Requirement for p34cdc2 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 p34cdc2 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 p34cdc2 and the human cyclins A and B1. The results show that p34cdc2 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 p34cdc2. The cyclin A-associated kinases are active during S and G2 phases and earlier in the division cycle than the p34cdc2-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 p34cdc2cdc2 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, p34cdc2 (reviewed in Nurse, 1985). Genetic analyses of cdc2 and cdc28 mutants have clearly established two control points for p34cdc2 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 cdcl3+ as a G2-specific suppressor of a cdc2 mutant demonstrated that the mitotic functions of p34cdc2 are distinct from those acting in G1 (Booher and Beach, 1987).

The p34cdc2 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., 1974a,b). p34cdc2 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, 1990a; Minshull et al., 1989, 1990; reviewed in Hunt, 1989). The activity of p34cdc2 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, 1990a; Minshull et al., 1989, 1990; reviewed in Hunt, 1989). Three mitotic cyclins, A, B1, and B2, associate with p34cdc2 during the cell cycle. B-type cyclins form a complex with p34cdc2 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-
tion of p34cdc and B-type cyclins (Gautier et al., 1988, 1990; Labbé et al., 1989) as components of highly purified M-phase promoting factor (MPF) (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 p34cdc also associates with a 60-kD protein (p60; Harlow et al., 1986) during S phase (Giordano et al., 1989, 1990b; Wittenberg and Reed, 1991). In subsequent work, Pines and Hunter (1990a) identified p60 as cyclin A and found that cyclin A is present in a more abundant complex with a 33-kD cdcl2-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 cdcl3 (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, 1990a). More recent evidence indicates that cyclin A is essential for DNA synthesis in mammalian cells (Girard et al., 1991).

In S. cerevisiae, p34cdc is thought to regulate the GI-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, 1990b; Reed, 1991). GI-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 p34cdc, and this complex exhibits weak histone H1 kinase activity in vitro and is maximally active during GI phase of the budding yeast cell cycle (Wittenberg et al., 1990).

Cell cycle control by the p34cdc kinases at two distinct phases of the division cycle is thought to be conserved in higher eukaryotes. The human cdcl homolog rescues both temperature-sensitive cdcl28 and cdcl2 mutants (Lee and Nurse, 1987; Wittenberg and Reed, 1989). Human p34cdc copurifies with a factor that stimulates DNA replication (D'Urso et al., 1990) and disruption of p34cdc expression with antisense oligonucleotides delays the onset of S phase following activation of human lymphocytes from G0 (Furu-kawa et al., 1990). In cell-free cycling Xenopus extracts, p34cdc depletion with antisemur or with immobilized p3cdc1 inhibits DNA synthesis (Blow and Nurse, 1990). These findings lend support to the notion that p34cdc, or a closely related gene, regulates the onset of DNA replication in metazoan cells.

cdcl2-like genes are expressed in higher eukaryotic cells (Lehner and O'Farrell, 1990b; Paris et al., 1991; Elledge et al., 1991; Tsai et al., 1991; Ninomiya-Tsuji et al., 1991; reviewed in Pines and Hunter, 1991b). cdcl2-like gene products have also been found by immunochemochemical means coprecipitated with antisera against human cyclin A (Pines and Hunter, 1990a) and p36cdc, a murine cyclin protein (Matsushima et al., 1991) that is also expressed in human cells (PRAD1/cyclin DI) (Motokura et al., 1991; Xiong et al., 1991). Significantly, Fang and Newport (1991) have recently shown that only one of two cdcl2-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 cdcl2 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 cdcl2 gene on the mammalian cell division cycle. While the mitotic functions of p34cdcls have been conserved in higher eukaryotes through evolution, we find that p34cdclc kinase activity is apparently not required during the GI and S phases of the FT210 cell cycle. Using standard fractionation procedures and a synthetic peptide substrate containing a consensus p34cdclc kinase phosphorylation site (Langan, 1978; reviewed in Pines and Hunter, 1990b), we also find that FT210 cells possess three H1 kinase activities, two of which are temperature-sensitive p34cdclc 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 cdcl2-like protein as subunits. Though it is not known how the permanent proliferative state of these cells will affect cell cycle control during GI, our results indicate that p34cdlc kinases functions are restricted to mitosis in mouse FT210 cells.

