Casein Kinase II Is a Predominantly Nuclear Enzyme

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Abstract. Casein kinase II (CK II) has been implicated in regulating multiple processes related to cell growth, proliferation, and differentiation. To better understand the function(s) and regulation of this ubiquitous kinase, it is important to know its subcellular distribution. However, this issue has been the subject of contradictory reports. In this study, we have used indirect immunofluorescence microscopy and cell fractionation to study the subcellular distribution of all three subunits of chicken CK II, α, α', and β. We examined primary chick embryo fibroblasts, virally transformed chicken hepatoma cells, as well as HeLa cells transiently transfected with cDNAs encoding chicken CK II subunits. We found that each of the three CK II subunits was located predominantly in the cell nucleus, irrespective of the cell type analyzed or the procedure used for cell fixation. No major differences were detected in the subcellular distributions of individual CK II subunits, and no evidence was obtained for subunit redistributions during interphase of the cell cycle. During mitosis, the bulk of the enzyme was dispersed throughout the cell, though a fraction of all three subunits was associated with the mitotic spindle. Biochemical studies based on mechanical enucleation of chicken cells confirmed the predominantly nuclear location of all three CK II subunits. Finally, immunoblotting experiments were carried out to study the expression of CK II subunits. A survey of different adult chicken tissues revealed substantial tissue-specific differences in the levels of CK II protein, but no evidence was obtained for pronounced tissue specificity in the expression of individual CK II subunits. These results strongly suggest that CK II functions primarily in regulating nuclear activities, and that the two catalytic subunits, α and α', may carry out overlapping functions.

CASEIN kinase II (CK II)[1] is a cyclic nucleotide- and calcium-independent serine/threonine-specific protein kinase. While little is known about the regulation of this ubiquitous enzyme, multiple functions have been proposed (for reviews see Edelman et al., 1987; Pinna, 1990; Tuazon and Traugh, 1991). In particular, current evidence suggests that CK II may serve to integrate multiple cellular activities related to cell growth and differentiation (for reviews see Krebs et al., 1988; Carroll et al., 1988; Schneider and Issinger, 1989). In support of a pleiotropic role of CK II, its proposed physiological substrates include metabolic enzymes, cytoskeletal proteins, transcription factors, as well as the products of several oncogenes and tumor suppressor genes (for reviews see Pinna, 1990; Tuazon and Traugh, 1991). Furthermore, CK II activity is increased in neoplastically transformed cell lines (Prowald et al., 1984), as well as in tumors (Münstermann et al., 1990). In cultured cells, CK II activity was shown to change during cell differentiation (Sommercorn and Krebs, 1987), and it was reported to be stimulated in response to various mitogens and growth factors (Sommercorn et al., 1987; Klarlund and Czech, 1988; Carroll and Marshak, 1989; Ackerman and Osheroff, 1989). These findings raise the possibility that CK II may be part of a protein kinase cascade involved in relaying signals from the plasma membrane to the cell nucleus.

Biochemical studies on CK II isolated from several organisms have revealed heterotetramers of the structure $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ (for reviews see Hathaway and Traugh, 1982; Edelman et al., 1987; Pinna, 1990). The two larger subunits α and α' have molecular masses between 36 and 44 kD, while the β subunit is ~25 kD. It is well established that the α and α' subunits provide the catalytic activity but the role of the β subunit remains poorly defined. Current evidence indicates that the β subunit stimulates CK II activity, and thus presumably plays a regulatory role (Lin et al., 1991; Grankowski et al., 1991). The recent sequencing of CK II cDNA clones from different species revealed a very high degree of structural conservation of the α, α', and β subunits during evolution (Padmanabha and Glover, 1987; Saxena et al., 1987; Chen-Wu et al., 1988; Heller-Harrison et al., 1989; Meisner et al., 1989; Jakobi et al., 1989; Hu and Rubin, 1990; Kopatz et al., 1990; Lozeman et al., 1990; Boldyreff et al., 1991; Maridor et al., 1991). The α and α' subunits are closely related to each other, but differ in their carboxyl-termini. While it is certain that α and α' subunits are derived from separate genes, it remains unclear whether or not they carry out distinct functions. Gene disruption experiments in *Saccharomyces cerevisiae* demonstrate that expression of at least one catalytic subunit of CK II is essential for cell viability (Padmanabha et al., 1990).

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblasts; CK II, casein kinase II.

Based on extensive studies with peptide substrates, the sequence specificity of CK II in vitro is well defined (Meggio et al., 1984; Kuenzel et al., 1985; Marchiori et al., 1988; Carroll et al., 1988; Pinna, 1990). However, primary sequence constraints are not the only parameters dictating kinase specificity in vivo. Another major factor determining the target range of a protein kinase is its subcellular location (for a discussion see Lohmann and Walter, 1984). Although several recent studies deal with the subcellular distribution of CK II, results obtained in different laboratories are highly contradictory (see Discussion). In an attempt to resolve this controversy, we have raised antibodies specific for individual subunits of chicken CK II. The subcellular distribution of CK II was then examined using immunocytochemical techniques as well as an approach based on cell fractionation. We have investigated whether or not the two catalytic subunits α and α' display distinct subcellular distributions, and we have examined to what extent they show differential expression in adult chicken tissues. Our results indicate that CK II is a predominantly nuclear kinase. Moreover, since the two catalytic subunits of CK II displayed no major differences with respect to either subcellular location or tissue distribution, we conclude that they are likely to carry out closely related if not identical functions.

Materials and Methods

Cell Culture and Metabolic Labeling

Chicken embryo fibroblasts (CEF) and HeLa cells were prepared and cultured as reported previously (Krek and Nigg, 1991*a*). MC-29 transformed chicken DU249 hepatoma cells (Langlois et al., 1974) were grown as described by Nakagawa et al. (1989).

For in vivo labeling of proteins with [^{35}S]methionine, subconfluent 10-cm dishes of DU249 cells were incubated for 30 min in methionine-free DME, then for 4 h in 5 ml of methionine-free DME containing 10% dialyzed FCS, 1% dialyzed chicken serum, and 0.1 mCi/ml [^{35}S]methionine (Amersham Corp., Arlington Heights, IL).

Overexpression of CK II α and β Subunits in Escherichia coli

For expression of the chicken CK II α and β subunits in *E. coli*, the corresponding cDNAs were subcloned into the bacterial expression vectors pET-3b and pET-3a, respectively (Rosenberg et al., 1987; Novagen, Inc., Madison, WI). These vectors carry the promoter for the gene 10 of the T7 bacteriophage (Studier and Moffat, 1986). For cloning, the following constructions were carried out: the chicken CK II α cDNA was excised from the pGEM-3Zf(−) plasmid (Maridor et al., 1991) by digestion with SmaI. The resulting 1,337-bp fragment was ligated into pET-3b, which had been digested with BamHI and treated with Klenow polymerase. The protein expressed from this plasmid is a fusion protein containing 12 residues encoded by the pET-3b vector, followed by amino acids 21 to 391 of the chicken CK II α protein. The chicken CK II β cDNA was excised from the pGEM-3Zf(−) vector (Maridor et al., 1991) by (partial) NarI and (complete) EcoRI digestion. The resulting 831-bp fragment was treated with Klenow polymerase and then ligated into pET-3a, which had been prepared as described above. The protein expressed from this construct contains 12 residues encoded by the pET-3a vector, followed by one amino acid (alanine) arising from the untranslated leader sequence of the chicken CK II β cDNA, and the entire coding region of the β subunit. Expression plasmids were introduced into the *E. coli* strain DH5α. Overexpression of the CK II α and β fusion proteins was induced by infection of cells with the phage CE6 (i.e., a lambda derivative carrying the gene encoding the bacteriophage T7 RNA polymerase), using the conditions described in Krek and Nigg (1989). After induction, cells were harvested by centrifugation and the bacterial pellet was frozen at −70°C.

For purification of the CK II fusion proteins, frozen bacterial pellets (from 50-ml cultures) were thawed on ice and resuspended in 1.8 ml of lysis-buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 1% aprotinin (Sigma Chemical Co., St. Louis, MO), 0.3% Triton X-100, and 1 mg/ml lysozyme. After incubation at room temperature for 20 min, cells were centrifuged at 12,000 *g* for 10 min. The pellet was resuspended in ice-cold 100 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0, and incubated on ice for 10 min. $MgCl_2$ and DNase I were added to final concentrations of 8 mM and 10 μg/ml, respectively, and the incubation on ice was continued for 30 min. Inclusion bodies were collected by centrifugation at 12,000 *g* for 15 min and solubilized in 3× gel sample buffer. Following boiling for 10 min, samples were loaded onto 10% preparative SDS-polyacrylamide gels. Relevant protein bands were visualized by staining of the gels with an ice-cold solution containing 250 mM KCl and 1 mM β-mercaptoethanol. They were excised and transferred to a dialysis bag containing 0.5× SDS-PAGE running buffer (10× stock: 10 g/l SDS, 30 g/l Tris-base, 144 g/l glycine) and proteins were electro-eluted for 3 h at 4°C using a minigel apparatus (Bio-Rad Laboratories, Cambridge, MA) at 100 V. Elution efficiency and purity of the corresponding proteins were determined by subsequent analysis on SDS-PAGE, followed by Coomassie blue staining.

