Human Cyclins A and B1 Are Differentially Located in the Cell and Undergo Cell Cycle–Dependent Nuclear Transport

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Abstract. We have used immunofluorescence staining to study the subcellular distribution of cyclin A and B1 during the somatic cell cycle. In both primary human fibroblasts and in epithelial tumor cells, we find that cyclin A is predominantly nuclear from S phase onwards. Cyclin A may associate with condensing chromosomes in prophase, but is not associated with condensed chromosomes in metaphase. By contrast, cyclin B1 accumulates in the cytoplasm of interphase cells and only enters the nucleus at the beginning of mitosis, before nuclear lamina breakdown. In mitotic cells, cyclin B1 associates with condensed chromosomes in prophase and metaphase, and with the mitotic apparatus. Cyclin A is degraded during metaphase and cyclin B1 is precipitously destroyed at the metaphase→anaphase transition. Cell fractionation and immunoprecipitation studies showed that both cyclin A and cyclin B1 are associated with PSTAIRE-containing proteins. The nuclear, but not the cytoplasmic form, of cyclin A is associated with a 33-kD PSTAIRE-containing protein. Cyclin B1 is associated with $p34^{cdc2}$ in the cytoplasm. Thus we propose that the different localization of cyclin A and cyclin B1 in the cell cycle could be the means by which the two types of mitotic cyclin confer substrate specificity upon their associated PSTAIRE–containing protein kinase subunit.

CYCLINS are proteins which have been implicated in the control of mitosis in all eukaryotes. They were first identified in rapidly cleaving embryonic cells, and were distinguished by their steady accumulation in interphase, followed by specific and rapid proteolysis at mitosis (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987; Westendorf et al., 1989; for review see Hunt, 1989; Pines, 1991). Mitotic cyclin cDNAs have been cloned and sequenced from a variety of organisms, and on the basis of sequence homologies in the most conserved central 200 amino acids (the cyclin box) have been subdivided into two classes, A type and B type. Synthetic cyclin A or B mRNAs will cause meiosis when microinjected into *Xenopus* oocytes (Swenson et al., 1986; Pines and Hunt, 1987; Westendorf et al., 1989), and periodic cyclin B synthesis and destruction is necessary and sufficient to cause a *Xenopus* egg cell-free system to oscillate between S and M phases (Minshull et al., 1989; Murray and Kirschner, 1989).

A second class of cyclins have been identified in budding yeast. These are the G1 cyclins which associate with $p34^{CDC28}$ (the functional homologue of $p34^{cdc2}$ in fission yeast) and are required at START in G1 phase for the cell to enter S phase (Hadwiger et al., 1989; Richardson et al., 1989; Wittenberg et al., 1990). G1 cyclins are ~25% homologous in the cyclin box to the mitotic cyclins and have been identified in budding and fission yeast (Hadwiger et al., 1989; Forsburg and Nurse, 1991), and candidate cDNAs have been isolated from mammalian cells (Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991; Lew et al., 1991). *Saccharomyces cerevisiae* has recently been shown to have 4 mitotic cyclins (Ghiara et al., 1991; Surana et al., 1991), thereby demonstrating that G1 cyclins are a separate family from mitotic cyclins.

Mitotic cyclins are believed to act through association with the highly conserved protein-serine/threonine kinase $p34^{cdc2}$, the product of the cell division cycle gene *cdc2* in the fission yeast *Schizosaccharomyces pombe* (reviewed in Norbury and Nurse, 1989; Nurse, 1990; Pines and Hunter, 1990*b*). There is strong genetic and biochemical evidence for this association for the B-type cyclins. On the basis of sequence comparison the product of the essential *Schizosaccharomyces pombe* $cdc13^{+}$ gene was identified as a B-type cyclin (Booher and Beach, 1988; Goebel and Byers, 1988; Hagan et al., 1988; Solomon et al., 1988). In *S. pombe* a *cdc13* allele suppresses a cold-sensitive *cdc2* mutant in its G2 to M function (Booher and Beach, 1987), and conversely overexpression of $p34^{cdc2}$ suppresses a temperature-sensitive *cdc13* mutant (Booher and Beach, 1987; Booher and Beach, 1988; Hagan et al., 1988). There is biochemical evidence that $p34^{cdc2}$ and $p63^{cdc13}$ can be co-immunoprecipitated (Booher et al., 1989; Moreno et al., 1989), and B-type cyclins can also be co-immunoprecipitated with $p34^{cdc2}$ homologues from clams, *Xenopus*, and human cells (Draetta et al., 1989; Pines and Hunter, 1989; Gautier et al., 1990). Evidence for the physiological significance of the association between cyclin B and $p34^{cdc2}$ comes from the identification

of both these proteins as components of purified M phase promoting factor (MPF)[1], and of the cell cycle–dependent histone H1 kinase (Draetta et al., 1989; Labbé et al., 1989; Meijer et al., 1989; Chambers and Langan, 1990: Gautier et al., 1990). In *Xenopus* two types of cyclin B have been cloned (Minshull et al., 1990), designated cyclins B1 and B2. By sequence homology, the human cyclin B clone we isolated is a cyclin B1, and a human cyclin B2 has since been cloned by S. Reed and colleagues (S. Reed, personal communication).

We have recently shown that human cyclin A associates with $p34^{cdc2}$ and with a related protein of ~33 kD that we have termed p33 (Pines and Hunter, 1990a). p33 is related in structure to $p34^{cdc2}$; it contains the PSTAIRE epitope found in the cdc2 family of protein kinases, and *N*-chlorosuccinimide treatment of p33 generates the same size fragments as $p34^{cdc2}$. These data suggest that p33 is likely to be a protein kinase. Both A- and B-type cyclin immunocomplexes display in vitro protein kinase activity and have very similar in vitro substrate specificities (Minshull et al., 1990). Cyclin A levels rise and fall in advance of cyclin B; cyclin A levels increase throughout S and G2 phases and decline as cells begin metaphase, whereas cyclin B accumulates in G2 phase and persists to mid-mitosis (Minshull et al., 1990; Pines and Hunter, 1990*a*). The associated in vitro protein kinase activities of both cyclin A and cyclin B peak during late G2/M phase, which might suggest that they serve similar, if not interchangeable functions. Some support for this idea is the observation that either cyclin A or cyclin B alone can cause *Xenopus* oocytes to mature and can elicit repeated rounds of mitosis in a *Xenopus* cell-free system. However, there is genetic evidence from *Drosophila* that cyclin A and B cannot substitute for each other in the early embryonic cell cycle (Lehner and O'Farrell, 1990). As yet no A-type cyclin homologs have been identified in yeast to confirm this observation.

It seemed possible that A- and B-type cyclins might perform different, essential roles by localizing their respective associated protein kinases to different parts of the cell. Therefore we undertook a study by immmunofluorescence staining of the intracellular distribution of human cyclins A and B1 throughout the cell cycle. We show here that in human cells cyclin B1 is predominantly cytoplasmic until just before mitosis, whereas cyclin A accumulates in the nucleus. In metaphase, cyclin B1 binds to the spindle and to condensed chromosomes.

Materials and Methods

Cell Culture and Synchronization

HeLa S3 TK^- cells were cultured at 37°C on plates in DME supplemented with 10% calf serum (complete medium). Human foreskin fibroblasts (gift of Dr. R. Allen, Salk Institute) were cultured at 37°C, 7.5% CO_2 in DME/F12 medium.

Cells were synchronized at the G1/S boundary by sequential thymidine (Sigma Chemical Co., St. Louis, MO) and aphidicolin (Sigma Chemical Co.) treatment according to Heintz et al. (1983), and as previously described (Pines and Hunter, 1989). Cells were synchronized in G2 phase by the addition of 0.15 μg/ml Hoechst 33342 dye (Calbiochem-Behring Corp., La Jolla, CA) according to Tobey et al. (1990), or in pseudo-metaphase by the addition of 0.4 μg/ml nocodazole (Sigma Chemical Co.), for 12 h after release from a thymidine block.

Antibodies

Rabbit polyclonal antibodies specific for cyclin A (Pines and Hunter, 1990*a*) or for cyclin B1 (Pines and Hunter, 1989) were raised in rabbits against the entire cyclin A or B1 protein expressed as nonfusion proteins in *Escherichia coli*, and characterized as previously described. These antisera were shown not to cross react on immunoblots or in immunoprecipitations. Both antisera recognize single proteins on an immunoblot of a whole-cell lysate. Other antibodies are documented in the immunofluorescence section.

Cell Fractionation, Immunoprecipitation, and Immunoblotting

Two ~80% confluent 10-cm dishes of HeLa cells were arrested in G2 phase with 0.15 μg/ml Hoechst 33342 dye (Tobey et al., 1990) and the cytoplasmic and nuclear fractions isolated by hypotonic lysis followed by treatment with 0.5% NP-40, according to Boyle et al. (1985). Immunoprecipitation and immunoblotting were carried out as described in Pines and Hunter (1989), except that samples for the anti-PSTAIRE immunoblot were run in 100 mM NEM instead of 2-mercaptoethanol so that most of the IgG molecules ran at >100 kD. The nuclear and cytoplasmic fractions were normalized to contain equal numbers of cell equivalents. When the anti-PSTAIRE mAb was used as a probe, 400 ng/ml of rabbit anti-mouse IgG (Organon Teknika, West Chester, PA) was included with the [^{125}I]protein A.

