Requirement for p34[cdc2] Kinase Is Restricted to Mitosis in the Mammalian cdc2 Mutant FT210

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Abstract. The mouse FT210 cell line is a temperature-sensitive cdc2 mutant. FT210 cells are found to arrest specifically in G2 phase and unlike many alleles of *cdc2* and *cdc28* mutants of yeasts, loss of p34[cdc2] at the nonpermissive temperature has no apparent effect on cell cycle progression through the G1 and S phases of the division cycle. FT210 cells and the parent wild-type FM3A cell line each possess at least three distinct histone H1 kinases. H1 kinase activities in chromatography fractions were identified using a synthetic peptide substrate containing the consensus phosphorylation site of histone H1 and the kinase subunit compositions were determined immunochemically with antisera prepared against the "PSTAIR" peptide, the COOH-terminus of mammalian p34[cdc2] and the human cyclins A and B1. The results show that p34[cdc2] forms two separate complexes with cyclin A and with cyclin B1, both of which exhibit thermal lability at the nonpermissive temperature in vitro and in vivo. A third H1 kinase with stable activity at the nonpermissive temperature is comprised of cyclin A and a cdc2-like 34-kD subunit, which is immunoreactive with anti-"PSTAIR" antiserum but is not recognized with antiserum specific for the COOH-terminus of p34[cdc2]. The cyclin A–associated kinases are active during S and G2 phases and earlier in the division cycle than the p34[cdc2]-cyclin B1 kinase. We show that mouse cells possess at least two cdc2-related gene products which form cell cycle regulated histone H1 kinases and we propose that the murine homolog of yeast p34[cdc/CDC28] is essential only during the G2-to-M transition in FT210 cells.

THE *cdc2⁺* gene of *Schizosaccharomyces pombe* and its functional and structural homolog *CDC28* of *Saccharomyces cerevisiae* encode the protein kinase catalytic subunit, p34[cdc2] (reviewed in Nurse, 1985). Genetic analyses of *cdc2* and *cdc28* mutants have clearly established two control points for p34[cdc2] during the cell cycles of both fission and budding yeasts, one during G1 phase in committing cells to the division cycle at "start" (Hartwell et al., 1974; Nurse and Bissett, 1981), and another during G2 phase in controlling the onset of mitosis (Nurse et al., 1976, 1980; Reed and Wittenberg, 1990). The isolation of *cdc13⁺* as a G2-specific suppressor of a *cdc2* mutant demonstrated that the mitotic functions of p34[cdc2] are distinct from those acting in G1 (Booher and Beach, 1987).

The p34[cdc2] kinase is found in all eukaryotes and controls the onset of M phase (reviewed in Nurse, 1990). Early studies from this laboratory established a correlation between mitotic chromosome condensation and the hyperphosphorylation of histone H1 and showed that addition of growth-associated H1 kinase activity to macroplasmodia of *Physarum polycephalum* 3 h before mitosis advanced mitosis by up to one hour. These studies led to the proposals that G2 phase to metaphase H1 hyperphosphorylation initiates chromosome condensation (Bradbury et al., 1973) and that H1 kinase activity controls entry into mitosis (Bradbury et al., 1974*a*,*b*). p34[cdc2] kinases from evolutionarily distant organisms phosphorylate histone H1 in vitro (Arion et al., 1988; Brizuela et al., 1989; Meijer et al., 1989; Pines and Hunter, 1989; Labbé et al., 1989; Reed and Wittenberg, 1990). The activity of p34[cdc2] is regulated in part by its physical association with mitotic cyclins, a class of proteins which accumulate during interphase and which undergo periodic degradation during mitosis (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987; Westendorf et al., 1989; Pines and Hunter, 1989, 1990*a*; Minshull et al., 1989, 1990; reviewed in Hunt, 1989). Three mitotic cyclins, A, B1, and B2, associate with p34[cdc2] during the cell cycle. B-type cyclins form a complex with p34[cdc2] that is most active as an H1 kinase at metaphase of mitosis during the human somatic cell cycle (Pines and Hunter, 1989) and at meiotic and mitotic metaphase of clams (Draetta et al., 1989) and *Xenopus* (Gautier et al., 1990; Minshull et al., 1990). The identifica-

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tion of p34[cdc2] and B-type cyclins (Gautier et al., 1988, 1990; Labbé et al., 1989) as components of highly purified M-phase promoting factor (MPF)[1] (Lohka et al., 1988; Labbé et al., 1989) provided biochemical evidence that MPF is an H1 kinase which acts as a mitotic inducer.

Two additional cyclin-associated kinases from human cells exhibit H1 kinase activity in vitro. A small fraction of human p34[cdc2] also associates with a 60-kD protein (p60; Harlow et al., 1986) during S phase (Giordano et al., 1989). In subsequent work, Pines and Hunter (1990*a*) identified p60 as cyclin A and found that cyclin A is present in a more abundant complex with a 33-kD *cdc2*-like protein recently identified as CDK2 (Tsai et al., 1991). Both the A- and B-type cyclins can induce M-phase (Swenson et al., 1986; Westendorf et al., 1989; Murray and Kirschner, 1989; Roy et al., 1991), but their specific functions in mediating the G2-to-M transition are not known. The B-type cyclins of higher eukaryotic cells are homologs of *cdc13* (Hagan et al., 1988; Solomon et al., 1988; Goebl and Byers, 1988), which mediates an essential function during the G2-to-M transition in *S. pombe* (Booher and Beach, 1988; Hagan et al., 1988). Cyclin A serves an essential role during the cell cycle which is not complemented by the B-type cyclins, since a null mutation in the cyclin A gene of *Drosophila* is lethal (Lehner and O'Farrell, 1989, 1990*a*). More recent evidence indicates that cyclin A is essential for DNA synthesis in mammalian cells (Girard et al., 1991).

In *S. cerevisiae*, p34[cdc2] is thought to regulate the G1-to-S transition separately from the G2-to-M transition by combining with distinct classes of cyclins at appropriate stages of the cell cycle (reviewed in Pines and Hunter, 1990*b*; Reed, 1991). G1-specific cyclins (encoded by *CLN1*, *CLN2*, and *CLN3*) are essential for entry into S phase in the budding yeast (Richardson et al., 1989; Wittenberg et al., 1990). Antiserum against the *CLN2* gene product coprecipitates p34[CDC28], and this complex exhibits weak histone H1 kinase activity in vitro and is maximally active during G1 phase of the budding yeast cell cycle (Wittenberg et al., 1990).

Cell cycle control by the p34[cdc2] kinases at two distinct phases of the division cycle is thought to be conserved in higher eukaryotes. The human cdc2 homolog rescues both temperature-sensitive *cdc28* and *cdc2* mutants (Lee and Nurse, 1987; Wittenberg and Reed, 1989). Human p34[cdc2] copurifies with a factor that stimulates DNA replication (D'Urso et al., 1990) and disruption of p34[cdc2] expression with antisense oligonucleotides delays the onset of S phase following activation of human lymphocytes from G0 (Furukawa et al., 1990). In cell-free cycling *Xenopus* extracts, p34[cdc2] depletion with antiserum or with immobilized p13[suc1] inhibits DNA synthesis (Blow and Nurse, 1990). These findings lend support to the notion that p34[cdc2], or a closely related gene, regulates the onset of DNA replication in metazoan cells.

cdc2-like genes are expressed in higher eukaryotic cells (Lehner and O'Farrell, 1990*b*; Paris et al., 1991; Elledge et al., 1991; Tsai et al., 1991; Ninomiya-Tsuji et al., 1991; reviewed in Pines and Hunter, 1991*b*). *cdc2*-like gene products have also been found by immunochemical means coprecipitated with antisera against human cyclin A (Pines and Hunter, 1990*a*) and p36[CYL], a murine cyclin protein (Matsushime et al., 1991) that is also expressed in human cells (PRAD1/cyclin D1) (Motokura et al., 1991; Xiong et al., 1991). Significantly, Fang and Newport (1991) have recently shown that only one of two *cdc2*-like proteins in *Xenopus* is needed for the initiation of DNA synthesis in a cell-free cycling extract.

Studies from two laboratories established the mouse FT210 cell line as a temperature-sensitive *cdc2* mutant (Th'ng et al., 1990; Yasuda et al., 1991). The cell line provides the first opportunity to monitor the effects of a genetic defect in the *cdc2* gene on the mammalian cell division cycle. While the mitotic functions of p34[cdc2] have been conserved in higher eukaryotes through evolution, we find that p34[cdc2] kinase activity is apparently not required during the G1 and S phases of the FT210 cell cycle. Using standard fractionation procedures and a synthetic peptide substrate containing a consensus p34[cdc2] kinase phosphorylation site (Langan, 1978; reviewed in Pines and Hunter, 1990*b*), we also find that FT210 cells possess three H1 kinase activities, two of which are temperature-sensitive p34[cdc2] kinases. The third H1 kinase is activated normally during the cell cycle at the nonpermissive temperature and is found to possess cyclin A and a *cdc2*-like protein as subunits. Though it is not known how the permanent proliferative state of these cells will affect cell cycle control during G1, our results indicate that p34[cdc2] kinase functions are restricted to mitosis in mouse FT210 cells.

