thus, both cyclin D/Cdk4 and cyclin E/Cdk2 contribute to inactivation of pRB and $p27^{Kip1}$.

Although this simple model is attractive and supported by a considerable body of evidence, the relative contributions of cyclin E and D to inactivation of pRB remain controversial. Much evidence indicates that cyclin E acts, in large part, downstream of or in parallel to cyclin D.[77−80] However, other evidence suggests that cyclin D is not sufficient for complete inactivation of pRB and therefore that cyclin E/Cdk2 is likely to be required for full inactivation.[81,82] Taken

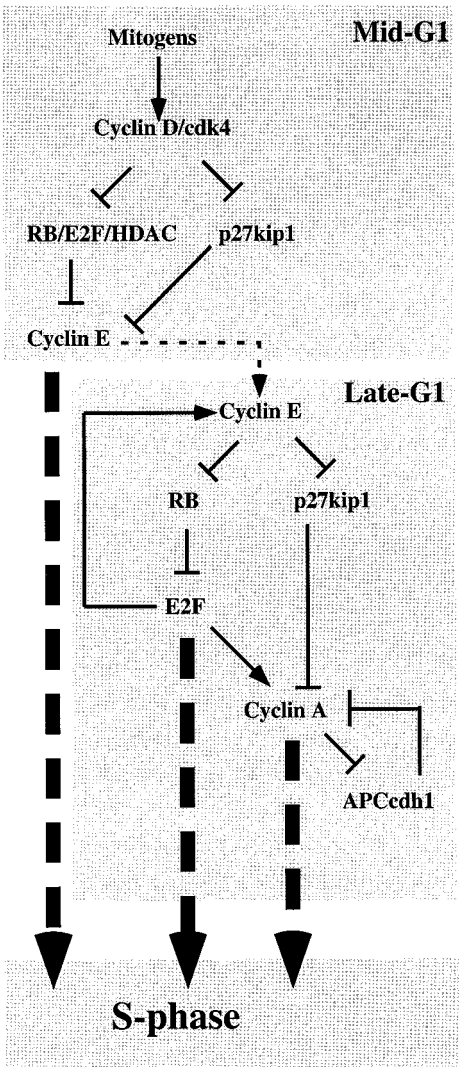

Figure 5. G1 switch. Cyclin D/Cdk4 kinase activity accumulates through the G1-phase in a mitogen-dependent manner. This initiates inactivation of pRB and p27Kip1, which, in turn, promotes expression of cyclin E in late G1. Cyclin E/Cdk2 activates a positive feedback loop that increases cyclin/Cdk2 kinase activity by further inactivating pRB and $p27^{Kip1}$ and promoting expression of cyclin A. Cyclin A/Cdk2 inhibits APC^{Cdh1}, thus completing the switch to a high cyclin/Cdk activity/low APC activity state. Concerted action of cyclin E/Cdk2-, cyclin A/Cdk2-, and E2F-dependent transcription activates the S-phase.

together, it seems likely that cyclins E and D cooperate to inactivate pRB in vivo, but the only essential consequence of cyclin-D-mediated phosphorylation of pRB is to trigger cyclin E expression.[78] Assuming that cyclin E/Cdk2 and cyclin D/Cdk4 cooperate to inactivate pRB, there are two alternative models. In the first, cyclin D/Cdk4 and cyclin E/Cdk2 both inactivate the same molecular function(s) of pRB and cyclin E/Cdk2 merely completes the process begun by cyclin D/Cdk4. In the second model, cyclin D/Cdk4 and cyclin E/Cdk2 inactivate different functions of pRB. pRB has many potential effector proteins other than E2F, such as HDAC and members of the SWI/SNF chromatin-remodeling family.[83−86] Indeed, an HDAC/RB/E2F complex actively represses transcription of E2F target genes in the G1-phase. Biochemical studies showing that different cyclin/Cdks phosphorylate different sites on pRB together with evidence

indicating that phosphorylation of different sites inactivates different functions supports the second model.[87−91] For example, it has been proposed that cyclin D/Cdk4 phosphorylates pRB and inactivates pRB/HDAC-mediated transcriptional repression of some E2F target genes, including cyclin E. However, repression of the cyclin A promoter is maintained by pRB/SWI−SNF-mediated transcriptional repression. Subsequently, cyclin E/Cdk2 phosphorylates pRB and causes release of "free" transcriptionally active E2F. This activates expression of cyclin A and other E2F target genes.[92] Expression of cyclin A and activation of cyclin A/Cdk2 indicates the transition to high S-, G2-, and M-phase kinase activity in mammalian cells.

Interestingly, recent evidence suggests that inactivation of pRB by cyclin D/Cdk4 is not required for S-phase entry in cells lacking both of the pRB-related proteins, p107 and p130.[93] Previous studies have shown that p107 is a likely in vivo substrate of cyclin D/Cdk4 and it inactivates p107-mediated repression of E2F activity.[94,95] This suggests that cyclin D/Cdk4 activity is required for inhibition of pRB or both p107 and p130 together. pRB and p107/p130 have been previously shown to regulate distinct sets of E2F-responsive genes.[96] Thus, the pathways by which cyclin D/Cdk4-mediated inactivation of pRB or p107/p130 promote S-phase are likely to be subtly different. Presumably, both pathways require activation of cyclin E and A/Cdk2.

