

Immunocytochemical Localization of Casein Kinase II during Interphase and Mitosis

Il Je Yu, David L. Spector, Young-Seuk Bae, and Daniel R. Marshak

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Abstract. We have developed specific antibodies to synthetic peptide antigens that react with the individual subunits of casein kinase II (CKII). Using these antibodies, we studied the localization of CKII in asynchronous HeLa cells by immunofluorescence and immunoelectron microscopy. Further studies were done on HeLa cells arrested at the G1/S transition by hydroxyurea treatment. Our results indicate that the CKII α and β subunits are localized in the cytoplasm

during interphase and are distributed throughout the cell during mitosis. Further electron microscopic investigation revealed that CKII α subunit is associated with spindle fibers during metaphase and anaphase. In contrast, the CKII α' subunit is localized in the nucleus during G1 and in the cytoplasm during S. Taken together, our results suggest that CKII may play significant roles in cell division control by shifting its localization between the cytoplasm and nucleus.

CASEIN kinase II (CKII)¹ is a ubiquitous protein serine/threonine kinase found in eukaryotic cells (Edelman et al., 1987) and highly conserved among eukaryotic organisms, including *Drosophila*, yeast, *C. elegans*, bovine, and human (Saxena et al., 1987; Chen-Wu et al., 1988; Hu and Rubin, 1990a; Lozeman, 1990). Casein kinase II from several species share a common polypeptide subunit structure, $\alpha_2\beta_2$, with α of M_r 37,000–44,000 and β of M_r 24,000–28,000 by electrophoresis (Edelman et al., 1987). Two forms of α are known designated α (M_r 41,000–44,000) and α' (M_r 37,000–42,000). The α and α' subunits are thought to be the catalytic subunits based on their kinase activity in the absence of the β subunit and sequences common to other protein kinases (Hathaway et al., 1981; Cochet and Chambaz, 1983; Chen-wu et al., 1988; Meisner et al., 1989; Hu and Rubin, 1990a). The function of the β subunit is unknown, and it shares no extensive homology to other known protein sequences (Jakobi et al., 1989). The β subunit has a high degree of polarity with clusters of negative charges in the amino-terminal region and positive charge clusters in the carboxy-terminal region (Takio et al., 1987). The basic compounds, spermine, spermidine, and polylysine, stimulate activity by interacting at least in part with the β subunit (Traugh et al., 1990). This subunit may have a regulatory role in the holoenzyme (Takio et al., 1987), and evidence supporting this has been found in A-431 cells (Ackerman et al., 1990). The comparative studies using the native CKII holoenzyme and the bacterially expressed α subunit show that the expressed α is inhibited by heparin, but it is not stimulated by polyamines and has 9% of K_{cat} of the holoenzyme (Hu and Rubin, 1990b). This

further suggests that CKII holoenzyme is stabilized by interaction with the β subunit.

The structures of cDNA clones for human CKII α and α' have been determined, and the molecular weights of the α and α' predicted from the human cDNAs were 45,160 and 41,450, respectively (Lozeman et al., 1990). The amino acid sequence of the β subunit was initially derived by sequencing the isolated subunit from bovine lung (Takio et al., 1987), and the human CKII β cDNA has been isolated as well, giving the predicted molecular weight of 24,925 (Jakobi et al., 1989).

The enzyme has a variety of known substrates, including enzymes, cytoskeletal proteins, transcription factors, a phosphatase inhibitor, and oncoproteins (Table I). The substrate specificity of CKII is for serine or threonine residues aminoterminal to acidic amino acids (Meggio et al., 1984; Kuenzel et al., 1987). The enzyme has been shown to be activated following the addition of growth factors, such as insulin and epidermal growth factor, or serum to cells (Sommercorn et al., 1987; Klarlund and Czech, 1988; Carroll and Marshak, 1989). It is also induced during differentiation of 3T3-L1 cells into adipocytes (Sommercorn and Krebs, 1987).

CKII or a similar enzymatic activity has been identified in both the cytoplasm and nucleus of mammalian cells (Hathaway and Traugh, 1982). In rat liver, CKII activity is predominantly (90%) cytosolic, with the remaining activity divided among the nuclear, mitochondrial, and microsomal fractions (Singh and Huang, 1985; Edelman et al., 1987). However, in *Rana temporaria* oocytes, the enzyme is found exclusively in the cytoplasm (Kandrór et al., 1989). The CKII activity purified from nuclei has similar characteristics to those used to define cytoplasmic CKII (Edelman et al., 1987), including (a) cAMP independence; (b) similar K_{ms}

1. Abbreviation used in this paper: CKII, casein kinase II.

Table I. Selected Substrates of CK II

Enzymes	DNA topoisomerase II (Ackerman et al., 1985) RNA polymerase I and II (Stetler and Rose, 1982) Glycogen synthase (Picton et al., 1982) DNA polymerase α (Podust et al., 1990) Ornithine decarboxylase (Peng and Richards, 1988)
Cytoskeletal proteins	Tubulin (Serrano et al., 1987; Diaz-Nido et al., 1990a) Brain myosin heavy chain (Murakami et al., 1990) MAP-1A, MAP-1B (Diaz-Nido et al., 1988) Clathrin (Cantournet et al., 1987)
Transcription factors	Serum response factor (Manak et al., 1990)
Oncoproteins	Myc and Myb (Lüscher et al., 1989, 1990) SV40 large T antigen (Grässer et al., 1988)
Other	Nucleolin (Schneider and Issinger, 1988) cAMP-regulated phosphoprotein (Girault et al., 1989)

for ATP and GTP as phosphate donors; (c) inhibition by heparin; and (d) stimulation by polyamines (Thornburg et al., 1979; Matthews and Huebner, 1984). Nucleolus-associated CKII has been identified, has been shown to phosphorylate nucleolin as a substrate, and may be involved in ribosome assembly (Caizergues-Ferrer et al., 1987; Schneider and Issinger, 1988) and regulation of rDNA transcription (Belenguer et al., 1989).

The distribution of the subunits of CKII between the nucleus and cytoplasm is not known. In fact, the exact subcellular location of the subunits of CKII (α , α' , and β), the distribution of the holoenzyme, and the regulation of these subunits are also unknown. Several localization studies have been done by subcellular fractionation (Thornburg et al., 1979; Singh and Huang 1985; Hathaway and Traugh, 1982; Filhol et al., 1990). Such studies involve isolating subcellular fractions by differential centrifugation and subsequent measurement of their CKII activity. It is difficult to assess whether CKII activity arises from a single subunit or a combination of subunits. In addition, these studies have not addressed the localization of the enzyme during specific stages of the cell division cycle.

Immunocytochemical localization studies using antibodies against holoenzyme have been published (Pfaff and Anderer, 1988; Serrano et al., 1989; Belenguer et al., 1989; Filhol et al., 1990). CKII was detected by immunocytochemistry in the cytoplasm, nuclei, and nucleoli of growing cells, while it was no longer present in nucleoli of confluent cells of adult aortic endothelial cells (Belenguer et al., 1989). In addition, the distribution of CKII exhibited a shift toward an increased nuclear concentration during active proliferation of bovine adrenocortical cells in primary culture (Filhol et al., 1990). Furthermore, CKII is localized to some microtubule arrays in neuronal and nonneuronal cells. This localization is to microtubule bundles within the mitotic spindle of dividing cells and within the neurites of differentiating neuroblastoma cells (Serrano et al., 1989). Immunohisto-

chemical localization studies using antibodies against holoenzyme do not specify the localization and interaction of CKII subunits. Thus, the development of specific antibodies against CKII subunits and their localization by immunocytochemistry is necessary to further address the role of CKII during cell growth and differentiation. In addition, the localization of CKII subunits will help to identify potential roles for CKII during the cell cycle.

In this report, we describe the development of specific antibodies to synthetic peptide substrates representing epitopes on CKII α , α' , and β subunits. These reagents were used to investigate the localization of CKII in asynchronous and synchronous cells using subunit-specific antibodies. Our current studies of CKII localization using immunocytochemistry and EM indicate that the CKII α and β subunits are predominantly localized in the cytoplasm, while the localization of the α' subunit is nuclear and appears to be regulated during the cell cycle. Finally, our results also suggest that there is translocation of CKII between the nucleus and the cytoplasm in S phase of the cell cycle.

Materials and Methods

Cells

HeLa cells were grown in 24-well culture dishes containing 12-mm circular glass coverslips in DME supplemented with 1% (wt/vol) penicillin and streptomycin, and 5% (vol/vol) FBS at 37°C. For cell division cycle experiments, HeLa cells were exposed to 1.5 mM hydroxyurea (Sigma Chemical Co., St. Louis, MO) for 14 h to arrest cells at the G1/S transition (Ashihara and Baserga, 1979). Furthermore, the arrested cells were allowed to proceed through their cell cycle by removing the hydroxyurea and by resupplying fresh medium.