**Materials and Methods**

**Immunological Reagents**

Antisera used to investigate the subunit composition of the HI kinases were generated in rabbits against bacterially expressed human cyclin A (Pines and Hunter, 1990a), human cyclin BI (Pines and Hunter, 1989), and a PSTAIR-peptide antigen (EGVPSTAIREISLKE; kindly provided by P. Russell, Research Institute of Scripps Clinic, La Jolla, CA). Antiserum against p34cdclc was prepared as described previously (Dracotta and Beach, 1988) against a synthetic peptide antigen (C-DNQIKKM) containing the COOH-terminal seven amino acid sequence of human and mouse p34cdcl (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. 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 × 10⁶ 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 × 10⁶ cells/ml and at 0.3 × 10⁶ cells/ml from synchronized FT210 cultures. Asynchronous 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 GI. The cells were then resuspended in 32° or 39°C complete medium supplemented with 5 μg/ml aphidicolin for 11 h to accumulate

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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
pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM DTT, 0.1 mM
pelleted by centrifugation. The packed cell volume (PCV) was ~1 ml per
Hoechst 33342 and incubated for 9 h at 32 °C or for 6 h at 39 °C to accumu-
late cells at G2 (see Figs. 3 and 9). Comparative analyses of the historic
HI.'9°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, G2 + M or S were calculated from DNA distributions with the aid of
the Multicycle Computer Program (Phoenix Flow Systems, San
Diego, CA) developed by Dr. P. S. Rabinowitz (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 chromo-
somes 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 x 10^6 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 pestle and the extract was supplemented with one PCV of 5% extraction buffer (final concentration 50 mM Tris-HCl, pH 7.4, 0.1 mM NaF, 5 mM EGTA, 10 mM NaF, 0.4 M. Chromatin precipitated upon the addition of NaCl and was removed from the nuclei essentially as described (Norton et al., 1989), with minor
change to lysing nuclei suspension was centrifuged at 1 OD00 g for 10 rain. To calculate
the yield of soluble chromatin in the supernatant, the absorbance at 260 nm
were eluted with a 40 ml linear gradient with column buffer containing 0
with an additional 15 ml of column buffer and proteins bound to the column
pellets were assayed using 100 pg/ml of the $1 peptide (see Fig. 2). 20-/~l
samples of the crude F1 and F2 extracts (1.1 and 0.8 mg/ml of protein,
with these samples as described above, except the protein A-Sepharose
tubes were centrifuged at 25,000 g for 30 min at 4°C and then added to the samples.

Immunoprecipitation of HI Kinase Activity

Preimmune serum or anti-COOH-terminal p34^cdx antisemur was used at a final dilution of 1:100. Anti-cyclin A and anti-cyclin B antisera (Pines and Hunter, 1989, 1990a) 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-ml 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 HI kinase activity by the standard assay procedure outlined above. Samples were spun in a microcen-
trifuge 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 by using 100 µg/ml of the SI 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 as-
sayed for HI kinase activity using the standard assay conditions. In reactions
with competing peptide antigen, antisemur specific for the p34^cdx 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 HI 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 microcen-
trifuge, 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 (Laemmlli, 1970) and transferred
to nitrocellulose membranes in a semidy 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 incu-
bated for 2 h with goat anti-rabbit secondary antibody conjugated to HRP
(Calbiochem) diluted 1:7500 in blocking buffer. Membranes were washed four times in blocking buffer and once in PBS. The immunoactive 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

HI Kinase Activity Assays
A synthetic peptide substrate was constructed to identify H1 kinases with specificity for the lysine-rich NH2- and COOH-terminal tails of histone H1. The sequence of the SI peptide, A-A-K-A-K-K-T-P-K-K-A-K-K, contains the consensus phosphorylation site for the p34\textsuperscript{cdc2} kinases (K/R-S/T-P-[X]-K) (reviewed in Pines and Hunter, 1990b) and is similar to the sites phosphorylated by growth-associated H1 kinase in the NH2- and COOH-terminal domains of H1 (Langan, 1978).

p34\textsuperscript{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 G1-to-S transition, a strategy which proved useful for clearly elucidating the G1 arrest properties of cdc2 S. pombe mutants (Nurse and Bissett, 1981). FT210 cells were presynchronized in G1 in isoleucine-deficient medium and then resuspended in either 32°C (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 G1 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 G1 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\textsuperscript{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\textsuperscript{cdc2} throughout the FT210 cell cycle, since a transient stabilization of p34\textsuperscript{cdc2} during the G1-to-S transition could explain why FT210 cells do not arrest in G1. Whole cell extracts were prepared from both the 32°C 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\textsuperscript{cdc2} kinases in human cells.