Once the S-phase cyclin/Cdks have been activated by the G1-phase cyclin/Cdks, they, in turn, inactivate the G1 APC^{Cdh1}, thus completing the switch from high APC/low kinase to low APC/high kinase activity. The G1-phase APC^{Cdh1} is inactivated by phosphorylation and inactivation of the activating subunit, Cdh1. In *S. cerevisiae*, both Clb/Cdc28 and Cln/Cdc28 kinases have been reported to inactivate Cdh1.[97] Thus, inactivation of the APC^{Cdh1} begins before the S-phase and, by stabilizing the cyclins, contributes to the rapid accumulation of Clb5, 6/Cdc28 kinase activity. Likewise in *S. pombe*, the G1 cyclin/Cdk, Puc1/Cdc2, has been suggested to inititiate inactivation of the APC.[45,98,99] In contrast, in mammalian cells, S-phase cyclin A/Cdk2 but not G1-phase cyclin E/Cdk2 is able to phosphorylate and inactivate Cdh1.[100] Thus, in mammalian cells, the APC is not inactivated until the S-phase cyclin/Cdks are activated. The inability of mammalian G1-phase cyclin/Cdks to inactivate the APC presumably extends the period of high APC activity in these cells, allowing a longer period of time to perform events that require the high APC/low kinase state.

C. Step 3: Initiation of Biochemical Processes During the S-, G2-, and M-Phases, e.g., DNA Replication and Histone Synthesis

The start of the S-phase is defined as the start of DNA replication. However, other processes, such as histone synthesis and chromatin assembly, accompany DNA replication. Subsequently, the chromosomes condense, a mitotic spindle forms, and the chromosomes are segregated. These events, and others during this period, are driven by high S-, G2-, and M-phase cyclin/Cdk activity. As examples, we will consider the role of the cyclin/Cdk kinases in two of these: initiation of DNA replication and activation of histone gene expression.

As stated previously, the pre-RC complex assembles in the G1-phase in the period of low S-phase kinase activity. Activation of S-phase cyclin/Cdks is the cue for initiation of DNA replication, and it has been suggested that direct phosphorylation of components of the pre-RC by these kinases triggers origin firing. Indeed, components of the pre-RC, such as Cdc6, serve as substrates of cyclin/Cdks in vitro and in vivo. However, as discussed below, this may be linked to inhibition of re-replication rather than initiation of replication. This issue is further complicated by the fact that another cell-cycle-regulated kinase, Cdc7/Dbf4, is also known to play a direct role in initiation of DNA replication. Therefore, establishing which component of the pre-RC is the physiological target of the cyclin/Cdks has been difficult.[52]

At least one step at the pre-RC prior to initiation of DNA replication is known to be dependent upon S-phase cyclin/Cdk activity. This is conversion of the pre-RC to the preinitiation complex (pre-IC) by recruitment of Cdc45.[101,102] However, Cdc45 has not been shown to be an in vivo substrate of a cyclin/Cdk, so the basis of the requirement for cyclin/Cdk activity is not clear. In *S. cerevisiae*, loading of Cdc45 does not require Cdc7/Dbf4 kinase activity, indicating that the Clb5/Cdc28 is probably acting directly upon the Cdc45 or the pre-RC rather then through Cdc7/Dbf4.[102] In contrast, in *Xenopus* Cdc7 is required for the Cdk-dependent loading of Cdc45.[103]

Cln/Cdc28 kinases are unable to activate the S-phase in the absence of Clb/Cdc28 complexes.[4] Likewise, inactivation of cyclin A and E/Cdk2 kinases blocks entry into the S-phase, showing that cyclin D/Cdk4 kinases cannot initiate DNA replication.[104] This is presumably a consequence of the restricted substrate specificity of the G1 cyclins. For example, in contrast to the Clb/Cdc28 kinases, Cln2/Cdc28 does not interact with the Cdc6 protein.[105] Similarly, cyclin D/Cdk4 kinases have very restricted substrate specificity.[106,107] Thus, it appears that the G1 cyclin/Cdks are designed to activate the G1 switch but are incapable of activating DNA replication.

To maintain proper nuclear organization, newly replicated DNA must be bound to histones and packaged into chromatin. This requires production of new histones, the synthesis of which coincides with DNA replication. As cells progress through the late G1-phase and into the S-phase, there is an increase in the rate of transcription and an increase in the efficiency of processing of the pre-mRNA to the mature cytoplasmic mRNA.[108] Presumably these increases are coupled to periodic activation of cyclin/Cdks. However, little is known of the coupling mechanism. An understanding of control of histone gene transcription is hindered by the large number of copies of each gene that are clustered at few sites throughout the genome. The two major clusters of cell-cycle-regulated human histone genes are at chromosome 1q21 (at least 6 genes) and 6p21 (∼50 genes).