Preparation and Affinity Purification of Anti-Chicken CK II α, α', and β Antibodies

For intramuscular injection into rabbits, gel-purified CK II α and β proteins were emulsified with either Freund's complete adjuvant (for the first injection) or Freund's incomplete adjuvant (for all subsequent injections). Approximately 0.3–1.0 mg of protein were administered at 2-wk intervals, for a total of 2 mo. Bleedings were done at 2-wk intervals.

For affinity purification of antibodies, 1 mg of bacterially expressed, purified α or β subunit protein was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), as described by the manufacturer. Following pre-equilibration of the affinity matrices in 0.2% Tween 20 in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2), 3 ml of anti-CK II α or 8 ml of anti-CK II β immune serum were diluted with 2 vol of 0.2% Tween 20 in PBS and added to the corresponding affinity matrices. Samples were agitated gently overnight at 4°C. Then, the material was poured into a column (Bio-Rad Econocolumn; 1 × 13 cm), and washed twice with 20 ml of 0.2% Tween 20 in PBS, followed by 40 ml of PBS. Specifically bound antibodies were eluted with 0.2 M glycine-HCl (pH 2.2). Each 0.75-ml fraction was immediately neutralized by the addition of 0.25 ml of 1 M K_2HPO_4. Finally, antibodies were dialyzed against cold PBS containing 50% glycerol and stored at 4°C.

Antibodies against an 18 amino acid peptide corresponding to the carboxy-terminus of the chicken CK II α' subunit were prepared using the carrier-coupling and immunization procedures described in Krek and Nigg (1989). Anti-peptide antibodies were affinity purified as follows: 1 g of CH-Sepharose 4B (Pharmacia Fine Chemicals) was activated following the protocol of the manufacturer. Then, 20 mg of synthetic peptide was added to the matrix and coupled by bis-diazo-benzidine cross-linking (Bassiri et al., 1979). Subsequently, the matrix was washed extensively with 0.2% Tween 20 in PBS; 6 ml of anti-CK II α' serum were applied to the affinity matrix, and specific IgGs were bound and eluted as described above.

Preparation of Protein Extracts and Immunochemical Techniques

Protein extracts from different adult tissues were prepared for immunoblotting experiments exactly as described previously (Krek and Nigg, 1989). To solubilize tissue culture cells, 3× sample buffer was added directly to monolayer cultures. After boiling for 10 min, the cellular proteins were resolved by SDS-PAGE. Electrophoretic transfer to nitrocellulose membranes (Schleicher & Schüll, Inc., Keene, NH) was carried out for 3–4 h at 200 mA at room temperature, using 1× blotting buffer (10× stock: 30 g/l Tris-Base, 144 g/l glycine). Nitrocellulose membranes were blocked with 5% nonfat dry milk in PBS, either overnight at 4°C, or for 3 h at room temperature. Then, they were incubated for 3–4 h at room temperature (or overnight at 4°C) with affinity purified anti-CK II α (1:1,000 dilution of 0.3 mg/ml specific IgG), anti-CK II α' (1:300 dilution of 0.2 mg/ml specific IgG) or anti-CK II β IgGs (1:300 dilution of 0.2 mg/ml specific IgG), washed three times for 15 min with PBS, and incubated for 2–3 h at room temperature with 5 μCi of ^{125}I-labeled donkey anti-rabbit IgG (Amersham Corp.) diluted in 10 ml of PBS containing 5% nonfat dry milk. Finally, membranes were washed three times for 15 min with PBS, dried, and exposed for autoradiography.

For immunoprecipitation, cells grown on a 10-cm dish were washed twice in ice-cold PBS and lysed in 1 ml of phosphate-buffered RIPA (P-RIPA; 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate [pH 7.2], 100 mM NaCl, 20 mM NaF, 0.3 mM sodium orthovanadate, 0.02% NaN_3) containing 1% aprotinin, 1 mM PMSF, 30 μg/ml DNase I, and 30 μg/ml RNase A. After 30 min on ice, lysates were centrifuged for 10 min at 15,000 *g*, and supernatants were pre-incubated for 60 min at 4°C with 50 μl of a 50% (wt/vol in P-RIPA) suspension of protein A-Sepharose (Pharmacia Fine Chemicals), followed by centrifugation. Then, 2 μl of anti-CK II α or 4 μl of anti-CK II β antisera were added to the lysates. After incubation on ice for 1 h, 50 μl of protein A-Sepharose was added and incubation was continued for another hour, under continuous gentle shaking at 4°C. Immune complexes were collected by centrifugation and washed four times with P-RIPA, and once with PBS. Then, they were resuspended in 50 μl of 3× gel sample buffer and boiled for 10 min. Following removal of the Sepharose beads by centrifugation, immunoprecipitated proteins were analyzed by SDS-PAGE (10% gels) and fluorography, using En^{3}Hance (New England Nuclear, Boston, MA).

In Vitro Kinase Assays

Immunoprecipitates of CK II α or CK II β were prepared as described above, except that immune complexes were washed three times in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT). Kinase reactions were carried out for 20 min at 30°C in assay buffer supplemented with 4 μM ATP and 20 μCi [γ-^{32}P]ATP (Amersham Corp.), in a total volume of 50 μl. Where indicated, dephosphorylated casein (Sigma Chemical Co., C-4032) was included at 0.5 mg/ml, to provide an exogenous substrate. In the case of these latter samples, reactions were stopped by addition of 50 μl of 3× gel sample buffer. In the other cases, samples were washed three times with P-RIPA and once with PBS before the addition of 3× gel sample buffer. Reaction products were visualized by SDS-PAGE and autoradiography.

Immunofluorescent Experiments

Cells were prepared and processed for indirect immunofluorescence microscopy as described previously (Krek and Nigg, 1991*b*). For pre-extraction, cells were treated for exactly 30 s with ice-cold Triton X-100 buffer, before being fixed with paraformaldehyde (Nigg et al., 1985). Affinity-purified primary antibodies were used at the following concentrations: 5 μg/ml of anti-CK II α, 10 μg/ml of anti-CK II β, and 15 μg/ml of anti-CK II α′ IgG. Mouse hybridoma supernatant CTR 2611, containing antibodies against a centrosomal antigen (Buendia et al., 1990), was used undiluted. Secondary reagents were affinity-purified rhodamine-conjugated goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) and fluorescein-conjugated goat anti-mouse IgG (Sigma Chemical Co.). Hoechst dye 33258 was used at a dilution of 1:1,000 from a 5 mg/ml stock solution. All dilutions were prepared in PBS. For microscopy, cells were mounted in 90% glycerol/10% 1 M Tris-HCl, pH 9.0. They were examined with a Reichert-Jung Polyvar fluorescence microscope, using a 100× oil immersion objective.

Plasmid Constructions and DNA Transfections into HeLa Cells

To achieve transient expression of chicken CK II subunits in mammalian cells, the 1,512-bp EcoRI fragment encoding chicken CK II α was isolated from the original recombinant pGEM-3Zf(−) plasmid (Maridor et al., 1991), overhangs filled in with Klenow polymerase and blunt end ligated into the HpaI-digested, alkaline phosphatase–treated mammalian expression vector pCMVneo (Krek and Nigg, 1991*b*). In the case of the chicken CK II β subunit, a 760-bp AvaII fragment was isolated from the original recombinant pGEM-3Zf(−) plasmid (Maridor et al., 1991), filled in with Klenow polymerase and blunt end ligated into the pCMVneo vector as described above. The resulting plasmids were propagated in DH5α bacteria and purified using the Qiagen plasmid purification kit. Transfections of HeLa cells were carried out exactly as described previously (Krek and Nigg, 1991*b*).

Cell Fractionation by Mechanical Disruption and Enucleation

For cell fractionation, DU249 cell monolayers were washed twice with ice-cold PBS, cells were harvested with a rubber policeman and taken up in ice-cold hypotonic lysis buffer (20 mM Hepes-KOH, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1% aprotinin). After incubation on ice for 10 min, cells were homogenized by 15 strokes with a tight-fitting Dounce homogenizer. The homogenate was subsequently centrifuged at 2,000 rpm (HB4 rotor; Sorvall Instrument, Newton, CT) for 10 min to recover a nuclear fraction (pellet) and a cytoplasmic fraction (supernatant).