Materials and Methods

Immunological Reagents

Antisera used to investigate the subunit composition of the H1 kinases were generated in rabbits against bacterially expressed human cyclin A (Pines and Hunter, 1990*a*), human cyclin B1 (Pines and Hunter, 1989), and a PSTAIR-peptide antigen (EGVPSTAIREISLLKE; kindly provided by P. Russell, Research Institute of Scripps Clinic, La Jolla, CA). Antiserum against p34[cdc2] was prepared as described previously (Draetta and Beach, 1988) against a synthetic peptide antigen (C-DNQIKKM) containing the COOH-terminal seven amino acid sequence of human and mouse p34[cdc2] (Lee and Nurse, 1987; Cisek and Corden, 1989; Th'ng et al., 1990). The peptide was coupled to keyhole limpet hemocyanin (KLH) and the peptide-KLH conjugates were isolated as previously described (Green et al., 1982). Rabbits were immunized with 200-μg peptide-KLH emulsified with complete Freund's adjuvant and boosted every 3-4 wk thereafter with 200-μg peptide-KLH emulsified with incomplete Freund's adjuvant. Serum was prepared from 20 ml of blood collected 7 d after each boost.

Cell Culture

FM3A and FT210 cells (Mineo et al., 1986) were maintained in spinner flasks in RPMI-1640 buffered with 25 mM Hepes to pH 7.4 and supplemented with 10% calf serum. Stable growth rates were observed at 0.2-1.2 $\times$ 10^6 cells/ml. Population doubling times four FM3A and FT210 cells were 16 h and 20 h, respectively, under permissive culture conditions at 32°C. Cells were harvested from asynchronous cultures with 0.25-1 $\times$ 10^6 cells/ml and at 0.3 $\times$ 10^6 cells/ml from synchronized FT210 cultures.

Synchronous cultures of FT210 cells were obtained by applying multistep induction synchrony protocols. Asynchronous cells were resuspended in 39°C medium supplemented with 0.2 μg/ml Hoechst 33342 (Tobey et al., 1990) and incubated at 39°C for 12 h. These conditions result in ~80% of the cells in G2 (data not shown). G2 phase cells were resuspended in isoleucine-deficient medium supplemented with 10% dialyzed and heat-inactivated calf serum (Tobey, 1973) and incubated at 32°C for 11 h to obtain cells in early G1. The cells were then resuspended in 32° or 39°C complete medium supplemented with 5 μg/ml aphidicolin for 11 h to accumulate

1. *Abbreviations used in this paper*: KLH, keyhole limpet hemocyanin; MPF, M-phase promoting factor; PCV, packed cell volume; PEI, polyethyleneimine.

cells at the G1/S boundary (see Figs. 3 and 9), or released into complete 39° or 32°C medium supplemented with 75 ng/ml nocodazole (Janssen Pharmaceuticals, Beerse, Belgium) for a total of 20 h (see Figs. 1 and 2). G1/S blocked cells were resuspended in medium containing 0.2 μg/ml Hoechst 33342 and incubated for 9 h at 32°C or for 6 h at 39°C to accumulate cells in G2 (see Figs. 3 and 9). Comparative analyses of the histone H1 kinases were performed on synchronous cultures prepared in parallel at 32° and 39°C.

Cell cycle progression was monitored by population DNA distributions of cell samples fixed in cold 70% ethanol in PBS, stained with mithramycin and then analyzed by flow cytometry (Tobey, 1973). The percentage of cells in G1, S and G2 + M were calculated from the DNA distributions with the aid of the Multicycle Computer Program (Phoenix Flow Systems, San Diego, CA) developed by Dr. P. S. Rabinovitz (University of Washington, Seattle, WA). Mitotic indexes were determined from cell samples spread on slides, fixed in 3.7% formaldehyde for 10 min, and stained with 50 or 100 ng/ml Hoechst 33342. The proportion of cells with metaphase chromosomes was determined by visual inspection of 500 cells.

Cell Extracts and Chromatography

Cells were harvested from suspension cultures by centrifugation at 800 *g*. The cells were washed twice in 10 ml of cold PBS per liter of culture and pelleted by centrifugation. The packed cell volume (PCV) was ~1 ml per liter of culture with a cell density of 5×10^5 cells/ml. The cells were resuspended in 4 PCVs of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT) and incubated on ice for 20 min. Cells were homogenized in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with 20 strokes with a tight-fitting B pestle and the extract was supplemented with one PCV of 5× extraction buffer (final concentration 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin, 10 mM 2-glycerophosphate, 10% glycerol) and with Triton X-100 to a final concentration of 0.2%. The cells were homogenized with 20 more strokes to obtain extracts with lysed cytoplasmic and nuclear membranes. Polyethyleneimine (PEI) was added to 0.04% to precipitate chromatin (Jendrisak and Burgess, 1975) and the extract was stirred for 15 min. The PEI precipitate was collected by centrifugation for 15 min at 25,000 *g* and the resulting supernatant was designated fraction F1. The PEI pellet was resuspended in one PCV of 1× extraction buffer supplemented with 0.4 M NaCl and centrifuged for 1 min at 25,000 *g*. The resulting supernatant was designated fraction F2. Ammonium sulfate was added to both the F1 and F2 extracts to 40% saturation. The extracts were stirred for 30 min and the precipitates were collected by centrifugation for 30 min at 20,000 *g*. The pellets were resuspended in one PCV of column buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM NaF, 10 mM 2-glycerophosphate, 0.1% polyoxyethylene 23-lauryl ether [BRIJ-35], 10% glycerol) and dialyzed for 4 h to remove excess salts. Extracts were stored in liquid nitrogen.

Frozen extracts were thawed and centrifuged for 10 min at 25,000 *g* to remove insoluble material and then filtered through a 0.2-μm membrane. Each extract was applied to a 1 ml Mono S cation exchange chromatography column (Pharmacia-LKB, Bromma, Sweden). The column was washed with an additional 15 ml of column buffer and proteins bound to the column were eluted with a 40 ml linear gradient with column buffer containing 0 to 0.25 M NaCl at a flow rate of 1 ml/min. 1-ml fractions were collected.

Whole cell extracts were prepared as described above but with the following modifications. The PEI precipitation step was omitted and sodium chloride was added to the homogenate directly to a final concentration of 0.4 M. Chromatin precipitated upon the addition of NaCl and was removed from the homogenate by centrifugation at 25,000 *g* for 30 min.

H1 kinase extracts from chromatin fractions were prepared as follows. Nuclei were prepared from FT210 cells as described (Roberge et al., 1990) in 1× extraction buffer (without EDTA and EGTA) supplemented with 0.2% Triton X-100 and 3 mM $MgCl_2$. Soluble chromatin was prepared from the nuclei essentially as described (Norton et al., 1989), with minor modifications. The nuclei were resuspended in the same buffer at 2 mg/ml DNA and incubated at 32°C for 10 min and then 10 U of micrococcal nuclease were added per mg DNA and incubated for 5 min more. EDTA and EGTA were added to a final concentration of 5 mM each to inhibit nuclease activity and to lyse the nuclear membrane. After 1 h on ice, the lysed nuclei suspension was centrifuged at 10,000 *g* for 10 min. To calculate the yield of soluble chromatin in the supernatant, the absorbance at 260 nm was determined for samples of the soluble chromatin and the nuclei suspension diluted in salt-saturated urea. H1 kinase activity was extracted from the soluble chromatin with the addition of NaCl to 0.4 M and PEI to 0.04%. The chromatin precipitate was removed by centrifugation at 25,000 *g* for 1 h, and the supernatant was dialyzed and then fractionated by Mono S chromatography as described above.

Protein concentrations of the chromatography fractions and the crude F1 and F2 extracts were determined by the method of Schaffner (1973). Protein concentrations in the whole cell extracts were determined using the BCA protein assay system from Pierce Chemical Co. (Rockford, IL). BSA was used as the protein standard.

H1 Kinase Activity Assays

The standard assay was performed in a total volume of 50 μl containing 50 mM Tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 10 mM 2-glycerophosphate, 0.1 mM EDTA, 0.1 mM γ^{32}P-ATP (1,000 cpm/pmole; ICN Radiochemicals, Irvine, CA), 5–20 μl of enzyme, and the synthetic peptide substrate S1 (AAKAKKTPKKAKK) at 20 μg/ml. The reactions were incubated for 10 min at 32°C then stopped with the addition of 5 μl of 825 mM phosphoric acid (final concentration 75 mM). Incorporation of $^{32}PO_4$ was determined by spotting 25-μl aliquots of assay reactions onto P81 phosphocellulose papers (Whatman Laboratory Products Inc., Clifton, NJ) which were washed four times in 75 mM phosphoric acid, once in acetone, dried, and counted in 5 ml of liquid scintillation cocktail (Glass et al., 1978). Specific incorporation of $^{32}PO_4$ into the S1 peptide was determined as the difference in counts per minute of reactions containing substrate and reactions performed in the absence of exogenous substrate.

Immunoprecipitation of H1 Kinase Activity

Preimmune serum or anti-COOH-terminal $p34^{cdc2}$ antiserum were used at a final dilution of 1:100. Anti-cyclin A and anti-cyclin B1 antisera (Pines and Hunter, 1989, 1990*a*) were used at a final dilution of 1:250. Reactions were incubated on a rotating plate at 4°C for 2 h. The samples were transferred to tubes containing 25-μl pellets of protein A–Sepharose resin equilibrated in immunoprecipitation buffer (column buffer supplemented with 0.2 M NaCl and 0.1% Triton X-100) and incubated for 1 h on a rotating plate at 4°C. After a 1-min centrifugation in a microcentrifuge, the protein A–Sepharose pellets were washed twice with immunoprecipitation buffer. The protein A–Sepharose pellets were assayed for H1 kinase activity by the standard assay procedure outlined above. Samples were spun in a microcentrifuge for 1 min, then 25 μl of the supernatants were spotted onto P81 papers.

