# cdc25 Is a Nuclear Protein Expressed Constitutively throughout the Cell Cycle in Nontransformed Mammalian Cells

**Franck Girard, Ulrich Strausfeld, Jean-Claude Cavadore, Paul Russell,* Anne Fernandez, and Ned J. C. Lamb**

Cell Biology Unit, Centre de Recherche en Biochimie Macromolèculaire, Centre National de la Recherche Scientifique-Institut National de la Santé Et de la Recherche Médicale, BP5051 34033 Montpellier Cedex, France; and * Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

***Abstract.*** A family of proteins homologous to the cdc25 gene product of the fission yeast bear specific protein tyrosine phosphatase activity involved in the activation of the $p34^{cdc2}$-cyclin B kinase. Using affinity-purified antibodies raised against a synthetic peptide corresponding to the catalytic site of the cdc25 phosphatase, we show that cdc25 protein is constitutively expressed throughout the cell cycle of nontransformed mammalian fibroblasts and does not undergo major changes in protein level. By indirect immunofluorescence, cdc25 protein is found essentially localized in the nucleus throughout interphase and during early prophase. Just before the complete nuclear envelope breakdown at the prophase-prometaphase boundary, cdc25 proteins are redistributed throughout the cytoplasm. During metaphase and anaphase, cdc25 staining remains distributed throughout the cell and excludes the condensed chromosomes. The nuclear locale reappears during telophase. In light of the recent data describing the cytoplasmic localization of cyclin B protein (Pines, J., and T. Hunter. 1991. *J. Cell Biol.* 115:1-17), the data presented here suggest that separation in two distinct cellular compartments of the cdc25 phosphatase and its substrate $p34^{cdc2}$-cyclin B may be of importance in the regulation of the cdc2 kinase activity.

$p34^{cdc2}$ is an implicit component in the regulatory mechanisms which bring about the coordinate division of cells. Present throughout the eukaryotic phyla, $p34^{cdc2}$ appears to undergo distinct activation coincident with the entry of cells into mitosis (Dorée, 1990; Nurse, 1990). This activation event involves its association with one or more regulatory proteins termed cyclins. At least five of such proteins have been reported, although little is known of their precise functions or relationship in the events that bring about $p34^{cdc2}$ kinase induction (Hunt, 1991; Hunter and Pines, 1991; Pines, 1991). In addition to the association with cyclin proteins, $p^{34cdc2}$ kinase activation also involves changes in the phosphorylation state of the $p34^{cdc2}$ catalytic subunit. While different phosphorylation levels can be identified for $p34^{cdc2}$, recent mutational analysis revealed that the dephosphorylation of two particular aminoacid residues at the $NH_2$-terminus of the protein was implicit in the activation of the mitotic form of the $p34^{cdc2}$ kinase (Clarke and Karsenti, 1991; Krek and Nigg, 1991; Norbury et al., 1991). The human homologue of cdc25 has been shown to bring about the specific dephosphorylation and activation of $p34^{cdc2}$-cyclin B kinase in an in vitro assay involving highly purified protein components (Strausfeld et al., 1991). Indeed, dephosphorylation of tyrosine 15 is directly controlled by $p34^{cdc2}$ specific protein phosphatase homologous to the cdc25 gene product of *Schizosaccharomyces pombe* (Dunphy and Kumagai, 1991; Gautier et al., 1991; Lee et al., 1992; Millar et al., 1991; for review see Millar and Russell, 1992).

We have previously shown that the presence of activated $p34^{cdc2}$ kinase in synchronized mammalian cells was sufficient to induce phenotypic changes similar to those observed during prophase (Lamb et al., 1990). Since $p34^{cdc2}$ protein levels appear to remain constant throughout the cell division cycle of mammalian cells, this would imply that regulation of $p34^{cdc2}$ kinase activity must involve, in part, differential synthesis of factors which bring about the activation of the kinase. In the present study, we have examined the expression and cellular distribution of cdc25 protein to determine if fluctuations during the cell cycle may play a role in the control of $p34^{cdc2}$ activity in nontransformed mammalian cells. Immunoblotting and indirect immunofluorescence analysis reveals that as in the transformed HeLa cells (Millar et al., 1991), cdc25 protein levels remain constant throughout the cell cycle, implying that posttranslational modifications and/or compartmentalization mechanisms must be involved in the activation of cdc25 phosphatase.

## Materials and Methods

### Peptide Synthesis

Solid phase synthesis of CEFSSKRGPDLLR peptide (referred to as C5XR1)

Franck Girard and Ulrich Strausfeld have contributed equally to this paper.

was performed on a 2050 Milligen Synthesizer (Millipore, France) with the use of polyacrylic resin (Calas et al., 1985), 9-fluorenyl methyl oxycarbonyl as temporary α-amino acid group protection, and benzotriazol-l-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate as coupling reagent. Peptide was desalted on Sephadex G10 column and purified on reverse phase high pressure liquid chromatography on a Nucleosil C18 column using a linear gradient of 0.1% aqueous trifluoroacetic acid with acetonitrile. Purified peptide was coupled to thyroglobulin or to BSA by the use of m-maleydobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce Chemical Co., Arlington Heights, IL). Peptide-protein conjugates were filtered on PD10 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in PBS.

### *Polyclonal Antibody Production and Purification*

Male New Zealand rabbits were injected subcutaneously with 300 μg of thyroglobuline-C5XR1 peptide conjugate in complete Freund adjuvant. Animals were boosted every 2 wk with 100 μg of the same preparation in incomplete Freund adjuvant, and bled 8–12 d after each injection. Antisera were purified using BSA–peptide conjugate coupled to CNBr-sepharose 4B (Pharmacia Fine Chemicals) as described elsewhere (Girard et al., 1991).

### *Cell Culture and Synchronization*

Rat embryonic fibroblasts REF52 (McClure et al., 1982) and human fibroblasts HS68 (CRL-1365) were cultured in DME supplemented with 7% FCS, Hela cells in 10% FCS DME, in 60-mm plastic dishes and acid-washed glass coverslips. Cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$/95% air, until they reached 75% confluence. REF52 and HS68 cells were then made quiescent through 30 h of serum deprivation. Synchronized cells were prepared as follows: quiescent cells were restimulated through serum addition, and grown at 37°C then prepared for Western blot or immunofluorescence at 1-h intervals following serum stimulation.

### *Western Blotting*

Synchronized cells obtained as mentioned above were washed in PBS at room temperature and scraped in SDS boiling sample buffer (Lamb et al., 1990). Cell extracts were separated on 12% SDS-PAGE and proteins were transferred to nitrocellulose. Nitrocellulose sheets were saturated in 3% nonfat dried milk in 25 mM Tris-HCl, pH 7.5, 200 mM NaCl (TBS) for 1 h, then incubated with anti-cdc25 peptide antibody αC5XR1 (concentration 0.67 mg/ml) diluted 1:2,000 in the same buffer for 1 h at room temperature. After washes in TBS containing 0.05% Tween 20, HRP-conjugated anti-rabbit (Amersham Corp.) was added 1:10,000 for 1 h. Detection of the immune signal was done using ECL Western blotting detection system (Amersham Corp.). For competition experiments, 7 μg of αC5XR1 were reacted with 75 μg of the immune peptide in a final volume of 200 μl for 1 h at room temperature, then diluted 1:2,000 in TBS and used for Western blotting as described above.