![Figure 1. Cell cycle progression of FT210 cells. FT210 cells were synchronized in G1 in isoleucine-deficient medium at 32°C as described in the Materials and Methods. The G1 culture (0 h) was divided and the cells were resuspended in complete media at 32°C 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.](https://jcb.rupress.org/content/117/4/1044)
Figure 2. Cell cycle analysis of p34<sup>cdc2</sup>, cyclin A, and cyclin B1. GI 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 HI kinase activity immunoprecipitated from the extracts (right) are shown, using antisera specific for the COOH-terminal sequence of p34<sup>cdc2</sup> (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<sup>cdc2</sup> (A), human cyclin A (B) or human cyclin B1 (C) as indicated. Lanes I and 9, GI 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 9-16 are from separate gels. (Right) Samples of the extracts from the 32°C control culture (O) or the 39°C culture (●) containing 200 μg of total protein were immunoprecipitated with immune sera against the COOH-terminus of p34<sup>cdc2</sup> (I:100 dilution) or the human cyclins A and B1 (I:250 dilution) and then bound to protein-A-Sepharose beads. HI kinase activity recovered in the protein-A-Sepharose pellets was determined using 100 μg/ml of the SI 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<sup>cdc2</sup> peptide antigen was incubated with the anti-p34<sup>cdc2</sup> antiserum and was then added to samples from the 32°C culture (A, □); or preimmune serum (I:100) was used instead of the immune sera (C, □).
icle 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, 1990a, 1991a; Minshull et al., 1990). Antisera against human cyclin A and cyclin B1 (Pines and Hunter, 1989, 1990a) 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, 1990a) 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\textsuperscript{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, o). 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, o and lanes 4–7). 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, e). 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\textsuperscript{cdc2} kinase activity (Fig. 2 C, e). These results and those presented below indicate that temperature-sensitive p34\textsuperscript{cdc2} is the catalytic subunit for only part of the cyclin A-associated H1 kinase, whereas p34\textsuperscript{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\textsuperscript{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.
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 p34cdc2, 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 p34cdc2 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 both cyclins A and B1 as well as p34cdc2. 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 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.

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). 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.

Figure 4. Mono S fractionation of FT210 cell extracts. Crude extracts from one liter of asynchronous FT210 cells (7.5 × 10⁶ 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 × 10⁶ 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.

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.
subunits of the H1 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 p34\textsuperscript{cdk2}, with antiseria against the human cyclins A and B1, 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 pellets were assayed with open symbols. H1 kinase activity initially recovered from the Mono S column (10-\mu l aliquots per assay) is shown as a plot without symbols superimposed over the preimmune control (top left).

Figure 7. Immunoprecipitation of fractionated H1 kinase activities. H1 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-\mu l aliquots of the column fractions were immunoprecipitated with 5 \mu l of diluted preimmune control serum (1:100 final dilution) or with diluted antisera against the COOH-terminal sequence of p34\textsuperscript{cdk2} (1:100 final dilution), human cyclin A (1:250 final dilution) or human cyclin B1 (1:250 final dilution). Activity recovered in the protein A-Sepharose pellets are plotted with closed symbols. 10 \mu l of the protein A-Sepharose supernatants were assayed and these activities are plotted with open symbols. H1 kinase activity initially recovered from the Mono S column (10-\mu l aliquots per assay) is shown as a plot without symbols superimposed over the preimmune control (top left).

The PSTAIR region of p34\textsuperscript{cdk2} is a highly conserved 16-amino acid sequence of the cdc2 gene (EGVPSTAIRESLKE) that is now known to occur in genes closely related in sequence to cdc2 (Lehner and O’Farrell, 1990b; 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, 1991b). 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 p34\textsuperscript{cdk2} antisem 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 H1 kinases, each with a distinct subunit composition. p34\textsuperscript{cdk2} forms two complexes, one with cyclin A (peak A) and one with cyclin B1 (peak C), and these H1 kinases from FT210 cells have temperature-sensitive activities. Cyclin A also copurifies with a PSTAIR-reactive protein (peak B) and has H1 kinase activity which is stable at the nonpermissive temperature.

Finally, we assessed the in vivo stability of the H1 kinases at the G1/S boundary separately from G2 in synchronized FT210 cells exposed to the nonpermissive temperature. In the first experiment, FT210 cells were synchronized at the G1/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 H1 kinase activity first appears in the F1 extract through S phase, then accumulates in the F2 extract in G2 phase cells. In Fig. 9, the Mono S elution profiles of the F1 extracts from G1/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 p34cdc2, 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 x 10^8 cells) were synchronized in early G1 by isoleucine deprivation and then released for 11 h into either 32°C 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 x 10^8 cells) were synchronized at the G1/S boundary with aphidicolin at 32°C and then released into either 32°C 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.
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^cd2 kinase activity.