For enucleation experiments, cell monolayers were grown on round (18-mm diam) plastic supports that had been cut from tissue culture dishes. Cells were washed twice in prewarmed DME (Gibco Laboratories, Grand Island, NY) and placed, cell side down, into Corex® glass tubes containing 10 ml of pre-warmed DME (without serum) with cytochalasin B (5 μg/ml) and immediately centrifuged for 8 min at 30–37°C (9,500 rpm; in an HB4 rotor; Sorvall Instruments). The extent of enucleation was monitored by subsequent fixation of one sample of cytoplasts with 3% paraformaldehyde, 2% sucrose and staining of DNA with Hoechst dye 33258. In parallel, cytoplasts as well as the corresponding nuclear pellets were solubilized with 3× gel sample buffer and subjected to SDS-PAGE. The partitioning of individual proteins was then determined by immunoblotting.

Results

Production and Characterization of Antibodies Monospecific for Chicken CK II Subunits α, α′, and β

To produce monospecific antisera against individual chicken CK II subunits, α and β subunits were expressed as fusion proteins in *E. coli* (Studier and Moffat, 1986), and the purified proteins were used for immunization of rabbits. In the case of the α′ subunit, rabbit antibodies were raised against an 18 amino acid synthetic peptide corresponding to the predicted carboxy-terminus (Maridor et al., 1991). Figs. 1 and 2 illustrate the specificity of the various anti-CK II antibodies. By immunoblotting on total cell lysates of either chicken DU249 hepatoma cells (lanes *1*) or chick embryo fibroblasts (lanes *2*), affinity purified anti-α (Fig. 1 *A*), anti-α′ (Fig. 1 *B*) and two different anti-β antibodies (Fig. 1, *C* and *D*) recognized single proteins of the expected molecular weights, i.e., 42 (α), 38 (α′), and 25 kD (β), respectively. All three CK II subunits were found to be more abundant in the transformed DU249 cell line than in primary fibroblasts (Fig. 1, compare lanes *1* and *2*).

The results of immunoprecipitation experiments are summarized in Fig. 2. From lysates of [^{35}S]methionine-labeled DU249 cells, anti-α antibodies specifically precipitated two polypeptides (Fig. 2 *A*, lane *1*). These correspond to the α and β subunits of CK II, respectively, as confirmed by immunoblotting experiments (not shown). To test for activity of the immunoprecipitated holoenzyme, we also carried out in vitro kinase assays, using immunoprecipitates prepared from unlabeled cells. In the absence of exogenous substrate, strong phosphorylation of the β subunit could be seen, while phosphorylation of the α subunit occurred to a much lesser extent (Fig. 2 *A*, lane *3*). High kinase activity could also be demonstrated using dephosphorylated casein as an exogenous substrate (Fig. 2 *A*, lane *5*). In similar experiments with anti-β antibodies, one major protein of the expected molecular weight was specifically immunoprecipitated from [^{35}S]methionine-labeled cells (Fig. 2 *B*, lane *1*, *arrowhead*). A second protein of lower molecular weight could also be seen (Fig. 2 *B*, lane *1*, *star*). We presume that this protein represents a proteolytic degradation product of the β subunit, although we cannot exclude that it may represent a tightly associated, as yet unidentified protein. Co-precipitation of CK II catalytic subunits was not very prominent in the β immunoprecipitates from [^{35}S]methionine-labeled

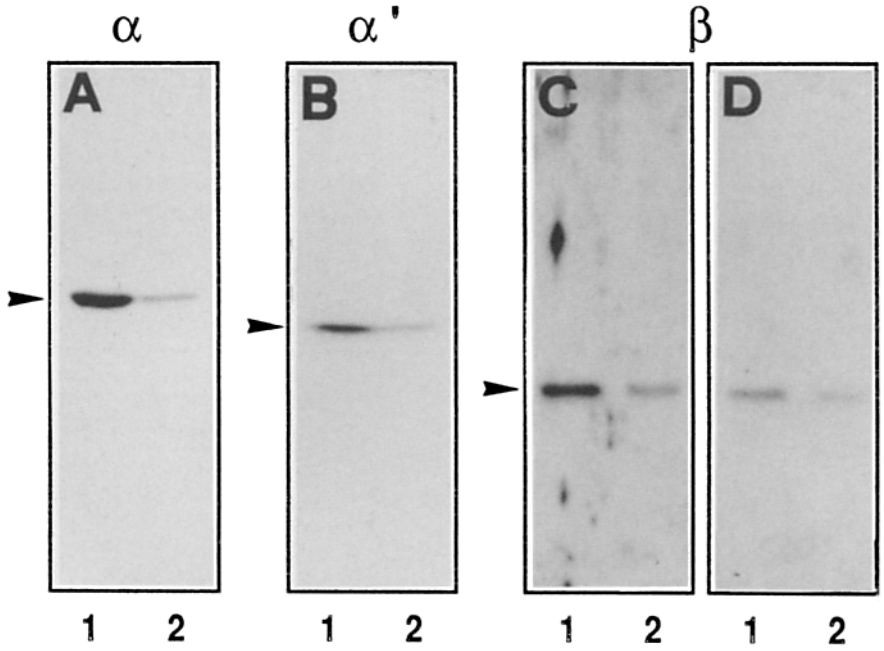

Figure 1. Specificity of antibodies against individual chicken CK II subunits α, α′ and β. Whole-cell extracts were prepared from chicken DU249 cells (lanes *1*) and chicken embryo fibroblasts (lanes *2*). Proteins were resolved by SDS-PAGE on 10% (*A* and *B*) or 12% (*C* and *D*) gels and transferred to nitrocellulose. Filters were probed with affinity-purified antibodies directed against bacterially expressed chicken CK II (*A*), a carboxy-terminal peptide of CK II α′ (*B*), or bacterially expressed CK II (*C* and *D*), followed by ^{125}I-labeled secondary antibodies. The two preparations of anti CK II β antibodies were obtained from two rabbits. The position of the respective CK II subunits are indicated by arrowheads.

cells, but could be demonstrated by kinase assays, monitoring phosphorylation of either the β subunit (not shown) or exogenous casein (Fig. 2 *B*, lane *3*). No CK II subunits and no significant kinase activities were precipitated when using pre-immune sera for control immunoprecipitations (Fig. 2 *A*, lanes *2*, *4*, and *6*; Fig. 2 *B*, lanes *2* and *4*). From these experiments we conclude that active CK II could be specifically immunoprecipitated using either anti-α or anti-β subunit antibodies. In contrast, the anti-α′ peptide antibody was not reactive in immunoprecipitation experiments (not shown).

The monospecificity of the anti-α′ antibody seen in immu-

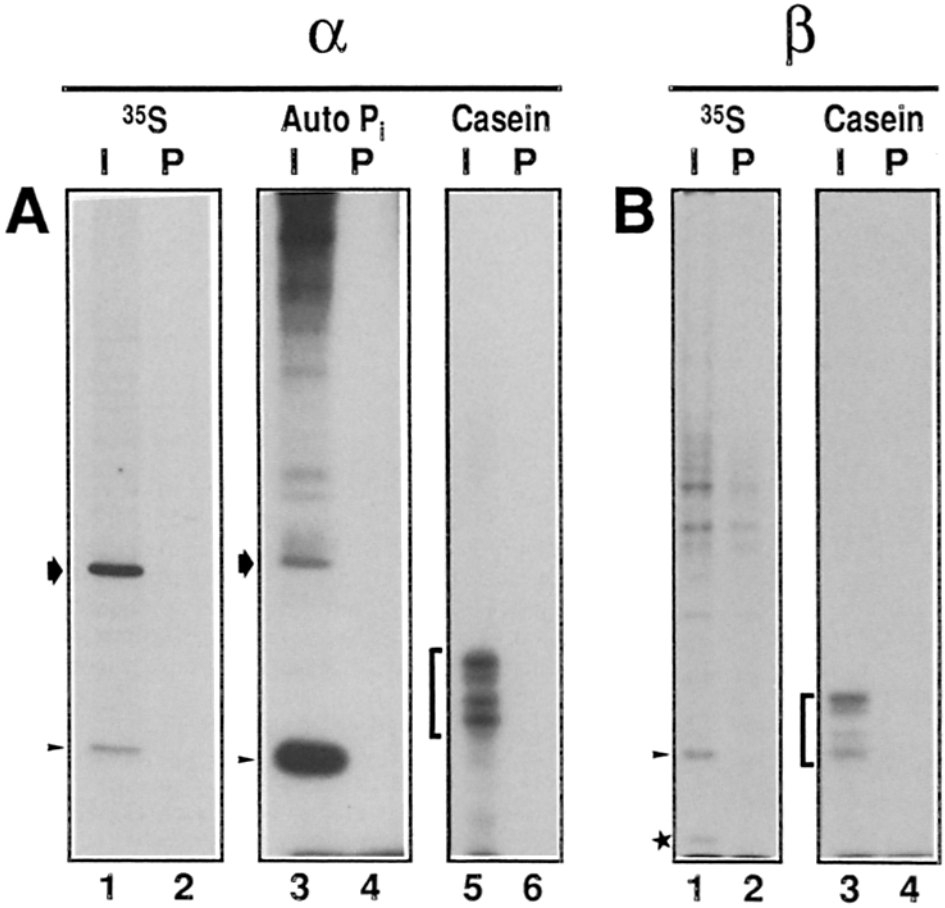

Figure 2. Anti-α and anti-β antibodies immunoprecipitate active CK II holoenzyme. Immunoprecipitations of CK II subunits from chicken DU249 cell lysates were carried out as described in Materials and Methods, using antibodies specific for CK II α (*A*) and β (*B*), or the corresponding pre-immune sera. CK II subunits were immunoprecipitated either from [^{35}S]methionine-labeled cells (*A* and *B*, lanes *1* and *2*), or from unlabeled cells (*A*, lanes *3–6*; *B*, lanes *3* and *4*). The latter samples were then assayed for CK II kinase activity in the absence (*A*, lanes *3* and *4*) or presence (*A*, lanes *5* and *6*; *B*, lanes *3* and *4*) of dephosphorylated casein as an exogenous substrate. All experiments were carried out using immune (*I*) and pre-immune sera (*P*) in parallel. The position of CKII α is indicated by arrows, whereas CKII β is marked by arrowheads. The star next to lane *1* in *B* indicates an unidentified protein.