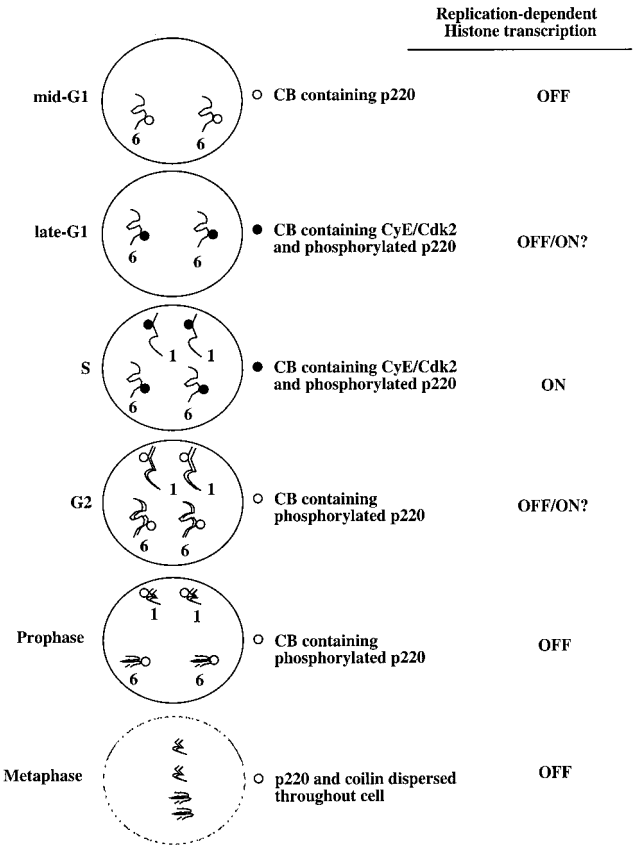

Figure 6. p220^{NPAT} links cell cycle-regulated transcription of histone gene clusters to cyclin E/Cdk2 kinase activity. In G1, coiled bodies (CBs) contain coilin and p220 and are physically tethered to the histone gene cluster on chromosome 6p21 in diploid cells. In late G1, as cyclin E/Cdk2 accumulates, this kinase is localized in CBs and phosphorylates p220. Cells in the S-phase contain CBs tethered to histone gene clusters on both chromosomes 6p21 and 1q21, and chromosome 1 tethered CBs also contain cyclin E/Cdk2 and phosphorylated p220. Phosphorylated p220 containing CBs remain tethered to histone gene clusters until the prophase−metaphase transition, while cyclin E/Cdk2 is absent from CBs in G2. At the metaphase, p220 and coilin are released from foci and p220 is presumably dephosphorylated prior to reassembly into CBs in the next cell cycle. p220 phosphorylation promotes histone transcription during the S-phase.

Recently, insight has been gained into regulation of histone transcription in mammalian cells through a protein called p220^{NPAT}. The gene encoding p220^{NPAT} was originally sequenced by virtue of its physical proximity in the genome to the gene encoding the ATM kinase (mutational inactivation of which results in ataxia telangiectasia in humans).[109] Subsequently, it was independently cloned as a novel cyclin E/Cdk2 substrate[110] and causes G1-phase acceleration when overexpressed.[110] Recent studies suggest that p220^{NPAT} plays a role in activation of histone gene transcription.[111,112] Immunofluorescence showed that p220^{NPAT} is found in discrete nuclear foci that are coincident with Cajal bodies and contain cyclin E. Cajal bodies are subnuclear organelles that are known to associate with cell-cycle-regulated histone gene clusters on chromosomes 1 and 6 and are thought to play a role in assembly of transcription complexes or RNA transcript processing.[113] The p220^{NPAT} foci are present at chromosome 1 only during the S-phase, when histones are expressed, but at chromosome 6 throughout the cell cycle. Therefore, in primary human fibroblasts, the number of p220^{NPAT} foci alternates between 2 and 4 in a cell-cycle-dependent manner (Figure 6). p220^{NPAT} was shown by chromatin immunoprecipitation assays to be present at the histone H2B, H3, and H4 promoters in vivo and to activate the histone H2B and H4 promoters in reporter assays. In each case, transcription activation required the so-called subtype-specific consensus element (SSCS) within the promoter. These are DNA sequences, specific to each histone promoter, that have been previously shown to be required for cell-cycle-dependent activation of transcription. Consistent with the observed colocalization of cyclin E and p220^{NPAT} in Cajal bodies, transcriptional activation by p220^{NPAT} also required phosphorylation by cyclin E/Cdk2. Thus, p220^{NPAT} appears to form a link between activated cyclin E/Cdk2 and activation of histone gene transcription. However, p220^{NPAT} has not been shown to directly bind to histone promoter DNA and its precise mechanism of action is unclear. One possibility is that p220^{NPAT} regulates transcription by binding to proteins that bind to the SSCS of each promoter, such as Oct-1 in the case of the histone H2B promoter.[114] Alternatively, the concentration of p220^{NPAT} within foci adjacent to histone gene clusters might indicate that p220^{NPAT} is involved in assembly of transcription complexes for histone promoters or that it participates in a large transcription complex that coordinately regulates expression of many histone genes within a cluster.

D. Step 4: Inhibition of DNA Re-replication To Ensure That Each DNA Sequence Replicates only Once per Cell Cycle

Faithful transmission of genetic information from mother to daughter cells requires that each DNA sequence replicates only once per cell cycle. Thus, controls exist to ensure that each replication origin only fires once per cycle (Figure 4). As discussed above (step 1), formation of pre-RCs is restricted to the G1-phase, when S-, G2-, and M-phase cyclin/Cdk2 kinase activity is low. Therefore, once a replication origin has fired, it is not possible for a pre-RC to reform until kinase activity is low again in the subsequent G1-phase. However, if cyclin/Cdk activity is artificially repressed in the G2-phase, by ectopic expression of a Cdk inhibitor (e.g., p25^{rum1} in *S. pombe*), then a second round of DNA replication occurs without an intervening mitosis.[115] Alternatively, if inactivation of cyclin/Cdks is uncoupled from cytokinesis, by disruption of the mitotic spindle or DNA damage, in a cell lacking proper checkpoints, then re-replication can occur.[116,117]

At the molecular level, inhibition or re-replication is relatively simple in *S. pombe* but more complicated in *S. cerevisiae* and metazoan cells. In *S. pombe*, expression of Cdc18 (the *S. pombe* homologue of Cdc6) peaks at the G1/S transition and then decreases. Cdc18 is a substrate of Cdc2/Cdc13 and phosphorylation targets the protein for degradation. Overexpression of Cdc18 is sufficient to promote DNA

re-replication, and mutation of a single Cdk phosphorylation site in the protein enhances re-replication.[118] Together, these data indicate that in *S. pombe* inhibition of DNA re-replication is largely due to phosphorylation of Cdc18 by Cdc2/Cdc13. However, recently Cdt1 has been shown to play a role in this process. When ectopically expressed, Cdc18 and Cdt1 cooperate to induce DNA re-replication. Cdt1 is removed from the chromatin in the S-phase in both *Xenopus* and *S. pombe*, suggesting that it might be a target of Cdk-dependent inhibition re-replication.[53,54]