Synthetic Peptides

Peptides, CSH 118 RRREEETEEE; CSH 122 acetyl-SGPVPSRARVYT-DVNTHRPREC (2–22 of CKII α); CSH 123 CVVKILKPKKKKIKREIKILE (65–86 of CKII α and 66–87 of CKII α'); CSH 124 HSENQHLVSPALDFLDKLLRYDHQC (286–310 of CKII α and 287–311 α'); CSH 125 CMDVYTPKSSRRHHTDGAYFGTG (140–162 of CKII β) were synthesized by solid phase methods (Barany and Merrifield, 1979) on p-methylbenzylhydramine polystyrene resin using pre-formed, symmetric anhydrides and hydroxybenzotriazole-activated esters of N- α -Boc protected amino acids on an automated peptide synthesizer (model 430A; Applied Biosystems Inc., Foster City, CA). Couplings were done in dimethylformamide and dichloromethane as solvents, and unreacted peptide was capped with acetic anhydride. The side chain-protected amino acids were: serine-O-benzyl; glutamyl- γ -O-benzyl ester, aspartyl- β -O-cyclohexyl ester; N- π -benzyloxycarbonyl histidine; N⁸-Tosyl arginine; and O-2-bromobenzyloxycarbonyl tyrosine. The peptides were deprotected and cleaved from the resin with liquid HF at -10°C for 2 h in the presence of 5% (vol/vol) anisole and 5% (vol/vol) dimethyl sulfide. The peptide was precipitated with ethyl ether, and solubilized in 0.1% (wt/vol) aqueous trifluoroacetic acid. The solution was subjected to HPLC using a Waters Delta Prep 3000 instrument on a column (4.9 \times 30 cm) of 300 Å, C₁₈ silica (Waters Division, Millipore Corp., Milford, MA) and eluted with 0.1% (wt/vol) trifluoroacetic acid with a linear gradient of acetonitrile (Burdick and Jackson Laboratories, Muskegon, MI). The peptide was further purified by HPLC on a column (2.2 \times 25 cm) of silica using C₁₈-bonded, 300 Å pore size silica, 10 μm in diameter (Vydac, The Separations Group, Hesperia, CA). The structure of the final peptide was verified by amino acid analysis, automated sequence analysis, plasma desorption mass spectrometry, and analytical microprobe HPLC as described (Marshak and Carroll, 1991).

Antibody Preparation

The peptides, CSH 122, 123, 124, and 125 were coupled to keyhole limpet hemocyanin (Sigma Chemical Co.) or ovalbumin (Sigma Chemical Co.) via maleimidobenzoyl-N-hydroxysuccinimide (Pierce Chemical Co., Rock-

ford, IL) as described (Harlow and Lane, 1988). Antisera against the complexes were raised as follows. The complexes (0.5 mg) in 0.5 ml of PBS was mixed with 0.5 ml of Freund's complete adjuvant and injected subcutaneously. Booster injections were given with incomplete adjuvant at 2-wk intervals. Additional booster injections were given at 2-wk intervals until reaching maximum serum titer. Serum antibody titer was determined by RIA. Wells of 96-well polyvinylchloride microtiter plates were coated with purified CKII. The plates were washed and unbound sites were saturated with 3% (wt/vol) BSA. Dilutions of immune and preimmune serum were added to wells. The plates were washed with PBS and 50,000 cpm of ^{125}I -labeled goat anti-rabbit IgG F(ab) $_2$ added per well. After washing, the specific activity of each serum sample was determined by measuring radioactivity in a gamma radiation counter (LKB Instruments, Gaithersburg, MD). Immune rabbit IgGs were prepared using a Protein G or A Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ) according to the instructions of the manufacturer. Affinity-purified TRITC-labeled goat anti-rabbit IgG was purchased from Cappel Laboratories (Malvern, PA). 245 Ab was further purified using a CNBr-activated Sephadex column (Pharmacia Fine Chemicals) containing bound peptide CSH 122.

Protein Kinase Assay

The peptide RRREETEEE (CSH 118) was used as a CKII-specific substrate (Kuenzel and Krebs, 1985). Protein kinase activity was assayed as described (Carroll and Marshak, 1989). Briefly, CKII activity was determined by measuring the incorporation of ^{32}P into CKII-specific peptide substrate, as follows. Samples (5 μl) were incubated in a final volume of 30 μl at 37°C for 30 min in the presence of 20 mM Hepes, pH 7.4, 10 mM MgCl_2 , 100 μM [γ - ^{32}P]ATP (1,000–3,000 cpm/pmol, Amersham Corp., Arlington Heights, IL) with 5 mM peptide. Reactions were started by adding radioactive ATP. Assays using the peptide RRREETEEE were stopped by adding TCA to a final concentration of 10% (wt/vol), and phosphate incorporation was determined by adsorption to phosphocellulose paper according to previously described protocols (Kuenzel and Krebs, 1985; Marshak and Carroll, 1991).

Purification of CKII from Bovine Brain and Liver

Fresh bovine brain or liver (~1,500 g) was homogenized in 3 L of buffer A (20 mM Tris-Cl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, and 10% (vol/vol) glycerol). The homogenates were centrifuged at 11,000 g, 4°C for 1 h. Then supernatants were collected and diluted in buffer A to a conductivity less than 2 mS/cm. The supernatants were mixed with 250 g of diethylaminoethyl cellulose (Whatman DE-52) for 2 h. The resin was then washed through a Buchner glass funnel with 5 liters of Buffer A containing 10 mM MgCl_2 . The washed resin was packed into a 5 \times 40-cm column, and a gradient of a total of 2,000 ml of KCl (50–400 mM) in buffer A containing 10 mM MgCl_2 eluted the column at a rate of 100 ml/h, 15 ml per fraction. Every third fraction was assayed for CKII activity. The active fractions were pooled and applied to a 100-ml (2.5 \times 20 cm) phosphocellulose (Whatman P11) column previously equilibrated with buffer A containing 10 mM MgCl_2 and 400 mM KCl. After washing the P11 column with the equilibration buffer, proteins were eluted with a gradient of 500 ml of KCl (400–1,000 mM) in buffer A containing 10 mM MgCl_2 . The fractions were assayed for CKII activity and the active fractions were pooled. This pool was diluted with buffer A to a conductivity equal to that of buffer A containing 300 mM KCl. The solution was applied to a hydroxyapatite (BioRad HTP; Bio-Rad Laboratories, Richmond, CA) column equilibrated in buffer A containing 300 mM KCl. The column was washed with buffer A containing 50 mM KCl, and the CKII was eluted with a gradient of 500 ml potassium phosphate (0–300 mM, pH 7.9) buffer. The fractions were assayed for CKII activity and the active fractions were pooled. Finally, the pooled solution was applied to a column of heparin-Sepharose CL-6B (Pharmacia Fine Chemicals) equilibrated in buffer A. CKII was eluted with a gradient of 50 ml KCl (0–800 mM) in buffer A. The fractions were assayed for CKII activity and the active fractions were pooled and stored at -70°C. Electrophoretic analysis of this material revealed homogeneous CKII containing α , α' , and β subunits as described (Hathaway and Traugh, 1982; Kuenzel and Krebs, 1985).

Gel Electrophoresis and Immunoblotting

Electrophoresis was performed on 12.5% (wt/vol) polyacrylamide gels in the presence of SDS using the buffer system of Laemmli (1970). Proteins were transferred electrophoretically to nitrocellulose (Towbin et al., 1979; Towbin and Kurstak, 1984). The nitrocellulose was blocked with 3% BSA

(wt/vol) in PBS and then incubated with antiserum at desired dilutions in the same buffer. ^{125}I -labeled goat anti-rabbit IgG F(ab) $_2$ was used as secondary antibody. The gels were stained with Coomassie blue, dried and exposed to film (Kodak X-Omat).

Flow Cytometry

Flow cytometric analysis was done as described by Buchkovich et al. (1989). Cells (5×10^6) were washed and resuspended in 0.5 ml of ice-cold PBS. The cells were fixed by gradual addition of 95% ethanol (-20°C) while vortexing. The fixed cells were washed with PBS and resuspended in 1 ml of PBS containing 10 $\mu\text{g/ml}$ boiled RNAase A (Sigma Chemical Co.) and 5 $\mu\text{g/ml}$ of propidium iodide. After a 30-min digestion and staining period at 37°C, the fluorescence intensities of the samples were measured by quantitative flow cytometry using an EPICS C system (Coulter Electronics Inc., Hialeah, FL).

Indirect Immunofluorescence

Indirect immunofluorescence was performed as described by Spector and Smith (1986). Cells (10^5) grown on glass coverslips (12 mm) or mitotic cells attached onto poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde in PBS. Cells were permeabilized with 0.2% Triton X-100 on ice in PBS containing 0.5% normal goat serum and reacted with respective primary antibodies for 1 h. Bound antibodies were detected with rhodamine conjugated goat anti-rabbit secondary antibody (Cappel Laboratories). The cells were stained with affinity-purified TRITC goat anti-rabbit IgG. Samples were examined with a Zeiss Axiophot microscope equipped with epifluorescence illumination (Carl Zeiss Inc., Thornwood, NY).

Immunoelectron Microscopy

Cells were fixed in 2% formaldehyde plus 0.1% glutaraldehyde or with just 0.5% glutaraldehyde for 15 min at 20°C and prepared for immunoelectron microscopy according to published procedures (Spector, 1984; Spector and Smith, 1986). Briefly, fixed cells were washed in 0.5 mg/ml sodium borohydride, permeabilized in 0.2% Triton X-100 and washed in PBS, pH 7.4, containing 1% normal goat serum before incubation in the specific purified IgG at a concentration of 100 $\mu\text{g/ml}$ or the corresponding preimmune IgG for 1 h at 20°C. Cells were then extensively washed in PBS and incubated in peroxidase-conjugated secondary antibody at a dilution of 1:20 for 1 h at 37°C. The peroxidase reaction product was developed by incubation in 0.5 mg/ml DAB containing 0.01% H_2O_2 for 20 min at 20°C. Cells were then fixed in 2% osmium tetroxide and prepared for EM by standard methods (Spector and Smith, 1986). Thin sections (100 nm) cut on a Reichert Ultracut E ultramicrotome with a Diatome diamond knife were examined with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Results

Development of Antibodies to Peptides

CKII activity has been found in both the nucleus and cytoplasm, and the enzyme has been implicated in the cell division cycle and in hormonal stimulation of cell growth. Substrates for the enzyme are found both in the cytosol and nucleus. To learn how the enzyme is regulated during cell growth, it appeared necessary to localize CKII within the cell. Because of its ubiquitous nature and highly conserved structure, our numerous attempts to obtain antibodies against the intact holoenzyme have not been successful. Therefore, we adopted the strategy of using synthetic peptides as antigens which allow us to prepare several site-directed antibodies to individual subunits of CKII.