### *Immunofluorescence*

Cells growing onto acid-washed coverslips were fixed in 3.7% formalin in PBS for 5 min, followed by extraction in −20°C acetone for 1 min. After washes in PBS and saturation in PBS containing 1% BSA for 5 min, incubation with anti-cdc25 antibody αC5XR1 was performed for 1 h at 37°C. Reaction was followed by incubating biotinylated antirabbit (Amersham Corp.) for 1 h then streptavidin Texas red conjugated (Amersham Corp.) for 30 min. After washes in PBS, cells were incubated 1 min in PBS/BSA containing Hoechst dye (1 μg/ml) to visualize DNA, washed in water, and mounted in Airvol. Fluorescent and phase-contrast micrographs were recorded on Zeiss Axiophot using Kodak Tri X pan and Technical pan, respectively.

For competition experiment, 20 μg of cdc25 peptide were incubated with anti αC5XR1 (concentration 0.67 mg/ml, dilution 1:100, final volume 100 μl) for 1 h at room temperature. This solution was then used as primary antibody and the immunofluorescence reaction was continued as described above. For double immunofluorescence staining for cdc25 and lamins A and C, serum synchronized HS68 cells were fixed in formalin and acetone extracted 22 h after serum restimulation, then incubated with αC5XR1 antibodies and monoclonal antihuman lamins A and C (Heald and McKeon, 1990). Reaction was continued with the incubations of biotinylated antirabbit antibodies followed by streptavidin Texas red, and fluorescein-conjugated goat antimouse antibodies (Organon Technika, Fresnes, France). Cells were stained for DNA with Hoechst dye before mounting as described elsewhere (Girard et al., 1991).

## *Results*

### *Expression of cdc25 Protein in Nontransformed Mammalian Fibroblasts*

To examine the expression and cellular distribution of the cdc25 gene product in mammalian cells, we have raised antibodies to a peptide sequence of cdc25 protein conserved among various species. This sequence CEFSSKRGPDLLR (referred to as C5XR1) (Fig. 1 *B*) has been shown to belong to the phosphatase consensus site of cdc25 (Gautier et al., 1991; Millar et al., 1991); particularly, the motif C5XR is conserved throughout evolution and is also present in other protein phosphatases such as human T cell PTP or vaccinia virus PTP (Guan et al., 1991; Streuli et al., 1990), and represents the catalytic site of the cdc25 phosphatase activity. The C5XR1 sequence is identical to the corresponding region of a starfish cdc25 protein that was determined by DNA sequence analysis of starfish cdc25 gene, cloned by PCR using degenerate primers as previously described (Sadhu et al., 1990). We have also synthesized a second peptide, -C5XR2 (Fig. 1 *B*), representing the consensus sequence for the cdc25 family in this region. Rabbits were immunized with peptide C5XR1 coupled to thyroglobuline, and antiserum was affinity purified using C5XR1–BSA conjugates coupled to Sepharose. The specificity of the resulting antibody (referred to as αC5XR1) is shown in Fig. 1 *A*. By Western blot, αC5XR1 detects a major protein species of relative molecular mass of 67 kD in G2-phase human fibroblasts HS68 cell extract (Fig. 1, lane *1*), a second smaller protein species at 38 kD as revealed by a longer exposure of the ECL blot (Fig. 1, lane *5*), and three protein species in G2-phase REF52 cell extract (Fig. 1, lane *2*) of relative molecular mass 75, 67, and 38 kD. The same three bands are also observed in HeLa cell extract. (data not shown). Preincubation of αC5XR1 antibodies with C5XR1 and C5XR2 peptides before Western blot completely abolished all three protein species in REF52 cells (Fig. 1, lane *4*), and both the 38 and 67 kD form in HS68 cells (Fig. 1, lane *3*), confirming the specificity of the affinity-purified antiserum. As presented in Fig. 1 (lane *6*), αC5XR1 antibody is also able to detect bacterially expressed human $p55^{cdc25}$. αC5XR1 antibodies were also specifically reactive on immunoblot against human cdc25 protein, immunoprecipitated from HeLa cell extracts with different polyclonal antibodies raised against the entire human cdc25 protein. Furthermore, these antibodies are also capable of inhibiting cdc25 phosphatase activity in a $p34^{cdc2}$-cyclin B kinase dephosphorylation assay (data not shown). The major 67-kD form probably represents the full-length cdc25 gene product, while the 38-kD form is likely to be a fragment resulting from partial proteolysis. The upper 75-kD form seen in REF52 cells might be another form of cdc25 protein, since a protein species of similar molecular mass is observed in HeLa cells (Galaktionov and Beach, 1991), and several cdc25 genes have been isolated in human cells (cdc25 A and B, Galaktionov and Beach, 1991; cdc25 C, Sadhu et al., 1990; Nagata et al., 1991). All these results have been confirmed with two other polyclonal anti-cdc25 antibodies, raised against bacterially expressed hu-