In *S. cerevisiae*, DNA re-replication is inhibited through multiple mechanisms. First, as the S-phase proceeds, the Mcm proteins are excluded from the chromatin and cell nucleus in a cyclin/Cdk-dependent manner. A number of the Mcm proteins are likely in vivo substrates of cyclin/Cdks, and nuclear exclusion might therefore be a direct result of phosphorylation.[119,120] Second, phosphorylation of Cdc6 by S-phase cyclin/Cdks promotes its ubiquitination by SCF^{cdc4} and subsequent degradation by the 26S proteasome.[121] Consistent with this, if Clb2/Cdc28 is artificially activated in the G1-phase, prior to expression of Cdc6, then pre-RCs are not assembled.[55,56] However, yeast expressing high levels of a stable Cdc6 protein and Mcm proteins that are constitutively localized to the nucleus do not re-replicate their DNA, indicating that at least one more level of control is present.[56]

Restricting replication of each DNA sequence to once per cell cycle is further complicated by the fact that eukaryotic cells assemble more pre-RCs in the G1-phase than are used in the S-phase. The additional, normally dormant, pre-RCs are known to be potentially functional because they can be activated under certain conditions, for example, by deletion of adjacent normally active origins. Therefore, a sequence of DNA could potentially be replicated twice from two forks originating at separate pre-RCs. To overcome this problem, a dormant pre-RC is inactivated when it is passively replicated by a replication fork from another origin.[122]

In mammalian cells, the Mcm proteins are constitutively nuclear and inhibition of re-replication appears to depend, at least in part, on Cdc6. However, rather than being degraded, human Cdc6 is exported from the nucleus during the S-phase as a consequence of phosphorylation by cyclin A/Cdk2.[123,124]

Nasmyth and co-workers showed that only the Clb/Cdc28 kinases, but not the G1-phase Cln/Cdc28 kinases, are able to block Cdc6 function and, therefore, pre-RC formation.[55] Consistent with this, Elsasser et al. showed that Clb/Cdc28 complexes but not Cln2/Cdc28 interact with Cdc6.[105] In addition, Labib et al. showed that Clb/Cdc28 can exclude chromatin-bound Mcms from the nucleus but Cln/Cdc28 can only exclude nonchromatin-bound Mcms.[119] Thus, the S-phase cyclin/Cdks are designed to inhibit pre-RC formation and block re-replication whereas the G1-phase cyclin/Cdks are designed to permit pre-RC formation.

E. Step 5: Inhibition of the M-Phase Until the Completion of the S-Phase

For each daughter cell to acquire a full complement of genetic material, mitosis must not occur until the entire genome has been replicated. In principle, any event that is associated with normal passage through the S-phase, such as activation of S-phase kinases, might be involved in inhibition of premature mitosis (Figure 4). Genetic analysis of yeast indicates that checkpoints exist to prevent the premature onset of mitosis in the presence of stalled replication forks induced by hydroxyurea (HU). These checkpoint pathways are quite well defined, and there is sparse evidence that S-phase cyclin/Cdk activity is required to prevent mitotic entry.[125,126] However, many of the checkpoint genes that are required to prevent mitosis in the presence of an HU-induced S-phase arrest, such as Rad3 in *S. pombe*, are not essential genes. This indicates that they are not required for passage through a normal unperturbed cell cycle. Thus, the controls that link mitosis to completion of the S-phase in a normal cell cycle are relatively undefined. Interestingly, in yeast meiosis, the S-phase cyclins, Clb5 and Clb6, are required for completion of DNA replication and to prevent premature meiotic division. When induced to sporulate, yeasts that lack Clb5 and Clb6 arrest midway through the S-phase and then attempt to undergo a meiotic division. Whether Clb5 and Clb6 are part of the signaling pathway that prevents meiotic division or whether they are just required for proper assembly of replication structures which are, in turn, required for inhibition of meiotic division is not clear.[127] Consistent with a role for S-phase cyclin/Cdks in inhibition of mitosis in *X. laevis*, Walker and Maller showed that ablation of cyclin A in the S-phase induces the premature onset of mitosis.[128]

In summary, although a role for S-phase kinases in inhibition of premature mitosis is an attractive model, most evidence is not consistent with such an idea. For example, rather than S-phase cyclin/Cdks delaying activation of mitotic cyclin/Cdks as would be predicted by such a model, the available evidence suggests that the former are required for activation of the latter. For example, inactivation of the APC by the S-phase kinases is required for accumulation of cyclin B in mammalian cells and in *X. laevis* Cdk2 kinase has been shown to be required for activation of cyclin B/Cdc2.[100,129]

Once the S-phase is completed, the process of cyclin B/Cdc2 activation is initiated. Cyclin B/Cdc2 complexes are formed during the late S-phase and G2 but are held in an inactive form through Tyr-15 phosphorylation. This modification results from the activity of the Wee1 tyrosine kinase and related protein kinases. Activation of cyclin B/Cdc2 occurs via dephosphorylation of Tyr-15 of Cdc2 by the Cdc25 protein phosphatase (reviewed in ref 1). Cdc25 activity may be at least one of the targets of S-phase signals that block mitosis prior to completion of the S-phase. Cdc25 is phosphorylated on Ser-216, and in this form, it is associated with 14−3−3 proteins.[130] The combination of phosphorylation and 14−3−3 binding lead to inhibition of phosphatase activity

toward Cdc2 and also restrict the localization of Cdc25 to the cytoplasm in human cells. The importance of this regulatory connection is emphasized by the fact that the G2/M DNA damage checkpoint pathway functions through the same Ser-216 phosphorylation event. In response to DNA damage, the Chk1 protein kinase is activated and can phosphorylate Ser-216, leading to binding of 14−3−3.[131] It is possible that the essential function of Chk1 for cell division is maintenance of Cdc25 Ser-216 phosphorylation during the S-phase. Still unanswered is the question of how Ser-216 is reversed and how Cdc25 activation via polo-mediated phosphorylation and feedback phosphorylation by Cdc2 might be linked to disruption of the interaction of 14−3−3 with Cdc25.