Immunofluorescence Microscopy

Cells were plated at low density onto sterile, 10-mm round glass coverslips. Cells were either fixed at room temperature with 50% vol/vol methanol/acetone for 2 min or with 3% formaldehyde for 5 min followed by permeabilization in 0.5% Triton X-100 for 10 min according to Nigg et al. (1985). Anti-cyclin antibodies were used at a dilution of 1:2,000 in 3% BSA in PBS. A mAb to mouse lamin A (gift of L. Gerace, Research Institute of Scripps Clinic), a mAb to canine signal recognition particle (SRP) receptor protein (gift of D. Meyer, University of California, Los Angeles, CA), and a mAb to β-tubulin (Amersham Corp., Arlington Heights, IL) were used at a dilution of 1:200; all three mAbs recognize the cognate human protein. The anti-PSTAIRE mAb was the kind gift of M. Yamashita, Okasaki, Japan, and was used at a dilution of 500 ng/ml. mAb 12CA5 (gift of I. Wilson, Research Institute of Scripps Clinic) was used at a dilution of 120 ng/ml. Cells were incubated with primary or secondary antibodies for 1 h at room temperature. FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG secondary antibodies were obtained from Cappel Laboratories (Cochranville, PA), and used at a dilution of 1:200. Rhodamine-labeled wheat germ agglutinin (Calbiochem-Behring Corp.) was used at a dilution of 1:200. Cells were also routinely stained with Hoechst dye 33258. The chromosome staining was used to judge the stage of mitosis. Cells described as being in S phase are from samples taken 2 to 4 h after release from a thymidine/aphidicolin block. Cells described as being in G2 phase are from samples arrested in G2 with Hoechst 33342. These blocks were >80% effective as judged by flow cytometry. Specimens were observed and photographed with a standard microscope (Zeiss, Oberkochen, Germany) and M35 camera, equipped with a Neofluor X100/1.30 lens. Confocal microscopy was performed using a Zeiss Axiphot equipped with a MRC Lasersharp confocal imaging system (Bio-Rad Laboratories, Cambridge, MA).

Results

Cyclin A Is Predominantly Nuclear Whereas Cyclin B1 Is Predominantly Cytoplasmic

We determined the subcellular distribution of cyclins A and B1 in asynchronous populations of growing HeLa cells, using antibodies raised in rabbits to the entire cyclin A or B1 protein expressed in *E. coli* (Pines and Hunter, 1989; Pines and Hunter, 1990a) (see Materials and Methods). The specificity of these antibodies has been tested in a variety of ways. Each of these antibodies recognizes a single protein on an immunoblot of a whole-cell lysate (Pines and Hunter,

1. *Abbreviations used in this paper*: MPF, M phase promoting factor; SRP, signal recognition particle.

1989; Pines and Hunter, 1990*a*; and data not shown), and neither antibody cross reacts with the other type of cyclin on immunoblots, or in immunoprecipitations from cell lysates or from in vitro translation products (data not shown). We have affinity purified these antibodies, but the highly denaturing conditions required to elute them from their respective antigens make them very poor reagents for immunofluorescence staining, and in consequence we have mostly used whole serum. Both sera were used at a dilution of 1:2,000, and the immunofluorescence patterns for both cyclins were independent of the method used to fix the cells. Similar results were obtained with fixation by methanol/acetone or by formaldehyde followed by solubilization in Triton X100, except that anti-cyclin B1 antibodies stained condensed chromosomes more strongly in methanol/acetone-fixed cells (see below). We demonstrated that the immunofluorescence signal obtained with both sera was specific by preincubating each antiserum with gel-purified antigen (either cyclin A or B1 as appropriate), which completely blocked immunofluorescence with formaldehyde-fixed cells (Fig. 1, *A* and *B*), and with methanol/acetone-fixed cells (data not shown). In addition no signal was obtained with the preimmune serum from either the cyclin A- or cyclin B1-immunized rabbit (data not shown). Perhaps most tellingly, we find that both our anti-cyclin A and B1 antibodies strongly stain cells in the cell cycle from S phase to metaphase, but do not stain cells in anaphase (Fig. 1, *C* and *D*), telophase or most of G1 (data not shown), exactly in accord with the amount of cyclin protein estimated by immunoblotting through the cell cycle (Pines and Hunter, 1990*a*).

In an asynchronous population of HeLa cells we found that cyclin A was predominantly nuclear in many cells (Fig. 1, *C* and *E*), whereas cyclin B1 accumulated in the cytoplasm, most notably in the perinuclear region (Fig. 1, *D* and *F*). The specificity of the anti-cyclin A serum was further confirmed by showing that its immunofluorescence pattern was identical in double immunofluorescence experiments to that observed with the mouse mAb C160 (data not shown; see Fig. 1 *E* for the immunofluorescence pattern obtained with the C160 mAb) which we have previously shown to recognize cyclin A (Pines and Hunter, 1990*a*). The specificity of the immunofluorescence pattern obtained with the B-type serum was confirmed by transfecting cells with a *Xenopus* cyclin B2 clone (gift of Dr. T. Hunt) tagged at the NH_2 terminus with the influenza HIA peptide. In double immunofluorescence experiments with the anti-human cyclin B1 serum, the immunofluorescence pattern obtained with the mAb 12CA5 (gift of Dr. I. Wilson), which recognizes the HIA epitope, mirrored that seen with the anti-cyclin B1 serum (data not shown). We have tested the distribution of cyclins A and B1 in both an immortalized cell line (HeLa S3), and in primary human cells (human foreskin fibroblasts), and the results are essentially identical for the two types of cells.

Cyclin A Enters the Nucleus at S Phase and Is Degraded during Metaphase

Our initial studies suggested that the two types of cyclin are transported to the nucleus at different times in the cell cycle. A double immunofluorescence experiment with anti-human cyclin B1 serum and the mAb C160 showed that cyclin A is nuclear at the time when cyclin B1 is perinuclear (Fig. 1, *E* and *F*). To determine the exact times at which cyclin A and B1 enter the nucleus we synchronized a population of cells at the beginning of S phase with a thymidine/aphidicolin block (see Materials and Methods), released the cells and looked at the distribution of the cyclins through the cell cycle. The low level of cyclin A detected at the beginning of S phase was largely nuclear. Cyclin A continued to accumulate predominantly in the nucleus of S phase and G2 phase cells (Figs. 2 *A*), where it was excluded from the nucleoli. A comparison of the Hoechst DNA stain with anti-cyclin A staining suggests that cyclin A was associated with some chromatin in prophase (Fig. 2, *B* and *C*). Although a three-dimensional reconstruction of such images or evidence that cyclin A is associated with isolated chromatin would be needed to establish this interaction, and some evidence for this is the observation that cyclin A is found in preparations of growth-associated histone H1 kinase isolated from rat chromatin (Chambers and Langan, 1990). After prophase cyclin A became dispersed throughout the cell, and apparently was not associated with fully condensed chromosomes (Figs. 2, *D* and *E*), although we cannot rule out that the more condensed chromatin structure in metaphase prevented the antibodies from binding cyclin A. By contrast, cyclin B1 does associate with condensed chromosomes in both prophase and metaphase (see below). During metaphase the intensity of staining with anti-cyclin A antibodies declined compared with the maximum observed in G2 and at prophase (Fig. 1 *C*), which was because of cyclin A degradation. At anaphase cyclin A staining had disappeared from the whole of the cell. These changes in the intensity of cyclin A immunofluorescence throughout the cell cycle paralleled the change in the level of cyclin A determined by immunoblotting (Pines and Hunter, 1990*a*). It is notable with respect to the behavior of cyclin B1 (see below) that at no time does cyclin A appear to associate specifically with the mitotic apparatus.

Cyclin B1 Only Moves to the Nucleus at M Phase

Cyclin B1 was first visible in the cytoplasm of early S phase cells, but in contrast to cyclin A it continued to accumulate exclusively in the cytoplasm of S and G2 phase cells, especially around the nucleus (Fig. 1, *D* and *F*). Cyclin B1 staining was initially particulate, but eventually became mostly perinuclear. At present the cytoplasmic distribution of cyclin B1 is not readily explained by any recognized cytoplasmic structure. Although there is a general correspondence between the microtubule network and cyclin B, this is not true colocalization (Fig. 3, *A* and *B*). However, the location of cytoplasmic cyclin B1 is influenced by the integrity of the microtubule network, because nocodazole treatment disrupted cyclin B1 distribution (data not shown). The perinuclear distribution of cyclin B1 was not a result of association with the Golgi apparatus, as revealed by double immunofluorescence staining with rhodamine-labeled wheat germ agglutinin (Fig. 3, *C* and *D*), nor obviously to association with the ER as shown by co-immunofluorescence with an anti-SRP receptor antibody (data not shown). At the beginning of prophase, before nuclear envelope breakdown, almost all the cyclin B1 underwent a dramatic redistribution into the nucleus (Fig. 4 *A* and 6 *C*). Despite the change observed in cyclin B1 localization upon microtubule disruption in interphase, the translocation of cyclin B1 into the nucleus was independent of