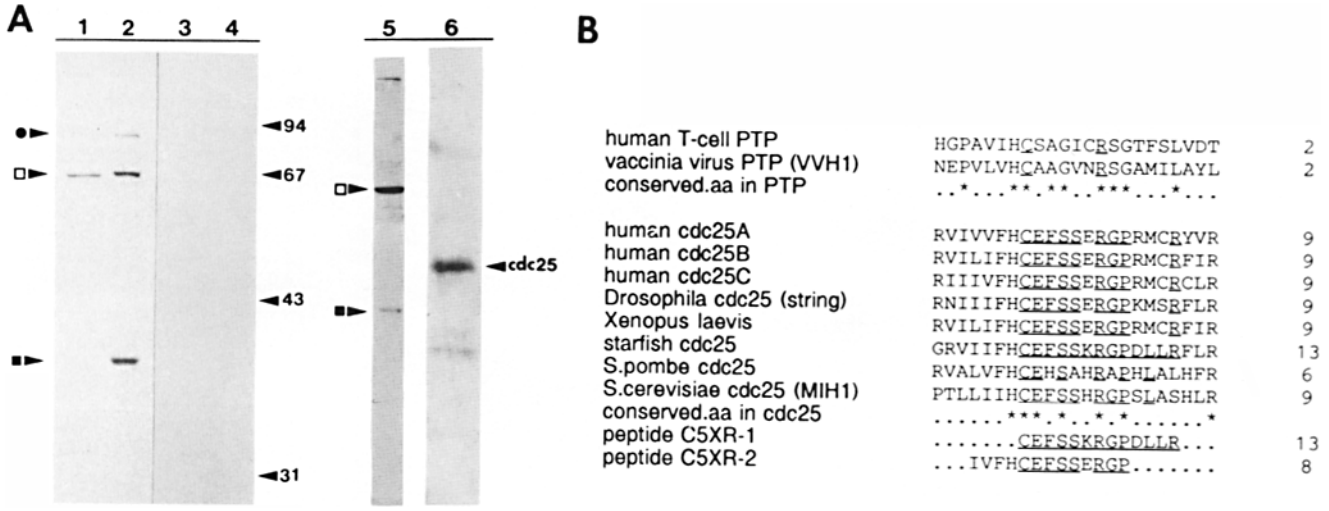


*Figure 1.* Characterization of the affinity-purified anti-cdc25 peptide antibodies. Peptide sequence termed C5XR1, corresponding to the putative catalytic site of the starfish cdc25 protein phosphatase, was synthesized as described in Materials and Methods and used to immunize rabbits. Polyclonal antiserum was affinity purified using C5XR1-BSA conjugated coupled to sepharose, and used for Western blotting as described in Materials and Methods, using the ECL system (Amersham Corp.) for detection of the immune signal. (*A*) Western blot of cell extracts from G2-phase human HS68 fibroblasts (lane *1*) and G2-phase REF52 cells (lane *2*) using anti-cdc25 peptide antibody αC5XR1. Arrowed on the left is the main protein species detected both in REF52 and HS68: open square at 67 kD together with two additional bands detected in REF52 cells, one at 75 kD (*closed circle*) and one at 38 kD (*closed square*), also detected in HS68 cells after a longer exposure of the ECL blot (lane *5*). Molecular masses are given in kilodaltons. Preincubation of anti-cdc25 antibodies with peptides C5XR1/C5XR2 prior to Western blot completely abolished immune signals in both G2-phase HS68 (lane *3*) and REF52 cells (lane *4*). (Lane *6*) Western blot of bacterial lysate containing human $p55^{cdc25}$ protein using anti-cdc25 peptide antibody αC5XR1. Arrowhead is the $p55^{cdc25}$ band. (*B*) Sequences of the peptides C5XR1 and C5XR2 are shown in comparison with cdc25 protein sequences from various species, and with different tyrosine phosphatases in the region corresponding to the catalytic site. Conserved amino acids are indicated by an asterisk. Amino acids identical to C5XR1 sequence are underlined. Also given on the right is the total number of amino acids identical to the one in the C5XR1 sequence.

man cdc25 protein (Millar et al., 1991). Finally, we have controlled the absence of cross-reactivity of αC5XR1 towards other nonrelated tyrosine phosphatase. In the conditions used to detect by blot cdc25 proteins, we have observed no cross-reactivity with tyrosine phosphatase type 1B (data not shown).

To assess the expression profile of cdc25 protein during the cell cycle of nontransformed mammalian cells, we have used REF52 and HS68 cells synchronized through serum deprivation for 30 h. Under these conditions for cell synchronization, we have shown elsewhere (Lamb et al., 1990; Girard et al., 1991) that G1-phase covers the period 0–16 h in REF52, S-phase the period 16–19 h (peaking at 18 h), and G2-phase the period 19–24 h when cells enter mitosis. At different times after serum addition, cells were lysed and the presence of cdc25 protein was examined through immunoblotting using αC5XR1. Fig. 2 shows a typical expression profile for cdc25 protein in HS68 (Fig. 2 *A*) and REF52 cells (Fig. 2 *B*). In HS68, the major 67-kD protein species is detected at all times after serum addition (Fig. 2 *A*), together with the less abundant 38-kD protein (data not shown), a result also observed in REF52 cells together with the 38- and 75-kD bands (Fig. 2 *B*). Quantification of the immunoblot by scanning revealed that no major change in the level of the cdc25 67-kD protein occurs during the cell cycle (Fig. 2 *A*). In contrast, the smaller band at 38 kD appears to undergo changes in levels with a detectable increase at a time corresponding to S-phase, and a second increase at 24 h, corresponding to mitosis in REF52 cells. Such changes, however, are not detected in the levels of the 38-kD protein present as a minor band in HS68 cells (data not shown). While the significance of these variations in REF52 cells is not clear, they might be relevant if this 38-kD fragment still carries the catalytic activity of cdc25 protein, a point that requires further investigations. To confirm the good synchronization of the cells in this study, we blotted the same cell extracts with anticyclin A antibody, and showed that cyclin A is detected from 16–24 h in REF52 cells, and 14–24 h in HS68 (not shown). Similarly, the levels of $p34^{cdc2}$ show little or no changes throughout the cell cycle in these synchronized REF52 and HS68 cell extracts (not shown).

### *Cytolocalization of cdc25 Protein in Nontransformed Mammalian Cells*

Since we have observed no changes in cdc25 protein levels during the cell cycle of nontransformed fibroblasts, we next examined cdc25 intracellular distribution by indirect immunofluorescence using the same αC5XR1 antibody. REF52 and HS68 cells growing onto glass coverslips and synchronized exactly as described above were used. In REF52 cells, cdc25 is essentially localized in the nucleus at all stages of interphase, with a low level of cytoplasmic staining similar in intensity to the background cytoplasmic staining obtained with the preimmune serum (data not shown). Fig. 3 shows the staining obtained with anti-cdc25 peptide antibody in G1- (*A* and *B*) and S-phase cells (*C* and *D*). Detailed analysis revealed cdc25 staining distributes evenly throughout the nuclear material. We observed no evidence that this staining was excluded from the nucleoli (in marked contrast with cyclin A protein). Furthermore, cdc25 staining also ap-