Whole cell extract samples were normalized by dilution to contain 200 μg of total protein in 40 μl. Immunoprecipitation reactions were performed with these samples as described above, except the protein A–Sepharose pellets were assayed using 100 μg/ml of the S1 peptide (see Fig. 2). 20-μl samples of the crude F1 and F2 extracts (1.1 and 0.8 mg/ml of protein, respectively) were immunoprecipitated as described above and were assayed for H1 kinase activity using the standard assay conditions. In reactions with competing peptide antigen, antiserum specific for the $p34^{cdc2}$ COOH terminus was diluted 10-fold, incubated with 1 μM of the peptide antigen for 1 h at 4°C and then added to the samples.

Immunoblot Analysis of H1 Kinases

Total protein extracts were prepared by boiling 10^6 cells per 10 μl of SDS-PAGE sample buffer for 3 min. The samples were pushed through small-bore syringe needles to shear the DNA and to reduce sample viscosity. Mono S column fractions were precipitated with 20% TCA on ice for 1 h. The precipitates were recovered by a 10-min centrifugation in a microcentrifuge, washed twice with acetone, dried, dissolved in SDS-PAGE sample buffer, and heated to 90°C for 2 min.

Proteins were resolved by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes in a semidry blotter for 1 h at 0.8 mA/cm^2 (Towbin et al., 1979). Membranes were blocked with 3% milk in PBS for 2 h, and then incubated with antisera diluted 1:500 in 1% milk in PBS for 3 h. Immunoblots were washed three times in blocking buffer and then incubated for 2 h with goat anti-rabbit second antibody conjugated to HRP (Calbiochem) diluted 1:7500 in blocking buffer. Membranes were washed four times in blocking buffer and once in PBS. The immunoreactive protein bands were visualized by soaking the blot in the chemiluminescent reagent ECL (Amersham Corp., Arlington Heights, IL) for 1 min and exposing X-ray film to the blot for 5–30 s. All manipulations with immunoblots were performed at room temperature.

Results

Hl Kinase Activity Assays

A synthetic peptide substrate was constructed to identify Hl kinases with specificity for the lysine-rich NH_2- and COOH-terminal tails of histone H1. The sequence of the S1 peptide, A-A-K-A-K-K-T-P-K-K-A-K-K, contains the consensus phosphorylation site for the $p34^{cdc2}$ kinases (K/R-S/T-P-[X]-K) (reviewed in Pines and Hunter, 1990*b*) and is similar to the sites phosphorylated by growth-associated Hl kinase in the NH_2- and COOH-terminal domains of Hl (Langan, 1978).

$p34^{cdc2}$ Is Not Required during G1 and S Phases of the FT210 Cell Cycle

The mouse FT210 cell line was isolated as a G2 phase cell cycle mutant (Mineo et al., 1986). Asynchronous cultures shifted to the nonpermissive temperatures of 39°C results in G2 arrest for the majority of the cells (Mineo et al., 1986; Hamaguchi, J. R., and E. M. Bradbury, unpublished observations). We have investigated the possibility that FT210 cells require presynchronization to observe an effect of the mutant cdc2 gene on the cell cycle progression through the Gl-to-S transition, a strategy which proved useful for clearly elucidating the Gl arrest properties of *cdc2 S. pombe* mutants (Nurse and Bissett, 1981). FT210 cells were presynchronized in Gl in isoleucine-deficient medium and then resuspended in either 32° (control culture) or 39°C medium and incubated for the length of one cell cycle. The control culture was supplemented with nocodazole to accumulate these cells in mitosis. The DNA distributions for both cultures (Fig. 1) showed that the cells traversed Gl and S phases and arrested with a 4C content of DNA. The cells incubated at the nonpermissive temperature arrested in G2 phase with interphase nuclei, while the control culture accumulated cells with metaphase chromosomes (Fig. 1). In other experiments, we presynchronized FT210 cells in metaphase with nocodazole, then shifted the culture to 39°C for 2 h before the removal of the drug. Again, we found that the nonpermissive temperature had no effect on cell cycle progression through the Gl and S phases of the subsequent cell cycle, and that FT210 cells arrested exclusively in G2 phase (data not shown).

We have previously shown that $p34^{cdc2}$ is degraded in vivo when asynchronous cultures of FT210 cells are incubated at the nonpermissive temperature (Th'ng et al., 1990). We were particularly interested in the stability of $p34^{cdc2}$ throughout the FT210 cell cycle, since a transient stabilization of $p34^{cdc2}$ during the Gl-to-S transition could explain why FT210 cells do not arrest in Gl. Whole cell extracts were prepared from both the 32° and 39°C cultures during the time courses shown in Fig. 1 using a hypertonic buffer supplemented with 0.2% Triton X-100. This buffer is similar to that used for studies of $p34^{cdc2}$ kinases in human cells

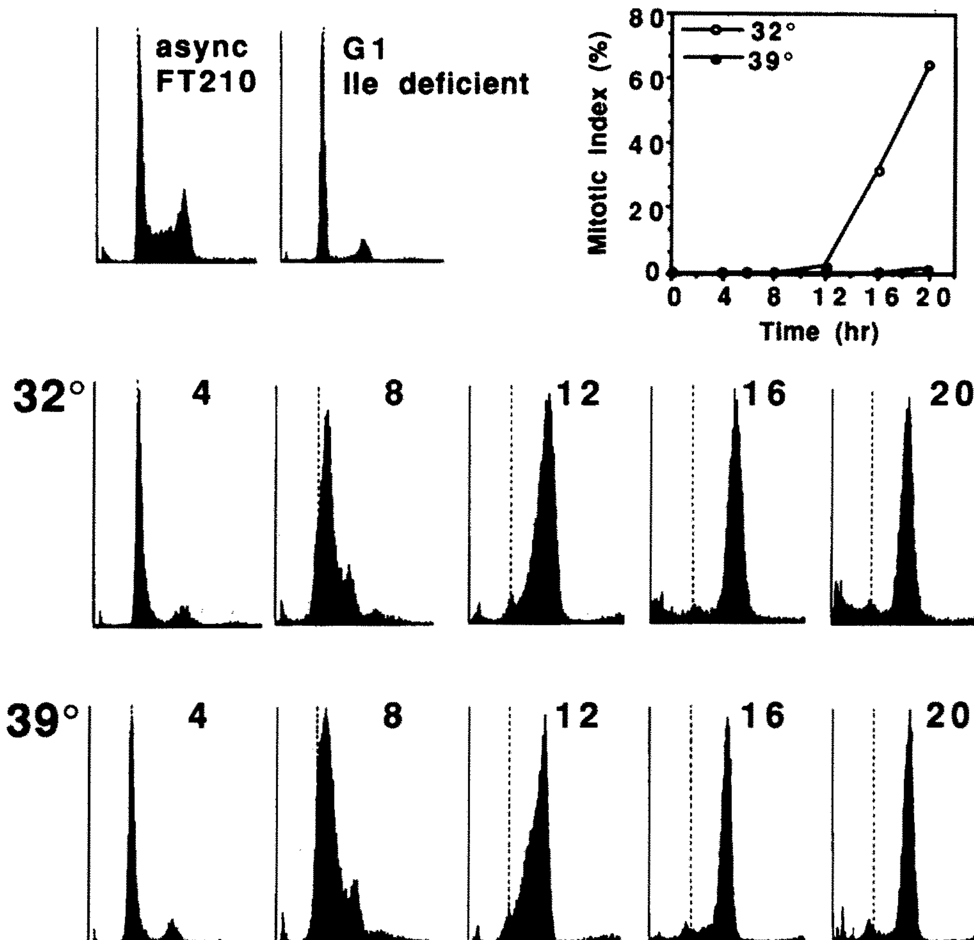

Figure 1. Cell cycle progression of FT210 cells. FT210 cells were synchronized in Gl in isoleucine-deficient medium at 32°C as described in the Materials and Methods. The Gl culture (*0 h*) was divided and the cells were resuspended in complete media at 32° or 39°C and incubated for the length of one cell cycle. The control 32°C culture was supplemented with nocodazole to accumulate these cells in metaphase. Cells were collected at 0, 4, 6, 8, 12, 16, and 20 h after release, and the cellular DNA content of the culture samples were determined by flow cytometry (6 h samples not shown). The percentages of cells with mitotic chromosomes were determined by visual inspection of cells stained with Hoechst 33342 and these values are shown with the DNA distributions in the graph. The vertical dotted line in each flow cytometric profile denotes a cellular DNA content of 2C.