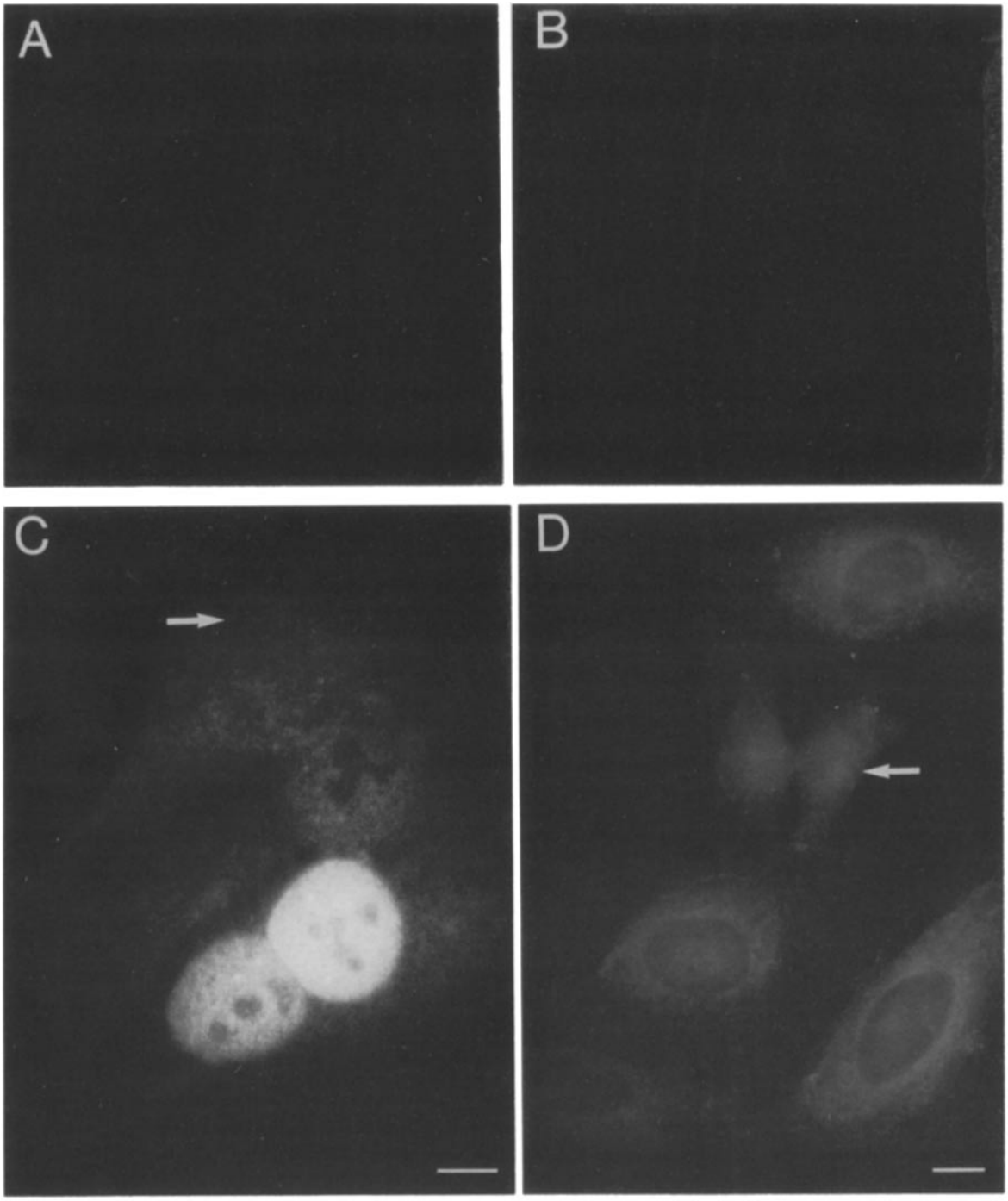


Figure 1. Immunofluorescence staining of asynchronized HeLa cells. (*A*) HeLa cells fixed with formaldehyde and stained with polyclonal anti-cyclin A antibodies pre-incubated with gel purified cyclin A protein. (*B*) HeLa cells fixed with formaldehyde and stained with anti-cyclin B1 antibodies pre-incubated with gel purified cyclin B1 protein. (*C*) HeLa cells fixed with formaldehyde and stained with polyclonal anti-cyclin A antibodies. A cell in metaphase is arrowed. (*D*) HeLa cells fixed with formaldehyde and stained with anti-cyclin B1 antibodies. Arrowed is a cell completing telophase. The staining here is artefactually high because of the geometry of the cell. (*E*) HeLa cells fixed with formaldehyde and stained with anti-cyclin A mAb C160. (*F*) HeLa cells fixed with formaldehyde and stained with polyclonal anti-cyclin B1 antibodies, showing the same field as in *A*. Bars, 10 μm.

intact microtubules, because it still occurred in nocodazole-treated cells.

A similar redistribution to the nucleus at prophase has previously been described for a 57-kD Golgi-associated protein (McMorrow et al., 1990), and it was suggested that the relocation could be because of phosphorylation at mitosis. Cyclin B1 is also phosphorylated at mitosis, and in cleaving sea urchin embryos this phosphorylation event causes a shift in the mobility of cyclin B1 on SDS-PAGE and is correlated with the appearance of histone H1 kinase activity (Meijer et

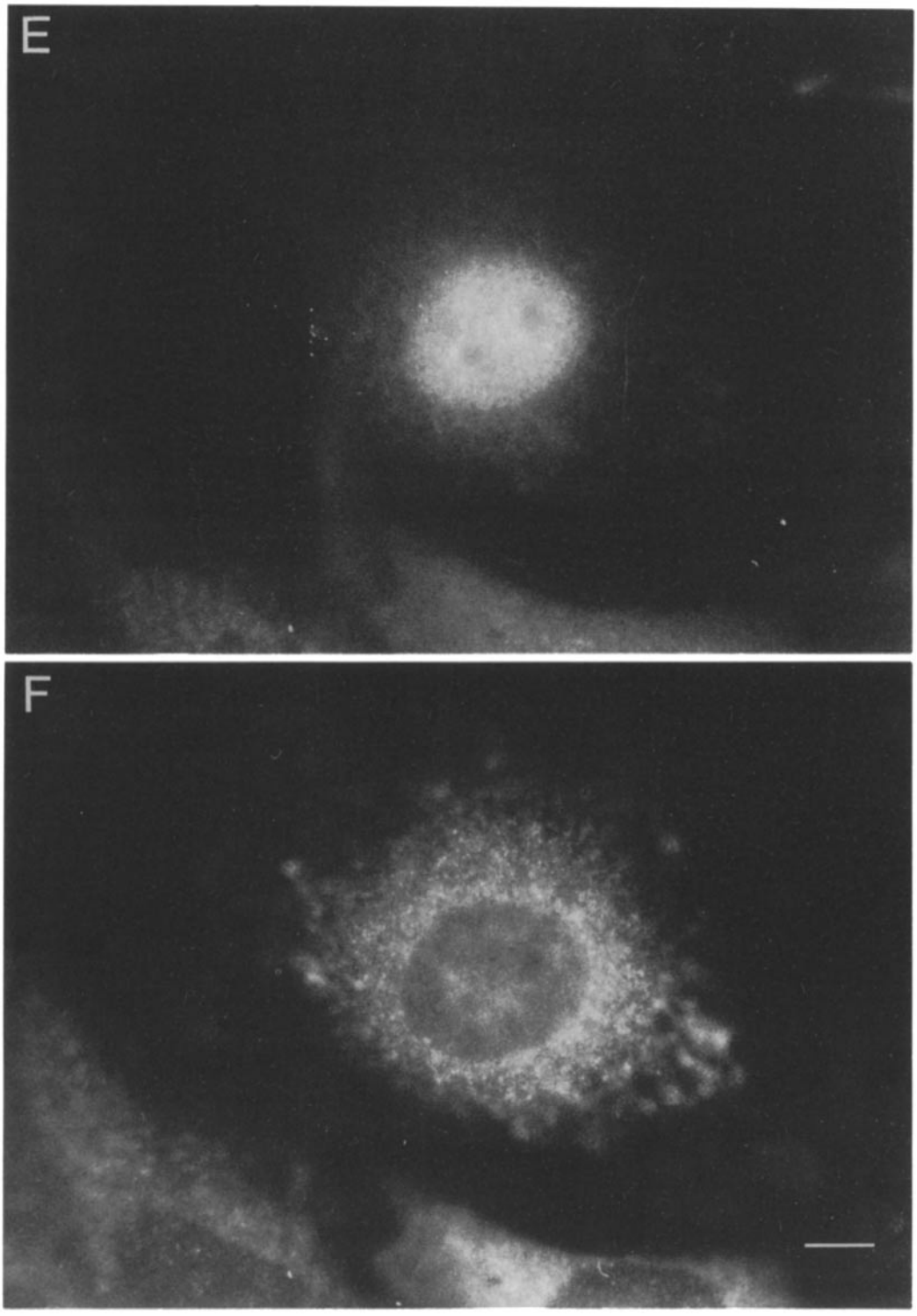


Figure 1.

al., 1989). The serine residue that causes this shift in mobility upon phosphorylation was identified in sea urchin cyclin B1 and *Xenopus* cyclin B2 by Tim Hunt and colleagues, who mutated this serine (serine 90) to an alanine. We tagged the wild-type and the mutant B2 *Xenopus* cyclins with the H1A epitope, expressed them in HeLa cells from a retroviral vector and studied their localization by immunofluorescence. Double immunofluorescence studies with the 12CA5 mAb and anti-human cyclin B1 antiserum to monitor the endogenous protein showed that both the mutant and the wild-type *Xenopus* cyclin relocated to the nucleus at the beginning of prophase, at the same time as the endogenous cyclin B1 (data not shown). This demonstrates that the nuclear localization signal is conserved between species, and, that if cyclin phosphorylation is involved in its nuclear transport, it must be at a residue other than serine 90.