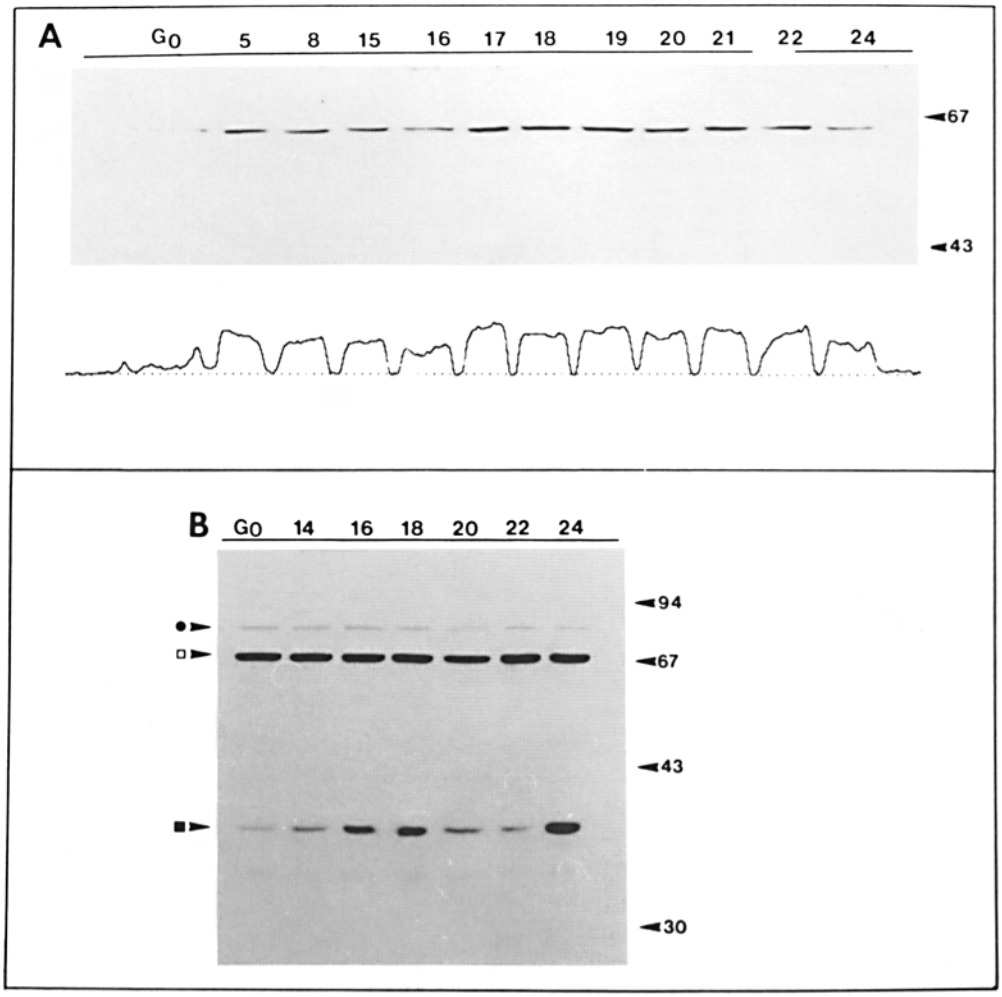


*Figure 2.* cdc25 is constitutively expressed throughout the cell cycle of nontransformed mammalian fibroblasts. Cell cycle expression profile of cdc25 protein was examined through Western blotting of whole cell extracts from HS68 (*A*) and REF52 cells (*B*), prepared from synchronized populations of cells. Synchronization was achieved through 30 h of serum deprivation, then restimulation through serum addition. Cells were scraped in boiling SDS sample buffer at the indicated times (in hours) following serum stimulation. Proteins were separated on 12% SDS-PAGE, transferred to nitrocellulose, and probed for the presence of cdc25 proteins with αC5XR1. The symbols represent the different protein species detected in REF52 and HS68 cells as described in Fig. 1. Molecular masses are given in kilodaltons. Quantification of the immune signal obtained in HS68 cell extracts was done using Shimadzu Scanner, and is referred to the Western blot in *A*, at the same indicated times.

pears to concentrate at the periphery of the nucleus as if associated with the nuclear envelope. More detailed analysis revealed that this particular staining represents a fine punctate pattern made up of numerous spots of fluorescence, particularly discernable in G2-phase and early prophase before the nuclear envelope breakdown (Figs. 4, *A* and *B* and 5, *A* and *B*). Similar staining is also observed in human HS68 fibroblasts at all stages of interphase (shown in Fig. 4, *E* and *F* are G2-phase HS68 cells stained with αC5XR1). To confirm this nuclear localization, we incubated affinity-purified antiserum with peptide C5XR1 alone, or with a mixture of both C5XR1 and C5XR2 peptides, before immunofluorescence experiments. As shown in Fig. 4 (*C* and *D*), preincubation completely eliminates nuclear staining, including that associated with the nuclear envelope, thus, strongly suggesting that both the staining within the nucleus and at the nuclear periphery represent the true locale of the cdc25 protein in REF52 cells. This localization of cdc25 protein was confirmed using the antibodies directed against the purified human cdc25 protein using REF52, HS68 and HeLa cells (data not shown). If the conditions were changed to use either simultaneous extraction/fixation in −20°C methanol or formalin fixation followed or preceded by extraction in 1% Triton X100, we observed no difference in the nuclear pattern of distribution of cdc25 staining (not shown).

To examine the distribution of cdc25 protein during mitosis, REF52 cells were fixed 24 h after serum stimulation and stained by indirect immunofluorescence with anti-cdc25 peptide antibodies. Fig. 5 shows fluorescent micrographs obtained with αC5XR1 and Hoechst DNA staining of the same cells at various stages of mitosis. During early prophase, as chromatin condensed, cdc25 staining remains essentially nuclear, retaining the more intense peripheral nuclear staining (Fig. 5, *A* and *B*). We observed no evidence of an association with chromatin as it condenses, as judged by comparing cdc25 staining (*A*) and Hoechst DNA staining (*B*) of the same cell. As nuclear envelope completely dissolves, cdc25 staining spreads throughout the cytoplasm, with a more intense staining in the region corresponding to the mitotic spindle (shown in Fig. 5 are a prometaphase [*C* and *D*] and a metaphase cell [*E* and *F*]). As the duplicated chromosomes move apart during anaphase, cdc25 staining remains distributed in the whole cell, with a more intense staining of the material associated with the peripheral chromatin (Fig. 5, *G* and *H*). Once anaphase is completed and the two daughter cells begin to reform a nuclear envelope, cdc25 staining again becomes clearly relocalized within the nucleus, as the level of cytoplasmic staining diminishes to a level similar to that observed in interphase cells (Fig. 5, *I* and *J*). These observations have been reproduced using the polyclonal anti-