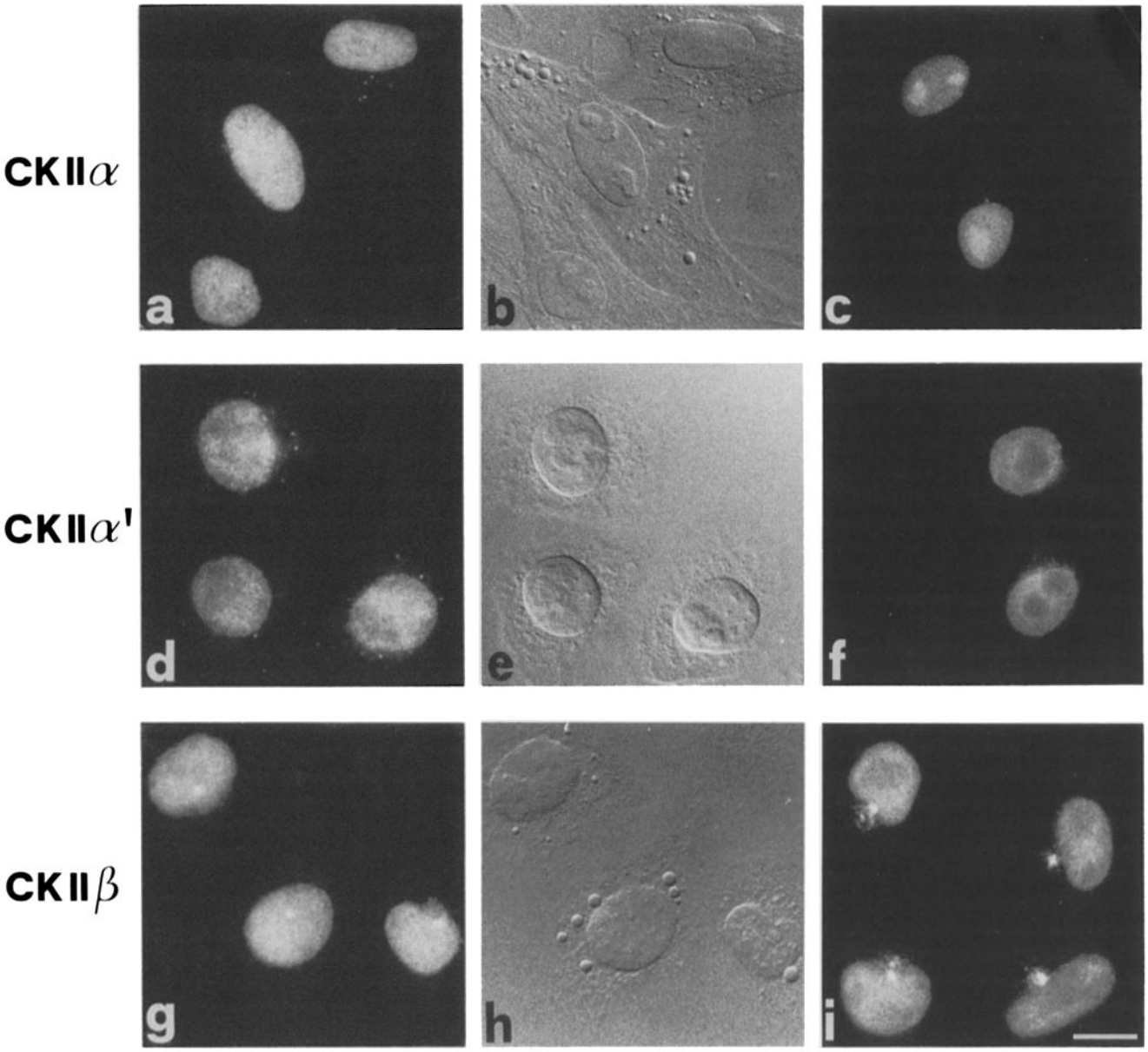

Figure 3. Immunofluorescent localization of CK II subunits in chicken cells. Chicken embryo fibroblasts (*a–c*) or DU249 cells (*d–i*) were fixed and permeabilized using either formaldehyde/Triton X-100 (*a*, *d*, and *g*) or pre-extracted with Triton X-100 buffer (see Materials and Methods) before formaldehyde fixation (*c*, *f*, and *i*). They were then incubated with affinity-purified anti-chicken CK II α (*a* and *c*), anti-chicken CK II α′ (*d* and *f*) or anti-chicken CK II β (*g* and *i*) antibodies, followed by rhodamine-conjugated goat anti-rabbit IgG. *b*, *e*, and *h* show differential interference contrast pictures corresponding to *a*, *d*, and *g*, respectively. Bar, 15 μm.

noblotting experiments was expected in view of the unique sequence of the α′ carboxy-terminal peptide used for immunization. However, it is remarkable that the antibody raised against the bacterially expressed α subunit did not detectably cross-react with the α′ subunit, irrespective of whether its specificity was assayed by immunoblotting (Fig. 1) or immunoprecipitation (Fig. 2). This observation indicates that the anti-α subunit antibodies were directed predominantly, if not exclusively, against regions where the α subunit differs from the α′ subunit (see Maridor et al., 1991).

Immunofluorescent Localization of Casein Kinase II Subunits in Interphase and Mitotic Chicken Cells

To determine the subcellular distribution of individual CK II subunits, primary chick embryo fibroblasts and transformed DU249 hepatoma cells were examined by indirect immunofluorescence microscopy. Since previous immunocytochemical localization studies yielded conflicting data, great care was taken to control for the specificity of the observed staining. In particular, several different fixation–permeabilization procedures were used for sample preparation, and antibody specificity was controlled by blocking with antigen. In a first series of experiments (Fig. 3), cells were fixed with paraformaldehyde before being permeabilized with non-ionic detergent (left and center panels); alternatively, they were treated briefly with detergent before fixation (right panels). In additional experiments, cells were subjected to aldehyde fixation for different lengths of time (Fig.

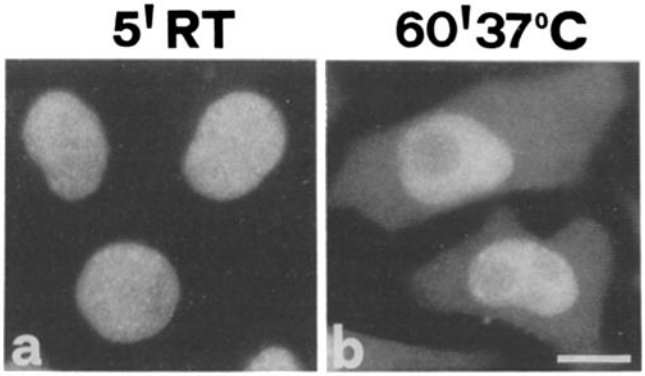

Figure 4. Distribution of CK II α subunit after prolonged fixation of DU249 cells with formaldehyde. Chicken DU249 cells were fixed with formaldehyde for either 5 min at room temperature (*a*) or for 60 min at 37°C (*b*). Then they were permeabilized with Triton X-100 and processed for indirect immunofluorescence microscopy, using affinity-purified anti-CK II α antibodies. Bar, 15 μm.

4), or they were fixed and permeabilized using a methanol–acetone procedure instead of the aldehyde–detergent protocols (Fig. 5, *a–d*). In all cases, affinity-purified anti-CK II antibodies were used at low concentrations (5–15 μg/ml), and staining specificity was controlled by pre-incubation of the antibodies with either bacterially expressed antigen (Fig. 5, *e–j*) or, in the case of the α′ subunit, antigenic peptide (not shown). Pre-absorption of antibodies on their respective antigens abolished staining of methanol/acetone as well as formaldehyde-fixed cells.