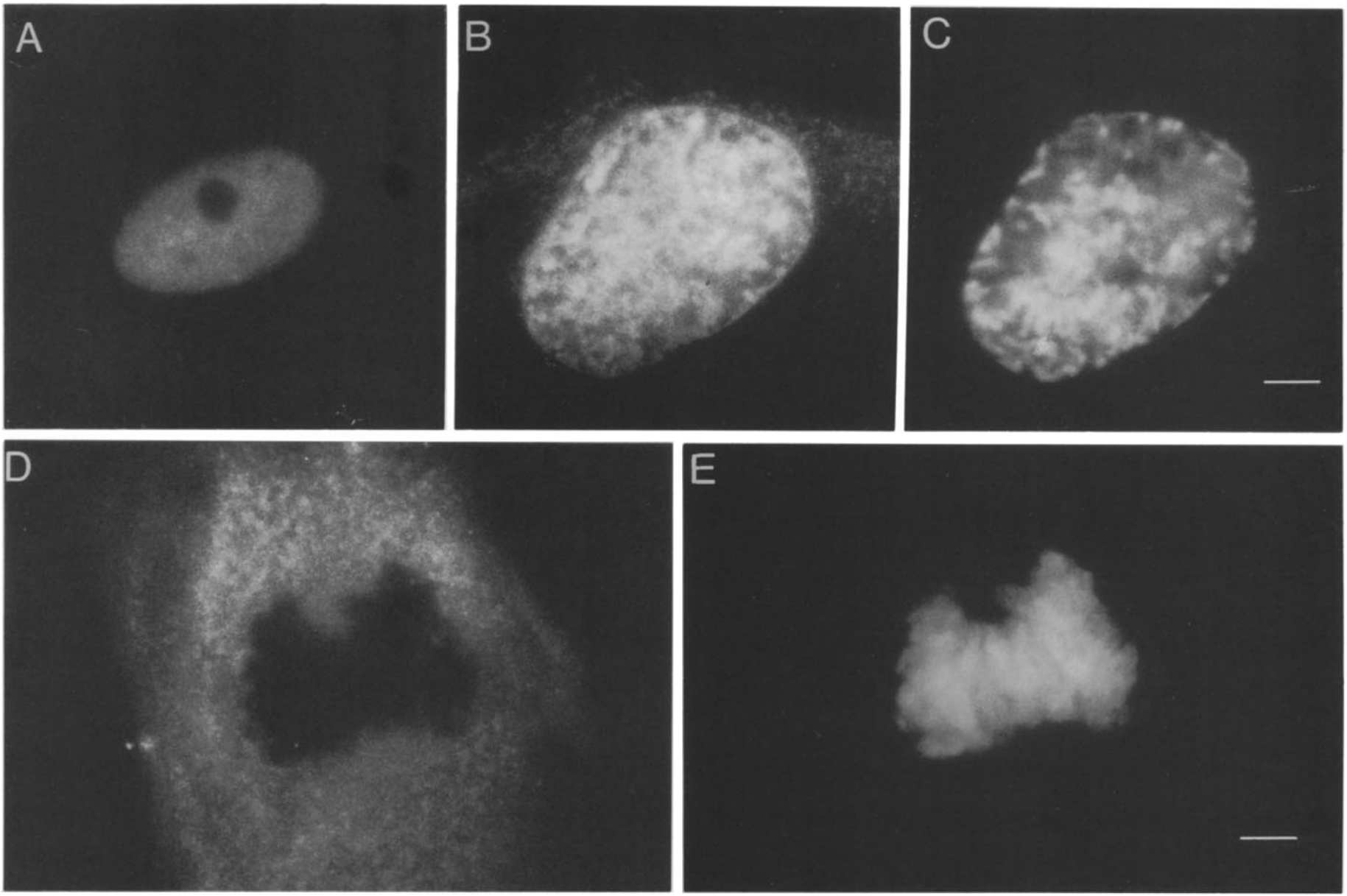


Figure 2. Cyclin A is nuclear in S phase and excluded from condensed chromosomes. (*A*) S phase HeLa cell fixed with formaldehyde and stained with polyclonal anti-cyclin A antibodies. (*B*) Prophase human foreskin fibroblast fixed with formaldehyde and stained with polyclonal anti-cyclin A antibodies. (*C*) Same cell as in *B* stained with Hoechst 33258 to visualize the chromosomes. (*D*) Metaphase human foreskin fibroblast fixed with formaldehyde and stained with polyclonal anti-cyclin A antibodies. (*E*) Same cell as in *D* stained with Hoechst 33258 to visualize the chromosomes. Bars, 10 μm.

Cyclin B1 Associates with Condensed Chromosomes and is Degraded at the Metaphase–Anaphase Transition

Although the anti-cyclin B1 antiserum did not stain chromosomes strongly in formaldehyde-fixed cells, it prominently stained chromosomes in methanol/acetone-fixed cells (Fig. 4, *A* and *C*). The cyclin B1 staining pattern on chromosomes differed from that of cyclin A in remaining associated with condensed chromosomes in metaphase (compare Fig. 2 *D* with Fig. 4, *A* and *C*). Another difference between cyclin A and B1 staining patterns was in the manner of cyclin's destruction. Cyclin A staining gradually decreased through mitosis, such that metaphase cells were less strongly stained than prophase cells (Fig. 1 *C*), and anaphase cells seemed to have little or no cyclin A. However, there was no sharp decrease in the intensity of the anti-cyclin A stain at any stage. By contrast, throughout metaphase cells stained strongly with anti-cyclin B1 antibodies, but as soon as cells entered anaphase it appeared that most, if not all cyclin B1 was degraded (Fig. 1 *D* and Fig. 5 *A*). This provides strong evidence for the reports from other systems that cyclin B destruction is tightly correlated with the metaphase–anaphase transition (Minshull et al., 1988; Murray and Kirschner, 1989; Ghiara et al., 1991).

Cyclin B1 Associates with the Mitotic Spindle

We noticed that some cyclin B1 seemed to be concentrated near to the condensed chromosomes in mitotic cells (Fig. 4, *A* and *C*), and a careful examination of these pictures suggested that cyclin B1 was localized to the mitotic spindle. To confirm that this was due to an association with the mitotic apparatus we co-stained cells with anti-cyclin B1 antibodies and an anti-β tubulin mAb. We found that in prophase cells cyclin B1 was associated with the mitotic asters (Fig. 5, *C* and *D*). In metaphase cells, cyclin B1 localized to the spindle poles, spindle pole caps, and to the main spindle fibers (Fig. 5, *E* and *F*). Cyclin B1 was strongly associated with microtubules in mitotic cells, but not in interphase cells (Fig. 3, *A* and *B*). When cells were permeabilized for 30 s with Triton X100 in a buffer which stabilizes microtubules, and then fixed in formaldehyde, cyclin B1 staining was almost completely eliminated from interphase cells and the cytoplasm of M phase cells, but was still present on the mitotic apparatus in prophase and metaphase cells (data not shown).

Cyclin B1 Enters the Nucleus before Nuclear Lamina Breakdown

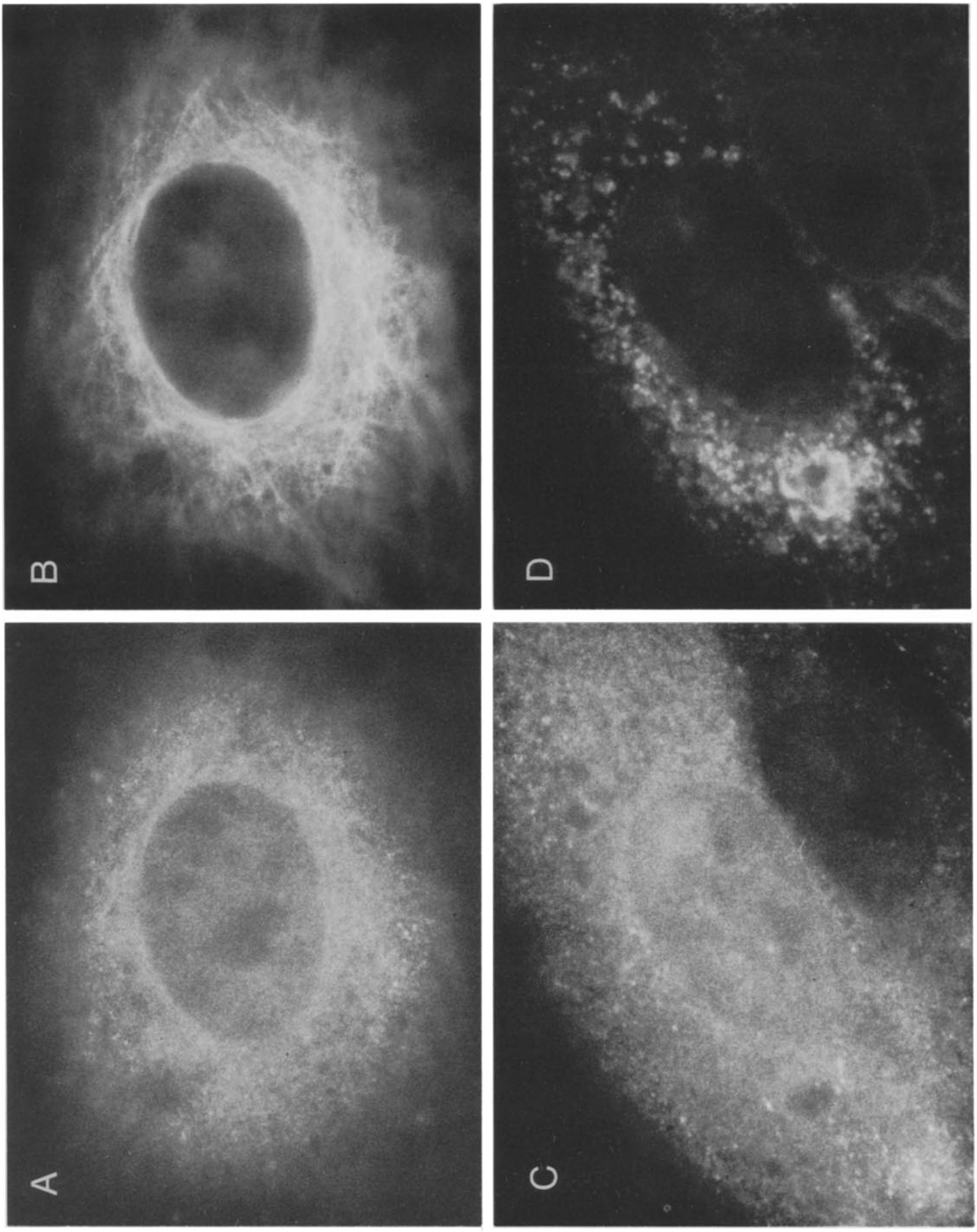
It has recently been shown that purified MPF, which is composed of cyclin B and $p34^{cdc2}$, is able to phosphorylate lamin B at the same sites in vitro as are specifically phosphorylated in mitosis in vivo (Peter et al., 1990; Ward and Kirschner, 1990). It has also been shown that in a cell-free system MPF will cause purified nuclei to undergo nuclear lamina breakdown (Peter et al., 1990). For cyclin B/$p34^{cdc2}$ to be instrumental in lamina disassembly in vivo, the complex would have to enter the nucleus while the lamina was still intact. To determine whether this is the case, we co-stained HeLa cells with anti-cyclin B1 antiserum and an anti-lamin A mAb (gift of Dr. L. Gerace). In cells in late G2, cyclin B1 had accumulated around the nucleus, which displayed a bright, nuclear lamin ring (Fig. 6, *A* and *B*). In prophase a significant proportion of cyclin B1 had entered the nucleus, but the nuclear lamina was still detected as an intact ring around the nucleus (Fig. 6, *C* and *D*).