F. Step 6: Inactivation of the S-, G2-, and M-Phase Associated Kinase Activity, Activation of APC-Dependent Proteolysis, and Exit from Mitosis

Events during the S-, G2-, and M-phases are driven by high cyclin/Cdk activity. In contrast, exit from mitosis requires inhibition of cyclin/Cdk activity. Thus, during mitosis, a second switch occurs, reciprocal to the switch that occurs in the G1-phase, that converts the cell from a state of high kinase/low APC activity to low kinase/high APC activity. As well as promoting exit from mitosis, this restores the low kinase state that is permissive for assembly of pre-RCs. Hence, the stage is simultaneously set for a new round of DNA replication (Figure 7).

In the S-phase, the APC is inactive due to the phosphorylation of APC activator, Cdh1, by the S-phase kinases (see step 2). The first event leading to reactivation of the APC is increased expression of another activator of the APC, Cdc20. Genetic analysis of *S. cerevisiae* indicates that APC^{Cdc20} has two essential substrates, Clb5 and Pds1.[132] Degradation of the former is required, but not sufficient, for inactivation of mitotic cyclin/Cdk activity and exit from mitosis.[50] Degradation of the latter is required for timely release of cohesion between sister chromatids and hence the onset of anaphase.[133] However, inactivation of Pds1 also has a second function. Degradation of Pds1 initiates activation of the mitotic exit network and ultimately activation of the phosphatase Cdc14.[134,135] This phosphatase has three major substrates, phosphorylated $p40^{Sic1}$, Cdh1, and the transcription factor, Swi5.[50,130] Dephosphorylation of $p40^{Sic1}$ prevents its ubiquitination and degradation by the SCF^{Cdc4}; dephosphorylation of Cdh1 activates the APC toward the mitotic cyclins, such as Clb2; dephosphorylation of Swi5 activates this transcription factor and facilitates transcription of $p40^{Sic1}$. All three events contribute toward inactivation of mitotic cyclin/Cdks.[50,136] Moreover, all three phosphoproteins are themselves substrates of cyclin/Cdks, and phosphorylation by these kinases antagonizes the effect of dephosphorylation by Cdc14. Therefore, dephosphorylation of these proteins by Cdc14 and partial inactivation of cyclin/Cdks activates a feedback loop that increasingly tips the balance toward inactivation of cyclin/Cdk activity. This APC-triggered inactivation of cyclin/Cdk activity promotes exit from mitosis. Moreover, it is the only function that is required of the APC to allow reassembly of the pre-RCs.[137]

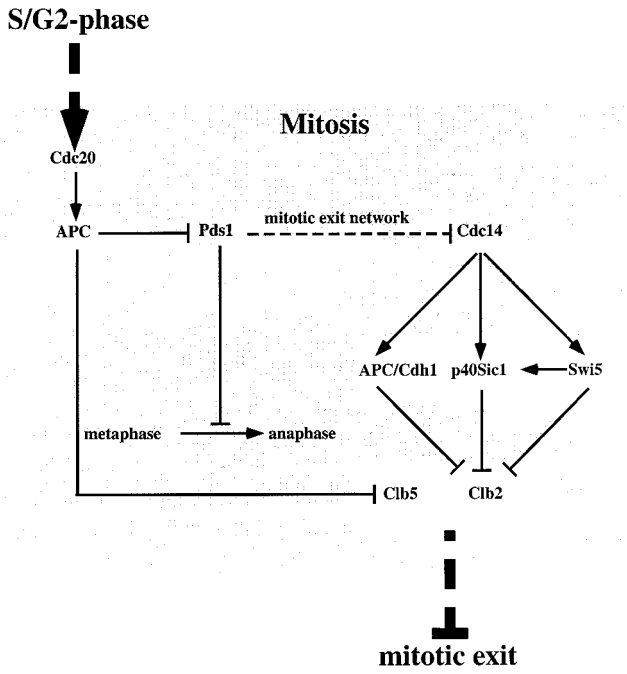

Figure 7. Exit from mitosis and the M-phase switch. In the S- and G2-phase, the cell is in a state of high cyclin/Cdk kinase activity and low APC activity. Accumulation of the APC activator, Cdc20, beginning in late S- and G2-phase triggers a reversal of this state. Cdc20 activates the APC toward its substrates Clb5 and Pds1. Degradation of the former is required for eventual exit from mitosis. Degradation of the latter promotes sister chromatid separation and the metaphase to anaphase transition. In addition, it activates the mitotic exit network and the phosphatase, Cdc14. Dephosphorylation of Cdc14 substrates, Cdh1, $p40^{Sic1}$, and Swi5 facilitates degradation and inactivation of the mitotic cyclins, particularly Clb2. Inactivation of mitotic cyclin/Cdks triggers exit from mitosis and reassembly of pre-RCs in the G1-phase.

Proper timing of activation of APC^{Cdc20} is crucial to orderly progression through mitosis. Little is known of the mechanisms that control expression of Cdc20, except that transcription begins in late S- and G2-phases and requires members of the forkhead transcription factor family.[138] A number of other genes are transcribed in the G2-phase and also require forkhead proteins and other associated proteins, such as Mcm1.[139] These proteins constitute a G2-phase-activated transcription complex that plays a central role in cell cycle control. In addition, Cdc20 is unstable throughout the cell cycle but particularly in the G1-phase.[140,141] Activation of APC^{Cdc20} also requires phosphorylation of Cdc20 and/or subunits of the APC itself. The precise role of phosphorylation and the kinases responsible remain controversial. At least three studies found that activation of APC^{Cdc20} requires phosphorylation of subunits of the APC.[142−144] One of these showed that mutation of residues in subunits of the APC (Cdc16, Cdc23, Cdc27) that are phosphorylated by Cdc28 in vivo impaired the mitotic activity of APC^{Cdc20}.[142] However, another study found that activation of APC^{Cdc20} requires phosphorylation of Cdc20,[145] and yet another found no requirement for phosphorylation.[146] Taken together, these results

indicate that phosphorylation of subunits of the APC and Cdc20 itself are likely required for activity of APC^{Cdc20}. The mitotic cyclin/Cdk kinases appear to be responsible for at least some of these activating phosphorylations .[142] However, it has been suggested that the polo-like kinase 1 (PLK1)/Cdc5 might also play a role.[147] An understanding of the mechanisms that result in the proper temporal activation of Cdc20 will increase our understanding of the M-phase switch that allows mitotic exit and re-assembly of pre-RCs.