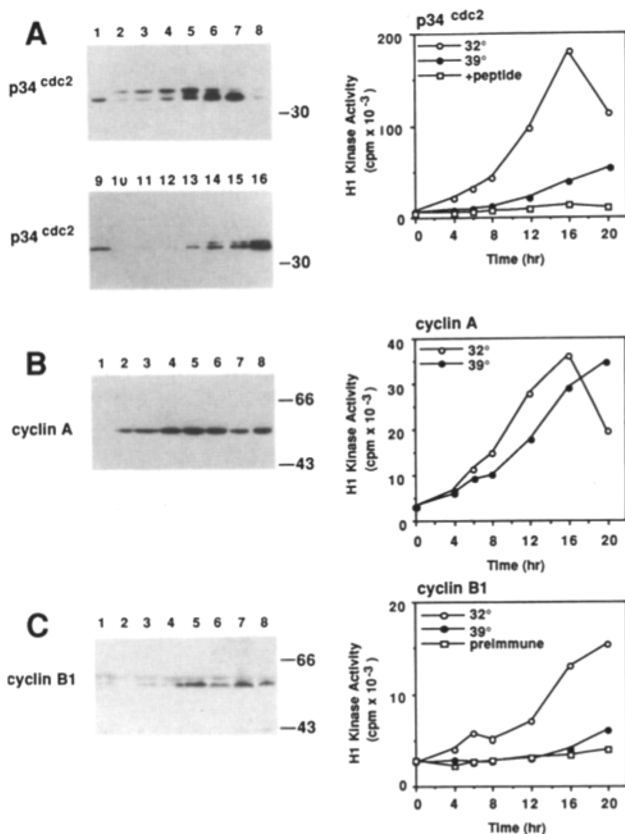

Figure 2. Cell cycle analysis of p34^cdc2^, cyclin A, and cyclin B1. G1 cells arrested in isoleucine deficient medium at 32°C (*0 h*) were released into 32°C medium supplemented with nocodazole or 39°C medium. Cells were collected at 0, 4, 6, 8, 12, 16, and 20 h after release and whole cell extracts were prepared as described in the Materials and Methods. The DNA distributions and mitotic indexes for the culture samples are shown in Fig. 1. Immunoblot analyses of the whole cell extracts (*left*) and H1 kinase activity immunoprecipitated from the extracts (*right*) are shown, using antisera specific for the COOH-terminal sequence of $p34^{cdc2}$ (*A*) human cyclin A or human cyclin B1 (*C*). (*Left*) Samples containing 100 μg of total protein were resolved in 10% acrylamide gels by SDS-PAGE. The proteins were transferred to nitrocellulose and the membranes were cut into halves at the position where a 40-kD protein would migrate. The membrane sections were probed with antisera against the COOH-terminal sequence of $p34^{cdc2}$ (*A*), human cyclin A (*B*) or human cyclin B1 (*C*) as indicated. Lanes *1* and *9*, G1 cells arrested in isoleucine deficient medium at 32°C. Lanes *2-7*, cells released into 32°C complete medium supplemented with nocodazole for 4, 6, 8, 12, 16, and 20 h, respectively. Lanes *10-15*, cells released into 39°C complete medium for 4, 6, 8, 12, 16, and 20 h, respectively. Lanes *8* and *14*, cells released into 39°C medium for 16 h. Lanes *7* and *16*, cells released into 32°C medium for 20 h. The position of molecular weight standards (in kD) are indicated to the right of the gels. Lanes *1-8* of *A* and *B* are from the same set of lanes from one gel; lanes *1-8* of *C* and *9-16* are from separate gels. (*Right*) Samples of the extracts from the 32°C control culture (○) or the 39°C culture (●) containing 200 μg of total protein were immunoprecipitated with immune sera against the COOH-terminus of $p34^{cdc2}$ (1:100 dilution) or the human cyclins A and B1 (1:250 dilution) and then bound to protein A-Sepharose beads. H1 kinase activity recovered in the protein A-Sepharose pellets was determined using 100 μg/ml of the S1 synthetic peptide substrate (A-A-K-A-K-K-T-P-K-K-A-K-K) in the assay. Two control immunoprecipitation reactions were performed: the $p34^{cdc2}$ peptide antigen was incubated with the anti-$p34^{cdc2}$ antiserum and was then added to samples from the 32°C culture (*A*, □); or preimmune serum (1:100) was used instead of the immune sera (*C*, □).

(Draetta and Beach, 1988), except that the salt concentration was raised to 0.4 M (from 0.25 M), which increased our recovery of mouse H1 kinase activity (Hamaguchi, J. R., and E. M. Bradbury, unpublished observations). Samples of these extracts were either resolved by SDS-PAGE and immunoblotted to determine the abundance of $p34^{cdc2}$ (Fig. 2 *A*, *left*) or subjected to immunoprecipitation to determine the H1 kinase activity of $p34^{cdc2}$ (Fig. 2 *A*, *graph*). As a probe for the mutant cdc2 gene product, we used an antiserum specific for the COOH terminus of mammalian $p34^{cdc2}$ (Lee and Nurse, 1987; Cisek and Corden, 1989; Th'ng et al., 1990).

In G1 cells arrested by isoleucine deficiency at 32°C, $p34^{cdc2}$ was present as a major band of fastest mobility (Fig. 2, *A*, lane *1*) with no detectable H1 kinase activity (Fig. 2 *A*, ○). In cells released into the cell cycle at the permissive temperature, most of the $p34^{cdc2}$ was converted to slower mobility bands in late G1 and S phase cells (Fig. 2 *A*, lanes *2-4*) when there was a gradual increase in $p34^{cdc2}$ kinase activity (Fig. 2 *A*, ○). The sharp rise in $p34^{cdc2}$ kinase activity in late S and G2 phase cells (12 and 16 h) was accompanied by a slight increase in $p34^{cdc2}$ abundance and a shift in $p34^{cdc2}$ mobility to faster migrating forms (Fig. 2 *A*, lanes *5* and *6*). The changes in $p34^{cdc2}$ mobility during the FT210 cell cycle is probably related to different $p34^{cdc2}$ phosphorylation states that regulate its H1 kinase activity. Phosphorylated forms of $p34^{cdc2}$ migrate slower in SDS-PAGE gels (Draetta and Beach, 1988; Morla et al., 1989) and partial $p34^{cdc2}$ dephosphorylation correlates with higher H1 kinase activity and entry into mitosis (Gould et al., 1989, 1991; Krek and Nigg, 1991; Norbury et al., 1991). The antiserum was specific for $p34^{cdc2}$, since the peptide antigen effectively inhibited antibody binding to the $p34^{cdc2}$ kinase from the 32°C culture (Fig. 2 *A*, □).

In FT210 cells released from the isoleucine-deficiency block into 39°C medium, $p34^{cdc2}$ was degraded in vivo and was not present in detectable quantities in G1 or early S phase cells (Fig. 2 *A*, lanes *10-12*). After 12 h at 39°C, some $p34^{cdc2}$ (Fig. 2 *A*, lane *13*) and its associated H1 kinase activity (Fig. 2 *A*, ●) was present but at levels severalfold lower than those in the control culture and after the majority of the cells had cleared G1 and most of S phase. The cells arrested in G2 phase 16-20 h after release from the isoleucine deficiency block (see Fig. 1) even though some $p34^{cdc2}$ protein was clearly present (Fig. 2 *A*, lanes *14* and *15*). The $p34^{cdc2}$ protein expressed in FT210 cells after 12 h at 39°C apparently lacked sufficient activity to promote the onset of mitosis. The resynthesis of $p34^{cdc2}$ at 39°C appears to be an adaptive response of the cells, as we have previously shown that the cdc2 mRNA levels are elevated in 39°C cultures (Th'ng et al., 1990). Though we cannot rule out the possibility that a very minor fraction of $p34^{cdc2}$ forms a G1-specific kinase at 39°C, the results show that the nonpermissive temperature causes $p34^{cdc2}$ destabilization at all phases of the cell cycle and results in a dramatic decrease in $p34^{cdc2}$ abundance. Our results indicate that $p34^{cdc2}$ is not required during G1 and S phases of the FT210 cell division cycle.

We also investigated two cyclins during the FT210 cell cy-

cle and monitored the effects of the nonpermissive temperature on their associated H1 kinase activities. The A- and B-type mitotic cyclins accumulate gradually during interphase and then abruptly degrade during mitosis. Cyclin A degradation occurs during metaphase, while the B-type cyclins accumulate until metaphase and are then degraded at the metaphase–anaphase transition (Westendorf et al., 1989; Pines and Hunter, 1989, 1990*a*, 1991*a*; Minshull et al., 1990). Antisera against human cyclin A and cyclin B1 (Pines and Hunter, 1989, 1990*a*) reacted with mouse proteins of 55 and 60 kD, respectively, which changed in abundance during the FT210 cell cycle (Fig. 2, *B* and *C*). Anti-cyclin B1 antiserum also reacted weakly with a 62-kD protein that was present throughout the cell cycle and is probably not a B-type cyclin. We found that the expression of mouse cyclin A preceded that of cyclin B1, consistent with the temporal pattern of expression of the A- and B-type cyclins in human (Pines and Hunter, 1990*a*) and frog cells (Minshull et al., 1990). Both cyclins were expressed normally at the nonpermissive temperature in FT210 cells (Figs. 2, *B* and *C*, lane *8*), since they were as abundant in G2 phase temperature-arrested cells as in the control G2-enriched culture (compare lanes *8* and *6*). Thus, the stability of cyclins A and B1 during interphase do not depend on the presence of active $p34^{cdc2}$.