In both cell types examined, all three CK II subunits were located predominantly in the cell nucleus, irrespective of the chemical procedure used for cell fixation (compare Figs. 3–5). Previous studies had emphasized nucleolar staining by anti-CK II antibodies (Pfaff and Anderer, 1988; Belenguer et al., 1989), but our results do not support the notion of a preferential association of CK II with nucleoli. The only instance we detected some nucleolar staining by anti-CK II antibodies was when anti-α (but not α′) antibodies were used

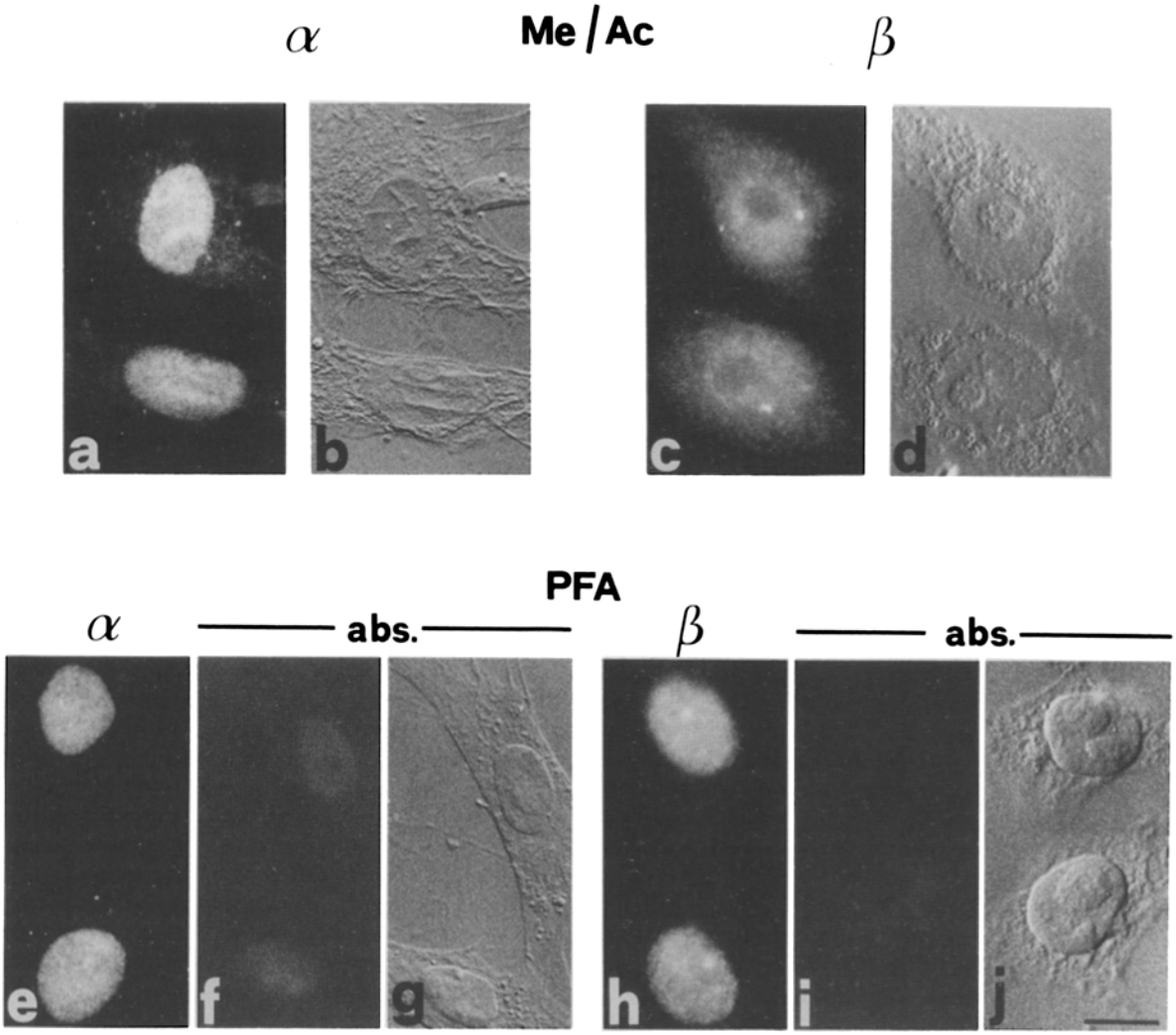

Figure 5. Nuclear staining by anti-CK II α and β antibodies is independent of fixation procedure, but abolished by antibody preabsorption. Chicken embryo fibroblasts (*a*, *b*, *e*, *f*, and *g*) or DU249 cells (*c*, *d*, *h*, *i*, and *j*) were fixed and permeabilized using either methanol/acetone (*a–d*) or formaldehyde/Triton X-100 (*e–j*). They were then incubated with affinity-purified antibodies against CK II α (*a* and *e*), or β (*c* and *h*), or with antibodies that had been preabsorbed on the corresponding bacterially expressed α (*f*) or β (*i*) subunits. Secondary antibodies were rhodamine-conjugated goat anti-rabbit IgG. *b*, *d*, *g*, and *j* show differential interference contrast pictures corresponding to *a*, *c*, *f*, and *i*, respectively. Bar, 15 μm.

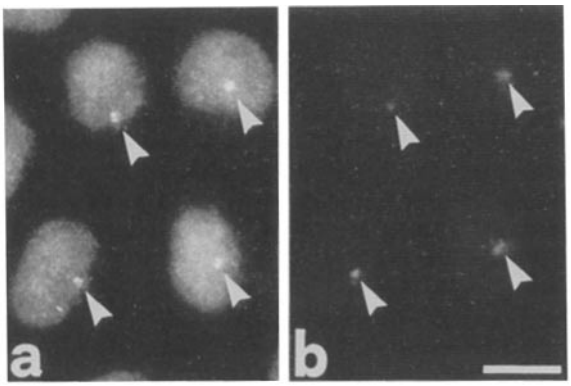

Figure 6. The β subunit of CK II is associated with centrosomes. Double indirect immunofluorescent labeling of formaldehyde/Triton X-100–treated chicken DU249 cells by affinity-purified anti-CK II β antibodies (*a*) and a mouse mAb (CTR 2611) directed against a centrosomal protein (*b*). Arrowheads in *a* and *b* point to centrosomes. Bar, 15 μm.

on preextracted cells (Fig. 3 *c*). This procedure involves substantial extraction of soluble proteins, and is therefore expected to allow preferential visualization of tightly bound antigens. However, since extraction of unfixed cells may also lead to artefactual protein redistribution, results obtained with this technique need to be interpreted with caution. When cells were prefixed according to standard protocols, staining of nucleoli was about equally intense as that of the surrounding nucleoplasm, and prolonged fixation of cells led to exclusion of anti-α antibodies from nucleoli (Fig. 4 *b*), presumably because extensive cross-linking prevented antigen accessibility.

In the case of anti-β antibodies, prominent staining of one or two cytoplasmic spots located in close proximity to nuclei could be detected, in addition to the pronounced staining of nuclei (e.g., Figs. 3 *i* and 5 *c*). As indicated by double immunofluorescent labeling of cells with anti-CK II β antibodies and a mAb (CTR2611) specific for a centrosomal antigen (Buendia et al., 1990), these fluorescent spots correspond to