To determine more precisely the location of cyclin B1 with respect to the nuclear lamina, we used confocal microscopy to visualize cells stained for cyclin B1 and lamin A. This confirmed that in interphase cyclin B1 accumulated all around the nucleus with no detectable cyclin B1 within the nucleus (data not shown). At prophase cyclin B1 entered the nucleus and dispersed throughout the nucleoplasm, but the nuclear lamina still stained as an intact sphere. Shortly after the translocation of cyclin B1, the nuclear lamins dispersed throughout the cell. Thus the cell cycle–dependent movement of cyclin B1, presumably with its associated $p34^{cdc2}$ subunit, is entirely consistent with this complex acting as the mitosis-specific lamin kinase.

PSTAIRE-containing Proteins Are Present in both the Nucleus and the Cytoplasm, and Associate with the Mitotic Spindle

Cyclins A and B1 are presumed to function by activating and targetting their associated kinase, either $p34^{cdc2}$ or p33, to its substrates. For this reason, it is important to determine where the cyclin–protein kinase complexes are located in the cell. Therefore we compared the localization of cyclin A and B1 through the cell cycle with PSTAIRE-containing proteins by double immunofluorescence stainings with anti-cyclin antibodies and with a mAb raised to the conserved "PSTAIRE" peptide motif found in all known members of the cdc2 protein kinase family (gift of Dr. M. Yamashita). This mAb recognizes both $p34^{cdc2}$ and p33 on immunoblots (see Fig. 7 *E*), and presumably any other PSTAIRE-containing protein in the cell. We demonstrated the specificity of the anti-PSTAIRE mAb in immunofluorescence by pre-incubating the antibody with the PSTAIRE peptide which completely eliminated cell staining (data not shown). Both nuclear cyclin A in S phase (Fig. 7, *A* and *B*) and cytoplasmic cyclin B1 in G2 phase (Fig. 7, *C* and *D*) colocalized with proteins recognized by the anti-PSTAIRE antibody. In addition, in mitotic cells both cyclin B1 and PSTAIRE-containing proteins localized to the mitotic spindle (data not shown).

These immunofluorescence data do not prove that the colocalized PSTAIRE-containing protein and cyclin are physically associated, nor can they address which PSTAIRE-containing protein is being detected. To determine whether cyclin–PSTAIRE protein complexes exist in different parts of the cell, we fractionated G2 phase HeLa cells into crude nuclear and cytoplasmic fractions, followed by immunoprecipitation with anti-cyclin A or B1 antibodies, and then immunoblotting with the anti-PSTAIRE mAb (Fig. 7 *E*) and anti-cyclin antibodies (data not shown). We found that most of cyclin A was nuclear, consistent with the immunostaining data, and that p33 was almost exclusively associated with the nuclear form of cyclin A. Very little $p34^{cdc2}$ associated with cyclin A in either the nucleus or the cytoplasm of HeLa cells, whereas $p34^{cdc2}$ co-immunoprecipitated with cyclin B1 in proportion to the level of cyclin B1, which was mostly cytoplasmic, in keeping with the immunofluorescence results.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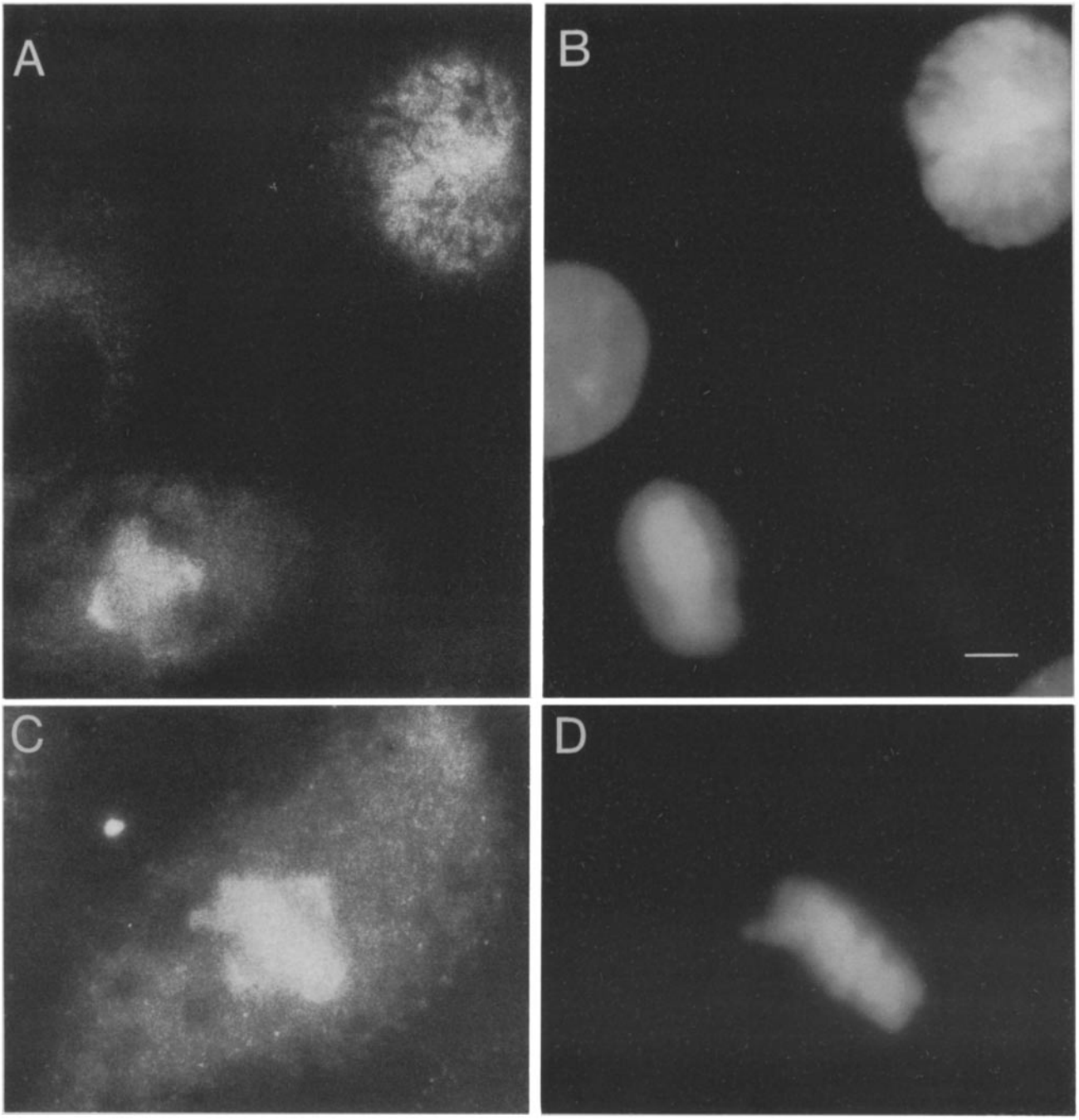


Figure 4. Cyclin B1 associates with condensed chromosomes and the mitotic spindle. (*A*) Prophase (*top right*), G2 phase (*center left*), and metaphase (*bottom left*) HeLa cells fixed in methanol–acetone and stained with anti-cyclin B1 antibodies. Cyclin B1 accumulates in the perinuclear region in a G2 phase cell and subsequently stains the chromosomes and the spindle in prophase and metaphase. (*B*) Hoechst 33258 stain of the same cells as in *E*, showing the diffuse DNA in the G2 cell and the condensed chromosomes in prophase and metaphase cells. (*C*) Metaphase HeLa cell fixed in methanol–acetone and stained with anti-cyclin B1 antibodies. Cyclin B1 is localized on the spindle and on the chromosomes. (*D*) Hoechst 33258 stain of the same cell as in *C*, showing the condensed chromosomes. Bar, 10 μm.

Figure 3. Cyclin B1 distribution in interphase cells. (*A*) G2 phase HeLa cell fixed with formaldehyde and stained with polyclonal anti-cyclin B1 antibodies. (*B*) The same cell as in *A* stained with a monoclonal anti-β tubulin antibody. (*C*) HeLa cells fixed with formaldehyde and stained with polyclonal anti-cyclin B1 antibodies. (*D*) The same cells as in *C* stained with rhodamine-wheat germ agglutinin to visualize the Golgi apparatus. Bars, 10 μm.