At the permissive temperature, cyclin A-associated kinase activity correlated with cyclin A expression, which was detected at the first timepoint in late G1/early S phase cells and peaked in G2/M phase cells 16 h after release from G1 (Fig. 2 *B*, ○). With further increases in the number of metaphase cells (from 31% metaphase cells 16 h after release to 64% metaphase cells 20 h after release), cyclin A was partially degraded (Fig. 2 *B*, lane *7*) and its associated kinase activity declined. Like human cyclin B1 (Pines and Hunter, 1989), mouse cyclin B1 expression during S phase preceded its activation as an H1 kinase and its activity was highest in G2 and M phase cells (Fig. 2 *C*, ○ and lanes *1–7*). At the nonpermissive temperature, the cyclin A-associated kinase was active during late G1 and S phase, but the activity was lower than the control culture throughout most of the time course (Fig. 2 *B*, ●). In contrast, the nonpermissive temperature inhibited cyclin B1-associated kinase activation until late in the time course when there was a small increase in $p34^{cdc2}$ kinase activity (Fig. 2 *C*, ●). These results and those presented below indicate that temperature-sensitive $p34^{cdc2}$ is the catalytic subunit for only part of the cyclin A-associated H1 kinase, whereas $p34^{cdc2}$ is a subunit of essentially all of the cyclin B1-associated H1 kinase.

Fractionation of H1 Kinases from FT210 Cells

A two-step extraction procedure was developed to purify H1 kinase activity from FT210 cells and the parent wild-type cell line, FM3A. Whole cell homogenates prepared in the presence of high chelator concentrations and 0.2% Triton X-100 contained broken nuclei and dispersed chromatin aggregates. To obtain a fraction of nonhistone chromosomal proteins which is enriched in H1 kinase activity (Lake and Salzman, 1972; Langan, 1978; Woodforde and Pardee, 1986; Chambers and Langan, 1990), we adapted procedures from those used to purify RNA polymerase II (Jendrisak and Burgess, 1975). Chromatin was precipitated from the homogenates with PEI and the supernatant of the PEI pellet was designated F1. The F1 extract contains proteins from the cytoplasm and those weakly associated with the nucleus. A second extract, designated F2, was prepared by extracting the PEI pellet with buffer containing 0.4 M sodium chloride. H1 kinase activity eluted from the PEI pellet between 0.2 and 0.4 M sodium chloride (data not shown).

Initial attempts to fractionate H1 kinases from FT210 cells showed that the relative amount of activity recovered in the F1 and F2 extracts was dependent on the cell cycle distribution of the culture. We prepared a synchronous 32°C culture of FT210 cells arrested at the G1/S boundary with aphidicolin, then released the cells into medium supplemented with Hoechst 33342 to accumulate the cells in G2 phase at the permissive temperature. Cells were collected at 3-h intervals through 9 h, and F1 and F2 extracts were prepared. The DNA distributions were determined from flow cytometric analyses of the culture samples (data not shown). The cells had a 2C content of DNA at the beginning of the time course, then traversed S phase and accumulated with a 4C content

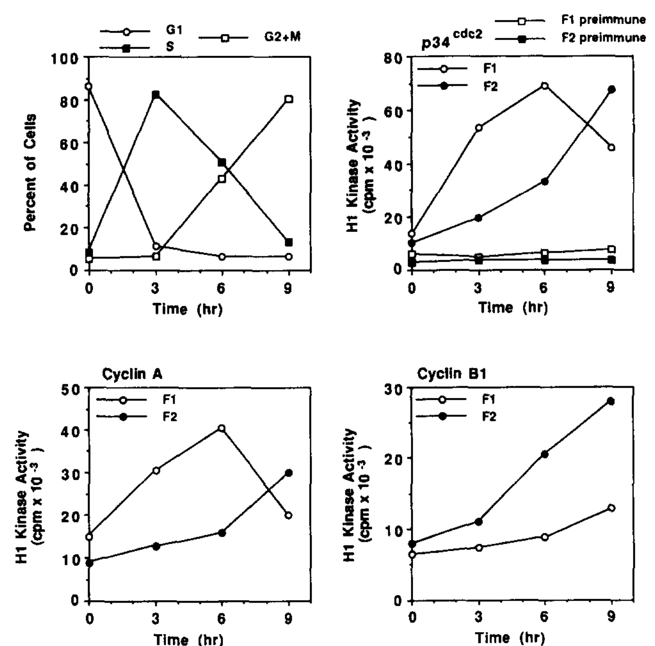

Figure 3. H1 kinase activity immunoprecipitated from crude F1 and F2 extracts. FT210 cells were synchronized with aphidicolin at the G1/S boundary at 32°C using sequential induction synchrony protocols (see Materials and Methods). The culture was released from the aphidicolin block into 32°C medium supplemented with Hoechst 33342 to obtain G2 phase cells. Culture samples were collected at 0, 3, 6, and 9 h after release from the aphidicolin arrest, then F1 and F2 extracts were prepared. Cell samples were analyzed by flow cytometry and the number of cells in G1, S and G2+M were calculated from the DNA distributions (*upper left*). Twenty μl samples of the F1 and F2 extracts (containing 1.1 and 0.8 mg/ml of total protein, respectively) were immunoprecipitated with antisera against the COOH-terminus of $p34^{cdc2}$ (*upper right*), cyclin A (*lower left*), or cyclin B1 (*lower right*) or preimmune serum as control (*upper right*, □,■). The immune complexes were adsorbed to protein A-Sepharose resin, then assayed for H1 kinase activity using the standard assay conditions. (○,□) H1 kinase activity immunoprecipitated from the F1 extract, which contains proteins solubilized at low ionic strength and 0.2% Triton X-100. (●,■) H1 kinase activity immunoprecipitated from the F2 extract which contains proteins solubilized from a polyethyleneimine pellet with buffer containing 0.4 M NaCl.

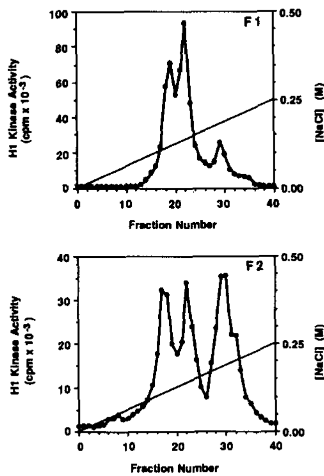

Figure 4. Mono S fractionation of FT210 cell extracts. Crude extracts from one liter of asynchronous FT210 cells (7.5 × 10^8 cells) were resolved on a one ml Mono S cation exchange column. Histone H1 kinase activity was eluted with a 40-ml gradient from 0 to 250 mM NaCl. 1-ml fractions were collected and 10 μl of each fraction were assayed for H1 kinase activity. The activity peaks recovered from the F1 (*top*) and F2 (*bottom*) extracts were designated A, B, and C, in order of their elution from the Mono S column.

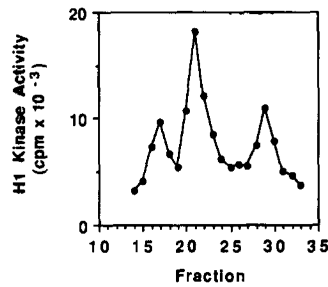

Figure 5. H1 kinase activities from chromatin. Nuclei were isolated from FT210 cells (9.0 × 10^8 cells) and incubated with micrococcal nuclease at 32°C. After the nuclei were lysed with buffers supplemented with EGTA, the soluble chromatin was recovered in the supernatant of a 10,000 *g* centrifugation. H1 kinase activity was eluted from the chromatin with the addition of 0.4 M NaCl. Chromatin was removed by the addition of polyethyleneimine followed by centrifugation at 25,000 *g*. The salt extract of the chromatin was dialyzed, then resolved by Mono S chromatography. The H1 kinase activity in the column fractions eluted between 90 and 200 mM NaCl of the gradient are shown here.

of DNA at the end of 9 h (Fig. 3). The cells with a 4C content of DNA were arrested in G2 phase (verified by visual inspection of fixed cells stained with Hoechst 33342), since Hoechst 33342 inhibits cells from entering M phase (Tobey et al., 1990). Samples of the F1 and F2 extracts were immunoprecipitated with antisera against the COOH terminus of $p34^{cdc2}$, cyclin A, cyclin B1, or preimmune serum as a control. The immunoprecipitates were then assayed for H1 kinase activity. In Fig. 3, the results show that the H1 kinase activities associated with $p34^{cdc2}$ and with cyclin A are recovered in the F1 extract through much of S phase, then accumulate in F2 extract in G2 phase cells. In contrast, the cyclin B1-associated kinase is largely confined to the F2 extract and has high H1 kinase activity in G2 phase cells. Thus, the F1 extract is enriched in S phase H1 kinases mediated by $p34^{cdc2}$ and by cyclin A, and the F2 extract contains G2 phase H1 kinases mediated by both cyclins A and B1 as well as $p34^{cdc2}$.

We fractionated the F1 and F2 extracts from asynchronous FT210 cells by Mono S cation exchange chromatography. Three peaks of H1 kinase activity were recovered from both extracts which eluted over a narrow range of the sodium chloride gradient between 90 and 200 mM and were designated A, B, and C in order of their elution from the column (Fig. 4). We did not detect significant amounts of H1 kinase activity in the flow-through fractions or in 1 M sodium chloride eluates of the Mono S column (data not shown). All of the Mono S fractionated kinases phosphorylate histone H1 as well as the S1 peptide (data not shown). Fractionation of wild-type FM3A cell extracts give similar results, but the activity peaks A and C, which we show below are two kinases containing $p34^{cdc2}$ (see Figs. 6–8), are about threefold more abundant (data not shown).