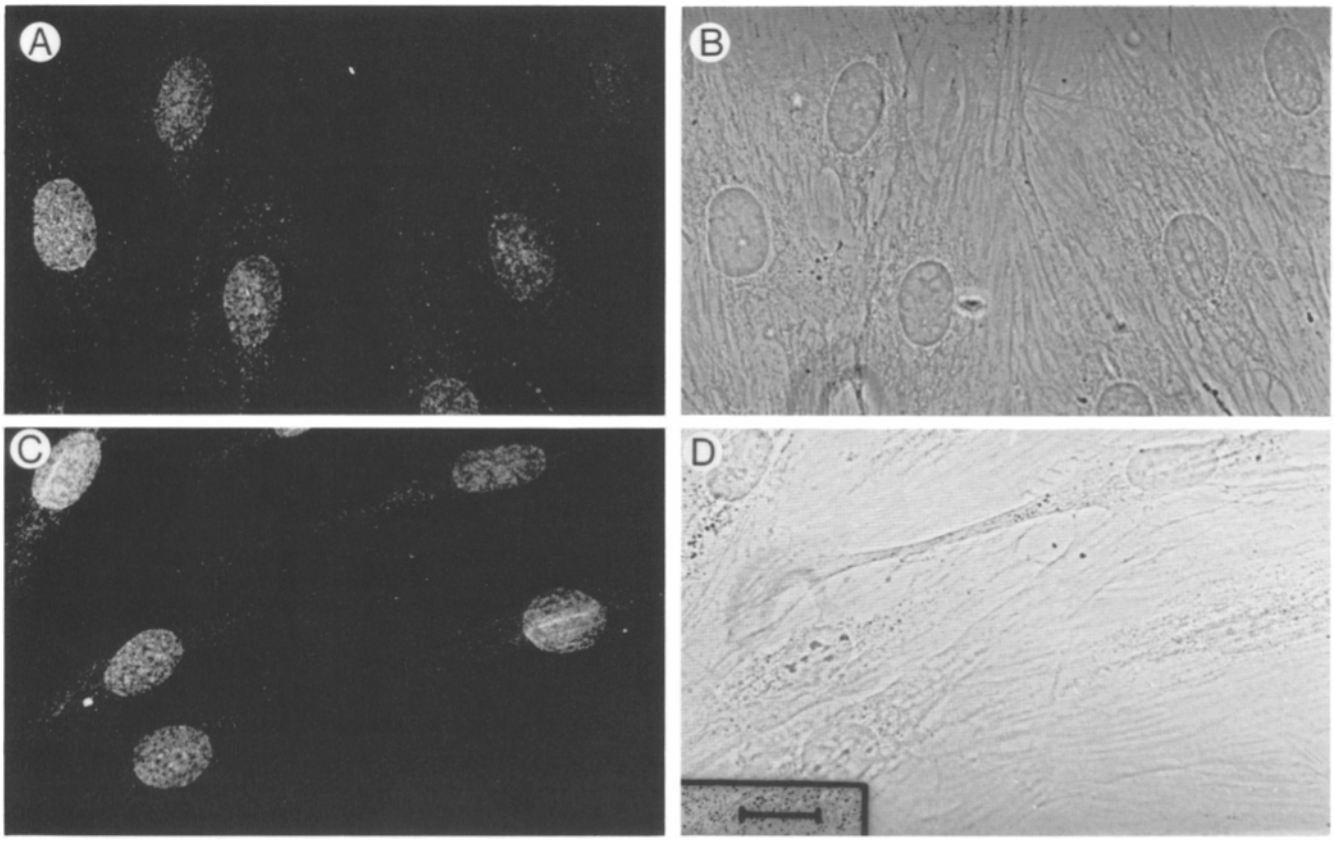


*Figure 3.* Nuclear distribution of cdc25 protein during G1 and S-phase. To determine the intracellular distribution of cdc25 protein during the mammalian cell cycle, REF52 cells were synchronized through 30 h of serum deprivation. Subsequently, cells were restimulated through serum addition and formalin fixed at different intervals after serum addition. Cells were stained by indirect immunofluorescence with αC5XR1, followed by incubations with biotinylated anti-rabbit and streptavidin Texas red as described in Materials and Methods. Shown are fluorescent micrographs (*A* and *C*) and phase contrast (*B* and *D*) of G1-phase (*A* and *B*) and S-phase REF52 cells (*C* and *D*), fixed and stained, respectively, 7 and 17 h after serum stimulation. Bar, 5 μm.

bodies against human cdc25. We have also found a similar profile of distribution with HS68 cell line using αC5XR1 (data not shown). To examine in more detail the kinetics of the changes in cdc25 protein distribution during prophase, we have performed double immunofluorescence experiments in which HS68 cells were stained for cdc25 protein with αC5XR1 together with staining for lamins with a mAb against human lamins A and C. Results are presented in Fig. 6. cdc25 staining is still essentially localized in the nucleus in early prophase (Fig. 6 *A*), as judged by the low level of chromatin condensation (Fig. 6 *C*) and lamin staining restricted to the nucleus (Fig. 6 *B*). At the transition from prophase to prometaphase as some lamin staining begins to distribute throughout the cytoplasm with the nuclear envelope still clearly defined (Fig. 6 *E*), there is already a dramatic decrease in the cdc25 nuclear staining (Fig. 6 *D*). In this late prophase cell, in which chromatin is highly condensed (Fig. 6 *F*) and nuclear envelope not completely dissolved, we observe a quantitative increase in the cytoplasmic cdc25 staining (Fig. 6 *D*). These observations suggest that part of cdc25 proteins leaves the nucleus to be redistributed into the cytoplasm before the complete loss of the nuclear envelope.

Since there appears to be a close liaison between the localization of cdc25 in the nuclear periphery and the nuclear envelope, cells were analyzed by confocal scanning laser microscopy to identify differences in locale between cdc25 and the nuclear envelope. Cells were fixed and stained for the locale of cdc25 as described in Materials and Methods. After determining the overall sectional thickness of the nucleus (locating the uppermost point and lowest point at which cdc25 fluorescence could be observed, on average 2.2–2.5 μm), sectional planes of 300 nm were taken every 0.5 μm through the nucleus (six on average). For each sectional plane, a summation of 70 images was taken. Shown in Fig. 7 are three sections through a single nucleus at, respectively, point 0 (the uppermost section which just cuts into the nucleus) (*A*), 1.5 μm (the center of the nucleus) (*B*), and point 2.5 μm (the lowest section of the nucleus) (*C*). Clearly, comparing *B* to *A* and *C*, cdc25 can be localized both throughout the nucleus and to the nuclear envelope. Since this is a single section through the center of the nucleus, there can be no contribution of the fluorescence in either upper or lower planes. From these data we can conclude that cdc25 protein effectively localized both intranuclearly and at the periphery of the nucleus. In addition, section in Fig. 7 *B* shows that there is little cdc25 staining in the nucleus.

## Discussion

Proteins homologous to the product of the *S. pombe* cdc25 gene are protein phosphatases that can activate the $p34^{cdc2}$/