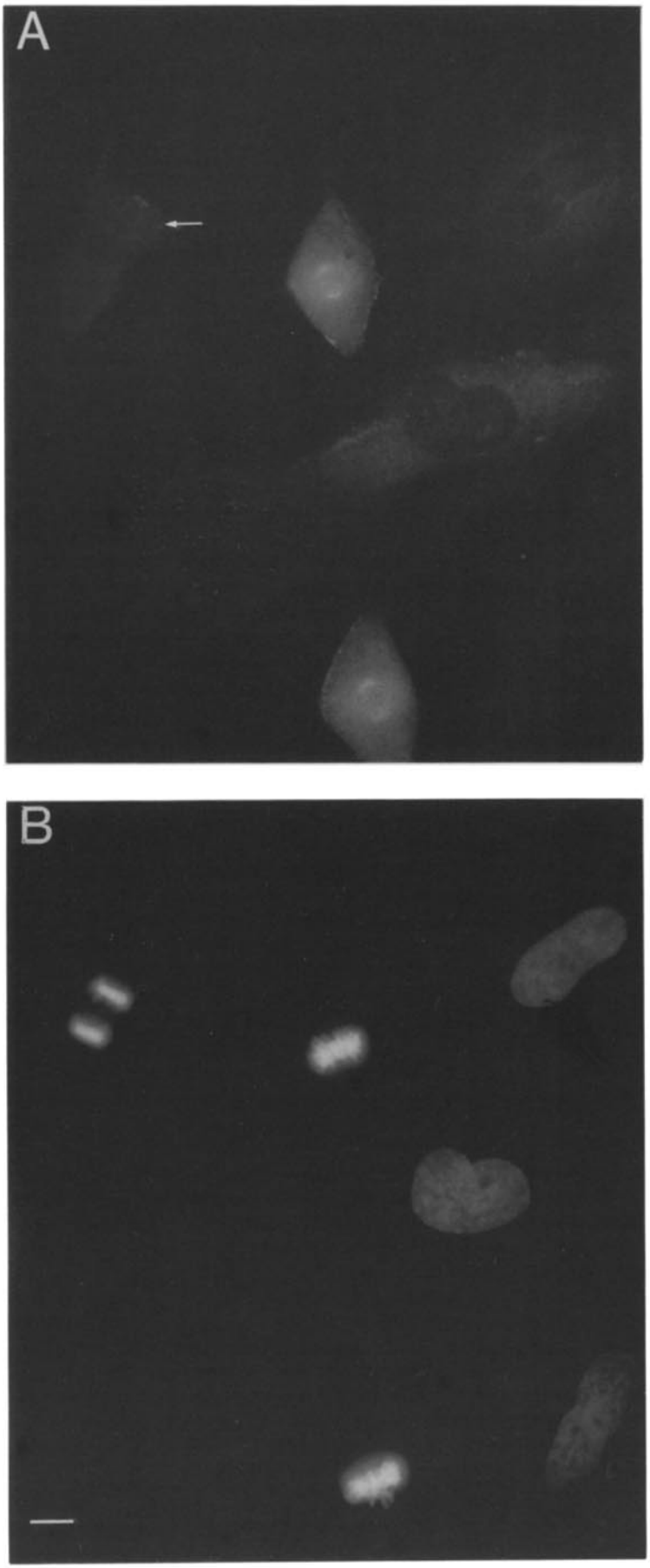


Figure 5. Cyclin B1 associates with the spindle in mitosis. (*A*) M phase HeLa cells fixed with formaldehyde and stained with anti-cyclin B1 antibodies. Two metaphase cells displaying bright spindle staining are shown, one in the center and one at bottom center of the panel. A late anaphase cell with very little cyclin B1 staining is arrowed at the top left of the panel. (*B*) Hoechst 33258 chromosome stain of the cells in *A*. (*C*) Prophase HeLa cell fixed with formaldehyde and stained with anti-cyclin B1 antibodies. (*D*) Anti-β tubulin mAb staining of the same cell as in *C*. (*E*) Metaphase HeLa cell fixed with formaldehyde and stained with anti-cyclin B1 antibodies. (*F*) Anti-β tubulin monoclonal antibody staining of the same cell as in *E*.

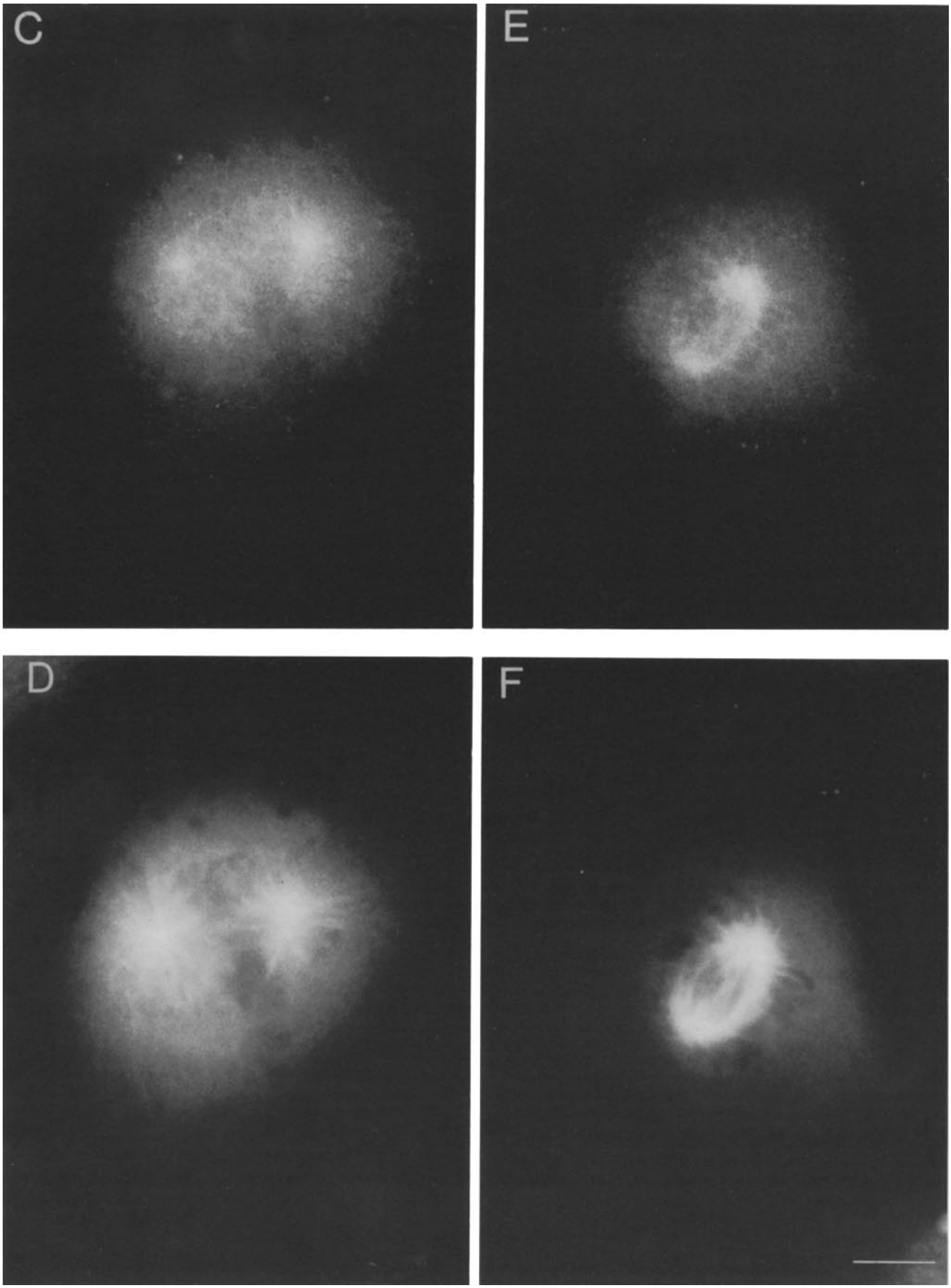
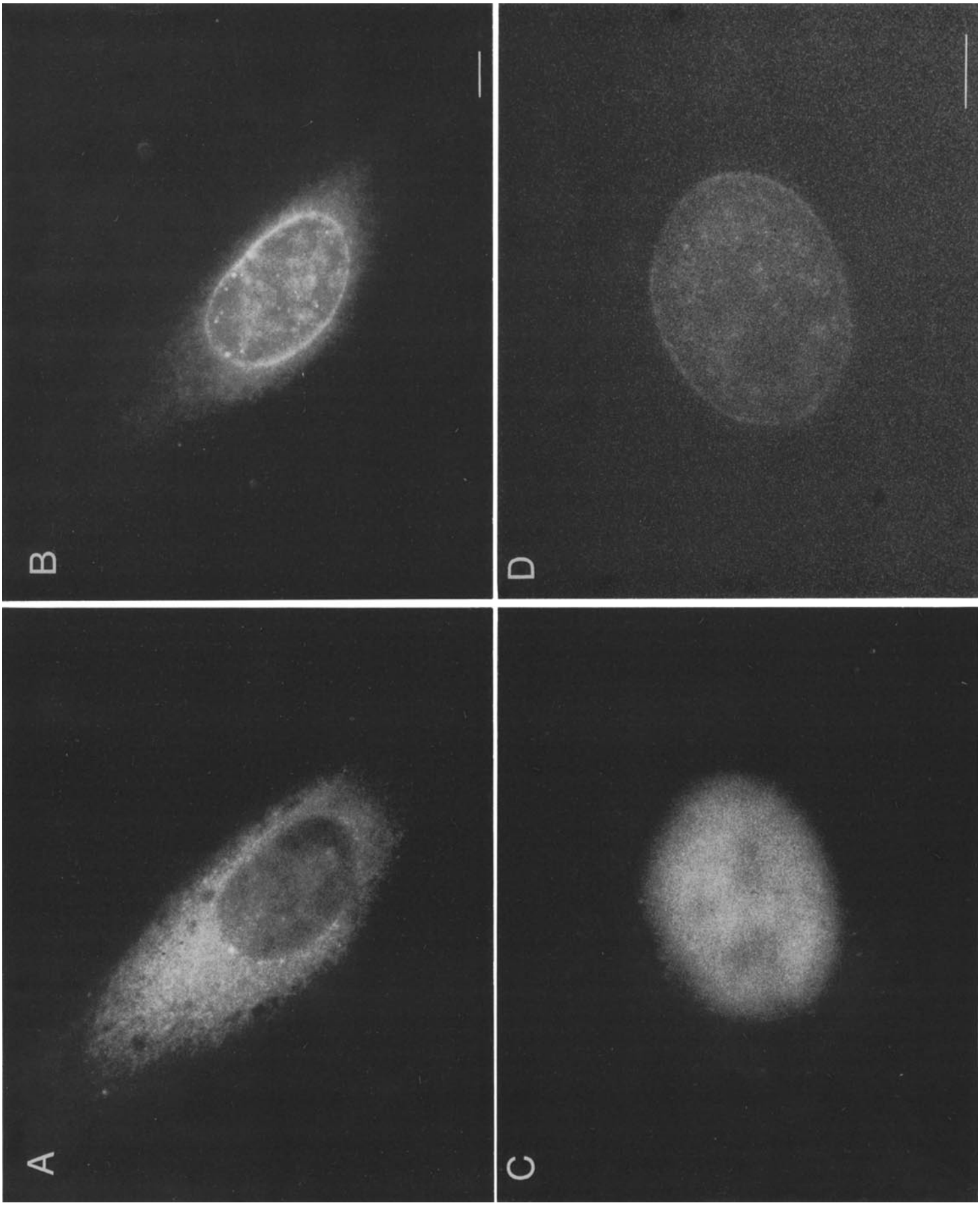