Synthetic peptides were designed to encompass three regions of CKII α and one of CKII β subunits (Fig. 1), containing amino acid sequences that are highly conserved among species. These regions consist of predominantly polar and charged residues, and are likely to lie on the surface of the proteins in solution (Rose et al., 1985). Such hydrated

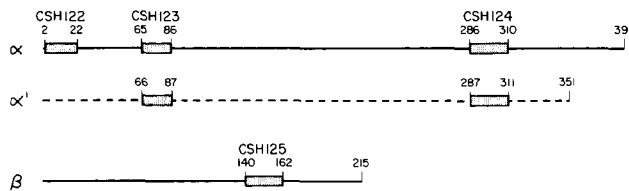


Figure 1. Sequences of synthetic peptides in CKII. The sequences of CKII α and α' subunits were deduced from human cDNA clones by Lozeman et al. (1990). The sequence of CKII β subunit was derived from human cDNA from HeLa cell by Jakobi et al. (1989). The shaded boxes represent the residues corresponding to the respective synthetic peptide.

Table II. Polyclonal Antisera Raised Against Synthetic Peptides

	Peptide Antigen	Specificity
Ab 245	CSH 122	CKII α
Ab 247	CSH 123	CKII α & α'
Ab 276	CSH 124	CKII α & α'
Ab 278	CSH 125	CKII β

regions are thought to be highly antigenic (Hopp and Woods, 1981).

As shown in Fig. 1 and Table II, antibody (Ab) 245 was raised by immunizing rabbits with peptide CSH 122, which represents a unique sequence at the amino terminus of CKII α (2–22). The methionine initiator was not included and the peptide was acetylated, because the amino terminus of α is blocked to Edman degradation. The peptide contained a cysteine at the carboxy terminus so it would be oriented properly on coupling. Ab 247 was raised by using peptide CSH 123 which contains an internal CKII α/α' sequence that

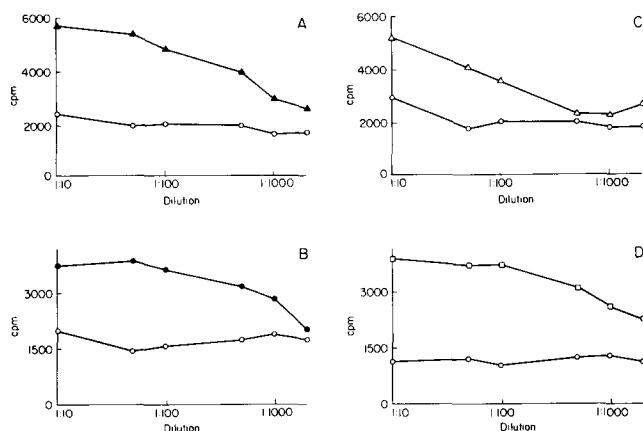


Figure 2. Titration of immune serum to purified CKII. Serum antibody titer was determined by RIA. Wells of 96-well polyvinyl microtiter plates were coated with purified CKII. Dilutions of immune and preimmune serum (*open circles*) were added to wells. The plates were washed with PBS and ^{125}I -labeled goat anti-rabbit IgG F (*ab*)₂ added per well. After washing the titer of serum was determined by measuring radioactivity in a gamma counter. Results represent the means of experiments in duplicates. *A*, Ab 245; *B*, Ab 247; *C*, Ab 276; *D*, Ab 278.

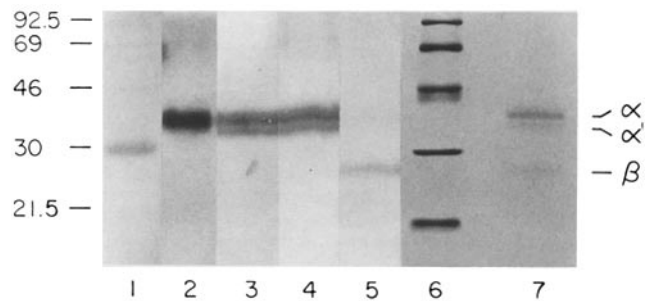


Figure 3. Immunoblotting of peptide antibodies to purified bovine liver CKII. Purified bovine liver CKII was subjected to electrophoresis on 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with peptide antibodies. Ab 245 specific for α (lane 2), Ab 247 (lane 3) and 276 (lane 4) for α and α' , and Ab 278 (lane 5) for β . The C^{14} -labeled molecular masses (in kilodaltons) of marker proteins are shown at the left (lane 1) and lane 6. Phosphorylase b (M_r 92,500), BSA (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and trypsin inhibitor (M_r 21,500). Coomassie staining of the molecular marker proteins and CKII α , α' , and β was shown in lane 6 and lane 7, respectively. The relative migration of the α , α' , and β subunits of bovine CKII are shown at the right (*arrowheads*).

is similar to a nuclear localization signal (Dang and Lee, 1989) and is common to both α and α' (Fig. 1). Ab 276 was raised against CSH 124 which spans sequences near the carboxy terminus and shares common sequence for α and α' . Finally, Ab 278 was raised by injecting CSH 125 conjugates that contain a sequence of β (140–162). All of these peptides were conjugated to carrier proteins (keyhole limpet hemocyanin or ovalbumin) and the conjugates were used to immunize rabbits as described under Materials and Methods.

Dilutions of the sera were tested for their ability to detect purified bovine liver or brain CKII on solid phase RIA. All

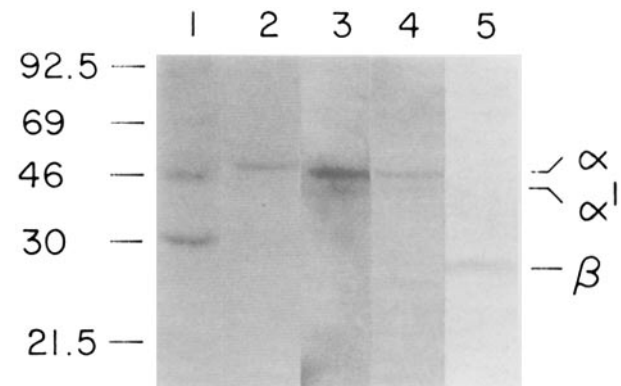


Figure 4. Immunoblotting of peptide antibodies to HeLa cell extracts. HeLa cell extracts were subjected to electrophoresis on 12.5% polyacrylamide gel, transferred to nitrocellulose, and probed with peptide antibodies. Ab 245 specific for α (lane 2), Ab 247 (lane 3) and 276 (lane 4) for α and α' , and Ab 278 (lane 5) for β . The C^{14} -labeled molecular masses (in kilodaltons) of marker proteins are shown at the left (lane 1). Phosphorylase b (M_r 92,500), BSA (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and trypsin inhibitor (M_r 21,500). The relative migration of the α , α' , and β subunits of bovine CKII are shown at the right (*arrowheads*).

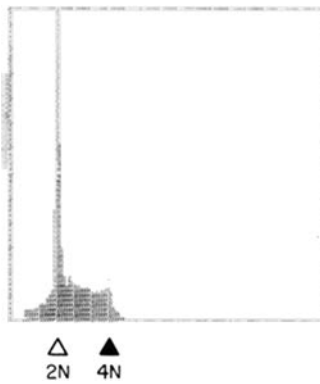


Figure 5. Flow cytometric profiles of asynchronous HeLa cells. Asynchronous HeLa cells were harvested, and then fixed in ethanol and stained for flow cytometry analysis. The open and closed triangles mark 2N and 4N DNA content, respectively.

the sera reacted with purified CKII and had $>1:1,000$ titer in this assay, indicating antibody reactivity to native CKII molecules (Fig. 2). All the sera reacted positively with CKII on immunoblots after polyacrylamide gel electrophoresis of the purified enzyme, demonstrating that the antibodies react with denatured CKII molecules as well. In control experiments, interactions between the antisera and CKII enzyme could be blocked by incubation with excess free peptides (data not shown). Ab 245 reacted to CKII α (M_r 42,000) exclusively (Fig. 3, lane 2), while Ab 247 and 276 reacted with both CKII α and α' (M_r 39,000) (Fig. 3, lanes 3 and 4) Ab 278 reacted with CKII β (M_r 29,000) (Fig. 3, lane 5). All of the immunoreactive bands comigrated with the corresponding band of purified CKII α , α' , or β visualized by Coomassie blue staining (Fig. 3, lane 7). The antibodies were further characterized by immunoblotting of HeLa cell lysates to test whether they are specific for CKII. Ab 245 only reacted to CKII α (M_r 46,700) (Fig. 4, lane 2), while Ab 247 and 276 reacted with both CKII α and α' (M_r 42,600) (Fig. 4, lanes 3 and 4). Ab 278 reacted with CKII β (M_r 27,000) (Fig. 4, lane 5). No other immunoreactive proteins were detected. These antibodies thus provided specific reagents to examine the subcellular localization of CKII.

Localization of CKII in Asynchronous HeLa Cells

In this study we have used the antibodies characterized above to localize CKII in HeLa cells by immunocytochemistry. Asynchronous HeLa cells consisted of a cell population $52.8 \pm 6.2\%$ of G1, $18.1 \pm 2.6\%$ of S, and $22.8 \pm 3.0\%$ of G2/M by FACS analysis based on an average of five separate experiments. One example of the FACS analysis is shown in Fig. 5. The open and closed triangles designate 2N and 4N DNA content, respectively.