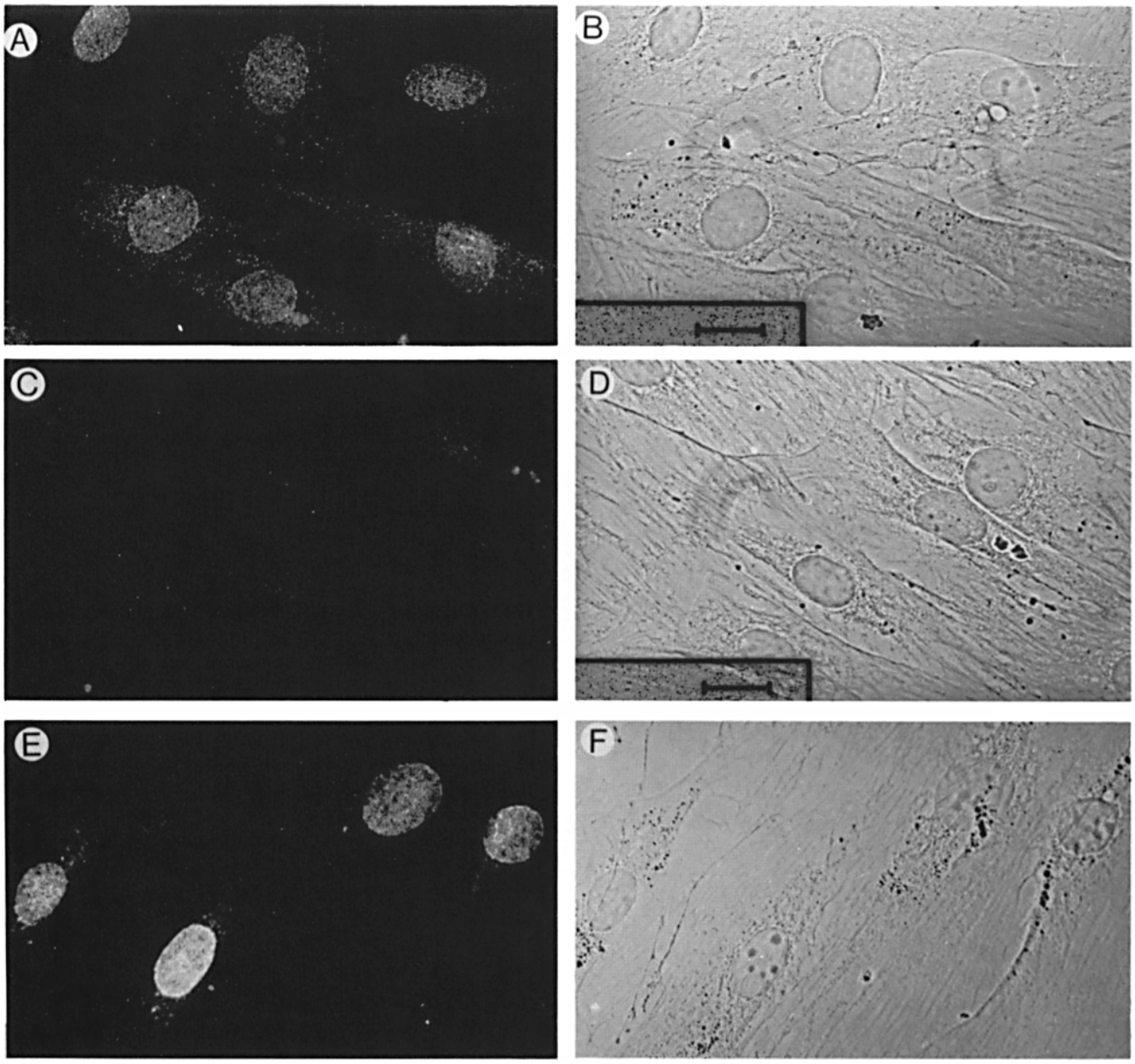


*Figure 4.* Nuclear distribution of cdc25 protein during G2-phase. Cellular distribution of cdc25 protein was examined during G2-phase of the cell cycle. Serum synchronized REF52 and HS68 cells were formalin fixed during G2, respectively, 22 and 20 h after serum stimulation, and stained with αC5XR1 as described in Fig. 3. (*A* and *B*) fluorescent micrographs and phase contrast of G2-phase REF52 cells stained with αC5XR1. (*C* and *D*) G2-phase REF52 cells stained with αC5XR1 preincubated with C5XR1 peptide. (*E* and *F*) Fluorescent micrographs and phase contrast of G2-phase HS68 cells stained with αC5XR1. Bar, 5 μm.

cyclin B kinase by dephosphorylation of the $p34^{cdc2}$ subunit. To characterize both the expression and cellular localization of cdc25 proteins during the cell cycle of nontransformed mammalian cells, we have developed antibodies directed against a peptide sequence C5XR-1, a highly conserved motif within the catalytic domain of the cdc25 gene family present from yeast to man. Between the three different isoforms of human cdc25 proteins recently identified (named cdc25-A, -B, -C; Sadhu et al., 1990; Galaktionov and Beach, 1991; Nagata et al., 1991), this region is 100% identical. In light of this high level of conservation we anticipated that antibodies directed against C5XR-1 would detect different forms of cdc25 in different mammalian species with similar sensitivity. Two amino acids within this region (cysteine and arginine in position 1 and 7 of C5XR-1, respectively) are not only conserved among members of the phosphotyrosine phosphatase gene family, but have also been shown necessary for both the biological function and tyrosine phosphatase activity of cdc25 (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991), and for the activity of tyrosine phosphatases in general (Streuli et al., 1990; Guan et al., 1991). It is believed that these amino acids are directly involved in binding and releasing of phosphate groups by tyrosine phosphatases (Streuli et al., 1990; Guan and Dixon, 1991). Consequently, we have based our choice of peptide on raising antisera that recognize this putative catalytic site