Figure 5.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Discussion

In this paper we have reported the localization of cyclins A and B1 in the somatic cell cycle, and have compared cyclin A and B1 intra-cellular localization with that of PSTAIRE-containing proteins. We have shown that cyclins A and B1 are differentially localized in human somatic cells, both with respect to each other and to the stage of the cell cycle. Cyclin A appears to be nuclear from the time its synthesis begins in S phase, and remains predominantly nuclear in S and G2 phase cells. Cyclin A is distributed in the nucleus in a fairly uniform fashion, but does not associate with the nucleoli. In prophase cyclin A possibly associates with condensing chromosomes, but by metaphase cyclin A is dispersed throughout the cell and is not bound to condensed chromosomes. At no time does cyclin A appear to associate strongly with the mitotic spindle. Cyclin A is degraded during metaphase before the metaphase→anaphase transition.

In contrast, cyclin B1 accumulates around the nucleus throughout S and G2 phases. The cytoplasmic location of human cyclin B1 agrees with the observation that cyclin B is cytoplasmic in frog (Gautier and Maller, 1991) and starfish (T. Kishimoto, personal communication) oocytes, which are naturally arrested at the start of first meiotic prophase. As a human cell enters mitosis, after chromatin has begun to condense but before nuclear lamina breakdown, a large proportion of cyclin B1 enters the nucleus. In agreement with this, cyclin B enters the nucleus just before germinal vesicle breakdown in maturing starfish oocytes (T. Kishimoto, personal communication). Unlike human cyclin A, in metaphase cells human cyclin B1 is found associated with the chromosomes and the spindle, especially the spindle poles, but there is some cyclin B1 distributed throughout the rest of the cell. Although anti-cyclin B1 antibodies stain chromosomes more strongly in methanol/acetone fixed cells, they do stain chromosomes in formaldehyde-fixed cells, and this staining is enhanced in mitotic nocodazole-treated cells (data not shown). It may be that this difference in cyclin B1 staining according to the fixation technique is due to a difference in the state of the mitotic apparatus in formaldehyde versus methanol/acetone-fixed cells. After cyclin A has been degraded, cyclin B1 is very rapidly destroyed at the metaphase→anaphase transition.

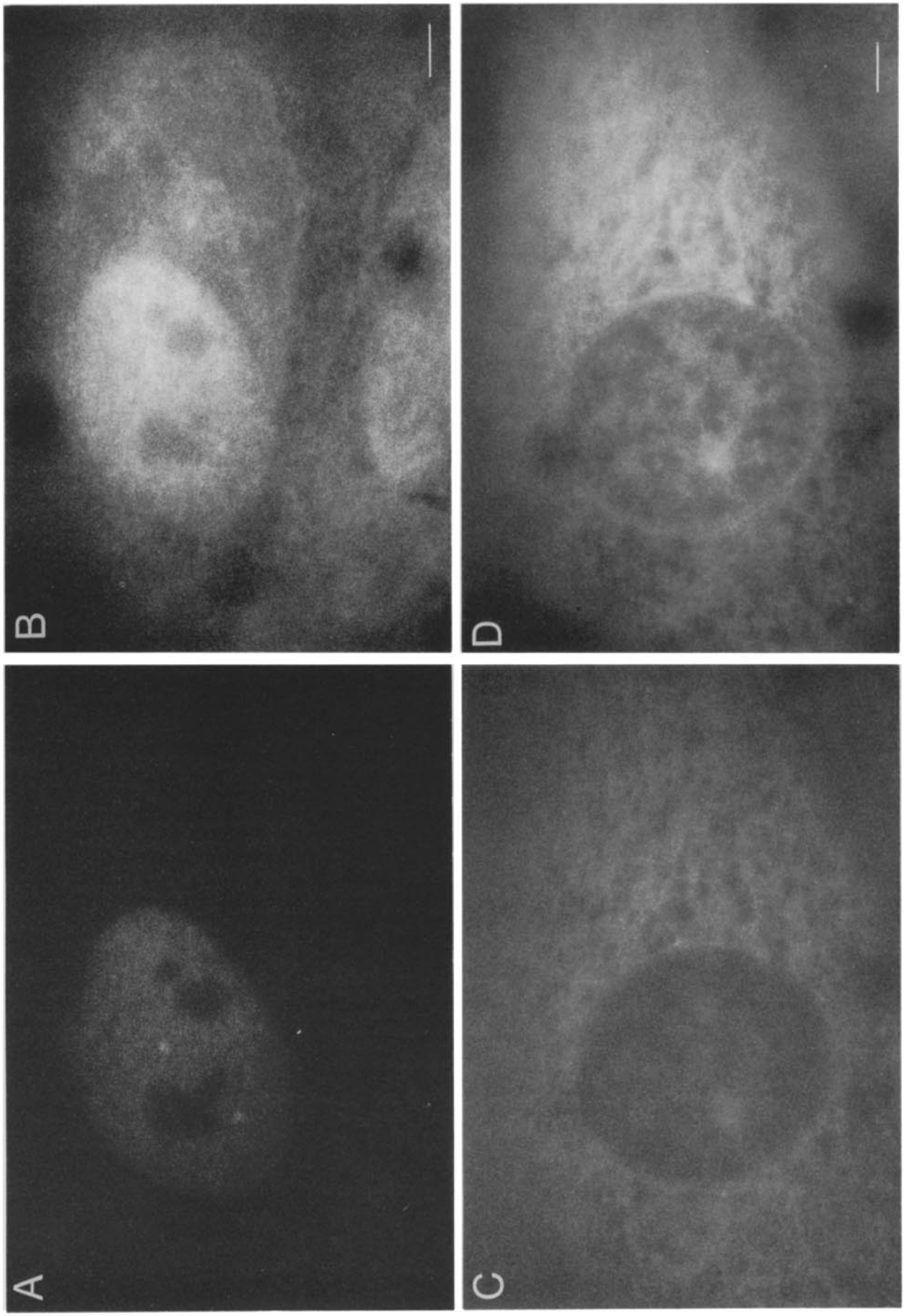
Human cyclin B2 is also expressed in HeLa cells (S. Reed, personal communication), but as our antibodies do not cross react with human cyclin B2 (data not shown), we cannot say whether cyclin B1 and B2 will behave in the same way in the cell. However, preliminary observations on human and chicken cyclin B2 indicate that cyclin B2 may undergo cell cycle–dependent movement similar to that of cyclin B1 (S. Reed, P. Gallant, and E. Nigg, respectively, personal communications), as do our own observations on the behaviour of *Xenopus* cyclin B2 transfected into HeLa cells.

From these studies it is obvious that there is precise control of the cellular distribution of the different cyclins throughout the mammalian cell cycle. Our findings for mammalian cyclins in somatic cells differ to some extent from the reported localizations of cyclin during early *Drosophila* developmental cell cycles (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990). After nuclear migration in the *Drosophila* embryo, cyclin A is cytoplasmic in interphase and nuclear in prophase, unlike the predominantly nuclear human cyclin A. *Drosophila* cyclin B accumulates in the cytoplasm of interphase cells, except for one uncharacterized nuclear dot, and in prophase diffusely stains around condensing chromosomes. Like human cyclin B, *Drosophila* cyclin B appears to associate with the spindle poles (D. Glover, personal communication). The results from the *Drosophila* and human systems also agree in finding that cyclin A is degraded before cyclin B, and that cyclin B destruction is correlated with the metaphase→anaphase transition. Differences in cyclin localization in the two systems are to be expected given the probable differences in the control of cell division in very rapidly dividing syncytial embryos (average cell cycle time ~10 min) versus the much more lengthy cell cycle in vertebrate somatic cells (average cell cycle time ~22 hr). There may even be specialized cyclins for embryonic and somatic cell cycles in the same species.