These cells were fixed, permeabilized, and stained with antibodies described above to α , α' , and β . The immunostaining pattern was visualized by indirect immunofluorescence using rhodamine-conjugated secondary antibodies. Cells stained with Ab 245, which specifically recognizes the α subunit, showed predominant localization to the cytoplasm (Fig. 6, C and D). The staining intensity of the nucleus is very low to negative as compared to the cytoplasmic staining. The staining is absent in vacuoles and on the plasma membrane. In contrast to Ab 245, Abs 247 and 276, which recognize the α and α' subunits, stained both the cytoplasm

and nucleus of $\sim 80\%$ of the cell population (Fig. 6, E-H), the other 20% of the cells were negative for nuclear staining. The β subunit-specific antibody, Ab 278, stained the cytoplasm (Fig. 6, I and J) similar to Ab 245; however, nuclei were not stained. The immunofluorescence of subunit-specific antibodies is blocked by their respective peptides (data not shown). From these results, it appears that CKII α and β are mainly localized to the cytoplasm in asynchronously growing cells. CKII α' , however, appears to be localized to the nucleus and possibly to the cytoplasm. The negative nuclear staining pattern apparent in $\sim 20\%$ of the cells immunostained with Ab 247 and 276, which both recognize the α' subunit of CKII, suggested that the localization of CKII α' may be regulated as a cell cycle-dependent phenomenon.

Localization of CKII by Immunoelectron Microscopy

To further identify the subcellular localization of CKII, immunoelectron microscopic studies were performed on asynchronous HeLa cells. As shown in Fig. 7, cells immunostained with Ab 245 showed exclusive cytoplasmic staining (Fig. 7 B) compared to control cells incubated in preimmune IgG followed by peroxidase-conjugated secondary antibody (Fig. 7 A). The EM study also revealed that the β subunit-specific antibody, Ab 278, immunostained the cytoplasm and showed little to no nuclear immunoreactivity (Fig. 7 C). These findings further confirm the immunofluorescence staining pattern observed and our notion that CKII α and β subunits are localized to the cytoplasm of interphase cells. In contrast to Ab 245 and Ab 278, the α - and α' -specific Abs 247 and 276 stained both the cytoplasm and nucleus by immunoelectron microscopy (Fig. 8, A and B). It is also notable that some cells are nuclear negative for both Ab 247 and 276. These findings support our initial immunofluorescence results (Fig. 6), reinforcing the idea that CKII α' is localized to the nucleus and, possibly, cytoplasm, and that α' staining is more intense in the nucleus than α staining.

Localization of CKII in HeLa Cells In Mitotic Cells

The results of CKII localization obtained using asynchronous cells did not provide us with enough information about the regulation of distribution of CKII during the cell division cycle. In addition, the roles of CKII in cellular regulation that involve ubiquitous and multiple phosphorylation reactions, in both the cytoplasm and nucleus, needed to be investigated in synchronous cell populations to clarify the actual distribution of CKII in each phase of the cell cycle.

Since mitotic cells are round and loosely attached to the growing surface, it was difficult to precisely assess the localization of CKII by immunofluorescence microscopy. Therefore, electron microscopic investigations were done to more fully elucidate the localization of CKII during mitosis.

Examples of the electron microscopic analysis of mitotic cells are shown in Fig. 9. Fig. 9 a shows a cell at metaphase, when the chromosomes become aligned at the metaphase plate half-way between the spindle poles. Fig. 9 c shows a cell in anaphase in which each pair of chromosomes has separated and movement to opposite poles has begun. In these micrographs, CKII α is diffusely localized (Ab 245) throughout the cytoplasm. It is notable, however, that there is very strong staining, compared to the control (Fig. 9 d), associated with spindle fibers (Fig. 9, arrowheads) whose

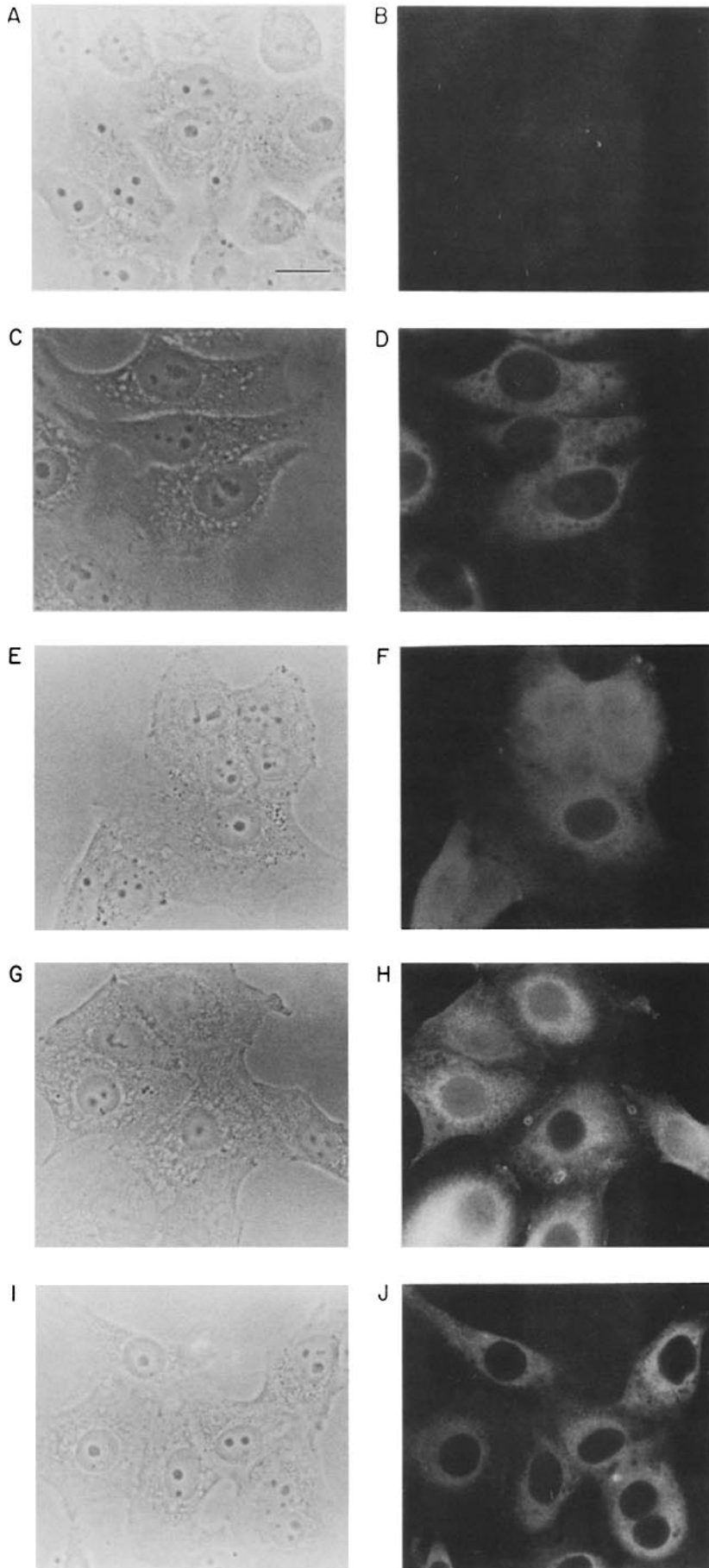


Figure 6. CKII α , α' , and β immunofluorescence in asynchronous HeLa cells. HeLa cells were fixed and stained as described in Materials and Methods using purified IgG of preimmune (A and B); Ab 245 (C and D); Ab 247 (E and F); Ab 276 (G and H); and Ab 278 (I and J). Bar, 20 μ m.