**Discussion**

**HI Kinases from FT210 Cells**

We have identified and characterized three different histone HI kinases in the mouse cdc2 mutant cell line, FT210. FT210 cells have one cyclin Bi-associated kinase and two abundant cyclin A-associated kinases. Our results indicate that cyclin Bi forms an HI kinase only with p34^cd2. This conclusion is based on the observations that the cyclin Bi-associated kinase is not activated in FT210 cells traversing G1 and S phases at the nonpermissive temperature (39°C) and when fractionated from control cultures, the activity of the p34^cd2-cyclin Bi kinase is unstable at 39°C in vitro.

Our results show that cyclin A forms two separate HI kinases in mouse cells, one containing p34^cd2 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 HI 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^cd2 immunoprecipitates only the temperature-sensitive p34^cd2 kinases. We have also found that the temperature-stable cyclin A kinase is active during S and G2 phases and, similar to the p34^cd2 kinases and p33-cyclin A in human cells (Pines and Hunter, 1990a), it binds to immobilized pl^cd3 (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, 1990a). 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; Tasi et al., 1991; Ninomiya-Tsuji et al., 1991) or another cdc2-like gene. We have found in preliminary experiments that antisera 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 HI kinases from FT210 cells were separated into two pools, a chromatin-bound fraction (F2) and an unbound fraction (F1). F1 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 (FI). In G2 phase cells, the cyclin A-associated kinases and much of the p34^cd2-cyclin B1 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 Bi, which are found associated with the chromosomes at prophase and metaphase of mitosis, respectively (Pines and Hunter, 1991a). 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 HI 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 F1 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^cd2 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^cd2 Kinase Activities**

The mitotic functions of the cdc2 gene have been conserved in mammalian cells, as mutations in the cdc2 gene cause cell cycle arrest in G2 and inhibits the entry of FT210 cells into mitosis (T'ng et al., 1990, Yasuda et al., 1991; Fig. 1). This conclusion is supported by the observation that p34^cd2 is 80–85% less active in FT210 cells temperature arrested in G2 phase relative to control cultures, and that the activities of the p34^cd2-cyclin A and p34^cd2-cyclin B1 kinases are temperature sensitive and do not form complexes in FT210 cells cultured at 39°C. Both p34^cd2 kinases have high HI 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^cd2-cyclin A and p34^cd2-cyclin B1 kinases bind to chromosomes in G2 phase in preparation for histone HI hyperphosphorylation and chromosome condensation during mitosis. Neither activity is apparently required during late G1 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^cd2 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 G1 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 G1 arrest of FT210 cells, as cells proceed through G1 and S phases at comparable or slightly faster rates at the nonpermissive temperature relative to controls. In the cdc28-IN mutant, the GI 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 (T'ng et al., 1990; Yasuda et al., 1991) affect p34^cd2 kinase activity only during the G2-to-M transition. We have shown here that p34^cd2 kinase activity is detected in FT210 cells at the permissive temperature in late G1 and...
gradually rises through S phase. At the nonpermissive temperature, p34^{cd2} is inactive as an H1 kinase in G1 and S phase cells. In vivo, p34^{cd2} 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^{cd2} kinase activity is present at the nonpermissive temperature, it is unlikely that there is adequate p34^{cd2} 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^{cd2} kinases are restricted to mitosis in the FT210 cell cycle.

We have estimated the detection limit of the anti-p34^{cd2} antiserum used in these studies by comparing the amount of p34^{cd2} needed to detect by Western Blotting to that amount needed to silver stain a band of p34^{cd2} in SDS-PAGE gels. We purified the p34^{cd2}-cyclin A kinase (from the Mono S peak A) through a Mono Q column, bound this material to p1\textsuperscript{3His}-Sepharose, eluted p34^{cd2} 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^{cd2} 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 \( \sim 3.5-35 \times 10^3 \) molecules of p34^{cd2}. This corresponds to 3,500-35,000 molecules of p34^{cd2} per cell, since FT210 cell lysates containing 10\(^5\) cell equivalents is needed to detect an immunoreactive band of p34^{cd2}. 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^{cd2} per cell which would not be detected by immunoblot analysis. Furthermore, a large proportion of this small number of p34^{cd2} 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^{cd2}. There is a strong probability that cdc2-like proteins identified with anti-PSTAIR antisera in human (Pines and Hunter, 1990a; Matsushima et al., 1991) and mouse cells (Fig. 8) and those encoded by cdc2-1ike genes (Lehner and O'Farrell, 1990b; 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, 1991b). 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 antisemur made against sequences unique to the Xenopus CDK2 gene (formerly designated Egl; 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 EZF 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^{cd2} 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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