Since the PEI pellet may contain other cellular structures in addition to chromatin, we prepared a chromatin fraction to determine if all or only a subset of the H1 kinases associate with chromosomes. We incubated nuclei with micrococcal nuclease (Ausio and Van Holde, 1986; Norton et al., 1989), which solubilized ~60% of the chromatin (see Materials and Methods). Analysis of this chromatin fraction by agarose gel electrophoresis showed that the DNA was 0.4 to 20 kbp in length and was present as a ladder of 200-bp increments (data not shown). We extracted the chromatin with 0.4 M sodium chloride, resolved the extract by Mono S chromatography, and found that all three peaks of H1 kinase activity were present (Fig. 5). These results indicate that the H1 kinase activities in the F2 extract bind to chromosomes in vivo.

Subunit Composition of H1 Kinases

From the following experiments, a minimum subunit composition of the Mono S fractionated kinases was obtained for the F2 extract. The same results were obtained for the F1 extract (data not shown).

The thermal stability of the Mono S fractions was used to identify the H1 kinases from FT210 cells with the temperature-sensitive $p34^{cdc2}$ catalytic subunit. Peak activity fractions from the Mono S column were incubated at the nonpermissive temperature, then assayed for residual kinase activity. As shown in Fig. 6, the kinases in peaks A and C from FT210 cells have temperature-sensitive activities at 39°C, whereas the corresponding kinases from the wild-type FM3A cells are relatively stable. All of the H1 kinases from both wild-type and FT210 cells had stable activity when preincubated at the permissive temperature (data not shown; Th'ng et al., 1990). In addition, we found that the kinase activity of peak B from FT210 cells is stable at 39°C, which clearly indicates that the catalytic subunit of this kinase is a gene product distinct from cdc2.

We used immunoprecipitation protocols to identify the

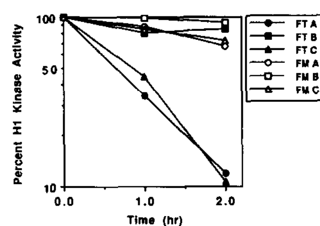

Figure 6. Temperature-sensitive H1 kinase activities from FT210 cells. Mono S column fractions *A*, *B*, and *C* from F2 extracts of FT210 cells (●, ■, ▲) or from wild-type FM3A cells (○, □, △) were preincubated at the nonpermissive temperature for 1 and 2 h and then assayed for residual H1 kinase activity. The amount of H1 kinase activity after preincubation is expressed as a percentage of the activity initially recovered from the Mono S column. The average values from two experiments are shown.

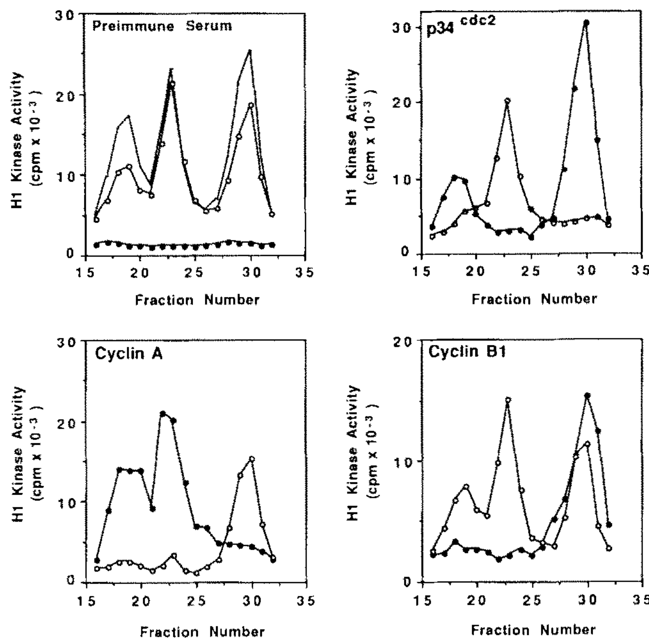

Figure 7. Immunoprecipitation of fractionated Hl kinase activities. Hl kinase activities in the F2 extract were fractionated by Mono S chromatography and the activity in those fractions collected between 90 and 200 mM NaCl of the gradient are shown. 45-μl aliquots of the column fractions were immunoprecipitated with 5 μl of diluted preimmune control serum (1:100 final dilution) or with diluted antisera against the COOH-terminal sequence of p34cdc2 (1:100 final dilution), human cyclin A (1:250 final dilution) or human cyclin Bl (1:250 final dilution). Activity recovered in the protein A-Sepharose pellets are plotted with closed symbols. 10 μl of the protein A-Sepharose supernatants were assayed and these activities are plotted with open symbols. Hl kinase activity initially recovered from the Mono S column (10-μl aliquots per assay) is shown as a plot without symbols superimposed over the preimmune control (*top left*).

subunits of the Hl kinase activities in the fractions resolved by Mono S Chromatography. Samples of the column fractions were immunoprecipitated with antiserum specific for the COOH terminus of mammalian p34cdc2, with antisera against the human cyclins A and Bl, or with preimmune serum as a control. The immune complexes were adsorbed to protein A-Sepharose and both the supernatants and the protein A-Sepharose beads were assayed for Hl kinase activity. The results in Fig. 7 show that the elution profile for each antigen superimposes a subset of the three Hl kinase activities resolved by Mono S chromatography (Fig. 7, •). We verified the presence of activities not bound by antisera in the supernatants of the immunoprecipitates (Fig. 7, ○). The Hl kinase activity in peak A is immunoprecipitated by antisera against both p34cdc2 and cyclin A, whereas the activity in peak B is immunoprecipitated only by antiserum against cyclin A. The activity recovered in peak C may contain two forms of Hl kinase, in that the activity is immunoprecipitated efficiently by antiserum against p34cdc2, but antiserum against cyclin Bl immunoprecipitates only one-half of the total activity. A twofold increase or decrease in the amount of anti-cyclin Bl antiserum immunoprecipitated a similar amount of Hl kinase activity from peak C (data not shown). The partial immunodepletion of Hl kinase activity with anti-cyclin Bl antiserum is not yet understood, but the results may be due to the coelution of an additional heteromeric kinase mediated by p34cdc2 but complexed with another B-type cyclin. In highly purified preparations of *Xenopus* MPF (Lohka et al., 1988), both cyclin Bl and B2 form complexes with p34cdc2 that copurify through several fractionation steps (Gautier et al., 1990). In addition, the anti-cyclin Bl antiserum reacts with only one of two B-type cyclins expressed in human cells (Pines and Hunter, 1991*a*; Lew et al., 1991).

Immunoblots of the Mono S fractions showed distributions of the cyclin proteins and p34cdc2 that are consistent with the results obtained by immunoprecipitation (Fig. 8). Cyclin Bl elutes with the Hl kinase activity of peak C. Cyclin A is detected across the elution profile of all three kinases, but the immunoreactive protein is most abundant in the activity peaks A and B. p34cdc2 elutes with the temperature-sensitive activities in peaks A and C. The Hl kinase activity of peak B does not, however, elute with protein bands that are detectable with the antiserum specific for the COOH terminus of p34cdc2.

The PSTAIR region of p34cdc2 is a highly conserved 16-amino acid sequence of the cdc2 gene (EGVPSTAIREISL-LKE) that is now known to occur in genes closely related in sequence to cdc2 (Lehner and O'Farrell, 1990*b*; Paris et al., 1991; Elledge et al., 1991; Tsai et al., 1991; Ninomiya-Tsuji et al., 1991; Pines, J., and T. Hunter, unpublished observations; reviewed in Pines and Hunter, 1991*b*). We used anti-PSTAIR antiserum to probe for cdc2-like proteins, and we found that a protein of 34-kD elutes from the Mono S column with the activity peak B (Fig. 8). As expected, anti-PSTAIR antiserum also reacted with 34-kD protein bands that are indistinguishable from those detected with the COOH-terminal-specific p34cdc2 antiserum in total protein extracts (Fig. 8, lanes *E*) and in the Mono S peaks A and C. We conclude that mouse cells possess at least three Hl kinases, each with a distinct subunit composition. p34cdc2 forms two complexes, one with cyclin A (peak A) and one with cyclin Bl (peak C), and these Hl kinases from FT210 cells have temperature-sensitive activities. Cyclin A also copurifies with a PSTAIR-reactive protein (peak B) and has Hl kinase activity which is stable at the nonpermissive temperature.

Finally, we assessed the in vivo stability of the Hl kinases at the Gl/S boundary separately from G2 in synchronized FT210 cells exposed to the nonpermissive temperature. In the first experiment, FT210 cells were synchronized at the Gl/S boundary with aphidicolin at either the control temperature or at 39°C (Fig. 9, *top*). In the second experiment, synchronized 32° and 39°C cultures arrested in G2 phase with Hoechst 33342 were prepared in parallel (Fig. 9, *bottom*). G2 phase FT210 cells were harvested from the 39°C culture 6 h after release from the aphidicolin block, and 9 h after release at 32°C, since the cells traversed S phase at a faster rate at 39°C. The extracts from the 39°C cultures were fractionated by Mono S chromatography and compared to the 32°C culture extracts.

We showed in Fig. 3 that Hl kinase activity first appears in the Fl extract through S phase, then accumulates in the F2 extract in G2 phase cells. In Fig. 9, the Mono S elution profiles of the Fl extracts from Gl/S phase cells (*top*) and the F2 extracts from G2 phase cells (*bottom*) are shown.