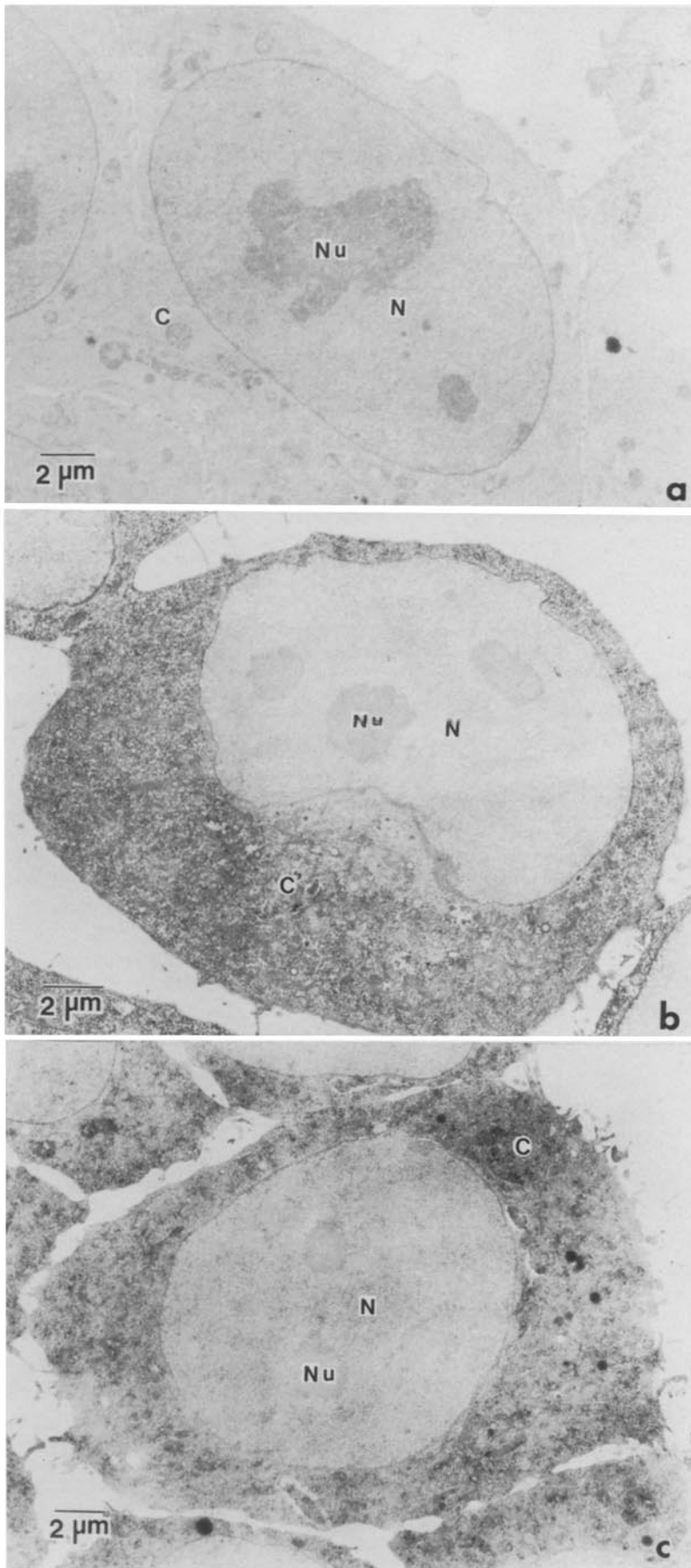


Figure 7. Immunoelectron microscopic analysis of asynchronous HeLa cells. HeLa cells were immunostained as described by using preimmune IgG (a); Ab 245 (b); and Ab 278 (c). Note the immunoreactivity is predominantly in cytoplasm (C), not in nuclei (N) or nucleoli (Nu) compared to the control.

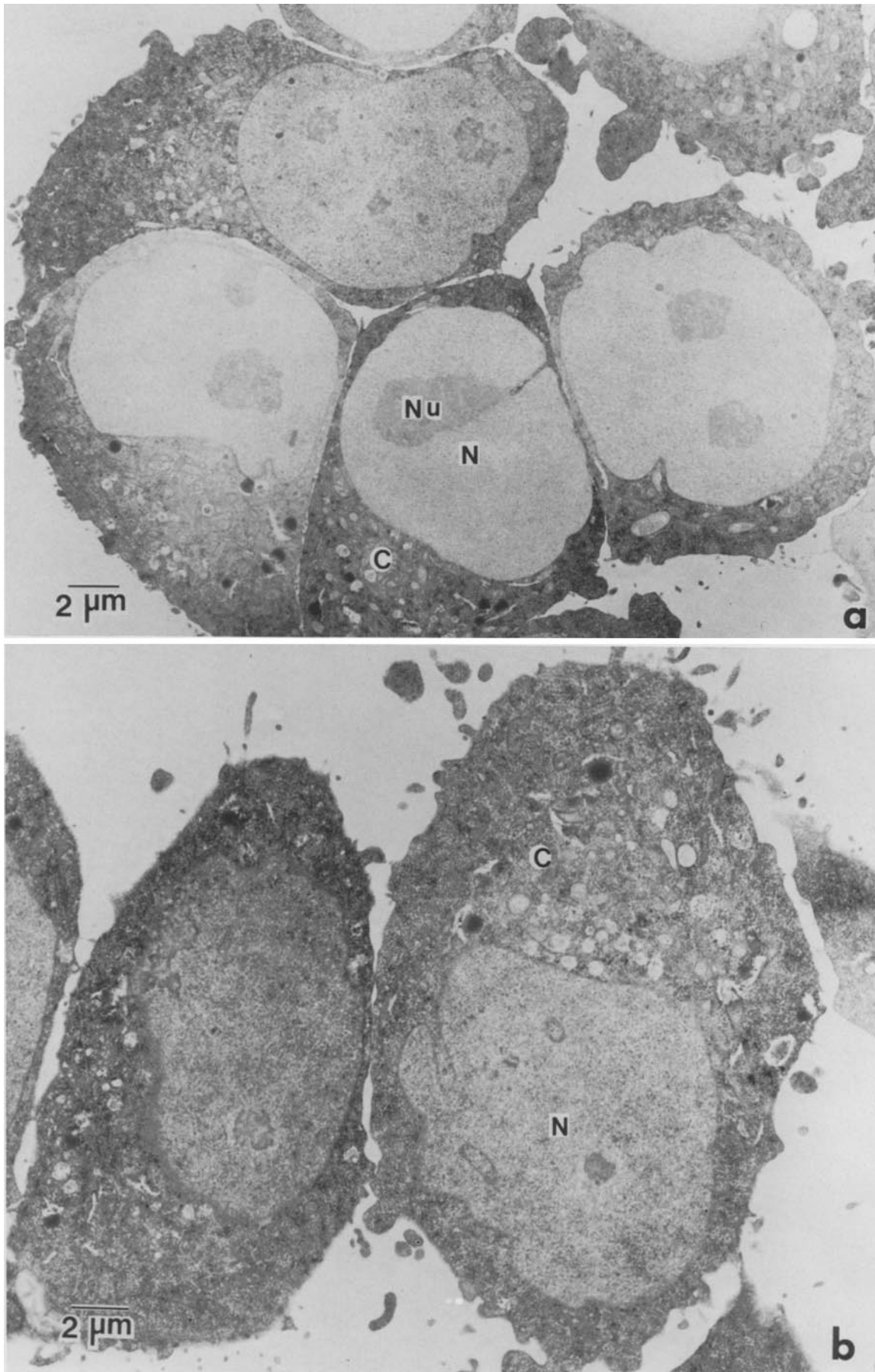


Figure 8. Immunoelectron microscopic analysis of asynchronous HeLa cells. HeLa cells were stained with Ab 247 (a) and Ab 276 (b). The immunoreactivity is present in either cytoplasm (C) exclusively or cytoplasm and nucleus (N).

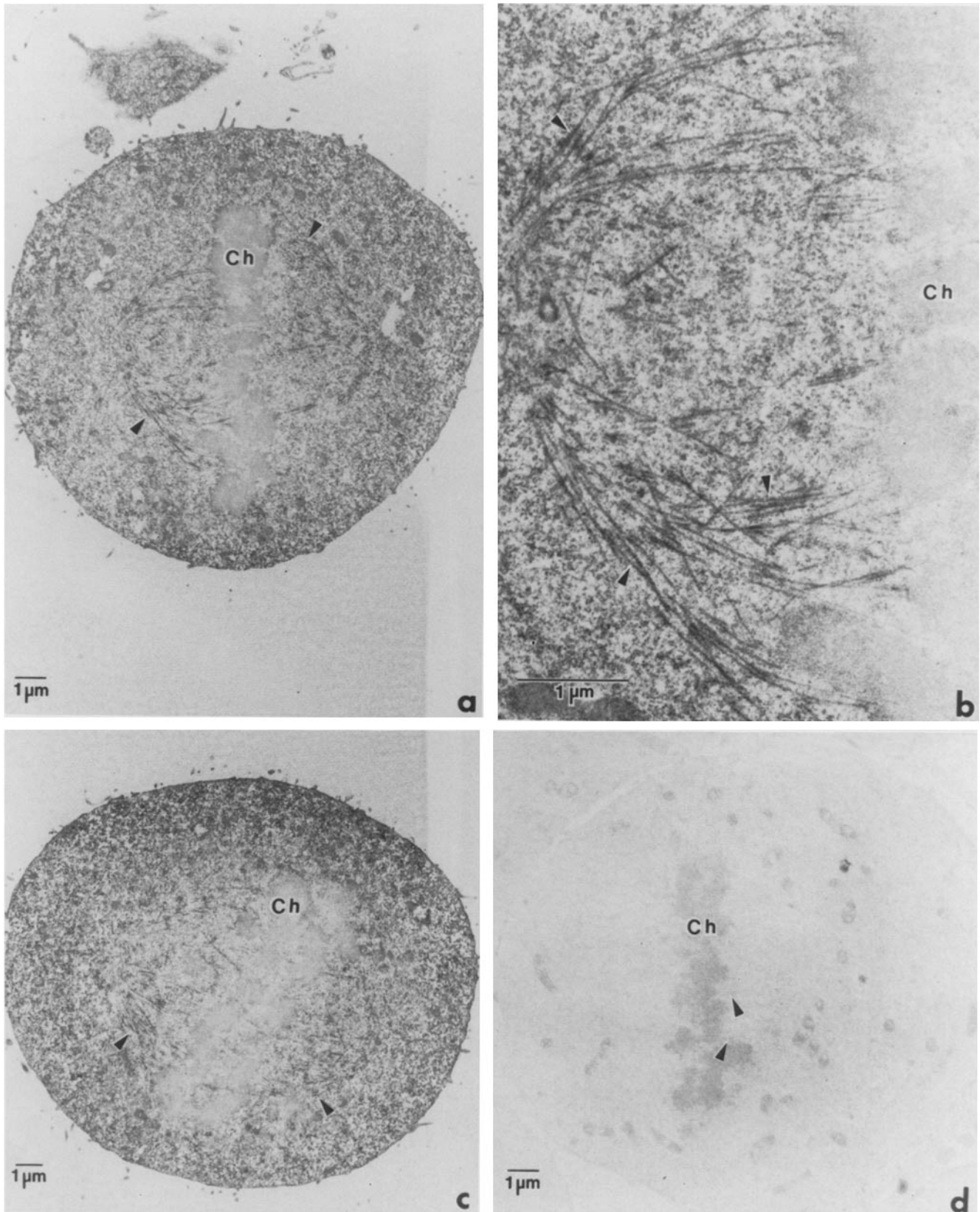


Figure 9. Immunoelectron microscopic analysis of mitotic HeLa cells. HeLa cells in metaphase (*a*, *b*, and *d*) and anaphase (*c*) were stained with Ab 245 (*a-c*) or preimmune IgG (*d*). The photograph in *b* is a higher magnification of that in *a*. Note the diffuse cytoplasmic immunoreactivity and very strong staining of spindle fibers (*arrowheads*).