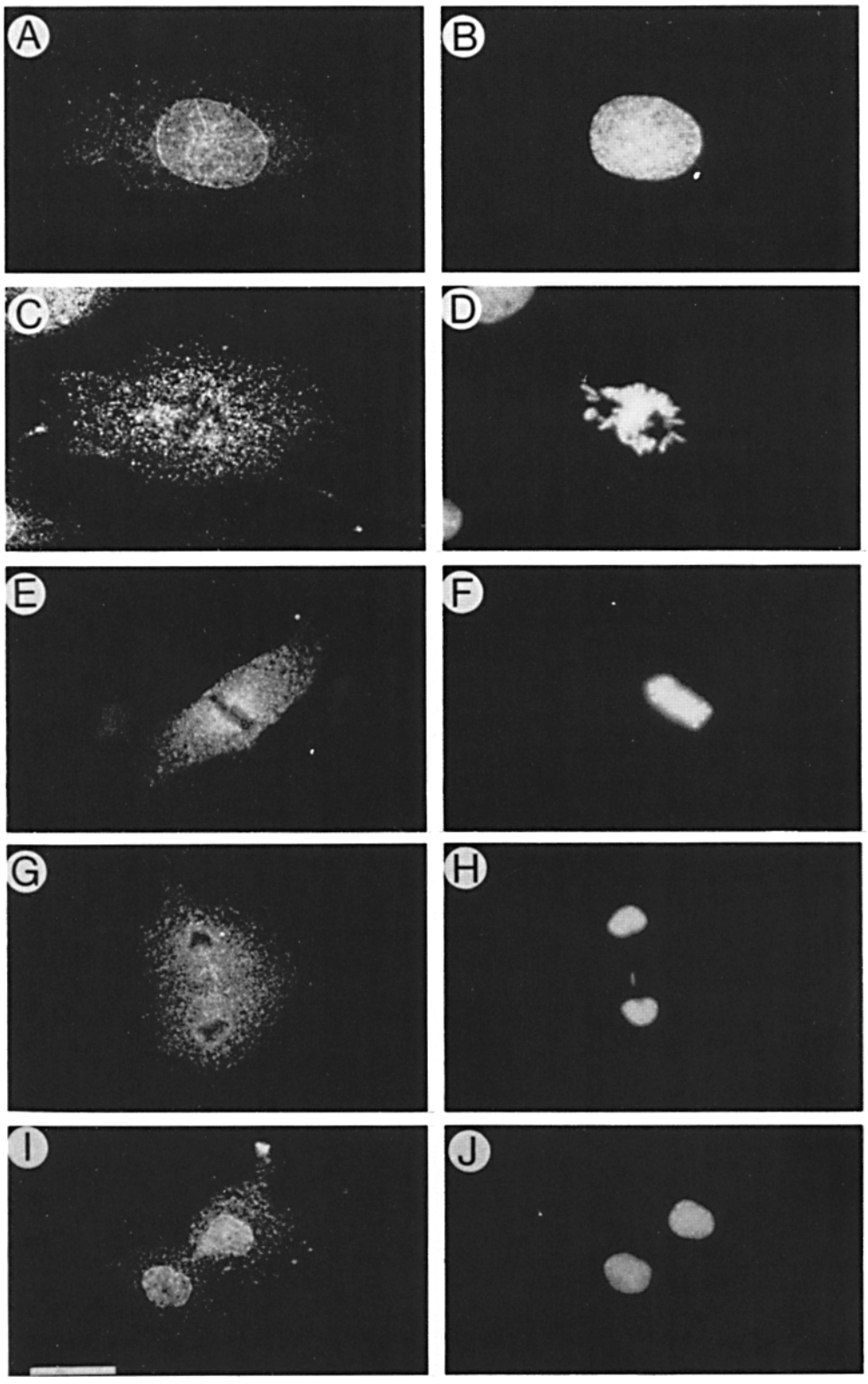


*Figure 5.* Cellular distribution of cdc25 protein during mitosis. To examine the intracellular locale of cdc25 protein during the different phases of mitosis, REF52 cells were synchronized through 30 h of serum deprivation, then restimulated upon serum addition, and formalin fixed followed by acetone extraction 24 h thereafter. Localization was determined by indirect immunofluorescence with αC5XR1, followed by incubations with biotinylated antirabbit and streptavidin Texas red as described in Materials and Methods. Before mounting, cells were stained with Hoechst dye to visualize the DNA. Shown are fluorescent micrographs (*A, C, E, G, I*) and DNA staining (*B, D, F, H, J*) of an early prophase cell (*A* and *B*), a prometaphase cell (*C* and *D*), a metaphase cell (*E* and *F*), an early anaphase cell (*G* and *H*) and a telophase cell (*I* and *J*), respectively. Bar, 5 μm.

on cdc25 proteins. Indeed, as expected we observed that binding of anti-C5XR-1 antibodies to cdc25 results in strong inhibition of the phosphatase activity as assayed by dephosphorylation of paranitrophenylphosphate (pNPP) or activation of highly purified starfish p34cdc2/cyclin B prokinase by cdc25 (U. Strausfeld, F. Girard, and N. Lamb, unpublished observations).

We have used these affinity-purified antibodies to determine both the timing of expression and cellular distribution of the cdc25 protein in two nontransformed mammalian fibroblast cell lines: rat REF52 and human HS68. Our results clearly show that the expression of the cdc25 67 kD protein is constant throughout the cell cycle. Furthermore, we have shown that in these cells, cdc25 proteins are essentially

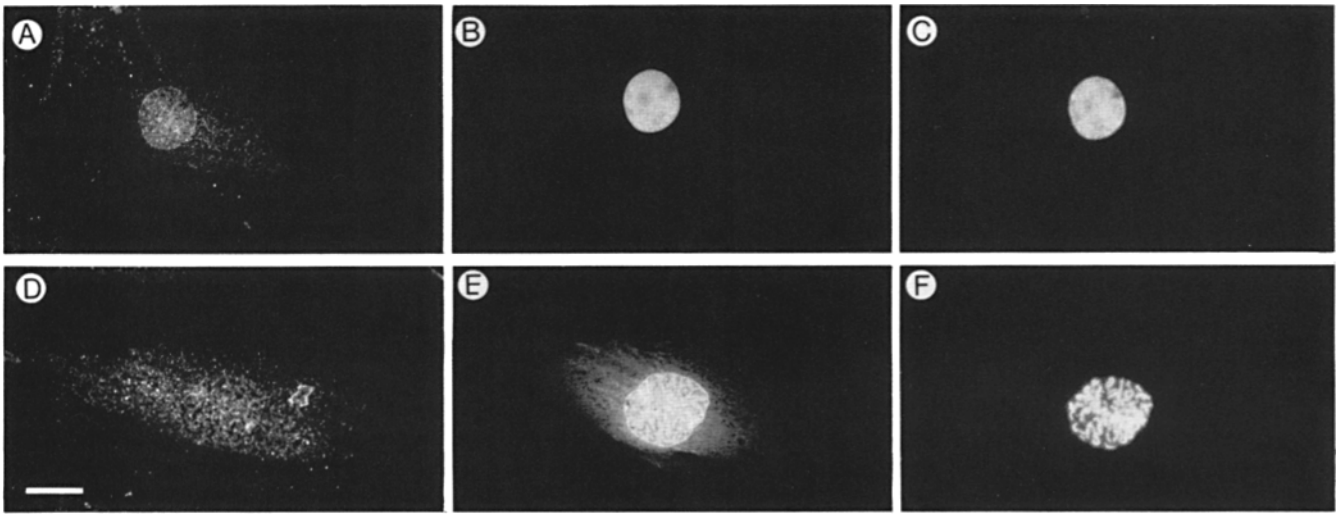


*Figure 6.* A proportion of cdc25 proteins leave the nucleus before the complete nuclear envelope breakdown. Cellular distribution of cdc25 protein during the early phases of mitosis was examined in serum-synchronized human HS68 cells, formalin fixed and acetone extracted 22 h after serum stimulation. Cells were costained for both cdc25 using αC5XR1 and lamins using monoclonal antihuman lamins A and C as described in Materials and Methods, and stained for DNA with Hoechst dye before mounting. (*A* and *D*) cdc25 staining; (*B* and *E*) lamin staining; and (*C* and *F*) DNA staining of prophase HS68 cells. *A–C* show staining of an early prophase cell; *D–F* show a late prophase cell. Bar, 5 μm.

localized into the nucleus throughout interphase. This nuclear locale remains until just before the complete nuclear envelope breakdown. At this time (end of prophase), staining for cdc25 becomes dispersed throughout the cytoplasm of these mitotic cells. During telophase, the cdc25 proteins relocate into the reforming nuclei. Although it has previously been shown that the levels of cdc25-C transcription change during the course of the HeLa cell cycle with an increase in cdc25 mRNA in G2-phase (Sadhu et al., 1991), an observation also made in fission yeast for both protein and mRNA levels (Moreno et al., 1990), our results are in agreement with the recent report of Russell and co-workers describing no major change in the level of cdc25 protein during the HeLa cell cycle (Millar et al., 1991). These discrepancies might arise partly from the nature of cdc25 protein species analyzed, since it is now evident that cdc25 proteins form part of a gene family, with three different genes identified to date in human cells (Sadhu et al., 1990; Galaktionov and Beach, 1991; Nagata et al., 1991).