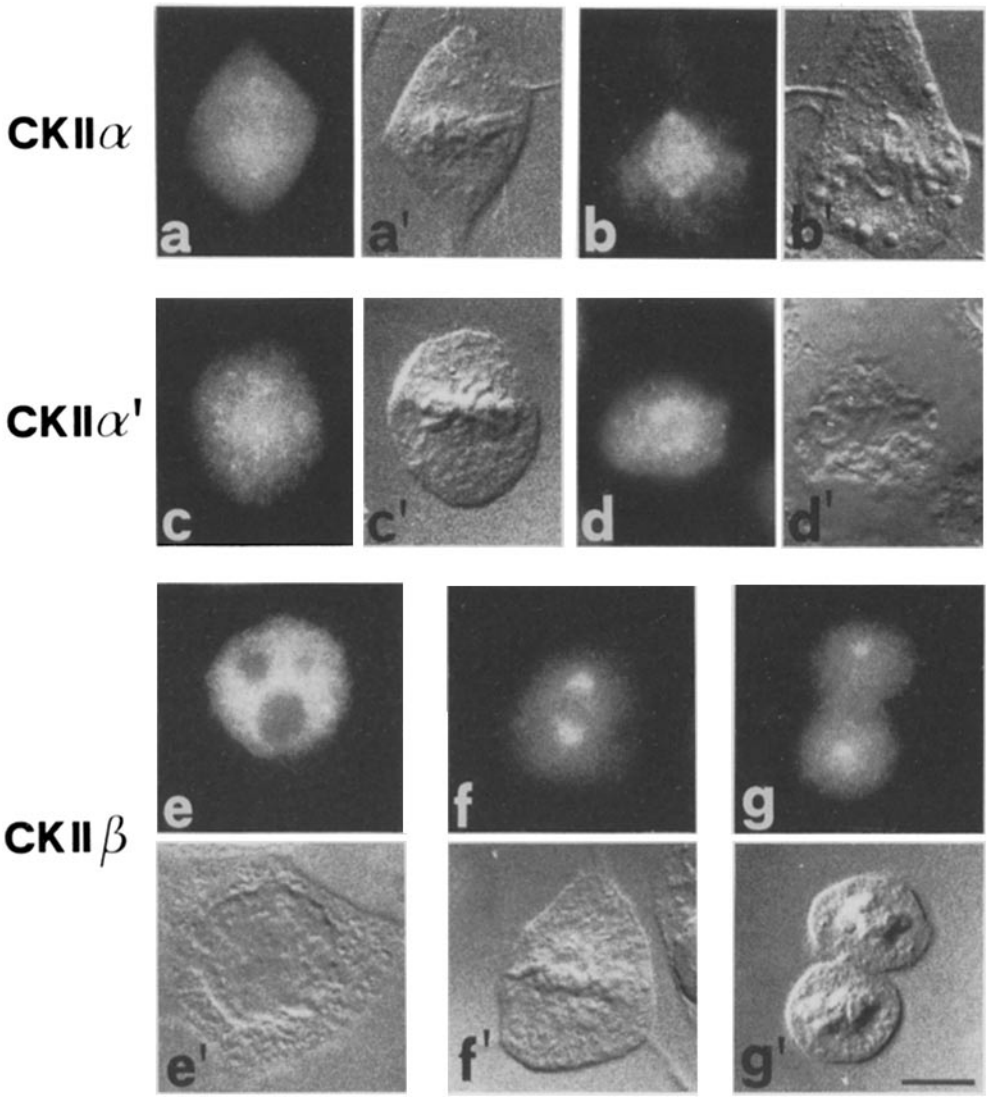

Figure 7. Distribution of CK II α, α', and β subunits during mitosis. Asynchronously growing chicken embryo fibroblasts (*a* and *b*) or chicken DU249 cells (*c–g*) were either fixed with formaldehyde and subsequently permeabilized with Triton X-100 (*a*, *c*, *e*, *f*, and *g*) or pre-extracted with Triton X-100, and then fixed with formaldehyde (*b* and *d*). Cells were stained with affinity-purified antibodies against CK II α (*a* and *b*), α' (*c* and *d*), or β (*e*, *f*, and *g*). Appropriate mitotic stages were identified under the microscope: (*a*, *b*, *c*, *d*, and *f*) metaphase; (*e*) prophase; (*g*) telophase. (*a'–g'*) Show differential interference contrast pictures corresponding to *a–g*, respectively. Bar, 10 μm.

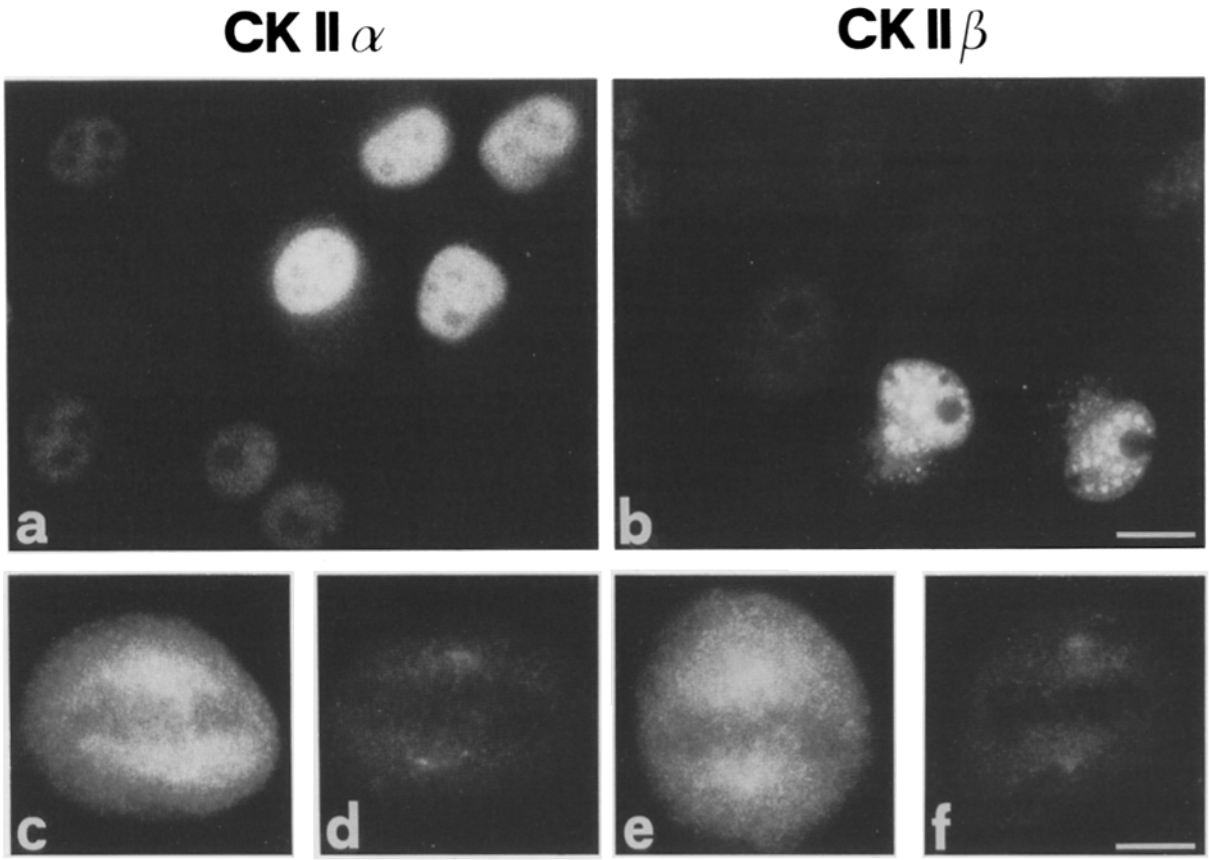

Figure 8. Localization of ectopically expressed chicken CK II α and β subunits in HeLa cells. HeLa cells were transfected with either cDNAs encoding chicken CK II α (*a* and *c*) or β subunits (*b* and *e*). Expressed proteins were visualized using the appropriate affinity-purified anti-CK II α or β antibodies for indirect immunofluorescence microscopy. Transfected interphase cells are readily identified on the basis of bright nuclear fluorescence (*a* and *b*). In transfected mitotic cells, chicken α and β subunits are dispersed (*c* and *e*), consistent with their distribution in chicken cells. *d* and *f* show untransfected metaphase HeLa cells stained with affinity-purified anti-chicken CK II α and β antibodies, respectively. Bars: (*b*) 20 μm; (*f*) 10 μm.

centrosomes (Fig. 6). We note that two different rabbits immunized with β subunits produced antibodies reacting strongly with centrosomes. In contrast, little or no staining of interphase cell centrosomes was detectable with anti-α and anti-α′ antibodies. We do not know whether this reflects the existence of a pool of free β subunits at the centrosome, or alternatively, is because of technical limitations (e.g., steric hindrance of antigen accessibility). Biochemical studies with highly purified centrosomes will be required to resolve this issue.

Although all three CK II subunits were predominantly nuclear, we emphasize that some cytoplasmic staining could also be seen, particularly when prolonged fixation conditions were used to minimize extraction of soluble cytoplasmic protein (see Fig. 4 *b* for the α subunit; not shown for α′ and β). Staining for CK II subunits was rather uniform among different cells in a population, suggesting that no major redistributions occurred during interphase stages of the cell cycle. In contrast, all three CK II subunits were distributed throughout the cell during mitosis (Fig. 7). In addition, CK II α and α′ could be seen to associate with mitotic spindles, particularly in pre-extracted cells (Fig. 7, *b* and *d*, respectively). While anti-β antibodies produced a strong labeling of the nucleus during early prophase (Fig. 7 *e*), they stained spindle caps in metaphase (Fig. 7 *f*) and telophase (Fig. 7 *g*) cells. These observations suggest that a subpopulation of CK II may interact with mitotic microtubules.