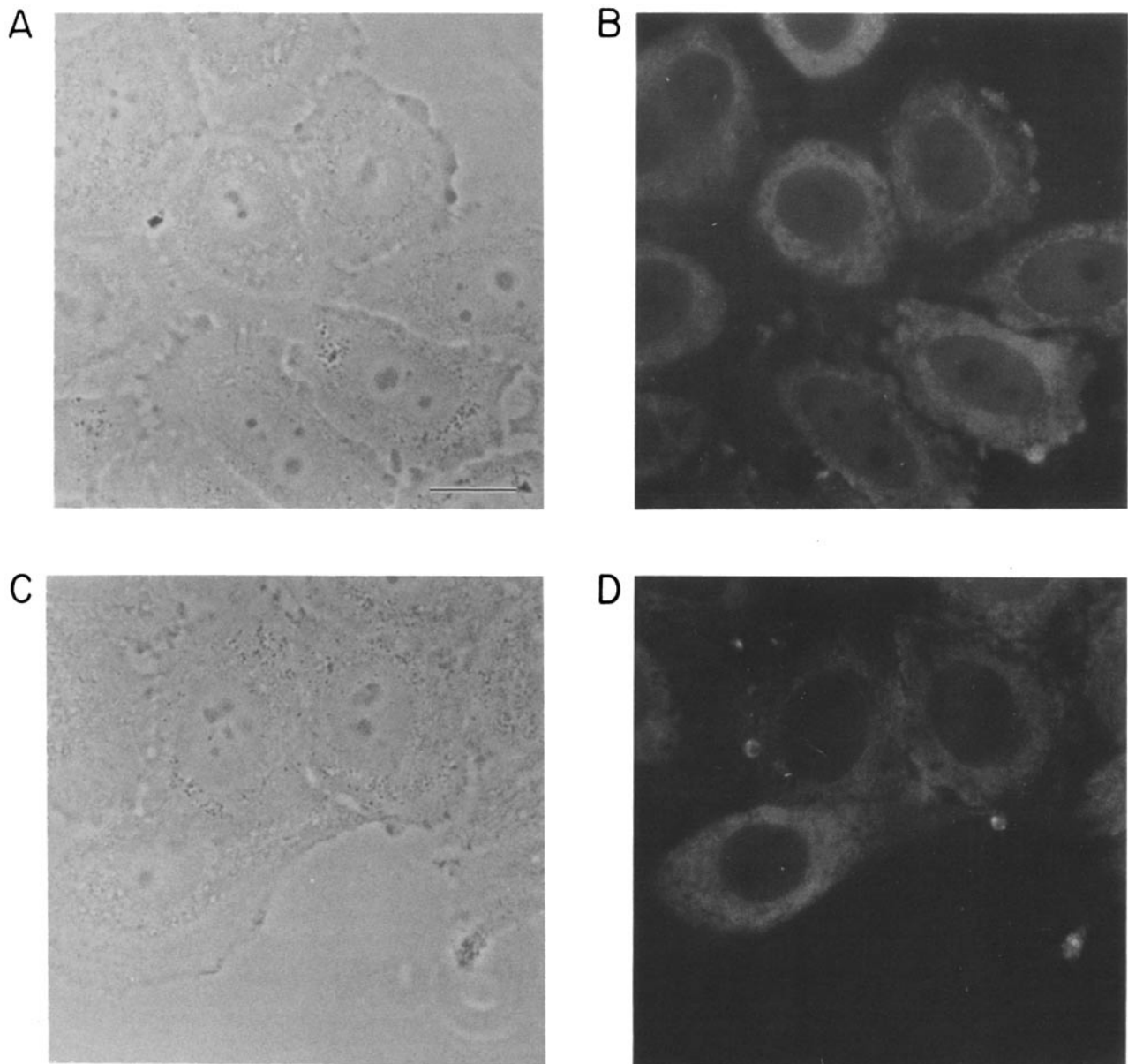


Figure 10. CKII α , and β immunofluorescence in hydroxyurea-treated HeLa cells. HeLa cells grown on 12-mm coverslips in 24-well tissue culture dishes were treated with hydroxyurea for 18 h. HeLa cells were fixed and stained as described in Materials and Methods using Ab 245 (*A* and *B*), Ab 278 (*C* and *D*). Bar, 20 μ m.

major component is tubulin. Fig. 9 *b* is a high magnification view showing the intense immunostaining of microtubules and the negative staining of the chromosomes. The intensity of the control (Fig. 9 *d*) was so weak that the negative needed four times longer exposure than experimental samples to visualize, thus indicating the specificity of the cytoplasmic and microtubule staining. In addition, the microtubules are present in the control section but hardly recognizable (*arrowheads*), indicating that the antibodies have substantial reactivity to microtubules or microtubule-associated proteins in the immunostained samples. The chromosomes themselves are not stained with anti-CKII α subunit antibody (Fig. 9, *a-d*). It is likely, therefore, that CKII is associated with microtubules in mitotic cells, and may be an integral part of the mitotic apparatus.

Localization of CKII in Synchronous HeLa Cells

To study cellular localization of CKII in synchronous cells, HeLa cells were exposed to 1.5 mM hydroxyurea for 14 h to arrest cells at the G1/S transition (Ashihara and Baserga, 1979). The results of FACS analysis indicated that >70% of the cells are in G1/S transition, whose DNA content was intermediate between 2N and 4N within 4 h after release. As shown in Fig. 10 (*A* and *B*), the staining pattern of hydroxyurea-treated cells stained with Ab 245 showed predominant staining of the cytoplasm as seen in asynchronous cells. The cytoplasmic staining is again absent in vacuoles and on the plasma membrane. The β subunit-specific antibody, Ab 278, exhibits cytoplasmic staining with little to no nuclear immunoreactivity (Fig. 10, *C* and *D*). The α and α -specific

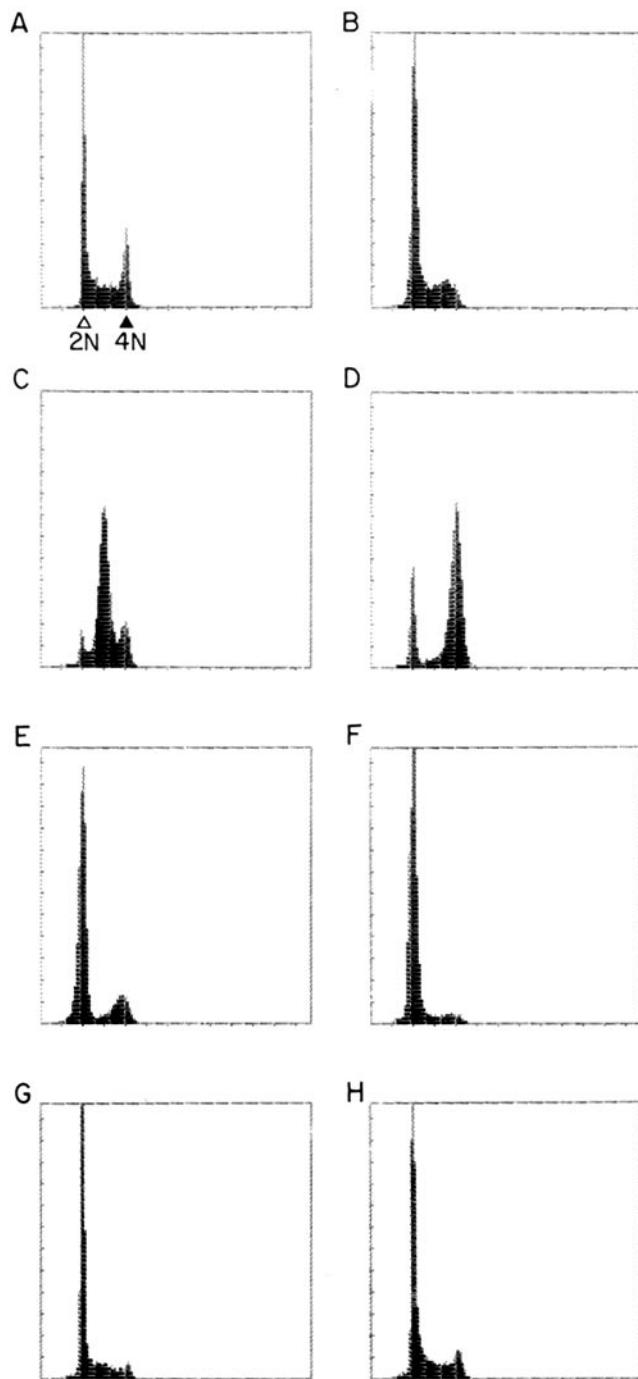


Figure 11. FACS analysis of hydroxyurea arrested HeLa cells. Asynchronous HeLa cells arrested by hydroxyurea for 14 h were washed and incubated with new medium. Samples were collected every 4 h and processed for FACS analysis. *A*, asynchronous HeLa cells; *B*, 0 h after release; *C*, 4 h; *D*, 8 h; *E*, 12 h; *F*, 16 h; *G*, 20 h; *H*, 24 h after release. The open and closed triangles mark 2 N and 4 N DNA content, respectively.