The cyclin immunostaining studies detect the whole population of cyclin. However, the active species of cyclin is believed to be associated with $p34^{cdc2}$ or $p34^{cdc2}$-related protein kinases. For this reason, we have compared the localization of cyclins with that of PSTAIRE-containing proteins by both immunofluorescence and cell fractionation. By immunofluorescence staining we find that there are PSTAIRE-containing proteins in both the nucleus and the cytoplasm, throughout the cell cycle, which could be associated with cyclins. However, this does not show that the PSTAIRE-containing proteins and cyclins are physically associated, and it is possible that there are significant unassociated pools of both types of molecule even in regions of the cell where they co-localize. In the absence of monoclonal antibodies specific for $p34^{cdc2}$ and $p34^{cdc2}$-related proteins we have not been able to determine which PSTAIRE-containing protein colocalizes with cyclin A and cyclin B1. However, our fractionation studies on G2 phase cells suggest that only the nuclear form of cyclin A is associated with a PSTAIRE-containing protein, and that in HeLa cells this is primarily p33, rather than $p34^{cdc2}$. By comparison, cyclin B1 only binds $p34^{cdc2}$, and this is mostly in the cytoplasmic fraction.

There have been several previous, conflicting reports of the cellular localization of $p34^{cdc2}$, and none of these studies addressed the issue of whether the $p34^{cdc2}$ detected was associated with cyclin. $p34^{cdc2}$ has been variously reported to be exclusively nuclear, with some specific centrosomal staining at mitosis (Riabowol et al., 1989), or cytoplasmic and strongly perinuclear (Akhurst et al., 1989), or both nuclear and cytoplasmic throughout the cell cycle with some specific centrosomal association (Bailly et al., 1989). In contrast to these studies with mammalian cells, $p34^{cdc2}$ and $p63^{cdc13}$ in *S. pombe* are reported to be nuclear proteins throughout the cell cycle (Booher et al., 1989), and $p63^{cdc13}$

Figure 6. Cyclin B1 enters the nucleus before nuclear lamina breakdown. (*A*) G2 phase HeLa cell fixed with formaldehyde and stained with anti-cyclin B1 antibodies. (*B*) Same cell as in *A* stained with an anti-lamin A mAb. (*C*) Prophase HeLa cell fixed with formaldehyde and stained with anti-cyclin B1 antibodies. (*D*) Same cell as in *C* stained with an anti-lamin A mAb. Bars, 10 μm.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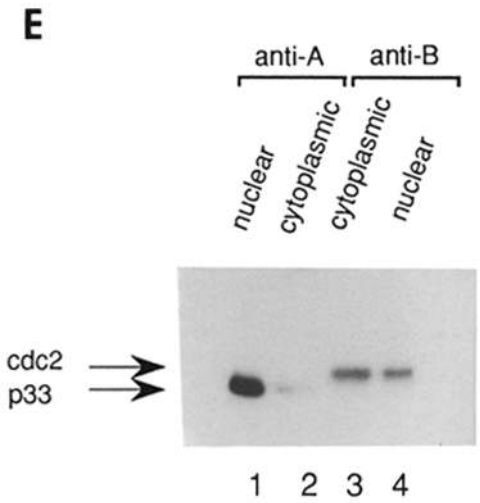

Figure 7.

has been shown to associate with the spindle poles (Alfa et al., 1990). Alfa et al. (1990) also showed that p34^{cdc2} is not activated if microtubules are disrupted before mitosis, which is in contrast to our observation, and those of others, that in human cells active cyclin B/p34^{cdc2} complex accumulates to high levels in the presence of the microtubule-disruptant, nocodazole.

Our observations on cyclin localization suggest a mechanism by which p34^{cdc2} and p34^{cdc2}-related protein kinases could perform different roles at various times in the cell cycle by phosphorylating distinct substrates. There is evidence that p34^{cdc2} can only be activated when associated with a cyclin (Solomon et al., 1990), and thus its activity as a protein kinase will be dependent on the localization of the cyclins (as well as the state of phosphorylation of p34^{cdc2}). For example, cyclin A is a nuclear protein from S phase onwards and is associated with detectable protein kinase activity. Therefore p34^{cdc2} or p33 bound to cyclin A could function in the nucleus in interphase and phosphorylate putative p34^{cdc2} interphase substrates such as the retinoblastoma protein, p53 and *c-abl* (reviewed in Pines and Hunter, 1990*b*), and perhaps proteins involved in the process of DNA replication (D'Urso et al., 1990). However, because cyclin A continues to accumulate until mitosis and can drive a *Xenopus* extract into mitosis, it is likely that cyclin A also plays a role in the initiation of M phase, which may involve interactions with chromatin or with the centromeres. Similarly, cyclin B1 associates with the mitotic spindle in prophase and metaphase, and so active p34^{cdc2} bound to cyclin B1 could play a role in maintaining the spindle in its pre-anaphase conformation. Thus there is no need to postulate that cyclins directly alter the substrate specificity of the protein kinase, simply that they activate p34^{cdc2} in different parts of the cell and the cell cycle.

The behavior of human cyclin B1/p34^{cdc2} is in exact accord with a role as a mitotic kinase. Cyclin B1/p34^{cdc2} accumulates outside the nucleus as an inactive complex. At the G2→M transition, p34^{cdc2} is activated by dephosphorylation on phosphothreonine and phosphotyrosine, and we show here that cyclin B1 enters the nucleus. Therefore, it is important to determine whether p34^{cdc2} is dephosphorylated before or after moving to the nucleus, and whether dephosphorylation is required for its nuclear transport. This in turn raises the question whether the currently unidentified protein kinases and phosphatases that act on p34^{cdc2} are both nuclear and cytoplasmic, or asymmetrically distributed. Pertinent to this question is the recent demonstration that in a *Xenopus* cell-free extract, p34^{cdc2} is not dephosphorylated on tyrosine if nuclear transport is inhibited with wheat germ agglutinin (Kumagai and Dunphy, 1991). One interpretation of this result is that p34^{cdc2} is dephosphorylated after transport to the nucleus by a nuclear phosphatase(s), which is further indicated by the observation that p80^{cdc25} is a nuclear protein (P. Russell, manuscript submitted for publication). Thus the regulated translocation of cyclin B1/p34^{cdc2} could be the critical step in the activation of the p34^{cdc2} protein kinase, and it will be important to determine how cyclin B1/p34^{cdc2} is retained in the cytoplasm during G2 and released at the start of prophase. So far we have not identified a cytoplasmic structure that corresponds to the localization pattern of cyclin B1 in the cytoplasm.

The cyclin B1/p34^{cdc2} complex enters the nucleus before detectable nuclear lamina breakdown, and very soon after this the lamina disperses. We have shown that cyclin A can enter the nucleus in interphase, thus establishing the precedent that cyclins can be transported into the nucleus via nuclear pores. In consequence we do not believe that the cyclin B1 complex leaks into the nucleus after the lamina has begun to disperse. Nuclear lamins have been shown to be phosphorylated by cyclin B1/p34^{cdc2} in vitro on the same sites that are phosphorylated in mitosis and which are necessary for lamina disassembly (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990), and so we propose that the nuclear lamina breaks down as soon as it becomes accessible to the active cyclin B1/p34^{cdc2} complex. The association of human cyclin B1 with the spindle and with chromosomes in mitosis suggests that the cyclin B1/p34^{cdc2} complex may be involved in regulating spindle architecture and

Figure 7. Cyclin A and cyclin B1 colocalize with PSTAIRE-containing proteins. (*A*) S phase HeLa cell (i.e., 2 h after release from an aphidicolin block) fixed with formaldehyde and stained with anti-cyclin A antibodies. (*B*) Same cell as in *A* stained with an anti-PSTAIRE mAb. (*C*) Late G2 phase (i.e., 2 h after release from a Hoechst 33342 block) HeLa cell fixed with formaldehyde and stained with anti-cyclin B1 antibodies. (*D*) Same cell as in *C* stained with an anti-PSTAIRE mAb. (*E*) HeLa cells were fractionated, immunoprecipitated with anti-cyclin A and B1 antibodies, and the immunoprecipitates were analyzed by immunoblotting for PSTAIRE-containing proteins as described in Materials and Methods. (Lane *1*) Immunoblot of an anti-cyclin A immunoprecipitate from G2 phase nuclei, probed with an anti-PSTAIRE mAb. (Lane *2*) Immunoblot of an anti-cyclin A immunoprecipitate from G2 phase cytoplasm, probed with an anti-PSTAIRE mAb. (Lane *3*) Immunoblot of an anti-cyclin B1 immunoprecipitate from G2 phase cytoplasm, probed with an anti-PSTAIRE mAb. (Lane *4*) Immunoblot of an anti-cyclin B1 immunoprecipitate from G2 phase nuclei, probed with an anti-PSTAIRE mAb. Blots were probed with ^{125}I-labeled protein A and autoradiography was for 3 d at −70°C with an intensifying screen.

perhaps chromosome alignment. Our observation that cyclin B1, but not cyclin A, binds to the mitotic spindle may be pertinent to the regulation of their destruction. Drugs that disrupt microtubules, and thus prevent the formation of an intact mitotic apparatus, are known to stabilize cyclin B but do not affect the destruction of cyclin A (Minshull et al., 1988; data not shown). This difference is especially striking now that it appears that both cyclin A and cyclin B1 are degraded by the same ubiquitin-mediated proteolysis pathway (Glotzer et al., 1991). The association of cyclin B1 with the spindle allied with the rapid degradation of cyclin B1 at the end of metaphase strongly implies that the cyclin B1/$p34^{cdc2}$ complex is involved in maintaining the mitotic apparatus, and thus the cell, in metaphase. One can postulate that once the chromosomes have been correctly aligned, cyclin B1 destruction is initiated and the cell exits into anaphase through dephosphorylation of $p34^{cdc2}$ substrates.

Thus we propose that the functions of the different cyclin-protein kinase complexes as regulators of mitosis are determined not only by the different times at which their associated protein kinases are activated, but to a very significant degree by their location in the cell.

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