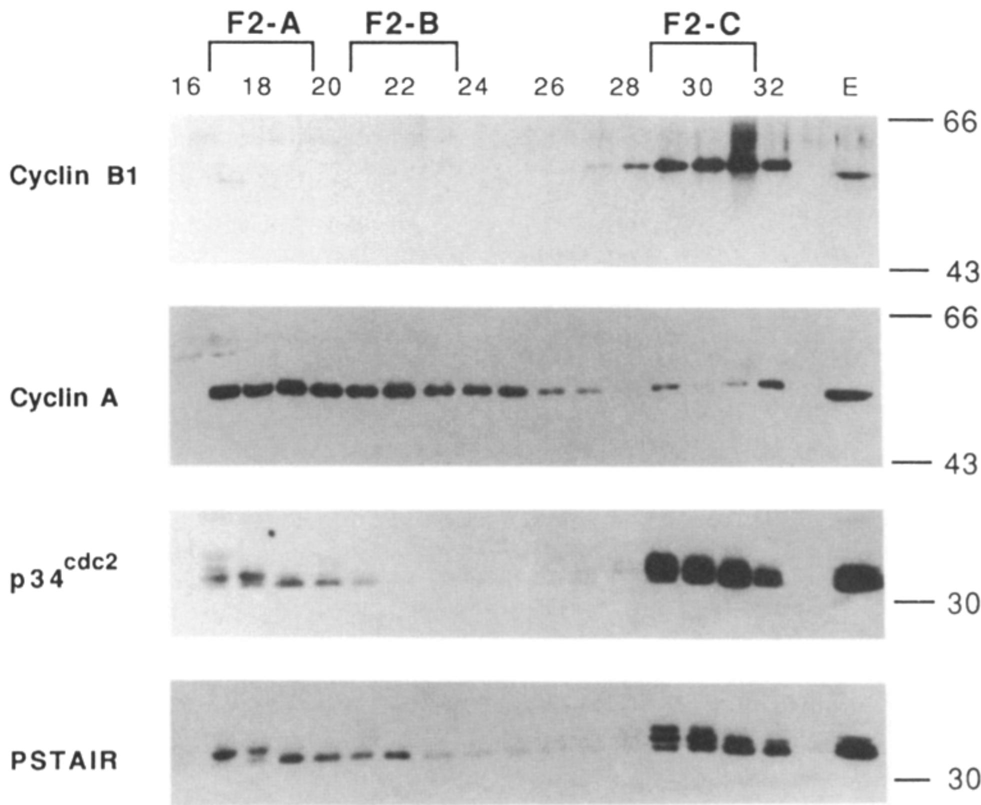

Figure 8. Immunoblot analysis of fractionated H1 kinase activities. Column fractions collected across the H1 kinase elution profile were resolved by SDS-PAGE in 8% acrylamide gels. The proteins were transferred to nitrocellulose and the membranes were cut into halves at the position where a 40-kD protein would migrate. The membrane sections were probed with antisera against human cyclin B1, human cyclin A, the COOH-terminal sequence of $p34^{cdc2}$, or the PSTAIR peptide as indicated. Total protein extracts from 10^6 FT210 cells boiled in SDS-PAGE sample buffer were resolved in the lanes marked *E*. The gel lane numbers correspond to the Mono S fraction numbers and the position of the activity peaks *A*, *B*, and *C* from the F2 extract are indicated. The Mono S activity profile for the immunoblotted fractions is shown in Fig. 4.

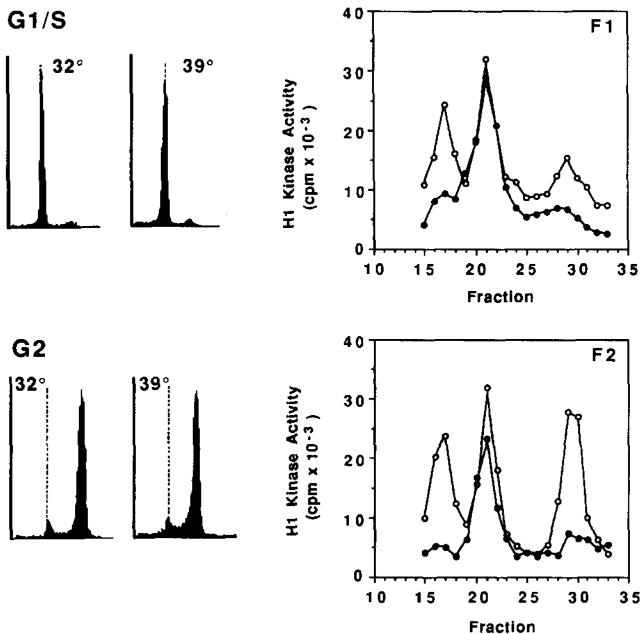

Figure 9. H1 kinase activities from permissive and nonpermissive cultures of FT210 cells. (*Top*) FT210 cells (1.5 × 10^8 cells) were synchronized in early G1 by isoleucine deprivation and then released for 11 h into either 32° or 39°C medium supplemented with aphidicolin. The flow cytometry profiles of the aphidicolin-blocked cultures are shown, in which the vertical dotted line denotes a DNA content of 2C. F1 extracts were prepared from both cultures, then resolved by Mono S chromatography. Only a portion of the elution gradient between 90 and 200 mM NaCl is shown here, in which 20 μl of each fraction were assayed for H1 kinase activity. (○) 32°C control culture. (●) 39°C culture. (*Bottom*) FT210 cells (1.5 × 10^8 cells) were synchronized at the G1/S boundary with aphidicolin at 32°C and then released into either 32° or 39°C medium supplemented with Hoechst 33342 to accumulate the cells in G2. The DNA distributions of the G2 phase cells collected 9 h after release from aphidicolin at the permissive temperature of 32°C and 6 h after release at the nonpermissive temperature of 39°C are shown. F2 extracts were prepared from both cultures, then resolved by Mono S chromatography. The portion of the elution gradient between 90 and 200 mM NaCl is shown, in which 20 μl of each fraction were assayed for H1 kinase activity. (○) 32°C control culture. (●) 39°C culture.

At the permissive temperature, it is apparent that all three H1 kinases are present in aphidicolin-blocked (*G1/S*) and in Hoechst 33342-blocked (*G2*) FT210 cells (Fig. 9, ○). FT210 and other rodent cell lines (Nishitani et al., 1991) appear to differ from HeLa cells, in which aphidicolin inhibits human cyclin A and cyclin B1 expression (Pines and Hunter, 1990*a*). In cells exposed to the nonpermissive temperature, the activities of both the $p34^{cdc2}$-cyclin A and $p34^{cdc2}$-cyclin B1 kinases are inhibited and probably do not form complexes at 39°C in vivo (Fig. 9, ●). $p34^{cdc2}$ kinase activity immunoprecipitated from the unfractionated extracts was 80–85% lower in the 39°C cultures relative to the control cultures (data not shown). In addition, we found that the activity of the temperature-stable cyclin A kinase was not affected by the nonpermissive temperature in vivo and was recovered at similar levels of activity from the 32° and 39°C cultures. These results indicate that the activation of the temperature-stable cyclin A kinase occurs independently of $p34^{cdc2}$ kinase activation during the cell cycle. It is also evi-

dent from this result and the results presented in Figs. 1 and 2 that cyclin A complexed with the PSTAIR-reactive protein cannot compensate for the loss of mitotic p34^{cdc2} kinase activity.

Discussion

Hl Kinases from FT210 Cells

We have identified and characterized three different histone Hl kinases in the mouse cdc2 mutant cell line, FT210. FT210 cells have one cyclin Bl-associated kinase and two abundant cyclin A-associated kinases. Our results indicate that cyclin Bl forms an Hl kinase only with p34^{cdc2}. This conclusion is based on the observations that the cyclin Bl-associated kinase is not activated in FT210 cells traversing Gl and S phases at the nonpermissive temperature (39°C) and when fractionated from control cultures, the activity of the p34^{cdc2}-cyclin Bl kinase is unstable at 39°C in vitro.

Our results show that cyclin A forms two separate Hl kinases in mouse cells, one containing p34^{cdc2} and the other containing a cdc2-like PSTAIR-reactive protein. Consistent with this conclusion is the observation that total cyclin A-associated kinase activity is only partially inhibited in FT210 cells incubated at the nonpermissive temperature. In fractionated extracts, we found that an Hl kinase with stable activity at the nonpermissive temperature is a complex containing cyclin A and a 34-kD PSTAIR-reactive protein. Antiserum specific for the COOH terminus of mouse and human p34^{cdc2} immunoprecipitates only the temperature-sensitive p34^{cdc2} kinases. We have also found that the temperature-stable cyclin A kinase is active during S and G2 phases and, similar to the p34^{cdc2} kinases and p33-cyclin A in human cells (Pines and Hunter, 1990*a*), it binds to immobilized p13^{suc1} (Hamaguchi, J. R., and E. M. Bradbury, unpublished observations). Thus, the temperature-stable cyclin A kinase is probably the murine homolog of human p33-cyclin A (Pines and Hunter, 1990*a*). We are presently working to identify the gene encoding the PSTAIR-reactive protein associated with cyclin A in FT210 cells, which may be the homolog of the human CDK2 gene (Elledge et al., 1991; Tsai et al., 1991; Ninomiya-Tsuji et al., 1991) or another cdc2-like gene. We have found in preliminary experiments that antiserum prepared against the human CDK2 gene product cross-reacts with the temperature-stable PSTAIR-reactive protein from FT210 cells (Hamaguchi, J. R., and E. M. Bradbury, unpublished observations).