Chicken CK II α and β Subunits Localize Predominantly to the Nucleus When Transiently Expressed in HeLa Cells

To provide a further control for the specificity of our anti-CK II antibodies, the subcellular distribution of chicken CK II subunits was examined following their transient expression in heterologous cells. The cDNAs coding for either the α or the β subunit of chicken CK II were cloned into the mammalian expression vector pCMVneo (Bender et al., 1989; Krek and Nigg, 1991*b*), and the constructs were transfected into HeLa cells. Although our anti-chicken CK II antibodies display some cross-reactivity with mammalian CK II subunits, they could readily be used to monitor the ectopic overexpression of chicken CK II subunits by indirect immunofluorescence microscopy (Fig. 8). Both chicken CK II α (Fig. 8 *a*) and β (Fig. 8 *b*) subunits were localized predominantly to the nucleus of transiently transfected HeLa cells. In the case of the anti-β antibodies, staining of nuclei was punctate rather than uniform, and labeling of a perinuclear area, probably corresponding to the region of the centrosome, could also be seen (Fig. 8 *b*). Note that non-

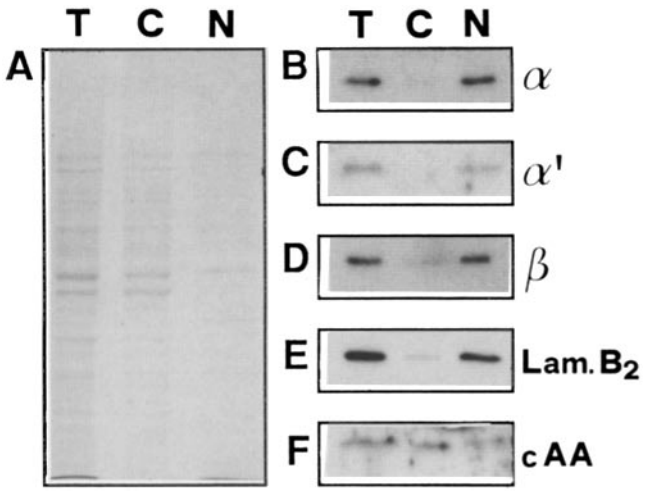

Figure 9. Partitioning of CK II subunits following fractionation of chicken DU249 cells by enucleation. Cytoplasmic and nuclear fractions of chicken DU249 cells were prepared by centrifugal enucleation as described in Materials and Methods. Aliquots of unfractionated cells (*T*), as well as cytoplasts (*C*), and nuclei (*N*) were resolved by SDS-PAGE on a 10% gel and either stained with Coomassie blue (*A*) or transferred to nitrocellulose and probed by immunoblotting (*B–F*). Lanes *1*, proteins of total fraction (*T*). Lanes *2*, proteins of cytoplasmic fraction (*C*). Lanes *3*, proteins of nuclear fraction (*N*). (*B–F*) Immunoblotting with antibodies against CK II α (*B*), CK II α′ (*C*), CK II β (*D*), nuclear lamin B_2 (*E*), and cytoplasmic aspartate aminotransferase (*F*).

transfected HeLa cells also displayed weak staining of nuclei, because of cross-reactivity of the anti-α and anti-β antibodies with endogenous CK II subunits (Fig. 8, *a* and *b*). For the same reason, weak staining of spindle poles was visible in non-transfected mitotic cells (Fig. 8, *d* and *f*).

Interestingly, transient overexpression of CK II α and β subunits did not detectably influence HeLa cell morphology. Moreover, we note that the transfected HeLa cells proceeded normally through mitosis (see Fig. 8, *c* and *e* for metaphase cells transfected with chicken α and β subunits, respectively). As shown above for chicken cells, CK II α and β subunits were distributed throughout mitotic HeLa cells, and there was evidence for a partial association with mitotic spindle caps (Fig. 8, *c* and *e*).

Biochemical Analysis of the Subcellular Distribution of CK II Subunits

Since immunocytochemical studies are prone to multiple types of artefacts (for a brief discussion see Nigg, 1988), we sought to corroborate the above localization data with independent biochemical evidence. Two different procedures were used for subcellular fractionation of chicken cells. In initial experiments, nuclei were separated from cytoplasm according to a conventional protocol based on cell homogenization and centrifugation (Scheidtmann, 1989). Under these conditions, up to 70% of the CK II subunits were found in the cytoplasm (not shown), consistent with the early literature describing CK II as a cytosolic enzyme (for review see Hathaway and Traugh, 1982). However, because many nuclear proteins are known to leak to the cytoplasm during cell homogenization (for discussion see Gordon et al., 1981; Bensch et al., 1982), we attribute this result to an artefactual redistribution of CK II subunits. To obtain more definitive biochemical evidence, we used an alternative procedure based on the rapid isolation of nuclei by cell enucleation (Gordon et al., 1981, Baeuerle and Baltimore, 1988). This technique yields intact nuclei with minimal contamination by cytoplasm, and cytoplasts with minimal contamination by intact cells. Following separation of nuclei and cytoplasts, the partitioning of CK II subunits was examined by immunoblotting. As controls, the distribution of a nuclear marker (lamin B_2; Lehner et al., 1986) and a cytoplasmic marker (cytosolic aspartate aminotransferase; Behra et al., 1981) were determined in parallel. The efficiency of the enucleation procedure was monitored by DNA staining of cytoplasts with Hoechst dye 33258 (not shown). Fig. 9 summarizes the results of these experiments. Efficient separation of nuclear and cytoplasmic fractions is illustrated by Coomassie blue staining (Fig. 9 *A*), as well as by the almost complete segregation of the two marker proteins, i.e., nuclear lamin B_2 (Fig. 9 *E*) and cytoplasmic aspartate aminotransferase (Fig. 9 *F*). As shown in Fig. 9, *B–D*, all three CK II subunits were located predominantly, though not exclusively, in the nuclear fraction. These results fully support and extend the immunocytochemical data shown above.

Expression of CK II Subunits α, α′, and β in Different Adult Tissues

The immunolocalization studies described above do not provide any evidence for major differences in the subcellular distribution of the α and α′ CK II subunits in primary fibroblasts or transformed hepatoma cells. However, we had previously observed that the two subunits differed dramatically in their expression in different tissues, when analyzed at the mRNA level (Maridor et al., 1991). In addition, we had found that in many tissues the mRNA levels for the β subunit were not balanced to match those of the transcripts for the two catalytic subunits (Maridor et al., 1991). With the availability of monospecific antibodies for the corresponding proteins, it became possible to examine whether or not the observed tissue-specific expression patterns of CK II transcripts were reflected at the protein level.

Protein extracts were prepared from those adult chicken tissues that we had studied previously by Northern analyses (Maridor et al., 1991), and similar amounts of total protein (see Coomassie blue staining in Fig. 10 *A*) were probed with affinity-purified antibodies specific for the individual CK II subunits (Fig. 10 *B*). As a control, cell extracts from DU249 cells were also analyzed (Fig. 10, *A* and *B*, lane *10*). From this study, the following conclusions may be drawn: first, all three CK II subunits are expressed at widely different levels in different tissues. For instance, CK II protein is abundant in brain (lanes *2*) but very scarce in liver (lane *1*). Second, despite variations in absolute amounts, the three subunits α, α′, and β, are expressed at comparatively constant ratios in all tissues examined. These results are in striking contrast to the previously observed tissue specificity of the α and α′ mRNAs and the unbalanced expression of the β mRNA (Maridor et al., 1991). The fact that large tissue specific differences in mRNA levels are not reflected at the protein

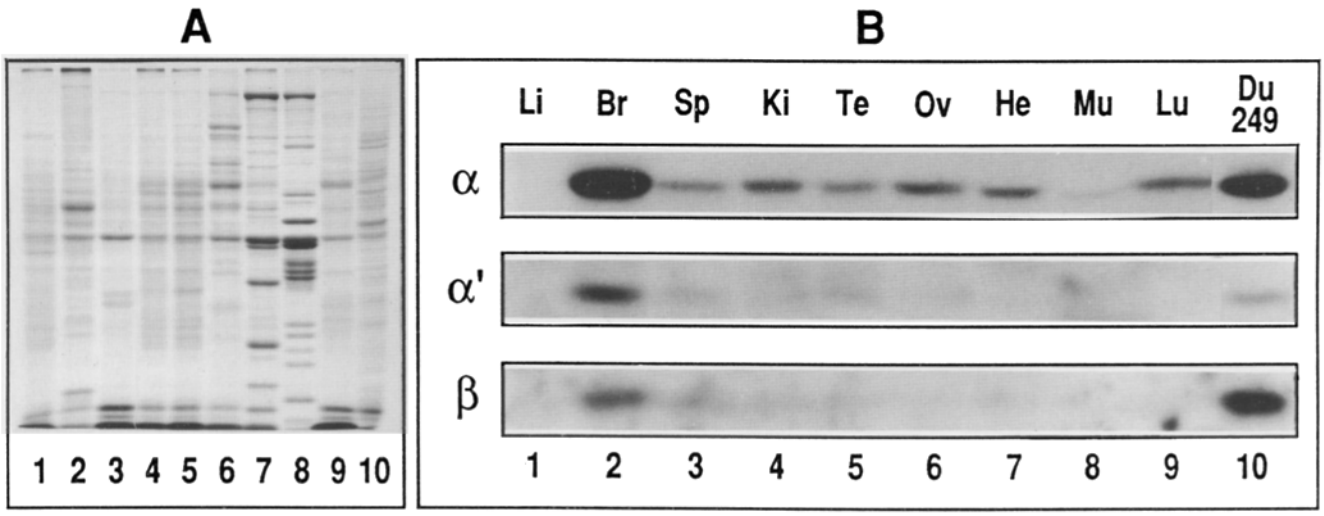

Figure 10. Expression of CK II subunits in selected adult tissues. Total cell extracts were prepared from the following adult tissues: liver (*Li*, lanes *1*); brain (*Br*, lanes *2*); spleen (*Sp*, lanes *3*); kidney (*Ki*, lanes *4*); testis (*Te*, lanes *5*); ovary (*Ov*, lanes *6*); heart (*He*, lanes *7*); muscle (*Mu*, lanes *8*); and lung (*Lu*, lanes *9*) as well as from DU249 cells (DU249, lanes *10*). Proteins were resolved on 10% gels by SDS-PAGE and either stained with Coomassie blue (*A*) or subjected to immunoblotting using antibodies directed against CK II α (*B*), CK II α′ (*B*), and CK II β (*C*).

level suggests that translational or posttranslational mechanisms contribute to control the expression of CK II subunits.