Ab 276 stained both cytoplasm and nucleus. However, in HeLa cells arrested by hydroxyurea, the percent of nuclear positive for Ab 276 increased slightly from 86 to 94%, based on analysis of >500 cells in each sample. The staining by Ab 247 to hydroxyurea-arrested cells appeared indistinguishable from the pattern seen for Ab 276 (data not shown).

These results further suggested that the expression of CKII α' might be regulated in a cell cycle-dependent manner.

To substantiate the findings, the HeLa cells arrested by hydroxyurea were released by washing out the hydroxyurea and by adding fresh culture medium. Samples were taken at time points from 0 h to 4, 8, 12, 16, 20, and 24 h, and processed for immunofluorescence microscopy. FACS analysis of cell samples was done at each time point. As shown in Fig. 11, FACS analysis indicated that cells proceeded through the cell cycle after removal of hydroxyurea. More than 500 cells were counted in continuous microscopic fields of each sample for nuclear positive cells by Ab 276 to detect changes of CKII α' in nucleus during cell cycle (Figs. 12 and 13). The percent of nuclear-positive cells in asynchronous cells increased from $86.4 \pm 0.3\%$ (Figs. 11 *A*; 12, *A* and *B*; and 13, *closed triangle*) to $94.7 \pm 1.7\%$ (Figs. 11 *B*, 12 *C* and *D*, and 13, *open triangles*) by the hydroxyurea arrest. After removal of hydroxyurea, cells become less nuclear positive ($60.7 \pm 3.3\%$; Fig. 12, *E* and *F*), and progress into S phase (Fig. 11 *C*). At 8 h, most cells are in G2/M phase (Figs. 11 *D* and 12, *G* and *H*). The cells are back to G1 phase at 12 h, maintaining up to 24 h, and are mostly nuclear positive (Figs. 11, *E-H*; 12, *I* and *J* to *O* and *P*; and 13, *open triangles*). These results further indicate the regulation of CKII α' localization during the cell cycle.

Discussion

In this report, we describe our investigations of the distribution of CKII in interphase and mitotic HeLa cells. Our findings of CKII α , α' , and β subunit localization by immunohistochemistry support the concept that CKII is regulated by transport between the cytoplasm and nucleus during the cell division cycle.

The localization studies indicated that the CKII α subunit, which is a catalytic subunit of the kinase, is localized to the cytoplasm throughout interphase. One of the functions of the CKII α subunit in mitotic and postmitotic cells might involve regulating the organization of the cytoskeleton. Both dividing cells and postmitotic cells undergo morphological changes that depend on the arrangement of the cytoskeleton. For example, mitotic cells become rounded and release from the substrate. After mitosis, cells reattach to the substrate and flatten out. In addition, postmitotic cells, such as neurons and macrophages, continue to reorganize the cytoskeleton to differentiate and maintain cellular functions (Mitchison and Kirschner, 1988; Didier et al., 1989). CKII α may be involved in this cytoskeletal reorganization by phosphorylation of protein components such as tubulin and myosin. Nonmuscle bovine brain myosin heavy chain can be phosphorylated by CKII, although the functional importance of the phosphorylation is not known (Murakami et al., 1990). Tubulin and microtubule-associated proteins also have been shown to be phosphorylated by CKII (Serrano et al., 1987; Diaz-Nido et al., 1990*a,b*), and endogenous CKII-like activity has been found in purified brain microtubule proteins (Serrano et al., 1987, 1989). The 68 K neurofilament protein also has a CKII site in its carboxy terminus (Lewis and Cowan, 1985), and there may be many other, yet unidentified, cytoskeletal proteins that are phosphorylated and regulated by CKII.

The β subunit of CKII showed a staining pattern similar

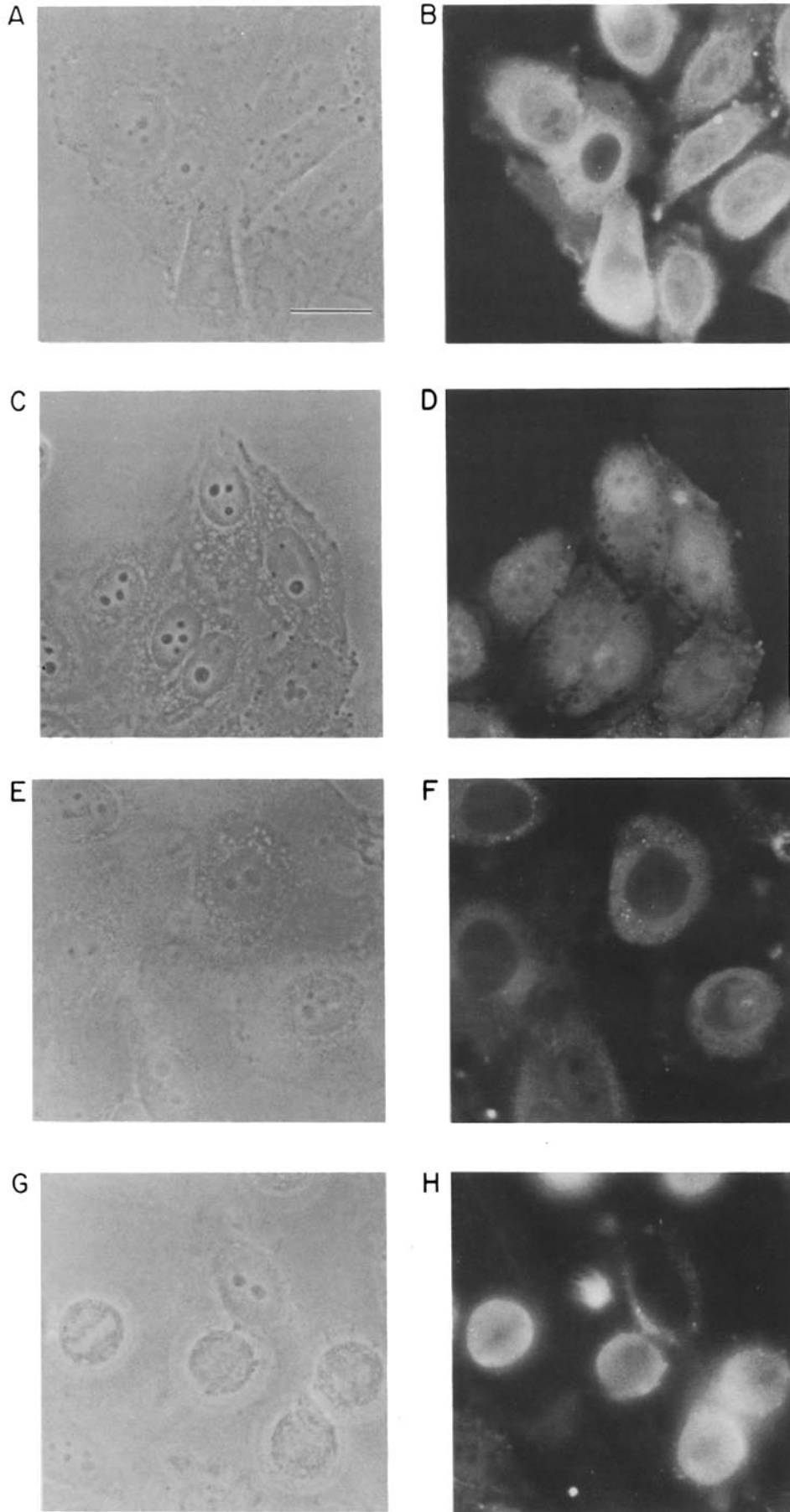


Figure 12. Immunofluorescence in hydroxyurea arrest released HeLa cells. HeLa cells grown on 12-mm coverslips in 24-well tissue culture dishes were treated with hydroxyurea for 14 h, then the cells were washed and incubated with new medium. HeLa cells were stained as described in Materials and Methods using Ab 276; *A* and *B*, asynchronous HeLa cells; *C* and *D*, 0 h after release; *E* and *F*, 4 h; *G* and *H*, 8 h; *I* and *J*, 12 h; *K* and *L*, 16 h; *M* and *N*, 20 h; *O* and *P*, 24 h. Bar, 20 μ m.