VII. Cdks and Transcription

The versatile nature of Cdks is exemplified by the finding that Cdk/cyclins are frequently found as part of the machinery unrelated to cell division. One prominent utilization of Cdks is in control of transcription (Figure 1). Some of the first evidence of roles for Cdks in transcriptional control came from work in budding yeast, where it was demonstrated that the cyclin-Cdk pair Pho80/Pho85 functions to control the activity of a transcription complex Pho2/Pho4 responsible for coordinating the response to alterations in phosphate.[148] We now know that Pho85 is a common Cdk subunit for a number of divergent cyclins, referred to as Pcls in budding yeast. Several Pcls have been shown to interact with and activate Pho85 and control different aspects of Pho85 function ranging from glycogen biosynthesis to actin regulation to cell cycle progression (reviewed in ref 149).

Cdk8/cyclin C (Srb10/Srb11 in budding yeast) is a component of the RNA polymerase II holoenzyme complex and is capable of phosphorylating the carboxyterminal domain (CTD) of the largest pol II subunit.[150] Cdk8/cyclin C is also a component of the mediator complex, which can function in vitro to repress transcription in a CTD-independent manner. Recent data indicates that Cdk8/cyclin C functions, in part, by phosphorylating another cyclin/Cdk complex, Cdk7/cyclin H.[151] As described above, Cdk7/cyclin H is responsible for activation of several Cdks involved in cell cycle control, but this complex also plays an independent role as a component of the general transcription initiation factor TFIIH.[18,19] The action of Cdk7 in transcription may reflect its ability to phosphorylate the CTD. Phosphorylation of cyclin H by Cdk8 blocks Cdk7-dependent CTD phosphorylation, and this correlates with inhibition of TFIIH-mediated transcriptional activity.[151] This provides a model wherein the mediator complex functions to directly regulate the basal transcription machinery via a Cdk signaling pathway. The involvement of Cdks in transcription extends to the transcriptional elongation complex p-TEFb (reviewed in refs 152 and 153). p-TEFb activity appears to be due to at least three distinct complexes that contain Cdk9 associated with three distinct cyclins: cyclinT1, cyclinT2a, and cyclin T2b.[154] The cyclins T2a and T2b are splice variants from the same gene, while cyclin T1 is from a different gene. Not surprisingly, Cdk9-containing complexes display CTD-kinase activity, which is thought to promote the elongation process. Importantly, Cdk9 has also been linked to HIV tat-dependent elongation of HIV transcripts.[152, 153, 155−157] The tat protein associates directly with cyclin T1 (but not T2a or T2b) and thereby recruits p-TEF and its associated Cdk9 to the 5′-end of nascent HIV transcripts. These transcripts contain an element referred to as TAR, which has been known for some time to associate with tat. Recent data indicate that recruitment of tat to TAR is greatly stimulated by phosphorylation of Cdk9, possibly through an autophosphorylation mechanism.[158] Moreover, recruitment of Cdk9 to TAR through tat appears to stimulate CTD phosphorylation, thereby providing a mechanistic explanation for tat-mediated elongation of HIV transcripts. There are likely to be other cyclin/Cdk complexes lurking in transcriptional pathways. For example, the cyclin C relative cyclin K is known to associate with RNA polymerase II[159] and can interact with Cdk9,[160] but its precise role has yet to be defined.

VIII. Recognition of Substrates by Cyclin/Cdk Kinases

Studies using model peptide substrates demonstrated that cyclin A, E, and B/Cdk kinases phosphorylate serine or threonine residues within the "best fit" consensus S/TPXK/R, although in physiological polypeptide substrates the requirement for K/R at the +3 position is apparently less stringent.[161,162] Recent structural studies have shown that this level of specificity is largely due to constraints imposed by the catalytic Cdk subunit. Brown et al. solved the crystal structure of human cyclin A3/Cdk2 (phosphorylated on T160) complexed to the substrate peptide, HHASPRK.[163] The strong specificity for proline at position +1 is explained by the inward-facing orientation of the main chain carbonyl of Val164 of Cdk2, making it unable to hydrogen bond with the substrate. Any residue other than proline, with its closed ring structure, would result in an uncompensated hydrogen bond from the substrate's main chain nitrogen. The +2 position makes no contact with the Cdk subunit, explaining the reduced specificity here. In contrast, the basic residue at the +3 position is hydrogen bonded to the phosphate group of phosphorylated T160 of Cdk2. This basic residue also makes a hydrogen bond with the main chain oxygen of I270 of cyclin A3. Consistent with the observation that cyclin A, E, and B/Cdk complexes all favor a basic residue at the +3 position, this region of cyclin A3 is highly conserved in each of these cyclins. Interestingly, the primary amino acid sequence differs in this region in the D-type cyclins. At least two studies, using pRB as a substrate, have demonstrated that, relative to cyclin A and E/Cdk2 complexes, cyclin D/Cdk4 complexes more efficiently phosphorylate S/TPX motifs that do not have a basic residue at the +3 position.[90,91] Thus, this region of the cyclin might play a role in determining specificity for the +3 position. Not all cyclin/Cdk complexes appear to be S/TP specific. For example, cyclin C/Cdk8 phosphorylates cyclin H/Cdk7 on SSQ and SKK motifs of cyclin H (where the underlined residue is the phosphoacceptor site).[164]

A well-defined function of the cyclin subunit is to further refine the substrate specificity of the core Cdk