Discussion

Based on a combination of immunocytochemistry and subcellular fractionation, we have shown that all three known subunits of chicken CK II, α, α′ and β, are located predominantly in the cell nucleus. No major changes in the subcellular distribution of CK II were observed during interphase of the cell cycle. In contrast, all three subunits were redistributed throughout the cell during mitosis, with evidence for association of a fraction of CK II with elements of the mitotic spindle. Our studies did not reveal major differences in the subcellular distribution of individual CK II subunits. In particular, no evidence was obtained for differential localizations of the two catalytic subunits α and α′. These results suggest, first, that CK II may function primarily to control nuclear activities and possibly mitotic events, and second, that the two CK II isoforms may carry out overlapping functions.

In additional studies, we have examined the tissue distribution of individual CK II subunits. These studies were of particular interest in view of previous data demonstrating a striking tissue specificity in the pattern of expression of CK II transcripts (Maridor et al., 1991). Our present results show that most of the tissue-specific variations in mRNA levels are not reflected at the level of the corresponding proteins. Although the absolute amounts of CK II displayed considerable variations among different tissues, the three CK II subunits α, α′, and β were found to be expressed at a fairly constant ratio in all tissues examined. Thus, our present results provide no evidence for tissue-specific expression of individual CK II subunits.

Definitive information about the subcellular distribution of the CK II subunits is indispensible for a better understanding of the function and regulation of this kinase. However, no consensus has been reached on this issue. Based on subcellular fractionation, some workers reported CK II activity to be predominantly cytoplasmic (Singh and Huang, 1985; Edelman et al., 1987; Kandor et al., 1989), while others emphasized its presence in nuclei (Thornburg et al., 1979; Hathaway and Traugh, 1982; see also Filhol et al., 1990). Since activity measurements do not necessarily reflect protein levels, several laboratories have applied immunocytochemistry to determine the subcellular distribution of CK II. Again, the reported results are confusing. According to some studies CK II is associated with the nucleolus (Pfaff and Anderer, 1988; Belenguer et al., 1989), while others describe it to be distributed between nucleus and cytoplasm (Filhol et al., 1990), or present almost exclusively in the cytoplasm (Yu et al., 1991).

It is difficult to definitively explain why different laboratories have obtained such widely different results. Although one could invoke differences between species and/or cell types, we consider this to be an unlikely explanation. Instead, we note that it has been notoriously difficult to raise high titer sera against mammalian CK II. As a consequence, these reagents were frequently used at rather low dilutions, and antibody specificity has rarely been documented. Most recently, antisera were raised against peptides synthesized according to the predicted sequences of CK II subunits (Yu et al., 1991). Based on the use of these tools, an almost exclusive cytoplasmic localization of CK II α and β subunits was reported. Only antibodies reacting with both α and α′ subunits displayed some staining of nuclei, at least at certain stages of the cell cycle (Yu et al., 1991). However, although the anti-peptide antibodies used in these studies were shown to recognize denatured CK II subunits in immunoblotting experiments on total cell lysates, it is not clear to what extent the various peptide epitopes were actually accessible to antibodies during the in situ immunolabeling experiments. A rigorous interpretation of these data will have to await molecular information on protein interactions involving CK II subunits.

We are confident that the immunofluorescent staining patterns reported here correctly describe the distribution of CK II α, α', and β subunits. This confidence is based on the following evidence: first, all antibodies used here were shown to be monospecific for their respective antigens by immunoblotting experiments on total cell lysates. Second, immunocytochemistry was carried out using affinity-purified immunoglobulins at very low concentrations (5–15 μg/ml). Third, very similar results were obtained irrespective of the chemical procedure used for cell fixation and permeabilization, and virtually identical results were obtained when analyzing either primary fibroblasts or an established, virally transformed hepatoma cell line. Fourth, immunostaining could be completely blocked by preincubation of antibodies with the respective antigen. Fifth, our immunocytochemical findings are fully supported by biochemical data obtained from cell enucleation experiments. Moreover, the anti-α and anti-β antibodies recognized active (and thus presumably native) CK II in immunoprecipitation experiments. In the case of the anti-α' peptide antibody, no evidence could be obtained for a positive reaction with native antigen. While one might argue therefore, that the immunofluorescence data obtained with this reagent should be interpreted with caution (see the above discussion of the data reported by Yu et al., 1991), we emphasize that the cell fractionation data shown in Fig. 9 are not affected by in situ epitope accessibility. Finally, chicken CK II subunits α and β were shown to localize predominantly to the cell nucleus when overexpressed in HeLa cells. This result is consistent with the observations made with chicken cells, and it provides a striking demonstration of the specificity of the anti-CK II antibodies used here.

Remarkably, transient overexpression of CK II α and β subunits did not detectably influence the morphology of the recipient cells. Moreover, we have not detected any effects of CK II subunit expression on either the frequency or structure of transfected mitotic HeLa cells. Although this preliminary finding indicates that overexpression of CK II does not interfere with normal cell cycle progression, definitive conclusions will have to await the results of further experiments.

While our studies emphasize a predominantly nuclear localization of CK II, we emphasize that minor amounts of CK II could be detected in the cytoplasm. These cytoplasmic pools of CK II may well be functionally important, as indicated by the existence of several cytoplasmic substrates of this kinase (for review see Pinna, 1990; Tuazon and Traugh, 1991). It remains an attractive possibility also, that cytoplasmic and nuclear pools of CK II may exist in a dynamic equilibrium, and that shuttling of CK II subunits may play a role in relaying signals from the cytoplasm to the nucleus (for discussion see Nigg, 1990, 1991). In this context, it will be of considerable interest to determine what signals specify the nuclear accumulation of CK II.

The present analysis of mitotic cells also provides evidence for an association of all three CK II subunits with the mitotic spindle apparatus. This observation is in agreement with previous studies (Serrano et al., 1989; Yu et al., 1991). Its physiological significance remains to be elucidated, but it is interesting that several microtubule associated proteins have been described as substrates of CK II (Serrano et al., 1987; Diaz-Nido et al., 1988). Also, we note that other protein kinases, including the cAMP-dependent protein kinase (Nigg et al., 1985), Ca^{2+}/calmodulin-dependent protein kinase II (Ohta et al., 1990), and $p34^{cdc2}$ kinase (Riabowol et al., 1989; Bailly et al., 1989; Krek and Nigg, 1991*b*), have been localized to centrosomes and mitotic spindle poles.

At the level of resolution provided by immunofluorescence microscopy, all three CK II subunits were found to colocalize. This observation is consistent with biochemical evidence indicating that CK II exists predominantly as a multisubunit enzyme with the structure $\alpha_2\beta_2$ (or $\alpha\alpha'\beta_2$) (for review see Pinna, 1990). Since we obtained no evidence for major differences in the subcellular distribution of α and α' subunits, it is likely that CK II holoenzymes phosphorylate substrates in the same subcellular compartments, regardless of whether they contain α or α' catalytic subunits. Although it would be premature to exclude the possibility that α and α' subunits may carry out subtly different functions, our present results provide no evidence for functional specialization. Instead, they suggest that α and α' subunits may carry out overlapping, if not identical functions. This conclusion is also supported by the previous finding that the two catalytic subunits of CK II can complement each other in *S. cerevisiae* (Padmanabha et al., 1990).

In conclusion, our present results indicate that CK II is likely to function predominantly in regulating the activities of nuclear proteins. This conclusion is consistent with current evidence indicating that nuclear substrates of CK II comprise multiple factors involved in controlling transcription and replication. Of particular interest, these include the products of several oncogenes, i.e., myc (Lüscher et al., 1989), myb (Lüscher et al., 1990), erb A (Glineur et al., 1989), the human papillomavirus (types 6, 16, and 18) E7 protein (Firzlaff et al., 1989; Firzlaff et al., 1991; Barbosa et al., 1990), the SV-40 large T antigen (Grässer et al., 1988), and the tumor suppressor gene product p53 (Meek et al., 1990). With the availability of both cDNA probes and monospecific antibodies for CK II subunits, the stage is set for future attempts to unravel the function(s) of this ubiquitous but enigmatic kinase. The finding that CK II is a nuclear kinase provides a provocative link between signal transduction pathways and nuclear proteins controlling cell proliferation and differentiation.

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