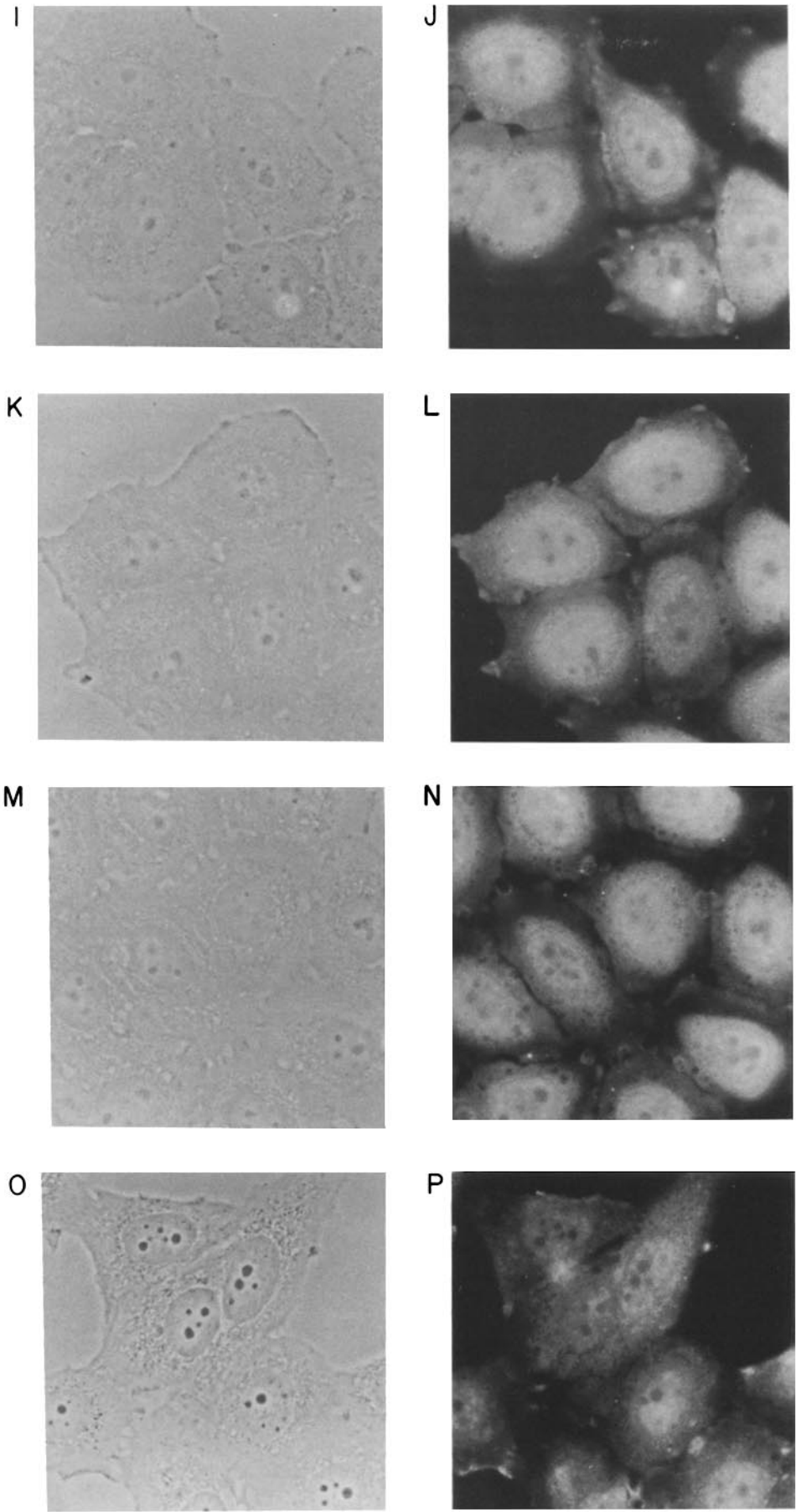


Figure 12.

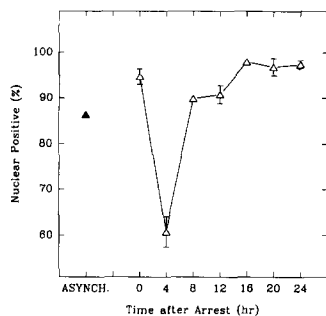


Figure 13. Percent of nuclear positive of hydroxyurea arrest released HeLa cells. Asynchronous HeLa cells (closed triangle) arrested by hydroxyurea for 14 h were washed and incubated with new medium. Samples were taken at 0, 4, 8, 12, 16, 20, and 24 h (open triangles) and processed for immunofluorescence microscopic evaluation. More than 500 cells were counted for the nuclear positive in continuous microscopic fields.

to that of CKII α . The CKII β subunit is mainly localized to the cytoplasm and to a much lesser degree in the nucleus of interphase cells. The function of the β subunit is not well understood, but it appears to have a regulatory role in the holoenzyme (Takio et al., 1987; Ackerman et al., 1990; Hu and Rubin, 1990b). No similarity has been found to any known proteins or protein sequences inferred from DNA sequences (Jakobi et al., 1989). The co-localization of the α and β subunits demonstrated here further supports the postulate that the β subunit is required for optimal activity of CKII holoenzyme and perhaps for regulation of the CKII α subunit (Cochet and Chambaz, 1983; Ackerman et al., 1990). One property of the β subunit is that overexpression of its cDNA in *xeroderma pigmentosa* fibroblasts makes the cells partially resistant to ultraviolet radiation (Teitz et al., 1990). This finding implies that the β subunit may have a function in DNA repair mechanisms, further emphasizing the need to characterize nuclear forms of CKII subunits.

The α' subunit of CKII showed a very different staining pattern compared to α and β subunits. The antibodies 247 and 276 that react with both α and α' subunits have provided useful information when interpreted with the data on the α subunit. It is likely that α' is the nuclear form of CKII, as suggested by the consistent presence of α in the cytoplasm rather than in the nucleus, and the nuclear staining pattern of Abs 247 and 276 which recognize both α and α' . Alternatively, the nuclear (α') form may be present in different complexes so the epitopes are not immunoreactive. However, Abs 247 and 276 recognize distinct epitopes on the molecule, strengthening our proposal that the differential staining represents true localization rather than differential immunoreactivity of protein complexes.

The immunocytochemical observations of Belenguer et al. (1989) and Filhol et al. (1990), showed reduced staining of confluent cells and an increased concentration of CKII in the nucleus of proliferating cells. Confluent bovine adrenocortical cells showed the presence of CKII in the cytoplasm and an absence of nuclear CKII, while actively growing cells showed the predominant CKII concentration in nuclei (Filhol et al., 1990). In confluent bovine aortic endothelial cells, CKII was barely detected in either the cytoplasm or the nucleus, while it was prominent in both the nucleus and the cytoplasm of exponentially growing cells (Belenguer et al., 1989). These variabilities in nuclear staining using antibodies against holoenzyme observed by those authors may be due to the movement or disintegration of CKII α' from the

nucleus in specific cell cycle stages. The different results may be due to the difference of their Ab's subunit specificity, of species, of cell type, or of cell culture stages such as G_0 vs G_1 , or quiescent vs arrested. Our data demonstrated that the major translocatable subunit of the enzyme in the nucleus of human cells is CKII α' . The activity of CKII is lower at S phase (Carroll, D., and D. R. Marshak, manuscript submitted for publication) or G_0 phase of cell cycle. Stimulation by serum or growth factors induces an enhanced activation of CKII (Carroll and Marshak, 1989). If the activation of CKII and translocation of CKII from cytoplasm to nucleus are a prerequisite for cell proliferation and onset of the cell cycle, the migration of CKII α' from the cytoplasm to the nucleus at G_1 and from the nucleus to the cytoplasm at S must be one of the important regulation steps of cell cycle control.

The nuclear form of the CKII α' subunit may not require the β subunit for its optimal activity or regulation in interphase cells. It seems possible, therefore, that the nuclear form of CKII may have a distinct physiological role from that of the α subunit. Notably, the β subunit is the site of autophosphorylation and, therefore, the α' subunit may be regulated by other means. The significance of α' in the cell cycle has been studied in the budding yeast, *Saccharomyces cerevisiae* (Padmanabha et al., 1990). Null mutations of the CKA1 (α) gene do not confer a detectable phenotype, and haploid cells in which the CKA2 (α') gene alone is disrupted show no detectable phenotype. However, haploid cells carrying disruptions in both the CKA1 and CKA2 genes are inviable, and exhibit growth arrest (Padmanabha et al., 1990). Cell cycle control of CKII activity in mammalian cells (Carroll, D., I. J. Yu, and D. R. Marshak, manuscript submitted for publication) might be regulated by the translocation phenomenon described here or by an endogenous inhibitor compound. The most distinct characteristic of the CKII α' subunit appears to be the regulation of its localization during the cell cycle.

As shown here, a small number ($\sim 20\%$) of cells are nucleus negative with Ab 276. CKII is known to phosphorylate numerous nuclear proteins in addition to cytoplasmic proteins (Edelman et al., 1987). The presence of the CKII α' subunit in the nucleus rather than the CKII α subunit, suggests that the CKII α' subunit may play a significant role in regulation of such processes as DNA replication, transcription, and nuclear and nucleolar reorganization. It further implies that signal transduction pathways from the cytoplasm to the nucleus by CKII are rather complicated. The advantages to the cell of utilizing the CKII α' subunit in the nucleus are not known at this time. The different properties may be provided by the carboxy terminus of the two polypeptides, since this is the region of vast sequence heterogeneity (Lozman et al., 1990). Absence of carboxy-terminal structures (~ 40 amino acids) in α' might provide a different means of regulatory transport of CKII across the nuclear envelope. Alternatively, the absence of these sequences may elevate the effect of the putative nuclear localization sequence that is recognized by Ab 247 and is essential for the inhibition mediated by polyanionic compounds, such as heparin (Hu and Rubin, 1990b). Furthermore, the absence of these sequences may inhibit association of the β subunit, and thus result in a different level of regulation.

The absence of CKII α , α' , and β subunits from the nucleus in some percentage of cells implies that there is a short

period in the nucleus when CKII is not required or is present as another form, which is not detected by conventional chromatography or by our immunological reagents. The regulation of the presence and absence of CKII α' in the nucleus during such critical phases is in need of further investigation.

In summary, our current working model of CKII localization in the cell cycle is as follows. During the G₁ phase of the cell cycle in proliferating cells, CKII activity is high, and the α and β subunits remain cytoplasmic, while the α' subunit exists as the nuclear form. At the S phase of cell cycle, the α' subunit translocates from the nucleus to the cytoplasm concomitant with a decrease in CKII activity. When cells reach mitosis and the nuclear envelope breaks down, all forms of the enzyme are diffusely distributed throughout the cell and are also associated with spindle fibers. The role of CKII in cell growth appear to be complex, with certain forms associated with cytoplasmic structures and other forms (α') associated with the nucleus. The studies presented here contribute to a greater understanding of the function of CKII subunits in the control of cell growth.

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