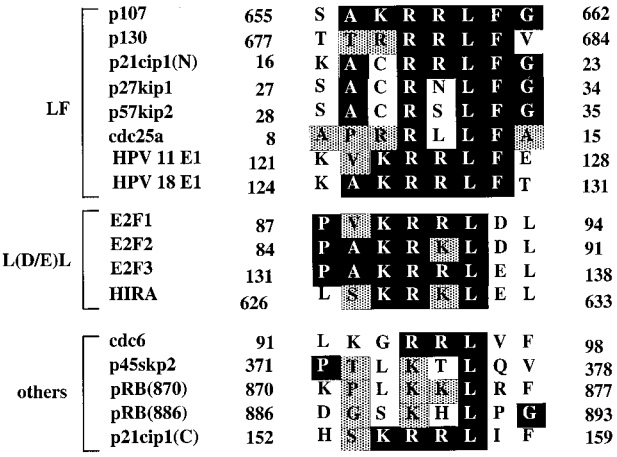

Figure 8. Alignment of RXL motifs. RXL motifs known to mediate interactions with cyclin A or E/Cdk2 are aligned. The numbers to the N- and C-termini of the aligned sequences indicate residue numbers in the full-length protein. Residues highlighted in black are identical to the majority sequence, PAKRRLFG. Residues highlighted in gray are conserved relative to this majority sequence. The RXL motifs are subdivided into three classes, depending upon the residues that follow the L of the conserved RXL.

subunit. Thus, different cyclin/Cdks have marked variation in substrate specificity when dealing with polypeptide substrates. For example, cyclin A/Cdc2 and cyclin A/Cdk2, but not cyclin B/Cdc2, phosphorylate the p107 protein;[165] cyclin A/Cdk2, but not cyclin E/Cdk2, binds to E2F1 and phosphorylates its heterodimeric partner, DP1.[166−168] Recent studies have shed light on the molecular mechanism by which cyclin subunits confer substrate specificity.

Cyclins A and E/Cdk2 recognize many of their substrates dependent upon an interaction between a conserved RXL motif in the substrate and a hydrophobic patch on the cyclin. The RXL motif was originally identified through biochemical studies that showed it to be necessary for a stable interaction between the substrate and kinase and also for phosphorylation of the substrate.[169−171] A number of proteins containing RXL motifs that have been shown to mediate interactions with cyclin/Cdk2 kinases are listed in Figure 8. Substrates include E2F family members, E2F-1, 2, and 3, pRB and related proteins, p107 and p130, other regulators of the cell cycle, Cdc25a, Cdc6, and HIRA, and the human papillomavirus E1 protein.[169−177] Interestingly, the $p21^{Cip1}$-like Cdk2 inhibitors also depend on RXL sequences for interaction with and inhibition of cyclin/Cdk2 kinases.[169−171] This implies that the $p21^{Cip1}$-like Cdk2 inhibitors function, at least in part, by competing with the substrate for access to the cyclin/Cdk2 kinase.

In support of the RXL motif being a cyclin/Cdk2 interaction motif, the crystal structure of cyclin A/Cdk2 complexed to $p27^{Kip1}$ showed that the RXL motif of $p27^{Kip1}$ makes major contacts with the cyclin A/Cdk2 complex.[21] Furthermore, consistent with the notion that substrate selection is largely a role for the cyclin subunit, the contacts made by the RXL are exclusively with cyclin A. Specifically, the residues RNLFG (where RNL comprises the RXL) of $p27^{Kip1}$ make both hydrogen bonds and hydrophobic interactions with cyclin A. For example, R30 and L32 of the RXL make hydrogen bonds with E220 and Q254 of cyclin A. In addition, M210, L214, and W217 of cyclin A make hydrophobic contacts with the RNLFG of $p27^{Kip1}$. Similar conclusions were drawn from the crystal structure of cylin A3/Cdk2 complexed to the p107-derived RXL-containing peptide, RRLFGEDPPKE.[163] Schulman et al. reasoned that these hydrophobic interactions would make a major contribution to the cyclin A/p27Kip1 interaction and converted these three residues of cyclin A to alanine.[178] This mutant of cyclin A efficiently interacted with Cdk2, indicating that it was not grossly conformationally distorted, but no longer interacted with RXL-containing proteins, p107, E2F-1, $p21^{Cip1}$, and $p27^{Kip1}$. As expected, cyclin A/Cdk2 complexes containing this mutant of cyclin A were impaired in their ability to phosphorylate p107, E2F-1, and pRB. Importantly, phosphorylation of p107 by the cyclin A mutant was restored when another p107-binding sequence that does not depend on a hydrophobic patch (the LXCXE motif, see below) was inserted into the cyclin A mutant. Thus, a heterologous p107-binding motif could functionally substitute for the RXL. Consequently, Schulman et al. proposed that the role of the RXL−hydrophobic patch interaction is to recruit the substrate to the general vicinity of the kinase and raise its local concentration. This conclusion is in accordance with the results of complementary experiments where an RXL was fused to a poorly phosphorylated substrate, thus potentiating the phosphorylation of that substrate.[175,177,179]

However, although the RXL motif plays an important role in substrate selection by cyclin A and E/Cdk2, it is clear that there are additional specificity determinants still to be identified. The residues mutated by Schulman et al. are conserved in most human cyclins, including cyclins D1 and B. Indeed, these residues comprise one of the most highly conserved cyclin signature motifs, the MRAIL motif (where M and L are M210 and L214 of cyclin A, respectively). Although these residues may play a role in substrate selection by cyclin D1, the substrate specificity of cyclin D/Cdk4 kinases is very different from cyclin A and E/Cdk2 (see below). Studies to date indicate that cyclin B/Cdc2 recognizes its substrates independent of an RXL motif.[169] Whether the MRAIL motif of cyclin B plays no role in substrate selection, perhaps being conserved for structural reasons or for binding to Cdk-inhibitor proteins such as $p21^{Cip1}$, or whether the MRAIL is involved in recognition of non-RXL proteins is not clear. Even in the case of cyclins A and E, there is evidence that residues outside of the RXL can modulate substrate specificity. For example, although both cyclin A and E interact with a short peptide spanning the RXL motif of E2F-1,[169] only cyclin A interacts with full-length E2F-1.[166−168] Thus, the hydrophobic patch of cyclin A and the RXL motif of cyclin A and E/Cdk2 substrates make an interaction that is necessary for efficient substrate selection. However, other interactions clearly come into play, particularly in the case of cyclins B and D (see below).