The nuclear distribution of cdc25 protein phosphatase is of importance with respect to the time and locale at which $p34^{cdc2}$-cyclin B kinase is activated through dephosphorylation by cdc25. Cyclin B appears to be localized in the cytoplasm of HeLa cells, until just before nuclear envelope breakdown at prophase, when cyclin B proteins translocate to the nucleus, and is found associated to the mitotic spindle at metaphase (Pines and Hunter, 1991). We have observed similar results in two different human cell lines (human embryonic fibroblasts and HS68) with the difference that we never saw any association of cyclin B with the condensed chromatin in the mitotic cells (F. Girard, U. Strausfeld, A. Fernandez, and N. Lamb, unpublished observations). Our

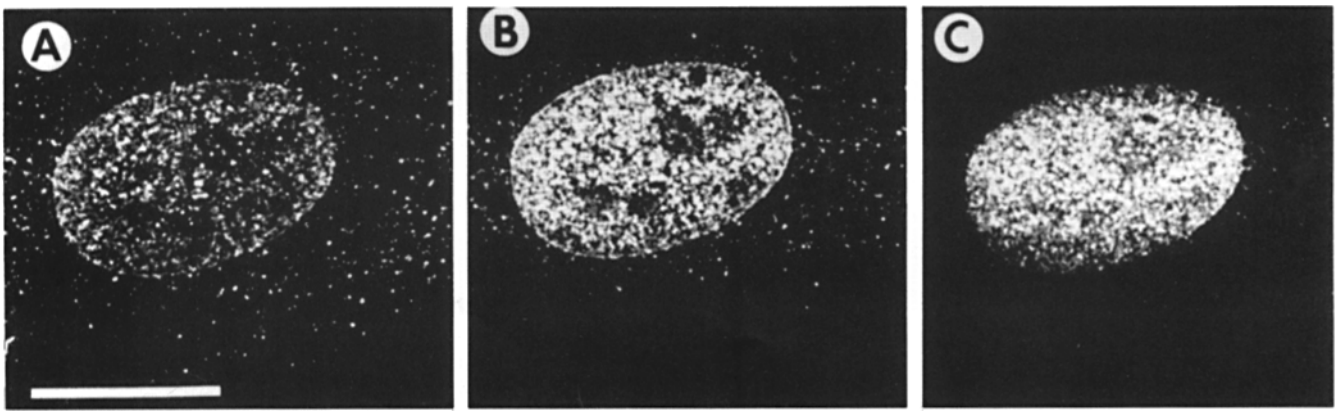


*Figure 7.* Analysis of the intranuclear localization of cdc25 protein by confocal scanning laser microscopy. G2-phase human fibroblasts HS68 were formalin fixed and acetone extracted as described in Materials and Methods. Cells were sequentially incubated with anti-cdc25 antibody (αC5XR1), biotinylated antirabbit antibody, and Streptavidine-conjugated Texas red. Cells were examined by confocal scanning laser microscopy (Biorad MRC5 microscope). Shown are three sections over a single nucleus. (*A*) Upper section; (*B*) section corresponding to the center of the nucleus; (*C*) lower section. Bar, 5 μm.

data precisely document the distribution of cdc25 protein in the course of the different phases of mitosis. In particular, we have observed two features. First, cdc25 protein seems to be excluded from the condensed chromosomes in all stages of mitosis. Second, it appears to transiently localize in the region of the mitotic spindle. These two features of cdc25 staining at mitosis are similar to the localization of cyclin B we have observed during mitosis in the same cell lines (F. Girard, U. Strausfeld, A. Fernandez, and N. Lamb, unpublished observations). With respect to the mitotic spindle distribution, this is also in agreement with the recent report in HeLa cells (Pines and Hunter, 1991). However, this report also states that cyclin B associates to the chromosomes, while we have clearly observed the opposite. A close correlation between the locale of cdc25 phosphatase and cyclin B in mitotic mammalian cells would be in good agreement with a recent report showing the physical association of cyclin B and cdc25 protein as a complex during mitosis in *Xenopus* oocytes (Jessus and Beach, 1992). Therefore, $p34^{cdc2}$-cyclin B complexes must accumulate in an inactive form into the cytoplasm during G2-phase. In addition, traffic through the nuclear pores is essential for the activation of $p34^{cdc2}$ by cdc25-dependent tyrosine dephosphorylation, as shown by blocking nuclear transport with wheat germ agglutinin, a lectin that binds to glycoproteins of the nuclear pores, which inhibits $p34^{cdc2}$ dephosphorylation, but has no effect on $p34^{cdc2}$ tyrosine phosphorylation (Dunphy and Kumagai, 1991). We can postulate that $p34^{cdc2}$-cyclin B kinase activation by cdc25 phosphatase involves one of two possibilities. Either it takes place in the cytoplasm, following the diffusion of cdc25 proteins from the nuclear fraction, an event detected only after the nuclear envelope has begun to disassemble (Fig. 6); or it takes place in the nucleus where $p34^{cdc2}$-cyclin B complex translocates just before any evidence of free lamin staining appears in the cytoplasm. The fine timing of these changes in locale detected for cyclin B and cdc25 would more favor the second possibility. However, it is certainly harder to detect precisely the time at which cdc25 first appears in the cytoplasm, since it involves an immediate volume dilution, than it is to assess the reciprocal change involving staining for a protein in the nucleus. We have indeed observed a substantial decrease in the levels of nuclear cdc25 staining as cells progress from early to late prophase, with both the affinity-purified αC5XR1 antibodies and polyclonal antiserum against human cdc25 protein (IgG fraction). However, since the anti-cdc25 antibodies we have used in these studies are raised against the catalytic site of cdc25 protein phosphatase, and presumably dephosphorylation of $p34^{cdc2}$-cyclin B kinase involves the binding of the cdc25 phosphatase to $p34^{cdc2}$, thus masking the C5XR1 epitope, we may explain the decrease in the cdc25 staining observed in late prophase as resulting from this binding event. Therefore, more detailed examination would be necessary to determine where the cdc25-dependent activation of $p34^{cdc2}$-cyclin B kinase occurs.

Like cdc25 protein phosphatase and in contrast with cyclin B-dependent kinase, cyclin A-dependent kinase is also strictly localized into the nucleus (Girard et al., 1991; Pines and Hunter, 1991). It is still unclear if cdc25 phosphatase is also involved in the process of dephosphorylation/activation of p33cdk2-cyclin A kinase. This might be of importance in the understanding of the mechanisms that coordinate the distinct roles of both cyclins A- and B-dependent kinases in the course of the cell cycle.

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