The Hl kinases from FT210 cells were separated into two pools, a chromatin-bound fraction (F2) and an unbound fraction (Fl). Fl is probably a mixture of cytoplasmic proteins and proteins weakly associated with the nucleus. The cyclin A-associated kinases in aphidicolin blocked and S phase cells were extracted with a low ionic strength buffer containing 0.2% Triton X-100 (Fl). In G2 phase cells, the cyclin A-associated kinases and much of the p34^{cdc2}-cyclin Bl kinase were extracted with a high salt buffer from a polyethyleneimine precipitate enriched in chromatin (F2). All three of the kinases bind to chromosomes in vivo, since the kinases are recovered from chromatin solubilized by micrococcal nuclease treatment of nuclei.

Our findings are consistent in part with the subcellular localization of human cyclin A and cyclin Bl, which are found associated with the chromosomes at prophase and metaphase of mitosis, respectively (Pines and Hunter, 1991*a*). However, the cyclin A-associated kinases may be both cytoplasmic and nuclear in FT210 cells, whereas human cyclin A-associated kinase activity as well as cyclin A staining is predominantly nuclear. Additional studies will be needed to determine more precisely the subcellular localization of the mouse Hl kinases, particularly during S phase. It is also of considerable interest to fully characterize these kinases, since it is not clear at this time whether the kinases of the Fl and F2 extracts are identical, or whether they differ in the identity or posttranslational modification states of the subunits. Human cyclin A has been shown to interact with other proteins of both viral and cellular origin in addition to p34^{cdc2} and PSTAIR-reactive proteins (Harlow et al., 1986; Giordano et al., 1989, 1991; Mudryj et al., 1991; Shirodkar et al., 1992; Devoto et al., 1992).

G2 Arrest of FT210 Cells Correlates with Loss of p34^{cdc2} Kinase Activities

The mitotic functions of the cdc2 gene have been conserved in mammalian cells, as mutations in the cdc2 gene causes cell cycle arrest in G2 and inhibits the entry of FT210 cells into mitosis (Th'ng et al., 1990, Yasuda et al., 1991; Fig. 1). This conclusion is supported by the observation that p34^{cdc2} is 80-85% less active in FT210 cells temperature arrested in G2 phase relative to control cultures, and that the activities of the p34^{cdc2}-cyclin A and p34^{cdc2}-cyclin Bl kinases are temperature sensitive and do not form complexes in FT210 cells cultured at 39°C. Both p34^{cdc2} kinases have high Hl kinase activity in G2 phase cells at the permissive temperature, and one or both of the kinases may be required for entry into mitosis. Perhaps the p34^{cdc2}-cyclin A and p34^{cdc2}-cyclin Bl kinases bind to chromosomes in G2 phase in preparation for histone Hl hyperphosphorylation and chromosome condensation during mitosis. Neither activity is apparently required during late Gl or S phase. The fact that the temperature-stable cyclin A kinase is fully activated in FT210 cells temperature-arrested in G2 phase indicates that it cannot complement the loss of p34^{cdc2} kinase function at mitosis.

cdc2 Control of the FT210 Cell Cycle Is Restricted to the G2-to-M Transition

Our results show that the temperature-sensitive cdc2 gene of FT210 cells causes cell cycle arrest only in G2 phase. Synchronized FT210 cells released from a mitotic block (data not shown) or from an isoleucine deficiency block in early Gl are able to progress through S phase and arrest only in G2 phase at the nonpermissive temperature. We also found no evidence for a transient Gl arrest of FT210 cells, as cells proceed through Gl and S phases at comparable or slightly faster rates at the nonpermissive temperature relative to control cultures.

In the *cdc28-1N* mutant, the Gl functions of the gene are not affected by the mutation and the cells arrest only in G2 phase (Piggott et al., 1982; Surana et al., 1991). We investigated the possibility that the point mutations in the FT210 cdc2 gene (Th'ng et al., 1990; Yasuda et al., 1991) affect p34^{cdc2} kinase activity only during the G2-to-M transition. We have shown here that p34^{cdc2} kinase activity is detected in FT210 cells at the permissive temperature in late Gl and

gradually rises through S phase. At the nonpermissive temperature $p34^{cdc2}$ is inactive as an H1 kinase in G1 and S phase cells. In vivo, $p34^{cdc2}$ is unstable and is degraded at the nonpermissive temperature (Th'ng et al., 1990; Fig. 2). Thus, although we cannot rule out the possibility that a minor fraction of $p34^{cdc2}$ kinase activity is present at the nonpermissive temperature, it is unlikely that there is adequate $p34^{cdc2}$ kinase activity to allow FT210 cells to efficiently pass through the G1/S boundary. From these results, we suggest that the functions of the $p34^{cdc2}$ kinases are restricted to mitosis in the FT210 cell cycle.

We have estimated the detection limit of the anti-$p34^{cdc2}$ antiserum used in these studies by comparing the amount of $p34^{cdc2}$ needed to detect by Western Blotting to that amount needed to silver stain a band of $p34^{cdc2}$ in SDS-PAGE gels. We purified the $p34^{cdc2}$-cyclin A kinase (from the Mono S peak A) through a Mono Q column, bound this material to $p13^{suc1}$-Sepharose, eluted $p34^{cdc2}$ and cyclin A with Laemmli gel sample buffer, resolved the proteins by SDS-PAGE and then silver stained the gel (Hamaguchi, J. R., and E. M. Bradbury, unpublished results). Assuming that a silver-stained band of $p34^{cdc2}$ corresponds to 1–10 ng of protein, and the finding that 1/50th of this amount is detected by our immunoblot protocols, we estimate the detection limit of our immunoblot protocols to be $\sim$3.5–35 $\times$ 10^8 molecules of $p34^{cdc2}$. This corresponds to 3,500–35,000 molecules of $p34^{cdc2}$ per cell, since FT210 cell lysates containing 10^5 cell equivalents is needed to detect an immunoreactive band of $p34^{cdc2}$. The lysates analyzed by Western Blot shown in Fig. 2 contained the cell equivalent of approximately 2 $\times$ 10^6 FT210 cells per gel lane. Thus, FT210 cells incubated at the nonpermissive temperature may contain 175–1,750 molecules of $p34^{cdc2}$ per cell which would not be detected by immunoblot analysis. Furthermore, a large proportion of this small number of $p34^{cdc2}$ molecules per cell at 39°C would likely have reduced activity in vivo at the nonpermissive temperature. Thus, within the detection limits of our immunoblotting and activity assays, we feel it is reasonable to conclude that the G1-to-S transition in FT210 cells is mediated by genes distinct from cdc2.

There is now evidence indicating that higher eukaryotic cells have evolved cell cycle controls that are more complex than those of yeasts. FT210 cells possess a PSTAIR-reactive protein complexed with cyclin A that is clearly independent of $p34^{cdc2}$. There is a strong probability that cdc-2-like proteins identified with anti-PSTAIR antisera in human (Pines and Hunter, 1990*a*; Matsushime et al., 1991) and mouse cells (Fig. 8) and those encoded by cdc2-like genes (Lehner and O'Farrell, 1990*b*; Paris et al., 1991; Elledge et al., 1991, Tsai et al., 1991; Ninomiya-Tsuji et al., 1991) mediate essential functions during the G1 and S phases of the cell cycle (reviewed in Pines and Hunter, 1991*b*). Elledge et al. (1991) isolated the human CDK2 gene by its ability to weakly suppress the phenotype of a budding yeast *cdc28* conditional mutant. Using a similar approach, Ninomiya-Tsuji et al. (1991) also isolated the human CDK2 gene and further suggested that CDK2 functions more efficiently in G1 than in G2 when expressed in a null *cdc28* mutant of *S. cerevisiae*. Finally, Fang and Newport (1991) have reported that antiserum made against sequences unique to the *Xenopus* CDK2 gene (formerly designated Eg1; Paris et al., 1991) immunodepleted *Xenopus* cycling cell-free extracts of an activity required for DNA synthesis, but had no effect on the onset of mitosis. Together, these findings indicate that metazoan cells require at least two cdc2-related proteins to complete the cell cycle.

DNA replication in mammalian cells is also dependent upon cyclin A. Girard et al. (1991) has shown that depletion of cyclin A by expression of the antisense cyclin A mRNA, and by microinjection of anti-cyclin A antibodies, inhibits DNA replication in rat fibroblasts. One possible form of cyclin A that may regulate DNA synthesis is the p33-cyclin A kinase. During S phase, the p33-cyclin A kinase is in a tetrameric complex with the E2F transcription factor and p107, a protein of similar structure and protein-binding properties to the retinoblastoma gene product (Mudryj et al., 1991; Devoto et al., 1992). Earlier observations that human cyclin A–associated kinase activity copurifies with a replication factor that stimulates DNA synthesis in cell-free extracts supports this proposal (D'Urso et al., 1990). If indeed the cyclin A–associated kinase is required for DNA replication, the results presented here show that $p34^{cdc2}$ is not one of the essential components.

It should be noted that the potent protein kinase inhibitor, staurosporine, arrests normal mammalian cells in G1 phase, whereas it has no effect on the progression of transformed cells through G1 (Crissman et al., 1991). Control of G1 phase progression by staurosporine-sensitive kinases is apparently lost during transformation, and the kinases involved may include the cell cycle control kinases.

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