The RXL-binding hydrophobic patch of mammalian cyclins is conserved in yeast Clbs. Interestingly, it is required for binding of Clb5 to mammalian $p27^{Kip1}$ but not to the unrelated yeast Cdk inhibitor, $p40^{Sic1}$.[180] Like the hydrophobic patch of human cyclin A, the hydrophobic patch of Clb5 is not required for phosphorylation of histone H1 in vitro. However, the hydrophobic patch is required for a number of biological functions of Clb5, such as promotion of DNA replication and rescue of viability of a *clb3, 4, 5, 6* mutant strain. Likewise, mutation of the hydrophobic patch of Clb2 impairs various biological functions of this cyclin.[48,180] Taken together, the results suggest that the hydrophobic patches of Clb5 and Clb2 are required for interaction with particular, as yet undefined, in vivo substrates. However, it appears that the ability of mammalian Cdk inhibitors, such as $p27^{Kip1}$, to interact with the hydrophobic patch is a relatively recent evolutionary development and yeast inhibitors, such as $p40^{Sic1}$, function in a different way.

Compared to cyclin/Cdk2 kinases, cyclin D/Cdk4 kinases have highly restricted substrate specificity. In vitro, they efficiently phosphorylate pRB but not histone H1 and the E2F-1/DP1 heterodimer.[179,181,182] Likely in vivo substrates of cyclin D/Cdk4 include pRB and the related proteins, p107 and p130.[93−95,183−185] It is therefore of note that all of the D-type cyclins stably interact with pRB and that they all contain a known pRB binding sequence, the LXCXE motif.[182,183] This motif was originally identified in the viral oncoproteins adenovirus E1a, SV 40 large T-antigen, and human papillomavirus E7. In each of these viral proteins the LXCXE motif is required for interaction with pRB and cell transformation.[186] Likewise, initial reports suggested that the LXCXE motif of the D-type cyclins was necessary for their phosphorylation of pRB.[182] However, subsequent reports have shown that cyclins D1 and D2 lacking the LXCXE motif can still efficiently phosphorylate pRB in vitro and in vivo.[87,187] Consistent with the notion that an LXCXE-dependent interaction is not necessary for inactivation of pRB by cyclin D/Cdk4, the cell cycle arrest induced by a mutant of pRB that can no longer interact with LXCXE proteins is still efficiently over-ridden by cyclin D/Cdk4 kinases.[188] Thus, the role of the LXCXE in the function of the D-type cyclins is not known.

Chen et al. showed that cyclin D1/Cdk4 interacts with $p21^{Cip1}$, at least in part, through the RXL motif of $p21^{Cip1}$.[171] This raises the possibility that cyclin D1/Cdk4 complexes might also recognize their substrates dependent upon RXL sequences. In support of this proposal, phosphorylation of pRB by cyclin D1/Cdk4 in vitro is mediated, at least under certain conditions, by an RXL motif.[177] Evidence that D-type cyclins interact with substrates and $p21^{Cip1}$-like proteins through both LXCXE and RXL-based interactions is consistent with the possibility that cyclin D/Cdk4 complexes recognize different proteins through distinct targeting mechanisms (or even that they phosphorylate different sites in the same protein through distinct mechanisms). However, at present, neither the LXCXE nor the RXL has been clearly shown to be necessary for phosphorylation of a cyclin D/Cdk4 substrate in vivo. Clearly, the mechanism of targeting of cyclin D/Cdk4 kinases to their substrates is an area for future research.

IX. Summary

In the preceding decade, much has been learned about cyclin-dependent kinases. These versatile enzymes form the core of the cell division cycle. Moreover, Cdks are used in transcriptional processes as well as in post-cell-division settings. We now understand to a large degree how Cdks are regulated and how certain Cdk family members recognize substrates. Moreover, the regulatory circuits that control and are controlled by cyclin/Cdks are beginning to be understood. Nevertheless, there are still many puzzles yet to be solved. In particular, what are the key Cdk substrates required for activation of DNA replication and activation of mitosis and how does Cdk-mediated phosphorylation alter the activities of these key targets? What is the role of subcellular localization in controlling access of Cdks to substrates and to the regulatory machinery that controls the levels and activities of Cdks? Several of the known Cdk substrates seem to have a precise subcellular localization that presumably reflects their function. Moreover, there is evidence that there is spatial control of cyclin levels. Thus, there must be control of access of ubiquitin ligases to cyclins and possibly to Cdk inhibitors that is required to precisely coordinate the timing of destruction. The use of real-time dynamic experiments as recently done for cyclin B[189] will no doubt lead to a more precise description of when and where cyclins and their substrates meet. A more basic question concerns to what extent substrate turnover is important for Cdk function. Since many substrates bind cyclins very tightly and there is little evidence, at least in vitro, that phosphorylation causes dissociation of substrates, it seems possible that some cyclin/substrate complexes may exist for an extended period of time. In some cases, the substrate may be held in a particular location in the cell through interactions with other proteins. In these situations, one can envision that substrates may also target cyclin/Cdk complexes to other substrates. Another question concerns the coordination of various cell cycle events. Cyclin/Cdks are already known to play a major role in this. For example, DNA synthesis and histone transcription are coordinately activated by cyclin/Cdks so that the two are initiated within the same time frame. However, coordination extends beyond the simple temporal coincidence of their activation. Specifically, if DNA synthesis is blocked with hydroxyurea, then histone synthesis is also repressed.[108] The molecular mechanism underlying this coordinated down-regulation of histone expression is not known. The role played by cyclin/Cdks in ongoing coordination of biosynthetic processes through the cell cycle is unclear.

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XI. References

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